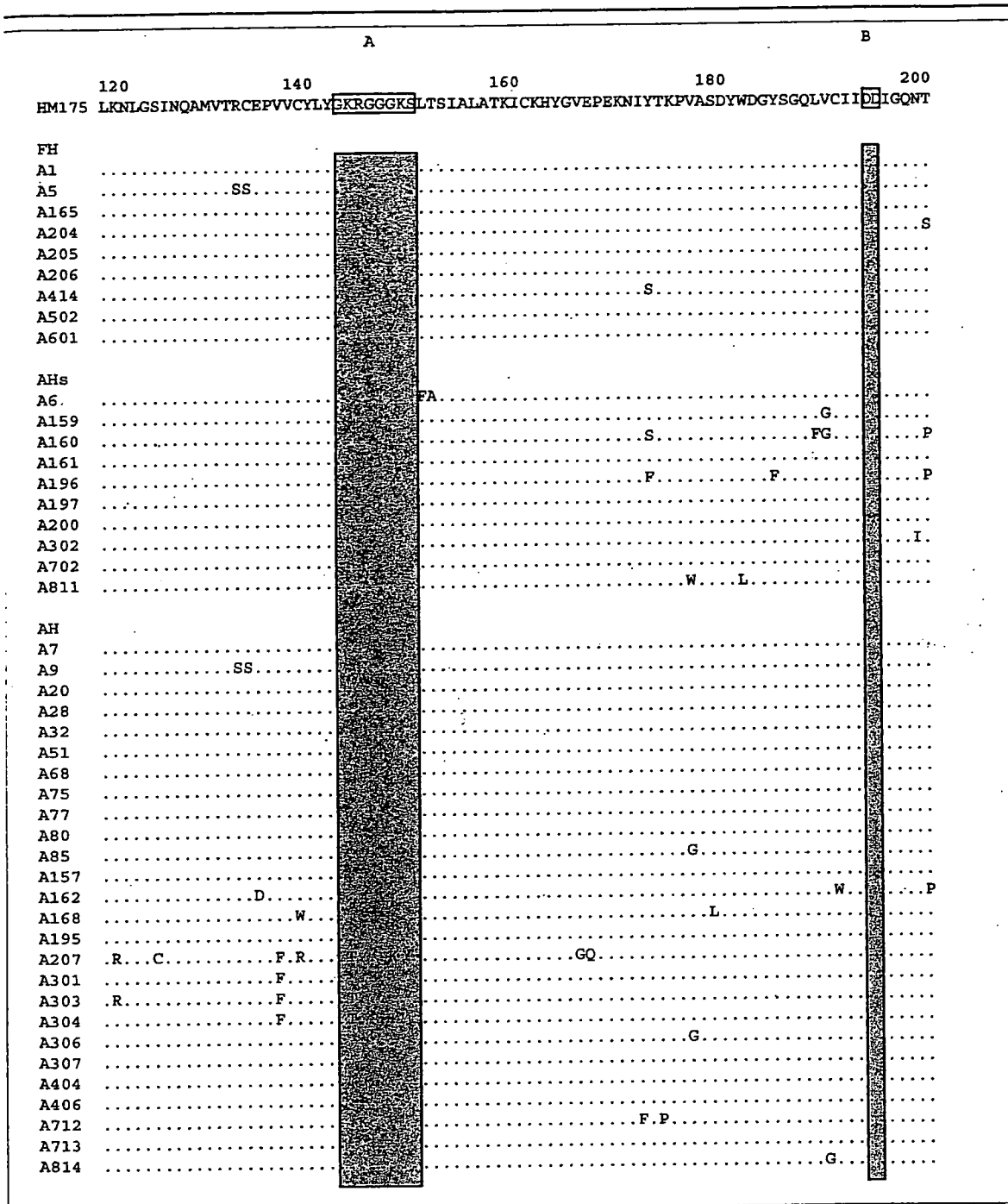

FIGURE 2
Alignment of amino acid sequences of the 2C regions. Numbers above the HM-175 sequence represent the amino acid positions from the amino terminus of HM-175 2C. The proposed NTP-binding motifs A and B are boxed.

Three of the AH cases had a K to T substitution at amino acid position 144, and one AH case had a R to K substitution at position 145 outside the conserved residues of the A site. No substitution was found in the B site of any of the cases. Interestingly, one FH case and one AH case had an amino acid substitution in the conserved residue of the A site (G to K at amino acid position 148).

DISCUSSION

The severity of hepatitis A varies among patients, but the reason for this is not clear. It is thought that disease severity may depend on the characteristics of individual patients including aging and underlying chronic liver disease (14,15). Willner *et al.* (16) reported that hepatitis A caused serious illness and death during an urban epidemic in the United States and that complications were more frequent in patients 40 years of age and older, although young healthy persons were also at risk for severe complications.

Durst *et al.* recently reported a cluster of fulminant hepatitis A (3). They related the severity of the infection in three siblings to the virulence of HAV, as the patients were all healthy before the infection and their illness followed a similar course.

In the 1990s, the numbers of patients with severe

forms of hepatitis A visiting our hospital were on the increase. However, our analyses of factors possibly contributing to the severity of the disease revealed no significant differences in patient characteristics including age (1,2), suggesting that viral factors might determine the severity of the disease. Therefore, to identify possibile differences in hepatitis A viruses for different types of hepatitis, we analyzed the HAV genome in sera from hepatitis A patients with various clinicopathological features (2,7-9,17).

HAV 2C protein encoded by the P2 region of the polyprotein is highly conserved among picornaviruses (4). 2C is a multifunctional protein, with some of its involvements including ATPase and GTPase (18), membrane-binding (19) and RNA-binding activities (20).

Polyprotein 2C, a nonstructural protein, is involved in replication of the viral genome. Analysis of the primary amino acid sequence of 2C shows homology with a family of proteins that contains a nucleoside-triphosphate (NTP)-binding motif. This motif consists of elements "A" (2/5 hydrophobic stretch) G/AXXGXGKS/T, where X stands for any amino acid, and "B" (3/5 hydrophobic stretch) D or DD/E. The residues mutated within the conserved A and B sites of the NTP-binding motif of 2C are critical in RNA replication and virus proliferation. 2C can tolerate an amino acid substitution outside the conserved residues (21). The A site forms an α helical loop, which places it in close proximity to the B site located at the carboxyl end of a β strand. The A site is critical for interaction with the phosphate group of GTP, and the Asp residue of the B site interacts with the magnesium cation and is critical for the GTP-dependent conformational shift and GTPase activity (22,23).

An ultrastructural analysis of HAV-infected cells revealed a tubular-vesicular network in close proximity to the rough endoplasmic reticulum. Protein 2BC is found on the network, and it is suggested to be involved in the rearrangement of cellular membranes (24).

The 2C protein anchors the negative-strand RNA to the membrane, and this anchoring may be crucial for the synthesis of positive-strand RNA from the negative-strand RNA template. 2C specifically interacts with the 3'-terminal sequences of the negative-strand RNA, but not with the 5'-terminal sequences of the positive-strand RNA (25). This interaction requires a stable stem-loop structure at the 3'-terminus of the negative-strand RNA.

In the present study we found the 2C gene of HAV obtained from patients with fulminant hepatitis A to have fewer amino acid substitutions than that from patients with ordinary self-limited acute hepatitis. These findings together indicate that viruses with fewer amino acid substitutions in this region may be more virulent in comparison to strains with more amino acid substitutions.

It has been reported that HAV strains adapted to cell culture systems have mutations in the 5'NTR and the P2 region (26,27). The simian HAV 2C gene was reported to be related to virulence in tamarins (28). Thus, subtle substitutions in 2C might influence the replication capability of the virus and thereby affect virulence.

Although our study demonstrated a possible association between the severity of hepatitis A and amino acid variations in 2C of HAV, we do not know whether the amino acid sequence in 2C of HAV is directly related to the virulence of the virus. HAV strains with fewer amino acid substitutions did not always cause severe hepatitis. Unlike the many reports of clusters of fulminant hepatitis B, there has been only one report about a cluster of fulminant hepatitis A (3).

Our current study of the 2C gene and our studies of the 5'NTR and genotype (9,17) have also suggested that both host immune responses and viral factors should be considered and examined when discussing the mechanisms responsible for the severity of hepatitis A. There are reports that the pathogenicity of HAV could be related to mutations within 5'NTR, P2 and P3 (28-30). When analyzing viral factors, rather than focusing on one specific region, perhaps several portions of the HAV genome should be investigated.

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Short Communication

Low serum level of hepatitis B core-related antigen indicates unlikely reactivation of hepatitis after cessation of lamivudine therapy

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Aim: The clinical significance of hepatitis B virus (HBV) core-related antigen (HBcrAg) in predicting the reactivation of hepatitis after halting lamivudine administration was analyzed.

Methods: A total of 34 patients with chronic hepatitis B were enrolled. Lamivudine was administered for at least 6 months before cessation, and reactivation of hepatitis was defined as elevation of alanine aminotransferase levels to more than 80 IU/L within 12 months of cessation.

Results: In total, 20 (59%) patients experienced hepatitis reactivation. Although concentrations of HBV DNA and HBcrAg in serum did not differ between the two groups of patients at the onset of lamivudine administration, HBcrAg serum levels were significantly higher ($P = 0.009$) in the reactivation patients (median 4.9, 25–75% range 4.7–5.9 log unit/mL) than the non-reactivation patients (median 3.2, 25–75% range <3.0–4.5 log unit/mL) post-lamivudine treatment. The concentration of HBV DNA did not differ between the two groups (median <3.7, 25–75% range <3.7–<3.7 log copy/mL in the reactivation group vs. median <3.7, 25–75% range <3.7–<3.7 log copy/mL in the non-reactivation group). Receiver operating characteristic analysis of HBcrAg concentration showed an area under the curve of 0.764 in predicting patients without reactivation of hepatitis.

Conclusion: HBcrAg can be a useful marker to identify patients who are not at risk of reactivation of severe hepatitis after discontinuation of lamivudine administration.

Key words: chronic hepatitis B, hepatitis B virus core-related antigen, hepatitis B virus DNA, hepatitis reactivation, lamivudine

INTRODUCTION

LAMIVUDINE, A NUCLEOSIDE analog that inhibits reverse transcriptase, has been found to inhibit the replication of hepatitis B virus (HBV), reduce hepatitis, and improve histological findings of the liver in long-term treatment.[1,2] Furthermore, it has been shown that lamivudine treatment improves the long-term outcome of patients with chronic hepatitis B.[3,4] However, there are a number of problems with lamivudine therapy, including hepatitis relapse due to the appearance of YMDD mutant viruses and the reactivation of hepatitis after its discontinuation.[5,6]

During lamivudine administration, the concentration of serum HBV DNA decreases, and usually becomes undetectable to even high sensitivity HBV DNA assays. However, this undetectable level is an inadequate indicator for safely discontinuing lamivudine

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administration as active hepatitis often recurs in patients post-treatment.

Previously, a chemiluminescence enzyme immunoassay (CLEIA) was developed by our laboratory to detect of hepatitis B core-related antigen (HBcrAg).[7,8] This HBcrAg CLEIA simultaneously measures the serum levels of hepatitis B core (HBc) and e (HBe) antigens using monoclonal antibodies, which recognize common epitopes of these two denatured antigens because both proteins are transcribed from the precore/core gene and their first 149 amino acids are identical.[9–11] Although this assay reflects the viral load of HBV in a similar manner to HBV DNA assays during disease progression, HBcrAg CLEIA shows characteristics different from HBV DNA assays under lamivudine administration since HBcrAg levels decrease more slowly than HBV DNA after treatment begins.[12] In the present study, we analyzed the clinical significance of the HBcrAg assay in predicting the likelihood of non-reactivation of hepatitis after discontinuing lamivudine administration in HBV treatment.

METHODS

Patients

A TOTAL OF 34 patients with chronic hepatitis B who were treated with lamivudine for at least 6 months were enrolled in the present study. The patients comprised 20 men and 14 women with a median age of 46 years (range 23–65 years), and were selected retrospectively from five medical institutions in Japan (Shinshu University Hospital, Kyoto Prefectural University Hospital, National Nagasaki Medical Center, Toranomon Hospital, and Hiroshima University Hospital). Written informed consent was obtained from each patient.

Of the 27 patients whose HBV genotype was determined, 25 (93%) were genotype C and the remaining two (7%) were genotype B. Serum HBV DNA was detectable in all patients, and HBe antigen was positive in 16 (47%) of the 34 patients before lamivudine administration.

For treatment of HBV infection, daily doses of 100 mg lamivudine were administered for at least 6 months. Lamivudine administration was stopped when alanine aminotransferase (ALT) levels were reduced to 40 IU/L or less in at least three separate tests. Serum samples were taken at several time points during and after lamivudine administration, and patients were seen at least once a month for at least 12 months after cessation of lamivudine. Estimated duration of HBV DNA level <3.7 log copy/mL before stopping lamivudine was a median 10 months (range 0–29 months).

Reactivation of hepatitis was defined as elevation of ALT to more than 80 IU/L within 12 months of stopping lamivudine treatment.

Serological markers for HBV

Serum hepatitis B surface antigen, HBe antigen, and anti-HBe antibody were measured by commercially available CLEIA kits (Fujirebio, Tokyo, Japan). Six major genotypes (A–F) of HBV are detectable using the method reported by Mizokami *et al.*[13] in which the surface gene sequence is amplified by polymerase chain reaction (PCR) and analyzed by restriction fragment length polymorphism. Serum concentration of HBV DNA was determined using a transcription mediated amplification (TMA) assay kit (Chugai Diagnostics Science, Tokyo, Japan) which has a quantitative range of 3.7–8.7 log copy/mL.

Serum concentration of HBcrAg was measured using a CLEIA developed by Fujirebio, as described previously.[7] Briefly, 150 μL of serum was incubated with 150 μL of pretreatment solution containing 15% sodium dodecylsulfate at 60°C for 30 min. After incubation, 120 μL of pretreated specimen was added to a ferrite microparticle solution in an assay tube. Ferrite microparticles were coated with monoclonal antibodies (HB44, HB61, HB114) against denatured HBc and HBe antigens. After washing, two other monoclonal antibodies against denatured HBcAg and HBeAg (HB91 and HB110) labeled with alkaline phosphatase were added as secondary antibodies. After further washing, 200 μL of AMPPD (3-(2′-spiroadamantan)-4-methoxy-4-(3″-phosphoryloxy) phenyl-1, 2-dioxetane disodium salt; Applied Biosystems, Bedford, MA) solution was added as substrate, and the assay tube was incubated for 5 min at 37°C.

From this, the relative chemiluminescence intensity was measured, and HBcrAg concentration was determined by comparison with a standard curve generated using recombinant pro-HBe antigen (amino acids, 10–183 of the precore/core gene product). The HBcrAg concentration was expressed as units/mL (U/mL) and a immunoreactivity of recombinant pro-HBe antigen of 10 fg/mL was defined as 1 U/mL. In the present study, the cutoff value of HBcrAg concentration was set at 3.0 log U/mL.

Statistical analysis

The Mann–Whitney *U*-test was used to analyze quantitative data, and Fisher's exact test was used for

qualitative data. Receiver operating characteristic (ROC) curve analysis was used to analyze cut-off levels of HBcrAg concentration for prospective recurrence of hepatitis. Statistical analyses were performed using the SPSS 14.0 J statistical software package (SPSS, Chicago, IL, USA), and a *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

TWENTY (59%) OF the 34 patients enrolled in the present study showed reactivation of hepatitis within 12 months after discontinuing lamivudine administration, with 15 (75%) showing reactivation within 6 months. The peak serum ALT levels in the 20 reactivation patients ranged from 103 to 1019 IU/L, with a median of 308 IU/L. After lamivudine cessation, the maximum serum HBV DNA was significantly higher ($P < 0.001$) in the reactivation patients (median 7.8, 25–75% range 7.4–8.1 log copy/mL) than in the non-reactivation patients (median 4.8, 25–75% range 4.1–5.9 log copy/mL).

Table 1 shows a comparison of the clinical backgrounds at the onset and completion of lamivudine administration between the two groups of patients. Although backgrounds were similar between the two groups just prior to lamivudine administration, HBcrAg levels were significantly higher in the reactivation patients after treatment. Both HBV DNA levels and positive rates of HBe antigen were similarly low between the two groups. The duration of undetectable HBV DNA before stopping lamivudine administration was also similar ($P > 0.2$) between the two groups (reactivation patients, median 11 months, 25–75% range 8–13 months vs. non-reactivation patients, median 6 months, 25–75% range 5–13 months).

In 23 patients who were negative for HBe antigen after treatment, HBcrAg levels were significantly higher ($P = 0.011$) in the reactivation patients ($n = 12$, median 4.8 log U/mL, 25–75% range 4.0–5.0 log U/mL) than in non-reactivation patients ($n = 11$, median 3.0 log U/mL, 25–75% range 2.5–4.4 log U/mL). In contrast, levels were similar ($P > 0.2$) between the two groups in 11 patients who were positive for HBe antigen after treatment (reactivation patients $n = 8$, median 5.9 log U/mL, 25–75% range 5.1–6.1 log U/mL vs. non-reactivation patients $n = 3$, median 5.6 log U/mL, 25–75% range 2.5–8.0 log U/mL).

The ability of HBcrAg concentration to predict non-recurrence of hepatitis was analyzed using a ROC curve (Fig. 1), and the area under the curve was as wide as 0.764. The point at which specificity was 0.8 and sensi-

Table 1 Comparison of clinical characteristics at the onset and cessation of lamivudine administration between patients with and without reactivation of hepatitis

Characteristics	Reactivation of hepatitis		*P*-value†
	Positive ($n = 20$)	Negative ($n = 14$)	
Demographics			
Age (years)	44 (38–51)	50 (35–59)	NS
Sex (male/female)	13/7	7/7	NS
HBV genotype (B/C)	0/16	2/9	NS
At onset of lamivudine administration			
ALT (IU/mL)	103 (57–234)	211 (76–515)	NS
HBeAg (positive)	12 (60%)	4 (29%)	NS
HBV DNA (log copy/mL)	7.1 (6.1–8.1)	6.0 (5.3–7.4)	NS
HBcrAg (log unit/mL)	6.2 (5.6–7.7)	6.4 (5.0–6.6)	NS
At cessation of lamivudine administration			
Duration of lamivudine (months)	12.7 (10.4–16.3)	10.3 (6.4–17)	NS
ALT (IU/mL)	30 (15–36)	21 (15–24)	NS
HBeAg (positive)	8 (40%)	3 (21%)	NS
HBV DNA (log copy/mL)	<3.7 (<3.7–<3.7)	<3.7 (<3.7–<3.7)	NS
HBcrAg (log unit/mL)	4.9 (4.7–5.9)	3.2 (<3.0–4.5)	0.009

†Analysis of continuous variables performed using Mann–Whitney *U*-test; analysis of dichotomous variables performed using Fisher's exact test. Values shown as median (25–75% range) or *n* (%).
ALT, alanine aminotransferase; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NS, not significant.

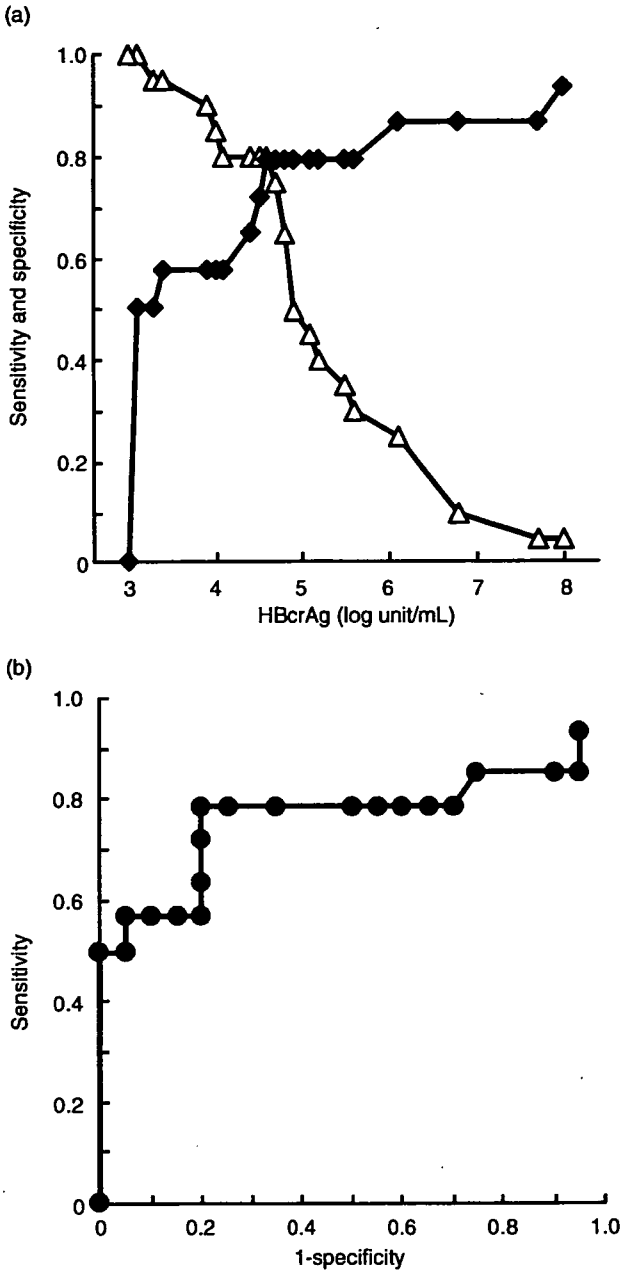

Figure 1 Receiver-operator characteristic (ROC) analysis of hepatitis B core-related antigen (HBcrAg) concentration for predicting patients without risk of reactivation of hepatitis within 12 months after halting lamivudine administration. (a) Sensitivity (■) and specificity (△) curves according to concentration of HBcrAg. (b) The ROC curve with the area under curve of 0.764.

tivity approximately 0.8 was deemed best for halting treatment without the risk of hepatitis recurrence. This point corresponds to an HBcrAg concentration of 4.1–4.6 log unit/mL.

DISCUSSION

THE REACTIVATION OF hepatitis following lamivudine administration was defined in the present study as an elevation of serum ALT level to more than 80 IU/L because we sought to find a more reliable indicator for safer discontinuation of lamivudine administration. Under these conditions, the majority (20/34) of patients showed reactivation of hepatitis within 12 months, as has been previously reported.[5,6] HBV DNA levels at the time of discontinuing lamivudine were similarly low between the two groups of patients, which is understandable as an undetectable reading typically indicates HBV remission following lamivudine therapy. However, HBcrAg levels were significantly higher in reactivation patients, implying that HBcrAg level is a better marker than HBV DNA level for predicting non-reactivation of hepatitis after discontinuing lamivudine administration especially in patients without HBe antigen.

In this study, ROC curve analyses showed a wide area under the curve of 0.764 in predicting the non-reactivation of HBV with HBcrAg level. If the corresponding cutoff is set at 4.5 logU/mL, then both specificity and sensitivity are as high as approximately 0.8. To obtain a higher specificity of 0.9, the cutoff value of HBcrAg concentration should be set at 4.0 log unit/mL. In this case, the sensitivity would still be nearly 0.6. The cutoff value of HBcrAg for predicting the non-relapse of hepatitis in our study is a little higher than that reported by Shinkai *et al.* (3.4 logU/mL).[14] Because numbers of patients analyzed were small in both studies, further studies are required to confirm the most appropriate cutoff value. It is noteworthy that this cutoff value may also differ among genotypes, which have been reported to be correlated with outcome of chronic HBV infection.[15] However, as over 90% of the patients had genotype C in this study, reactivation could not be analyzed in relation to HBV genotypes.

The HBV is an enveloped DNA virus containing a relaxed circular DNA genome which is converted into a covalently closed circular DNA (cccDNA) episome in the nucleus of infected cells and serves as transcriptional template for the production of viral RNA.[11,16,17] Reverse transcription of pregenomic RNA and second-strand DNA synthesis then occur in the cytoplasm within viral

capsids formed by the HBV core protein. Because lamivudine inhibits reverse transcription of pregenomic RNA, it directly suppresses production of HBV virions, and serum HBV DNA levels decrease rapidly after the initiation of lamivudine administration. However, the production of viral proteins is not suppressed by lamivudine as this process does not include reverse transcription. Furthermore, it has been reported that the amount of cccDNA, which also serves as a template for mRNAs, decreases quite slowly after commencement of administration of nucleoside analogs.[18,19] Thus, it is possible that serum HBcrAg levels reflect the cccDNA level in hepatocytes more accurately than serum HBV DNA. High levels of cccDNA are considered to be associated with hepatitis reactivation because they preceed reactivation of viral replication and consequent elevation of HBV DNA level in serum.

Lamivudine has already been eliminated from first line therapy in naïve chronic hepatitis B patients due to a higher incidence of developing resistant mutations than new antiviral agents, such as adefovir dipivoxil and entecavir.[20] However, the distinct characteristic of the HBcrAg assay under lamivudine therapy that is different from other HBV DNA assays is that lamivudine suppresses production of HBV virions by inhibiting reverse transcription of pregenomic RNA, but does not suppress the production of viral proteins, in which reverse transcription is unnecessary. Thus, it is possible that the HBcrAg assay may also useful for patients undergoing entecavir or adefovir dipivoxil administration because the main mechanism of suppressing HBV replication is similar between lamivudine and other antiviral agents. As a considerable number of patients who started lamivudine administration in the past are still taking this treatment now, the present study may be valuable for such patients when they consider changing therapies in the future. Additionally, further studies are required to determine whether the HBcrAg assay is indeed applicable to antiviral agents other than lamivudine.

In conclusion, significant markers that can predict reactivation of hepatitis after discontinuing lamivudine administration are clinically valuable because the reactivation of hepatitis is a fundamental problem in lamivudine therapy. Our results suggest that patients with an HBcrAg level of less than 4.5 log unit/mL may stop lamivudine administration with a lower risk of reactivation. The present study is a preliminary one because the patients enrolled were selected retrospectively without standardized criteria for stopping lamivudine and the number of patients enrolled was not large; however, the results may be valuable for patients with hepatitis B undergoing lamivudine therapy as such a diagnostic marker has rarely been reported. Further studies are required to establish the clinical significance of the HBcrAg assay in the treatment of hepatitis B.

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Hepatocyte growth factor accelerates thrombopoiesis in transgenic mice

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Hepatocyte growth factor (HGF) is one of the potent growth factors for liver regeneration and has a strong effect on epithelial and nonepithelial cells. As one of the pleiotropic functions, HGF acts as a hematopoietic regulator in the proliferation and differentiation of hematopoietic progenitors. However, the effect of HGF on the thrombopoietic function remains unclear. The correlation between HGF and thrombopoiesis was investigated in transgenic (TG) mice overexpressing murine HGF controlled by the murine HGF by the metallothionein promoter. Furthermore, the mechanism of thrombocytosis induced by HGF *in vitro* was analyzed in hepatoma cell line HepG2. Both the platelet count and the serum thrombopoietin (TPO) concentration were significantly higher in TG than in the wild type (WT) control mice. In the liver and spleen, the expression of TPOmRNA in TG was higher than that in WT by real-time polymerase chain reaction. The expressions of transcriptional factor of TPO, GABP-alpha/beta were more increased in TG liver compared to WT. In an *in vitro* study, HGF induced TPO and GABP-alpha/beta expression and enhanced TPO promoter activity. Therefore, HGF induced thrombopoiesis accompanied with the overexpression of TPO through GABP stimulation.
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KEYWORDS: hepatocyte growth factor; thrombopoietin; GABP; Ets; c-Mpl

Hepatocyte growth factor (HGF) is a polypeptide originally characterized as a highly potent hepatocyte mitogen.[1,2] Recent studies have revealed HGF to be a multifunctional cytokine which can elicit mitogenic, motogenic and morphogenic responses in a variety of cultured epithelial cells expressing the transmembrane tyrosine kinase receptor, c-Met.[3,4]

HGF plays an essential role in hematopoiesis.[5–8] HGF or c-met null mice showed defects in the liver and placental development and the migration of myogenic precursor cells into the limb bud.[9–11] The erythrocyte count in embryonal blood has been reported to decrease in HGF null mice, although fetal hematopoiesis occurred in the impaired liver. This might be reflected in the reduction of the size of the liver and extensive cell death in HGF null mice.[11] On the other hand, the effect of HGF on thrombopoiesis and thrombopoietin (TPO) remains controversial.[12–14]

TPO is the most potent factor promoting megakaryocyte growth and platelet production and is mainly synthesized in the liver.[15,16] Several groups reported the cloning of the gene encoding TPO in 1994.[15,17–19] TPO binds to its receptor, c-Mpl, which is expressed on the megakaryocytes and platelets. The serum TPO levels are mainly regulated by the platelet count, because the platelets express a significant number of high-affinity c-Mpl receptors[20–22] that can capture and degrade TPO.[23–25]

We therefore investigated the correlation of HGF and thrombopoiesis. We thus found a new phenotype of HGF transgenic (TG) mouse thrombocytosis, although the number of white blood cells and erythrocytes did not change. We herein describe thrombocytosis in HGF TG mice and investigate the mechanism of thrombocytosis during the overexpression of HGF.

MATERIALS AND METHODS

Generation of HGF TG Mice and Control Mice

TG mouse, in which the expression of a murine HGF cDNA was driven by the metallothionein promoter and locus control regions, were generated on the inbred albino FVB/NCr genetic background (hereafter referred to as FVB) as previously described.[26] Briefly speaking, the transgene expressed a characteristic 2.4-kb RNA in virtually all adult tissues, at

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level between 3- and 50-fold higher than the major 6-kb endogenous HGF transcript. In addition, serum HGF level of TG was about four times higher than that of WT.[26] Six to 8-week-old male and female HGF TG mice and FVB wild type (WT) mice were used. WT and TG were placed at weaning (3 week of age) on a normal chow diet. No additional zinc was added because the transgene HGF induced by metallothionein promoter was sufficiently driven by the zinc in the usual diet. All animal studies were performed according to the guidelines for animal care and use as established by the Gunma University Graduate School of Medicine.

Peripheral Blood Cell Count and Histological Analysis of Bone Marrow and Liver

Peripheral blood was collected from axillary artery and blood cells were counted by auto-cell counter (SE-9000, Sysmex, Kobe, Japan). The femoral bone marrows of mouse were obtained and fixed in 10% formalin. The bone marrow samples were then stained with hematoxylin and eosin (HE) staining. We counted the number of megakaryocytes per high-power fields ($\times$ 400). To examine the difference of the cell proliferation between WT and TG megakaryocytes, we performed PCNA staining[27,28] on paraffin section of mice bone marrow using monoclonal mouse anti-PCNA antibody (PC-10; Dako Japan, Tokyo, Japan). The PCNA positive megakaryocytes were scored by counting 30 high-power light microscope fields ($\times$ 400) for each group. The liver was stained with HE and then specimens were compared between WT and TG.

Measurement of Murine TPO

The plasma TPO concentrations in mice were measured by an enzyme linked immunosorbent assay.[29] Microtiter black plates (Sumilon, Osaka, Japan) were incubated with 5 μg/ml rabbit anti-rmTPO IgG antibody at 4°C overnight. After washing, the bound murine TPO was detected using biotinylated chicken anti-mTPO IgG antibody (1 μg/ml), followed by alkaline phosphatase-labelled streptavidin and Lumigen PPD (Wako Chemical Co., Osaka, Japan), a chemiluminescent reagent, as the substrate. The chemiluminescent light emission was measured in a luminometer (Top Count; Packard, Meriden, CT, USA). The lower limit of sensitivity of the assay was approximately 50 pg/ml.

TPO Receptor Mpl Expression on the Platelet Surface

The peripheral blood was hemolysed with PharmLyse (BD Biosciences, San Jose, CA, USA), washed with the buffer (PBS containing 1 mmol/l of EDTA, 0.05% NaN_3, and 0.3% BSA) and incubated with 10 μg/ml of rabbit anti-murine c-Mpl polyclonal antibody (Amgen Inc., Wet Greenwich, RI, USA) plus 10 μg/ml of FITC-labeled hamster anti-murine glycoprotein V monoclonal antibody (Seikagaku Corp., Tokyo) for 30 min at 4°C. Blood cells were washed and incubated with PE-labeled goat anti-rabbit immunoglobulin polyclonal antibody (Dako, Glostrup, Denmark) for 30 min at 4°C. The cells were washed and analyzed on FACS Calibur (BD Bioscience, San Jose, CA USA). The expression of platelet surface c-Mpl was analyzed by measuring the fluorescence intensity of the cells in the glycoprotein V-positive fraction.

Cell Lines

The human hepatocarcinoma cell, HepG2 used in this study was originally obtained from the American Type Culture Collection (Manassas, VA 20108, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (Equitech-Bio, Ingram, TX, USA), 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco BRL). Cells were incubated at 37°C in a humidified chamber of 5% CO_2.

Reagent

Recombinant Human HGF was purchased from R&D Systems Inc., Minneapolis, MN, USA. In order to confirm the effect of HGF signal, a specific mitogen-activated protein kinase/extracellular signal-related kinase (MEK) inhibitor PD98059 (Sigma-Aldrich Japan, K.K.Tokyo, Japan) was co-cultured with HGF.

Transient Transfection and Reporter Assay

To determine whether HGF induction of TPO gene expression is regulated at the transcriptional level, we performed promoter studies using the human TPO promoter. We used human TPO promoter-luciferase reporter plasmids with the 5′-flanking sequence of the TPO gene using pLUC-basic plasmid (Promega, Madison, WI, USA), constructed and kindly provided by Professor Kitajima.[30]

The reporter plasmid (pLUC-Basic, pLUC-T58, pLUC-T88 and pLUC-T158) were transiently transfected into HepG2 cells with the control hpRL-tk vector (Promega) by FuGENE 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA). At 3-h after transfection, 40 ng/ml of HGF was added to the dishes followed by incubation for 24 h. PD98059 was added 1 h before HGF treatment. The cells then were lysed and subjected to luciferase-based reporter assays using a dual luciferase assay system (Promega).

mRNA Analysis

Cells (2×10^5) were seeded in 35 mm-dish for 24 h then the medium were exchanged with serum-free DMEM and incubated over night. Furthermore these cells were incubated for 6 h with 40 ng/ml of the recombinant Human HGF, without or with 20 μM of PD98059 for 1 h before HGF stimulation.

Cells and mouse liver were trimmed and the total RNAs were extracted using ISOGEN (Nippon Gene, Tokyo) according to the protocol supplied by the manufacturer. Complementary DNA was synthesized from 5 μg of total RNA using SuperScript II reverse transcriptase and random hexamers (Invitrogen, Carlsbac, CA, USA).

Mouse *TPO*, *GABP-alpha/beta* which is the *Ets* family transcription factors were essential for the expression of the *TPO* in the liver,[30] and human *TPO*, *GABP-alpha/beta* mRNA were quantitated using real-time polymerase chain reaction (PCR) using TaqMan® fluorogenic probes (*TPO* and 18S ribosomal RNA; Applied Biosystems, Foster City, CA, USA) or SYBRgreen® detection (Mouse *GABP-alpha/beta* and human *TPO*, *GABP-alpha/beta*, Applied Biosystems). All primers except mouse *TPO* were designed using the Primer Express™ design software (Applied Biosystems); sequence details are given in Table 1. Only mouse *TPO* primers and probe were obtained from Applied Biosystems assay on demand. PCR reactions and analyses were carried out using the ABI Prism 7700 Sequence Detector and software (Applied Biosystems) using mouse *actin* or *human glyceraldehyde-3-phoaphate dehydrogenase (GAPDH)* as internal control. The relative copy numbers mRNA were calculated as recommended by the manufacturer.

Statistical Analysis

The values are the mean ± s.d. of triplicate analysis from three independent experiments. Differences between the groups were analyzed by the Student's *t* test or one-way ANOVA. Comparisons between the groups are illustrated with box-plot graphics, where the dotted line within the box indicates the median value, and the box boundaries represent 50% of the values of non-outliners. The threshold for significance was set at $P<0.05$.

RESULTS

Blood Cell Count

Table 2 shows both the blood white cell count and red cell count were not significantly different between WT and TG (2.96 ± 1.74 *vs* $3.25\pm1.24\times10^3$ cells/mm^3, 7.63 ± 0.59 *vs* $7.29\pm0.70\times10^6$ cells/mm^3, NS, respectively). The hemoglobin concentration and hematocrit were also similar between both mice. On the other hand, the platelet count was higher in TG than WT (2219 ± 386 *vs* $967\pm145\times10^3$/mm^3, $P<0.0001$).

Microscopic Findings of Bone Marrow and the Number of Megakaryocytes

To investigate *in vivo* hematopoiesis, bone marrow was decalcified and stained with HE (Figure 1a) and counted the number of megakaryocytes in high-power fields. The number of megakaryocytes of TG was significantly higher than that of WT (20 ± 2.5 *vs* 10.8 ± 1.55 per field, $P=0.0003$) shown as Figure 1b.

PCNA Staining of Bone Marrow Cells

The number of PCNA positive megakaryocytes in TG bone marrow was clearly higher than that of WT (4.4 ± 0.55 *vs* 1.4 ± 0.55 per field, $P<0.0001$) shown as Figure 1c and d.

Liver and Spleen Weight/Body Weight Ratio (Table 3) and Histological Analysis of the Liver

To rule out any basal characteristics in TG and WT, we measured the liver, spleen and body weight, and calculated the ratio between them. As a result, the liver weight was higher in TG than WT (2.00 ± 0.44 *vs* 1.23 ± 0.13 g, $P=0.015$). However, the spleen weight was not different

Table 2 Hemogram of WT and TG

	WT	TG	*P*
WBC ($\times10^3$ cells/mm^3)	2.96 ± 1.74	3.25 ± 1.24	0.77
RBC ($\times10^6$ cells/mm^3)	7.63 ± 0.586	7.29 ± 0.700	0.43
Hb (g/dl)	11.7 ± 0.740	11.0 ± 1.21	0.31
Ht (%)	36.1 ± 2.10	35.3 ± 3.63	0.70
Plt ($\times10^3$/mm^3)	967 ± 145	2219 ± 386	<0.0001

Table 1 Sequences of primer pairs used for amplification of mRNA by real-time PCR

mRNA	Accession number	Primer sense (5′–3′)	Primer antisence (3′–5′)
Mouse			
actin	NM_007393	GGCTCCTAGCACCATGAAGA	ACATCTGCTGGAAGGTGGAC
GABP alpha	NM_008065	GTACCAGATTATTATGCAAGACCG	TAAAGAAGATCGCCTACTGAGC
GABP beta	NM_207669	AGACCAACAAAGAAGCCG	TTAAGTCCCGTTATCAAGCTGTAG
Human			
GAPDH	BC023632	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
TPO	NM_199228	AGGTCCCTGGACCAAATC	TGGAAGAGGGAAGAGCG
GABP alpha	NP_002031	AAAGAGCGCCGAGGATTTCAG	CCAAGAAATGCAGTCTCGAG
GABP beta	NP_005245	CCCAGAGAGTCCTGACACT	TCTGAAGAATTGGACAATGG

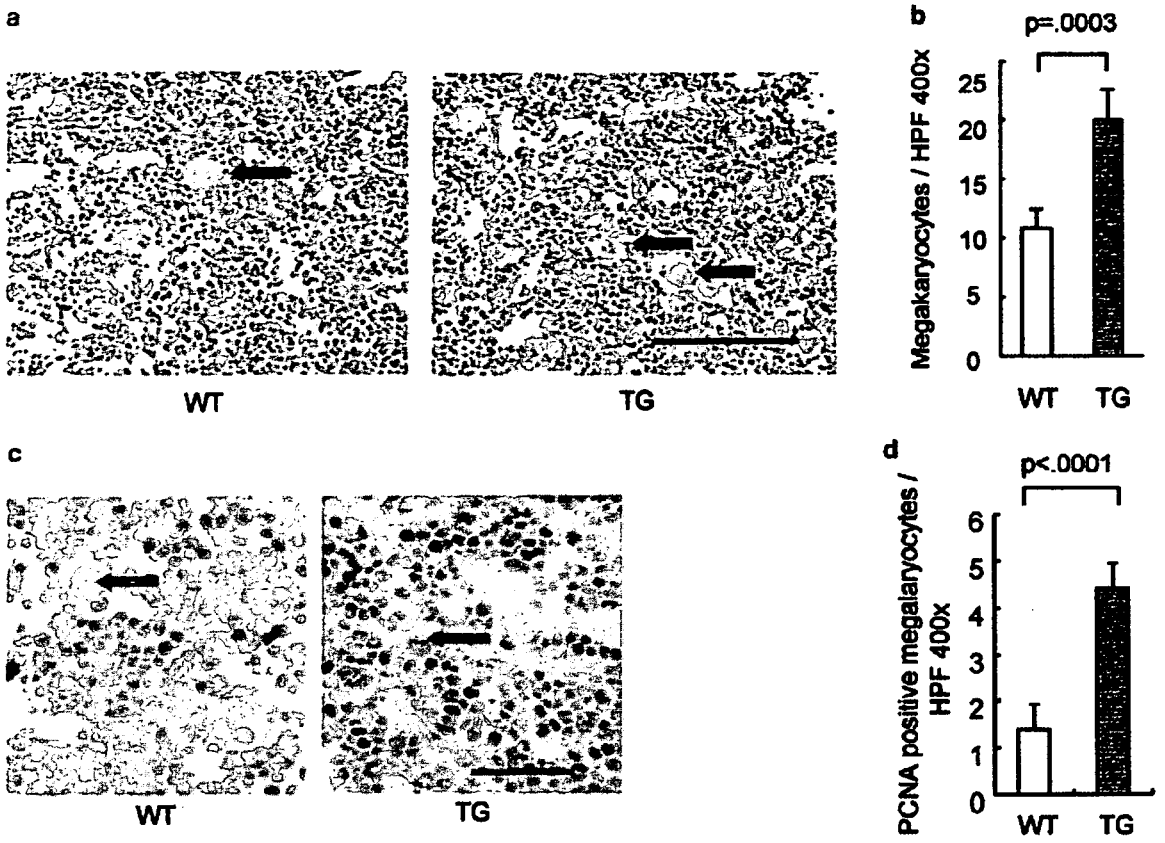

Figure 1 Histological findings of bone marrow. (**a**) Bone marrows of WT and TG were stained with hematoxylin and eosin. The megakaryocytes (arrows) showed a greater increase in TG bone marrow than in that of WT. × 400, scale bars: 100 μm. (**b**) The number of megakaryocytes in TG bone marrow was statistically higher than that of WT. $P=0.0003$. (**c**) PCNA staining was clearly demonstrated more megakaryocytes were positive in TG than in WT. Bone marrow DNA synthesis in WT mice (left panel) and TG mice (right panel). Arrows show megakaryocytes. × 400, scale bars: 50 μm. (**d**) The number of PCNA positive megakaryocytes in the WT or TG bone marrow. WT = white bars, TG = shaded bars. Error bars represent the standard deviation of triplicate experiments. Similar results were obtained in three independent experiments. $P<0.0001$.

Table 3 Liver and spleen weight

	WT	TG	*P*
Liver weight (g)	1.23±0.13	2.00±0.44	0.015
Spleen weight (mg)	108.8±13.9	99.9±24.0	0.21
Liver/body weight ratio (%)	5.06±0.8	7.26±1.1	0.0005
Spleen/body weight ratio (%)	0.386±0.03	0.431±0.06	0.11

between TG and WT (99.9±24.0 *vs* 108.8±13.9 mg, $P=0.21$). The liver/body weight ratio was higher in TG than WT (7.26±1.1 *vs* 5.06±0.8 %, $P=0.0005$). On the other hand, there was no difference between the TG and WT in spleen/body weight ratio (0.431±0.06 *vs* 0.386±0.03%, $P=0.11$). At 8 weeks of age, the histological findings of TG liver did not grossly differ from that of WT liver. Moreover, no extramedullary hematopoiesis was demonstrated in both WT and TG liver (see Supplementary Information). The weights of other organs (brain, femur muscle, heart, small intestine and kidney) were also not different between WT and TG (data not shown).

TPO Level in the Serum, c-Mpl Expression on Platelet and the Expression of TPO and GABPs in the Liver

The serum TPO concentration was higher in TG than WT (Figure 2a, 134±49.3 *vs* 59.4±16.5 pg/ml, $P=0.0012$). Furthermore, a positive correlation was recognized between the blood platelet count and the serum TPO level. Figure 2b shows that serum TPO level, which correlates with the liver weight, to be higher in TG than in WT (84.2±40.4 *vs* 49.4±17.5 pg/ml/g liver weight, $P=0.042$).

The mean value of c-Mpl expression of TG shifts to the left, in comparison to WT on flow cytometry. This result indicated that in TG the expression of c-Mpl is lower than that of WT (Figure 2c and d). Furthermore, the c-Mpl expression negatively correlated to the platelet count and serum TPO level (data not shown).

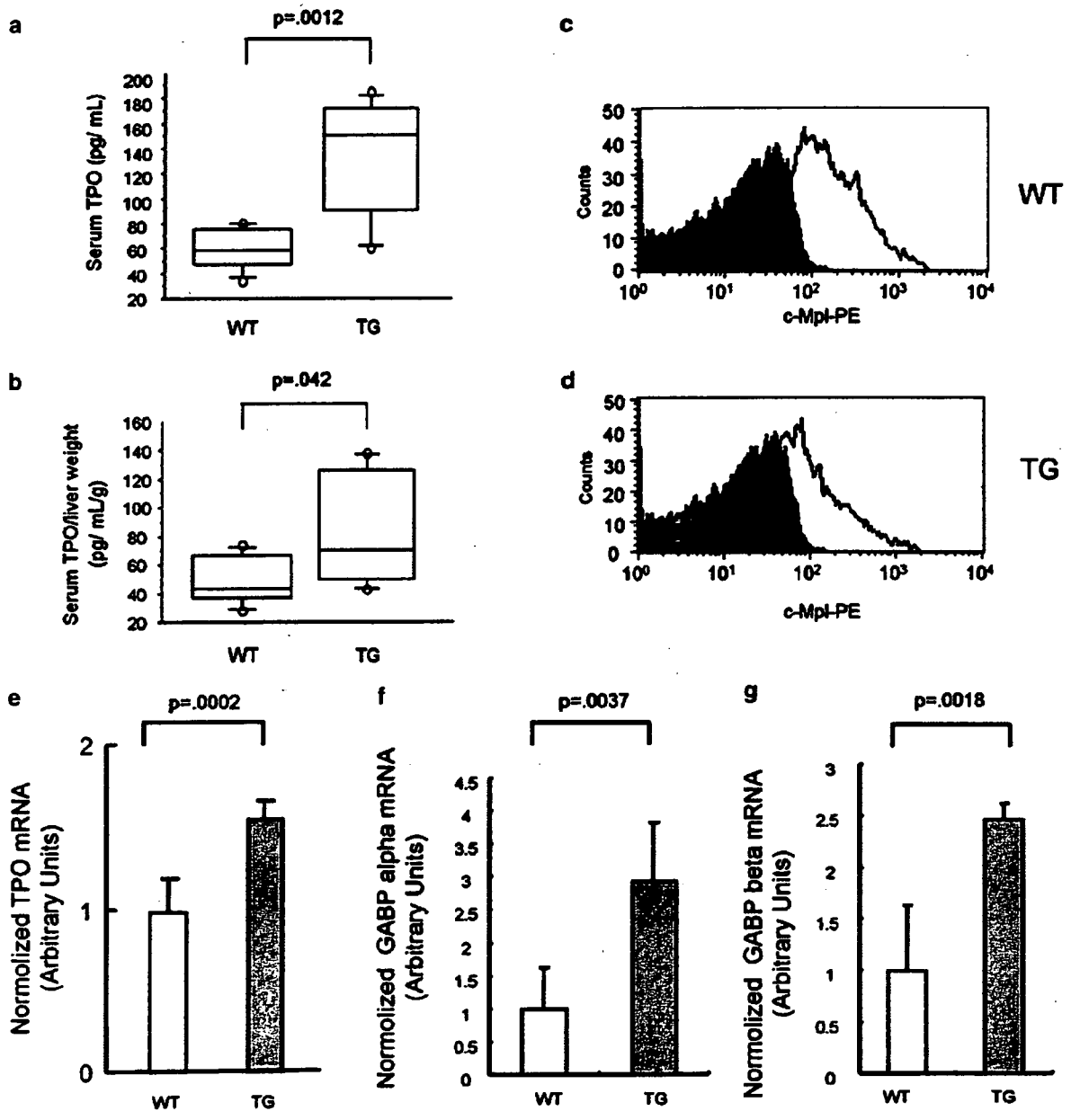

Figure 2 The serum TPO levels in WT and TG. (**a**) Serum TPO was compared between WT and TG. TG showed significantly higher TPO than WT. $P = 0.0012$. (**b**) Serum TPO per liver weight was compared between WT and TG because the liver is always larger in TG than WT. Comparisons between WT and TG are illustrated with box-plot graphics and the box boundaries represent 50% of the values of non-outliners. $P = 0.042$. Expression of c-Mpl on the platelets surface in WT (**c**) and TG mice (**d**). The expression of c-Mpl on the platelets analyzed with flow cytometry. The cells were stained with PE-labeled anti-c-Mpl (open area) or the isotype control antibody (filled area). The peak of Mpl expression in TG shifted left namely decreased in comparison to WT. The expression of TPO in the liver in WT and TG (**e**). TPOmRNAs were assessed by real-time PCR, and are expressed as the fold-induction relative to the WT liver. TPOmRNAs of TG were significantly higher than those of WT in the liver ($P = 0.0002$). Transcriptional factor GABP-alpha/beta expression in the liver. GABP-alpha (**f**) and -beta (**g**) mRNA were assessed by real-time PCR, and were expressed as the fold-induction relative to the WT liver. $P = 0.0037$, 0.0018, respectively, WT mice = white bars, TG mice = shaded bars.

The TPO expressions in TG was significantly higher than those in WT in the liver (Figure 2e, $P = 0.0002$).

The Ets family transcription factors GABP-alpha and beta were more markedly upregulated in the TG liver than in WT (Figure 2f and g, 2.9- and 2.5-fold, respectively).

Expression of TPO, GABP-Alpha and -Beta Induced by HGF Stimulation

We investigated TPO induction by HGF *in vitro* using human hepatoma cell line HepG2. In addition, to examine the HGF/c-Met signaling, we used a MEK inhibitor, PD98059. TPO

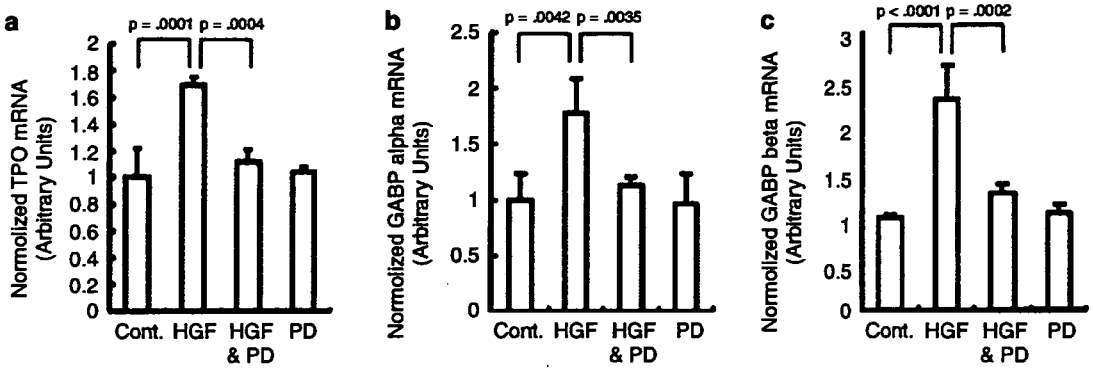

Figure 3 Effect of HGF and PD on the TPO and GABP mRNA expression in HepG2 cells. Real-time PCR analysis of mRNA of TPO (a), GABP-alpha (b) and GABP-beta (c). Lane 1, serum-free DMEM alone (Cont.); lane 2, HGF 40 ng/ml; lane 3, HGF 40 ng/ml and PD 20 μM; lane 4, PD 20 μM alone. Error bars represent the s.d. of triplicate experiments. Similar results were obtained in three independent experiments.

was induced 1.7-fold by HGF stimulation and a MEK inhibitor, PD98059 reduced the expression of TPO (Figure 3a). PD98059 alone had no effect on TPO.

Furthermore, Ets family transcription factor GABP-alpha (Figure 3b) and -beta (Figure 3c) were similarly induced by HGF stimulation (1.8- and 2.3-fold, respectively) and reduced by PD98059.

Effect of HGF and PD on TPO Promoter Activity in HepG2

To localize the region essential for human TPO gene expression, a series of 5′- to 3′-luciferase reporter plasmids were transiently expressed in HepG2 cells. As shown in Figure 4a, the plasmids containing 5′ deletions of both lengths −158 and −88 promoted high level expression of luciferase activity in comparison to the background of pLUC-Basic. Moreover, HGF treatment strengthened these expressions. In contrast, a further deletion to −58 hardly promoted them. Furthermore, PD98059 inhibited TPO promoter induction by HGF, although not to the baseline. PD98059 alone had no effect on the TPO promoter activity (Figure 4b).

DISCUSSION

Thrombocytosis accompanied with TPO overexpression was clearly demonstrated in HGF TG in the present study.

In TG, HGF was always highly expressed in almost all internal organs including the liver and blood and the signal transduction of HGF through c-Met could be activated.[26,31,32] On the other hand, TPO was reported to be primarily expressed in the liver and to a lower extent, in the kidneys, bone marrow and other organs.[18,33,34] In a similar manner, we demonstrated the overexpression of TPO in the liver (Figure 2e). Hepatomegaly is one of the phenotypes of TG.[31] Sakata *et al*[31] had demonstrated that adult TG hepatocytes contained 4.7-fold greater proliferation index than WT by quantitating BrdUrd incorporation. Larger TG liver may induce a large amount of TPO in comparison to WT but

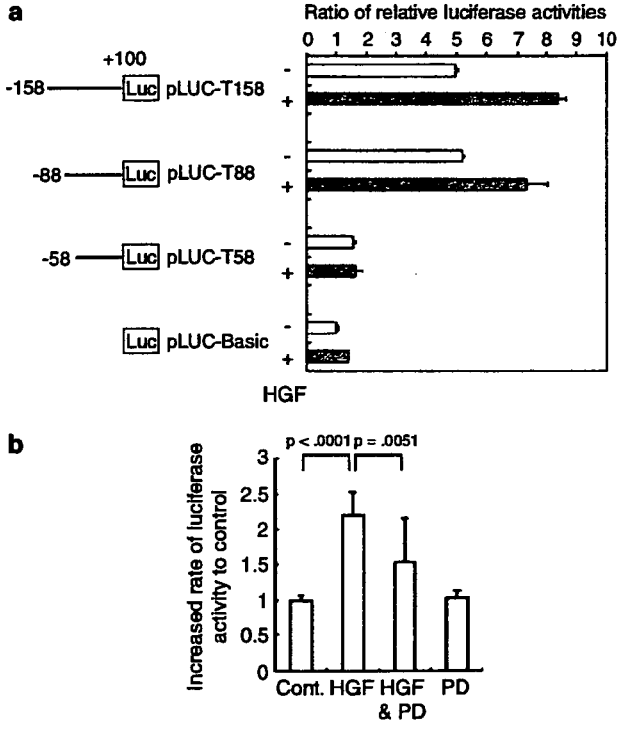

Figure 4 (a) Expression analysis of the various human TPO gene promoters constructs. Two micrograms of each reporter plasmid was transiently transduced into HepG2 cells with control vector to normalize the transfection efficiency. Luciferase assay was preformed at 24 h after stimulation with or without 40 ng/ml HGF. Scheme of TPO promoter-luciferase fusion constructs (left panel). Relative luciferase activities using various plasmids are shown in the right panel. The relative luciferase activity of each construct was expressed as the fold-induction relative to non-treatment pLUC-Basic. Control group = white bars, HGF treatment group = shaded bars. (b) Effect of HGF and PD on the TPO promoter assay (pLUC-T158). Lane 1, serum-free DMEM alone (Cont.); lane 2, HGF 40 ng/ml; lane 3, HGF 40 ng/ml and PD 20 μM; lane 4, PD 20 μM alone. The relative luciferase activity of each construct was expressed as the fold-induction relative to control. Error bars represent the s.d. of triplicate experiments. Similar results were obtained in three independent experiments.

the TPO level was still higher in TG even when it was corrected by the weight of the liver (Figure 2b). As a result, the volume of the liver alone was thus not considered to be the cause of thrombopoiesis in TG.

Primary or essential thrombocytosis is a myeloproliferative disease caused by monoclonal or polyclonal abnormalities in hematopoietic cells. On the other hand, secondary or reactive thrombocytosis results from megakaryopoiesis and thrombopoiesis caused by infection, chronic inflammation, tissue damage (trauma, surgery, burns) or neoplasia.[35,36] Neither monoclonal nor polyclonal abnormalities in hematopoietic cells were observed in TG. In addition, TG did not suffer from infection, inflammation or tissue damage. Although the mice could bear the liver tumor as the mice age,[31,32] we used TG at a younger age before development of lover tumors. The cause of the thrombocytosis of our TG was none of the above.

Regarding the various functions of HGF, the thrombopoietic effect has yet to be fully elucidated. Banu *et al*[37] reported that HGF had no direct effect on megakaryocytes. The TPO induction by HGF *in vitro* has been controversial.[12–14] In this study, we confirmed TPO induction by HGF with HepG2 cell line (Figure 3).

Furthermore, in our study *in vivo*, we demonstrated that platelet count, serum TPO level and TPOmRNA expression in liver were more increased in TG than in WT mice. Masunaga *et al*[38] and Yamashita *et al*[14] also reported that HGF treatment caused thrombocytosis. Yamashita *et al*[14] demonstrated that the administration of HGF to the cirrhotic rats stimulated TPO mRNA expression in the livers and resulted in significant increases of peripheral platelets and bone marrow megakaryocytes. However, the mechanism of TPO induction by HGF has yet to be clarified.

As one of the mechanisms of TPO mRNA induction by HGF, a transcription factor, Ets-1 is considered to be involved.[39–41] As a direct pathway, HGF activates Ets-1 *in vitro*.[39,40] Furthermore, Ets-1 is a downstream target of HGF acting through a RAS-REF-MEK-ERK pathway, could activate a signal transduction leading to the gene expression by HGF.[41] The binding of Ets family transcription factors to the sequence 5′-ACTTCCG-3′ in the human TPO promoter has been implicated in the expression of the TPO gene in the liver.[30] In TG liver, the expressions of Ets family, GABP-alpha and -beta, were significantly increased (Figure 2f and g). Kamura *et al*[30] revealed that GABP-alpha/beta were critical for the expression of the TPO gene in liver.

As shown in Figure 4a, positive regulatory elements were located from −88 to −58, and this region is essential for optimal transcription of the TPO gene. This result confirmed with the Ets motif recognized by E4TF1/GABP, which was regarded as the essential region for the high expression of the human TPO gene in the liver.[30] On the other hand, we demonstrated that HGF accelerated the expression of TPO, GABP-alpha/beta mRNA expression and the TPO promoter activity and additionally, MEK inhibitor, PD98059 inhibited this expression and decreased promoter activity (Figures 3 and 4). These results implicated direct induction of TPO by HGF resulting thrombocytosis via the stimulation of Ets through activating MEK pathway.

HGF has been considered for the treatment of patients with liver cirrhosis,[42] fulminant hepatic failure[43] and liver transplantation[44,45] as well as arteriosclerosis.[46] As thrombocytopenia is often complicated in liver cirrhosis patients, HGF could thus be a choice for the treatment of thrombocytopenia as a therapeutic option for intractable diseases. However, we have previously shown that aberrant HGF expression induces several anomalies.[26,31,32,47–50] The development of hepatic carcinoma makes use of HGF clinically not feasible.

In conclusion, HGF induced thrombocytosis with megakaryocytosis associated with the elevation of TPO in the TG liver and serum. As a part of the mechanism of this phenomenon, Ets family transcription factor GABP was induced by HGF in the liver. This is the reason why thrombocytosis was therefore accompanied with a high level of TPO induced by HGF. Taken all together, HGF induced thrombopoiesis accompanied with the overexpression of TPO through GABP stimulation. Therefore, HGF TG mice may thus be appropriate models of secondary thrombocytosis caused by a TPO overexpression induced by HGF.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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HEPATOLOGY

Preventive effects of vitamin K on recurrent disease in patients with hepatocellular carcinoma arising from hepatitis C viral infection

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Abstract

Background: Despite the progression of therapeutic approaches, a high frequency of recurrence is what determines the long-term prognosis of patients with hepatocellular carcinoma (HCC). In this study, the chemopreventive effects of vitamin K2 on the recurrence and survival of patients with HCC after curative therapy were evaluated.

Methods: Sixty patients who were diagnosed to be free of HCC after radiofrequency ablation therapy or surgery were randomly assigned to either the vitamin K2 group ($n = 30$ patients) or the control group ($n = 30$ patients). All patients were positive for the hepatitis C virus (HCV) antibody and hepatitis B surface antigen positive patients were excluded from this study. Patients in the vitamin K2 group received an oral dose of menatetrenone at 45 mg per day. Disease recurrence and the survival rates were analyzed in patients with HCC.

Results: The cumulative recurrence-free rates in the vitamin K2 group were 92.3% at 12 months, 48.6% at 24 months and 38.8% at 36 months; and those in the control group were 71.7%, 35.9% and 9.9%, respectively ($P = 0.045$). The cumulative survival rates in the vitamin K2 group were 100% at 12 months, 95.0% at 24 months and 77.5% at 36 months; and those in the control group were 95.8%, 90.2% and 66.4%, respectively ($P = 0.70$).

Conclusions: Vitamin K2 may have a suppressive effect on the recurrence of HCC and a beneficial effect on tumor recurrence. However, there was no significant difference in the survival rates. The chemopreventive effects of vitamin K2 are not sufficient. The development of a further regimen such as combination therapy is required.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide and is closely associated with chronic liver disease. Approximately 80-90% of cases occur in patients with liver cirrhosis, which is believed to be the most important risk factor for HCC. Despite the progression of therapeutic approaches, including radiofrequency ablation, the long-term prognosis for patients with HCC is still poor.[1] One of the major difficulties for patients with HCC is the high frequency of recurrence in the remnant liver, even after curative treatment.[1,2] Therefore, the development of effective chemoprevention for the recurrence of HCC after curative treatment is considered important. Interferon (IFN) has been shown to be a promising chemopreventive agent for HCC recurrence.[3,4] However, most cases of HCC are complicated with liver cirrhosis and thrombocytopenia. Adverse effects thus sometimes prevent us from continuing to use IFN as a chemopreventive agent.

Vitamin K is a fat-soluble vitamin that regulates clotting factor production by acting as a coenzyme for vitamin K-dependent carboxylase.[5] Vitamin K2 and its derivatives have previously demonstrated antiproliferative effects against leukemia and hepatoma cell lines.[6–8] Vitamin K2 also has the ability to induce differentiation of leukemic and hepatoma cells[6] and it has been used in the treatment of myelodysplastic syndrome.[9] Abnormal, uncarboxylated prothrombin, protein induced by vitamin K absence or the antagonist II (PIVKA-II) are increased in the serum of patients with HCC.[10] The administration of vitamin K2 suppresses plasma PIVKA-II concentrations in patients with HCC.[11,12]

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Recently, Mizuta *et al.*[13] reported the preventive effects of vitamin K2 on disease recurrence and survival in patients with HCC after they underwent curative resection or percutaneous local ablation therapy. We also conducted a similar study after radiofrequency ablation therapy or curative resection with a randomized controlled study. The current study's findings suggest that vitamin K2 may have a suppressive effect on the recurrence of HCC and a beneficial effect on tumor recurrence.

Methods

Patients and study protocol

Patients were newly diagnosed with HCC and received radiofrequency ablation therapy or surgery from September 2002 to December 2003 at the Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine and four other affiliated hospitals; Kiryu Kousei General Hospital, Tone Chuo Hospital, Public Tomioka General Hospital, and Fuji Heavy Industries, Health Insurance Society General Ota Hospital. Diagnosis of HCC was confirmed histopathologically from biopsy specimens or diagnostic imaging findings. All patients underwent curative treatment for HCC with either radiofrequency ablation or surgical resection. Patients were confirmed to be free of HCC according to a computed tomography (CT) that was obtained within 1 month after treatment. Cases of local recurrence after radiofrequency ablation within 3 months were excluded from the analysis because the initial treatment was a failure. Patients were excluded if they were receiving medications, such as warfarin and/or vitamin K analogs, that had the potential to influence the effects of the vitamin K metabolism. Patients were also excluded from the study if they had any of the following traits: obvious tumor invasion into the portal vein, extra-hepatic metastasis, uncontrollable ascites, or encephalopathy. All patients tested positive for the hepatitis C virus antibody (HCVAb) and hepatitis B surface antigen (HBsAg) positive patients were excluded from this study. In total, 65 patients were enrolled in this study after their informed consent was obtained. The patients were randomly assigned into one of two groups, namely, the vitamin K2 group or the control group. Patients in the vitamin K2 group were given oral menatetrenone (at a dose of 45 mg per day) (Eisai, Tokyo, Japan) continuously during the follow-up period. No placebo was used in the control group. Until the discovery of recurrent HCC, no patient in either group received any type of chemopreventive therapy, including chemotherapy or IFN. The protocol of this study was approved by the Ethics Committee of Gunma University Hospital. Sixty of the 65 patients, 30 patients in each group, met the above criteria.

For each patient, data were recorded including age, sex, HCVAb, biochemical analysis (total bilirubin, albumin, alanine aminotransferase [ALT] and prothrombin time), serum alpha-fetoprotein (AFP), PIVKA-II, diameter and number of tumors, Child-Pugh grading, tumor staging, recurrence and survival. Habitual heavy drinking was defined as an average daily consumption of an amount equivalent to 65 g of pure ethanol over a period of >5 years. The AFP level was divided into two categories: 20 ng/mL or less and more than 20 ng/mL. The PIVKA-II level was also divided into two categories: 40 mAU/mL or less and more than 40 mAU/mL. The diameter of the largest tumor was measured in its greatest dimension if the patient had two or more tumors. The number of HCC was divided into two groups: solitary and non-solitary tumors.

The end-points of the current study were disease recurrence of HCC and survival. Ultrasonography and CT scans were obtained within 3 months after curative treatment and every 3–4 months thereafter. Examinations were continued until the detection of recurrent HCC. If atypical or non-enhanced nodules were observed, then a tumor biopsy was obtained for histological examination. After recurrence was detected, appropriate treatment was selected and survival was evaluated.

Statistical methods

Differences in the proportions were evaluated by Fisher's exact probably test. Differences in the means were also evaluated by Student's *t*-test. The survival curves according to the Kaplan-Meier method were compared with the log-rank test. A multivariate analysis using Cox's proportional hazard model was performed to evaluate the prognostic factor. A P-value <0.05 was considered to indicate statistical significance.

Results

Characteristics

Patient's characteristics are shown in Table 1. There were no significant differences between the groups with regard to age, sex, habitual heavy drinking, tumor stage (tumor size and number), AFP and PIVKA-II levels, treatment modalities, and the Child-Pugh classification. No problematic adverse effects were observed as a result of vitamin K2 treatment and no patients were withdrawn during the follow-up period. Figure 1 shows the cumulative

Table 1 Characteristics of the patients enrolled in the present study

Characteristic	Control	Vitamin K2 group	*P*-value
Number of patients	30	30	NS
Age (years)	69.0 ± 7.0	69.1 ± 5.9	NS
Males/females	18/12	17/13	NS
Habitual heavy drinking (yes/no)	3/27	6/24	NS
Tumor stage (I/II/III)	8/18/4	12/14/4	NS
Tumor size (mm)	25.0 ± 9.4	20.4 ± 11.6	NS
Number of tumors (solitary/multiple)	22/8	19/11	NS
AFP (≤20 ng/mL/>20 ng/mL)	16/14	15/15	NS
PIVKA-II (≤40 mAU/mL/ >40 mAU/mL)	8/22	10/20	NS
Total bilirubin (mg/dL)	1.06 ± 0.52	1.02 ± 0.50	NS
Alb (g/dL)	3.63 ± 0.56	3.8 ± 0.4	NS
PT (%)	81.1 ± 12.1	80.6 ± 10.8	NS
ALT (IU/L)	66.9 ± 41.8	68.9 ± 48.5	NS
Child-Pugh classification (A/B)	22/8	22/8	NS
Treatment (surgery/RFA)	7/23	4/26	NS

AFP, alpha-fetoprotein; Alb, albumin; ALT, alanine aminotransferase; PT, prothrombin time; RFA, radiofrequency ablation therapy.

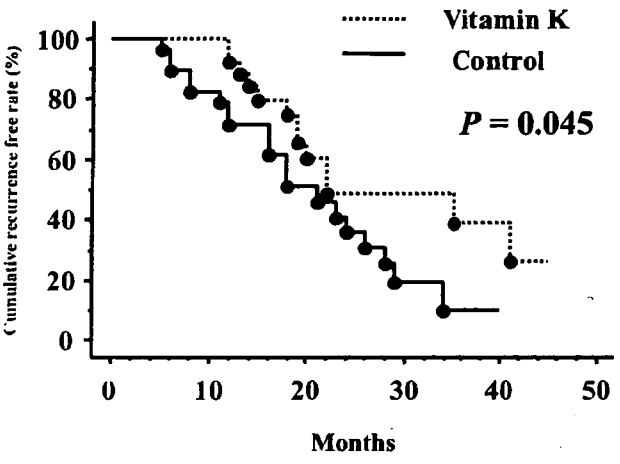

Figure 1 Recurrence-free rates of patients with hepatocellular carcinoma from the vitamin K2 group (. . .) and the control group (-) after curative treatment. Disease recurrence was found to be significantly lower in the vitamin K2 group (P= 0.045; log-rank test).

recurrence-free rate of the two groups. Vitamin K2 decreased the cumulative incidence of HCC recurrence (log-rank test; $P = 0.045$). The cumulative recurrence-free rates in the vitamin K2 group were 92.3% at 12 months, 48.6% at 24 months and 38.8% at 36 months; and those in the control group were 71.7%, 35.9% and 9.9%, respectively. The mean time of tumor recurrence was 20.8 ± 8.8 months (median 19 months) in the vitamin K2 group, whereas it was 16.0 ± 8.8 months (median 16 months) in the control group, respectively. During the follow-up period, recurrent HCC was observed in 20 patients in the control group and in 14 patients from the vitamin K2 group. Multivariate Cox proportional hazard models were used to analyze if each variable was related to the recurrence rate of HCC (Table 2). There were no significant predictive factors related to the recurrence rate of HCC with the multivariate Cox proportional hazard models. Although the administration of vitamin K2 tended to decrease the risk of recurrence, it did not reach statistical significance according to the multivariate Cox proportional hazard models.

The cumulative survival rates (Fig. 2) for the vitamin K2 group were 100% at 12 months, 95.0% at 24 months and 77.5% at 36 months; whereas the corresponding rates for the control group were 95.8%, 90.2% and 66.4%, respectively (log-rank test; $P = 0.70$). There was no significant difference in the survival rates between the vitamin K2 and control groups. During the follow-up period, six patients from the control group and five patients from the vitamin K2 group died. Five patients from the control group and four patients from the vitamin K2 group died because of advanced cancer, and one patient each from the both groups died because of hepatic failure. No patient died of any causes unrelated to liver disease. There was no difference in the cause of death between the two groups.

Discussion

To the best of our knowledge, the current study is the second clinical trial to examine if menatetrenone, a vitamin K2 compound, can suppress the recurrence of HCC and improve survival rates after patients receive curative treatment. Mizuta *et al.*[13] reported the first clinical trial of the chemopreventive effects of menatetrenone. They reported the beneficial preventive effects of vitamin K2 on disease recurrence and survival in patients with HCC.[13] Our result in this study also showed the beneficial preventive effects of vitamin K2 on disease recurrence. Mizuta *et al.* acknowledged three problems with their study. The first problem was the baseline difference in serum PIVKA-II levels between the treatment group and the control group. The second problem was that the treatment methods for HCC were heterogeneous, including surgical resection, percutaneous ethanol injection, percutaneous microcoagulation and percutaneous radiofrequency ablation. The third problem with their study was the method of diagnosing HCC before entry and at the time of disease recurrence. In our study, there were no differences in PIVKA-II levels. Regarding Mizuta *et al.*'s second problem, our study included only curative treatments of surgical resection and radiofrequency ablation. Thus, we confirmed their results by more homogenous subjects. Although our study is also a pilot study with small numbers of patients, both studies produced results indicating the possible applicability of vitamin K2 as a chemopreventive drug for HCC.

Among vitamin K properties, vitamin K3 has a strong inhibitory effect on the proliferation of tumor cells *in vitro*.[6–8,14] To the best of our knowledge, the mechanisms involved in this antiproliferative activity of vitamin K are not fully understood. Wang *et al.*[9] showed that vitamin K3 exerted antiproliferative effects on hepatoma cells in a free radical-dependent manner, whereas the effects of vitamin K2 were not mediated by free radicals. Bouzahazah *et al.*[15] reported that vitamin K2 increased c-Jun and c-Myc mRNA expression in hepatoma cells, suggesting the induction of apoptosis through vitamin K-dependent proteins.

The chemoprevention of tumor recurrence is an important strategy for the treatment of HCC, due to the high frequency of recurrence even after patients undergo curative therapy.[1,2] IFN is effective in both the primary and secondary prevention of HCC in patients with hepatitis C[3,4] and an acyclic retinoid has been indicated as being effective in preventing the development of secondary HCC.[15] However, IFN has frequent adverse effects that are not tolerated by all patients. Although vitamin K3 and its derivatives have been shown to be effective *in vitro*, to the best of our knowledge they have yet to be tested in human studies. Conversely, vitamin K2 is used currently and safely for the treatment of osteoporosis.[16] Indeed, Habu *et al.*[17] reported a preventive role of vitamin K2 for HCC during an assessment on the long-term effects of vitamin K2 on bone loss in women with viral liver cirrhosis. In this trial, there were no adverse effects related to the daily oral administration of 45 mg menatetrenone, the same dosage used in the treatment of osteoporosis. The longest case was administered for 45 months. Because dose-dependent antiproliferative effects of vitamin Ks have been reported,[6–8,14] it may be possible to adjust the dose of vitamin K2 administered to increase its chemopreventive effects on HCC recurrence. However, increasing the dosage of vitamin K2 should be performed gradually and carefully in order to avoid any adverse effects.

Although high PIVKA-II levels tended to be a risk factor of recurrence, it did not reach statistical significance based on the multivariate Cox proportional hazard models in this study. There