にて外科的切除率は初発時31.3%であるのに対し，再発時では2.2%しか外科的切除が行われていなかったと報告している[18]ように，肝予備能などの問題から，肝細胞癌を2度以上外科的切除することはまれである．RFA治療後の異所再発率と，外科的切除後の異所再発率が大きく違わないこと，またRFA治療後の異所再発予測因子は外科的切除後の異所再発予測因子でもあることを考えれば，(b)群ではたとえ外科的切除をしたとしても数年で高率に再発し，その再発癌のほとんどに対して非外科的治療が施行されることとなる．さらに今回の検討で，(b)群は(a)群に比して有意にAlb値やPT値が低く，ALT値には差がなかったもののAST値が高い傾向を示していたことから，(b)群では(a)群に比して残存肝機能の低下した症例が多いものと考えられる．つまり高再発群は治療時の肝機能も低再発群に比し低下しており，高再発群を初発時に肝切除することは，肝予備能をさらに低下させ，数年以内に起こる可能性の極めて高い再発時の治療の選択肢を狭めてしまうのではないかと危惧される．今後生存率の検討は必要であるが，再発予防治療が確立されるまでは，多発例または血小板低値例において，RFA適応病変であればRFAを積極的に選択するべきではないかと考える．逆に，RFA治療後の局所再発率が限りなく0であると予測される病変でなければ，低再発群は手術を積極的に選択するべきではないかと考える．

腫瘍個数は血管内浸潤などと違い特殊な検査機材を必要とせず，血小板値は安価かつ簡便に測定できる検査データの一つである．C型慢性肝疾患を背景肝とする症例の多い日本では，これら2つの指標によりRFA適応病変の異所再発のリスクが今回の結果のように大きく分かれるということから，初発肝細胞癌に対してRFA治療すべきか外科的切除をすべきか悩む症例において治療法を選択する上で腫瘍個数と血小板値が重要な判断指標となり得ると考える．

結　語

当科における初発肝細胞癌患者に対するRFA後の異所再発に関して検討を行った．累積異所再発率は1年12.0%，2年36.3%，3年53.5%であり，報告されている外科的切除後の異所再発率と差はなかった．また，治療時腫瘍多発例，血小板値$10.0\times10^4/\mu l$未満例はRFA治療後の異所再発率が非常に高かった．治療時腫瘍多発例や血小板低値例は外科的切除でも異所再発率が非常に高いことから，再発予防治療が確立されるまでは，安全にRFAで根治可能な病変であればRFAを積極的に治療法として選択すべきではないかと考えられた．

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Predictive factors for intrahepatic distant recurrence of hepatocellular carcinoma after radiofrequency ablation for primary tumors

Hayato Hikita, Yoshio Tanaka, Minoru Shigekawa, Kaori Mukai, Keiko Nakamura, Changho Song, Osamu Tatsumi, Yuko Inoue, Akira Sasakawa, Fumihiko Nakanishi, Eiji Masuda, Taizo Hijioka

We studied 56 patients with primary hepatocellular carcinoma (HCC) treated with radiofrequency ablation (RFA) in our hospital to clarify the predictive factors for intrahepatic distant recurrence. The observation period was 16.4 months. Cumulative rate of recurrence was 12.0% in a year and 53.5% in 3 years. Multivariate analysis revealed that multinodular HCC (odds ratio 4.78, $p = 0.01$) and platelet count $<10.0 \times 10^4/\mu l$ (odds ratio 5.25, $p<0.01$) were considered to be useful predictive factors for recurrence. These recurrence rate and factors after RFA were similar to those after surgical resection. Therefore, patients showed uninodular HCC and whose platelet count was $10.0 \times 10^4/\mu l$ and over before treatment might not be treated with RFA, but surgical resection if possible. However, considering remnant liver function and recurrence rate, we think patients either showed multinodular HCC or whose platelet count was less than $10.0 \times 10^4/\mu l$ before treatment should be treated with RFA if possible.

Kanzo 2006; 47: 209—216

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HEPATOLOGY RESEARCH
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Usefulness of elastometry in evaluating the extents of liver fibrosis in hemophiliacs coinfected with hepatitis C virus and human immunodeficiency virus

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Received 31 October 2005; received in revised form 13 February 2006; accepted 15 February 2006

Abstract

The newly developed elastometer, FibroScan®, was utilized to evaluate liver fibrosis in hepatitis C virus (HCV)- and human immunodeficiency virus (HIV)-coinfected 33 hemophiliacs and HIV-uninfected 24 patients with chronic hepatitis C. Chronicity in the liver was categorized into 4 stages by abdominal ultrasound (AUS): 1 (normal or fatty liver); 2 (chronic liver disease, mild); 3 (moderate); and 4 (severe). Stiffness of the liver was significantly increased as AUS stages advanced: 5.4 ± 2.2 ($N=3$) versus 7.5 ± 2.7 ($N=9$), in stage 1; 4.9 ± 1.7 ($N=2$) versus 9.9 ± 6.0 ($N=10$), in stage 2, 13.5 ± 4.7 ($N=5$) versus 12.9 ± 5.9 ($N=6$), in stage 3, and 22.0 ± 9.5 ($N=14$) versus 28.1 ± 21.3 ($N=8$), in stage 4, in non-HIV group and in HIV group, respectively ($P=0.004$ and 0.007). Stiffness was correlated with AUS stages ($r=0.740$, $P<0.001$), platelet counts (PLT; $r=-0.642$, $P=0.001$) and 7S domain of type IV collagen (IV-coll; $r=0.480$, $P=0.024$) in non-HIV group, while in HIV group, with IV-coll ($r=0.801$, $P<0.001$), AUS stages ($r=-0.603$, $P<0.001$), procollagen type III peptides (P-III-P; $r=0.621$, $P=0.001$), PLT ($r=-0.480$, $P=0.005$), and hyaluronic acid ($r=0.433$, $P=0.027$). FibroScan® is absolutely noninvasive and can be the alternative to liver biopsy, especially in patients with bleeding tendency.

Keywords: Liver fibrosis; HCV; HIV; Stiffness

1. Introduction

It has been well documented that coinfection of hepatitis C virus (HCV) and human immunodeficiency virus (HIV) might accelerate the progression of liver fibrosis, as compared with HCV infection alone [1]. However, especially in patients with hemophilia coinfected with HCV and HIV, it is practically difficult to perform liver biopsy, the established gold standard to evaluate the extents of liver fibrosis, because of their bleeding tendency. Moreover, tiny biopsied specimens, corresponding to only one forty thousandth of entire liver volume, may sometimes cause misleading explanations. Although rough estimation of liver fibrosis can be performed by abdominal ultrasound (AUS), it would be somewhat subjective and dependent on technical experience. In addition, several serum markers for liver fibrosis such as procollagen type III peptides (P-III-P), 7S domain of type IV collagen (IV-coll), and hyaluronic acid, have been commercially available, however, those substances might just reflect overproduction of collagen in vivo.

Recently, a new noninvasive device to quantify liver fibrosis, FibroScan®, was developed by Echosens (Paris, France). This device is based on one-dimensional transient elastography, using both ultrasound (5 MHz) and low-frequency (50 Hz) elastic waves, whose propagation velocity is directly proportional to elasticity [2]. Usefulness of this device to assess the extents of liver fibrosis in patients with chronic hepatitis was already reported from France [3], and from Japan [4]. In this study, we attempted to evaluate its usefulness

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doi:10.1016/j.hepres.2006.02.006

in hemophilacs coinfected with HCV and HIV, in whom liver biopsy is ordinarily contraindicated, as well as in non-hemophiliacs infected by HCV alone.

2. Patients and methods

Hemophiliacs coinfected with HCV and HIV or non-hemophiliacs infected by HCV alone, who consulted Division of AIDS Research Center or Division of Gastroenterology, International Medical Center of Japan, from February to April, 2004, were randomly enrolled in this study, after informed consent was given. The patient received elastometry with FibroScan® version 2 (FibroScan® 502: Echosens, Paris, France), as previously reported [4], at the same time with B-mode AUS, by two hepatologists (N.M. and M.I.) with over 15-year experience. The elasticity of the liver was measured 10 times and the median value, which was automatically calculated, was considered as Stiffness (KPa). AUS findings of the liver were categorized into 4 stages, based on the extents of surface irregularity, dullness of the edge, heterogeneity of internal echogenecity, blurriness of hepatic veins, and imbalance in size of both lobes (hypertrophy of the caudate lobe and/or atrophy of the right lobe): (1) normal or fatty liver; (2) chronic liver disease, mild; (3) moderate; and (4) severe. On the same day, serum levels of albumin, alanine aminotransferase (ALT), and liver fibrosis markers (P-III-P, IV-coll, and hyaluronic acid), were measured. HCV genotype and peripheral platelet counts (PLT) were also determined. Only in HIV group, CD4 counts and HIV RNA concentrations were determined. The correlation between Stiffness and AUS stage, PLT, or serum liver fibrosis markers were investigated.

In patients, where the age of blood transfusion or the first infusion of clotting factor concentrates could be identified, annual progression rate of liver fibrosis (R-value: KPa/year) was arbitrarily calculated, according to the following equation. R-value = [Stiffness − 5.4]/[Age (at the elastometry) − Age (of the HCV infection)], where 5.4 is the mean value of Stiffness in patients with normal AUS finding (data shown in Section 3).

2.1. Statistical analysis

The data were shown as mean ± standard deviation. The correlation between the Stiffness and AUS stage was determined by Kruskal–Wallis test. The correlation between the parameters was determined by Pearson's correlation coefficient or Spearman's correlation coefficient. The comparisons between the data were analyzed by Mann–Whitney U-test or Fisher's exact test.

3. Results

Thirty-three hemophiliacs coinfected with HCV and HIV (HIV group; hemophilia A/B = 23 patients/10 patients; all males) and 24 non-hemophiliacs infected by HCV alone (non-HIV group; 12 males and 12 females), were enrolled in this study, and received B-mode AUS and elastmetry at the same time. The demographic features and laboratory data of both groups were shown in Table 1. All the patients in HIV group have been successfully treated by highly active antiretroviral therapy (HAART) since 1996, and in 23 patients, HIV RNA concentrations were maintained less than 50 copy/ml. Seven out of 21 (33%) patients in HIV group were infected by HCV with uncommon genotype in Japan (1a or 3a). The patients were classified into 4 stages on AUS: stage 1: 3 and 9; stage 2: 2 and 10; stage 3: 5 and 6; stage 4: 14 and 8, in non-HIV group and HIV group, respectively. Apparently, in non-HIV group, most of the patients were older and had more advanced stages of chronic liver disease, as compared with those in HIV group. Stiffness of the liver in each AUS stage was shown as follows: 5.4 ± 2.2 and 7.5 ± 2.7, in stage 1; 4.9 ± 1.7 and 9.9 ± 6.0, in stage 2, 13.5 ± 4.7 and 12.9 ± 5.9, in stage 3, 22.0 ± 9.5 and 28.1 ± 21.3, in stage 4,

Table 1
Demographic features and laboratory data of non-HIV group and HIV group

Parameters	Non-HIV group (N = 24)	HIV group (N = 33)	P-value
Age	69 ± 13	39 ± 11	0.000[a]
Gender (M/F)	12/12	33/0	0.000[b]
Albumin (g/dl)	4.0 ± 0.4	4.3 ± 0.3	0.004[a]
ALT (U/l)	60 ± 45	67 ± 54	NS[a]
PLT ($\times 10^4/\mu l$)	12.5 ± 5.2	19.0 ± 8.6	0.003[a]
CD4 counts (μl^{-1})	n.d.	442 ± 230	
P-III-P (U/ml)	1.2 ± 0.3	0.9 ± 0.3	0.003[a]
IV-coll (ng/ml)	8.3 ± 3.1	6.2 ± 2.7	0.013[a]
Hyaluronic acid (ng/ml)	335 ± 399	210 ± 190	NS[a]
HCV genotype (1/1a/1a + 1b/1b/2a/2b/3a/3a + 2b)	2/0/0/8/1/2/0/0	4/4/1/5/3/2/1/1	
AUS stage (1/2/3/4)	3/2/5/14	9/10/6/8	0.031[c]

n.d.: not determined, NS: not significant. The abbreviations used are: ALT, alanine aminotransferase; PLT, platelet counts; P-III-P, procollagen type III peptides; IV-coll, 7S domain of type IV collagen; AUS, abdominal ultrasound.
[a] Mann–Whitney U-test.
[b] Fisher's exact test.
[c] Pearson's χ^2-test.

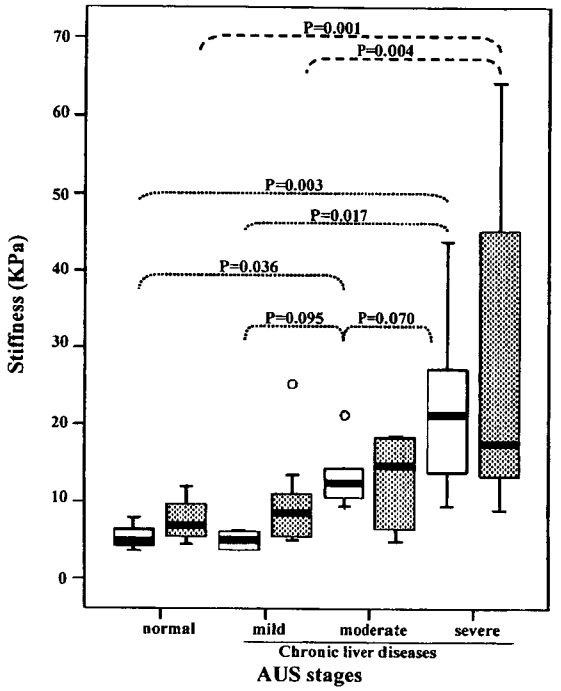

Fig. 1. Box plots of Stiffness for each AUS stage in non-HIV group and HIV group. The *top* and *bottom* of the boxes are the first and third quartiles, respectively. The length of the box represents the interquartile range within which 50% of the values were located. The *line* through the middle of each box represents the median. The *error bars* show the minimum and maximum values. Open boxes and dotted boxes show the data of non-HIV group and those of HIV group, respectively. The *P*-value was calculated by Mann–Whitney *U*-test.

in non-HIV group and in HIV group, respectively ($P=0.004$ and 0.007, by Kruskal–Wallis test in each group). There was no significant difference between non-HIV group and HIV group, in each AUS stage. Box plots of Stiffness for each AUS stage were demonstrated in Fig. 1. In non-HIV group, there existed significant difference in Stiffness between stages 4 and 2 ($P=0.017$), between stages 4 and 1 ($P=0.003$), and between stages 3 and 1 ($P=0.036$). In addition, there was marginal difference between stages 4 and 3 ($P=0.070$), and between stages 3 and 2 ($P=0.095$). While, in HIV group, Stiffness in stage 4 was significantly higher than that in stage 1 ($P=0.001$), and that in stage 2 ($P=0.004$).

To examine usefulness of elastometry in differentiating advanced stage of chronic liver disease from non-advanced one, similar analysis was conducted after patients of stages 2 and 3 were combined together. Chronicity in the liver was simply categorized into 3 modified AUS stages: normal (stage 1), non-advanced (stage 2 + 3) and advanced (stage 4). Stiffness of the liver in each modified AUS stage was shown as follows: 5.4 ± 2.2 ($N=3$) and 7.5 ± 2.7 ($N=9$), in stage 1 (normal), 11.1 ± 5.7 ($N=7$) and 11.1 ± 6.0 ($N=16$), in stage 2+3 (non-advanced), 22.0 ± 9.5 ($N=14$) and 28.1 ± 21.3 ($N=8$), in stage 4 (advanced), in non-HIV group and in HIV group, respectively ($P=0.003$ and 0.004, by Kruskal–Wallis test in each group). Again, there was no significant difference between non-HIV group and HIV group, in each modified AUS stage. In addition, as compared with other liver fibrosis markers such as P-III-P, IV-coll, hyaluronic acid, and PLT, Stiffness was found to be the most useful in differentiating advanced stage of chronic liver disease from non-advanced one, both in non-HIV group and in HIV group (Fig. 2).

3.1. Correlation between Stiffness and other parameters for liver fibrosis

To evaluate usefulness of elastometry, we investigated correlation between Stiffness and conventional liver fibrosis markers such as P-III-P, IV-coll, and hyaluronic acid, including AUS stage and PLT. In non-HIV group, Stiffness was significantly correlated with AUS stage ($r=0.740$, $P<0.001$), PLT ($r=-0.642$, $P=0.001$), and IV-coll ($r=0.480$, $P=0.024$). On the other hand, in HIV group, Stiffness was significantly correlated with IV-coll ($r=0.801$, $P<0.001$), AUS stage ($r=0.603$, $P<0.001$), P-III-P ($r=0.621$, $P=0.001$), PLT ($r=-0.480$, $P=0.005$), and hyaluronic acid ($r=0.433$, $P=0.027$).

3.2. Correlation between platelet counts and other parameters for liver fibrosis

It has been well established that PLT are inversely proportional to the extents of liver fibrosis [5]. In this study, we investigated correlation between PLT and conventional liver fibrosis markers, AUS stage or Stiffness. In non-HIV group, PLT were significantly correlated with AUS stage ($r=-0.647$, $P=0.001$), IV-coll ($r=-0.643$, $P=0.001$), Stiffness ($r=-0.642$, $P=0.001$), P-III-P ($r=-0.526$, $P=0.012$), and hyaluronic acid ($r=-0.424$, $P=0.049$). On the other hand, in HIV group, PLT were significantly correlated with Stiffness ($r=-0.480$, $P=0.005$), AUS stage ($r=-0.415$, $P=0.016$), and IV-coll ($r=-0.417$, $P=0.031$).

3.3. Annual progression rate of liver fibrosis

We attempted to evaluate whether HIV coinfection may affect progression of liver fibrosis in patients with chronic hepatitis C. In non-HIV group, the age of blood transfusion was identified in 9 patients. Two of them had received interferon (IFN) therapy: IFN monotherapy and IFN-α2b/ribavirin, each, without sustained virological response (SVR). On the other hand, in HIV group, they had received multiple infusions of clotting factor concentrates, it was practically impossible to determine the time of the first infection of HIV or HCV. We speculated the age of the first infusion of such concentrates as the time of HCV infection, in 23 patients, in order to arbitrarily calculate annual progression rate of liver fibrosis (*R*-value). Twelve out of them had history of IFN therapy: IFN monotherapy, IFN-α2b/ribavirin and Pegylated IFN-α2a in 3, 5 and 4 patients,

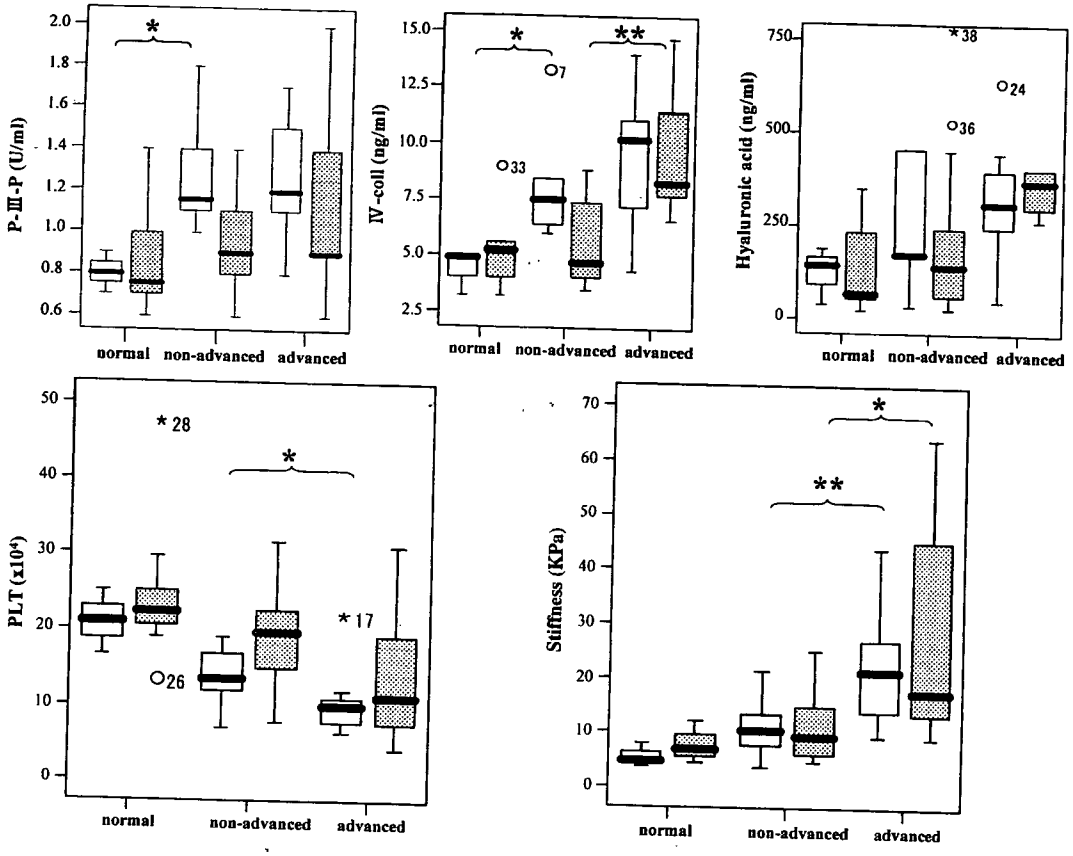

Fig. 2. Box plots of liver fibrosis markers (P-III-P, IV-coll, hyaluronic acid), PLT and Stiffness for each modified AUS stage in non-HIV group and HIV group. Open boxes and dotted boxes show the data of non-HIV group and those of HIV group, respectively. The P-value was calculated by Mann–Whitney U-test ($^{*}P<0.05$, $^{**}P<0.01$).

respectively. Four of them (33%) obtained SVR. There was no significant difference in R-value between non-HIV group and HIV group (0.32 ± 0.17 versus 0.38 ± 0.70; $P=0.125$, by Mann–Whitney U-test). In HIV group, there was no significant difference in R-value between IFN-treated ($N=12$) and untreated ($N=11$) patients (0.34 ± 0.50 versus 0.43 ± 0.90, $P=0.710$). Furthermore, in HIV group treated by IFN, there was no significant difference in R-value between patients with SVR ($N=4$, 0.08 ± 0.10) and those without SVR ($N=7$, 0.52 ± 0.60; $P=0.252$).

4. Discussion

In this study, we attempted to validate Stiffness measured by the newly developed elastometer, FibroScan® 502, in evaluating the extents of liver fibrosis in hemophiliacs coinfected with HIV and HCV (HIV group). Non-hemophiliacs infected by HCV alone (non-HIV group), could be regarded as a control, since hemophilia per se may not affect inflammation or fibrosis in the liver. It has been recently established that Stiffness is strongly correlated with liver fibrosis markers as well as F score evaluated by liver biopsy, in non-HIV group [3,4]. Liver biopsy could not be practically performed in clinical settings, however, such correlation was similarly, and even more significantly, confirmed also in HIV group. In this study, correlation between Stiffness and other parameters for liver fibrosis was rather weak in non-HIV group where more than half of the patients might have liver cirrhosis, probably because the variations of such liver fibrosis markers were greater in more advanced stage of chronic liver disease as shown in Fig. 2, as well as described previously [4]. Although AUS findings might be somewhat subjective, Stiffness in stage 4 was significantly higher than those in stages 1 and 2, both in HIV group and in non-HIV group, in this study (Fig. 1). In addition, when the patients of stages 2 and 3 were combined together, Stiffness was the only tool in differentiating advanced stage of chronic liver disease from non-advanced one, both in non-HIV group and in HIV group, as shown in Fig. 2. Accordingly, measurement of Stiffness could be a very useful tool to identify patients, at least, with advanced liver fibrosis. In contrast, PLT, which have been widely accepted as one of the very sensitive markers for liver fibrosis [5], seemed to be less reliable in HIV group than in

non-HIV group. Possibility of influence of HAART regimens on PLT counts should be further investigated in a future study.

It has been occasionally described that HIV coinfection increases the risk of cirrhosis and even hepatocellular carcinoma (HCC) in HCV-infected patients [6]. In addition, whether introduction of HAART or IFN therapy could affect these situations would be undoubtedly our major concern. Kramer et al. [7] recently reported that HIV coinfection accelerated liver fibrosis in pre-HAART era, but not in HAART era, while it did not affect the risk of HCC in HCV infected U.S. veterans in both eras. Similarly, Marine-Barjoan et al. [8] found that early HAART may slow liver fibrosis progression in coinfected French patients. On the contrary, Martinez-Sierra et al. [9] showed that the immune response to HAART did not influence liver fibrosis progression rate in coinfected Spanish patients. Moreover, it has been recently pointed out that HAART regimens including nevirapine, one of nonnucleoside reverse-transcriptase inhibitors, may be associated with faster liver fibrosis progression in coinfected patients [10], especially with advanced stages of liver fibrosis [11]. In this study, we arbitrarily calculated annual progression rate of liver fibrosis, using Stiffness and duration of HCV infection. Interestingly, there was no significant difference between non-HIV group and HIV group receiving HAART. This may be quite consistent with our previous findings that plasma levels of TGF-β and IL-13, known cytokines to play pivotal roles in liver fibrosis, were significantly decreased in coinfected patients under HAART, as compared with those in patients infected HCV alone (unpublished results). Furthermore, we could not get any definitive results that IFN therapy may counteract progression of liver fibrosis, because of the small numbers of patients examined and short terms of follow-up after IFN therapy. Further regular check-ups of the elasticity of the liver would be prerequisite to settle these controversial issues.

In conclusion, FibroScan® is absolutely noninvasive and can be the alternative to liver biopsy, especially in patients with bleeding tendency. It is strongly suggested that HIV/HCV coinfection may not accelerate liver fibrosis progression in HCV-related chronic liver disease, at least in HAART era.

Acknowledgements

This work was supported by Grant-in-Aid for Health Sciences Research Grants of The Ministry of Health, Welfare and Labor, Japan.

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ONCOLOGY REPORTS 16: 389-392, 2006

Survivin downregulation by siRNA sensitizes human hepatoma cells to TRAIL-induced apoptosis

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Received December 2, 2005; Accepted January 23, 2006

Abstract. Survivin, an anti-apoptotic protein, is abundantly expressed in a variety of cancer cells, including hepatoma cells, resulting in the resistance of these cells to various apoptotic stimuli. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is known to induce cancer cell-specific apoptosis, but hepatoma cells are resistant to TRAIL-induced apoptosis. In the present study, we have examined whether the downregulation of survivin by short interfering RNA (siRNA) promotes spontaneous or TRAIL-induced apoptosis in Huh-7 human hepatoma cells. Survivin siRNA transfection downregulated the expression of survivin in Huh-7 cells and reduced cell viability by 20% through inducing spontaneous apoptosis. TRAIL (1 to 2 ng/ml) only slightly induced apoptosis in Huh-7 cells; however, survivin siRNA transfection apparently enhanced TRAIL-induced apoptosis. These results suggest that the level of survivin is linked to the susceptibility of Huh-7 cells to TRAIL. It is possible that survivin downregulation by siRNA combined with TRAIL administration may provide a new therapeutic strategy against hepatoma.

Introduction

Survivin, the smallest mammalian member of the inhibitor of apoptosis (IAP) family, plays a key role, not only in cell division but also in apoptosis inhibition, by repressing caspase activities (1-3). Survivin is abundantly expressed in a variety of cancer cells, including hepatoma cells, but not in normal cells (3-6), resulting in the resistance of cancer cells to various apoptotic stimuli (3,5,7,8). In addition, the overexpression of survivin in cancer is closely related to unfavorable disease outcome (3,6,9-11). Therefore, survivin is a possible target of cancer therapy. In fact, the downregulation of survivin by anti-sense oligonucleotides, short interfering RNA (siRNA) and dominant-negative mutant induces apoptosis of cancer cells or sensitizes cancer cells to chemotherapeutic agents and irradiation (12).

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily, induces apoptosis in a variety of cancer cells with little or no effect on normal cells (13,14). However, several cancer cells, including hepatoma cells, are resistant to TRAIL-induced apoptosis (15). Griffith *et al* have reported that the cellular level of survivin is linked to the resistance against TRAIL-induced apoptosis of renal cell carcinoma cells (16). It has been also reported that the downregulation of survivin by siRNA sensitizes resistant melanoma cells to TRAIL-induced apoptosis (17). Moreover, we previously reported that interferon (IFN)-α downregulated the survivin expression in human hepatoma cells and sensitized these cells to TRAIL-induced apoptosis, and ectopic expression of survivin partially rescued these cells from IFN-α TRAIL-induced apoptosis (18). Therefore, it is possible that survivin plays a role in the resistance to TRAIL-induced apoptosis in human hepatoma cells. To confirm this, in the present study, we examined whether downregulation of survivin by siRNA can sensitize human hepatoma cells to TRAIL-induced apoptosis.

Materials and methods

siRNAs. To select siRNA duplexes for survivin mRNA, we searched for sequences of AA and the N19 (N, any nucleotide) from the open reading frame of human survivin as described previously (19). siRNAs with the following sequences were used for experiments: surA, 5'-GGUUAAUUCUUCAAA CUGCTT-3' (antisense); surB, 5'-GCAAUUUUGUUCUUG GCUCTT-3' (antisense).

siRNA against green fluorescent protein (GFP) was also used as a control: siGFP, 5'-UGCGCUCCUGGACGUAGC CTT-3' (antisense). All siRNAs were purchased from Nihon Bioservice (Saitama, Japan).

Cell culture and transfection. Huh-7 human hepatoma cells were maintained in RPMI with 5% fetal bovine serum. Cells were plated on a 96-well plate or Lab-Tek chamber slide at

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Key words: survivin, siRNA, tumor necrosis factor-related apoptosis-inducing ligand, hepatoma

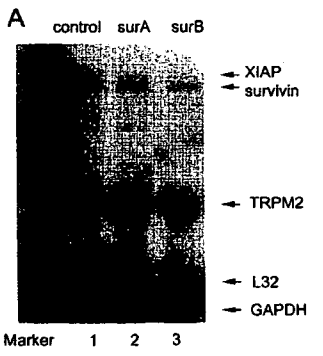

Figure 1. Effect of siRNA transfection on survivin expression in Huh-7 cells. Huh-7 cells were transfected with siGFP as a control (lane 1), surA (lane 2) or surB (lane 3), respectively. (A) The levels of indicated mRNAs were analyzed by RNase protection assay. (B) The expression of XIAP and survivin was analyzed by Western blotting. Results are from one representative experiment from a total of three performed.

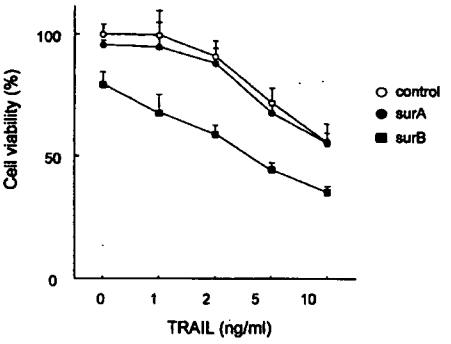

Figure 2. Effect of siRNA transfection with or without TRAIL on the viability of Huh-7 cells. Huh-7 cells were transfected with siGFP as a control (○), surA (●) or surB (■), respectively, and then treated with various concentrations of TRAIL for 12 h. Cell viability was determined by the colorimetric method. Data represent mean ± SD values of the four experiments.

an appropriate density. A day later, cells were transfected with each siRNA (4 pmol/10^4 cells) using oligofectamine (Gibco-Invitrogen, Rockville, MD, USA) according to the instructions provided by the manufacturer. The medium was replaced with fresh medium 6 h after transfection, and the cells were cultured for 36 h and treated with or without varying concentrations of TRAIL for 12 h.

RNase protection assay. The RNase protection assay was performed using a RiboQuant Multi-Probe RNase Protection Assay System (BD PharMingen, Franklin Lakes, NJ, USA). According to the instructions provided by the manufacturer, an hAPO5c (inhibitors of apoptosis) template set, including an L32 ribosomal protein and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) template as internal controls, was labeled with [α^{32}P]-UTP using T7 RNA polymerase. The labeled RNA probes were hybridized with 10 μg of total RNA from Huh-7 cells transfected with each siRNA. Samples were digested with RNase to remove single-stranded (non-hybridized) RNA. The remaining probes were resolved on 6% urea-polyacrylamide-bis-acrylamide gels. Gels were dried and analyzed using an image analyzer (BAS; Fuji Film Co., Tokyo, Japan).

Western blot analysis. Cells were lysed by adding lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 0.1% SDS, 1% NP40 and 100 μg/ml PMSF) for 10 min at 4°C and passed several times through a 25-gauge needle. The same amount of protein from each lysate (20 μg/well) was subjected to 12% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes that were then blocked for 1.5 h using 5% non-fat dried milk in PBS containing 0.1% Tween-20 (PBS-T), washed with PBS-T and incubated at room temperature for 1 h in the presence of each antibody (mouse monoclonal anti-human XIAP from MBL, Nagoya, Japan; and rabbit polyclonal anti-human survivin from Alpha Diagnostic International Inc., San Antonio, TX, USA). The membranes were washed with PBS-T and incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG for 1 h. Following washing with PBS-T, immunoreactive bands were visualized using the ECL chemiluminescence system (Amersham Life Science, Buckinghamshire, UK).

Analysis of cell viability and apoptosis. Cell viability was determined by the colorimetric method using a Cell Counting kit (Wako Life Science, Osaka, Japan). The absorbance of each well was measured at 405 nm with a microtiter plate reader (Multiskan JX; Thermo BioAnalysis Co., Tokyo, Japan). For the detection of apoptosis, TUNEL assay was performed using a DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI, USA). Briefly, the cells cultured in Lab-Tek chamber slides were fixed in 4% paraformaldehyde in PBS. The fragmented DNA of apoptotic cells was detected by catalytically incorporating fluorescein-12-dUTP at 3'-OH DNA ends using the terminal deoxynucleotidyl transferase. 1 μg/ml of propidium iodide (PI) was added for nuclear staining. Sample slides were directly visualized by fluorescence microscopy (BX 50; Olympus, Tokyo, Japan) at 520 nm for the green fluorescence of fluorescein and at >620 nm for the red fluorescence of PI.

Results

siRNA transfection downregulates survivin expression. To confirm that siRNA transfection downregulates survivin expression in Huh-7 cells, RNase protection assay and Western blotting were performed (Fig. 1). surA transfection did not repress the level of survivin mRNA, but slightly upregulated the level of XIAP mRNA. In contrast, surB transfection repressed the level of survivin mRNA, but did not upregulate the level of XIAP mRNA (Fig. 1A). The levels of TRPM2, L32 and GAPDH mRNAs were almost unchanged by the transfection of surA and surB. Similarly, surB transfection clearly repressed survivin expression, but did not repress XIAP expression (Fig. 1B).

Survivin downregulation by siRNA enhances TRAIL-mediated cytotoxicity. We examined the effect of survivin siRNA transfection with or without TRAIL on cell viability (Fig. 2). surB transfection alone repressed the viability of Huh-7 cells by almost 20% compared with the control but surA transfection

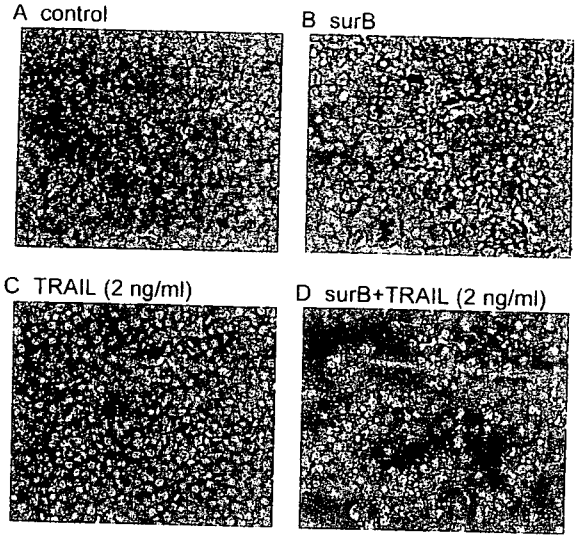

Figure 3. Effect of siRNA transfection with or without TRAIL on apoptosis in Huh-7 cells. Huh-7 cells were transfected with (A) siGFP alone as a control, (B) suB alone, (C) siGFP followed by TRAIL (2 ng/ml) treatment and (D) surB followed by TRAIL (2 ng/ml) treatment. Apoptotic cells were detected by TUNEL assay. Results are from one representative experiment from a total of four performed.

did not. Low concentrations of TRAIL (1 and 2 ng/ml) showed little effect on the viability of control cells, although high concentrations of TRAIL (5 and 10 ng/ml) reduced the viability. On the other hand, surB transfection sensitized Huh-7 cells to TRAIL-mediated cytotoxicity. In fact, the viability of cells transfected with surB and treated with TRAIL (1 or 2 ng/ml) was nearly equal to the viability of control cells treated with TRAIL (5 or 10 ng/ml), respectively.

Survivin downregulation by siRNA enhances TRAIL-induced apoptosis. To clarify whether surB transfection enhances TRAIL-induced apoptosis in Huh-7 cells, TUNEL assay was performed (Fig. 3). TUNEL-positive nuclei, small and bright fluorescence spots, were scattered in the cells either transfected with surB or treated with TRAIL (2 ng/ml), respectively. However, TUNEL-positive nuclei were apparently increased by combination of surB transfection with TRAIL treatment.

Discussion

We previously reported that IFN-α sensitized Huh-7 cells to TRAIL-induced apoptosis, in which IFN-α not only reduced survivin expression but also inhibited NF-κB activity and increased the expression of DR5, a death receptor of TRAIL (18). However, we could not address which was the key change directly relevant to the susceptibility to TRAIL, although ectopic expression of survivin partially rescued the IFN-α/TRAIL-induced apoptosis. In the present study, the downregulation of survivin by siRNA transfection sensitized Huh-7 cells to TRAIL-induced apoptosis as well as IFN-α, suggesting that downregulation of survivin is the major cause of IFN-α-mediated susceptibility of Huh-7 cells to TRAIL. Therefore, it is possible that the level of survivin is linked to the resistance against TRAIL-induced apoptosis in Huh-7 cells. This is consistent with previous observations in renal cell carcinoma cells and melanoma cells (16,17).

In this study, downregulation of survivin reduced the viability of Huh-7 cells through the induction of apoptosis. It was reported that an adenovirus expressing the dominant negative mutant of survivin caused spontaneous apoptosis in human cell lines of breast, cervical, prostate, lung and colorectal cancer but did not affect the viability of normal human cells, including fibroblasts, endothelium or smooth muscle cells (12,20). It was also reported that transduction of antisense oligonucleotides and siRNA against survivin induced apoptosis in HepG2 (21) and SMMC-7721 (22) human hepatoma cells, respectively. Therefore, it is likely that survivin supports the survival of hepatoma cells through inhibiting spontaneous apoptosis.

Survivin is abundantly expressed in hepatoma cells but not in normal hepatocytes (5,6), and TRAIL specifically induces apoptosis in cancer cells but not in normal hepatocytes (13,14). Therefore, the combination therapy of survivin knockdown and TRAIL administration may provide a new strategy for hepatoma treatment.

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DHMEQ, a novel NF-κB inhibitor, induces apoptosis and cell-cycle arrest in human hepatoma cells

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Received March 27, 2006; Accepted May 23, 2006

Abstract. Several reports have indicated that nuclear factor-κB (NF-κB) is constitutively activated in a variety of cancer cells including hepatoma cells and plays a key role in their growth and survival. Dehydroxymethylepoxyquinomicin (DHMEQ) derived from the structure of an antibiotic epoxyquinomicin C is a novel NF-κB inhibitor. In the present study, we evaluated the effect of DHMEQ on the NF-κB activity in human hepatoma cells, Huh-7, HepG2 and Hep3B, and the anti-tumor effect of DHMEQ on these cells *in vitro* and *in vivo*. DHMEQ inhibited the steady-state transcriptional activity of NF-κB in all hepatoma cells. DHMEQ blocked the constitutive DNA-binding activity and TNF-α-mediated nuclear translocation of NF-κB in Huh-7 cells. DHMEQ (5-20 μg/ml) dose-dependently reduced the viable cell number of all hepatoma cells. DHMEQ (20 μg/ml) induced apoptosis in all hepatoma cells, especially in Hep3B cells, and cell-cycle arrest in Huh-7 and HepG2 cells. These effects were accompanied by downregulation of proteins involved in anti-apoptosis (Bcl-xL, XIAP or c-IAP2) and cell-cycle progression (cyclin D1), and induction of proteins involved in pro-apoptosis (Bax) and cell-cycle retardation ($p21^{Waf1/Cip1}$), although the degree of changes by DHMEQ was different in each hepatoma cell type. Moreover, intraperitoneal administration of DHMEQ (8 mg/kg) significantly repressed the growth of Huh-7 tumor subcutaneously transplanted into BALB/c *nu/nu* athymic mice. Our results suggest that DHMEQ could qualify as a candidate for a new chemotherapeutic agent against human hepatoma.

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Abbreviations: ATL, adult T-cell leukemia; c-IAP, cellular inhibitor of apoptosis protein; DHMEQ, dehydroxymethylepoxyquinomicin; DMSO, dimethyl sulfoxide; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; FLIP, FLICE inhibitory protein; HCC, hepatocellular carcinoma; IκB-α, inhibitor of κB-α; NF-κB, nuclear factor-κB; PBS-T, phosphate-buffered saline containing 0.1% Tween-20; PMSF, phenylmethylsulfonyl fluoride; PTTG, pituitary tumor-transforming gene; TNF-α, tumor necrosis factor α; TRAF, TNFR-associated factor; XIAP, X-chromosome-linked inhibitor of apoptosis protein

Key words: NF-κB, hepatoma, apoptosis, cell-cycle arrest

Introduction

Hepatocellular carcinoma (HCC), hepatoma, is one of the most common malignancies worldwide. It is estimated that half a million cases occur annually worldwide (1). Several strategies have been implemented for the treatment of patients with HCC, such as surgical resection, percutaneous ethanol injection, radiofrequency ablation, transcatheter arterial embolization, and liver transplantation. However, reduced liver function derived from underlying liver cirrhosis has a profound effect on all treatment decisions and interferes with the use of these therapeutic modalities for HCC except liver transplantations (2). In addition, multicentric tumor recurrence rates are very high, even in patients with HCC who receive curative treatment. Therefore, HCC is a tumor with high lethality (1,3), and a novel approach for the treatment of HCC is needed.

The nuclear factor kappa B (NF-κB), a transcription factor family, consists of several structurally-related proteins such as RelA (p65), RelB, c-Rel, p50/p105 and p52/p100, which form homo- or heterodimers with each other and regulate the expression of a number of genes (4,5). NF-κB plays a well-known function in the regulation of immune responses and inflammation, but growing evidence supports a major role in oncogenesis. NF-κB regulates the expression of genes involved in many processes that play a key role in the development and progression of cancer such as proliferation, migration and apoptosis (6-8). Aberrant or constitutive NF-κB activation has been detected in many human malignancies including HCC (6-9).

Dehydroxylmethylepoxyquinomicin (DHMEQ) is a novel NF-κB inhibitor produced by Dr Kazuo Umezawa (Keio

University, Japan), based on the structure of epoxyquinomicin C originally isolated from Amycolatopsis (10,11). DHMEQ has been reported to repress renal inflammation in rats and osteoclastogenesis in cultured bone marrow cells through inhibiting NF-κB activity (12,13). In addition, DHMEQ have shown anti-tumor activity against several cancer cells including prostatic cancer (14,15), thyroid cancer (16), malignant myeloma (17,18), breast cancer (19) and adult T-cell leukemia (ATL) cells (20,21), in which NF-κB is constitutively activated and contributes to the growth and survival of these cells. The present study was set up to determine the effects of DHMEQ in human hepatoma cells both *in vitro* and *in vivo* and to additionally elucidate the molecular mechanisms underlying the action of this agent.

Materials and methods

Cell culture and viability assay. The human hepatoma cell lines, Huh-7, HepG2 and Hep3B were maintained in RPMI containing 10% fetal bovine serum (FBS). DHMEQ was a generous gift from Professor Kazuo Umezawa (Department of Applied Chemistry, Faculty of Science and Technology, Keio University, Yokohama, Japan). It was dissolved in DMSO to prepare a 10 μg/μl solution and subsequently diluted in culture medium to a final DMSO concentration of <0.2%. To analyze cell viability, $3x10^3$ cells were placed into 96-well multiplates. One day later, the medium was replaced with fresh medium containing varying concentrations of DHMEQ or vehicle (0.2% DMSO) alone, and the cells were incubated for 48 h. Following removal of the medium and dead cells, viable cells were counted with Particle counter Z1 (Beckman Coulter, Inc., Fullerton, CA).

Reporter gene transfection assay. The pNFκB-luc (Stratagene, La Jolla, CA) containing four copies of the binding sequence of NFκB and firefly luciferase gene and pRL-CMV-luc (Promega, Madison, WI) containing the cytomegalovirus immediate early enhancer/promoter and expressing renilla luciferase gene were used in the assay. Cells were grown in 24-well multiplates in triplicate one day before transfection. In the next step, 1 μg of pNFκB-luc and 10 ng of pRL-CMV-luc were transfected into the cells using Lipofectin (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol. After 6-h incubation, the medium was replaced with fresh medium containing varying concentrations of DHMEQ or vehicle (0.2% DMSO) alone, and the cells were incubated for 12 h. Luciferase activity in the cells was then determined by a dual-luciferase reporter assay system and a TD-20/20 luminometer (Promega).

Electrophoretic mobility shift assay (EMSA). Huh-7 cells were incubated with 20 μg/ml of DHMEQ for 1-24 h. The nuclear extract was prepared as described previously (22). As a positive control, the nuclear extract from HUT-78 cells containing NF-κB (23) was used. EMSA was performed using an EMSA kit (Panomics, Inc., Redwood City, CA) according to the manufacturer's protocol. Briefly, the same amount of protein from each nuclear extract was incubated with a biotin-tagged NF-κB probe; 5'-AGTTGAGGGGACTTTCCCAGGC-3' for 30 min at 15°C. The reaction mixture was electrophoresed using a 5% polyacrylamide gel containing 25 mM Tris-borate and 0.25 mM EDTA at 4°C and transferred to nylon membrane (Hybond-N$^+$, Amersham Biosciences AB, Uppsala, Sweden). The blots were incubated with a detection agent in the kit and visualized with SuperSignal® West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL).

Immunofluorescent histochemistry. Huh-7 cells were seeded onto 11-mm glass coverslips in 24-well plates at $2x10^4$ cells/well. The next day, the medium was replaced with serum-free medium, and the cells were pretreated with 20 μg/ml of DHMEQ, or vehicle (0.2% DMSO) alone for 2 h then stimulated with 200 U/ml of TNF-α for 60 min. The cells were fixed with 4% paraformaldehyde in PBS for 10 min at 4°C, immersed in -20°C methanol for 10 min, and incubated in blocking buffer (5% normal horse serum in PBS) for 1 h. The cells were incubated with polyclonal rabbit anti-human p65 (RelA) (Biogenesis, Poole, UK) for 1 h at room temperature, washed three times in PBS, incubated with rhodamine-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h, washed in PBS, and mounted in Vectashield® Mounting Medium (Vector Laboratories Inc., Burlingame, CA). Immunofluorescence for rhodamine was analyzed by Olympus BX50 microscope (Tokyo, Japan) and images were digitally captured using a Nikon DXM 1200 digital camera (Tokyo, Japan).

Western blotting. The following antibodies were used in the experiments; rabbit polyclonal anti-human Bcl-xL, rabbit polyclonal anti-human XIAP, mouse monoclonal anti-human cyclin D1, mouse monoclonal anti-human $p21^{Waf1/Cip1}$ (Cell Signaling Technology, Inc. Beverly, MA), rabbit polyclonal anti-human c-IAP2, mouse monoclonal anti-human Bax (B-9), rabbit polyclonal anti-human PTTG (Santa Cruz Biotechnology, Inc. Santa Cruz, CA), mouse monoclonal anti-human ß-actin (Sigma-Aldrich, Inc.). Cells were incubated with 20 μg/ml DHMEQ or vehicle (0.2% DMSO) alone for 24 h. Then cells were lysed by addition of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/ml PMSF, 1 μg/ml of aprotinin, 1% NP-40, 0.5% sodium deoxycholate) for 10 min at 4°C, and insoluble material was removed by centrifugation at 14,000 rpm for 30 min at 4°C. The same amount of protein from each lysate (20 μg /well) was subjected to 15% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membranes which were then blocked for 1.5 h using 5% non-fat dried milk in PBS containing 0.1% Tween-20 (PBS-T), washed with PBS-T and incubated at 4°C overnight in the presence of each primary antibody. The membranes were washed with PBS-T and incubated with sheep anti-mouse IgG or donkey anti-rabbit IgG coupled with horseradish peroxidase (Amersham Biosciences AB). The enhanced chemiluminescence system (SuperSignal® West Pico Chemiluminescent Substrate; Pierce Chemical Co.) was used for detection.

Determination of apoptosis and cell-cycle modulation. Cells ($4x10^5$) were plated in 100 mm dish, incubated overnight, and treated with 20 μg/ml of DHMEQ or vehicle (0.2% DMSO) alone for 24 h. Cells were washed in PBS and permeabilized with 70% ethanol at 4°C at least overnight. After centrifugation,

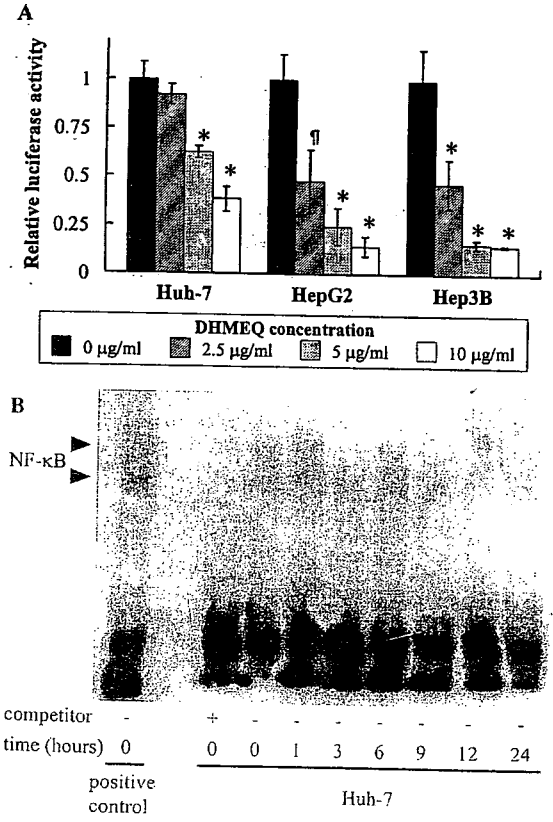

Figure 1. Effect of DHMEQ on NF-κB activity in human hepatoma cells. (A) pNFκB-luc was cotransfected with pRL-CMV-luc into Huh-7, HepG2, Hep3B cells. Six hours later, the cells were incubated with varying concentrations of DHMEQ for 24 h or vehicle (0.2% DMSO) alone as a control. Luciferase activity in the cells was analyzed by dual-luciferase assay. Data represent the ratios of firefly-luc activity derived from pNFκB-luc over renilla-luc activity derived from pRL-CMV-luc relative to the control, and are expressed as mean ± SD of three separate experiments, ¶P<0.01 versus control; *P<0.001 versus control. (B) Nuclear extracts from Huh-7 cells incubated with 20 μg/ml of DHMEQ for indicated periods were subjected to EMSA using a biotin-tagged NF-κB probe. For analysis of the specific binding to the κB sequence, a 100-times molar excess of the non-biotin-tagged NF-κB oligonucleotides was added to the sample as a competitor. The nuclear extract from HUT-78 cells containing NF-κB was used as a positive control. Results shown are from one representative experiment from a total of three performed.

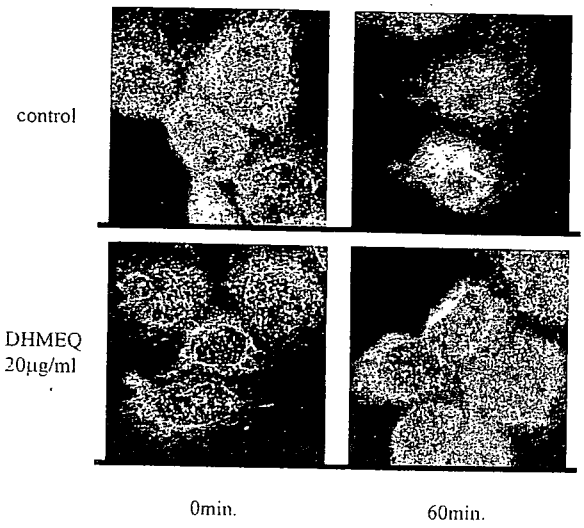

Figure 2. Inhibition of TNF-α-mediated nuclear translocation of p65 by DHMEQ in human hepatoma cells. Huh-7 cells were pretreated in the presence or absence of 20 μg/ml DHMEQ for 2 h and stimulated with TNF-α for the indicated time. Then the cells were fixed, permeabilized, processed for immunofluorescence using the p65-specific antibody, and visualized with fluorescence microscopy. Results shown are from one representative experiment from a total of three performed.

cell pellets were resuspended in 1 ml PBS, treated with 0.25 mg/ml Ribonuclease A (Sigma-Aldrich, Inc., St. Louis, MO, USA) at 37°C for 30 min, and stained with 50 μg/ml propidium iodide (Sigma-Aldrich, Inc.) for 30 min on ice. The DNA content in each cell nucleus was determined by an Epics XL flow cytometer (Beckman Coulter, Miami, FL).

In vivo study. All of the procedures involving animals and their care in this study were approved by the Ethics Committee of Nagasaki University in accordance with institutional and Japanese government guidelines for animal experiments. Four-week-old male BALB/c *nu/nu* athymic mice were obtained from Charles River Japan, Inc. Huh-7 cells ($3x10^6$) were implanted subcutaneously into the left thigh. Tumor volume was calculated according to the formula $a^2 \times b \times 0.5$, where a and b are the smallest and largest diameters, respectively. When the tumor volume reached 50 mm^3, mice were randomly assigned into two groups, and received intraperitoneal injection of 8 mg/kg DHMEQ or vehicle alone every other day for 18 days. Tumor size and body weight of mice were monitored at least every 4 days for 5 weeks.

Statistical analysis. The statistical analysis was performed using Student's t-test. Unless otherwise indicated, average values were expressed as mean values with SD. $P<0.05$ was considered as statistically significant.

Results

DHMEQ inhibits the constitutive NF-κB activity in human hepatoma cells. Effect of DHMEQ on the transcriptional activity of NF-κB in human hepatoma cells was determined by transient transfection assay using luciferase reporter plasmid, pNFκB-Luc, which contains four repeats of the binding sequence of NF-κB. DHMEQ dose-dependently repressed the transcriptional activity of NF-κB in Huh-7, HepG2 and Hep3B cells (Fig. 1A). These results suggest that DHMEQ inhibited the steady-state transcriptional activity of NF-κB in these cells. Next, NF-κB binding activity to the κB DNA site was analyzed by EMSA (Fig. 1B). Nuclear extracts from unstimulated Huh-7 cells and positive control cells formed two shifted bands, fast migrating and slow migrating. Addition of 100 times molar excess of unlabeled competitor DNA completely abrogated both bands, indicating that these bands corresponded to NF-κB-DNA complexes. Time-course study showed that 20 μg/ml of DHMEQ treatment diminished the NF-κB binding activity in Huh-7 cells in a time-dependent manner. These results suggested that NF-κB

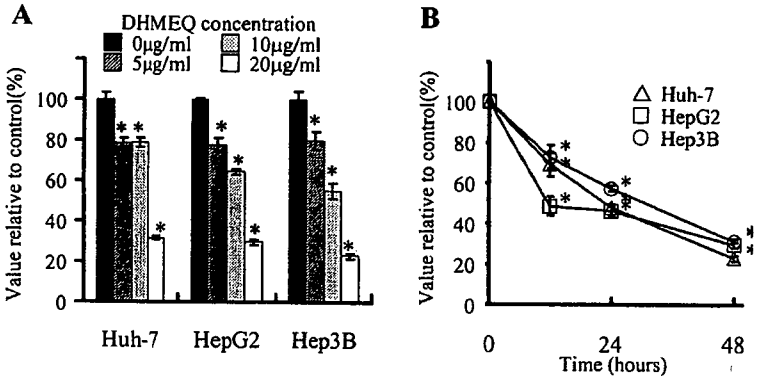

Figure 3. Cytotoxic effects of DHMEQ on human hepatoma cell. Huh-7, HepG2, Hep3B cells were treated with indicated concentrations of DHMEQ for 48 h (A), or the cells were incubated with 20 μg/ml of DHMEQ for indicated periods (B). Control cells were treated with the same concentration of DMSO as used in DHMEQ treatment. The cell numbers were counted with Particle counter. Each value represents the mean derived from at least four individual experiments, bars, ±SD. *P<0.001 versus control.

was constitutively activated in human hepatoma cells and DHMEQ inhibited its activity.

DHMEQ inhibits the TNF-α-mediated nuclear translocation of p65. We studied the effect of DHMEQ on the TNF-α-mediated nuclear translocation of p65, a component of NF-κB, in Huh-7 cells by immunofluorescence microscopy. After 60-min stimulation with 200 U/ml of TNF-α, p65 translocated from cytoplasm to nucleus. In contrast, pretreatment with 20 μg/ml of DHMEQ inhibited the TNF-α-mediated nuclear localization of p65 (Fig. 2).

DHMEQ induces apoptosis and cell-cycle arrest in human hepatoma cells. To elucidate the effect of DHMEQ on the viability of hepatoma cells, HuH-7, HepG2 and Hep3B were incubated with varying concentrations of DHMEQ for 48 h. The viable cell number was decreased in all hepatoma cells by DHMEQ in a dose-dependent manner (Fig. 3A). Similarly, DHMEQ at a concentration of 20 μg/ml decreased the viable cell number in a time-dependent manner (Fig. 3B). To examine whether DHMEQ induced apoptosis or cell-cycle arrest in hepatoma cells, we analyzed the contents of DNA in those cells using flow cytometry after propidium iodide staining. In all hepatoma cells, especially in Hep3B, DHMEQ increased the number of cells in the subG1 phase of cell-cycle, representing apoptotic cells (Fig. 4). In addition, Huh-7 and HepG2 cells treated with DHMEQ showed a decrease in the number of cells in the S-phase and an increase in the number of cells in the G0/G1 phase. These results suggest that DHMEQ reduced the viable cell number through inducing apoptosis and cell-cycle arrest at G0/G1 phase in hepatoma cells.

DHMEQ downregulates the expression of proteins involved in anti-apoptosis and cell-cycle progression. To elucidate the mechanism of cytotoxic effect of DHMEQ on human hepatoma cells, we examined the effect of DHMEQ on the expression of apoptosis-related proteins, including Bcl-xL, XIAP, c-IAP2, Bax by Western blotting. As shown in Fig. 5, Bcl-xL expression was downregulated by DHMEQ in Hep3B but not in HuH-7 and HepG2 cells. XIAP expression was downregulated by

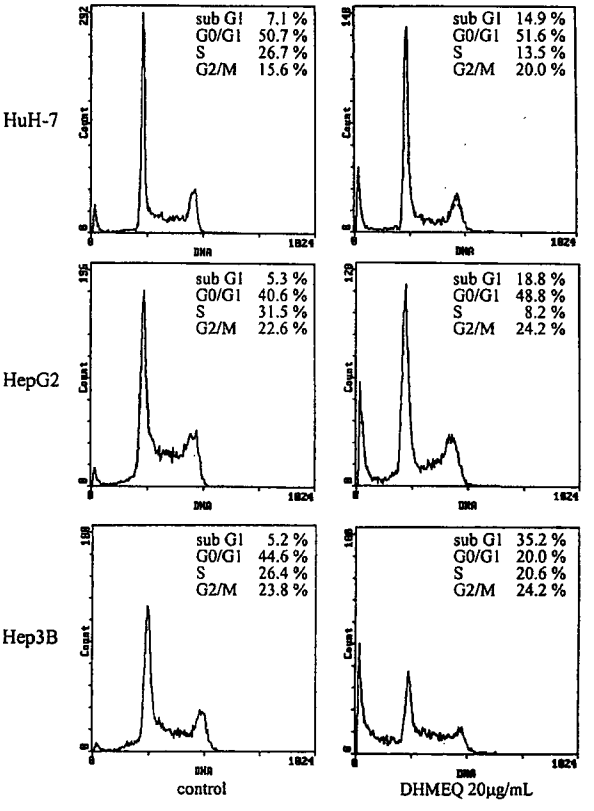

Figure 4. Determination of apoptosis and cell-cycle modulation by DHMEQ in human hepatoma cells. Huh-7, HepG2 and Hep3B cells were incubated with 20 μg/ml of DHMEQ or vehicle (0.2% DMSO) alone as a control for 24 h, cells were then stained with propidium iodide and subjected to DNA content analysis by flow cytometry. The percentages of cells in the sub G1, G0/G1, S and G2/M phase are indicated respectively. Results shown are from one representative experiment from a total of four performed.

DHMEQ in Huh-7 and HepG2 but not in Hep3B cells. c-IAP2 expression was downregulated by DHMEQ in Huh-7 and

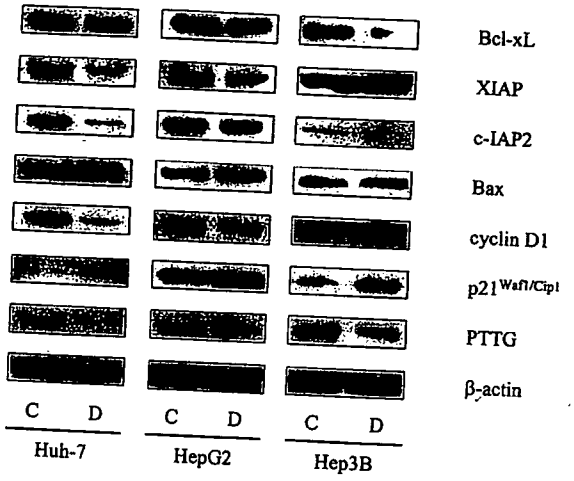

Figure 5. Effect of DHMEQ on the expression of several apoptosis-related and cell-cycle-related proteins in human hepatoma cells. Huh-7, HepG2 and Hep3B cells were incubated with 20 μg/ml of DHMEQ for 24 h or vehicle (0.2% DMSO) alone as a control, and the expression of apoptosis-related proteins [Bcl-xL, XIAP, c-IAP2 and Bax (B-9)] and cell-cycle-related proteins [cyclin D1, $p21^{Waf1/Cip1}$ and PTTG] and β-actin as an internal control in the cells was analyzed by Western blotting using the appropriate antibodies. Results shown are from one representative experiment from a total of four performed.

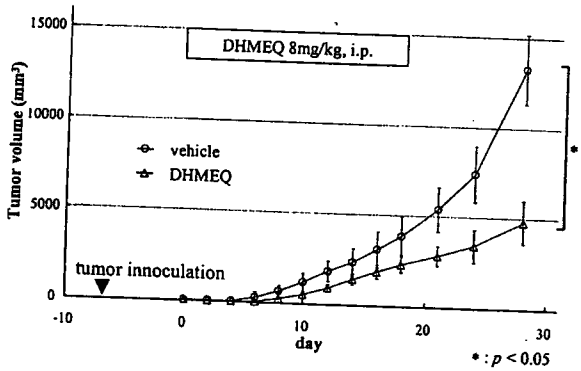

Figure 6. Anti-tumor effect of DHMEQ in xenograft models. Huh-7 cells were implanted into athymic mice subcutaneously, when the tumor volume reached 50 mm³, mice are treated with intraperitoneal injection of vehicle (0.1 ml of 50% DMSO) alone as a control; open circle, DHMEQ (8 mg/kg); open square, every other day for 18 days, respectively, then tumor growth was monitored as described in Materials and methods. Data represent the mean ± SD values (n=5), *P<0.05 versus control.

Hep3B but not in HepG2 cells. On the contrary, DHMEQ slightly stimulated Bax expression in all hepatoma cells. We next examined the effect of DHMEQ on the expression of cell-cycle regulating proteins, including cyclin D1, $p21^{Waf1/Cip1}$ and pituitary tumor transforming gene (PTTG). DHMEQ repressed the cyclin D1 expression in Huh-7 and HepG2 but not in Hep3B cells. In contrast, the expression of $p21^{Waf1/Cip1}$ which inhibits G0/G1 to S-phase transition (24) was upregulated by DHMEQ in all hepatoma cells. The expression of PTTG, a regulator of cell division (25), was downregulated by DHMEQ in all hepatoma cells.

Anti-tumor effect of DHMEQ in vivo. Huh-7 cells were subcutaneously implanted and tumors were established in athymic mice because Huh-7 cells were more efficiently transplantable than the other cells. After the tumor volume reached 50 mm³, a solution of DHMEQ (8 mg/kg) was injected into peritoneal space every other day for 18 days, and tumor size was monitored. Injection of DHMEQ significantly repressed the tumor growth compared with vehicle-injection (Fig. 6). DHMEQ treatment at the dosage used was well tolerated, and did not lead to weight loss or increase of serum transaminase attributable to toxicity (data not shown).

Discussion

In the present study, reporter assay using pNFκB-Luc revealed that DHMEQ dose-dependently inhibited the steady-state transcriptional activity of NF-κB in all hepatoma cells as reported in other cancer cells (14-21). The precise mechanism of inhibition of NF-κB activity by DHMEQ is still unclear, however, it has been reported that DHMEQ directly inhibits the nuclear translocation of NF-κB rather than the degradation of IκB-α which retains NF-κB in cytoplasm (11,26). EMSA assay showed that nuclear extracts from unstimulated Huh-7 cells formed NF-κB-DNA complexes which appeared as two shifted bands, and these complex formations were abolished by DHMEQ. We did not perform the super-shift assay to determine what components of NF-κB were included in these complexes. However, we have already reported using anti-p50 and p65 antibodies that fast migrating or slow migrating complex in Huh-7 cells corresponded to the p50/p50 homodimer or p65/p50 heterodimer, respectively (27). In addition, immunohistochemical study (Fig. 2) showed that DHMEQ inhibited the TNF-α-mediated nuclear translocation of p65 in Huh-7 cells. Taken together, it is possible that DHMEQ effectively inhibits constitutive and TNF-α-mediated nuclear translocation of NF-κB containing p65 in hepatoma cells.

In our study, the treatment of 20 μg/ml of DHMEQ for 48 h reduced the viable cell number to almost one third of control in all hepatoma cells. In addition, intraperitoneal injection of 8 mg/kg of DHMEQ significantly repressed the growth of Huh-7 hepatoma inoculated subcutaneously in athymic mice. Since the used concentrations of DHMEQ *in vitro* and *in vivo* were similar to the previous studies in prostatic cancer (14,15), thyroid cancer (16), multiple myeloma (17,18), breast cancer (19), and ATL cells (20,21), it is likely that the susceptibility of human hepatoma cells to DHMEQ is equivalent to other cancer cells. Previous studies have concluded that DHMEQ reduced the viable cell number through inducing apoptosis (14-21). In fact, 20 μg/ml of DHMEQ induced apoptosis in all hepatoma cells, especially in Hep3B cells. However, DHMEQ also reduced the number of cells in S-phase and increased that in G0/G1 phase in Huh-7 and HepG2 cells, suggesting that DHMEQ not only induced apoptosis but also inhibited the G0/G1 to S cell-cycle

progression in these cells. This was supported by the results from Western blotting (Fig. 5), in which the expression of cyclin D1, a regulator of G0/G1 to S progression, was downregulated by DHMEQ in Huh-7 and HepG2 cells. Our observation was consistent with the recent report that DHMEQ induced cell-cycle arrest at G0/G1 phase in ATL cells accompanying a decrease of cyclin D1 expression (21).

NF-κB such as a p65/p50 heterodimer regulates the expression of many genes involved in anti-apoptosis and cell-cycle progression (4-8). Of these, Bcl-xL, CIAP1,2, XIAP, FLIP, TRAF1,2 and cyclin D1 are well known NF-κB-target genes (4-8,28). In previous studies, DHMEQ repressed the expression of Bcl-xL, CIAP1, 2, XIAP, FLIP and cyclin D1 in several cancer cell types (14-21), by which DHMEQ could promote apoptosis and block cell-cycle progression in cancer cells. However, the effects of DHMEQ on the expression of these genes were different in the cancer cells used. For instance, DHMEQ downregulated the expression of Bcl-xL in thyroid cancer cells, in ATL, and U266 myeloma cells (16,17), but not in prostatic cancer cells and 12PE myeloma cells (14,17). Similar phenomenon was observed in our study. DHMEQ did not equally downregulate the expression of Bcl-xL, c-IAP2, XIAP and cyclin D1 in three hepatoma cell types. Since these genes are regulated not only by NF-κB but also by other transcriptional factors, the dependence of the gene expression on NF-κB may be different in each hepatoma cell type. In this study, in addition to cyclin D1, the expression of $p21^{Waf1/Cip1}$ and PTTG was modulated by DHMEQ. Although the expression of these genes is not directly regulated by NF-κB, altered expression of these proteins may, at least in part, mediate the anti-tumor effect of DHMEQ in hepatoma cells.

NF-κB is thought to a molecular target in the treatment of cancer (6,29,30). A phase I clinical study of PS341, a proteasome inhibitor, which represses the NF-κB activity through stabilizing IκB protein and induces apoptosis in cultured cancer cells including hepatoma cells (31,32) is ongoing in patients with advanced cancer (33). In the present study, we have demonstrated that DHMEQ exhibited anti-tumor activity against human hepatoma cells through inhibiting NF-κB activity as reported in other cancer cells. Therefore, DHMEQ is also a promising candidate as a therapeutic agent in patients with advanced cancer including HCC.

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Aging of patients with hepatitis C virus-associated hepatocellular carcinoma: Long-term trends in Japan

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Received March 21, 2006; Accepted June 13, 2006

Abstract. The incidence of hepatocellular carcinoma (HCC) in Japan has been increasing. The aim of the present study was to analyze epidemiological changes in Japanese HCC patients. A total of 463 patients with HCC diagnosed at our hospital between 1982 and 2001 were recruited for this study. Cohorts of patients with HCC were categorized into intervals of five years. The number of HBV- and HCV-associated HCC cases had decreased and increased in 1987-1991, respectively, and thereafter reached a plateau. The mean age of patients at diagnosis of HCV-associated HCC showed a steady significant increase from 60 to 68 years of age during the period, suggesting that these findings were associated with a shift toward an older-age group that had the highest rate of HCV infection. The mean age of patients with other types of HCC did not significantly change during the period. Since it is known that the prevalence of HCV infection in young Japanese persons is low and that the incidence of HCV infection is very low at present, our findings may indicate that the prevalence of HCC will decline in Japan, an advanced country with regard to HCV-associated HCC, in the near future.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary cancer of the liver. HCC accounts for approximately 6% of all human cancers. It is estimated that half a million cases occur worldwide annually, making HCC the fifth most common malignancy in men and the ninth in women (1-6). The age-adjusted HCC mortality rate has increased in recent decades in Japan (7). Similarly, a trend of increasing rates of HCC has been reported from several developed countries in North America, Europe and Asia (8,9). HCC often develops in patients with liver cirrhosis caused by hepatitis B virus (HBV), hepatitis C virus (HCV), excessive alcohol consumption or non-alcoholic fatty liver disease. Of the hepatitis viruses that cause HCC, HCV is more common than HBV in Japan (10-13).

Although the age-adjusted incidence rates of HCC have increased during the period of rising HCC mortality in Japan, sequential changes in background features of HCC patients are not fully understood (14). Yoshizawa *et al* report that deaths due to HCC in Japan have continued to increase in males, particularly in those older than 60 years of age in the past 3 decades, although the reasons for this are unclear (15). To clarify factors affecting epidemiological changes in Japanese HCC patients, especially the change in age distribution, we analyzed the underlying features of HCC patients in a single-center, hospital-based study, including demographic data, etiology and stage of liver disease, and tumor characteristics.

Patients and methods

Patients. A total of 463 patients with HCC diagnosed between January 1982 and December 2001 in the First Department of Internal Medicine, Nagasaki University School of Medicine, were recruited for this study. The diagnosis of HCC was based on AFP levels and imaging techniques including ultrasonography (USG), computerized tomography (CT), magnetic resonance imaging (MRI), hepatic angiography (HAG), and/or liver biopsy. The diagnostic criteria for HCC were either a confirmative liver biopsy or elevated AFP (≥20 ng/ml) and neovascularization in HAG and/or CT. Cohorts of patients with HCC were categorized into five-year intervals (1982-1986, 1987-1991, 1992-1996 and 1997-2001).

Of the persons who visited Nagasaki Tarami hospital for health screening during the period from January 1996 to December 2002, 13869 were first-time visitors. There are same region of Nagasaki University School of Medicine and Nagasaki Tarami hospital. All of them received blood screening for the anti-HCV antibody (HCVAb) and the data were

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Key words: hepatitis C virus, hepatocellular carcinoma, aging, Japan

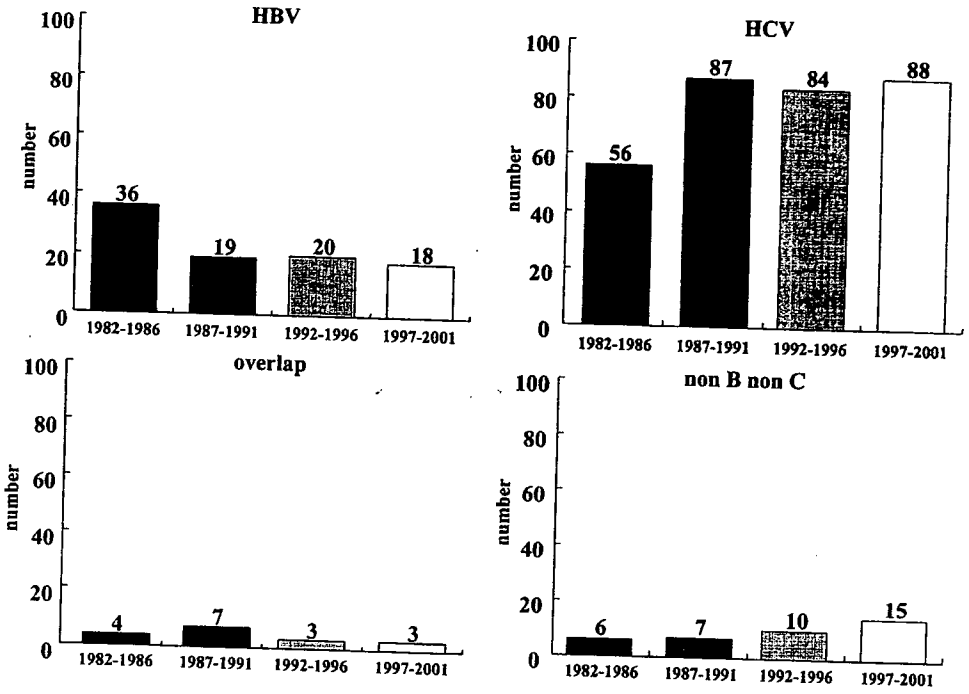

Figure 1. Sequential changes in the number of HCC patients categorized by etiology during the observation period. *P<0.05.

representative of the prevalence of HCV infection in the general population of Nagasaki prefecture, Japan.

Etiology of HCC. Sera were stored at -80°C. A diagnosis of chronic HCV infection was based on the presence of HCVAb (microparticle enzyme immunoassay; Abbott Laboratories) and HCV-RNA detected by polymerase chain reaction (PCR), whereas diagnosis of chronic HBV infection was based on the presence of hepatitis B surface antigen (HBsAg) (enzyme-linked immunosorbent assay; Abbott Laboratories). Serum AFP was measured by a radioimmunoassay (Abbott Laboratories). The history of alcohol intake was noted from medical records. Habitual drinking was defined as an average daily consumption of an amount equivalent to 80 g of pure ethanol over a period of more than 10 years.

Statistical analysis. The data were analyzed by the Mann-Whitney test for the continuous ordinal data between two qualitative variables. For multiple group comparisons, homogeneity of variance was assessed by the Levene test. Parametric comparisons used analysis of variance (ANOVA). The significance of individual differences was evaluated by using the Scheffé test. The standard deviation was calculated based on the binomial model for the response proportion. $P<0.05$ was considered statistically significant.

Results

Clinical features of the studied patients. A total of 463 patients with HCC were diagnosed at our hospital from 1982 to 2001. There were 362 male (78.2%) and 101 female (21.8%) patients, with a mean age of 63 years. The proportion of patients diagnosed with HBV-associated HCC was 20.1% (93 of 463), whereas 68.0% (315 of 463) had HCV-associated HCC, and an additional 3.7% (17 of 463) had HCC associated with both viruses. Seven of the other 38 patients had a history of significant alcohol intake and the remaining 31 had no known etiology.

As shown in Figs. 1 and 2, the number of HBV-associated HCC cases decreased in 1987 -1991 and thereafter stabilized, whereas HCV-associated HCC increased and reached a plateau in 1987-1991. On the other hand, the mean age at diagnosis of HCV-associated HCC steadily increased, although patients with other types of HCC had no significant change during the observation period. Fig. 3 shows the age distribution of patients with HBV- and HCV-associated HCC during the four 5-year periods. There was no difference in the age distribution of patients with HBV-associated HCC during these periods. In contrast, HCV-associated HCC obviously had an increase in the number of patients aged more than 60 years.

Background features for patients with HBV- and HCV-associated HCC. To examine the factors affecting the change in age distribution, the mean age of patients with HBV- and HCV-associated HCC was analyzed according to each background in Tables I and II, respectively. The mean age of patients with HBV-associated HCC was not significantly different except for gender. On the other hand, in HCV-associated HCC, patients with excessive alcohol consumption, diabetes mellitus or Child-Pugh stage C in addition to male gender were younger age than those without as described above.