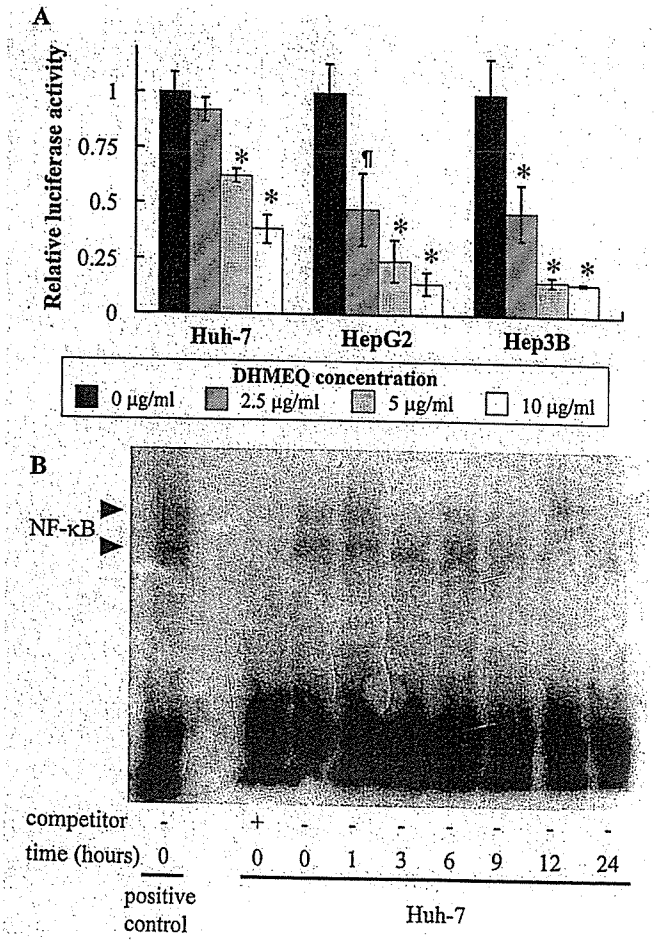

Figure 1. Effect of DHMEQ on NF-κB activity in human hepatoma cells. (A) pNFκB-luc was cotransfected with pRL-CMV-luc into Huh-7, HepG2, Hep3B cells. Six hours later, the cells were incubated with varying concentrations of DHMEQ for 24 h or vehicle (0.2% DMSO) alone as a control. Luciferase activity 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.

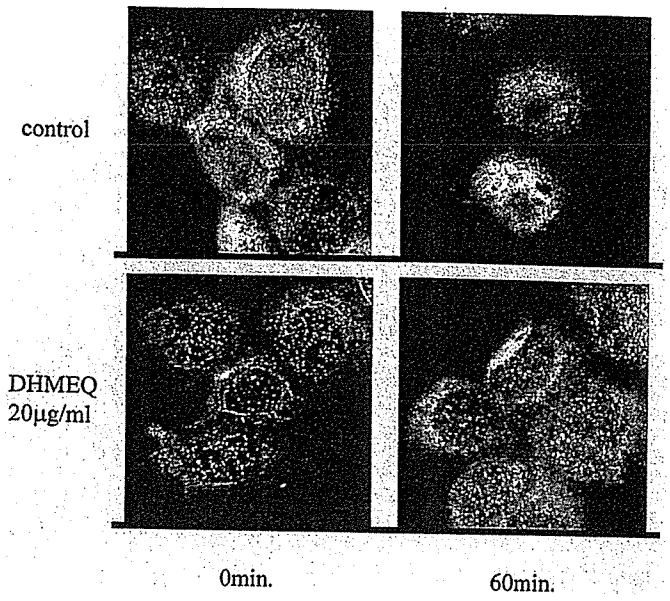

Figure 2. Inhibition of TNF-α-mediated nuclear translocation of p65 by DHMEQ in human hepatoma cells. Huh-7 cells were pretreated in the presence or absence of 20 μg/ml DHMEQ for 2 h and stimulated with TNF-α for the indicated time. Then the cells were fixed, permeabilized, processed for immunofluorescence 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 x b x 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

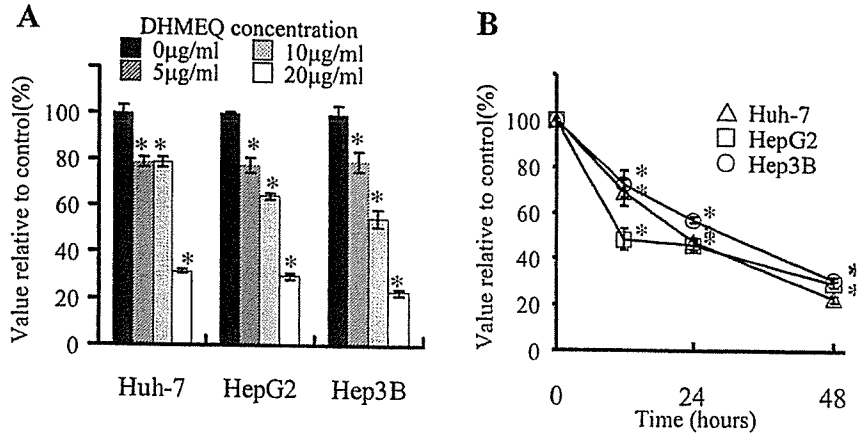

Figure 3. Cytotoxic effects of DHMEQ on human hepatoma cell. Huh-7, HepG2, Hep3B cells were treated with indicated concentrations of DHMEQ for 48 h (A), or the cells were incubated with 20 μg/ml of DHMEQ for indicated periods (B). Control cells were treated with the same concentration of DMSO as used 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

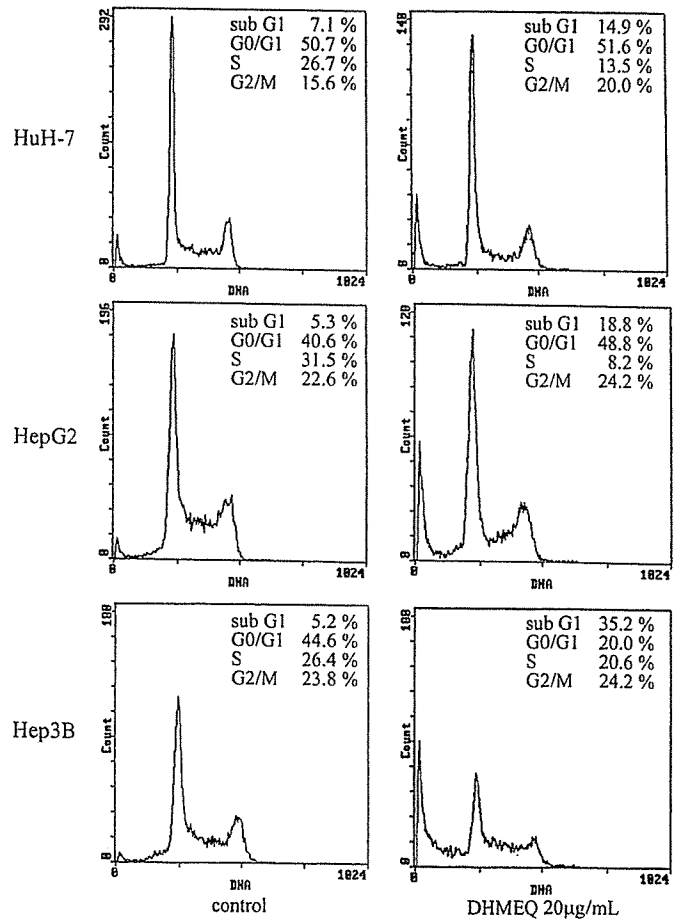

Figure 4. Determination of apoptosis and cell-cycle modulation by DHMEQ in human hepatoma cells. Huh-7, HepG2 and Hep3B cells were incubated with 20 μg/ml of DHMEQ or vehicle (0.2% DMSO) alone as a control for 24 h, cells were then stained with propidium iodide and subjected to DNA content analysis by 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

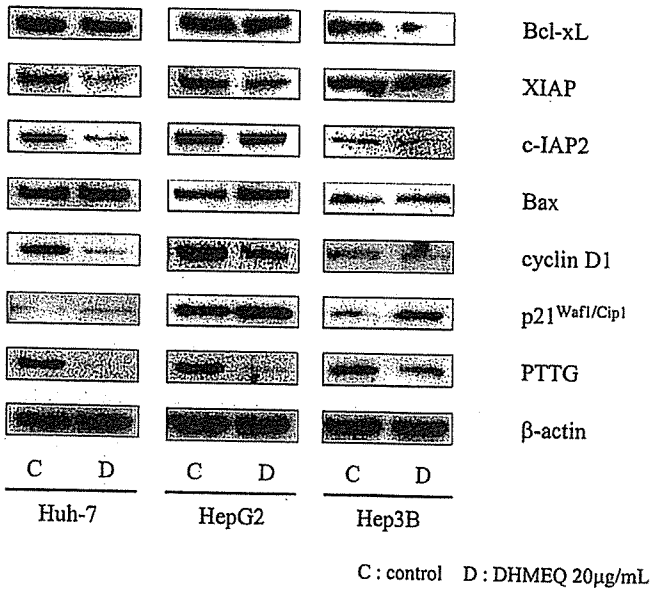

Figure 5. Effect of DHMEQ on the expression of several apoptosis-related and cell-cycle-related proteins in human hepatoma cells. Huh-7, HepG2 and Hep3B cells were incubated with 20 μg/ml of DHMEQ for 24 h or vehicle (0.2% DMSO) alone as a control, and the expression of apoptosis-related proteins [Bcl-xL, XIAP, c-IAP2 and Bax (B-9)] and cell-cycle-related proteins [cyclin D1, $p21^{Waf1/Cip1}$ and PTTG] and ß-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.

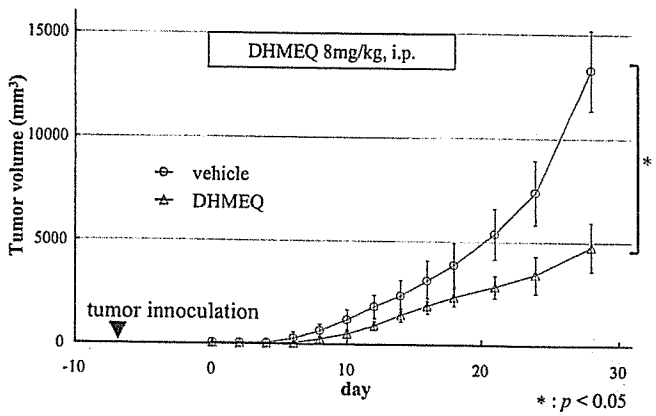

Figure 6. Anti-tumor effect of DHMEQ in xenograft models. Huh-7 cells were implanted into athymic mice subcutaneously, when the tumor volume reached 50 mm^3, mice are treated with intraperitoneal injection of vehicle (0.1 ml of 50% DMSO) alone as a control; open circle, DHMEQ (8 mg/kg); open square, 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 mm^3, 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 down-regulated 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.

References

1. Bosch FX, Ribes J, Diaz M and Cleries R: Primary liver cancer: worldwide incidence and trends. Gastroenterology 127: S5-S16, 2004.
2. Carr BI: Hepatocellular carcinoma: current management and future trends. Gastroenterology 127: S218-S224, 2004.
3. El-Serag HB: Hepatocellular carcinoma: an epidemiologic view. J Clin Gastroenterol 35: S72-S78, 2002.
4. Karin M and Lin A: NF-kappaB at the crossroads of life and death. Nat Immunol 3: 221-227, 2002.
5. Kucharczak J, Simmons MJ, Fan Y and Gelinas C: To be, or not to be: NF-kappaB is the answer - role of Rel/NF-kappaB in the regulation of apoptosis. Oncogene 22: 8961-8982, 2003.
6. Luo JL, Kamata H and Karin M: IKK/NF-kappaB signaling: balancing life and death - a new approach to cancer therapy. J Clin Invest 115: 2625-2632, 2005.
7. Pikarsky E, Porat RM, Stein I, *et al*: NF-kappaB functions as a tumour promoter in inflammation-associated cancer. Nature 431: 461-466, 2004.
8. Perkins ND: NF-kappaB: tumor promoter or suppressor? Trends Cell Biol 14: 64-69, 2004.
9. Tai DI, Tsai SL, Chang YH, *et al*: Constitutive activation of nuclear factor kappaB in hepatocellular carcinoma. Cancer 89: 2274-2281, 2000.
10. Matsumoto N, Ariga A, To-e S, *et al*: Synthesis of NF-kappaB activation inhibitors derived from epoxyquinomicin C. Bioorg Med Chem Lett 10: 865-869, 2000.
11. Ariga A, Namekawa J, Matsumoto N, Inoue J and Umezawa K: Inhibition of tumor necrosis factor-alpha-induced nuclear translocation and activation of NF-kappa B by dehydroxymethylepoxyquinomicin. J Biol Chem 277: 24625-24630, 2002.
12. Miyajima A, Kosaka T, Seta K, Asano T, Umezawa K and Hayakawa M: Novel nuclear factor kappa B activation inhibitor prevents inflammatory injury in unilateral ureteral obstruction. J Urol 169: 1559-1563, 2003.
13. Takatsuna H, Asagiri M, Kubota T, *et al*: Inhibition of RANKL-induced osteoclastogenesis by (-)-DHMEQ, a novel NF-kappaB inhibitor, through downregulation of NFATc1. J Bone Miner Res 20: 653-662, 2005.
14. Kikuchi E, Horiguchi Y, Nakashima J, *et al*: Suppression of hormone-refractory prostate cancer by a novel nuclear factor kappaB inhibitor in nude mice. Cancer Res 63: 107-110, 2003.
15. Kuroda K, Horiguchi Y, Nakashima J, *et al*: Prevention of cancer cachexia by a novel nuclear factor kappaB inhibitor in prostate cancer. Clin Cancer Res 11: 5590-5594, 2005.
16. Starenki DV, Namba H, Saenko VA, *et al*: Induction of thyroid cancer cell apoptosis by a novel nuclear factor kappaB inhibitor, dehydroxymethylepoxyquinomicin. Clin Cancer Res 10: 6821-6829, 2004.
17. Tatetsu H, Okuno Y, Nakamura M, *et al*: Dehydroxymethyl-epoxyquinomicin, a novel nuclear factor-kappaB inhibitor, induces apoptosis in multiple myeloma cells in an IkappaBalpha-independent manner. Mol Cancer Ther 4: 1114-1120, 2005.
18. Watanabe M, Dewan MZ, Okamura T, *et al*: A novel NF-kappaB inhibitor DHMEQ selectively targets constitutive NF-kappaB activity and induces apoptosis of multiple myeloma cells *in vitro* and *in vivo*. Int J Cancer 114: 32-38, 2005.
19. Matsumoto G, Namekawa J, Muta M, *et al*: Targeting of nuclear factor kappaB Pathways by dehydroxymethylepoxyquinomicin, a novel inhibitor of breast carcinomas: antitumor and anti-angiogenic potential *in vivo*. Clin Cancer Res 11: 1287-1293, 2005.
20. Ohsugi T, Kumasaka T, Ishida A, *et al*: *In vitro* and *in vivo* antitumor activity of the NF-kappaB inhibitor DHMEQ in the human T-cell leukemia virus type I-infected cell line, HUT-102. Leuk Res: 2005.
21. Watanabe M, Ohsugi T, Shoda M, *et al*: Dual targeting of transformed and untransformed HTLV-1-infected T-cells by DHMEQ, a potent and selective inhibitor of NF-kappaB, as a strategy for chemoprevention and therapy of adult T cell leukemia. Blood: 2005.
22. Nakao K, Nakata K, Yamashita M, *et al*: p48 (ISGF-3gamma) is involved in interferon-alpha-induced suppression of hepatitis B virus enhancer-1 activity. J Biol Chem 274: 28075-28078, 1999.
23. Beck Z, Bacsi A, Liu X, *et al*: Differential patterns of human cytomegalovirus gene expression in various T-cell lines carrying human T-cell leukemia-lymphoma virus type I: role of Tax-activated cellular transcription factors. J Med Virol 71: 94-104, 2003.
24. Weinberg WC and Denning MF: P21Waf1 control of epithelial cell cycle and cell fate. Crit Rev Oral Biol Med 13: 453-464, 2002.
25. Yu R, Ren SG, Horwitz GA, Wang Z and Melmed S: Pituitary tumor transforming gene (PTTG) regulates placental JEG-3 cell division and survival: evidence from live cell imaging. Mol Endocrinol 14: 1137-1146, 2000.
26. Yamamoto Y and Gaynor RB: IkappaB kinases: key regulators of the NF-kappaB pathway. Trends Biochem Sci 29: 72-79, 2004.
27. Shigeno M, Nakao K, Ichikawa T, *et al*: Interferon-alpha sensitizes human hepatoma cells to TRAIL-induced apoptosis through DR5 upregulation and NF-kappa B inactivation. Oncogene 22: 1653-1662, 2003.

28. Hinz M, Krappmann D, Eichten A, Heder A, Scheidereit C and Strauss M: NF-kappaB function in growth control: regulation of cyclin D1 expression and G0/G1-to-S-phase transition. Mol Cell Biol 19: 2690-2698, 1999.
29. Amit S and Ben-Neriah Y: NF-kappaB activation in cancer: a challenge for ubiquitination- and proteasome-based therapeutic approach. Semin Cancer Biol 13: 15-28, 2003.
30. Yamamoto Y and Gaynor RB: Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. J Clin Invest 107: 135-142, 2001.
31. Sunwoo JB, Chen Z, Dong G, *et al*: Novel proteasome inhibitor PS-341 inhibits activation of nuclear factor-kappa B, cell survival, tumor growth, and angiogenesis in squamous cell carcinoma. Clin Cancer Res 7: 1419-1428, 2001.
32. Chiao PJ, Na R, Niu J, Sclabas GM, Dong Q and Curley SA: Role of Rel/NF-kappaB transcription factors in apoptosis of human hepatocellular carcinoma cells. Cancer 95: 1696-1705, 2002.
33. Dy GK, Thomas JP, Wilding G, *et al*: A phase I and pharmacologic trial of two schedules of the proteasome inhibitor, PS-341 (bortezomib, velcade), in patients with advanced cancer. Clin Cancer Res 11: 3410-3416, 2005.

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

NAOTA TAURA[1], KEISUKE HAMASAKI[1], KAZUHIKO NAKAO[1], TATSUKI ICHIKAWA[1], DAISUKE NISHIMURA[1], TAKASHI GOTO[1], MARIKO FUKUTA[1], HIROSHI KAWASHIMO[1], HISAMITSU MIYAAKI[1], MASUMI FUJIMOTO[1], KOUICHIRO KUSUMOTO[1], YASUHIDE MOTOYOSHI[1], HIDETAKA SHIBATA[1], KAORU INOKUCHI[2] and KATSUMI EGUCHI[1]

[1]The First Department of Internal Medicine, Nagasaki University School of Medicine, Sakamoto 1-7-1, Nagasaki 852-8501; [2]Nagasaki Tarami Hospital, Kayana 986-2, Tarami, Isahaya, Nagasaki 859-0497, Japan

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

Correspondence to: Dr Naota Taura, The First Department of Internal Medicine, Nagasaki University School of Medicine, Sakamoto 1-7-1, Nagasaki 852-8501, Japan
E-mail: ntaura-gi@umin.ac.jp

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

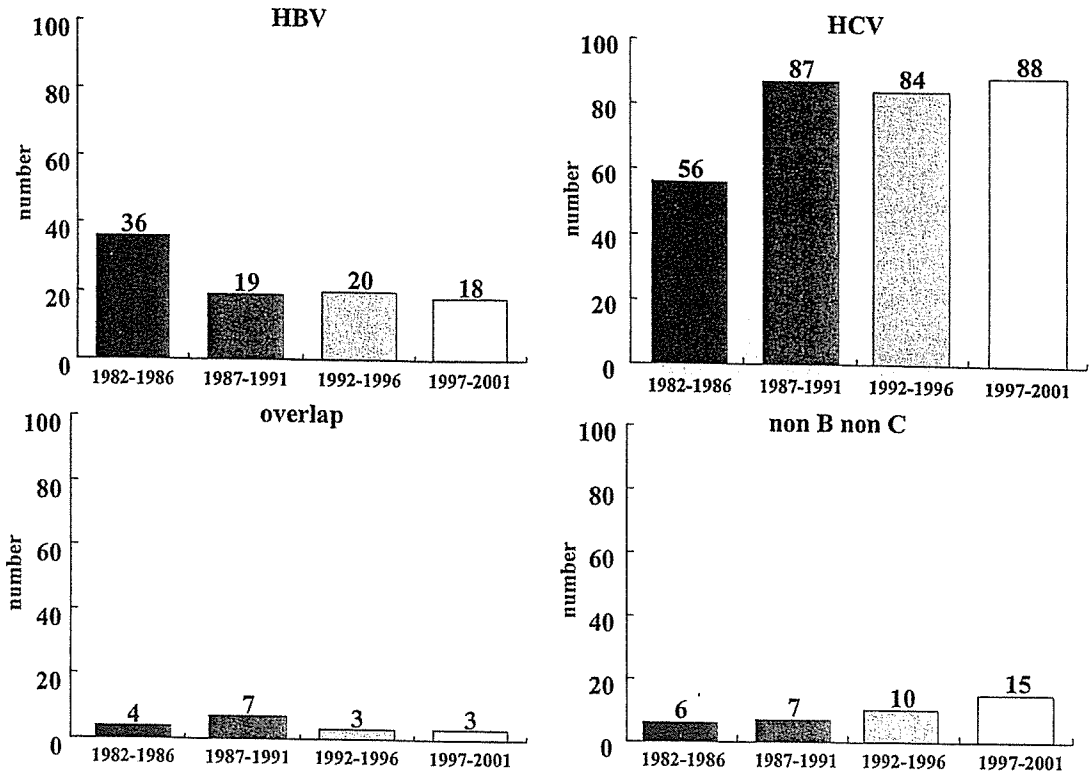

Figure 1. Sequential changes in the number of HCC patients categorized by etiology during the observation period. *P<0.05.

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.

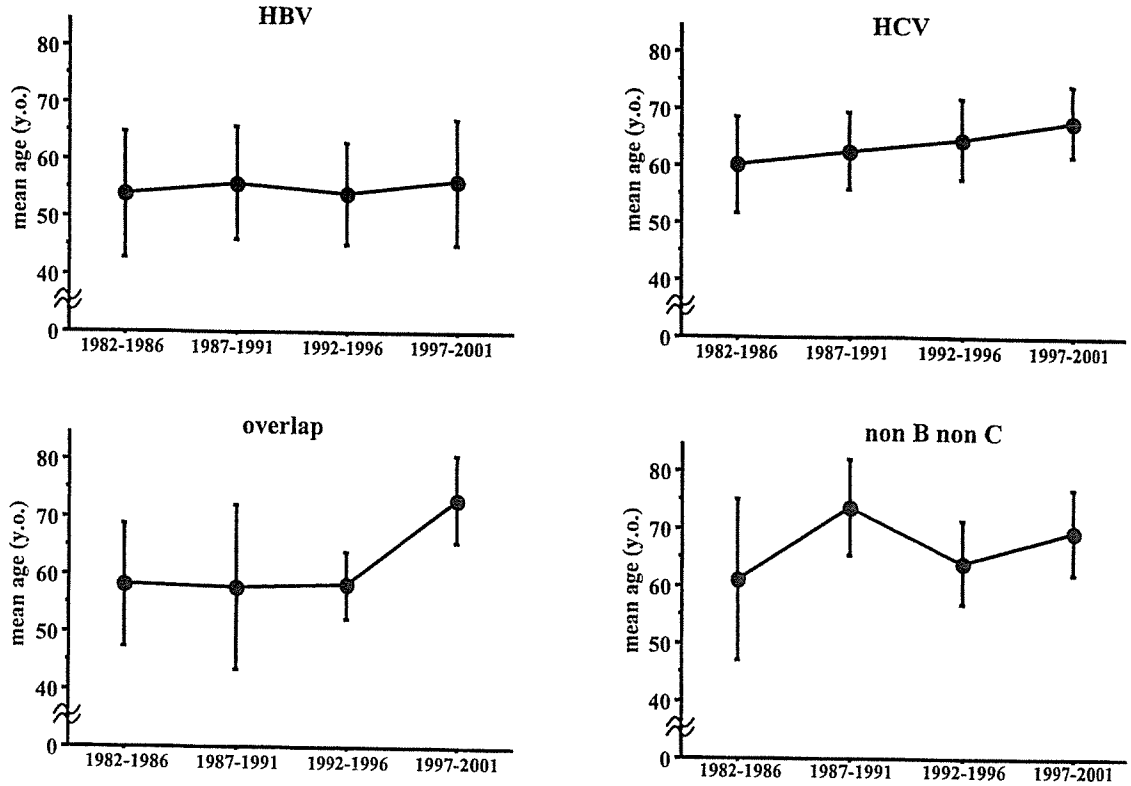

Figure 2. Sequential changes in the mean age of HCC patients categorized by etiology during the observation period. *P<0.05. The bars is standard deviation (SD).

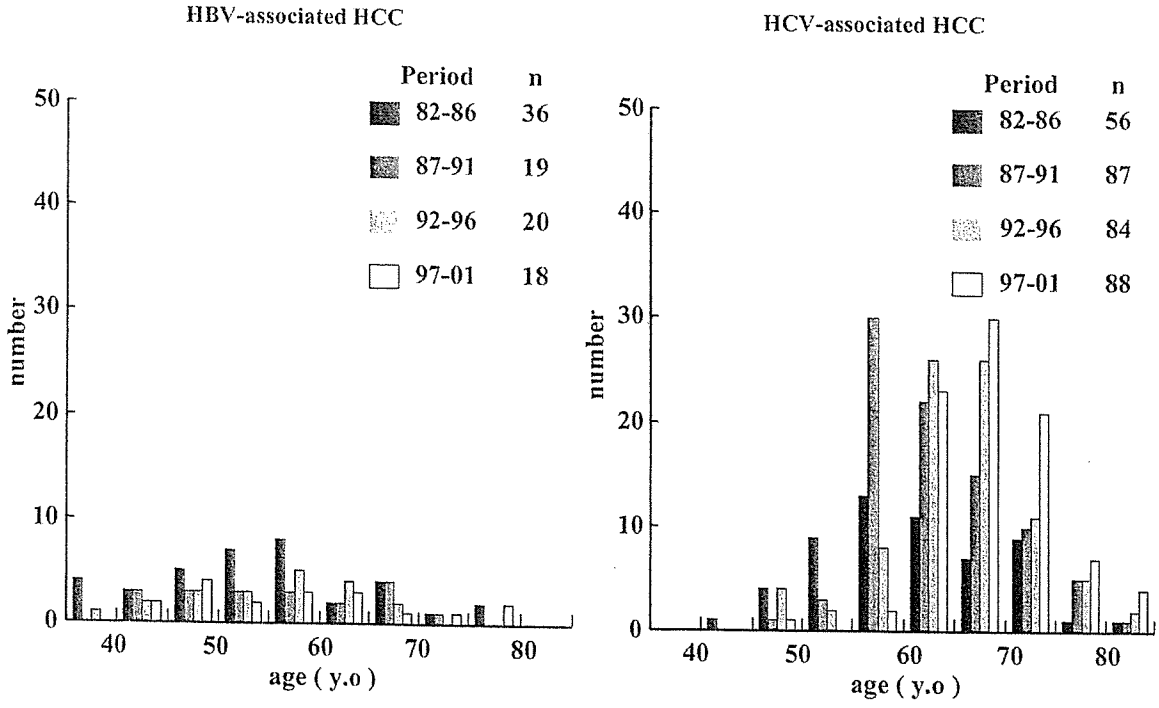

Figure 3. The age distribution of patients with HBV- and HCV-associated HCC during the four 5-year periods.

Table III shows the mean age and other characteristics at diagnosis of HCV-associated HCC in five-year intervals (1982-1986, 1987-1991, 1992-1996, and 1997-2001). In addition to mean age, the number of patients with Child-Pugh stage A showed a significant increase during the studied periods. Alcohol consumers significantly decreased during the periods.

In analysis of patients without alcohol consumption and Child-Pugh stage C in HCV-associated HCC, the mean ages in 1982-1986, 1987-1991, 1992-1996 and 1997-2001 were

Table I. The background of the 93 patients with HBV-associated HCC.

	No.	(%)	Mean age	(SD)	P-value
All	93	100	55	(10)	
Gender					
Male	71	76	53	(10)	
Female	22	24	60	(9)	0.0065[a]
Alcohol consumption					
Not excessive	84	90	55	(10)	
Excessive	9	10	52	(8)	NS[a]
IFN therapy					
(-)	91	98	55	(10)	
(+)	2	2	51	(3)	NS[a]
BMI					
<25	76	82	56	(10)	
≥25	17	18	54	(9)	NS[a]
Diabetes mellitus					
(-)	83	89	55	(11)	
(+)	10	11	58	(10)	NS[a]
Child-Pugh staging					
A	57	61	55	(1)	
B	28	30	56	(2)	
C	8	9	50	(3)	NS[b]
Tumor size					
<3 cm	38	41	56	(9)	
≥3 cm	55	59	54	(11)	NS[a]
Tumor no.					
Single	50	54	56	(10)	
Multiple	43	46	53	(10)	NS[a]

[a]Mann-Whitney U test. [b]ANOVA. SD, standard deviation; NS, not significant.

Table II. The background of the 315 patients with HBV-associated HCC.

	No.	(%)	Mean age	(SD)	P-value
All	315	100	64	(7)	
Gender					
Male	251	80	64	(7)	
Female	64	20	67	(7)	0.0032[a]
Alcohol consumption					
Not excessive	266	84	65	(7)	
Excessive	49	16	62	(7)	0.0107[a]
IFN therapy					
(-)	298	95	64	(8)	
(+)	17	5	66	(4)	NS[a]
BMI					
<25	255	81	63	(7)	
≥25	60	19	65	(8)	NS[a]
Diabetes mellitus					
(-)	229	73	65	(8)	
(+)	86	27	63	(7)	0.0173[a]
Child-Pugh staging					
A	207	66	65	(7)	
B	93	30	64	(8)	
C	15	4	60	(10)	0.0181[b]
Tumor size					
<3 cm	136	43	65	(7)	
≥3 cm	179	57	64	(8)	NS[a]
Tumor no.					
Single	165	52	65	(8)	
Multiple	150	48	64	(7)	NS[a]

[a]Mann-Whitney U test. [b]ANOVA. SD, standard deviation; NS, not significant.

62, 63, 64 and 68 years of age, respectively (1982-1986 vs. 1997-2001, p=0.0001) (Table IV).

Age-specific prevalence of HCV infection in the general population of studied area. The age-specific prevalence of HCVAb among the 13869 persons who visited Nagasaki prefecture Tarami Hospital for health screening from 1996 to 2002 is shown in Table V. Although the positive rate for HCVAb was 1.64% (277 of 13869) as a whole, it was higher in the group aged more than 60 years irrespective of gender.

Discussion

Our study was a single-center, hospital-based study designed to examine the sequential change in backgrounds among patients with HCC during the past 2 decades. More than 90% of our patients had chronic HBV or HCV infections. During the observation period, the number of HBV- and HCV-associated HCC cases decreased and increased in 1987-1991, respectively, and thereafter reached a plateau. These findings were consistent with previous reports from Japan (7,14). Additionally, the age-

Table III. The mean age and the other characteristics of HCV-associated HCC at diagnosis in 5-year intervals.

Period	1982-1986	1987-1991	1992-1996	1997-2001	Total	P-value
No.	56	87	84	88	315	
Age (years) (SD)	60 (8)	63 (7)	65 (8)	68 (6)	64 (7)	<0.0001
Gender						
Male	47	68	67	69	251	
Female	9	19	17	19	64	
Ratio	5.2	3.6	3.9	3.6	3.9	NS
Alcohol consumption						
Not excessive	42	68	75	81	266	
Excessive	14	19	9	7	49	
Ratio	3.0	3.6	8.3	11.6	5.4	0.0078
Diabetes mellitus						
(-)	39	64	64	62	229	
(+)	17	23	20	26	86	
Ratio	2.3	2.8	3.2	2.4	2.7	NS
Child-Pugh staging						
A	28	52	57	70	207	
B	25	32	22	14	93	
C	3	3	5	4	15	0.0160

SD, standard deviation; NS, not significant.

Table IV. Mean age of HCV-associated HCC without excessive alcohol consumers and Child-Pugh stage C.

Year	1982-1986	1987-1991	1992-1996	1997-2001	Total
No.	40	66	71	77	254
Mean age (years)	62	63	64	68	65
SD	8	7	7	6	7
	NS	NS	0.0338		
	NS				
	0.0012				
	0.0001				

SD, standard deviation; NS, not significant.

specific prevalence of HCV infection in the general population of the studied area was also in agreement with Japanese epidemiological studies which showed a high prevalence of HCVAb in the population, 60 years of age and older (15-17).

In analysis of background features among HCC patients, HBV-associated HCC cases revealed no significant change, whereas the mean age of patients with HCV-associated HCC steadily increased from 60 to 68 years of age during the studied period. In patients with HCV-associated HCC, factors such as male gender, excessive alcohol consumption and diabetes mellitus, which are known to be risk factors for HCC, contributed to lowering the age of HCC occurrence. Furthermore, patients with Child-Pugh stage C were younger than those with stages A and B. When the mean age of HCV patients without alcohol consumption and Child-Pugh stage C, which may contribute to aging of HCV-associated HCC, was

Table V. Age-specific prevalence of HCV infection in Nagasaki prefecture, Japan.

	Male			Female			Total		
Age	No.	HCVAb(+)	(%)	No.	HCVAb(+)	(%)	No.	HCVAb(+)	(%)
0-19	0	0	0.00	0	0	0.00	0	0	0.00
20-29	7	0	0.00	7	0	0.00	7	0	0.00
30-39	594	3	0.51	303	4	1.32	897	7	0.78
40-49	2553	29	1.14	1051	9	0.86	3604	38	1.05
50-59	3188	38	1.16	1445	19	1.31	4633	56	1.21
60-69	2517	58	2.30	1309	37	2.83	3826	95	2.48
≥70	515	21	4.08	380	10	2.63	895	31	3.46
Total	9374	148	1.58	4495	79	1.76	13869	277	1.64

analyzed, a significant increase was also found during the studied period. Since the size and number of HCC cases was not associated with the mean age of HCV-associated HCC patients, it is unlikely that the delay in diagnosis of HCC accounted for aging of HCV-associated HCC cases. Indeed, it is possible that other factors contributed to the steady increase in the mean age of patients with HCV-associated HCC.

Japan with overall HCV prevalence rates similar to that of the United States (approximately 1-2%) but with higher incidence rates of hepatocellular carcinoma (8-10 times lower in the United States) is thought to have had earlier onset and peaks of the HCV epidemic than the United States (5,8,9). A recent study examined the constant evolutionary rate of HCV over time ('the molecular clock') in retrospectively collected serum samples of HCV carriers in Japan and the United States (18). The study concluded that HCV first appeared in Japan around 1882 and in the United States around 1910, whereas widespread dissemination occurred from 1940s to 1960s in Japan and from 1960s to 1980s in the United States. Risk factors for transmitting HCV were rampant during this period (e.g, injection drug use, needle sharing, and transfusion of unscreened blood and blood products) (19). It is speculated that these modes of transmission are responsible for differences in the age-specific prevalence of HCV infection in the general population. In the United States, the incidence of HCC continues to increase with the fastest rate among 40- to 50-year-old persons who have the highest rate of HCV infection (20,21). Thus, it is likely that the increasing mean age of patients with HCV-associated HCC in our study was associated with a shift toward an older-age group who had the highest rate of HCV infection.

It is known that 2-4 decades of chronic HCV infection is required to develop cirrhosis and subsequent HCC (22-25). The number of HCC cases has increased in Japan, because individuals infected with HCV during the past have grown old and have reached the cancer-bearing age. The prevalence of HCV infection in young Japanese persons is low and the incidence of HCVAb is very low because of preventative actions against HCV infection such as the screening of blood products for HCV and the use of sterile medical equipment (26). Additionally, we showed that the number of patients with HCV-associated HCC cases reached a plateau together with an increase in the mean age, although the present study was a single-center, hospital-based study. These findings indicate that a decrease in the prevalence of HCC in Japan, a country that is far advanced with regard to HCV-associated HCC, is expected in the near future. We believe that long-term experience in Japan helps to plan strategies against HCV-associated HCC and to cope with its long-term sequelae in many other countries worldwide.

References

1. El-Serag HB and Mason AC: Risk factors for the rising rates of primary liver cancer in the United States. Arch Intern Med 160: 3227-3230, 2000.
2. El-Serag HB: Epidemiology of hepatocellular carcinoma. Clin Liver Dis 5: 87-107, 2001.
3. El-Serag HB, Hampel H, Yeh C and Rabeneck L: Extrahepatic manifestations of hepatitis C among United States male veterans. Hepatology 36: 1439-1445, 2002.
4. El-Serag HB: Hepatocellular carcinoma and hepatitis C in the United States. Hepatology 36: S74-S83, 2002.
5. El-Serag HB: Hepatocellular carcinoma: an epidemiologic view. J Clin Gastroenterol 35: S72-S78, 2002.
6. Hassan MM, Frome A, Patt YZ and El-Serag HB: Rising prevalence of hepatitis C virus infection among patients recently diagnosed with hepatocellular carcinoma in the United States. J Clin Gastroenterol 35: 266-269, 2002.
7. Kiyosawa K and Tanaka E: Characteristics of hepatocellular carcinoma in Japan. Oncology 62: 5-7, 2002.
8. McGlynn KA, Tsao L, Hsing AW, Devesa SS and Fraumeni JF Jr: International trends and patterns of primary liver cancer. Int J Cancer 94: 290-296, 2001.
9. Bosch FX, Ribes J, Diaz M and Cleries R: Primary liver cancer: worldwide incidence and trends. Gastroenterology 127: S5-S16, 2004.
10. Hamasaki K, Nakata K, Tsutsumi T, Tsuruta S, Nakao K, Kato Y, Shima M, Koji T and Nagataki S: Changes in the prevalence of hepatitis B and C infection in patients with hepatocellular carcinoma in the Nagasaki Prefecture, Japan. J Med Virol 40: 146-149, 1993.
11. Kato Y, Nakata K, Omagari K, Furukawa R, Kusumoto Y, Mori I, Tajima H, Tanioka H, Yano M and Nagataki S: Risk of hepatocellular carcinoma in patients with cirrhosis in Japan. Analysis of infectious hepatitis viruses. Cancer 74: 2234-2238, 1994.
12. Shiratori Y, Shiina S, Imamura M, Kato N, Kanai F, Okudaira T, Teratani T, Tohgo G, Toda N, Ohashi M, *et al*: Characteristic difference of hepatocellular carcinoma between hepatitis B- and C-viral infection in Japan. Hepatology 22: 1027-1033, 1995.

13. Shiratori Y, Shiina S, Zhang PY, Ohno E, Okudaira T, Payawal DA, Ono-Nita SK, Imamura M, Kato N and Omata M: Does dual infection by hepatitis B and C viruses play an important role in the pathogenesis of hepatocellular carcinoma in Japan? Cancer 80: 2060-2067, 1997.
14. Kiyosawa K, Umemura T, Ichijo T, Matsumoto A, Yoshizawa K, Gad A and Tanaka E: Hepatocellular carcinoma: recent trends in Japan. Gastroenterology 127: S17-S26, 2004.
15. Yoshizawa H: Hepatocellular carcinoma associated with hepatitis C virus infection in Japan: projection to other countries in the foreseeable future. Oncology 62: 8-17, 2002.
16. Moriya T, Koyama T, Tanaka J, Mishiro S and Yoshizawa H: Epidemiology of hepatitis C virus in japan. Intervirology 42: 153-158, 1999.
17. Okayama A, Stuver SO, Tabor E, Tachibana N, Kohara M, Mueller NE and Tsubouchi H: Incident hepatitis C virus infection in a community-based population in Japan. J Viral Hepat 9: 43-51, 2002.
18. Tanaka Y, Hanada K, Mizokami M, Yeo AE, Shih JW, Gojobori T and Alter HJ: Inaugural Article: a comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. Proc Natl Acad Sci USA 99: 15584-15589, 2002.
19. Zanetti AR, Romano L and Bianchi S: Primary prevention of hepatitis C virus infection. Vaccine 21: 692-695, 2003.
20. Alter MJ, Kruszon-Moran D, Nainan OV, McQuillan GM, Gao F, Moyer LA, Kaslow RA and Margolis HS: The prevalence of hepatitis C virus infection in the United States, 1988 through 1994. N Engl J Med 341: 556-562, 1999.
21. Armstrong GL, Alter MJ, McQuillan GM and Margolis HS: The past incidence of hepatitis C virus infection: implications for the future burden of chronic liver disease in the United States. Hepatology 31: 777-782, 2000.
22. Deuffic S, Poynard T and Valleron AJ: Correlation between hepatitis C virus prevalence and hepatocellular carcinoma mortality in Europe. J Viral Hepat 6: 411-413, 1999.
23. El-Serag HB and Mason AC: Rising incidence of hepatocellular carcinoma in the United States. N Engl J Med 340: 745-750, 1999.
24. Planas R, Balleste B, Antonio Alvarez M, Rivera M, Montoliu S, Anton Galeras J, Santos J, Coll S, Maria Morillas R and Sola R: Natural history of decompensated hepatitis C virus-related cirrhosis. A study of 200 patients. J Hepatol 40: 823-830, 2004.
25. Davila JA, Morgan RO, Shaib Y, McGlynn KA and El-Serag HB: Hepatitis C infection and the increasing incidence of hepatocellular carcinoma: a population-based study. Gastroenterology 127: 1372-1380, 2004.
26. Sasaki F, Tanaka J, Moriya T, Katayama K, Hiraoka M, Ohishi K, Nagakami H, Mishiro S and Yoshizawa H: Very low incidence rates of community-acquired hepatitis C virus infection in company employees, long-term inpatients, and blood donors in Japan. J Epidemiol 6: 198-203, 1996.

The Impact of Newer Treatment Modalities on Survival in Patients With Hepatocellular Carcinoma

NAOTA TAURA, KEISUKE HAMASAKI, KAZUHIKO NAKAO, TATSUKI ICHIKAWA, DAISUKE NISHIMURA, TAKASHI GOTO, MARIKO FUKUTA, HIROSHI KAWASHIMO, MASUMI FUJIMOTO, KOICHIRO KUSUMOTO, YASUHIDE MOTOYOSHI, HIDETAKA SHIBATA, and KATSUMI EGUCHI
First Department of Internal Medicine, Nagasaki University School of Medicine, Sakamoto, Nagasaki, Japan

Background & Aims: Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. However, although the therapeutic approaches for HCC have progressed rapidly, it remains unknown whether the current management of patients with HCC has reduced its mortality. We analyzed changes of survival rate in patients with HCC over a 20-year period. **Methods:** Between 1982 and 2001, 463 patients were diagnosed with HCC at our hospital. Subjects were enrolled in the current cohort according to the following inclusion criteria: HCC lesion measuring less than 3 cm in diameter, no evidence of extrahepatic metastasis, and no evidence of main portal vein infiltration/thrombosis. A total of 257 patients with HCC were recruited for this study, and categorized into 5-year intervals. **Results:** The survival rates improved significantly during the study period. When the patients were stratified according to Child-Pugh score, only patients with Child's B showed improved survival rates. Furthermore, patients with surgical resection or transarterial chemoembolization during the latter period had a better prognosis than those during the early period. **Conclusions:** Our findings suggest that the development of therapeutic interventions for HCC have led to improvements in the prognosis for HCC patients.

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. Estimates of the incidence of cancer in 2000 indicate that primary liver cancer represented the fifth and eighth most common malignancy in men and women, respectively. The number of new cases is predicted to be 564,000, with 398,000 cases in men and 166,000 in women.[1] The geographic areas at highest risk are Eastern Asia, Middle Africa, and some countries of Western Africa.[1] Recently, a trend of increasing rates of HCC has been reported from several of the developed countries in North America and Europe.[2,3]

Although the age-adjusted rates of HCC incidence have increased between 1958 and 2000 in Japan,[4,5] the changes in the prognosis of HCC patients over this period are not understood fully. In the present study, we examined the changes in the survival rates of HCC patients over a 20-year period.

Patients and Methods

Patients

Between 1982 and 2001, 463 patients were diagnosed with HCC in the First Department of Internal Medicine at the Nagasaki University School of Medicine. Subjects were enrolled in the current cohort according to the following inclusion criteria: (1) HCC lesion measuring less than 3 cm in diameter, (2) no evidence of extrahepatic metastasis, and (3) no evidence of main portal vein infiltration/thrombosis. A total of 257 patients with HCC were recruited for this study. The diagnosis of HCC was based on α-fetoprotein (AFP) levels and imaging techniques, including ultrasonography, computed tomography, magnetic resonance imaging, hepatic angiography, and/or liver biopsy examination. The diagnostic criteria for HCC included confirmative liver biopsy examination or increased AFP levels (>20 ng/mL) and neovascularization in hepatic angiography and/or computed tomography. The cohorts of patients with HCC were divided into 5-year intervals (1982–1986, 1987–1991, 1992–1996, and 1997–2001). Table 1 indicates the diagnostic procedure(s) of the studied patients during each of the 5-year intervals.

Cause of Hepatocellular Carcinoma

Sera were stored at −80°C until they were used for the following assays. The diagnosis of chronic hepatitis C virus infection was based on the presence of anti-hepatitis C virus antibodies (microparticle enzyme immunoassay; Abbott Laboratories, Abbott Park, IL) and hepatitis C virus RNA, as detected by polymerase chain reaction. The diagnosis of chronic hepatitis B virus infection was based on the presence of hepatitis B surface antigen (enzyme-linked immunosorbent assay; Abbott Laboratories). Serum AFP level was measured by 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 for a period of more than 10 years.

Treatment and Follow-Up Evaluation

All patients were assessed for surgical resection once they were diagnosed with HCC. These assessments were based on lobar involvement and liver functional status. The lobar involvement was evaluated by a combination of ultrasonography, computed tomography, magnetic resonance imaging, and hepatic angiography. Patients were considered not suitable for resection when they showed the following criteria: (1) bilobar involvement, (2) evidence of main portal vein infiltration/

Abbreviations used in this paper: AFP, α-fetoprotein; HCC, hepatocellular carcinoma; PEIT, percutaneous ethanol injection therapy; TACE, transarterial chemoembolization.

1542-3565/06/$32.00
doi:10.1016/j.cgh.2006.06.008

Table 1. Diagnostic Procedure of the Studied Patients in 5-Year Intervals

	1982–1986	1987–1991	1992–1996	1997–2001
Diagnostic method (%)				
Histologic diagnosis	14 (37.8)	28 (45.2)	47 (66.2)	52 (59.8)
Hepatic angiography	23 (62.2)	34 (54.8)	17 (23.9)	30 (34.5)
Computed tomography only	0 (0)	0 (0)	7 (9.9)	5 (5.7)

thrombosis, (3) evidence of extrahepatic metastases, (4) Child's C cirrhosis, or (5) poor cardiac and respiratory performance status. If the patients were deemed not suitable for surgery or did not agree to undergo surgery, percutaneous ethanol injection therapy (PEIT) was the second choice of treatment offered to patients with HCCs less than 3 cm in diameter. The remaining patients without main portal vein thrombosis or extrahepatic metastasis were advised to undergo transarterial chemoembolization (TACE) irrespective of the size and number of tumors.

After initial treatment, the studied patients underwent measurement of AFP levels and liver function biochemistry every 1–3 months of the follow-up period, and ultrasonography was performed every 3–6 months. Patients in whom recurrence of HCC was suspected were evaluated further by computed tomography and/or magnetic resonance imaging. The assessment of treatment for recurrent HCC was based on lobar involvement and liver functional status in the same manner as that for the initial treatment. Radiofrequency ablation or liver transplantation for HCC was started at our institution in 2002. Therefore, none of the patients were treated by radiofrequency ablation or transplantation between 1982 and 2001. Furthermore, none of the subjects in our study received either of these treatments for recurrent HCC during the follow-up period.

The closing date of the current study was April 2005 or the time of a patient's death. If a patient had not been monitored in our hospital for more than 1 year, the patient was considered lost to follow-up evaluation.

Statistical Analysis

The time of survival was measured from the time of HCC diagnosis to the time of death, or until the time of writing. The data were analyzed by the Mann-Whitney test for continuous ordinal data, χ^2 test with Yates' correction and the Fisher exact test for the association between 2 qualitative variables, and Kaplan-Meier survival analysis. Parametric comparisons were assessed by analysis of variance. The significance of individual differences was evaluated by use of the Scheffe's test. The standard error was calculated based on the binomial model for the response proportion. *P* values of less than .05 were considered statistically significant.

Results

Clinical Features of the Studied Patients

A total of 257 patients were enrolled in this study, and were followed up for a mean of 3.64 years (range, 1.41–22.38 y). Patient characteristics at the time of HCC diagnosis are presented in Table 2. There were 187 men (72.8%) and 70 women (27.2%) who were HCC patients (mean age, 63 y). The proportion of patients diagnosed with hepatitis B virus–associated HCC was 17.9% (46 of 257), whereas 72.3% (186 of 257) showed hepatitis C virus–associated HCC, and an additional 3.9% (10 of 257) showed HCC associated with both viruses. Three of the remaining 15 patients had a history of significant alcohol intake and 12 had no known cause. Child-Pugh grade A was recorded in 63.8% (164 of 257), grade B was noted in 30.7% (79 of 257), and grade C was noted in 5.5% (14 of 257) of the patients. Solitary HCC was detected in 61.1% (157 of 257) of all cases. The AFP values were normal (<20 ng/mL) in 36.6% (94 of 257), 21–200 ng/mL in 37.0% (95 of 257), and more than 201 ng/mL in 26.4% (68 of 257). Of the studied patients, 11.3% (29 of 257) underwent surgical resection, 43.6% (112 of 257) received PEIT, 34.6% (89 of 257) underwent TACE, and 10.5% (27 of 257) received palliative care only.

Table 3 presents the characteristics at diagnosis of HCC in 5-year intervals (1982–1986, 1987–1991, 1992–1996, and 1997–2001). The mean age at the time of HCC diagnosis increased steadily and the number of patients with Child-Pugh grade A or AFP value of less than 20 ng/mL showed a significant increase during the studied periods. There were no significant differences in the cause of liver disease between the 5-year

Table 2. Background Features of the Studied 257 Patients at Baseline

	Number	%
Patients	257	100
Average age at diagnosis (SD)	63 (9)	
Sex		
Male	187	72.8
Female	70	27.2
Cause of liver disease		
HBV	46	17.9
HCV	186	72.3
HBV + HCV	10	3.9
Alcohol	3	1.2
Unknown	12	4.7
Child-Pugh staging		
A	164	63.8
B	79	30.7
C	14	5.5
Tumor lesions		
Solitary	157	61.1
Not solitary	100	38.9
AFP level, *ng/mL*		
≤20	94	36.6
20–200	95	37.0
>200	68	26.4
Therapy		
Surgical resection	29	11.3
PEIT	112	43.6
TACE	89	34.6
Only palliative care	27	10.5

HBV, hepatitis B virus; HCV, hepatitis C virus.

Table 3. Characteristics of the Studied Patients in 5-Year Intervals

	1982–1986	1987–1991	1992–1996	1997–2001	*P* value
Number of patients	37	62	71	87	
Average age (SD)	58.5 (10.3)	61.8 (8.5)	62.9 (7.0)	66.6 (8.0)	<.0001
Male/female	25/12	44/18	51/20	67/20	.7026
Child-Pugh staging (%)					
A	15 (40.5)	37 (59.7)	47 (66.2)	65 (74.7)	
B	20 (54.1)	22 (35.5)	21 (29.6)	16 (18.4)	
C	2 (5.4)	3 (4.8)	3 (4.2)	6 (6.9)	.0387
Number of tumor lesions (%)					
Solitary	24 (64.9)	39 (62.9)	42 (59.2)	52 (59.8)	
Not solitary	13 (35.1)	23 (37.1)	29 (40.8)	35 (40.2)	.9239
AFP level, *ng/mL* (%)					
≤20	4 (10.8)	23 (37.1)	23 (32.4)	44 (50.6)	
21–200	14 (37.8)	23 (37.1)	27 (38.0)	31 (35.6)	
>200	19 (51.4)	16 (25.8)	21 (29.6)	12 (13.8)	<.0001

intervals (data not shown). As shown in Table 4, surgical resection for HCC has decreased drastically since 1989 when PEIT was introduced into our hospital. Patients who underwent liver transplantation or radiofrequency ablation were not included in this study because these therapies were only introduced into our hospital in 2002. When the patients were categorized according to the Japan integrated staging score,[6,7] which combines the Child-Pugh classification and TNM staging, there were no significant changes between the 5-year intervals (Table 5).

Independent Predictors of Survival Rate

Table 6 indicates the results of univariate and multivariate analyses using the Cox proportional hazards model. According to univariate analysis, 6 of 9 factors (Child-Pugh grade B or C, not solitary HCC, AFP level >200, treatment by PEIT, TACE, and study period between 1982 and 1991) significantly affected the survival rate in the patients with HCC. When multivariate analyses were performed on the 5 significant variables (Child-Pugh score, number of tumors, AFP level, treatment by TACE, study period), 4 of the factors (Child-Pugh grade B or C, not solitary tumor, AFP level >200, and study period between 1982 and 1991) were found to be independent prognostic indicators.

Change of Survival Rate in 5-Year Intervals

Figure 1 indicates the cumulative survival rates for cohorts of HCC patients during each of the 5-year intervals between 1982 and 2001, and shows improved prognosis during the studied periods (1982–1986 vs 1997–2001, $P < .0001$; 1982–

Table 4. Therapeutic Procedure of the Studied Patients in 5-Year Intervals

	1982–1986	1987–1991	1992–1996	1997–2001
Therapy (%)				
Surgical resection (%)	14 (37.8)	13 (20.9)	0	2 (2.3)
PEIT (%)	0	15 (24.2)	47 (66.2)	50 (57.5)
TACE (%)	17 (45.9)	25 (40.2)	17 (23.9)	30 (34.5)
Only palliative care (%)	6 (16.3)	9 (14.5)	7 (9.9)	5 (5.7)

Table 5. Categorization According to JIS Score of the Studied Patients in 5-Year Intervals

	1982–1986	1987–1991	1992–1996	1997–2001	Total
Number of patients	37	62	71	87	257
JIS score (%)					
0	9 (24)	16 (26)	22 (31)	27 (31)	74 (29)
1	11 (30)	22 (35)	22 (31)	31 (36)	86 (33)
2	12 (32)	14 (23)	15 (21)	21 (24)	62 (24)
3	4 (11)	8 (13)	5 (7)	5 (6)	22 (9)
4	1 (3)	2 (3)	7 (10)	3 (3)	13 (5)
5	0	0	0	0	0
	NS	NS	NS		
		NS			
			NS		
			NS		

JIS, Japanese integrated staging score (Scheffe's test).

Table 6. Univariate and Multivariate Analyses of Prognostic Factors for the Studied Patients

Variable	Univariate analysis		Multivariate analysis	
	P	Relative risk (95% CI)	*P*	Relative risk (95% CI)
Age, y: ≥63 vs <63	.6055	1.09 (.79–1.48)		
Sex: male vs female	.3484	1.18 (.83–1.67)		
Child–Pugh score: B or C vs A	<.0001	3.01 (2.18–4.15)	<.0001	2.58 (1.83–3.62)
Number of tumors: not solitary vs solitary	.0009	1.70 (1.24–2.32)	.0025	1.64 (1.91–2.27)
AFP level, ng/mL: >200 vs ≤200	<.0001	2.02 (1.46–2.80)	.0032	1.67 (1.19–2.34)
Therapy				
Surgical resection vs other therapy	.1729	.72 (.44–1.56)		
PEIT vs other therapy	.0028	.62 (.45–0.85)		
TACE vs other therapy	<.0001	1.91 (1.40–2.59)	.0691	1.65 (.96–2.83)
Period: 1982–1991 vs 1992–2001	.0005	1.72 (1.27–2.34)	.0090	1.63 (1.13–2.35)

CI, confidence interval.

1986 vs 1987–1991, $P = .0494$; 1987–1991 vs 1997–2001, $P = .0263$).

By stratification according to the Child-Pugh score at baseline, the survival rate of patients with Child's B increased significantly during the studied periods (1982–1986 vs 1997–2001, $P < .0001$; 1982–1986 vs 1992–1996, $P = .0029$; 1987–1991 vs 1997–2001, $P = .0429$; 1987–1991 vs 1997–2001, $P = .0001$) (Figure 2). However, the survival rates of patients with Child's A or Child's C were not significantly different throughout the studied periods.

Furthermore, when the patients were categorized according to the initial treatment, the survival rates of patients undergoing surgical resection (1982–1986 vs 1987–1991, $P = .0487$) or TACE (1982–1986 vs 1997–2001, $P < .0001$; 1987–1991 vs 1997–2001, $P = .0040$) showed a significant increase during the studied periods (Figure 3). However, there was no significant difference in the survival rate of patients undergoing PEIT.

Discussion

The prognosis for patients with HCC remains poor because recurrence of HCC is common and most patients with HCC also show underlying cirrhosis. Furthermore, these patients do not tolerate cytotoxic therapy or extensive resection. Possible curative therapies, including surgical resection, liver transplantation, or percutaneous treatments, benefit only a small proportion of patients and these therapeutic approaches can improve life expectancy.[8–13] In Japan and other countries, surveillance programs have led to an increase in the application of curative therapies.[14–16] In addition, several therapies have been proposed for patients who will not benefit from a radical approach. Of these approaches, only TACE has been shown to improve survival in properly selected candidates.[17,18] However, to date, no consensus agreement has been achieved on a common treatment strategy for patients with HCC worldwide.[19–21]

Tumor size can be crucial in the selection of therapeutic options and the prognosis of HCC patients. Although surgical resection and TACE are considered effective treatments for HCC irrespective of tumor size, PEIT has been used only as a potentially curative treatment for HCCs less than 3 cm in diameter in Japan.[22,23] This is likely because the total amount of ethanol injected is less than 10 mL, and it often is difficult to penetrate through all parts of the tumor nodules when the HCC is larger than 3 cm in diameter. Furthermore, in our institution PEIT was selected for patients with HCCs less than 3 cm in diameter throughout the studied periods. Therefore, to select for patients who were able to receive effective therapeutic options, including PEIT, we added HCC lesions measuring less than 3 cm in diameter to the inclusion criteria for the enroll-

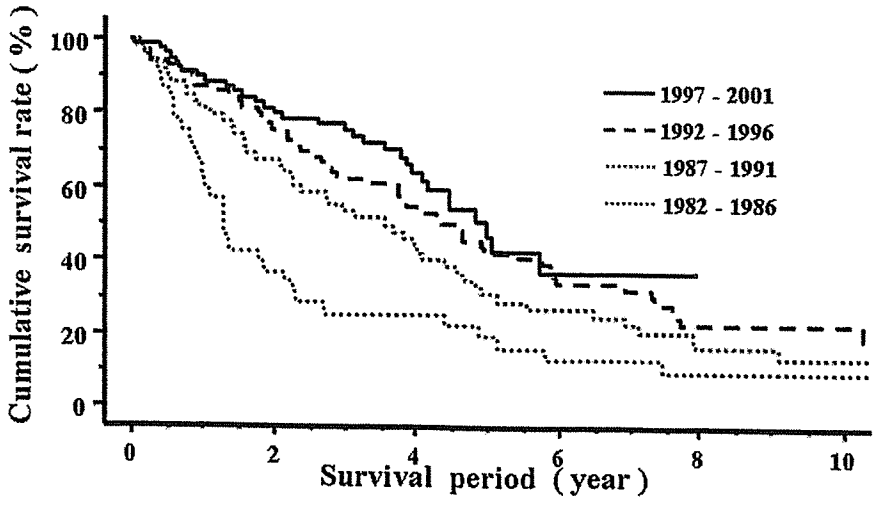

Figure 1. The cumulative survival rates in patients with HCC from 1982 to 1986 (· · ·), 1987 to 1991 (●●●), 1992 to 1996 (—), and 1997 to 2001 (—) are shown separately. For 1982–1986 vs 1997–2001, $P < .0001$; 1982–1986 vs 1987–1991, $P = .0494$; 1987–1991 vs 1997–2001, $P = .0263$.

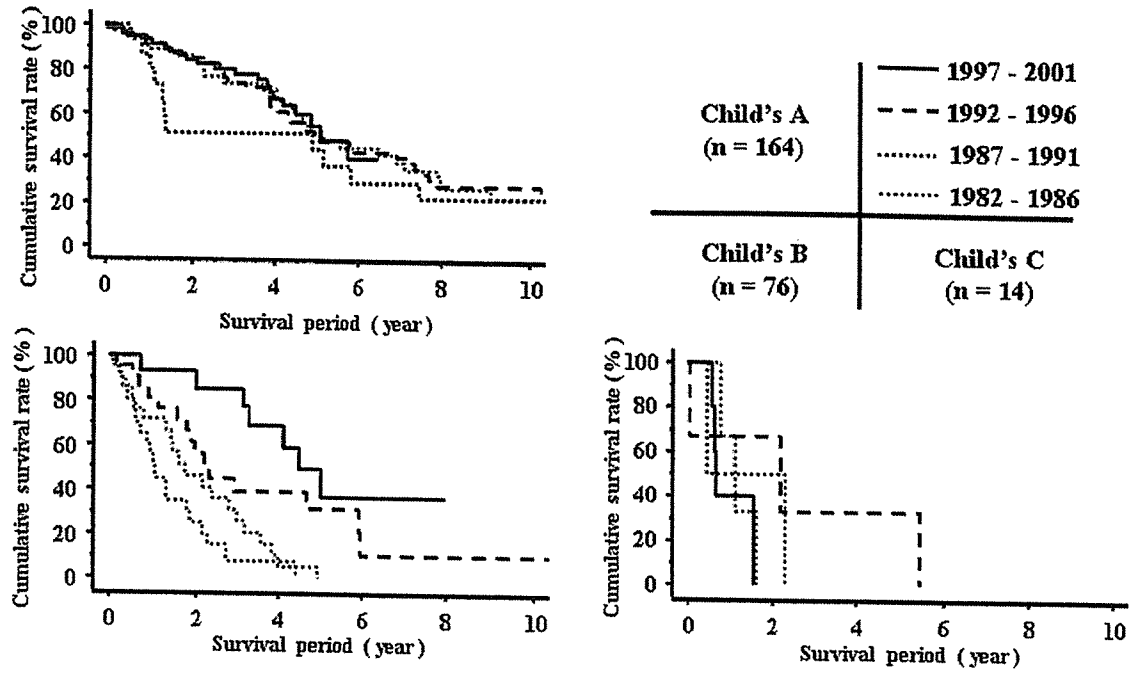

Figure 2. The cumulative survival rates in patients with HCC from 1982 to 1986 (· · ·), 1987 to 1991 (●●●), 1992 to 1996 (—), and 1997 to 2001 (—) according to Child-Pugh staging are shown separately. Child-Pugh A (n = 164), P = NS. Child-Pugh B (n = 76), 1982–1986 vs 1997–2001, $P < .0001$; 1982–1986 vs 1992–1996, $P = .0029$; 1987–1991 vs 1997–2001, $P = .0001$; 1987–1991 vs 1997–2001, $P = .0429$. Child-Pugh C (n = 14), P = NS.

ment of subjects in the current cohort. Although there were significant differences in several factors between the 5-year intervals, the Japan integrated staging score, which has been proposed as a useful prognostic staging system for HCC, showed no significant change.[7] In statistical analyses, the study period was found to be an independent prognostic indicator and the cumulative survival rates showed a significant increase during the studied periods. The survival of our subjects was consistent with previous reports.[3,24] Furthermore, categorized analyses indicated that patients with Child-Pugh grade B, surgical resection, or TACE showed improved prognosis during the studied periods.

It is extremely difficult to determine the optimal treatment choice for HCC patients with Child-Pugh grade B because

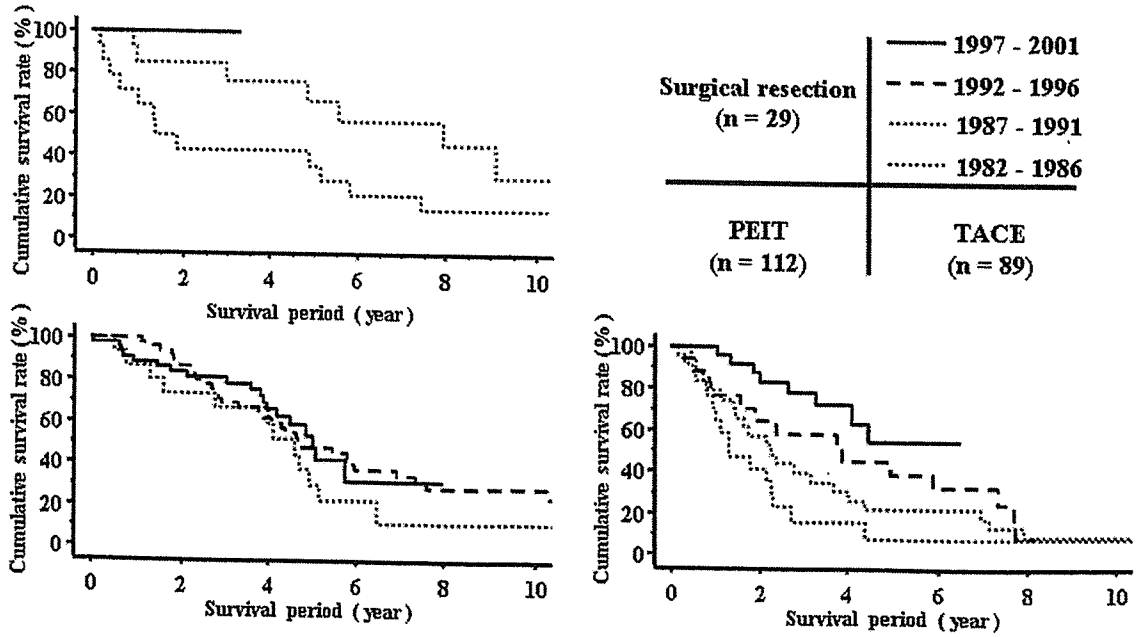

Figure 3. The cumulative survival rates in patients with HCC from 1982 to 1986 (· · ·), 1987 to 1991 (●●●), 1992 to 1996 (—), and 1997 to 2001 (—) according to their respective treatments are shown separately. Surgical resection (n = 29), 1982–1986 vs 1987–1991, $P = .0487$. PEIT (n = 112), P = NS. TACE (n = 89), 1982–1986 vs 1997–2001, $P < .0001$; 1987–1991 vs 1997–2001, $P = .0040$.

those patients progress to hepatic failure as a result of invasive therapy more often than those with grade A. Accordingly, our results may indicate that the choice of therapy for HCC patients during the latter period was more appropriate than that during the early period; this improvement in therapeutic choice occurred concurrently with advances in radiologic assessment. In addition, the improved prognosis in HCC patients with surgical resection or TACE may be associated with advances in surgical procedure and instrumentation. In fact, the surgical mortality rate for hepatectomy has decreased from the 10%–20% that was observed in the 1980s to less than 5% today.[8] However, the survival rates of the cohorts categorized according to the initial treatment do not necessarily reflect the efficacy of each treatment because patients with recurrent HCC commonly undergo additional therapies that are different from the initial treatment. On the other hand, the mortality from variceal hemorrhage in cirrhotic patients has decreased because of advanced management, such as variceal ligation and pharmacologic treatment. Furthermore, long-term nutritional supplementation with oral branched-chain amino acids has been useful in the prevention of progressive hepatic failure.[25–28] These supportive treatments may have contributed to improvement of the survival rate of our subjects.

Although our data showed an improvement in the prognosis of HCC patients over the past 20 years, HCC remains a fatal disease. Recently, radiofrequency ablation and liver transplantation have been added to the therapeutic options for HCC and new approaches also have been suggested, including proton beam irradiation, intrahepatic infusion of yttrium-90 microspheres, and immunotherapy.[10,29–33] In addition to the evaluation of these newer therapeutic modalities, advances in the prevention and early diagnosis of HCC also are needed to achieve further improvement of the prognosis of this disease.

References

1. Bosch FX, Ribes J, Cleries R, et al. Epidemiology of hepatocellular carcinoma. Clin Liver Dis 2005;9:191–211.
2. El-Serag HB, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. N Engl J Med 1999;340:745–750.
3. El-Serag HB, Mason AC, Key C. Trends in survival of patients with hepatocellular carcinoma between 1977 and 1996 in the United States. Hepatology 2001;33:62–65.
4. Kiyosawa K, Tanaka E. Characteristics of hepatocellular carcinoma in Japan. Oncology 2002;62(Suppl 1):5–7.
5. Kiyosawa K, Umemura T, Ichijo T, et al. Hepatocellular carcinoma: recent trends in Japan. Gastroenterology 2004;127:S17–S26.
6. Kudo M, Chung H, Osaki Y. Prognostic staging system for hepatocellular carcinoma (CLIP score): its value and limitations, and a proposal for a new staging system, the Japan Integrated Staging Score (JIS score). Hepatology 2003;40:207–215.
7. Kudo M, Chung H, Haji S, et al. Validation of a new prognostic staging system for hepatocellular carcinoma: the JIS score compared with the CLIP score. Prognostic staging system for hepatocellular carcinoma (CLIP score): its value and limitations, and a proposal for a new staging system, the Japan Integrated Staging Score (JIS score). Hepatology 2004;40:1396–1405.
8. Song TJ, Ip EW, Fong Y. Hepatocellular carcinoma: current surgical management. Gastroenterology 2004;127:S248–S260.
9. Omata M, Tateishi R, Yoshida H, et al. Treatment of hepatocellular carcinoma by percutaneous tumor ablation methods: ethanol injection therapy and radiofrequency ablation. Gastroenterology 2004;127:S159–S166.
10. Befeler AS, Hayashi PH, Di Bisceglie AM. Liver transplantation for hepatocellular carcinoma. Gastroenterology 2005;128: 1752–1764.
11. Lu DS, Yu NC, Raman SS, et al. Percutaneous radiofrequency ablation of hepatocellular carcinoma as a bridge to liver transplantation. Hepatology 2005;41:1130–1137.
12. Tateishi R, Shiina S, Teratani T, et al. Percutaneous radiofrequency ablation for hepatocellular carcinoma. An analysis of 1000 cases. Cancer 2005;103:1201–1209.
13. Lencioni R, Cioni D, Crocetti L, et al. Early-stage hepatocellular carcinoma in patients with cirrhosis: long-term results of percutaneous image-guided radiofrequency ablation. Radiology 2005; 234:961–967.
14. Taura N, Hamasaki K, Nakao K, et al. Clinical benefits of hepatocellular carcinoma surveillance: a single-center, hospital-based study. Oncol Rep 2005;14:999–1003.
15. Daniele B, Bencivenga A, Megna AS, et al. Alpha-fetoprotein and ultrasonography screening for hepatocellular carcinoma. Gastroenterology 2004;127:S108–S112.
16. Di Bisceglie AM. Issues in screening and surveillance for hepatocellular carcinoma. Gastroenterology 2004;127:S104–S107.
17. Llovet JM, Real MI, Montana X, et al. Arterial embolisation or chemoembolisation versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomised controlled trial. Lancet 2002;359:1734–1739.
18. Lo CM, Ngan H, Tso WK, et al. Randomized controlled trial of transarterial lipiodol chemoembolization for unresectable hepatocellular carcinoma. Hepatology 2002;35:1164–1171.
19. GETCH: A comparison of lipiodol chemoembolization and conservative treatment for unresectable hepatocellular carcinoma. Groupe d'Etude et de Traitement du Carcinome Hepatocellulaire. N Engl J Med 1995;332:1256–1261.
20. Bruix J, Llovet JM, Castells A, et al. Transarterial embolization versus symptomatic treatment in patients with advanced hepatocellular carcinoma: results of a randomized, controlled trial in a single institution. Hepatology 1998;27:1578–1583.
21. Bruix J, Sala M, Llovet JM. Chemoembolization for hepatocellular carcinoma. Gastroenterology 2004;127:S179–S188.
22. Fujimoto T. The experimental and clinical studies of percutaneous ethanol injection (PEIT) under ultrasonography for small hepatocellular carcinoma. Acta Hepatol Jpn 1988;29:52–59.
23. Shiina S, Teratani T, Obi S, et al. Percutaneous ethanol injection therapy for liver tumors. Eur J Ultrasound 2001;13:95–106.
24. Sangiovanni A, Del Ninno E, Fasani P, et al. Increased survival of cirrhotic patients with a hepatocellular carcinoma detected during surveillance. Gastroenterology 2004;126:1005–1014.
25. El-Serag HB, Everhart JE. Improved survival after variceal hemorrhage over an 11-year period in the Department of Veterans Affairs. Am J Gastroenterol 2000;95:3566–3573.
26. McCormick PA, O'Keefe C. Improving prognosis following a first variceal haemorrhage over four decades. Gut 2001;49:682–685.
27. Marchesini G, Bianchi G, Merli M, et al. Nutritional supplementation with branched-chain amino acids in advanced cirrhosis: a double-blind, randomized trial. Effects of hyperglycaemia and hyperinsulinaemia on plasma amino acid levels in obese subjects with normal glucose tolerance. Gastroenterology 2003; 124:1792–1801.
28. Marchesini G, Marzocchi R, Noia M, et al. Branched-chain amino acid supplementation in patients with liver diseases. J Nutr 2005;135:1596S–1601S.
29. Shiina S, Teratani T, Obi S, et al. A randomized controlled trial of radiofrequency ablation with ethanol injection for small hepatocellular carcinoma. Gastroenterology 2005;129:122–130.
30. Goin JE, Salem R, Carr BI, et al. Treatment of unresectable hepatocellular carcinoma with intrahepatic yttrium 90 microspheres: a risk-stratification analysis. J Vasc Interv Radiol 2005; 16:195–203.

31. Butterfield LH. Immunotherapeutic strategies for hepatocellular carcinoma. Gastroenterology 2004;127:S232–S241.
32. Geschwind JF, Salem R, Carr BI, et al. Yttrium-90 microspheres for the treatment of hepatocellular carcinoma. Gastroenterology 2004;127:S194–S205.
33. Bush DA, Hillebrand DJ, Slater JM, et al. High-dose proton beam radiotherapy of hepatocellular carcinoma: preliminary results of a phase II trial. Gastroenterology 2004;127:S189–S193.

Address requests for reprints to: Naota Taura, MD, The First Department of Internal Medicine, Nagasaki University School of Medicine, Sakamoto 1-7-1, Nagasaki, 852-8501 Japan. e-mail: ntaura-gi@umin.ac.jp; fax: (81) 95-849-7267.

Different mechanisms for anti-tumor effects of low- and high-dose cyclophosphamide

YASUHIDE MOTOYOSHI[1,2], KAZUHISA KAMINODA[1,3], OHKI SAITOH[1], KEISUKE HAMASAKI[2], KAZUHIKO NAKAO[3], NOBUKO ISHII[3], YUJI NAGAYAMA[1] and KATSUMI EGUCHI[2]

Departments of [1]Medical Gene Technology, Atomic Bomb Disease Institute; [2]Division of Immunology, Endocrinology and Metabolism, Department of Medical and Dental Sciences, and [3]Division of Clinical Pharmaceutics and Health Research Center, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8501, Japan

Received February 3, 2006; Accepted April 7, 2006

Abstract. It is known that, besides its direct cytotoxic effect as an alkylating chemotherapeutic agent, cyclophosphamide also has immuno-modulatory effects, such as depletion of $CD4^+CD25^+$ regulatory T cells. However, its optimal concentration has not yet been fully elucidated. Therefore, we first compared the effects of different doses of cyclophosphamide on T cell subsets including $CD4^+CD25^+$ T cells in mice. Cyclophosphamide (20 mg/kg) decreased the numbers of splenocytes, $CD4^+$ and $CD8^+$ T cells by ~50%, while a decline in $CD4^+CD25^+$ T cell number was more profound, leading to the remarkably lower ratios of $CD4^+CD25^+$ T cells to $CD4^+$ T cells. In contrast, 200 mg/kg cyclophosphamide severely decreased the numbers of all the T cell subsets by >90% although the decreased ratios of $CD4^+CD25^+$ T cells to $CD4^+$ T cells were still observed. Next, low-dose cyclophosphamide significantly inhibited *in vivo* growth of murine hepatoma MH129 tumor in immuno-competent but not immuno-deficient mice. This anti-tumor effect was abolished by $CD4^+CD25^+$ T cell repletion. In contrast, high-dose cyclophosphamide exhibited similar anti-tumor effects in both mice. In addition, contrary to antibody-mediated $CD4^+CD25^+$ T cell depletion, administration of low-dose cyclophosphamide after tumor inoculation was more efficacious than the prior administration. Our data show that low-dose cyclophosphamide selectively depletes $CD4^+CD25^+$ T cells, leading to enhanced anti-tumor effects against pre-existing tumors, while the anti-tumor effect of high-dose cyclophosphamide is solely attributed to its direct cytotoxicity. These findings appear to be highly crucial in a clinical setting of combined chemotherapy and immunotherapy for cancer treatment.

Correspondence to: Dr Yuji Nagayama, Department of Medical Gene Technology, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan
E-mail: nagayama@net.nagasaki-u.ac.jp

Key words: cyclophosphamide, $CD4^+CD25^+$ naturally occurring regulatory T cells, tumor immunity, MH129 hepatoma cells, effector T cells

Introduction

Chemotherapy and immunotherapy are generally regarded as unrelated or even mutually exclusive in cancer treatment, because chemotherapy kills not only target cancer cells but also immune cells, inducing systemic immune suppression and dampening the therapeutic efficacy of immunotherapy. In this regard, however, cyclophosphamide appears an exceptional chemotherapeutic agent (1,2). Besides its direct cytotoxic effect as an alkylating agent, cyclophosphamide is reported to modulate the immune system in hosts (1,2). Examples for this include (i) enhancement of dendritic cell-based anti-tumor immunity by increased tumor antigens released from tumor cells dying of cyclophosphamide-induced apoptosis (3), (ii) increased type-I interferon production and evolution of $CD44^{hi}$ memory T cell response by cyclophosphamide (4), (iii) induction of homeostatic T cell proliferation by cyclophosphamide-mediated lymphopenia that enhances some cancer vaccines (5,6), and (iv) down-regulation of T-cell derived IL-10 and TGF-β productions by cyclophosphamide (7). More importantly, recent studies show selective suppression by cyclophosphamide of $CD4^+CD25^+$ naturally occurring regulatory T cells (8-11), which are widely believed to play a key role in immune tolerance (12). Although it is widely believed that 'low-dose' cyclophosphamide augments the immune response, the optimal concentration has not yet been fully elucidated. Thus, the amounts of cyclophosphamide used vary from 10 to 300 mg/kg in studies on immuno-potentiation of low-dose cyclophosphamide (2) and from 30 to 200 mg/kg in those on cyclophosphamide-mediated $CD4^+CD25^+$ T cell suppression (8-11). In this article, therefore, we compared the effects of different doses of cyclophosphamide on T cell subsets including $CD4^+CD25^+$ T cells and also on tumor immunity in mice. Our results clearly demonstrate that low-dose (20 mg/kg), but not high-dose (200 mg/kg), cyclophosphamide selectively suppresses the number of $CD4^+CD25^+$ T cells but spares those of conventional $CD4^+$ and $CD8^+$ T cells in spleen, and efficiently inhibits, through $CD4^+CD25^+$ T cell