Table 3. Multivariate Analyses of Factors Associated with Patient Survival

Factor		Parameter Estimate	Standard Error	Chi	Risk Ratio (95% Confidence Interval)	*P* Value
Age		−0.0188	0.0158	1.41	0.9814 (0.9512–1.0122)	.2342
Sex	Male				1	
	Female	0.0378	0.1804	0.04	1.0385 (0.7096–1.4504)	.8353
Child-Pugh class	A				1	
	B	0.1316	0.1428	0.84	1.1406 (0.8580–1.5057)	.3602
Tumor size	≤ 2 cm				1	
	> 2 cm and ≤ 5 cm	0.2868	0.1688	2.98	1.3322 (0.9625–1.8733)	.0842
	> 5 cm	0.0282	0.1939	0.02	1.0286 (0.7029–1.5113)	.8843
Tumor number	Single				1	
	Multiple	0.3492	0.1516	5.71	1.4179 (1.0631–1.9331)	.0169
Portal vein invasion	Absent				1	
	Present	0.3970	0.1852	4.31	1.4874 (1.0232-2.1235)	.0379
Nucleotide analogue	No				1	
	Yes	−0.4420	0.1727	7.46	0.6428 (0.4483–0.8871)	.0063

Note–Data on Child-Pugh class, tumor size, tumor number, and portal vein invasion refer to the status at initial diagnosis of hepatocellular carcinoma.

side analogue intake and improved patient survival was likely mediated by the increased number of chemoembolization treatments. The use of nucleoside analogues may have slowed the progressive decline in liver function that occurs even with repeated chemoembolization treatments, potentially allowing more sessions of chemoembolization treatment in patients who would otherwise have been excluded from chemoembolization treatment because of progressive liver dysfunction. Additional chemoembolization sessions may have explained the improved patient survival, although not the improved progression-free survival. Several groups have reported on the beneficial survival effects nucleoside analogues exert by preserving liver function in patients with HCC and HBV who undergo curative treatment (29,30). Our experience may suggest that this finding also applies to patients receiving chemoembolization as palliative therapy.

Although previous studies reported that nucleoside analogues can suppress the development of HCC (17,31), it has not been confirmed that nucleoside analogues can suppress HCC recurrence after treatment (30,32–34). Because the patients in the present study had been treated for both initial and recurrent HCC solely by chemoembolization, which is not a curative treatment, it is difficult to determine the extent to which nucleoside analogues prevent HCC progression or recurrence. Although there was no difference in the progression-free survival rate after the initial HCC treatment based on nucleoside analogue intake, further studies are needed to investigate whether the suppressive effects of nucleoside analogues on HCC recurrence or progression play a role in improving the survival of HBV-infected patients with HCC.

There are several limitations to this study. This was a retrospective study, and the patients were not randomly assigned to treatment arms. There may have been selection bias toward the patients who were administered nucleoside analogues. In addition, the data on liver function deterioration during the course of HCC recurrence and retreatment were insufficient, and the mechanisms behind the effect of nucleoside analogues on patients with HCC treated with chemoembolization were not elucidated. Additional studies are necessary to elucidate these mechanisms.

In conclusion, administering nucleoside analogues for chronic hepatitis B was associated with longer survival and more chemoembolization treatments in patients with HCC who were treated solely with chemoembolization. Additional studies are needed to examine these findings further and to clarify the mechanisms underlying this association.

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Table E1. Pretreatment Characteristics of Study Patients (n = 81)

Age (mean ± SD, y) (range)	60.6 ± 9.2 (37–81)
Sex ratio (female/male)	14 (17.3%)/67 (82.7%)
Child-Pugh class (A/B)	49 (60.5%)/32 (39.5%)
Albumin (mean ± SD, g/dL)	3.42 ± 0.73
Total bilirubin (mean ± SD, mg/dL)	1.04 ± 0.82
15-minute retention rate of ICG (%)*	20.0 ± 13.5
Prothrombin (%)	80.1 ± 20.0
Platelet count (× 1,000/mL)	135 ± 77
Tumor size (mean ± SD, cm) (range)	4.38 ± 3.15 (1.0–15.9)
Tumor size (≤ 2 cm/> 2 cm and ≤ 5 cm/> 5 cm)	21 (25.9%)/36 (44.5%)/24 (29.6%)
Tumor number (single/multiple)	29 (35.8%)/52 (64.2%)
Portal vein invasion (absent/present)	63 (77.8%)/18 (22.2%)
AFP (median, ng/mL) (range)	61.4 (0.8–1,304,200)
AFP (≥ 20 ng/mL/< 20 ng/mL)	48 (59.3%)/33 (40.7%)
AFP-L3 (median, %) (range)	6.1 (0–64.0)
AFP-L3 (≥ 10%/< 10%)	31 (38.3%)/50 (61.7%)
DCP (median, mAU/mL) (range)	62.0 (10–75,000)
DCP (≥ 40 mAU/mL/< 40 mAU/mL)	54 (66.7%)/27 (33.3%)

AFP = alpha-fetoprotein; AFP-L3 = *Lens culinaris* agglutinin-reactive AFP; DCP = des-gamma-carboxy prothrombin; ICG = indocyanine green test.
* ICG test was not performed in 14 patients.

Table E2. Multivariate Analyses of Factors Associated with Patient Survival (including Number of Chemoembolization Treatments)

Factor		Parameter Estimate	Standard Error	Chi	Risk ratio (95% Confidence Interval)	*P* Value
Age		−0.0250	0.0150	2.79	0.9753 (0.9469–1.0047)	.0949
Sex	Male				1	
	Female	−0.0013	0.1794	0.00	0.9987 (0.6836–1.3912)	.9943
Child-Pugh class	A				1	
	B	−0.0173	0.1476	0.01	0.9828 (0.7329–1.3106)	.9064
Tumor size	≤ 2 cm				1	
	> 2 cm and ≤ 5 cm	0.2361	0.1668	2.06	1.2662 (0.9183–1.7740)	.1512
	> 5 cm	0.0940	0.1920	0.24	1.0986 (0.7529–1.6069)	.6242
Tumor number	Single				1	
	Multiple	0.4285	0.1562	8.23	1.5350 (1.1415–2.1140)	.0041
Portal vein invasion	Absent				1	
	Present	0.3841	0.1843	4.05	1.4683 (1.0107–2.0898)	.0440
Nucleotide analogue	No				1	
	Yes	−0.1040	0.1903	0.31	0.9013 (0.6067–1.2859)	.5793
No. chemoembolization procedures		−0.3658	0.1194	10.00	0.6936 (0.5450–0.8720)	.0016

Note–Data on Child-Pugh class, tumor size, tumor number, and portal vein invasion refer to the status at initial diagnosis of hepatocellular carcinoma.

ARTICLE

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Suppression by Heat Shock Protein 20 of Hepatocellular Carcinoma Cell Proliferation via Inhibition of the Mitogen-Activated Protein Kinases and AKT Pathways

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ABSTRACT

Heat shock protein (HSP) 20, one of the low-molecular weight HSPs, is known to have versatile functions, such as vasorelaxation. However, its precise role in cancer proliferation remains to be elucidated. While HSP20 is constitutively expressed in various tissues including the liver, we have previously reported that HSP20 protein levels in human hepatocellular carcinoma (HCC) cells inversely correlate with the progression of HCC. In this study, we investigated the role of HSP20 in HCC proliferation. The activities of extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and AKT were negatively correlated with the HSP20 protein levels in human HCC tissues. Since HSP20 proteins were hardly detected in HCC-derived cell lines, the effects of HSP20 expression were evaluated using human HCC-derived HuH7 cells that were stably transfected with wild-type human HSP20 (HSP20 overexpressing cells). In HSP20 overexpressing cells, cell proliferation was retarded, and the activation of the mitogen-activated protein kinases (MAPKs) signaling pathways, including the ERK and JNK, and AKT pathways, as well as cyclin D1 accumulation induced by either transforming growth factor-α (TGFα) or hepatocyte growth factor, were significantly suppressed compared with the empty vector-transfected cells (control cells). Taken together, our findings strongly suggest that HSP20 suppresses the growth of HCC cells via the MAPKs and AKT signaling pathways, thus suggesting that the HSP20 could be a new therapeutic target for HCC. J. Cell. Biochem. 112: 3430–3439, 2011. © 2011 Wiley Periodicals, Inc.

KEY WORDS: HSP20; HEPATOCELLULAR CARCINOMA; GROWTH FACTOR; MAPK; AKT

Small heat shock proteins (HSPs) have molecular masses from 15 to 30 kDa based on their apparent molecular sizes. These proteins have significant similarities in terms of their amino acid sequences, known as the α-crystallin domain [Kappé et al., 2003; Taylor and Benjamin, 2005]. HSPs, which are well recognized to function as molecular chaperones, have been reported to be involved in a wide range of human cancers such as skin, breast, lung, prostate, colon and liver, and implicated in tumor cell proliferation, differentiation, invasion, metastasis, death, and recognition by the immune system [Ciocca and Calderwood, 2005]. HSP20 (HSPB6) was first identified from skeletal muscle [Kato et al., 1994; Taylor and Benjamin, 2005], and it is highly expressed in normal skeletal and smooth muscle, heart and liver tissues, where it may be essential [Kato et al., 1994]. HSP20 shows versatile functions, such as suppression of platelet aggregation [Kozawa et al., 2002], association with insulin resistance [Wang et al., 2001], prevention of vasospasms [Flynn et al., 2005], and airway smooth muscle relaxation [Komalavilas et al., 2008]. In addition, recent studies

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showed that HSP20 has protective functions in the heart [Fan et al., 2006, 2008; Fan and Kranias, 2010].

Hepatocellular carcinoma (HCC) is the third of most lethal cancer worldwide [Greten et al., 2009]. Even after resection, the overall survival of patients with HCC is still unsatisfactory due to frequent recurrence [Ercolani et al., 2003]. There is increasing evidence that protumorigenic growth factor signaling pathways, such as the transforming growth factor-α (TGFα)/epidermal growth factor (EGF)-signaling and/or hepatocyte growth factor (HGF) signaling pathways, are dysregulated in human HCC [Daveau et al., 2003; Breuhahn et al., 2006]. TGFα has been reported to correlate with differentiation and proliferation, which is likely to affect the early stages of hepatocarcinogenesis [Breuhahn et al., 2006]. HGF is also a potent growth factor for hepatocytes, and has been shown to be increased in clinical HCC tissues. Moreover, the HGF levels in the serum negatively correlate with the survival of patients and positively correlate with tumor size [Breuhahn et al., 2006]. MET, a HGF receptor, is an oncogene, and has been reported to be dysregulated in a subset of human HCCs, and its expression is related to more aggressive phenotype and poor prognosis of HCC [Daveau et al., 2003].

Mitogen-activated protein kinases (MAPKs) and AKT are essential components of intracellular signal transduction, and are activated by phosphorylation in response to various extracellular stimuli, including growth factors, such as TGFα and HGF [Breuhahn et al., 2006]. Among the MAPK superfamily, extracellular signal-regulated kinase (ERK) and c-jun N-terminal kinase (JNK) are key molecules that transfer signals into the nuclei to induce proliferation and differentiation [Ito et al., 1998; Kojima et al., 2004]. ERK has been reported to act as a potent proliferative factor in HCC and to be constitutively activated in the human HCC cells and tissues [Ito et al., 1998; Matsushima-Nishiwaki et al., 2001; Kojima et al., 2004]. JNK is also known as a potent inducer of proliferation in HCC [Hui et al., 2008]. As for AKT, while it generally regulates multiple processes, such as apoptosis, cell proliferation, glucose utilization, and anchorage-dependency [Datta et al., 1999], increasing evidence suggests that it also plays a major role in carcinogenesis and drug resistance in numerous types of cancer, including HCC [Datta et al., 1999; Nakanishi et al., 2005]. In particular, AKT phosphorylation has been reported as a significant risk factor for early HCC recurrence and poor prognosis [Nakanishi et al., 2005]. By inhibiting glycogen synthase kinase (GSK)-3β, both AKT and ERK-mediated growth factor pathways promote dephosphorylation and stabilization of cyclin D1 [Cohen and Frame, 2001]. Therefore, cyclin D1 plays a crucial role in neoplastic transformation and growth [Kim and Diehl, 2009], and its expression has been reported to be increased in HCC [Zhang et al., 1993].

Our previous studies showed that the level of HSP20 in human HCC tissues inversely correlates with the tumor size and the TNM stage of HCC [Noda et al., 2007]. However, the exact role of HSP20 in cancer progression remains to be clarified. The present study aimed to unveil the role of HSP20 in HCC in vivo and in vitro. Using a HCC-derived cell line that was stably transfected with wild-type HSP20, we demonstrated that HSP20 expression affects the proliferation of HCC cells and both the MAPK and AKT signaling pathways induced by TGFα and HGF.

MATERIALS AND METHODS

ANTIBODIES, CHEMICALS, AND PLASMIDS

Anti-HSP20 antibodies were purchased from Stressgen Biotechnologies Corporation (Atlanta, GA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies against ERK (p44/p42 MAPK), phospho-ERK (threonine (Thr) 202/tyrosine (Tyr) 204), MEK, phospho-MEK (serine (Ser) 217/221), JNK, phospho-JNK (Thr183/Tyr185), c-Jun, phospho-c-Jun (Ser63), Elk-1, phospho-Elk-1 (Ser383), AKT, phospho-AKT (Thr308), GSK-3β, phospho-GSK-3β (Ser9), phosphoinositide-dependent protein kinase (PDK)1, phospho-PDK1 (Ser 241), and cyclin D1 were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Recombinant human TGFα and HGF were obtained from R&D systems Inc. (Minneapolis, MN). Wild-type human HSP20 cDNA (clone ID 6074542), which was obtained from Open Biosystems, Inc. (Huntsville, AL), was cut out using restriction enzymes (*EcoRI* and *Xho* I) and subcloned into the eukaryotic expression vector pcDNA 3.1(+) (Invitrogen Corp., Carlsbad, CA).

TISSUE SPECIMENS

HCC tissues were obtained by surgical resection from 23 patients in the Department of Surgery, Ogaki Municipal Hospital (Gifu, Japan) according to a protocol approved by the committee for the conduct of human research at Ogaki Municipal Hospital and at Gifu University Graduate School of Medicine. Informed consent was obtained from all of the patients.

CELL CULTURES

Human HCC-derived HuH7, HLE, HLF, and PLC/PRF/5 cells were obtained from the Health Science Research Resources Bank (Tokyo, Japan). HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA). HuH7 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 (Sigma–Aldrich, MO) supplemented with 1% fetal calf serum (FCS) (Hyclone Co, Logan, UT) and other cell lines were cultured in DMEM (Sigma–Aldrich) with 10% FCS.

ESTABLISHMENT OF A STABLY TRANSFORMED CELL LINE

We newly established two new HuH7 cell lines, which were transfected with either a wild-type HSP20 vector or an empty control vector (called HSP20 overexpressing cells and control cells, respectively), by means of Tet-Off™ gene expression systems (Clontech Laboratories Inc., Palo Alto, CA) according to the manufacturer's instructions. Induction of HSP20 protein expression in HSP20 overexpressing cells can be controlled by the presence of doxycycline (Sigma–Aldrich). HSP20 overexpressing cells and control cells were maintained in RPMI1640 supplemented with 1% FCS, 200 μg/ml G418 (Invitrogen), 100 μg/ml hygromycin B (Merck KGaA Co. Darmstadt, Germany) and 1 μg/ml doxycycline.

PREPARATION OF PROTEIN SAMPLES AND THE WESTERN BLOT ANALYSIS

In order to induce HSP20 expression, HSP20 overexpressing cells and control cells were incubated in RPMI1640 without doxycycline

for 24 h. The cells were then cultured under serum-starvation for another 24 h and subsequently stimulated with 20 ng/ml TGFα or 20 ng/ml HGF for the indicated periods. A Western blot analysis of the lysates from HCC tissues and the cultured cells was performed as described previously [Noda et al., 2007; Matsushima-Nishiwaki et al., 2008]. To quantify the protein from the HCC tissue extracts, 0.3 μl of MagicMark XP Western protein standard (Invitrogen), a marker protein, was run in every gel. The samples from the cell cultures to be quantitatively compared by the Western blot analyses were run in the same gel. The data of the normalized values of the protein bands were statistically analyzed as described in the Statistical analysis section.

CELL COUNTING ASSAY AND BROMODEOXYURIDINE (BRDU) INCORPORATION ASSAYS

HSP20 overexpressing cells and control cells were plated on 96-well dishes (3×10^3 cells/well) in RPMI1640 medium with 10% FCS without doxycycline. For the cell counting assay, the medium was exchanged for RPMI1640 with 1% FCS 24 h after seeding, and cells were incubated for the indicated periods. The cell numbers were then counted using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions and the trypan blue dye exclusion method. For the BrdU incorporation assay, the medium was exchanged for RPMI1640 with 1% FCS 24 h after seeding, and cells were incubated for an additional 24 h. BrdU incorporation was then quantified using Cell proliferation enzyme-linked immunosorbent assay (ELISA), BrdU (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer's instructions.

DENSITOMETRIC ANALYSIS

The densitometric analysis was performed using a scanner and an image analysis software program (image J version 1,32). The phosphorylated protein levels were calculated as follows: the background-subtracted signal intensity of each phosphorylation signal was normalized to the respective total protein signal and plotted as the fold increase in comparison to control cells without stimulation.

STATISTICAL ANALYSIS

Data are expressed as the means ± SD. The statistical significance of the data from the cell culture experiments was analyzed using the Mann–Whitney *U*-test, and the patient clinical data were analyzed using the Spearman's correlation coefficient (r). All *P* values were derived from two-tailed tests and $P < 0.05$ was accepted as statistically significant. A Spearman's correlation coefficient of $|r| > 0.400$ was accepted as a positive correlation.

RESULTS

THE LEVELS OF HSP20 INVERSELY CORRELATED WITH THE LEVELS OF ERK, JNK, AND AKT ACTIVATION IN HUMAN HCC TISSUES

In this study, we first examined the correlation between HSP20 protein levels and the expression levels of phosphorylated ERK, JNK, and AKT in the clinical HCC tissues. The clinical and pathological characteristics of the patients with HCC are shown in Figure 1A. We found that the HSP20 protein levels were negatively correlated with the phosphorylation level of ERK ($r = -0.730$, $P = 0.0006$; Fig. 1B), JNK ($r = -0.555$, $P = 0.0092$; Fig. 1C), and AKT ($r = -0.646$, $P = 0.0024$; Fig. 1D). These results led us to further investigate the role of HSP20 in vitro.

THE EXPRESSION OF THE HSP20 PROTEIN IN HCC CELL LINES

In our previous study [Noda et al., 2007], we performed an immunohistochemical analysis of HSP20 in HCC specimens from several patients with HCC. We showed that HSP20 expression was detected in both HCC cells and the hepatocytes of non-tumor tissue. We have also provided some Western blot analysis data about HSP20 expression in HCC tissues [Noda et al., 2007, Supplementary Fig. 1A). Therefore, we decided to use an HCC cell line in order to analyze the role of HSP20 in the present study. We examined the endogenous expression levels of the HSP20 protein in five HCC cell lines, HuH7, HepG2, HLE, HLF, and PLC/PRF/5. Unexpectedly, HSP20 protein was hardly detected in any of the HCC cell lines (Supplementary Fig. 1A).

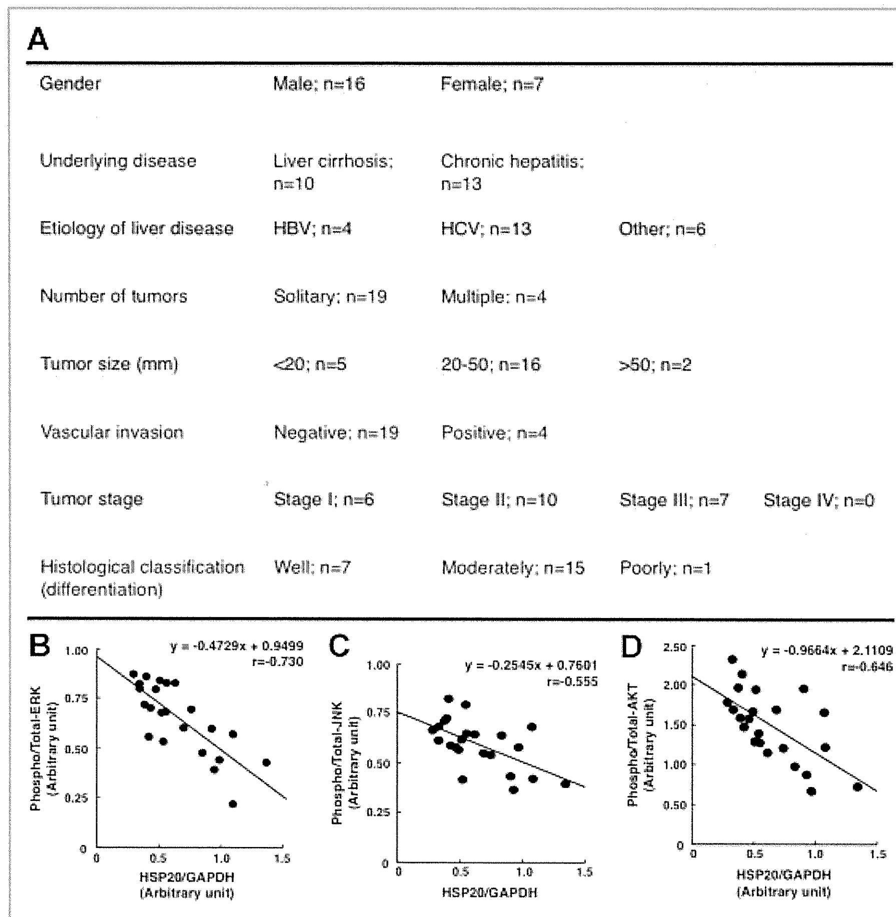
OVEREXPRESSION OF HSP20 DECREASES THE PROLIFERATION OF HCC CELLS

Based on our finding that HSP20 was absent or minimally expressed in HCC cell lines and tumor tissue specimens, we next established HuH7 cell lines which were transfected with wild-type HSP20 (HSP20 overexpressing cells) or an empty vector (control cells), as described in the Materials and Methods section and investigated the effect of HSP20 on cell proliferation in these HCC cells. A Western blot analysis demonstrated that HSP20 was overexpressed in the HSP20 overexpressing cells, but not control cells (Supplementary Fig. 1B). In our experiments, the results from the naive cells were almost the same as those from the control cells (data not shown).

To clarify the relationship between the expression of the HSP20 protein and cell growth, we first performed a cell counting assay using these two cell lines. The cell growth of the HSP20 overexpressing cells was remarkably suppressed in comparison to that of the control cells (Fig. 2). In addition to retarding cell proliferation, the cell viability of the HSP20 overexpressing cells also decreased more than that of the control cells. The percent cell viability of the HSP20 overexpressing cells and control cells were 50.5% and 85.9% after 7 days, and 50.3% and 71.0% after 8 days, respectively. To further investigate the inhibitory effect of HSP20 on HCC cell proliferation, BrdU incorporation, an indicator of cell proliferation, was analyzed. As expected, the BrdU incorporation in HSP20 overexpressing cells was significantly decreased in comparison to that of control cells (Supplementary Fig. 1C).

OVEREXPRESSION OF HSP20 REPRESSES THE ACTIVATION OF MEK-ERK AND JNK SIGNALING IN HCC CELLS

We hypothesized that the suppressive effect of HSP20 on the HCC cell proliferation was caused by a decrease in their response to growth factors. TGFα and MET, a unique HGF receptor, are frequently overexpressed in human HCCs, and enhanced signaling via these growth factor pathways correlates with cell proliferation [Breuhahn et al., 2006]. In addition, the expression of TGFα mRNA has been reported in HuH7 cells [Nakamura et al., 1996]. We



A

Gender	Male; n=16	Female; n=7		
Underlying disease	Liver cirrhosis; n=10	Chronic hepatitis; n=13		
Etiology of liver disease	HBV; n=4	HCV; n=13	Other; n=6	
Number of tumors	Solitary; n=19	Multiple; n=4		
Tumor size (mm)	<20; n=5	20-50; n=16	>50; n=2	
Vascular invasion	Negative; n=19	Positive; n=4		
Tumor stage	Stage I; n=6	Stage II; n=10	Stage III; n=7	Stage IV; n=0
Histological classification (differentiation)	Well; n=7	Moderately; n=15	Poorly; n=1	

Fig. 1. There is a significant inverse correlation between the levels of HSP20 and the activation of ERK, JNK, and AKT in human HCC tissues. A: The clinical and pathological characteristics of patients with HCC. B–D: The correlations between the expression levels of HSP20 and the levels of phopsho-ERK (B), phospho-JNK (C) or phospho-AKT (D) in human HCC tissues. The expression levels of HSP20, phospho- and total ERK, phospho- and total JNK, phospho- and total AKT, and GAPDH were determined by the band intensities obtained from the Western blot analyses. The band intensities of HSP20, phospho-ERK, phospho-JNK, and phospho-AKT were normalized to those of GAPDH, total ERK, total JNK and total AKT, respectively. The data were analyzed with Spearman's correlation coefficient (r), and $|r| > 0.400$ was accepted as a positive correlation. $P < 0.05$ was considered to be statistically significant.

observed that HuH7 cells indeed released TGFα, and that when the cells were treated with neutralizing antibodies against TGFα, the cell number and BrdU incorporation of the HuH7 cells were significantly inhibited by 11% and 19%, respectively [data not shown). Since HGF is also a potent growth factor for hepatocytes and we detected MET protein expression in HuH7 cells (data not shown), we examined the effects of TGFα and HGF on several molecules related to cell proliferation in both of the HSP20 overexpressing and control HuH7 cells. Twenty nanogram per millilitre of growth factors was found to be the minimum concentration that could be used to obtain a significant effect.

We examined whether the retardation of cell growth in HSP20 overexpressing cells correlates with the ERK activity in these cells. The basal levels of phosphorylated ERK were similar between HSP20 overexpressing cells and control cells (Fig. 3A,B, lane 1 compared with lane 2, respectively). Although the phosphorylation levels of ERK (phospho-ERK level) in HSP20 overexpressing cells and control cells were similarly increased after stimulation with 20 ng/ml of TGFα for 10 min, it in HSP20 overexpressing cells was significantly decreased in comparison to that in control cells for 30 or 60 min after the stimulation (Fig. 3A, lanes 3–8). With regard to HGF, while 20 ng/ml of HGF also induced the phosphorylation of ERK in HSP20 overexpressing cells and control cells, the level in HSP20 overexpressing cells was significantly lower than that in control cells when the cells were stimulated with HGF for 10, 30, or 60 min (Fig. 3B, lanes 3–8). Neither TGFα nor HGF affected the total ERK protein levels in HSP20 overexpressing cells or control cells.

We next focused on the upstream kinase, MEK, because it regulates ERK activity. MEK was not affected in a manner similar to ERK, and in particular, the inhibition of MEK by TGFα and HGF had different kinetics (Fig. 3C,D). Most probably, this was due to the different behavior of the controls. Additionally, we did not detect any differences in the phosphorylation levels of c-Raf, an upstream kinase of MEK, between HSP20 overexpressing cells and the control cells stimulated with TGFα or HGF (data not shown). We also examined whether the retardation of cell growth in HSP20 overexpressing cells correlated with the JNK activity. The basal levels of phosphorylated JNK were similar between HSP20

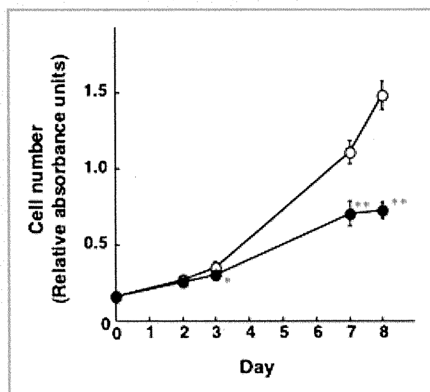


Fig. 2. The cell proliferation of HSP20-overexpressing HuH7 (HSP20 overexpressing) cells. The cell growth curves of HSP20 overexpressing cells (closed circle) compared with control cells (open circle). The data are the means ± SD (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$ versus curves form the control cells (open circle) at the indicated days.

overexpressing cells and control cells (Fig. 3E,F, lanes 1 and 2). TGFα or HGF caused a marked phosphorylation of JNK in the control cells at 10 min after stimulation (Fig. 3E,F, lane 3 compared to lane 1). The phospho-JNK levels in the control cells were greatly decreased starting from 30 min after stimulation. On the other hand, TGFα or HGF had little effect on the phosphorylation of JNK in the HSP20 overexpressing cells (Fig. 3E,F, lanes 4, 6, and 8). At 30 and 60 min, the levels between the control cells and HSP20 overexpressing cells were very similar. Neither TGFα nor HGF affected the total expression of JNK protein in either cell line.

HSP20 INHIBITS THE C-JUN AND ELK-1 ACTIVITIES IN THE HCC CELLS

ERK and JNK are known to activate the transcription factors Elk-1 and c-Jun by phosphorylation [Su and Karin, 1996]. Elk-1 contributes to an increased expression of the c-Fos oncoprotein [Su and Karin, 1996]. The heterodimerized complex of c-Jun and c-Fos is called activation protein-1 (AP-1), which is known as a potent activator of HCC proliferation [Koike and Moriya, 2005]. Therefore, we next examined whether the retardation of cell growth in HSP20 overexpressing cells correlated with the activities of c-Jun and Elk-1. We observed that the basal levels of phosphorylated c-Jun and Elk-1 were similar between HSP20 overexpressing and control cells (Fig. 4, lanes 1 and 2). However, the phospho-c-Jun levels in HSP20 overexpressing cells were markedly decreased in comparison to control cells when they were stimulated with TGFα (Fig. 4A, lanes 4, 6, and 8 compared to lanes 3, 5, and 7, respectively). As for HGF, while it also induced the phosphorylation of c-Jun in HSP20 overexpressing cells and control cells, the phosphorylation levels in HSP20 overexpressing cells were significantly lower than those in the control cells (Fig. 4B, lanes 4 and 6 compared to lanes 3 and 5, respectively). In addition, similar effects were observed for Elk-1 in cells that were stimulated with TGFα or HGF (Fig. 4C,D). Although

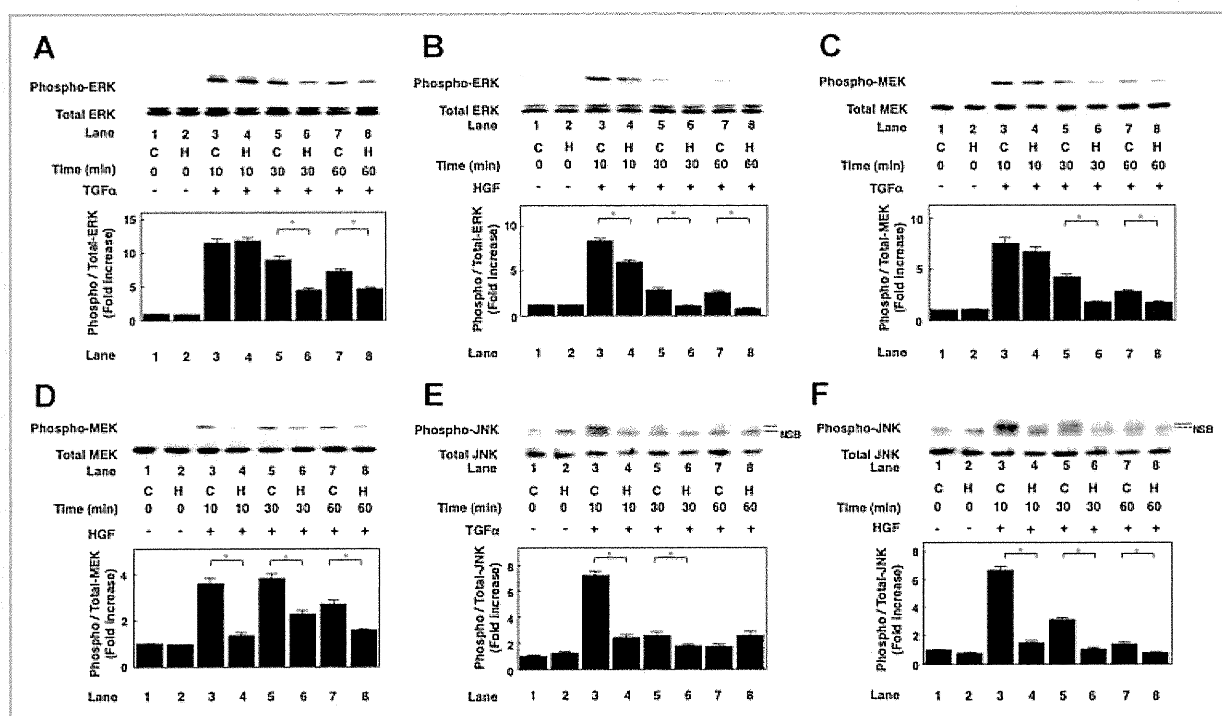


Fig. 3. The phosphorylation of MEK-ERK and JNK signaling induced by TGFα or HGF in HSP20 overexpressing cells (H) and control cells (C). HSP20 overexpressing cells and control cells were stimulated with 20 ng/ml TGFα (A, C, and E) or HGF (B, D, and F) for the indicated periods and the levels of phospho-ERK (A,B), phospho-MEK (C,D) and phospho-JNK (E,F) were determined by a Western blot analysis. The lower bar graph shows the quantification data for the relative levels of phospho-ERK, phospho-MEK, or phospho-JNK after normalization with respect to the total proteins, as determined by a densitometry analysis. The values are the means ± SD (n = 3). $^{*}P < 0.05$. NSB, non-specific band.

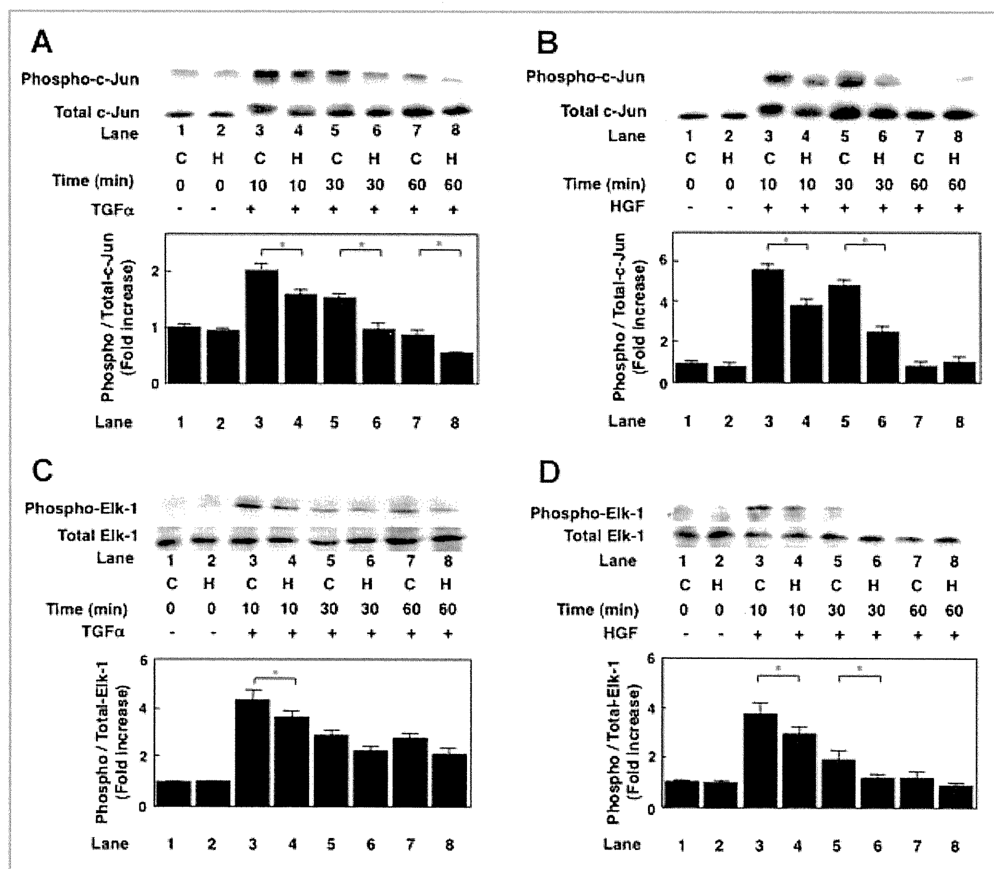


Fig. 4. The phosphorylation of c-Jun and Elk-1 after treatment with TGFα or HGF in HSP20 overexpressing cells (H) and control cells (C). HSP20 overexpressing cells and control cells were stimulated with 20 ng/ml TGFα (A,C) or HGF (B,D) for the indicated periods and the levels of phospho-c-Jun (A,B) and phospho-Elk-1 (C,D) were determined by a Western blot analysis. The lower bar graph shows the quantification data for the relative levels of phospho-c-Jun and phospho-Elk-1, after normalization with respect to the total protein, as determined by a densitometry analysis. The values are the means ± SD (n = 3). $^*P < 0.05$.

the bands of phosphorylated c-Jun were shifted and shown as broad bands by a Western blot analysis, the total c-Jun and Elk-1 proteins were expressed at similar level in both the HSP20 overexpressing cells and control cells, regardless of whether or not they were stimulated with TGFα or HGF.

HSP20 INHIBITS THE ACTIVATION OF THE AKT SIGNALING PATHWAY IN HCC CELLS

PDK1 plays a critical role in growth factor pathways [Datta et al., 1999]. The phosphorylation of the activation loop threonine 308 by PDK1 is necessary for AKT activity [Datta et al., 1999]. Therefore, we examined whether the retardation of cell growth in HSP20 overexpressing cells correlated with the phosphorylation status of PDK1. The basal level of phospho-PDK1 in HSP20 overexpressing cells was significantly lower than that in the control cells (Fig. 5A). Neither TGFα nor HGF affected the phosphorylation level of PDK1 (data not shown), which is consistent with a previous study showing that PDK1 is a constitutively active enzyme, and that its activity is not enhanced by AKT activators [Datta et al., 1999].

While TGFα caused a marked increase in the phosphorylation of AKT in control cells (Fig. 5B, lane 3 compared to lane 1), TGFα had little effect on the phospho-AKT levels in HSP20 overexpressing cells (Fig. 5B, lanes 4, 6, and 8). Similarly, HGF also induced the phosphorylation of AKT in control cells at a peak of 30 min after stimulation, and the phosphorylation of AKT in HSP20 overexpressing cells was induced to a much lower extent, compared to the control cells (Fig. 5C).

Unlike most protein kinases, GSK3β is constitutively active in unstimulated cells [Force and Woodgett, 2009]. AKT and ERK signaling cascades are known to phosphorylate and inactivate the growth suppressive activity of GSK3β [Cohen and Frame, 2001]. Surprisingly, the basal phosphorylation level of GSK3β was dramatically lower in HSP20 overexpressing cells than that in control cells (Fig. 5D,E, lane 1 compared with lane 2, respectively). The phosphorylation levels of GSK3β in HSP20 overexpressing cells seem to be lower than those in the control cells, regardless of the addition of TGFα or HGF.

CYCLIN D1 EXPRESSION IS SUPPRESSED IN THE HCC CELLS OVEREXPRESSING HSP20

Increased ERK and JNK activities and the downregulation of GSK3β contribute to the induction of AP-1 transcriptional activity, and AP-1 activates the cyclin D1 promoter to induce cell proliferation [Cohen and Frame, 2001; Lavoie et al., 1996]. Moreover, cyclin D1 is

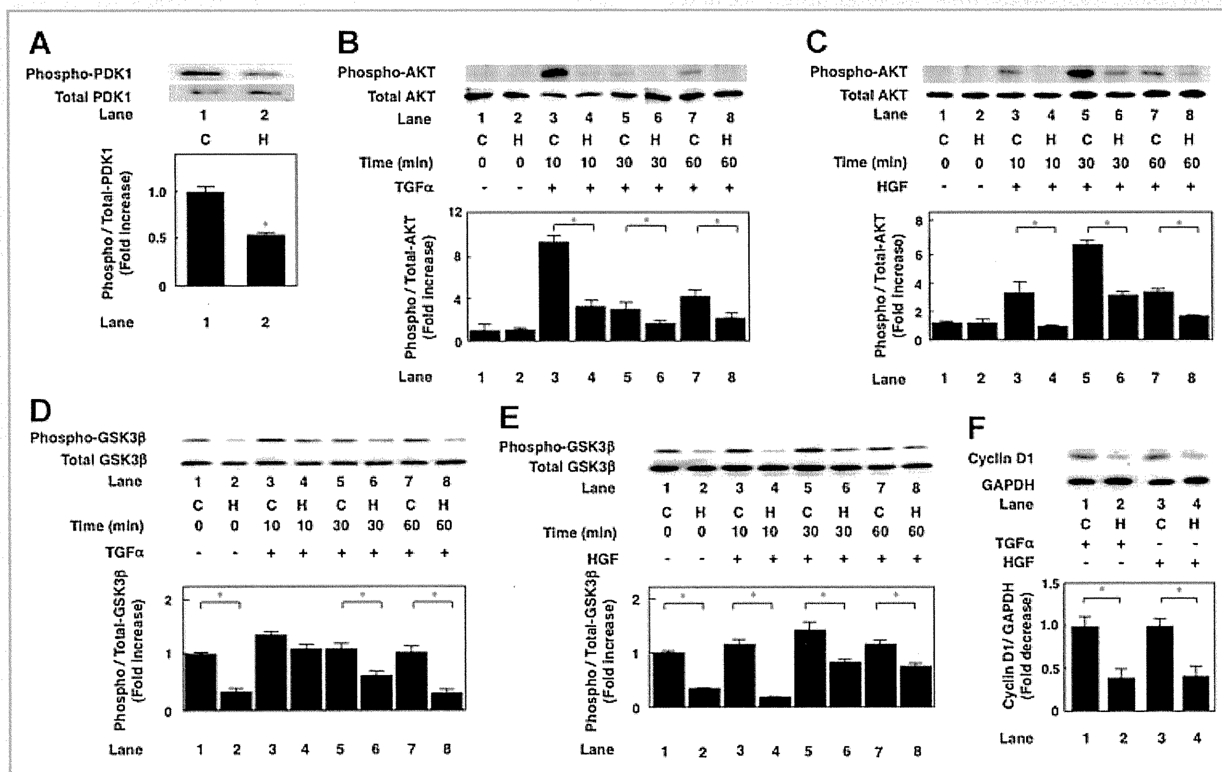


Fig. 5. The PDK1–AKT–GSK3β signaling pathway and cyclin D1 protein expression in HSP20 overexpressing cells (H) and control cells (C). A: The phosphorylation status of PDK1 in HSP20 overexpressing cells and control cells. HSP20 overexpressing cells and control cells were collected without stimulation, and protein levels were determined by a Western blot analysis. B–E: The phosphorylation of AKT and GSK3β by TGFα or HGF in HSP20 overexpressing cells and control cells. HSP20 overexpressing cells and control cells were stimulated with 20 ng/ml TGFα (B,D) or HGF (C,E) for the indicated periods, and the levels of phospho-AKT (B,C) and phospho-GSK3β (D,E) were determined by a Western blot analysis. F: The expression level of cyclin D1 in HSP20 overexpressing cells and control cells. HSP20 overexpressing cells and control cells were stimulated with TGFα or HGF for 24 h and protein levels were determined by a Western blot analysis. Each lower bar graph shows the quantification data for the relative phosphorylation level, after normalization with respect to the total protein. The values are the means ± SD (n = 3). *$P < 0.05$ versus control cells (A), *$P < 0.05$ (B–F).

phosphorylated by GSK3β at Thr286, which is a target for ubiquitination and subsequent proteolytic destruction [Cohen and Frame, 2001]. Therefore, we examined the involvement of HSP20 in the expression levels of cyclin D1 protein in HSP20 overexpressing cells and control cells. When cultured in the presence of either TGFα or HGF for 24 h, the expression level of cyclin D1 protein in HSP20 overexpressing cells was significantly lower than that in control cells (Fig. 5F), thus suggesting a suppressive role for HSP20 in cell proliferation.

DISCUSSION

Although a variety of functions of HSP20 have been previously reported, the precise role of HSP20 in tumor progression still remains unknown. We previously reported that the HSP20 levels in human HCC tissues are reduced compared with the non-tumor tissues, and that the expression levels of HSP20 in tumors are inversely correlated with the tumor stage based on the TNM classification, presence of microvascular invasion, and tumor size [Noda et al., 2007]. It is generally accepted that the activation of the ERK, JNK, and AKT signaling pathways lead to mitogenic effects in HCC [Ito et al., 1998; Datta et al., 1999; Llovet and Bruix, 2008; Min et al., 2011]. In this study, we demonstrated that the expression of HSP20 was inversely correlated with the activity of ERK, JNK, and AKT in the clinical specimens from patients with HCC, and when HSP20 was overexpressed in an HCC cell line (HSP20 overexpressing cells). Our present findings strongly suggest that the decrease in HSP20 protein expression in tumors accelerates HCC progression. The decreased expression of the HSP20 protein may result in an increased or infinite proliferative capacity of HCC cell lines.

The dysregulation of cell signaling caused by growth factors, such as TGFα and HGF, is commonly found in human HCC [Daveau et al., 2003; Llovet and Bruix, 2008; Greten et al., 2009]. Various substances that promote HCC proliferation, such as the hepatitis C virus core protein and ethanol, induce TGFα expression [Sato et al., 2006; Hennig et al., 2009], and HGF promotes hepatocarcinogenesis through MET activation in HCC [Horiguchi et al., 2002]. We therefore examined the function of HSP20 in HCC cells in the presence of these growth factors. The downregulation of the MAPKs and AKT pathways in HSP20 overexpressing cells were followed by attenuated cyclin D1 expression. A decrease in cyclin D1 due to inhibition of the MAPKs and AKT pathways might be a mechanism by which HSP20 controls of HCC proliferation. In contrast, whereas TGFα and HGF induced EGF receptor (EGFR) and

MET phosphorylation, respectively, there was little difference in the phosphorylation levels of these proteins between HSP20 and control cells (data not shown), which led us to speculate that HSP20 targets a protein downstream of the EGFR and MET. The potential mechanism by which HSP20 regulates the proliferation of HCC cells is summarized in Figure 6.

HSP20 was recently reported to form a complex with 14-3-3 protein, and this association affects the activities of other 14-3-3 binding proteins [Chernik et al., 2007; Dreiza et al., 2010]. MAPK/ERK kinase kinase 3 (MEKK3), a common upstream kinase of ERK and JNK, was also reported to bind 14-3-3, and its association with 14-3-3 is required for sustained MEKK3 kinase ativity [Fritz et al., 2006]. In our present study, HSP20 did not interfere with Raf-1 kinase activity, but did inhibit MEK activity in HCC cells, thus suggesting that HSP20 might compete for 14-3-3 binding with MEKK3 in HCC. Furthermore, 14-3-3 directly interacts with and regulates the activities of PDK1 and GSK3β [Sato et al., 2002; Agarwal-Mawal et al., 2003]. We showed that the phosphorylation levels of GSK3β in HSP20 overexpressing cells were lower than those in the control cells, regardless of TGFα or HGF. In addition, the basal level of phospho-PDK1 in HSP20 overexpressing cells was lower than that in the control cells. Therefore, it is likely that 14-3-3 might mediate the signaling between HSP20 and its targets proteins, such as GSK3β and PDK1, and HSP20 might regulate the activity of GSK3β or PDK1 via 14-3-3.

Besides 14-3-3, phosphorylated HSP20 has also been reported to interact with AKT, actin, apoptosis signal-regulating kinase 1 (ASK1), Bax, Bag3, Beclin-1 HSP22, and HSP27 [Fan and Kranias, 2010]. In addition, wild-type HSP27 has been reported to associate with and inhibit the rate of phosphorylation of HSP20 [Bukach et al., 2009]. Taken together with our previous study showing that phosphorylated HSP27 represses the growth of HCC cells via inhibition of the ERK signaling pathway [Matsushima-Nishiwaki et al., 2008], the interaction between HSP20 and HSP27 might have an effect on the ERK signaling in HCC. The attenuation of β-adrenergic agonist-mediated cardiac remodeling by HSP20 is associated with an inhibitory effect on the apoptosis signal-regulating kinase 1 (ASK1)-JNK/p38 MAPK signaling cascade [Fan et al., 2006]. In addition, phosphorylated HSP20 has been reported to interact with phospho-AKT and preserve AKT activities in cardiomyocytes, leading to cardioprotective effects against doxorubicin-triggered cardiac toxicity [Fan et al., 2008]. The overexpression of HSP20 also reportedly protects mesenchymal stem cells against oxidative stress-induced cell death, and this effect is associated with enhanced AKT activation and increased secretion of growth factors [Wang et al., 2009]. The HSP20 in cardiomyocytes and mesemchymal cells showed opposite activities on AKT activity from our results in HCC. These discrete effects of HSP20 might be caused by the differences in the phosphorylation status of HSP20 and/or the conditions of the other cellular proteins that form a complex with HSP20.

The activation of signaling pathways, such as the ERK or AKT pathways, predicts poor prognosis and early recurrence of HCC [Matsushima-Nishiwaki et al., 2001; Schmitz et al., 2008], and the development of targeted therapies against the ERK and AKT pathways are ongoing [Porta and Paglino, 2010]. AZX100, a cell permeable phosphopeptide analog of HSP20, is currently under development as a candidate therapeutic agent for its cardioprotective and smooth muscle relaxation effects [Lopes et al., 2009; Furnish et al., 2010]. The HSP20 expression has been recently reported to decrease not only in HCC, but also in melanoma, cervical squamous carcinoma, lung adenoma and in glioma [Edwards et al., 2011]. HSP20 could therefore represent a new therapeutic target for cancer. Although further detailed analyses, especially on the phosphorylation status and activities of HSP20 and its associated proteins are necessary, our findings suggest that the inhibition of MAPK and AKT signaling pathways by HSP20 in HCC could represent a novel therapeutic strategy.

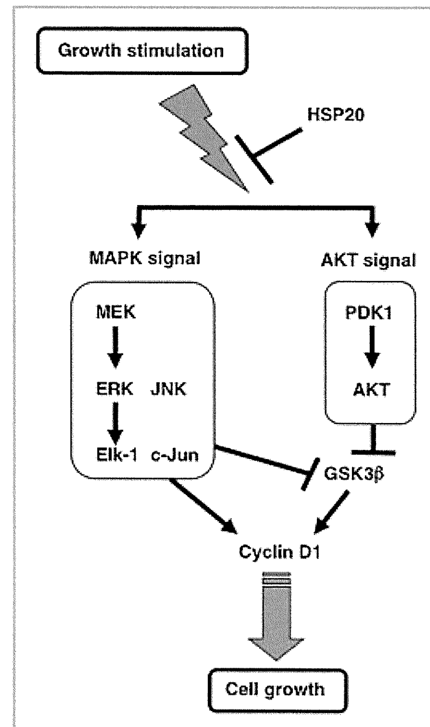


Fig. 6. Role of HSP20 in HCC cell growth. In a liver with HCC, growth factors, such as TGFα or HGF, cause the activation of the ERK/JNK (MAPK) signaling pathway and/or AKT signaling pathway, leading to the subsequent increase in the expression of cyclin D1. We speculate that HSP20 functions as a negative regulator upstream of MEK or PDK1.

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HEPATOLOGY

Characteristics and prognosis of patients with hepatocellular carcinoma after the year 2000 in Japan

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after the year 2000, early detection, hepatocellular carcinoma, liver function, prognosis, surveillance.

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Abstract

Background and Aim: The survival rate of patients with hepatocellular carcinoma (HCC) improved through the 1990s in Japan, primarily due to advances in the detection of small HCC under the establishment of surveillance systems. We investigated how the characteristics of patients with HCC changed and whether this trend is continuing after the year 2000.

Methods: The characteristics and survival rates of patients with initial HCC (not a recurrence) who were diagnosed after the year 2000 until 2008 were analyzed and compared with those of patients in whom HCC was diagnosed in the 1990s or before.

Results: In comparison to 8 years before the year 2000, the percentage of patients with better liver function at diagnosis of HCC increased after the year 2000, whereas the size of maximal HCC tumors did not change in comparison to patients before the year 2000. The survival rate of patients continued increasing after the year 2000.

Conclusions: The prognosis of patients with HCC continues to improve after the year 2000. This is not due to further improvements in the detection of small-sized HCC; the detection of small HCC had reached a plateau in the 1990s. Rather, this improvement appears to be due in part from the continued increase in the distribution of patients with better liver function at diagnosis.

Introduction

Hepatocellular carcinoma (HCC) is among the most common cancers worldwide. It is the sixth most common cancer in the world, and the third most common cause of cancer-related death.[1,2] In Japan, HCC is the third most common cause of death from cancer in men, and the fifth in women.[3] The prognosis of patients with HCC has improved due to improvements in the management of such patients, including the development of novel treatment options or techniques and increased early detection of HCC.

We previously observed the improvement of the survival rate of patients with HCC during the years 1976–2000, particularly in the 1990s.[4] In that observation, we found that the increase in the early detection of HCC associated with the establishment of surveillance systems for HCC was the strongest contributing factor in the improvement of patient survival.[4,5] However, it has not been revealed whether this trend persists after the year 2000 into the 21st century.

In the present study, we investigated how the characteristics of patients with HCC changed and whether the improvement of patient survival continues after the year 2000.

Methods

Patients and analyses

The entire protocol was approved by the hospital ethics committee and carried out in compliance with the Declaration of Helsinki. Between 1981 and 2008, a total of 2013 patients were diagnosed as having initial HCC (not a recurrence) at Ogaki Municipal Hospital (Ogaki, Japan). Diagnosis was confirmed by histological findings on the basis of resected hepatic tumors or ultrasonography-guided needle biopsy specimens. In cases in which resection was not indicated and it was necessary to avoid biopsy of the tumor because of the possibility of needle tract seeding of the cancer cells in association with biopsy, especially in patients with advanced tumors, the diagnosis of HCC was based on the imaging findings of selective hepatic angiography and computed tomography (CT). These included hypervascularity on angiographic images and a high-density mass on arterial-phase dynamic CT images, and a low-density mass on portal-phase dynamic CT images. When findings indicative of HCC were not obtained by means of dynamic CT or angiography, CT during hepatic arteriography and CT during arterial portography or T1- and T2-weighted imaging

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associated with superparamagnetic iron oxide-enhanced magnetic resonance imaging (MRI) were performed after the 1990s.

Individual decisions regarding treatment were made primarily on the basis of the treatment guidelines for HCC in Japan. Patients were initially assessed for eligibility for hepatic resection. In hepatic resection, the tumor was resected with an ample margin as hepatectomy, and enucleation of the HCC tumor without margin was not performed as surgical treatment. Only patients who had class A liver function by Child–Pugh classification[6] (with some exceptions) and 15-min retention of indocyanin green test of ≤30%, and had no more than three HCC tumors, were considered for surgical treatment. When patients declined or were deemed ineligible for surgical treatment, they underwent non-surgical treatment. Patients were first considered to be offered locoregional ablative therapies (LAT). Patients who had no more than three HCC tumors with a maximal tumor size ≤3 cm were considered for LAT. Before the year 1995, percutaneous ethanol injection (PEIT) was performed for all patients as LAT, because other modalities for LAT were not available. Some patients underwent percutaneous microwave thermocoagulation (PMCT) during 1996–2000. After the year 2000 when radiofrequency ablation (RFA) became available for LAT, all patients underwent RFA with some exceptions. Patients who were ineligible for both surgery and LAT were offered transcatheter arterial chemoembolization (TACE). No patient underwent liver transplantation as a treatment, because it is extremely difficult to find a cadaveric donor for transplantation in Japan due to religious reasons. In addition, living-donor liver transplantation was not performed at our institution during the study period. No patients received molecular-targeted drugs during the study period.

The etiology of underlying liver disease, characteristics and the progression of HCC, liver function at the time of HCC diagnosis, and patient survival rates were analyzed on the basis of clinical records. The Child–Pugh classification was determined as an indicator of liver function. Tumor staging was performed according to the American Joint Committee on Cancer (AJCC) classification system.[7] In cases in which pathological evaluation was not available, vascular invasion was assessed by means of dynamic CT and angiography. The initial treatment for HCC was also investigated. Patients were stratified into seven periods by year of HCC diagnosis: 1981–1984, 1985–1988, 1989–1992, 1993–1996, 1997–2000, 2001–2004, and 2005–2008.

All patients were followed up from 0.1 months to 241.1 months (median follow-up period: 19.1 months) at our institution after diagnosis and treatment. Patients were followed up with ultrasonography, and CT or MRI was performed every 3–6 months. In addition, regular monitoring of serum tumor markers (α-fetoprotein [AFP] and des-gamma-carboxy prothrombin [DCP]) was performed every 3 months. When the elevation of tumor markers was observed, additional imaging examinations (usually by CT or MRI) were performed to check the presence of HCC. When the recurrence of HCC was confirmed, patients underwent treatment for recurrent HCC, as well as the treatment for initial HCC.

Statistical analysis

Values were expressed as mean ± standard deviation, unless otherwise indicated. Differences in percentages between groups were analyzed by the χ^2-test. Differences in mean quantitative values were analyzed by Mann–Whitney U-test. The date of HCC diagnosis was defined as time zero in the calculation of patient survival rates. Surviving patients and patients who died from a cause other than liver disease were censored. Patients who died from an HCC-related cause or liver failure were not censored. The Kaplan–Meier method[8] was used to calculate survival rates, and the log–rank test[9] was used to analyze differences in survival.

The Cox proportional hazards model[10] was used for the multivariate analysis of factors related to survival. The variables analyzed were the period of the diagnosis of HCC (1981–2008), patient age and sex, Child–Pugh class, tumor stage by AJCC, and initial treatment. Data analyses were performed with the JMP statistical software package (version 6.0, Macintosh version; SAS Institute, Cary, NC, USA). All P-values were derived from two-tailed tests, and $P < 0.05$ was accepted as statistically significant.

Results

Patient characteristics and HCC

The demographic characteristics of the 2013 patients included in this study are summarized in Table 1. The study patients included 1495 men and 518 women, with a mean age of 65.0 ± 9.6 (range: 21–93) years. Liver function at diagnosis of HCC was Child–Pugh class A in 1137 (56.5%) patients. HCC was stage I in 797 (39.6%) patients and stage II in 574 (28.5%) patients, according to the TNM stage classification of the AJCC.

With the exception of 356 (17.7%) patients who had not received treatment, all patients underwent treatment for HCC within 2 weeks after the diagnosis of HCC. Treatment included hepatectomy in 459 (22.8%) patients and LAT in 392 (19.5%) patients. Among patients receiving LAT, 190 patients were treated by PEIT and 189 patients were treated by RFA. HCC was treated by TACE in 618 (30.7%) patients. The diagnosis of HCC in 459 patients who underwent hepatectomy was based on a histological examination of tumor tissue taken from resected specimens. In patients treated by LAT, the diagnosis of HCC was made based on fine-needle biopsy of specimens from 162 of the 392 patients (41.3%). In the remaining 230 patients treated by LAT, the diagnosis was made based on the imaging findings. HCC was diagnosed by the imaging findings in all 618 patients who underwent TACE. A histological diagnosis was made in 21 of the 188 patients (11.2%) who underwent treatment other than surgery, LAT, or TACE, and 20 of the 356 patients (5.6%) who did not undergo treatment. In total, HCC was diagnosed histologically in 662 (32.9%) patients.

Characteristics and treatment for HCC by period

We analyzed the trends in the characteristics of patients with HCC by period. The numbers of patients who were diagnosed as having initial HCC (not a recurrence) were 141 patients during the period 1981–1984, 220 during 1985–1988, 292 during 1989–1992, 305 during 1993–1996, 334 during 1997–2000, 366 during 2001–2004, and 355 during 2005–2008. This number increased during the 1980s and 1990s and peaked during 2001–2004. Patient age at the diagnosis was increasing throughout the study period. The

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Table 1 Clinical characteristics of study patients ($n = 2013$)

Age (mean ± SD, years) (range)	65.0 ± 9.6 (21–93)
Sex ratio (female/male)	518 (25.7%)/1495 (74.3%)
Etiology of underlying liver disease (HBV/HCV/HBV, HCV/non-HBV, non-HCV/non-HBV)	368 (18.3%)/1175 (58.4%)/23 (1.1%)/223 (11.1%)/224 (11.1%)
Child–Pugh class (A/B/C)†	1137 (56.5%)/650 (32.3%)/226 (11.2%)
Albumin (mean ± SD, g/dL)	3.50 ± 0.56
Total bilirubin (mean ± SD, mg/dL)	1.19 ± 1.28
Diagnostic modality (histology/other)	662 (32.9%)/1351 (67.1%)
AJCC tumor stage (I/II/III/IV)	797 (39.6%)/574 (28.5%)/554 (27.5%)/88 (4.4%)
Tumor size (mean ± SD, cm) (range)	5.70 ± 3.37 (0.5–29.4)
Tumor size (≤ 2 cm/> 2 cm and ≤ 5 cm/> 5 cm)	572 (28.4%)/677 (33.6%)/764 (38.0%)
Tumor number (single/multiple)	870 (43.2%)/1143 (56.8%)
Vascular invasion (absent/present)	1398 (69.4%)/615 (30.6%)
Initial treatment	
No treatment	356 (17.7%)
Hepatectomy	459 (22.8%)
LAT	392 (19.5%)
TACE	618 (30.7%)
Other	188 (9.3%)

†Category of Child–Pugh class A includes patients without cirrhosis. Other treatment included repeated arterial infusion chemotherapy ($n = 93$), one-shot arterial infusion of anticancer drug ($n = 61$), systemic chemotherapy ($n = 26$), and radiation ($n = 8$). AJCC, American Joint Committee on Cancer; HBV, hepatitis B virus; HCV, hepatitis C virus; LAT, locoregional ablative therapy, including percutaneous ethanol injection, percutaneous microwave thermocoagulation, and radiofrequency ablation; non-HBV, hepatitis B virus was negative (hepatitis C virus was not tested before 1990); non-HBV, non-HCV, both hepatitis B virus and hepatitis C virus were negative; SD, standard deviation; TACE, transcatheter arterial chemoembolization.

Table 2 Clinical characteristics of study patients between periods 1992–2000 and 2001–2008

	Periods 1992–1996 and 1997–2000 ($n = 639$)	Periods 2001–2004 and 2005–2008 ($n = 721$)
Age (mean ± SD, years) (range)[1]	64.7 ± 8.8 (36–93)	68.2 ± 9.3 (21–91)
Sex ratio (female/male)	172 (26.9%)/467 (73.1%)	203 (28.2%)/518 (71.8%)
Etiology of underlying liver disease (HBV/HCV/HBV, HCV/non-HBV, non-HCV)	94 (14.7%)/463 (72.5%)/12 (1.9%)/70 (10.9%)	114 (15.8%)/503 (69.8%)/9 (1.2%)/95 (13.2%)
Child–Pugh class (A/B/C)[2]	380 (59.5%)/197 (30.8%)/62 (9.7%)	497 (68.9%)/169 (23.5%)/55 (7.6%)
Albumin (mean ± SD, g/dL)[3]	3.31 ± 0.62	3.59 ± 1.09
Total bilirubin (mean ± SD, mg/dL)	1.33 ± 1.76	1.20 ± 1.37
AJCC tumor stage (I/II/III/IV)[4]	266 (41.6%)/190 (29.7%)/157 (24.6%)/26 (4.1%)	369 (51.2%)/199 (27.6%)/124 (17.2%)/29 (4.0%)
Tumor size (mean ± SD, cm) (range)	4.28 ± 3.39 (0.5–19.0)	4.07 ± 3.25 (0.5–19.2)
Tumor size (≤2 cm/>2 cm and ≤5 cm/>5 cm)	221 (34.6%)/221 (34.6%)/197 (30.8%)	237 (32.9%)/300 (41.6%)/184 (25.5%)
Tumor number (single/multiple)[5]	282 (44.1%)/357 (55.9%)	392 (54.4%)/329 (45.6%)
Vascular invasion (absent/present)[6]	487 (76.2%)/152 (23.8%)	599 (83.1%)/122 (16.9%)
AFP (median, ng/mL) (range)[7]	38.0 (0.0–595 000)	24.7 (0.8–2 402 000)
DPC (median, mAU/mL) (range)[8]	62.0 (10.0–8 000)	38.2 (10.0–75 000)
Antiviral therapy for HBV infection[9]	8 (7.5%)	72 (58.5%)
Antiviral therapy for HCV infection[10]	36 (7.6%)	73 (14.3%)
Eradication of HCV by antiviral therapy	8 (1.7%)	17 (3.3%)

[1] $P < 0.0001$; [2] $P = 0.0013$; [3] $P < 0.0001$; [4] $P = 0.0011$; [5] $P = 0.0002$; [6] $P = 0.0020$; [7] $P = 0.0003$; [8] $P = 0.0027$; [9] $P < 0.0001$; [10] $P = 0.0012$. †Category of Child-Pugh class A includes patients without cirrhosis. AFP, α-fetoprotein; AJCC, American Joint Committee on Cancer; DCP, des-gamma-carboxy prothrombin; HBV, hepatitis B virus; HCV, hepatitis C virus; non-HBV, non-HCV, both hepatitis B virus and hepatitis C virus were negative; SD, standard deviation.

mean age was 60.6 ± 9.1 during the period 1981–1984, 61.4 ± 10.0 during the period 1985–1988, 62.3 ± 9.2 during the period 1989–1992, 63.8 ± 8.5 during the period 1993–1996, 65.5 ± 9.0 during the period 1997–2000, 68.0 ± 9.1 during the period 2001–2004, and 68.5 ± 9.5 during the period 2005–2008. The prevalence of patients with Child–Pugh class A liver function at diagnosis and the prevalence of patients with AJCC tumor stage I continued increasing after the period 1985–1988. In contrast, the prevalence of patients with maximal tumor size < 2 cm markedly increased between the period 1985–1988 and the period 1989–

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1992, but reached a plateau after the period 1989–1992 (Fig. 1). The median serum AFP value at a diagnosis continued decreasing throughout the study period, and the median serum DCP value also continued decreasing after the period 1989–1992 (data not shown).

When the characteristics were compared between two periods (8 years) before the year 2000 (1992–1996 and 1997–2000, $n = 639$) and two periods after the year 2000 (2001–2004 and 2005–2008, $n = 721$) to elucidate the characteristics of patients with HCC after the year 2000, the age of the patients, as well as their serum albumin levels, were significantly higher after the year 2000 (64.7 ± 8.8 years *vs* 68.2 ± 9.3 years and 3.31 ± 0.62 g/dL *vs* 3.59 ± 1.09 g/dL, both $P < 0.0001$). After the year 2000, the percentage of patients with Child–Pugh class A liver function was significantly higher (59.5% *vs* 68.9%, $P = 0.0013$), and the percentage of patients with AJCC tumor stage I was significantly higher (41.9% *vs* 51.2%, $P = 0.0011$). Additionally, the percentage of patients with single HCC tumors (44.1% *vs* 54.4%, $P = 0.0002$) and the percentage of patients with HCC that lacked vascular invasion (76.2% *vs* 83.1%, $P = 0.0020$) were significantly higher after the year 2000. The serum AFP value at diagnosis was significantly lower after the year 2000 (38 ng/mL *vs* 24.7 ng/mL, $P = 0.0003$). Also, the serum DCP value at diagnosis was significantly lower after the year 2000 (62 mAU/mL *vs* 38.2 mAU/mL, $P = 0.0027$). In contrast, there was no decrease in maximal tumor size after the year 2000 in comparison to the periods before ($P = 0.4301$). The percentage of patients with hepatitis B virus (HBV) infection who were administered a nucleoside analog against HBV was significantly higher after the year 2000 (7.5% *vs* 58.5%, $P < 0.0001$). The percentage of patients with hepatitis C virus (HCV) infection who had undergone interferon-based antiviral therapy against HCV was significantly higher after the year 2000 (7.6% *vs* 14.3%, $P = 0.0012$), although the rate of patients in whom HCV was eradicated by antiviral therapy (i.e. sustained virological responders) was not significantly different.

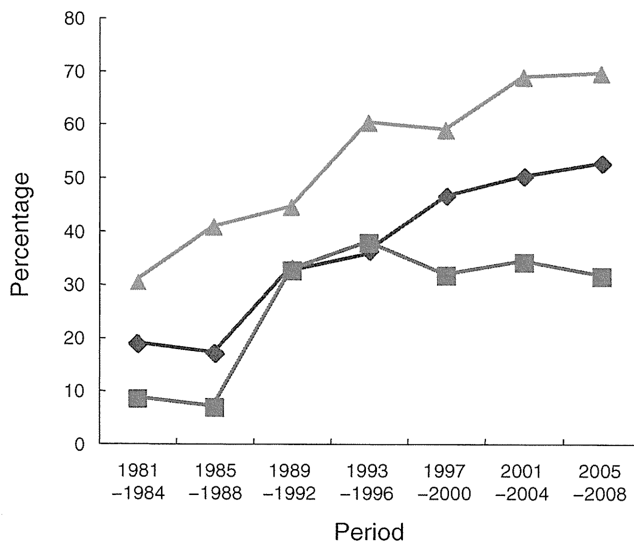

Figure 1 Changes in the percentage of patients with Child–Pugh class A or those without cirrhosis (green line), the percentage of patients with hepatocellular carcinoma (HCC) of maximal tumor size < 2 cm (red line), and the percentage of patients with HCC of American Joint Committee on Cancer (AJCC) tumor stage I (blue line) at the first diagnosis of HCC. Patients with Child–Pugh class A include patients with cirrhosis. ▲, Child–Pugh class A; ■, maximal tumor size < 2 cm; ◆, AJCC tumor stage I.

An analysis of the initial treatment selected (Table 3) demonstrated that the percentage of patients who underwent surgery as an initial treatment increased from the period 1997–2000, and was approximately 35% in the periods after the year 2000 (1997–2000 *vs* 2001–2004, $P = 0.0002$). In contrast, patients who underwent TACE decreased from over 50% in the period 1985–1988, to approximately 20% in the periods after the year 2000 (1997–2000 *vs* 2001–2004, $P < 0.0001$). LAT treatment increased from the period 1989–1992 (1989–1992 *vs* 1993–1996, $P = 0.0004$), and was almost constant after this period, with some fluctuations. However, when the details of the treatment were analyzed in patients who underwent LAT, the percentage of patients who underwent RFA as LAT markedly increased in the period after the year 2000 (1997–2000 *vs* 2001–2004, $P < 0.0001$), and most patients underwent RFA in this period. In six patients who underwent PEIT as LAT after the year 2000, a HCC tumor was located just beside the bowel (2 patients), gallbladder (1 patient), and main trunk of the intrahepatic bile duct (3 patients), and therefore, RFA should have been avoided. We found no patient who underwent RFA before the period 1993–1996. The percentage of patients who underwent RFA among patients treated by LAT was 21.9% in the period 1997–2000. It was 95.1% and 98.7% in the periods 2001–2004 and 2005–2008, respectively.

Table 3 Initial treatment for hepatocellular carcinoma by periods

Periods	Surgery	Locoregional ablative therapies				TACE	Others	None
		Total	PEIT	PMCT	RFA			
1981–1984 ($n = 141$)	19 (13.5)	0	0	0	0	39 (27.7)	38 (26.9)	45 (31.9)
1985–1988 ($n = 220$)	25 (11.4)	6 (2.7)	6 (2.7)	0	0	115 (52.3)	31 (14.1)	43 (19.5)
1989–1992 ($n = 292$)	46 (15.8)	50 (17.1)	50 (17.1)	0	0	114 (39.0)	28 (9.6)	54 (18.5)
1993–1996 ($n = 305$)	42 (13.8)	91 (29.8)	90 (29.5)	1 (0.3)	0	108 (35.4)	23 (7.5)	41 (13.5)
1997–2000 ($n = 334$)	72 (21.5)	64 (19.2)	38 (11.4)	12 (3.6)	14 (4.2)	109 (32.6)	25 (7.5)	64 (19.2)
2001–2004 ($n = 366$)	127 (34.7)	103 (28.2)	5 (1.4)	0	98 (26.8)	59 (16.1)	22 (6.0)	55 (15.0)
2004–2008 ($n = 355$)	128 (36.1)	78 (22.0)	1 (0.3)	0	77 (21.7)	74 (20.8)	21 (5.9)	54 (15.2)

PEIT, percutaneous ethanol injection therapy; PMCT, percutaneous microwave thermocoagulation therapy; RFA, radiofrequency ablation; TACE, transcatheter arterial chemoembolization.

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Survival rate for patients with HCC by period

The survival rates were compared according to the periods of initial HCC diagnosis (Fig. 2). The survival rates increased throughout the periods 1981–1984, 1985–1988, and 1989–1992 (1981–1984 *vs* 1985–1988, $P = 0.0003$; 1985–1988 *vs* 1989–1992, $P = 0.0009$; 1989–1992 *vs* 1993–1996, $P = 0.0383$). We found no difference in the survival rates between the periods 1993–1996 and 1997–2000 ($P = 0.5887$). In contrast, the survival rate markedly increased in the period 2001–2004 in comparison to the period 1997–2000 ($P < 0.0001$). Again, we found no difference in the survival rates between the periods 2001–2004 and 2005–2008 ($P = 0.5151$).

When we compared the survival rates according to treatment, it was highest in patients treated by surgery, followed by patients treated by LAT, by TACE, by other treatment, and patients without treatment in this order (data not shown). In patients who underwent LAT, the survival rate was higher in patients treated by RFA than those treated by PEIT ($P = 0.0073$, Fig. S1).

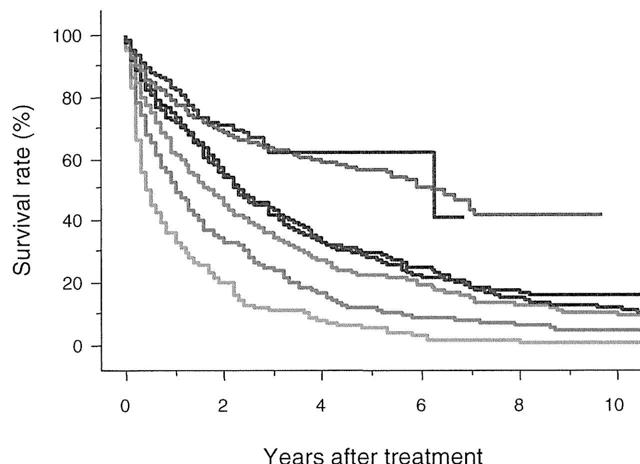

Figure 2 Survival rate of patients with hepatocellular carcinoma (HCC) according to the period of initial HCC diagnosis. ▬, 1981–1984 ($n = 141$); ▬, 1985–1988 ($n = 220$); ▬, 1989–1992 ($n = 292$); ▬, 1993–1996 ($n = 305$); ▬, 1997–2000 ($n = 334$); ▬, 2001–2004 ($n = 366$); ▬, 2004–2008 ($n = 355$).

Multivariate analyses for factors affecting survival rates

We conducted a multivariate analysis to determine factors that were associated with patient survival rate (Table 4). The analysis revealed that Child–Pugh class (B and C), AJCC tumor stage (II and III), treatment (surgery, LAT, TACE, and other treatments), and the period of diagnosis (periods 1997–2000, 2001–2004, and 2005–2008) were independently associated with patient survival rate.

Discussion

In the present study, we analyzed several trends in patients with HCC, including the periods after the year 2000. The incidence of HCC is reportedly increasing in the USA and other Western countries.[11,12] In contrast, a recent study demonstrated that the incidence

Table 4 Multivariate analysis of factors associated with patient survival

Factor		Parameter estimate	Standard error	X	Risk ratio (95% confidence interval)	*P*-value
Age		0.0038	0.0033	1.36	1.0038 (0.9974–1.0102)	0.2428
Sex	Male				1	
	Female	−0.0566	0.0337	2.87	0.9449 (0.8845–1.0095)	0.0904
Child–Pugh class	A†				1	
	B	0.2379	0.0328	52.00	1.2686 (1.1895–1.3529)	< 0.0001
	C	0.4768	0.0481	89.41	1.6109 (1.4658–1.7703)	< 0.0001
AJCC tumor stage	Stage I				1	
	Stage II	0.2559	0.0380	45.36	1.2916 (1.1990–1.3915)	< 0.0001
	Stage III	0.6170	0.0431	206.14	1.8534 (1.7032–2.0168)	< 0.0001
	Stage IV	0.7973	0.0704	97.98	2.2196 (1.9334–2.5481)	< 0.0001
Treatment	No treatment				1	
	Surgery	−0.7011	0.0599	139.24	0.4960 (0.4411–0.5578)	< 0.0001
	LAT	−0.6365	0.0559	129.84	0.5291 (0.4742–0.5904)	< 0.0001
	TACE	−0.4039	0.0451	77.19	0.6677 (0.6113–0.7294)	< 0.0001
	Other	−0.1233	0.0530	5.49	0.8840 (0.7968–0.9808)	0.0192
Period	1981–1984				1	
	1985–1988	−0.0832	0.0569	2.12	0.9202 (0.8231–1.0287)	0.1456
	1989–1992	−0.0818	0.0543	2.23	0.9215 (0.8285–1.0249)	0.1350
	1993–1996	−0.0824	0.0557	2.16	0.9209 (0.8256–1.0272)	0.1419
	1997–2000	−0.1208	0.0553	4.67	0.8862 (0.7952–0.9877)	0.0306
	2001–2004	−0.2904	0.0621	21.52	0.7480 (0.6623–0.8448)	< 0.0001
	2005–2008	−0.2988	0.0707	18.33	0.7417 (0.6456–0.8520)	< 0.0001

†Category of Child–Pugh class A includes patients without cirrhosis. AJCC, American Joint Committee on Cancer; LAT, locoregional ablative therapies including percutaneous ethanol injection therapy, percutaneous microwave thermocoagulation therapy, and radiofrequency ablation; TACE, transcatheter arterial chemoembolization.

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of HCC in urban areas of Japan began to decrease by the year 2000.[13] However, we did not find a decrease in the number of patients with HCC during this period. This authors' institution is located in a county area, and the results indicated that the incidence of HCC has not started to decrease and remains constant even after the year 2000 for county regions of Japan.

In the comparisons based on the period of the HCC diagnosis in the present study, patient age continued to increase throughout the study period. One of the most important risk factors for the development of HCC worldwide[14,15] is chronic viral hepatitis, and the majority of patients with HCC in Japan have chronic HCV infection.[16] The age of Japanese individuals with HCV infection is increasing, thus contributing to the higher patient age found in this study.

When we analyzed the characteristics of patients with HCC who were diagnosed after the year 2000, in comparison to those diagnosed in the 1990s, the prevalence of patients with liver function of Child–Pugh class A significantly increased, along with the serum albumin level and patient age. This indicates the trend that patients had better liver function at first diagnosis of HCC after the year 2000. At the same time, it indicates that HCC develops more frequently in livers with less progressed fibrosis, and even in livers without cirrhosis, after the year 2000. The reason for this better liver function at diagnosis is unknown. It could be due to the increase in patient age at diagnosis in Japan, as it has been reported that HCC develops in the liver with less progressed fibrosis in cases of high-aged patients.[17] It could also be due to the increase in the number of patients with a history of several antiviral therapies, including nucleoside analogs for patients with HBV infection and interferon-based therapy for those with HCV infection. Indeed, the percentage of patients who underwent antiviral therapy markedly increased after the year 2000 in both patients with HBV and HCV infection. Further studies will be needed to clarify the reason for the increase of HCC patients with better liver function in the period after the year 2000.

With respect to HCC tumor progression at diagnosis, we did not find an improvement in the size of HCC tumor at diagnosis in the period after the year 2000. The size of maximal HCC tumor at diagnosis reached a plateau after the period 1993–1996. Tremendous effort has been made to increase the detection of small-size HCC tumors, especially during the 1990s. This has led to the development of various scanning techniques and imaging apparatuses,[18,19] identification of highly-sensitive and specific tumor markers,[20,21] and the establishment of surveillance for patients at high-risk of developing HCC.[5,22] It appears that this effort reached a limit during the 1990s, as it currently is difficult to detect smaller HCC tumors. In contrast to the size of maximal HCC tumors, the prevalence of single HCC tumors or HCC without vascular invasion significantly increased after the year 2000. This resulted in an increase in patients with an earlier tumor stage of HCC. Also, serum AFP and DCP values at diagnosis, which are reported to be a biomarker of biological malignant features of HCC, continued decreasing after the year 2000. Advances in techniques for the early detection of HCC continue to effect the improvement not of the size of HCC tumors but of these factors after the year 2000; less advanced HCC continues increasing, even in the same size HCC tumors.

In regards to patient prognosis, the survival rates were almost constant during the period 1993–2000. This is associated with the fact that the detection of small-sized HCC tumors reached a plateau during this period. In contrast, the survival rates significantly increased after the year 2000. The increase in patients with better liver function at diagnosis after the year 2000 resulted in an increase of the percentage of patients who underwent hepatectomy as an initial treatment in this period. This increase in the percentage of patients who underwent hepatectomy for the treatment for HCC might have contributed to the improvement in the survival rate of patients with HCC diagnosed after the year 2000. In addition, the percentage of patients who underwent RFA markedly increased after the year 2000. Considering the higher survival rate in patients who underwent RFA as LAT than that in patients treated by PEIT, the emergence of RFA as a treatment modality for HCC might also have played a role in the increasing survival rate after the year 2000.

In conclusion, the survival rate of patients with HCC continues to increase after the year 2000 in Japan. This is not due to improvements in the detection of small HCC tumors, as observed in 1990s, but is a consequence of the increase in the percentage of patients with better liver function and patients with single HCC tumors or HCC without vascular invasion with low serum tumor marker levels at first diagnosis. These changes resulted in an increase in the number of patients who underwent radical curative treatment and contributed to the continuing improvement of patient prognosis. Further studies will be necessary to elucidate the reasons for these changes in the characteristics of patients with HCC after the year 2000.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Survival rate of patients with hepatocellular carcinoma treated by percutaneous ethanol injection therapy and radiofrequency ablation as a locoregional ablative therapy.

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