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Gastrointestinal, Hepatobiliary and Pancreatic Pathology

## Pigment Epithelium-Derived Factor Inhibits Lysosomal Degradation of Bcl-xL and Apoptosis in HepG2 cells

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**Pigment epithelium-derived factor (PEDF) has several biological actions on tumor cells, but its effects are cell-type dependent. The aim of this study was to examine the pathophysiological role of PEDF in hepatocellular carcinoma (HCC). PEDF expression was examined in various hepatoma cell lines and human HCC tissues, and was seen in various hepatoma cell lines including HepG2 cells. In human HCC tissues, PEDF expression was higher than in adjacent non-HCC tissues. In addition, serum PEDF levels were higher in HCC patients than in non-HCC patients, and curative treatment of HCC caused significant reductions in serum PEDF levels compared with pretreatment levels. *In vitro* experiments, camptothecin (CPT) was used to induce apoptosis and the effect of PEDF was investigated by knockdown of the *PEDF* gene in CPT-treated HepG2 cells. Knockdown of the *PEDF* gene enhanced CPT-induced apoptosis, simultaneously down-regulating Bcl-xL expression in HepG2 cells. Expression of apoptosis-related molecules and effects of bafilomycin A1 on CPT-induced apoptosis were also examined in *PEDF* gene knockdown HepG2 cells. Treatment with bafilomycin A1 suppressed CPT-induced decreases in Bcl-xL expression and increases in apoptosis in**

***PEDF* gene knockdown HepG2 cells. PEDF may, therefore, exert anti-apoptotic effects through inhibition of lysosomal degradation of Bcl-xL in CPT-treated HepG2 cells. (Am J Pathol 2010, 176:168–176; DOI: 10.2353/ajpath.2010.090242)**

Hepatocellular carcinoma (HCC) is a common cancer that causes nearly 1 million deaths a year worldwide.<sup>1</sup> The incidence of HCC is predicted to continue to increase over the next 30 years.<sup>2</sup> To develop new therapeutic strategies, it is important to elucidate molecular mechanisms underlying hepatocarcinogenesis.

Pigment epithelium-derived factor (PEDF) is a 50-kDa glycoprotein initially isolated from fetal human retinal pigment epithelial cells.<sup>3</sup> PEDF exerts a range of biological effects depending on the type of the target cell. PEDF induces apoptosis of endothelial cells and results in inhibition of neovascularization.<sup>4</sup> Overexpression of PEDF causes a reduction in tumor microvessel density and subsequent anti-tumor effects in pancreatic adenocarcinoma and melanoma cells.<sup>5,6</sup> In contrast to its effects in endothelial cells, PEDF causes the opposite effect in other types of cells. PEDF protects granule cells against both natural and potassium-induced apoptosis through activation of prosurvival genes.<sup>7</sup> In cultured retinal pericytes, PEDF inhibits oxidative stress-induced apoptosis through an increased ratio of B-cell leukemia/lymphoma

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2 (Bcl-2)-associated X protein (bax) to bcl-2 mRNA levels with subsequent activation of caspase-3.<sup>8,9</sup> PEDF also inhibits light-induced apoptotic processes in photoreceptor cells *in vivo*.<sup>10,11</sup>

HCC is a hypervascular solid tumor, in which neovascularization plays an important role in disease progression and prognosis. However, changes in PEDF expression in human HCC have never been investigated and therefore, it is unclear whether PEDF is a useful target for therapeutic strategies in patients with HCC. Dysregulation of apoptosis is also deeply involved in hepatocarcinogenesis. Although HCC is known to be resistant to apoptosis,<sup>12</sup> it remains unknown whether PEDF has anti-apoptotic effects in HCC.

Thus, PEDF is a multifunctional protein with opposing activities, apoptotic and anti-apoptotic activities. These disparate effects depend on cell type. The aims of the present study were to investigate changes in PEDF expression and the role of PEDF in HCC.

## Materials and Methods

### Materials

All reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan) unless otherwise indicated.

### Cell Lines

Human hepatoma cell lines, HepG2, Hep3B, Huh-7, PLC/PRF/5, HLF and SK-Hep1, and the human hepatocyte cell line, OUMS-29,<sup>13,14</sup> were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 U/ml) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> as previously described.<sup>15</sup> Because PEDF is present in fetal bovine serum, each cell line was incubated for 48 hours with Dulbecco's modified Eagle's medium without fetal bovine serum to examine PEDF expression in culture medium.

### Human Samples

We obtained six pairs of HCCs and adjacent non-HCC liver tissues at the time of surgical resection for HCC. All tissues were stored at -80°C until used. Serum samples were obtained from cirrhotic patients with non-HCC ( $n = 25$ ) or HCC ( $n = 110$ ). Paired serum samples were also obtained from patients with HCC before and after complete treatment with surgical resection or percutaneous radiofrequency ablation ( $n = 15$ ). All serum samples were stored at -80°C until used. Informed consent in writing was obtained from each patient and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by the institutional review committee.

### RT-PCR

From each cell line or each tissue, total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA). Two hun-

dred fifty nanograms of RNA was used as a template for RT-PCR as previously described.<sup>16</sup> Expression of mRNA was evaluated by using a pair of unique primers for human *PEDF* (sense 5'-CCGGGCTCTCTACTATGACTTGAT-3' and antisense 5'-ACGGTCCTCTCTTATCCAAGTAG-3'),  *$\beta$ -actin* primer pair (Promega, Madison, WI), or *cyclophilin* (sense 5'-CCCACCGTGTCTTCGAC-3' and antisense 5'-ATCT-TCTGCTGGTCTTGCC-3'). The cycle numbers (24 cycles for *PEDF*; 20 cycles for  *$\beta$ -actin*; 22 cycles for *cyclophilin*) for amplification were chosen in the linear range. The above conditions were determined by plotting signal intensities as functions of the template amounts and cycle numbers, and reactions proceeded linearly.

### Immunohistochemistry

Cell lines were fixed with 100% acetone at -20°C for 20 minutes. Nonfixed human tissues were sectioned at a thickness of 6  $\mu$ m and fixed with 100% acetone at -20°C for 20 minutes. Cells or sections were washed three times for 5 minutes each in PBS (pH 7.4, 130 mmol/L NaCl, 2 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, and 7 mmol/L Na<sub>2</sub>HPO<sub>4</sub>) and then blocked with 10% skim milk in PBS for 30 minutes. Cells or sections were incubated overnight at 4°C with the monoclonal antibody to human PEDF (Chemicon International, Temecula, CA or TransGenic, Kumamoto, Japan) diluted 1:100 in PBS. After several washes with PBS, cells or sections were incubated for 1 hour with the secondary antibody, horseradish peroxidase-labeled anti-mouse IgG (Amersham Biosciences, Piscataway, NJ), or a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM (Cappel, Aurora, OH) diluted 1:100 in PBS at room temperature. Subsequently, cells or sections were washed with PBS. Immunostaining for PEDF was developed with 3,3'-diaminobenzidine or a confocal laser scanning microscope (FluoView FV 300; Olympus, Tokyo, Japan). In addition, propidium iodide was used concomitantly for nuclear staining. The stored images were overlaid to create a single integrated image referred to as a "volume projection" by using the manufacturer's proprietary software (Olympus) as previously described.<sup>17</sup>

### Immunoblotting and Quantitation

Conditioned media were quantitated by using DC protein assay kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instruction. Proteins were resolved by using 10% SDS-polyacrylamide gel electrophoresis gel. Following electrophoresis, proteins were transblotted onto polyvinylidene difluoride membranes (PolyScreen, PerkinElmer Life Sciences, Waltham, MA). The membranes were blocked with 5% fat-free skim milk powder in Tris-buffered saline containing 0.02% (v/v) Tween 20. The primary antibodies were monoclonal antibodies to human PEDF (Chemicon or TransGenic), p53 (Cell Signaling Technology, Danvers, MA), phospho-p53 (Ser46) (Cell Signaling Technology), Akt (Cell Signaling Technology), phospho-Akt (Cell Signaling Technology), cyclin D1 (Cell Signaling Technology), Bax (Cell Signaling Technology),

survivin (Cell Signaling Technology), or Bcl-xL (Cell Signaling Technology). The bound antibodies were detected with horseradish peroxidase-labeled anti-mouse or anti-rat IgG (Amersham Biosciences) by using an enhanced chemiluminescence detection system (ECL advanced kit, Amersham Biosciences) as previously described.<sup>18</sup> A positive signal from the target proteins was visualized by using an image analyzer LAS-1000 plus (Fujifilm, Tokyo, Japan). Band intensities were determined by using the Scion Image (Scion Corporation, Frederick, MD). Values were based on four different experiments.

### Assay for Serum or Medium PEDF

Serum or medium PEDF measurements were performed with a modified competitive enzyme-linked immunosorbent assay (ELISA) as previously described.<sup>19</sup> To dissociate PEDF from binding-proteins, 50  $\mu$ l of serum or medium was pretreated with 200  $\mu$ l of 8 M urea for 1 hour. Then, 100- $\mu$ l aliquots of standard recombinant human PEDF proteins (Chemicon International) or 50-fold diluted serum were added to wells that had been precoated with an anti-PEDF monoclonal antibody (TransGenic). Then, 50  $\mu$ l of biotinylated anti-human PEDF polyclonal antibody (R and D Systems, Minneapolis, MN) was added to each well, and the plate was read at 450 nm by using a microplate reader.

### Small Interfering RNA-Mediated Gene Knockdown and Evaluation of Apoptosis

*PEDF* gene was knocked down by using small interfering RNA (siRNA) in HepG2 cells. HepG2 cells were plated together with siRNA (individual siRNA serpinF1 or negative control number 2; Ambion, Austin, TX)-siPORT NeoFX complexes. Twenty-four hours later, siRNA-siPORT NeoFX complexes were removed by replacing them with basal medium containing 1% serum and an apoptosis inducer, camptothecin (CPT; 2  $\mu$ mol/L) and then cells were incubated for 24 hours. In some experiments, 10  $\mu$ mol/L of carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132; Peptide Institute, Osaka, Japan), a proteasomal proteolysis inhibitor or 100 nmol/L of bafilomycin A1, a lysosomal proteolysis inhibitor was mixed with CPT. Apoptosis was evaluated by visualization of caspase activity by using a FITC-labeled carbobenzoxy-valyl-alanyl-aspartyl-fluoromethylketone (CaspACE FITC-VAD-fmk *in situ* marker; Promega) according to the manufacturer's instructions and quantified by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay by using the Cell Death Detection ELISA kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Knockdown of the *PEDF* gene was verified by semiquantitative RT-PCR and by measuring medium PEDF levels.

### Statistical Analysis

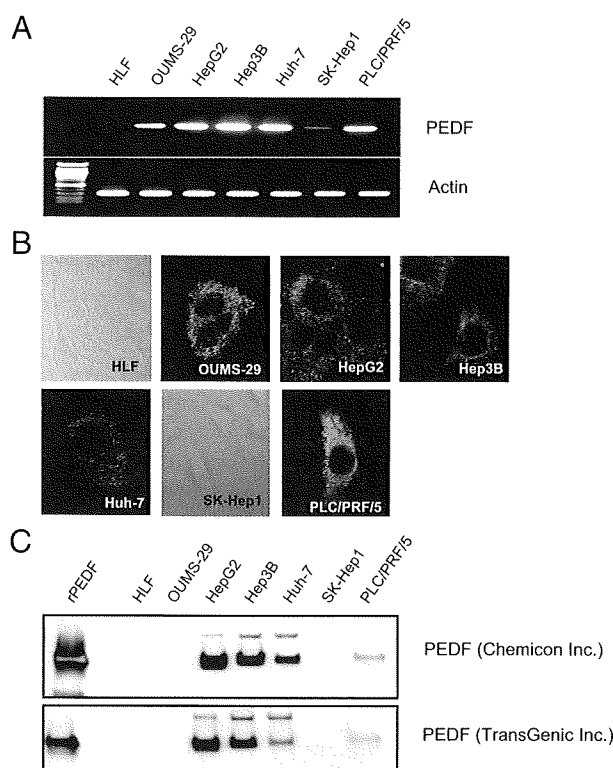
All data are expressed as mean  $\pm$  SD. Comparisons between any two groups were performed by using the

Mann-Whitney *U* test. Comparisons among multiple groups were analyzed by using the Kruskal-Wallis analysis of variance. A *P* value <0.05 was considered statistically significant.

## Results

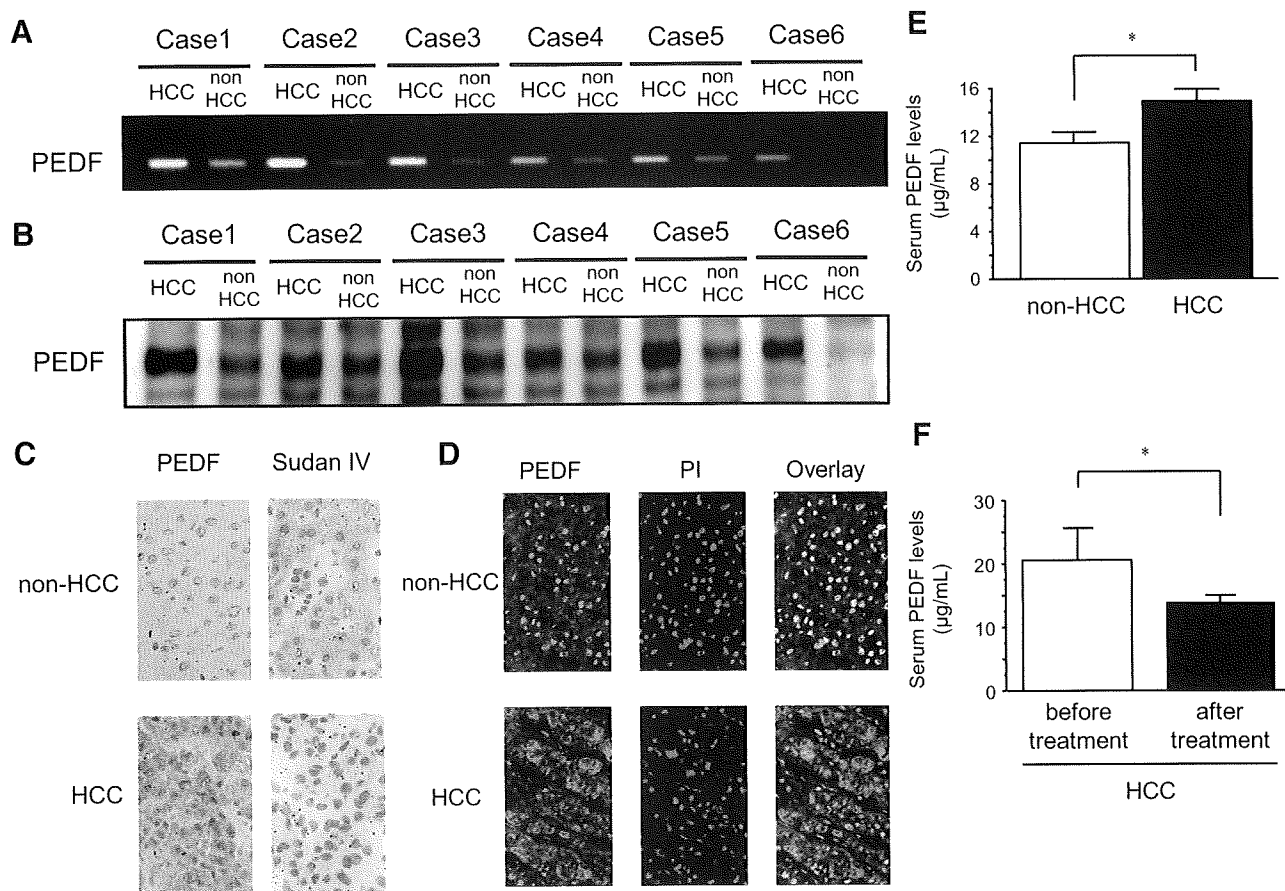
### *PEDF* mRNA and *PEDF* Expression in Human Hepatoma Cell Lines

No expression and weak expression of *PEDF* mRNA were seen in HLF and SK-Hep1 cells, respectively. However, expression of *PEDF* mRNA was seen in OUMS-29, HepG2, Hep3B, Huh-7, and PLC/PRF/5 cells (Figure 1A). Intracellular expression of *PEDF* was seen in OUMS-29, HepG2, Hep3B, Huh-7, and PLC/PRF/5 cells, but not in HLF and SK-Hep1 cells (Figure 1B). Secreted *PEDF* in the culture medium was evaluated by using two different primary antibodies. Expression of *PEDF* was seen in the culture medium of HepG2, Hep3B, Huh-7, and PLC/PRF/5 cells, but not in that of HLF, OUMS-29, and SK-Hep1 cells (Figure 1C, upper column). Similar results were obtained when a different primary antibody was used (Figure 1C, lower column).



**Figure 1.** Expression of *PEDF* mRNA and actin mRNA as a control in various human hepatoma cell lines (A), intracellular expression of *PEDF* (B), and secreted *PEDF* into the culture medium (C) were evaluated by RT-PCR, immunohistochemistry, and immunoblotting, respectively. In HLF and SK-Hep1 cells, *PEDF* expression was negative and cell morphology was demonstrated by phase contrast (B).





**Figure 2.** In human HCC tissues and adjacent non-HCC liver tissues, expression of PEDF mRNA ( $n = 6$ ) (A) and PEDF ( $n = 6$ ) (B) were evaluated by RT-PCR and immunoblotting, respectively. The distribution of PEDF was examined by immunostaining. Immunostaining for PEDF was developed with 3,3'-diaminobenzidine (C) and lipid accumulation was evaluated by Sudan IV staining (C). The distribution of PEDF was examined by a confocal laser scanning microscopy. Green color indicates expression of PEDF and red color indicates propidium iodide, a marker for the nucleus. Yellow color indicates co-localization of PEDF and propidium iodide (D). Serum PEDF levels were measured in non-HCC patients ( $n = 25$ ) or HCC patients ( $n = 110$ ) with a competitive ELISA (E). Changes in serum PEDF levels after HCC treatment were examined in HCC patients treated with surgical resection or percutaneous radiofrequency ablation ( $n = 15$ ) (F). \* $P < 0.01$ .

### PEDF mRNA and PEDF Expression in Human Liver Tissues and Serum Samples

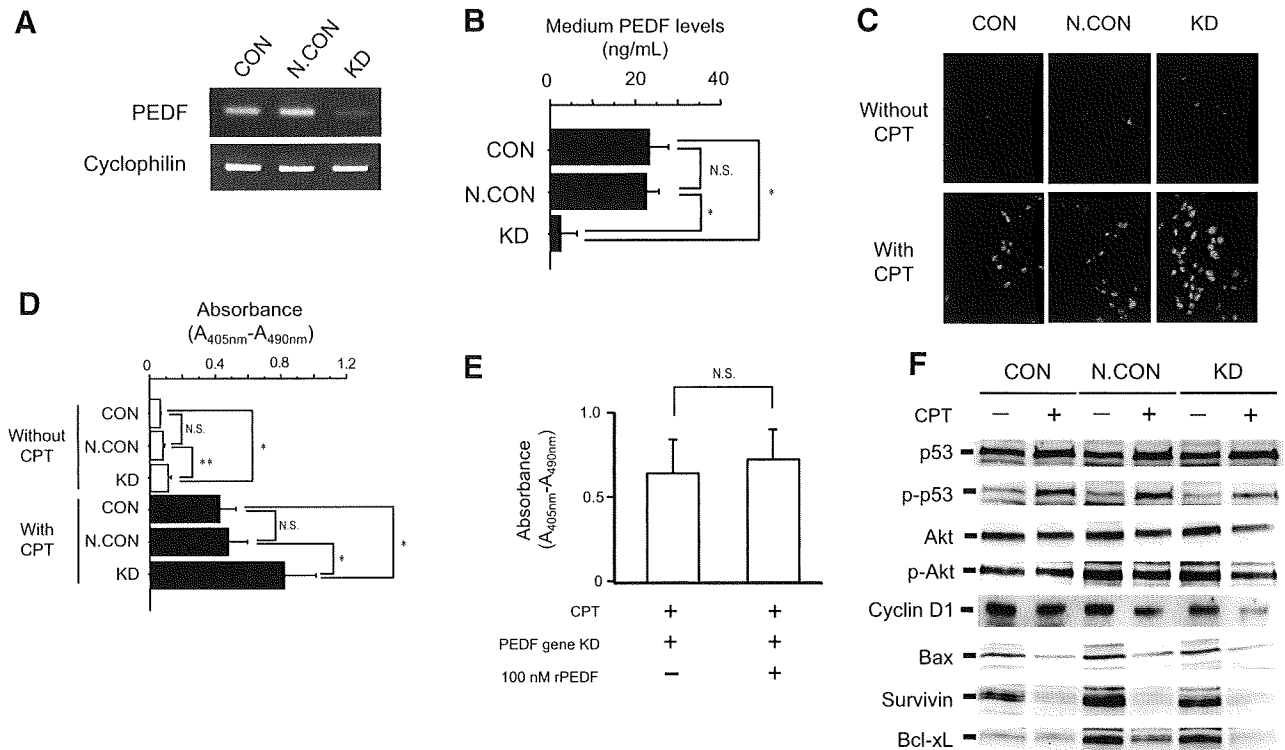
Expression of PEDF mRNA was higher in HCC tissue than in non-HCC tissue in all cases (Figure 2A). Similar to expression of PEDF mRNA, expression of PEDF was higher in HCC tissue than in non-HCC tissue in all cases (Figure 2B). When PEDF was visualized by 3,3'-diaminobenzidine, expression of PEDF was seen in nuclei of hepatocytes and mainly in the cytoplasm of HCC where no lipid accumulation was seen by Sudan IV staining (Figure 2C). Similar results were obtained by confocal laser scanning microscopy. In hepatocytes, PEDF (green color) was colocalized with propidium iodide (red color), showing a yellow color in the overlay image (Figure 2D, upper column). However, in HCC, PEDF (green color) was dissociated with propidium iodide (red color; Figure 2D, lower column). Serum PEDF levels were significantly higher in patients with HCC than in patients with non-HCC (Figure 2E). After complete treatment of HCC such as surgical resection or percutaneous radiofrequency ablation, serum PEDF levels were significantly lower than before treatment (Figure 2F).

### Effects of PEDF Gene Knockdown on CPT-Induced Apoptosis in HepG2 Cells

Knockdown of the *PEDF* gene was verified by semiquantitative RT-PCR (Figure 3A) and PEDF levels in the culture medium (Figure 3B).

Apoptosis was visualized by FITC-conjugated VAD-FMK, which binds with activated caspase. Laser scanning microscopic images showed a slight increase of apoptosis in *PEDF* gene knockdown HepG2 cells compared with CON and N.CON (Figure 3C, upper column). By treatment with CPT, an apoptotic inducer, marked increase of apoptosis was seen in *PEDF* gene knockdown HepG2 cells compared with CON and N.CON (Figure 3C, lower column).

In addition, apoptosis was quantified by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-based ELISA. *PEDF* gene knockdown increased apoptotic cell numbers in HepG2 cells compared with CON and N.CON (Figure 3D, white bars). By treatment with CPT, a significant increase in apoptotic cell number was seen in knockdown compared with CON and N.CON (Figure 3D, black bars). Similar findings were found in Hep3B and Huh7 cells (Supplemental Figure 1, see <http://ajp.amjpathol.org>).



**Figure 3.** In *PEDF* gene knockdown HepG2 cells, expression of *PEDF* mRNA (A) and medium *PEDF* levels (B) were evaluated by semiquantitative RT-PCR and a competitive ELISA ( $n = 4$ ), respectively. Effects of *PEDF* gene knockdown on CPT-induced apoptosis was examined in HepG2 cells. Apoptosis was visualized by FITC-conjugated VAD-FMK, which binds with activated caspase and green color indicated apoptotic cells (C). Apoptosis was quantified by using Cell Death Detection ELISA kits (Roche Applied Science) and increases in absorbance ( $A_{405\text{nm}}-A_{490\text{nm}}$ ) indicate increased number of apoptotic cells ( $n = 4$ , D). Effects of r*PEDF* on CPT-induced apoptosis was examined in *PEDF* gene knockdown HepG2 cells. Apoptosis was quantified by using Cell Death Detection ELISA kits (Roche Applied Science) ( $n = 4$ , E). Effects of *PEDF* gene knockdown on cell cycle-related molecules (p53, Akt, and cyclin D1) and apoptosis-related molecules (Bax, survivin, and Bcl-xL) were evaluated by immunoblotting in HepG2 cells (F). \* $P < 0.01$ , \*\* $P < 0.05$ .

### Effects of Recombinant *PEDF* on CPT-Induced Apoptosis in *PEDF* Gene Knockdown HepG2 Cells

After addition of recombinant *PEDF* (r*PEDF*) (100 nmol/L) into the culture medium, no significant changes were seen in CPT-induced apoptotic cell numbers in *PEDF* gene knockdown HepG2 cells (Figure 3E).

### Effects of *PEDF* Gene Knockdown on Expression of Cell Cycle- and Apoptosis-Related Molecules in CPT-Treated HepG2 Cells

Although there was no significant decrease of p53 expression on CPT-treated knockdown cells compared with that in CPT-treated CON and CPT-treated N.CON cells, significant decrease was seen in phospho-p53, Akt, phospho-Akt, and cyclin D1 in CPT-treated knockdown cells compared with those in CPT-treated CON and CPT-treated N.CON cells (Figure 3F and Table 1). Treatment with CPT decreased expression of Bax and survivin in all groups (Figure 3F and Table 1). However, expression of Bcl-xL was markedly decreased in CPT-treated knockdown cells compared with that in CPT-treated CON and CPT-treated N.CON cells (Figure 3F). Decreased expression of Bcl-xL was also found in

Hep3B and Huh7 cells (Supplemental Figure 2, see <http://ajp.amjpathol.org>).

### Effects of *PEDF* Gene Knockdown on Bcl-xL mRNA in HepG2 Cells

Expression of Bcl-xL mRNA was higher in CPT-treated knockdown cells compared with that in CPT-treated CON and CPT-treated N.CON cells at 6 hours and 12 hours after *PEDF* gene knockdown (Figure 4A).

### Effects of MG132 or Bafilomycin A1 on Decreases in Expression of Bcl-xL and Increases in Apoptosis in CPT-Treated *PEDF* Gene Knockdown HepG2 Cells

After treatment with 10  $\mu\text{mol/L}$  of MG132, a proteasomal proteolysis inhibitor, no marked change in expression of Bcl-xL was seen in CPT-treated knockdown cells (Figure 4B). Treatment with 100 nmol/L of bafilomycin A1, a lysosomal proteolysis inhibitor, inhibited decreases in expression of Bcl-xL in CPT-treated knockdown cells (Figure 4B). Treatment with bafilomycin A1, but not MG132, also inhibited an increase of apoptosis in CPT-treated *PEDF* gene knockdown HepG2 cells (Figure 4C).

**Table 1.** Densitometric Analysis of Expression of Cell Cycle- and Apoptosis-Related Molecules in CPT-Treated HepG2 Cells

Molecule	CON (n = 4)		N.CON (n = 4)		kd (n = 4)	
	-	+	-	+	-	+
CPT						
P53	223 ± 24	235 ± 19	221 ± 21	232 ± 20	229 ± 21	238 ± 21
p-p53	136 ± 15	193 ± 22	106 ± 17	195 ± 18	101 ± 15	124 ± 26 <sup>†</sup>
Akt	173 ± 16	169 ± 19	186 ± 16	157 ± 17	182 ± 18	120 ± 25 <sup>†</sup>
p-Akt	194 ± 18	197 ± 21	242 ± 23	236 ± 22	244 ± 21	160 ± 28 <sup>†</sup>
cyclin D	215 ± 29	194 ± 31	209 ± 41	164 ± 43	193 ± 37	118 ± 39 <sup>†</sup>
Bax	132 ± 15	75 ± 11	156 ± 18	85 ± 11	149 ± 12	81 ± 10
Survivin	194 ± 18	56 ± 7	226 ± 21	61 ± 10	210 ± 18	54 ± 9
Bcl-xL	111 ± 13	130 ± 11	215 ± 19	159 ± 15	205 ± 21	87 ± 9 <sup>‡§</sup>

Data presented as mean ± SD.  
<sup>\*</sup>P < 0.05 compared with CON with CPT.  
<sup>†</sup>P < 0.05 compared with N.CON with CPT.  
<sup>‡</sup>P < 0.01 compared with CON with CPT.  
<sup>§</sup>P < 0.01 compared with N.CON with CPT.

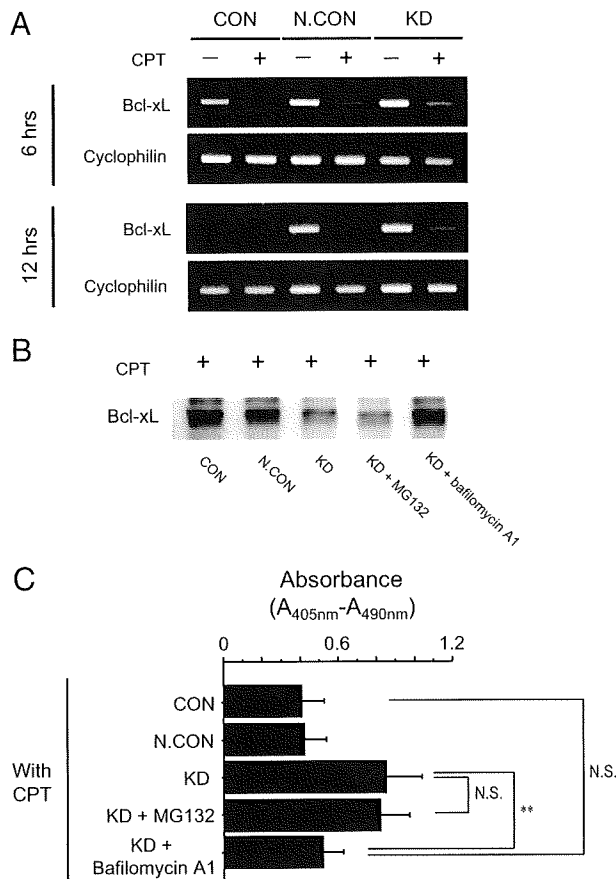
**Discussion**

In this study, we demonstrated that PEDF was overexpressed not only in human hepatoma cell lines, but also in human HCC tissues. Knockdown of the *PEDF* gene enhanced CPT-induced apoptosis with down-regulation

of Bcl-xL expression. Treatment with bafilomycin A1, a lysosomal proteolysis inhibitor, inhibited the CPT-induced decreases in expression of Bcl-xL and increases in apoptotic cell number in *PEDF* gene knockdown HepG2 cells. Thus, PEDF may exert anti-apoptotic effects by inhibiting lysosomal-mediated degradation of Bcl-xL in HepG2 cells.

Decreased expression of PEDF was reported in various cancers such as prostate,<sup>20</sup> pancreas,<sup>21</sup> breast,<sup>22</sup> and ovarian cancers.<sup>23</sup> PEDF has anti-angiogenic<sup>24</sup> and tumoricidal effects through induction of both apoptosis and differentiation.<sup>25</sup> In fact, treatment with exogenous PEDF has been shown to inhibit neovascularization and proliferation of malignant melanoma *in vivo*.<sup>5</sup> Generally, substitution of PEDF is considered as a novel therapeutic strategy against several cancers. However, there is some controversy about the pathophysiological role of PEDF in cancers because PEDF levels have been increased in some types of cancers.<sup>26,27</sup> Indeed, in this study, we found that PEDF was overexpressed in most of the human hepatoma cell lines and HCC tissues. Although PEDF mRNA was not seen only in HLF cell line, HLF cell line resembles fibroblast in morphology and does not produce alpha-fetoprotein.<sup>28</sup> Thus, HLF cell line shows different characteristics from other hepatoma cell lines and this may be a reason why PEDF mRNA was not seen only in HLF cell line.

More than 99% of PEDF cDNA from HepG2 cells was identical to human PEDF cDNA (data not shown) and therefore, PEDF in HepG2 cells seems to have biological activity. Although treatment with exogenous PEDF is reported as a potential therapeutic strategy for HCC,<sup>29</sup> endogenous expression of PEDF in human HCC has never been investigated and the validity of treatment strategies targeting PEDF has not been confirmed. Expression of PEDF was increased in all six HCC tissues that we examined, although all six HCCs were typical hypervascular tumors. PEDF is known to localize regions of steatosis.<sup>30,31</sup> However, no lipid accumulation was seen in the cytoplasm of HCC and therefore, high expression of PEDF seems to be a character of HCC. Moreover, serum PEDF levels were significantly higher in HCC patients than in non-HCC patients or in HCC patients who had complete treatment. Recently, we, along with others,



**Figure 4.** Effects of *PEDF* gene knockdown on Bcl-xL mRNA and cyclophilin mRNA as a control were evaluated by semiquantitative RT-PCR 6 and 12 hours after *PEDF* gene knockdown in CPT-treated HepG2 cells (A). In CPT-treated *PEDF* gene knockdown HepG2 cells, effects of 10 μmol/L of MG132 or 100 nmol/L of bafilomycin A1 on expression of Bcl-xL (B) and on CPT-induced apoptosis were evaluated by immunoblotting and Cell Death Detection ELISA kits (Roche Applied Science), respectively. Increases in absorbance ( $A_{405\text{nm}} - A_{490\text{nm}}$ ) indicate increases in number of apoptotic cells (n = 5, C). \*\*P < 0.05.

have found that adipocytes secrete PEDF and serum PEDF level is associated with metabolic syndrome.<sup>19,32-34</sup> However, in this study, enrolled patients were not obese and there was no significant difference in body mass index between HCC and non-HCC patients ( $22.5 \pm 2.1$  vs.  $22.3 \pm 1.9$ ; not significant). Taken together, PEDF expression levels were increased in HCC as a countersystem against neovascularization. Further, our present results suggest that elevation of PEDF may have other biological effects on HCC, ie, promotion of HCC growth and expansion by suppressing apoptosis.

Because PEDF was expressed in human hepatoma cell lines, knockdown of the *PEDF* gene seemed to be a better strategy for examining the role of PEDF in HCC than overexpression of PEDF or treatment with recombinant PEDF. Knockdown of the *PEDF* gene itself induced apoptosis and the apoptotic effect was enhanced by the co-treatment with CPT, an inducer of apoptosis, in various hepatoma cell lines. These findings indicate that PEDF has a protective role against CPT-induced apoptosis in HepG2 cells. Cao et al<sup>35</sup> reported that PEDF protects cultured retinal neurons against hydrogen peroxide-induced apoptosis. In good accordance with our results, a similar protective role of PEDF against apoptosis has been found in cerebellar granule cells,<sup>7,36-39</sup> retinal pericytes,<sup>8,9</sup> photoreceptor cells,<sup>10,11</sup> and retinal ganglion cells.<sup>40</sup> Addition of rPEDF into the culture medium did not protect CPT-induced apoptosis in *PEDF* gene knockdown HepG2 cells. Although PEDF is a secretory protein,<sup>41,42</sup> these data indicate that PEDF exerted its anti-apoptotic activity through intracellular interaction between PEDF and anti-apoptotic molecules, and not through autocrine or paracrine pathways in HepG2 cells. Precise mechanisms for PEDF-induced protection against apoptosis are unknown. However, several pathways have been suggested. PEDF is known to activate cAMP-responsive element binding protein, nuclear factor  $\kappa$ B, extracellular signal-regulated kinases 1/2, and glutathione peroxidase, resulting in protection against apoptosis.<sup>8,38-40</sup> In this study, we found that cell cycle-related molecules such as phospho-p53, Akt, phospho-Akt, and cyclin D1 were down-regulated by knockdown of the *PEDF* gene in CPT-treated HepG2 cells. These changes were in good accordance with previous reports<sup>43-47</sup> and suggest that cell cycle arrest may enhance CPT-induced apoptosis. In addition to these pathways, we first found that knockdown of the *PEDF* gene down-regulated Bcl-xL, an anti-apoptotic molecule, in CPT-treated HepG2 cells. Thus, PEDF may exert anti-apoptotic effects in HCC through up-regulation of Bcl-xL.

Then, we investigated mechanisms for down-regulation of Bcl-xL by *PEDF* gene knockdown. Expression of Bcl-xL mRNA was decreased in CPT-untreated CON cells at 12 hours after *PEDF* gene knockdown compared with that at 6 hours. Although the reason for down-regulation of Bcl-xL mRNA expression at 12 hours is unclear, the following is a possible reason: HepG2 cells were plated together with siRNA for PEDF. Twenty-four hours later, siRNA for PEDF were removed by replacing them with basal medium containing 1% fetal bovine serum. Fetal bovine serum contains various apoptosis-inducing cytokines including TNF- $\alpha$ . Those apoptosis-inducing cy-

tokines might temporary up-regulate Bcl-xL mRNA expression at 6 hours after *PEDF* gene knockdown and apoptotic stimuli might not last until 12 hours.

Expression of Bcl-xL mRNA was higher in CPT-treated knockdown cells compared with that in CPT-treated CON and CPT-treated N.CON cells at 6 hours and 12 hours after *PEDF* gene knockdown. However, the protein expression of Bcl-xL was lower in CPT-treated knockdown cells. Therefore, we assumed that high expression of Bcl-xL mRNA is a cellular adaptive response against decreased protein expression of Bcl-xL. The ubiquitin-proteasome pathway is a major mechanism for protein degradation. However, treatment with MG132, a proteasomal proteolysis inhibitor, showed no marked changes in expression of Bcl-xL in our study. In concordance with our results, Bcl-xL is not degraded by the ubiquitin-proteasome pathway in ischemic retinal injury.<sup>48</sup> Autophagy is another major mechanism for protein degradation through lysosomal proteolysis.<sup>49</sup> We found that treatment with bafilomycin A1, a lysosomal proteolysis inhibitor, prevented the CPT-induced decrease in expression of Bcl-xL. Treatment with bafilomycin A1 also prevented CPT-induced apoptosis in *PEDF* gene knockdown HepG2 cells. These findings led us to hypothesize that knockdown of the *PEDF* gene causes lysosomal proteolysis-mediated down-regulation of Bcl-xL and subsequent enhancement of CPT-induced apoptosis in HepG2 cells. In other words, PEDF may up-regulate Bcl-xL through inhibition of lysosomal proteolysis in HCC. Supporting our hypothesis, expression of Bcl-xL is known to be increased in human HCC.<sup>50</sup> There are significant correlations between expression of Bcl-xL and disease-free survival, and Bcl-xL is an independent prognostic factor for disease-free survival in patients with HCC.<sup>51</sup>

In the present study, we also examined the expression of PEDF in normal hepatocytes and found that high expression of PEDF was found in cytosol of normal hepatocyte cell line, OUMS-29, but not in non-HCC tissue. Although the reason for this discrepancy is unclear, the following is a possible explanation. PEDF is a secretory protein,<sup>41,42</sup> and cell-matrix interaction is one of the regulatory mechanisms for secretion of PEDF.<sup>52</sup> There is no cell-matrix interaction in culture dish and PEDF was not secreted in the culture medium of OUMS-29. Thus, accumulation of unsecreted PEDF may be a reason for high expression of PEDF in OUMS-29 cells.

A limitation of this study is that a hepatoma xenograft study using nude mice was not conducted to prove the anti-apoptotic property of PEDF. However, continuous and specific down-regulation of *PEDF* gene of hepatoma cell in nude mice is impossible at this point because of following reasons: (1) because effects of knockdown only last 3 days in HepG2 cells (data not shown) and none of *PEDF* knockout hepatoma cells survived for more than 7 days; and (2) PEDF occurs in various organs<sup>53</sup> and therefore, continuous knockdown by injection of siRNA for PEDF affects not only hepatoma cell line, but also various organ functions.

In conclusion, we found increased expression of PEDF in both human hepatoma cell lines and human HCC tissues. Knockdown of the *PEDF* gene enhanced CPT-

induced apoptosis with down-regulation of Bcl-xL expression. Treatment with bafilomycin A1 prevented CPT-induced decreases in expression of Bcl-xL and increases in apoptosis in *PEDF* gene knockdown HepG2 cells. Thus, increased expression of PEDF may exert anti-apoptotic effects in HCC through inhibition of lysosomal degradation of Bcl-xL.

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# Long-term trends of the incidence of hepatocellular carcinoma in the Nagasaki prefecture, Japan

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**Abstract.** The incidence of hepatocellular carcinoma (HCC) in Japan is still increasing. The aim of the present study was to analyze the epidemiological trend of HCC in the Western area of Japan, Nagasaki. A total of 1,807 patients with HCC diagnosed at our two hospitals between 1981 and 2005 were consecutively recruited for this study. Cohorts of patients with HCC were categorized into five-year intervals. The etiology of HCC was categorized into four groups: HCC-B: HBsAg positive, HCVAb negative, HCC-C: HCVAb positive, HBsAg negative, HCC-BC: both of HBsAg and HCVAb positive and HCC-nonBC: both of HBsAg and HCVAb negative. The number and proportion of HCC-B cases decreased from 1986 to 1990 and thereafter stabilized, whereas those of HCC-C reached the peak from 1995 to 2000 and thereafter decreased. On the other hand, the number and ratio of the HCC-nonBC cases continued to increase in the whole period. The male/female ratio of HCC-C patients decreased from 6.4 in the period 1981-1985 to 1.9 in 2001-2005, indicating clearly the increase of female patients. On the other hand, the male/female ratio of other types of HCC patients did not change during the period. HCC patients rapidly increased from 1981 to 2000 and this increase was originated from that of HCC-C. The increase of the median age and the number of female patients with HCC-C was also demonstrated. The increase in the number and the proportion of the HCC-nonBC patients was also significant.

## Introduction

Primary liver cancer is the most common primary cancer of the liver accounting for ~6% of all human cancers. It is estimated that half a million cases occur worldwide annually, making

primary liver cancer the fifth most common malignancy in men and the ninth in women (1-6). Hepatocellular carcinoma (HCC) accounts for 85 to 90% of primary liver cancers (7) and the age-adjusted HCC mortality rate has increased in recent decades in Japan (8). Similarly, a trend of increasing rates of HCC has been reported from several developed countries in North America, Europe and Asia (9,10). HCC often develops in patients with liver cirrhosis caused by hepatitis B virus (HBV), hepatitis C virus (HCV), excessive alcohol consumption, or nonalcoholic fatty liver disease. Of the hepatitis viruses which cause HCC, HCV is predominant in Japan (11-14).

Although the age-adjusted incidence of HCC has increased in Japan, sequential changes in background features of HCC patients are not fully understood (15). Yoshizawa reports 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 (16). To clarify factors affecting epidemiological changes in Japanese HCC patients, especially the change in age distribution and gender, we analyzed the underlying features of HCC patients in a two major liver center-based study.

## Patients and methods

**Patients.** A total of 1,807 patients with HCC diagnosed between January 1981 and December 2005 in the Liver Disease Center, National Nagasaki Medical Center and in the outpatient clinic of The First Department of Internal Medicine, Nagasaki University Hospital, were consecutively 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 tumor biopsy. The diagnostic criteria for HCC were either a confirmative tumor biopsy or elevated AFP ( $\geq 20$  ng/ml) and neovascularization in HAG and/or CT. Cohorts of patients with HCC were categorized into five-year intervals (1981-1985, 1986-1990, 1991-1995, 1996-2000 and 2001-2005).

**Etiology of HCC.** Sera were stored at  $-80^{\circ}\text{C}$  until use. A diagnosis of chronic HCV infection was based on the presence of HCVAb (microparticle enzyme immunoassay; Abbott

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

Table I. The characteristics of HCC patients, 1981-2005.

Period	1981-1985	1986-1990	1991-1995	1996-2000	2001-2005	Total
Number	240	316	369	419	463	1807
Gender						
Male	194	257	268	314	314	1347
Female	46	59	101	105	149	460
Ratio (male/female)	4.2	4.4	2.7	3.0	2.1	2.9
Age (y.o) (IQR)	57 (6.5)	61 (5.1)	63 (5.4)	66 (5.1)	68 (6.3)	64 (6.5)
Hepatitis virus						
HCC-B	95	70	80	67	100	412
HCC-C	111	213	240	292	278	1134
HCC-B+C	8	8	9	11	10	46
HCC-nonBC	26	25	40	49	75	215

Gender: 2000-2005 vs. 1981-1985  $p=0.0003$ ; 2000-2005 vs. 1986-1990  $p\leq 0.0001$ ; 2000-2005 vs. 1991-1995  $p=0.1330$ ; 2000-2005 vs. 1996-2000  $p=0.0197$ . Age: 2000-2005 vs. 1981-1985  $p\leq 0.0001$ ; 2000-2005 vs. 1986-1990  $p\leq 0.0001$ ; 2000-2005 vs. 1991-1995  $p\leq 0.0001$  and 2000-2005 vs. 1996-2000  $p=0.0292$ . IQR, interquartile range.

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).

*Statistical analysis.* The data were analyzed by the Mann-Whitney test for the continuous ordinal data between two qualitative variables. 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 1,807 patients with HCC were diagnosed at our hospital from 1981 to 2005. There were 1,347 male (75%) and 460 female (25%) patients, with a median age of 64 years. The proportion of patients diagnosed as HCC-B (HBV-associated: HBsAg positive, HCVAb negative) was 23% (412 of 1,807), whereas 63% (1,134 of 1,807) had HCC-C (HCV-associated: HCVAb positive, HBsAg negative) and an additional 3% (46 of 1,807) had HCC associated with both viruses. The remaining 215 patients (12%) showed both of the virus markers negative.

As shown in Table I and Fig. 1, the number and proportion of HCC-B cases decreased from 1986 to 1990 and thereafter stabilized, whereas those of HCC-C reached the peak in the period 1996-2000 and thereafter decreased. The number and proportion of the HCC-nonBC (HBsAg and HCVAb negative) cases continued to increase in the whole period.

*Background features for patients with HCC.* Fig. 2 shows the median age at diagnosis of HCC-B, HCC-C and HCC-nonBC in five-year intervals (1981-1985, 1986-1990, 1991-1995, 1996-2000 and 2001-2005). The median age of patients at diagnosis of HCC-C showed a steadily significant increase

from 58 to 69 years of age during the period. The median age of patients with HCC-B and HCC-nonBC did not significantly change during the period.

Fig. 3 shows the age distribution of patients with HCC-B and HCC-C with the five 5-year intervals. There was no difference in the age distribution of patients with HCC-B during these periods. In contrast, HCC-C obviously had a trend to increase in the number of patients aged >65 years.

Table I shows that the male/female ratio of HCC patients decreased from 4.2 in the period 1981-1985 to 2.1 in 2001-2005, indicating clearly the increase of female patients. In analysis of patients in HCC-C, the male/female ratio in the periods 1981-1985, 1986-1990, 1991-1995, 1996-2000 and 2001-2005 were 6.4, 4.8, 2.5, 2.7 and 1.9, respectively (1981-1985 vs. 2001-2005,  $p\leq 0.0001$ ) (Table II). The ratio became clearly smaller, indicates an increase in female patients with HCC-C. On the other hand, the male/female ratio of other types of HCC patients did not significantly change during the period.

## Discussion

This was a two major liver center-based study designed to examine the sequential change in the background of HCC patients during the past 25 years, 1981-2005. More than 80% of our patients had chronic HBV or HCV infections. During the observation period, the number and proportion of HCC-B cases decreased in the period 1986-1990 and thereafter reached a plateau, whereas HCC-C reached a peak in the period 1995-2000 and thereafter slightly decreased. On the other hand, the number and the proportion of HCC-nonBC gradually increased in the periods of 1981-1985, 1986-1990, 1991-1995, 1996-2000 and 2001-2005 being 26 (11%), 25 (8%), 40 (11%), 49 (12%) and 75 (16%), respectively. Previous studies from Japan reported that the proportion of HCC-C had been increased and reached a plateau in the



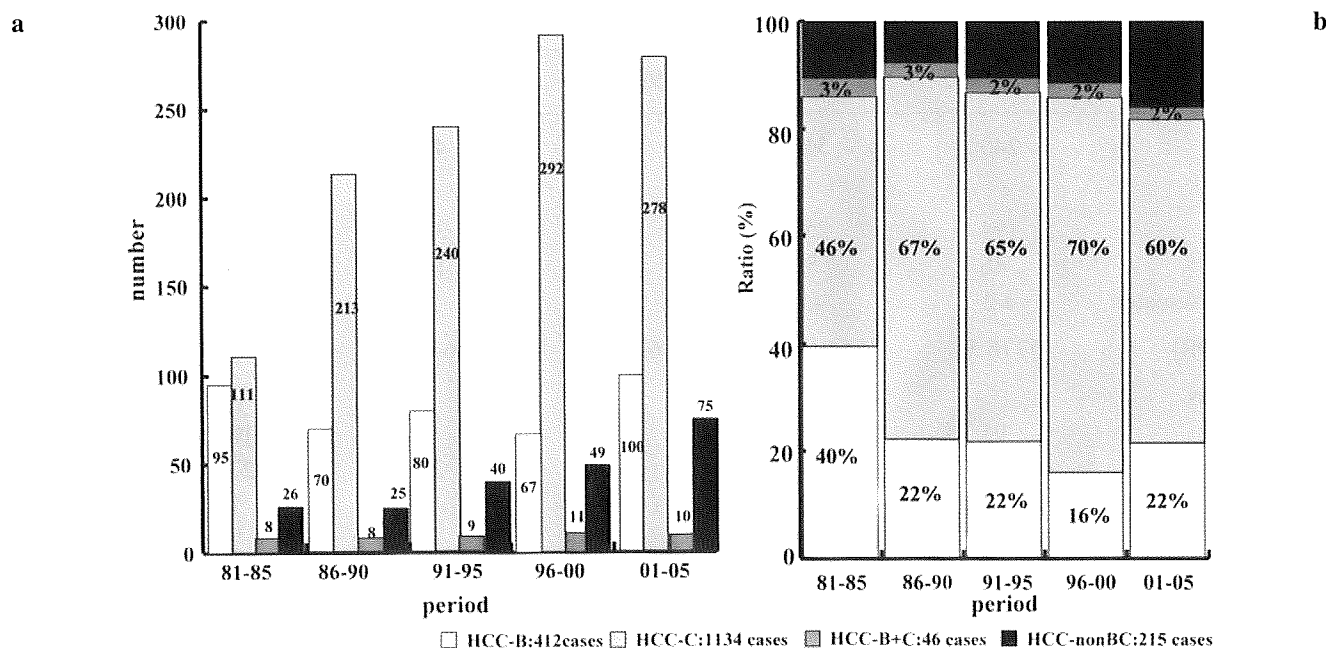


Figure 1. Sequential changes in the number (a) and ratio (b) of HCC patients categorized by etiology during the period 1981-2005 with 5-year intervals.

Table II. The number and male/female ratio of HCC patients during the period of 1981-2005.

Period	1981-1985	1986-1990	1991-1995	1996-2000	2001-2005	Total
Number	240	316	369	419	463	1807
Total						
Male	194	257	268	314	314	1347
Female	46	59	101	105	149	460
Ratio (male/female)	4.2	4.4	2.7	3.0	2.1	2.9
HCC-B						
Male	69	54	61	55	74	313
Female	26	16	19	12	26	99
Ratio (male/female)	2.7	3.4	3.2	4.6	2.9	3.2
HCC-C						
Male	96	176	172	212	182	838
Female	15	37	68	80	96	296
Ratio (male/female)	6.4	4.8	2.5	2.7	1.9	2.8
HCC-nonBC						
Male	21	20	29	40	51	1347
Female	5	5	11	9	24	460
Ratio (male/female)	4.2	4.0	2.6	4.4	2.1	2.9

HBV and nBnC: NS. HCV: 2000-2005 vs. 1981-1985  $p \leq 0.0001$ ; 2000-2005 vs. 1986-1990  $p \leq 0.0001$ ; 1996-2000 vs. 1981-1985  $p = 0.0033$ ; 1996-2000 vs. 1986-1990  $p = 0.0084$ ; 1991-1995 vs. 1981-1985  $p = 0.0024$  and 1991-1995 vs. 1986-1990  $p = 0.0058$ .

period of 1981-2001 (8,15,17-19). However, in our study, the number and proportion of HCC-C cases decreased in the period 2001-2005. This may be due to interferon therapy associated with a decreased incidence of HCC (20-24). Iron depletion for chronic hepatitis C patients is a promising modality for lowering the risk of progression to HCC

(25,26). Oral supplementation with oral branched-chain amino acids has been useful in the prevention HCC (27). Finally, the chronically HCV-infected population is aging in Japan. Yoshizawa reported that age-specific prevalence for the presence of HCVAb among ~300,000 voluntary blood donors from Hiroshima in 1999 clearly increased with the

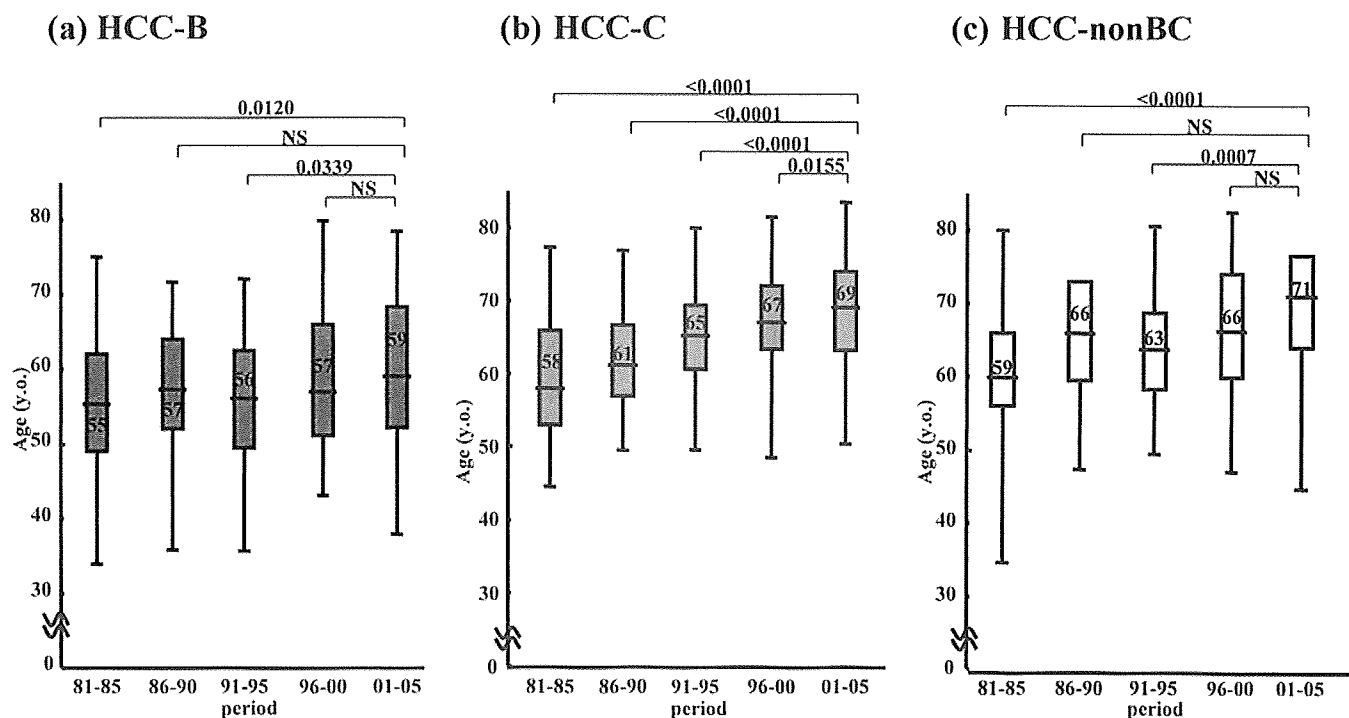


Figure 2. Sequential changes in the median age of HCC patients categorized by etiology during the period, 1981-2005 with 5-year intervals. (a) HCC-B, (b) HCC-C and (c) HCC-nonBC type  $p < 0.05$ .

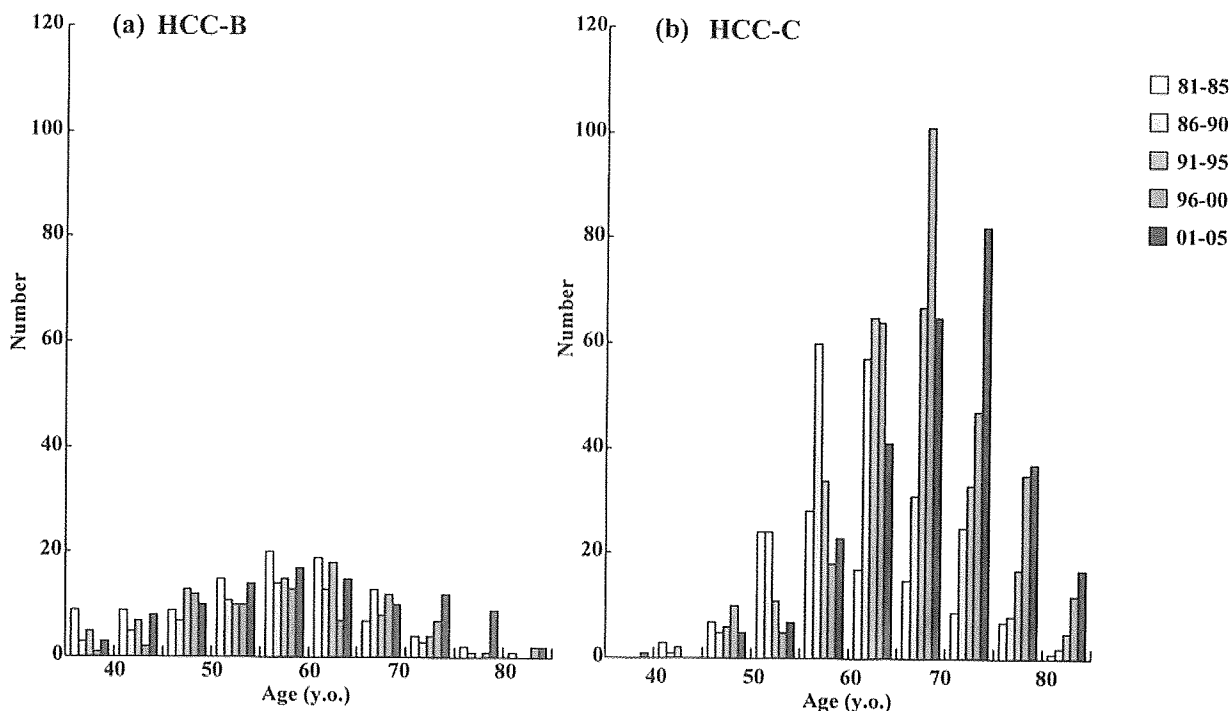


Figure 3. Changes in the age distribution of patients with HCC-B and HCC-C during the period, 1981-2005 with 5-year intervals.

age, reaching the highest proportion of 7% in individuals who were >70 years old (15,16). In this study, the median age of patients with HCC-C steadily increased from 58 to 69 years of age during the studied period. *i.e.* HCV infected people become older and they were regarded as a high risk for HCC.

In almost all populations, males have higher liver cancer proportions than females, with the male/female ratios usually

averaging between 2:1 and 4:1 (7). However, the male/female ratio of HCC in Japan was 4.5 in the period 1983-1985 and 2.57 in 2000-2001 (17). In analysis of background features among HCC patients, HCC-B and HCC-nonBC cases revealed no significant change, whereas the male/female ratio of patients with HCC-C steadily decreased from 6.4 to 1.9 during the period. We suggest that the increase of female

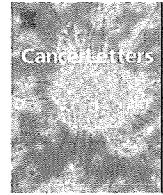
patient with HCC-C was caused by the aging of HCV infected people. The increase of females among HCC patients was considered to increase because of HCC-C.

It is known that 2 to 4 decades of chronic HCV infection are required to develop cirrhosis and subsequent HCC (28-31). The number of HCC cases has increased in Japan, because individuals infected with HCV in the past have grown old and have reached the cancer-bearing age. The prevalence of HCV infection in young Japanese individuals 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 (32). Additionally, we showed that the number and proportion of patients with HCC-C cases decreased together with an increase in the median age, whereas the number and ratio of HCC-nonBC steadily increased during the studied period. Based on these findings it may be expected that the incidence of HCC-nonBC in Japan may continue to increase even after the consequence of the HCV epidemic level off in the near future, although Japan is far advanced with regard to HCC-C.

In summary, HCC patients rapidly increased from 1981 to 2000 and this increase originated from HCC-C and the increase of the median age and the number of female patients with HCC-C. Increase in the number and proportion of the HCC-nonBC patients are also significant.

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## Mini-review

## Cancer stem cells in hepatocellular carcinoma: Recent progress and perspective

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## ABSTRACT

Although the “cancer stem cell (CSC)” hypothesis was first proposed roughly 50 years ago, recent progress in stem cell biology and technologies has successfully achieved the identification of CSCs in a variety of cancers. CSCs are defined as a minor population which possesses a prominent ability to generate new tumors that faithfully reproduce the phenotype of original tumors in xenotransplant assays. Additionally, CSCs are able to self-renew and generate differentiated progenies to organize a hierarchical cell system in a similar fashion to normal stem cells. Although not all types of cancer follow the CSC theory, it provides an attractive cellular mechanism to account for the therapeutic resistance and recurrence of the disease. A minor population with CSC properties has been detected in a number of established hepatocellular carcinoma (HCC) cell lines and extensive analyses characterizing the CSC system in primary HCC samples are now ongoing. Considering that HCC has high rates of recurrence and mortality, novel therapeutic approaches are urgently required. Although the clinical relevance of CSCs remains elusive, deep understanding of the cellular organization of HCC may allow us to develop therapies targeting specific cell types such as CSCs.

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## 1. Introduction

Cancer is usually unicellular in origin [1,2], although cancer cells constitute functional heterogeneity in a wide variety of cancers [3]. Classically, two general theories have been debated in terms of carcinogenesis [4]. The stochastic model indicates that a few cells which acquired proliferative potential via stochastic events are responsible for tumor formation. The alternative hypothesis, namely, the hierarchical model, postulates that a small subset of cells generates a hierarchical organization containing

varied downstream descendants, proliferates extensively, and initiates tumors at high frequency.

Stem cells, generally defined by an ability to differentiate into multiple cell lineages and self-renew, contribute to not only organogenesis but also regeneration in response to the injury of tissues and organs [5]. Recent advancements in stem cell biology have allowed for the identification and characterization of stem cells in a variety of tissues and organs. On the other hand, it has been documented that solid tumors such as breast cancer and colon cancer contain a small subset of tumorigenic cells which can generate new tumors in xenograft transplantation [6,7]. This minor population of cells, termed cancer stem cells (CSCs) or tumor initiating cells (TICs), possesses stem cell-like properties and contributes to a hierarchical structure containing varied progenies in a similar fashion to normal stem cells. Successful detection of CSCs in a wide variety of cancers supports the hierarchical carcinogenesis theory.

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