fibrosis or sever liver injury, and ≤ 40% in patients with cirrhosis.[55,58] Furthermore, Clavien *et al.* proposed the decision tree for major hepatectomy using CTP score, presence of portal hypertension, and ICG R15 together with future remnant liver volume.[59] In the review article, the authors proposed that the cutoff points of FRL for major hepatectomy was proposed 30% and 50% for patients with normal and cirrhotic liver, respectively, and that ICG R15 less than 14% is safety limit for major hepatectomy without PVE in patients with cirrhosis. As mentioned above, Makuuchi criteria have been expanded in both aspects of ICGR R15 and the rate of FRL volume in relation to the indication of PVE because the criteria is so strict that patients can underwent safe hepatic resection. However, extension of Makuuchi criteria should be carefully tried and validated in a large number of patients.

## 7. CONCLUSION

IN SUMMARY, THE CTP system is still useful for selecting surgical candidates for liver resection. The MELD score is helpful for predicting surgical outcome only in patients with advanced liver cirrhosis. The safety limit for the parenchymal resection rate can be determined using "criteria" based on the ICG-R15 score. GSA scintigrams provide data that complements the ICG test. Other quantitative liver function tests require further validation and simplification.

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ORIGINAL PAPER

# Des-γ-carboxy prothrombin stimulates human vascular endothelial cell growth and migration

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**Abstract** Des-γ-carboxy prothrombin (DCP) is an aberrant prothrombin produced by hepatocellular carcinoma (HCC) cells. Serum and tissue DCP expressions are thought to reflect the biological malignant potential of HCC. However, the role of DCP in the development of angiogenesis is not well understood. Herein, we report the effects of DCP on growth and migration of human vascular endothelial cells. DCP significantly stimulated the proliferation of HUVEC (ECV304) cells in a dose and time dependent manner, as measured by the MTT assay. A continuous rapid migration of ECV304 cells was observed in the presence of DCP measured by the scratch wound assay. The continuous rapid invasive activity, measured by transwell chamber assay also showed that DCP increased endothelial cells migration through the reconstituted extracellular matrix (Matrigel). Further, the tube formation of vascular endothelial cells on 3-D Matrigel showed an increased number of branch points of ECV304 cells induced by DCP in a dose dependent manner. The levels of vascular endothelial cell growth-related angiogenic factors and matrix metalloproteinase were also examined. DCP significantly stimulated the expression levels of epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), and matrix metalloproteinase (MMP)-2 (latent and active). Together, these data suggest that DCP is a novel type of vascular endothelial growth factor that possesses potent mitogenic and migrative activities in angiogenesis of HCC.

**Keywords** HCC · DCP · Human vascular endothelial cell · Angiogenesis · EGFR · VEGF · MMP-2

**Abbreviations**

| | |
|---|---|
| HCC | Hepatocellular carcinoma |
| DCP | Des-γ-carboxy prothrombin |
| HUVEC | Human umbilical vein vascular endothelial cell line |
| MTT | 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide |
| PBS | Phosphate buffered saline |
| EDTA | Ethylenediamine tetraacetic acid |
| HEPES | Hydroethyl piperazine-ethane sulfonic acid |
| ELISA | Enzyme-link immunosorbent assay |
| EGFR | Epidermal growth factor receptor |
| VEGF | Vascular endothelial growth factor |
| MMPs | Matrix metalloproteinase |
| ECM | Extracellular matrix |
| HGF | Hepatocyte growth factor |
| KDR | Kinase insert domain receptor |

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MAPK Mitogen-activated protein kinase
TGF-$\beta$ Transforming growth factor-$\beta$

## Introduction

Since Liebman et al. [1] reported an increased plasma Des-$\gamma$-carboxy prothrombin (DCP) level in patients with hepatocellular carcinoma (HCC), DCP has been found to be a useful diagnostic marker of HCC [2, 3]. DCP (also known as protein induced by vitamin K absence or antagonist-II, PIVKA-II) is an aberrant prothrombin produced in HCC cells [4]. Production of DCP is the result of an acquired defect in the post-translational carboxylation of the prothrombin precursor in HCC cells. Recently, it was reported that DCP is a potential autologous growth factor in development of HCC [5, 6]. Elevated serum DCP levels have been found in 44–81% of HCC patients [7]. Large tumour size has been associated with high levels of DCP in serum. Therefore, DCP is the novel clinical and molecular predictor of HCC outcome [8, 9].

However, the role of DCP in the development of HCC has not been completely defined and the mechanism underlying the processes of angiogenesis has remained largely unknown. Aberrant angiogenesis is an essential step for the invasion and metastasis of HCC [10]. It is hypothesized that DCP is secreted from HCC cells, where it works as an autologous growth factor for HCC development and a paracrine interaction factor between HCC cells and vascular endothelial cells [5, 6, 10]. In this study, we examined the effects of DCP on proliferation and tube formation of human vascular endothelial cells. The biochemical mechanisms behind the development of HCC by vascular endothelial cell growth-related angiogenic factors were investigated.

## Materials and methods

### DCP

Des-$\gamma$-carboxy prothrombin is a gift from Eisai Co., Ltd., Tokyo, Japan. The DCP was purified from the DCP-producing cell line PLC/PRF/5 in the conditioned media by affinity chromatography with an anti-prothrombin antibody [5]. DCP was distinguished from normal prothrombin by high performance liquid chromatography (HPLC) analysis [5].

### Cell line and cell culture

The human umbilical vein vascular endothelial cell line (HUVEC) ECV304 was purchased from Shanghai Cell Bank, Institute of Cell Biology, China National Academy of Sciences (Shanghai, China). Cells were maintained in RPMI-1640 media supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin–streptomycin (100 IU/ml–100 μg/ml), 2 mM glutamine, and 10 mM hydroethyl piperazine-ethane sulfonic acid (HEPES) buffer at 37°C in a humid atmosphere (5% $CO_2$-95% air) and were harvested by brief incubation in 0.02% (w/v) EDTA in PBS (ICN, Aurora, OH, USA).

### Cell proliferation assay

ECV304 cells (0.5–1 × $10^4$ per well) seeded in 96-well plates were incubated with different concentrations of DCP or human normal prothrombin (negative control, Sigma, St Louis, USA) for the indicated time. The medium was then removed and the wells were washed with PBS. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by adding 20 μl MTT (5 mg/ml, Sigma, St Louis, Missouri, USA) for 4 h. Light absorbance of the solution was measured at 570 nm on a THERMOmax microplate reader (Molecular Devices, Sunnyvale, California, USA). Triplicate experiments with triplicate samples were performed [11].

### Scratch assay

The scratch assay was performed by plating cells in 6-well culture dishes. After ECV304 cells were allowed to attach and reach 80% confluence, a scratch (1 mm) was made through the culture dish with a sterile plastic 200 μl micropipette tip to generate one homogeneous wound along each well. After wounding, the peeled off cells were removed with twice PBS washes. Cells were further incubated without or with different concentrations of DCP for 24 and 48 h and the wound widths were measured under the microscope using an ocular grid [12]. Three wounds were sampled for each treatment and experiments were carried out in triplicate. Cell migration = 0 time wound width (1 mm) − 24 or 48 h wound width. The migration thus observed is represented as percentage migration considering migration in untreated control as 100%. Images of wound were taken using a microscope at 100× magnification (Olympus IX51, Olympus Corporation, Tokyo Japan).

### Transwell chamber assay

The motility and invasive ability of ECV304 cells were performed in 24-well transwell plates (Corning, NY, USA) [13]. The upper surface of polycarbonate filters with 8 mm pores was coated with 100 μg of Matrigel (Sigma–Aldrich, USA). ECV304 cells were pre-incubated with different concentrations of DCP or 1% BSA (negative control) for

24 h at 37°C in a $CO_2$ incubator and then detached and resuspended in serum-free RPMI-1640. A suspension of cells ($2 \times 10^5$ cells/100 μl) was placed in the upper chambers. The lower chambers were filled with 600 μl of RPMI-1640 medium. Cells were allowed to migrate for 8 h at 37°C. Migration was terminated by removing the cells from the upper compartment of the filter with a cotton swab. Cells that had invaded through the Matrigel and reached the lower surface of the filter were quantified by counting the number of cells that migrated in five random microscopic fields per filter at a magnification of 200× (Olympus IX51, Olympus Corporation, Tokyo Japan).

### Capillary tube formation assay

The capillary tube formation assay was performed as described earlier [14]. Matrigel (100 μl, Sigma–Aldrich, USA) was added to each well of a 96-well plate and allowed to polymerize for 1 h at 37°C. ECV304 cells were suspended in medium at a density of $3 \times 10^5$ cells/ml, and 0.1 ml of cell suspension was added to each well coated with Matrigel, together with or without the indicated concentrations of DCP. It is considered that ECV304 cells on 3-D Matrigel spread out and generate lateral processes 3 h after being placed onto material [15]. Therefore, the capillary tube formations were visualized after 10 h. The images were captured by an inverted microscope using a 20× objective lens. Images from a total of five microscopic fields per well were analyzed by Motic Image Plus 2.0 software (Motic Instruments Inc., Richmond, Canada). The tube formation was defined by counting the branch points of the formed tubes and the average numbers of branch points were calculated. Each set of the conditions were repeated in triplicate.

### Western blot analysis

ECV304 cells ($3 \times 10^5$) seeded in 6-well plates were treated with different concentrations of DCP for 24 h. The medium was removed and the cells were washed with PBS. Cells were lysed with 200 μl of 5 g/l SDS (Shenggong Biological Technology Ltd, Shanghai, China) and centrifuged at 10,000*g*. Supernatant was collected and the total protein was determined using Bradford method [16]. Cell lysates (30 μg of protein per lane) were fractionated by 10% SDS–PAGE and then electrotransferred onto nitrocellulose membranes (Osmonics, USA). After blocking with TBST buffer (20 mM Tris-buffered saline and 0.1% Tween-20) containing 5% (w/v) nonfat dry milk (Wander Sun, Heilongjiang, China) for 1 h at room temperature, the membranes were incubated with specific antibodies against epidermal growth factor receptor (EGFR; rabbit polyclonal antibody against human EGFR, a peptide mapping near the C-terminus, Boster Biological Technology Ltd, Wuhan, China), vascular endothelial growth factor (VEGF; A-20; mouse monoclonal antibody against human VEGF, sc-152, Santa Cruz Biotechnology, USA), and matrix metalloproteinase (MMP)-2 (H-76; mouse monoclonal antibody against human MMP-2, sc-10736, Santa Cruz Biotechnology, USA) in dilution buffer [2% (w/v) BSA in TBS] for 2 h. The blots were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Santa Cruze Biotechnology, USA) at appropriate dilutions and room temperature for 1 h. The bound antibodies were visualized using an ECL system (Amersham Pharmacia Biotech, Piscataway, NJ; 2–5 min exposure) and quantified by densitometry using an electrophoresis image analysis system (FR980, Furi Science & Technology, Shanghai, China). The rates of stimulation were estimated by comparison to the untreated control for each individual protein [17].

### Enzyme-link immunosorbent assay

The secretion of VEGF was evaluated using commercially available sandwich enzyme-link immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). HCC cells were plated in 6-well plates and treated with different concentrations of DCP for 24 h. Assay was performed using 24-h-collected, serum-free medium. Results were normalized for the number of producing cells and reported as pg of VEGF/$10^6$ cells/24 h [6]. Duplicate experiments were performed.

### Statistical analysis

Data was described as the mean ± SD, and analyzed by Student's two-tailed *t*-test. The limit of statistical significance was $P < 0.05$. Statistical analysis was done with SPSS/Win11.0 software (SPSS, Inc., Chicage, Illinois). Dr. X. J. Qu is responsible for the statistical analysis.

## Results

### DCP stimulates the proliferation of HUVEC cells

ECV304 cells were treated with DCP (10–160 ng/ml) for up to 120 h and then the rates of cell growth stimulation were evaluated based on the viable cell number as estimated by MTT assay. As shown in Fig. 1, incubation with DCP significantly stimulated ECV304 cell proliferation, although the statistical significances were not seen in the low concentrations (10, 20 ng/ml, 24 h, $P > 0.05$). This mitogenic effect reached a plateau at 32.5% enhancement over untreated cells with the concentration of 160 ng/ml at 120 h incubation. Normal prothrombin did not affect cell growth in the experiment (data not shown).

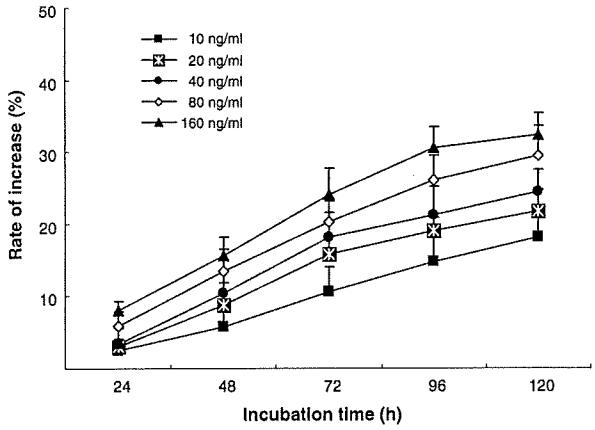


**Fig. 1** Stimulation of proliferation of HUVEC cells by DCP. ECV304 cells were cultured without (control) or with different concentrations (10–160 ng/ml) of DCP for up to 120 h. Cell growth was evaluated using the MTT assay and the percentage of stimulation was calculated by comparing to the control at the concurrent time point. The *bars* indicate means ± SD ($n = 3$)

## DCP promotes ECV304 cell migration

One of the earliest steps in angiogenesis is the invasion of basement membrane and migration of endothelial cells toward the angiogenic stimulants. We measured the migration distances of scratched cells into the cell-free 'scratch' region for a highly confluent (90–100%) monolayer of endothelial cells without or with DCP. As shown in Fig. 2, the migration distances of ECV304 cells was significantly increased in the presence of DCP (10–160 ng/ml) for 24 and 48 h, when compared with the spontaneous (absence of DCP) cell migration (Fig. 2a). At the concentrations range of 10–160 ng/ml of DCP, the rates of migration were increased from 30.0% to maximum increase of 169.5%, for 24 h of incubation and from 21.4% to maximum increase of 170.0%, for 48 h of incubation (Fig. 2b).

We further evaluated the motility and invasive ability of endothelial cells migrating through Matrigel. The ability of invasive and migrative of ECV304 cells was significantly increased by 24 h pre-incubation with DCP (Fig. 3a). At the concentrations range of 10–160 ng/ml of DCP, the number of cells migrating the Matrigel coated membrane were increased by 10.5, 15.0, 27.3, 53.1, and 86.4%, respectively (Fig. 3b).

## DCP increases capillary tube formation of HUVEC

The process of capillary tube formation of HUVEC involves ECV304 attachment, invasion and migration. To investigate the aberrant activity of DCP on angiogenesis, we examined the formation of capillary tube on 3-D Matrigel in the presence of DCP. DCP significantly increased the capillary tube formation of ECV304 after 10 h exposure to DCP. As shown in Fig. 4, the increasing number of branch points were DCP-concentration dependent when compared with un-treated control (Fig. 4b). At the concentrations of 10, 20, 40, 80 and 160 ng/ml of DCP, formation of branch points were 70.4, 100.0, 140.7, 188.9, and 225.9% of controls, respectively (Fig. 4b).

## DCP activates expressions of angiogenic proteins

We measured the levels of vascular endothelial cell growth-related angiogenic proteins in ECV304 cells using Western blotting analysis. DCP significantly increased the expressions of EGFR in vascular endothelial cells. As shown in Fig. 5a, EGFR was detected in the gel at a molecular weight of 170 kDa. The levels of EGFR were elevated by up to 116.2% compared with the control, after 24 h incubation with DCP (Fig. 5b).

We then examined the level of VEGF in ECV304 cells exposed to DCP. DCP significantly stimulated the expression of VEGF evaluated using Western blotting analysis (Fig. 6a). At the concentrations range of 10–160 ng/ml of DCP, the rates of stimulation were increased from 27% to maximum increase of 145.8% (Fig. 6b). We measured the level of secretion of VEGF by using ELISA assay. DCP treatment resulted in a markedly increased secretion of VEGF in a dose-dependent manner (Table 1).

## DCP stimulates expression of MMP-2

The stimulation of DCP on the activity of matrix metalloproteinase was also evaluated using Western blot analysis in ECV304 cells. As shown in Fig. 7, pro and active MMP-2 were measured in the gel at molecular weights of 72 and 63 kDa. DCP treatment of ECV304 cells resulted in a markedly increased expression of MMP-2 and remarkable in active MMP-2 more than in pro MMP-2. The increasing expression rates for pro MMP-2 were up to 50% increase and 160% increase for active MMP-2 by DCP treatment (Fig. 7b).

## Discussion

In this study, the function of DCP relating to the angiogenic process of invasion and metastasis of HCC was evaluated by different assays. The results showed that DCP significantly increased the proliferation, migration and capillary tube formation of vascular endothelial ECV304 cells. The expression levels of vascular endothelial cell growth-related angiogenic proteins, such as EGFR and VEGF levels were elevated in the presence of DCP. Moreover, high levels of expression of MMP-2 (latent and

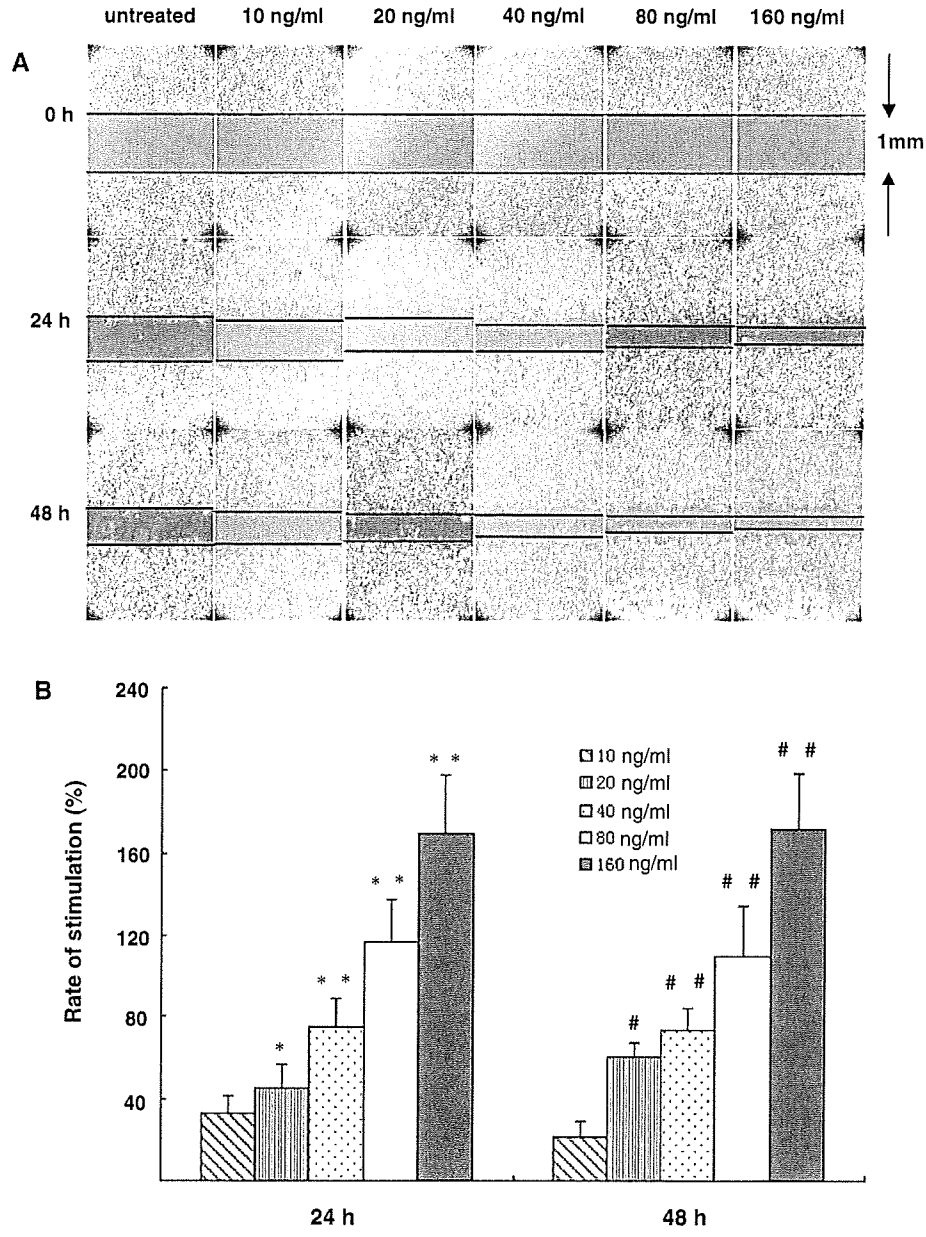


**Fig. 2** Increase of ECV304 cell migration by DCP. Scratch assays were performed by plating cells in 6-well culture dishes. Cells were scratched as described in "Materials and methods". Cells were then further incubated without (control) or with different concentrations of DCP for 24 and 48 h and the wound widths were measured under microscope using an ocular grid (magnification, ×100). The *bars* indicate means ± SD ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$ (24 h vs. control); $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ (48 h vs. control)

active) was observed in ECV304 cells exposed to DCP. This implies that DCP maybe involve in the process of angiogenesis in the development of HCC.

Des-γ-carboxy prothrombin is an abnormal prothrombin in which the 10 glutamic acid residues (Glu) are not completely carboxylated to γ-carboxyglutamic acid residues (Gla) in HCC cells [18]. Many studies consider DCP a prognostic indicator of HCC. For example, serum and tissue DCP expressions are thought to reflect the biological malignant potential of HCC, and serum DCP level is used as a clinical marker for the development of portal venous invasion of HCC. Cell proliferation markers have been found to correlate with tissue DCP expression in clinical pathological studies of HCC [5]. There are two kringle domains in DCP, are similar to those of hepatocyte growth factor (HGF), which has been identified as a member of angiogenic growth factors in HCC cells [5]. The kringle domains may bind with c-Met (HGF transmembrane tyrosine kinase receptor) to stimulate HCC cell proliferation [4]. DCP might work as a paracrine interaction factor between HCC cells and vascular endothelial cells [6, 10]. It was reported that DCP could promote vascular endothelial cell proliferation and migration via binding with kinase insert domain receptor (KDR) [10]. Consistent with the above, we observed the stimulation of growth and migration of vascular endothelial cells in the presence of DCP.

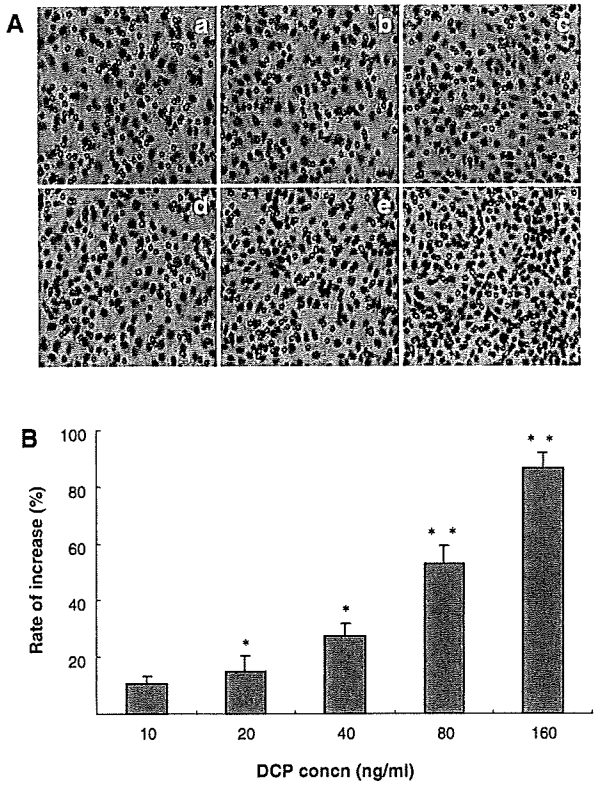


**Fig. 3** Stimulation of ECV304 cell invasion and migration induced by DCP. Cells pre-incubated with various concentrations of DCP were placed on Matrigel-coated filters and incubated for 8 h. The number of cells passing through the filter was counted after staining with hematoxylin. **a** Typical micrographs of invading ECV304 cells pre-treated with the indicated concentrations of DCP (original magnification, ×100). *a*, without DCP; *b–f*, cells pre-incubated with 10, 20, 40, 80, and 160 ng/ml of DCP, respectively. **b** Increase rates of cell invasion by various concentrations of DCP. *Bars*, SD ($n = 6$). $^*P < 0.05$; $^{**}P < 0.01$ versus without DCP treatment

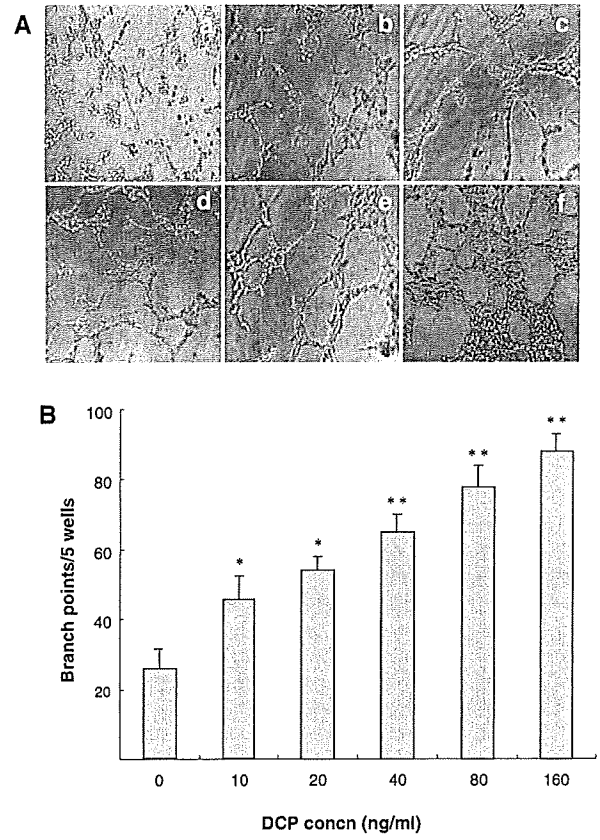


**Fig. 4** DCP stimulates vascular endothelial cell tube formation. ECV304 cells ($3 \times 10^4$ cells/well) were seeded into 96-well plate which had been pre-coated with Matrigel in medium for 10 h with the indicated concentrations of DCP. The images were captured by an inverted microscope using a ×10 objective (**a**). Tube formations on 3-D Matrigel were defined by counting the branch points of formed tubes. *a*, without DCP; *b–f*, cells pre-incubated with 10, 20, 40, 80, and 160 ng/ml of DCP, respectively. Five microscopic fields were counted for each treatment. The data represented mean ± SD from triplicate experiments (**b**). $^*P < 0.05$; $^{**}P < 0.01$ versus without DCP treatment

Hepatocellular carcinoma is a mainly hypervascular tumor and the progression of invasion and metastasis is highly correlated to the formation of angiogenesis [19]. Angiogenesis actually starts with cancerous tumor cells releasing molecules that send signals to vascular endothelial cells [14]. These signals activate the proliferation and migration of vascular endothelial cells leading to the formation of solid endothelial cell sprouts in the stromal space [20]. Currently, more than a dozen different molecules have been identified as angiogenesis-stimulating factors, including EGFR, VEGF, FGF and MMPs [21–24]. Among them, EGFR is known to be a major angiogenesis growth factor in predicting the proliferation and angiogenesis of vascular endothelial cells [24]. VEGF is the key mediator of angiogenesis in HCC [25]. MMPs could activate the angiogenic factors through proteolytic processing [24]. All three molecules have been shown to be closely correlated to HCC angiogenesis. Therefore, we focused on examining the expressions of the three molecules in vascular endothelial cells exposure to DCP.

The mechanism of stimulation of DCP on HCC angiogenesis remains unknown. It has been shown that increased expression of RGFR is the key factor to enhance vascular endothelial cell replication and migration [24]. High level of EGFR provides the basic drive for the ligand binding, such as epidermal growth factor (EGF), TGF-α, amphiregulin ß-cellulin (BTC), heparin-binding EGF-like growth factor, and epiregulin [26, 27]. These bindings may lead to activation of a cascade of biochemical and physiological responses involved in the mitogenic signal transduction of cells. Further, EGFR may mediate the signal transduction pathway of many other molecules in HCC angiogenesis, for example, EGFR activation has been shown to induce or up-regulate the expression of VEGF in capillary tube

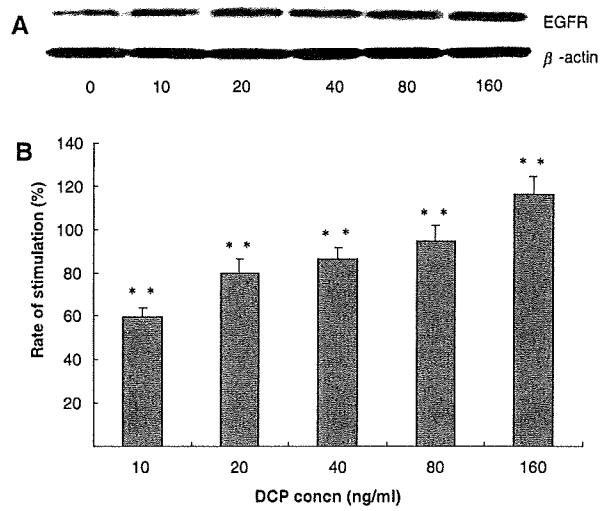


**Fig. 5** Western blot analysis of EGFR in ECV304 cells. ECV304 cells were incubated with different concentrations of DCP for 24 h. Total cell proteins (30 μg) were fractionated using 10% SDS–PAGE, transferred to nitrocellulose filters, and incubated with rabbit polyclonal antibody against human EGFR. The bound antibody was visualized using an ECL system (**a**) and quantified by densitometry using an electrophoresis image analysis system. Data are mean ± SD ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$ versus without DCP treatment (**b**)

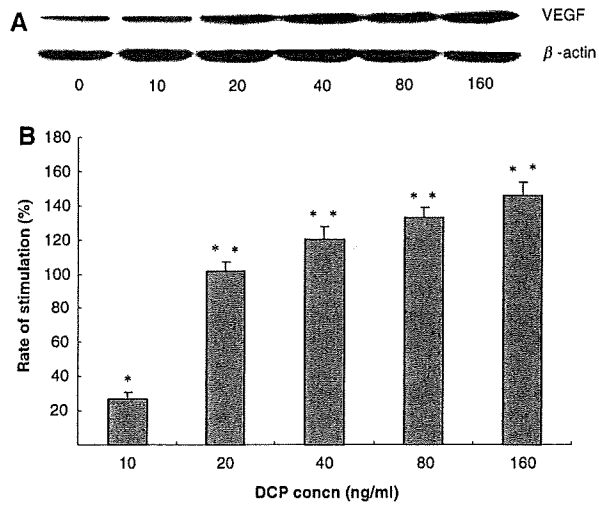


**Fig. 6** High expression of VEGF in ECV304 cells induced by DCP. The assay of Western blot analysis was described in Fig. 5 and the expression of VEGF was quantified by densitometry using an electrophoresis image analysis system (**a**). Data are mean ± SD ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$ versus without DCP treatment (**b**)

formation and invasion of HUVEC [28]. EGFR activation is considered to be the inducer of VEGF [24, 28, 29]. High level of EGFR may also upregulate the expression of MMPs through paracrine and/or autocrine mechanisms [24]. Therefore, VEGF and MMPs are considered to be the downstream signals of EGFR activation [24].

**Table 1** The levels of VEGF in ECV304 cells after treatment with DCP

| Dose of DCP (ng/ml) | VEGF (pg/$10^6$ cells/24 h) | $P$ (paired $t$-test) |
|---|---|---|
| 0 | 84 ± 9 | |
| 10 | 106 ± 12 | <0.05 |
| 20 | 122 ± 14 | <0.05 |
| 40 | 267 ± 23 | <0.01 |
| 80 | 271 ± 36 | <0.01 |
| 160 | 361 ± 41 | <0.01 |

The secretion of VEGF was determined on 24-h-collected serum-free medium by using ELISA kits, as described in "Material and methods" Results were normalized for the number of producing cells and reported as pg of VEGF/$10^6$ cells/24 h

The bars indicate means ± SD ($n = 3$). $P < 0.01$ versus without DCP treatment

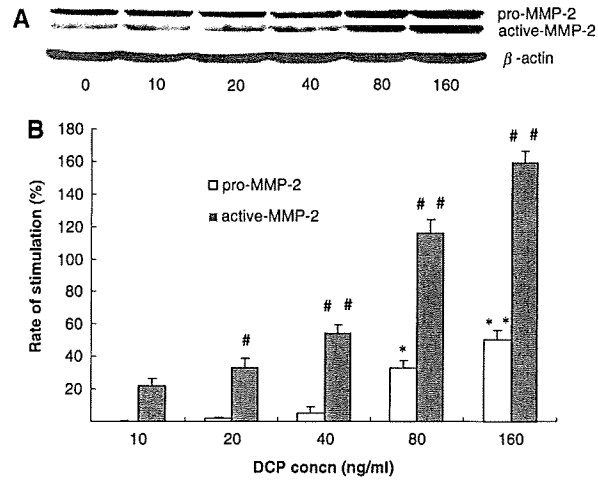


**Fig. 7** DCP stimulated high expression of MMP-2 in ECV304 cells. The assay of Western blot analysis was described in Fig. 5 and the expressions of MMP-2 were quantified by densitometry using an electrophoresis image analysis system (**a**). Data are mean ± SD ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus without DCP (pro MMP-2); $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus without DCP (active MMP-2) (**b**)

Vascular endothelial growth factor is first synthesized inside HCC cells and vascular endothelial cells and then secreted into the surrounding tissue induced by DCP [6]. In this study, we measured high levels of VEGF in both ECV304 cells and cell culture media. High levels of VEGF can induce the movement of endothelial cells, remodeling of the extracellular matrix, and the formation of capillary tubules via stimulation of tyrosine phosphorylation of focal adhesion kinase, activation of Akt and mitogen-activated protein kinase (MAPK), and promotion of thymidine incorporation into DNA [30].

Des-γ-carboxy prothrombin may also induce angiogenesis through the stimulation of MMPs in vascular endothelial cells. The activated endothelial cells produce

MMPs to break down the extracellular matrix (ECM) toward the source of the angiogenic stimulus for proliferation and migration [24, 28]. The activation of MMPs in endothelial cells release sequestered angiogenesis factors such as VEGF and bFGF to stimulate capillary tube formation [28]. The formation of tube-like structures by vascular endothelial cells in vitro depends on activation of MMP-2 and MMP-9 [31]. Consistent with these observations, we observed high level of MMP-2 expression in vascular endothelial cells after stimulation with DCP. MMP-2 activation has a great affinity for fibronectin, laminins, elastin and collagens to degrade and promote endothelial cell invasion into the surrounding interstitial matrix [32, 33]. MMP-2 may also activate angiogenic factors including VEGF, FGF-2 and TGF-$\beta$ during the degradation of ECM [34, 35]. The activated endothelial cells begin to divide and organize into capillary tubes that evolve gradually into a mature network of blood vessels.

In conclusion, we have found that DCP, the aberrant prothrombin produced in HCC cells, has a stimulatory activity on proliferation and migration in human vascular endothelial cells. The biochemical mechanism behind this activity of DCP may be related to the up-regulation of angiogenic factors. These results provide the information about the role of DCP in progression of HCC. Further work on mechanism of action of DCP is warranted.

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ORIGINAL ARTICLE – HEPATOBILIARY TUMORS

# Significance of Alpha-Fetoprotein and Des-γ-Carboxy Prothrombin in Patients with Hepatocellular Carcinoma Undergoing Hepatectomy

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## ABSTRACT

**Background.** Alpha-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP) are well-known tumor markers of hepatocellular carcinoma (HCC). The aims of this study are to calculate the sensitivity/specificity of AFP and DCP measurement for the diagnosis of HCC, measure response rates of the markers following curative-intent resections, determine the correlations between the marker levels and clinicopathological prognostic variables, and determine the correlations between the marker levels before hepatectomy and those at diagnosis of recurrence.

**Methods.** A retrospective cohort study of 714 consecutive patients with HCC undergoing hepatectomy was carried out.

**Results.** The areas under the receiver operating characteristic curves were 0.79 versus 0.91 for AFP and DCP, respectively ($P < 0.001$). Positive AFP and DCP status became negative at 6 months post surgery in 184/229 (80.3%) and 245/246 (99.6%) patients, respectively (cutoff values being 20 ng/ml for AFP and 40 mAU/ml for DCP; $P < 0.0001$). No correlation was found between marker levels ($rs = 0.23$). The level of DCP, but not that of AFP, showed a close correlation with tumor size ($rs = 0.51$ and 0.19, respectively). They were associated with indices of tumor invasiveness without showing any specific associations. AFP and DCP levels in patients showing recurrence in ≤6 months correlated with the levels measured before surgery ($rs = 0.78$ and 0.49, respectively) but not in those showing recurrence after 2 years ($rs = 0.31$ and 0.30, respectively).

**Conclusions.** DCP is a more accurate, albeit complementary, HCC marker than AFP. While the levels of both markers increased with advancing tumor growth, no specific associations were found. The marker values at recurrence indicated the type of recurrence.

Early diagnosis remains the key to effective therapy in cases of hepatocellular carcinoma (HCC).[1] Although serum alpha-fetoprotein (AFP), a biological tumor marker of HCC, has long been used as a tool for HCC surveillance, it is not an ideal screening test due to its low sensitivity/specificity.[2–5] Liebman et al. first reported, in 1984, an increase in the plasma levels of des-γ-carboxy prothrombin (DCP), which is an abnormal prothrombin and also otherwise known as protein induced by vitamin K deficiency or antagonist-II (PIVKA-II), in patients with HCC.[6] Since then, the significance of DCP has been examined by many investigators and it was introduced as a routine laboratory test for HCC during the early 1990s in Japan.[7–9] In addition, a two-step enzyme immunoassay method was developed and has been in use since 1997; it shows a tenfold higher sensitivity for detection as compared with the conventional enzyme immunoassay method.[10] Consensus appears to have been reached on both DCP and AFP being independent tumor markers in HCC.[8,11–17] However, it still remains controversial whether or not DCP is superior to AFP as a single marker.[12,16–22]

The second role of tumor markers is in the monitoring of response to therapy. Ideally, the levels of tumor markers should fall to within normal range after effective treatment. This aspect is especially important in the case of



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transcatheter arterial embolization, because radiological findings do not necessarily reflect the degree of biological remission achieved by necrosis or fibrosis.[23] Comparisons of AFP and DCP in this regard have not been conducted.

Thirdly, elevation of tumor marker levels reportedly represents specific clinicopathological variables identified as prognostic factors.[14,21,22,24–26] Although high plasma levels of DCP reportedly indicate the presence of portal venous thrombosis and increased serum AFP levels are associated with a poor degree of differentiation of the tumor cells, in particular, these studies failed to comprehensively investigate the relationships with various parameters.[14,21,22,24,27]

Finally, another use of tumor markers is in the prediction of tumor recurrence. In theory, patients with HCC with elevated levels of AFP and/or DCP before treatment should also show elevated levels of the respective markers at the time of recurrence if the recurrence is metastatic. On the other hand, de novo secondary tumors also contribute to postoperative intrahepatic HCC recurrence.

In the present study, taking into account these unaddressed aspects of tumor markers of HCC, we comprehensively investigated the clinical significance of measurement of two tumor markers in cases of HCC, i.e., AFP and DCP, in a large cohort.

## PATIENTS AND METHODS

### *Patients*

The base population consisted of 714 consecutive patients who underwent curative liver resections for HCC at the Division of Hepato-Biliary-Pancreatic Surgery, Tokyo University Hospital, between January 1998 and November 2006. Curative resection was defined as removal of all recognizable tumors with a clear margin. The diagnosis of HCC was finally confirmed by pathological examination of the resected specimens in all cases.

Background characteristics of the patients are presented in Table 1. After discharge, monthly follow-up by tumor markers (AFP and DCP) and ultrasound as well as dynamic computed tomography (CT) scan every 4 months were conducted for 1 year. Then, we screened patients by tumor marker measurement and ultrasound every 2 months and dynamic CT scan every 6 months thereafter. We defined recurrence as the appearance of new lesions with radiological features typical of HCC, as confirmed by at least two imaging methods.[28]

### *AFP and DCP Assay*

Samples for AFP and DCP were taken within 7 days prior to the liver resection. Serum AFP level was measured by commercially available immunometric assay (ST AIA-PACK AFP, Tosoh, Tokyo, Japan). Plasma DCP level was measured by two-step enzyme immunoassay (Picolumi PIVKA-II, Eizai, Tokyo, Japan).[10]

**TABLE 1** Background characteristics of 714 patients with HCC

| Variables | $n = 714$ |
|---|---|
| Sex | |
| Male | 556 (77.9%) |
| Female | 158 (22.1%) |
| Age (years)[a] | 67 (19–90) |
| Hepatitis B virus infection[b] | |
| No | 560 (78.4%) |
| Yes | 154 (21.6%) |
| Hepatitis C virus infection[b] | |
| No | 250 (35.0%) |
| Yes | 464 (65.0%) |
| Child–Turcotte–Pugh grade[c] | |
| A | 601 (84.2%) |
| B | 113 (15.8%) |
| Background liver status[d] | |
| Normal liver | 14 (2.0%) |
| Chronic hepatitis | 295 (41.3%) |
| Cirrhosis | 405 (56.7%) |

[a] Median with range

[b] Five patients were positive for both hepatitis B and C virus infections and 101 patients were negative for both hepatitis B and C virus infections

[c] No patient was Child–Turcotte–Pugh grade C

[d] Pathological findings assessed in the resected specimen

### *Assessment*

*Sensitivity/Specificity of AFP and DCP for Presence of HCC* At 6 months post surgery, 25 out of the 714 patients were lost to follow-up in terms of serial tumor marker measurements, 190 had developed recurrence, 9 were disease-free at <6 months of follow-up, and the remaining 490 patients were confirmed to be disease free at this time point. The AFP and DCP values in 714 patients before the liver resection were defined as those of patients with HCC, while the values of these 490 patients at 6 months post surgery were defined as those of patients without HCC. Using these values, receiver operating characteristic (ROC) curves were constructed. The diagnostic performance of AFP and DCP was evaluated and compared through their areas under the receiver operating characteristic curves (AUROC). The cutoff values for AFP and DCP used in this study are those that have been conventionally used and/or have been proposed in previous reports: 20 ng/ml for AFP and 40 mAU/ml for DCP.[29]

*AFP and DCP Levels as Tools for Evaluating Therapeutic Response to HCC* In these 490 patients, complete tumor remission was thought to be achieved at 6 months after the liver resection. We examined whether this treatment response was correctly reflected in the alterations in the marker values. According to the cutoff values defined above, we classified the 490 patients into marker-positive or marker-negative status both before and at 6 months after the liver resection. We then investigated the changes of AFP- and DCP-positive/negative status following the liver resection.

*AFP and DCP as Complementary Tumor Markers for HCC* We first evaluated the relationship between AFP and DCP values in a total of 714 patients. Second, we classified these patients into four categories according to their positive/negative status for AFP and/or DCP according to the cutoff values.

*AFP and DCP as Markers of Clinicopathological Variables Representative of Tumor Invasiveness and Prognosis* We assessed the association of AFP and DCP values with clinicopathological variables that have been reported as prognostic factors for HCC in the 714 patients. The variables investigated are shown in Table 2. All variables were assessed pathologically on the resected specimens. Vascular invasion was defined as presence of portal vein invasion, venous invasion or biliary invasion. Multiple primary tumor nodules and intrahepatic metastases were differentiated using the guidelines proposed by the Liver Cancer Study Group of Japan.[30]

*AFP and DCP Levels as Indices for Predicting the Pattern of Recurrence* At the time of data collection, recurrence was observed in 444 patients. We classified these patients with recurrence into two groups, i.e., a group in which the recurrence occurred ≤6 months post surgery ($n = 190$),

**TABLE 2** Tumor-related factors

| Variables | $n = 714$ | AFP (ng/ml)[a] | DCP (mAU/ml)[a] |
|---|---|---|---|
| *Tumor size (mm)* | | | |
| ≤20 | 223 (31.2%) | 18.0 (7.0–69.0) | 24.0 (16.0–61.0) |
| 20–50 | 335 (46.9%) | 22.0 (7.0–144.0) | 57.0 (21.0–328.0) |
| >50 | 156 (21.9%) | 57.0 (8.5–3007) | 1251.0 (118.5–7486.0) |
| | | $rs = 0.19$ | $rs = 0.51$ |
| *Tumor number* | | | |
| 1 | 483 (67.7%) | 19.0 (1.0–216.0) | 55.0 (20.0–456.0) |
| 2 | 138 (19.3%) | 26.0 (8.0–177.5) | 53.0 (19.50–254.0) |
| ≥3 | 93 (13.0%) | 49.0 (13.5–162.5) | 59.0 (19.5–329.5) |
| | | $P = 0.07$ | $P = 0.73$ |
| *Capsular formation* | | | |
| No | 169 (23.7%) | 25.0 (8.0–148.0) | 32.0 (18.0–163.0) |
| Yes | 545 (76.3%) | 21.0 (7.0–207.5) | 72.0 (21.0–489.5) |
| | | $P = 0.83$ | $P < 0.05$ |
| *Capsular infiltration*[b] | | | |
| No | 137 (25.1%) | 14.0 (6.0–78.5) | 64.0 (10.0–364.0) |
| Yes | 408 (74.9%) | 27.0 (7.0–278.0) | 83.5 (21.5–579.5) |
| | | $P < 0.01$ | $P = 0.21$ |
| *Vascular invasion*[c] | | | |
| No | 495 (69.3%) | 17.0 (7.0–76.0) | 38.0 (18.0–189.0) |
| Yes | 219 (30.7%) | 88.0 (12.0–1271.0) | 233.0 (31.0–2110.0) |
| | | $P < 0.0001$ | $P < 0.0001$ |
| *Intrahepatic metastases* | | | |
| No | 601 (84.2%) | 19.0 (7.0–137.0) | 44.0 (10.0–310.5) |
| Yes | 113 (15.8%) | 81.0 (9.5–1261.0) | 235.0 (40.0–2544.0) |
| | | $P < 0.001$ | $P < 0.0001$ |
| *Tumor differentiation* | | | |
| Well | 104 (14.5%) | 12.5 (6.0–31.0) | 29.0 (17.0–87.5) |
| Moderate | 511 (71.6%) | 20.0 (1.0–174.0) | 63.0 (10.0–441.0) |
| Poorly | 99 (13.9%) | 165.0 (25.0–2326.0) | 145.0 (26.0–2455.0) |
| | | $P < 0.0001$ | $P < 0.0001$ |

[a] Median with interquartile range

[b] We assessed 545/714 patients who had capsular formation

[c] Macroscopic invasion was observed in 45/219 (20.5%) patients, while microscopic invasion was found in 174/219 (79.5%) patients

and another in which the recurrence occurred >6 months post surgery ($n = 254$). We first compared the preoperative levels of AFP and DCP as well as the levels at time of recurrence between the two groups of patients. Then, we further classified the two groups of patients into two subgroups according to site of recurrence, i.e., intrahepatic or extrahepatic recurrence. We investigated the correlations between the preoperative marker values and the site of recurrence.

*Etiological Association Between the Primary and Recurrent Tumors Investigated Through AFP and DCP Marker Values* We investigated the correlations of the tumor marker values at the time of recurrence with those measured before the liver resection. We classified 444 patients who developed recurrences into four groups according to time to recurrence, as follows: recurrence at ≤6 months ($n = 190$), recurrence between 7 and 12 months ($n = 70$), recurrence between 13 and 24 months ($n = 70$), and recurrence after 2 years ($n = 114$). Then, we examined the chronological alterations in the correlation of values of the respective tumor markers measured before the liver resection with those measured at the time of recurrence.

*Statistical Analysis*

Marker values are expressed as median with interquartile range. The AUROC for markers was compared by Wilcoxon's rank-sum test.[31] Correlations between marker values were analyzed by Spearman's rank correlation. Categorical binary variables were compared by Fisher's exact test. Associations between marker values and clinicopathological variables were analyzed by Wilcoxon's rank-sum test or by the Kruskal–Wallis test, as appropriate. $P$ values of $< 0.05$ were accepted as statistically significant. All statistical analyses were performed using the GraphPad Prism® computer software, version 5 (GraphPad Software Inc., San Diego, CA).

## RESULTS

*Sensitivity/Specificity of AFP and DCP for Presence of HCC*

The median (interquartile range) AFP and DCP levels in 714 patients before liver resection were as follows: 22.0 (7.0–195.0) ng/ml and 55.0 (20.0–443.0) mAU/ml. The AFP and DCP levels in 490 patients who had no evidence of tumor recurrence at 6 months post surgery were 5.0 (3.0–9.0) ng/ml and 11.0 (10.0–15.0) mAU/ml, respectively. The sensitivity and specificity of AFP and DCP were assessed by ROC curves (Fig. 1). The AUROC (95% confidence interval, CI) for AFP and DCP were 0.79 (0.76–0.81) and 0.91 (0.89–0.92), respectively ($P < 0.001$). The sensitivities and specificities at various cutoff values including those adopted in the present study (AFP, 20 ng/ml; DCP, 40 mAU/ml) and proposed in previous reports are presented in Table 3.

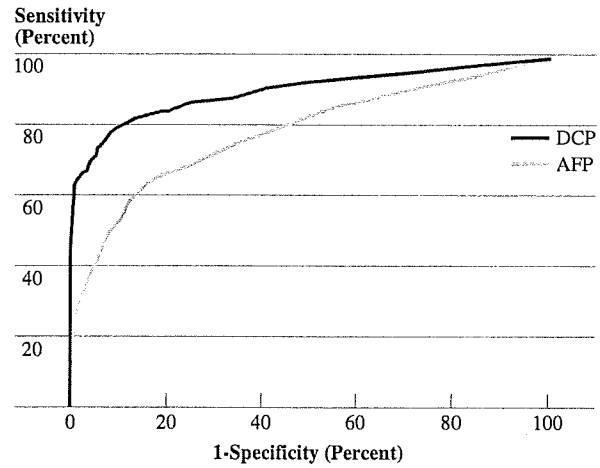


**FIG. 1** ROC curves for AFP and DCP. The yellow line represents AFP and the blue line represents DCP. The AUROC (95% CI) for AFP and DCP were 0.79 (0.76–0.81) and 0.91 (0.89–0.92), respectively ($P < 0.001$)

**TABLE 3** Sensitivities and specificities of AFP and DCP values according to various cutoff values

| AFP (ng/ml) | 11 | 13 | 20 | 100 | 200 |
|---|---|---|---|---|---|
| Sensitivity (%) | 64.9 | 60.8 | 51.3 | 30.4 | 24.7 |
| Specificity (%) | 82.9 | 86.1 | 90.8 | 98.6 | 99 |
| DCP (mAU/ml) | 20 | 30 | 40 | 100 | 125 |
| Sensitivity (%) | 73.4 | 62.8 | 55.9 | 41.9 | 39.1 |
| Specificity (%) | 94.7 | 99.4 | 99.8 | 100 | 100 |

In the present study, the cutoff values adopted were 20 ng/ml for AFP and 40 mAU/ml for DCP

*AFP* alpha-fetoprotein, *DCP* des-γ-carboxy prothrombin

*AFP and DCP as Tools for Evaluating Response to Therapy of HCC*

Among the 490 patients who were confirmed to be disease free at 6 months postoperatively, 229 (46.7%) and 246 (50.2%) were classified as AFP positive and DCP positive, respectively, before the liver resection under the present cutoff values. At 6 months post surgery, when complete tumor remission was thought to have been achieved, marker-negative status was achieved in 184/229 (80.3%) and 245/246 (99.6%) patients for AFP and DCP, respectively ($P < 0.0001$) (Table 4). Out of 45 patients

**TABLE 4** Pre- and postoperative marker status in 490 disease-free patients at 6 months

| Preoperative status | | Postoperative status | |
|---|---|---|---|
| *AFP* | | | |
| (+) | 229/490 (46.7%) | (−) | 184/229 (80.3%) |
| | | (+) | 45/229 (19.7%) |
| (−) | 261/490 (53.3%) | (−) | 261/261 (100%) |
| | | (+) | 0/261 (0%) |
| *DCP* | | | |
| (+) | 246/490 (50.2%) | (−) | 245/246 (99.6%) |
| | | (+) | 1/246 (0.4%) |
| (−) | 244/490 (49.8%) | (−) | 244/244 (100%) |
| | | (+) | 0/244 (0%) |

Cutoff values were set at 20 ng/ml for AFP and 40 mAU/mL for DCP, respectively

*AFP* alpha-fetoprotein, *DCP* des-γ-carboxy prothrombin

who showed AFP-positive status without recurrence at 6 months post surgery, 33 remained disease free at 12 months post surgery, whereas 12 had developed recurrence by this time point. In retrospect, the AFP values at 6 months post surgery were not thought to be indicative of recurrence at least in 6/12 patients. A single patient positive for DCP at 6 months post surgery was also disease free 5 years later. In all the 261 (53.3%) and 244 (49.8%) patients who were negative for AFP and DCP, respectively, before the surgery, the marker status for both of these markers remained negative at 6 months post surgery (Table 4).

### *AFP and DCP as Complementary Tumor Markers for HCC*

The correlation between the levels of these markers in the 714 patients is shown in Fig. 2; no association was seen ($rs = 0.23$). These patients were classified into four categories by the cutoff values used in the present study, as follows: AFP(+)/DCP(+): 229 (32.1%), AFP(+)/DCP(−): 137 (19.2%), AFP(−)/DCP(+): 170 (23.8%), and AFP(−)/DCP(−): 178 (24.9%) (Fig. 2).

### *AFP and DCP as Markers of Clinicopathological Variables Representative of Tumor Invasiveness and Prognosis*

The correlations of the AFP and DCP levels with clinicopathological findings are shown in Table 2. Although the DCP levels increased with increasing tumor size ($rs = 0.51$), this relationship was not found for AFP ($rs = 0.19$). While no statistical correlation was found between DCP levels and tumor number ($P = 0.73$), AFP levels tended to increase with increasing tumor number

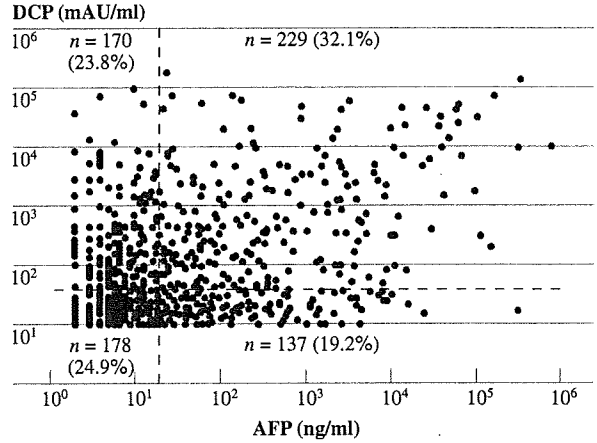


**FIG. 2** Correlation between AFP and DCP values in 714 patients. No correlation was found between the two markers ($rs = 0.23$, $P < 0.0001$). Dotted line represents cutoff values, i.e., 20 ng/ml for AFP and 40 mAU/ml for DCP. Patients were placed into four categories: either positive or negative for AFP and/or DCP according to these cut-off values. Number of patients in the each category was shown

($P = 0.07$). AFP and DCP levels increased to similar extent in the presence of indices of tumor invasiveness, such as vascular invasion and intrahepatic metastases. Likewise, both marker levels increased with increasing tumor cell differentiation.

### *AFP and DCP Levels as Indices for Predicting the Pattern of Recurrence*

The preoperative AFP and DCP values in HCC patients who developed recurrence ≤6 months ($n = 190$) versus patients who developed recurrence >6 months post surgery ($n = 254$) are shown in Table 5. Patients who developed recurrence ≤6 months post surgery showed higher preoperative AFP and DCP values than those who developed recurrence >6 months post surgery. Similarly, the AFP and DCP values measured at the time of recurrence in the two groups are shown separately in Table 5. Again, patients who developed HCC recurrence ≤6 months post surgery showed higher AFP and DCP values at the time of recurrence.

Out of 190 recurrences observed ≤6 months post surgery, 32 (16.8%) were extrahepatic: 18/32 (59 %) in the lung, 6/32 (19%) in the lymph node, 4/32 (13%) in the bone, 2/32 (6%) in the peritoneal membrane, and 1/32 (3%) in the adrenal gland.

On the other hand, the overall rate of extrahepatic recurrence in the patients who developed recurrence >6 months post surgery was 3/254 (1.2 %). Since extrahepatic recurrence was a rare event >6 months post surgery, we analyzed the correlations between the

**TABLE 5** AFP and DCP values in patients who developed HCC recurrence ≤6 months ($n = 190$) and >6 months ($n = 254$) post surgery

| | Preoperative values | | Values at recurrence | |
|---|---|---|---|---|
| | Recurrence ≤6 months | Recurrence >6 months | Recurrence ≤6 months | Recurrence >6 months |
| AFP (ng/ml) | 54.0 (9.0–624.5)[a] | 18.5 (7.0–76.0) | 17.5 (6.0–163.5)[a] | 13.0 (6.0–43.0) |
| DCP (mAU/ml) | 237.5 (22.8–2553.0)[b] | 37.5 (19.0–142.0) | 25.0 (14.0–131.0)[c] | 18.0 (13.0–34.3) |

Values are expressed as median (interquartile range)

[a] $P < 0.0001$ compared with recurrence >6 months

[b] $P < 0.005$ compared with recurrence >6 months

[c] $P < 0.0005$ compared with recurrence >6 months

**TABLE 6** Preoperative AFP and DCP values in patients who developed intrahepatic ($n = 158$) and extrahepatic ($n = 32$) recurrence ≤6 months post surgery

| | Intrahepatic recurrence | Extrahepatic recurrence |
|---|---|---|
| AFP (ng/ml) | 50.0 (9.0–337.8)[a] | 255.0 (10.8–9636.0) |
| DCP (mAU/ml) | 188 (22.8–184.0)[b] | 543.0 (34.3–10179.0) |

Values are expressed as median (interquartile range)

One patient who developed intra- and extrahepatic recurrences simultaneously was classified into those with extrahepatic recurrence

[a] $P < 0.05$ compared with extrahepatic recurrence

[b] $P = 0.08$ compared with extrahepatic recurrence

preoperative marker values and the site of recurrences exclusively in the 190 patients who developed recurrence ≤6 months post surgery (Table 6). Patients who developed intrahepatic recurrence ($n = 158$) showed higher preoperative marker values than those who developed extrahepatic recurrence ($n = 32$).

### *AFP and DCP as Markers Reflecting the Association Between the Primary and Recurrent Tumors*

The values of AFP and DCP measured before the liver resection are plotted against the values measured at the time of recurrence separately according to their time to recurrences in Fig. 3A–D and Fig. 4A–D, respectively. The AFP values in patients with recurrence at ≤6 months showed a close relationship with those measured before the liver resection ($rs = 0.78$, Fig. 3A). The strength of this relation became weaker in the groups with longer time to recurrence (Fig. 3B–D).

A similar trend was found in regard to the relationship of DCP values, although the correlations were weaker than those observed for AFP (Fig. 4A–D).

## DISCUSSION

The diagnostic accuracy of tumor markers should be evaluated on the basis of a trade-off between sensitivity and specificity, ideally by drawing ROC curves.[31] To date, three cross-sectional studies have compared the accuracy of AFP and DCP levels for the diagnosis of HCC through ROC curves, each using the present sensitive assay method for measuring DCP.[17,19,20] Two studies reported superiority of DCP.[17,20] However, a third reported better overall diagnostic accuracy of AFP.[19] The distribution of the etiology of the underlying liver disease in the present study population was similar to that in the populations studied by Marrero et al. and Nakamura et al., except that the former included a quantifiable proportion of alcoholic patients.[19,20] In regard to the distribution of the Child–Turcotte–Pugh (CPT) grade, our cohort is thought to lie in between the study cohorts of Marrero et al. and Nakamura et al., since 84.2% of our patients were classified into CPT grade A.[19,20]

In this study, we defined patients without recurrence at 6 months post surgery as a cohort without HCC. Although this approach may be different from that of former studies, this is advantageous in that the background characteristics are uniform in the patients with and without HCC.[17,19,20] This situation, which is an essential requirement in prospective screening studies of tumor markers, is not necessarily guaranteed in a cross-sectional study.[32] This study showed similar ROC results to those reported by Marrero et al. and Wang et al., which demonstrated superiority of DCP by approximately 10% (0.73–0.83 versus 0.85–0.93 for AFP versus DCP) (Fig. 1).[17,20]

In the present study, we used the cutoff values for AFP (20 ng/ml) and DCP (40 mAU/ml) proposed by previous studies and used most commonly in clinical settings.[29] Considering that much higher AFP values, e.g., 100 ng/ml or 200 ng/ml, have often been proposed as cutoff points, it is noteworthy that the present cutoff value showed better performance than these cutoff values, and even lower cutoff values can be adopted in terms of ROC performance (Table 3, Fig. 1). The cutoff value for DCP in the present study (40 mAU/ml), showing similar sensitivity to that of AFP, was thought to be the lowest among the values proposed until now (40–125 mAU/ml). Again, analysis of the ROC curve revealed that this value can be reduced even further in terms of a trade-off between sensitivity and specificity.

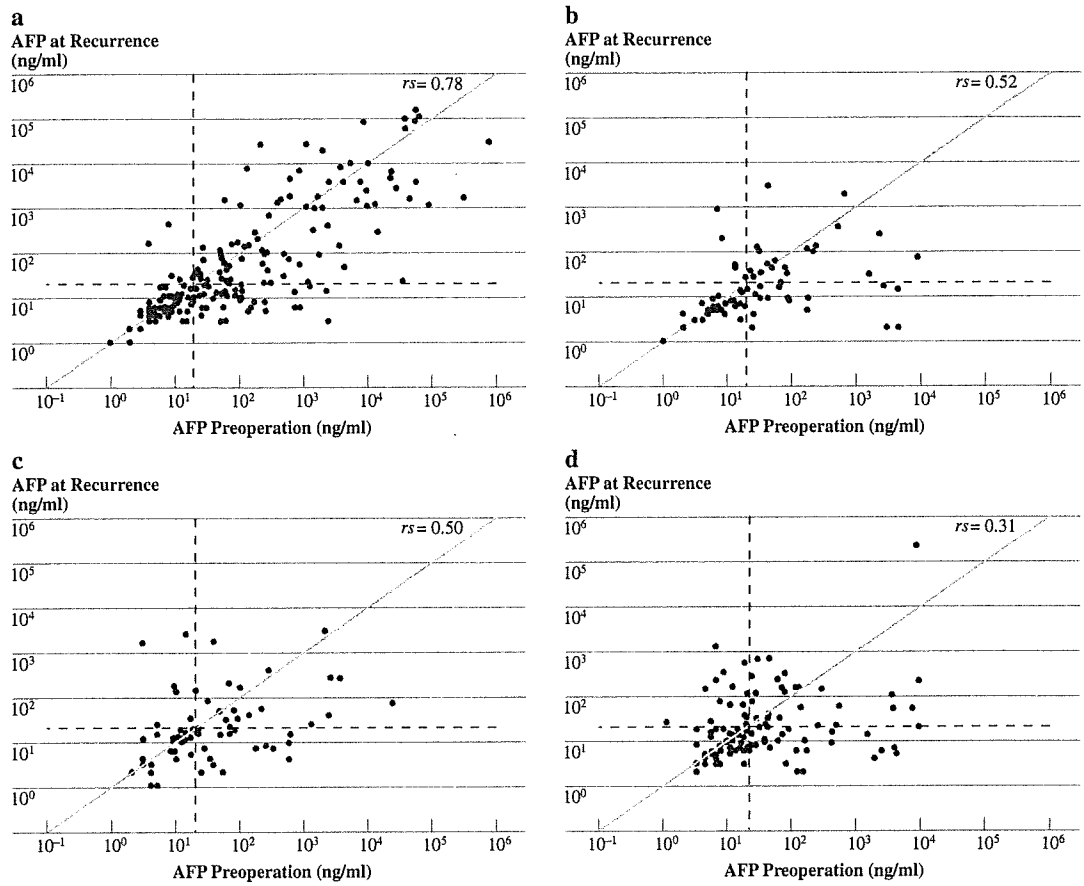


**FIG. 3** Correlations between preoperative AFP values and AFP values at recurrence stratified according to period of recurrence: (**a**) recurrence ≤6 months ($n$ = 190), (**b**) recurrence from 7 to 12 months ($n$ = 70), (**c**) recurrence from 13 to 24 months ($n$ = 70), and (**d**) recurrence >2 years ($n$ = 114). The dotted lines represent 20 ng/ml

In addition, DCP is a superior marker for monitoring response to therapy, that is, it was confirmed that positive DCP status converted to negative status in 99.6% (245/246) of patients at 6 months post surgery in the absence of tumor recurrence; in contrast, conversion from AFP-positive to AFP-negative status was achieved in only 80.3% of the patients (184/229). This high false-positive rate of AFP is thought to reflect the observed elevation in the levels of this marker also in conditions such as acute and/or chronic hepatitis and cirrhosis, which is an inherent drawback of AFP as a HCC-specific tumor marker.[3] Whereas high DCP values have been reported in patients with vitamin K deficiency, such as in cases of obstructive jaundice or cases receiving vitamin K antagonists, e.g., warfarin, these uncommon clinical situations can be easily discriminated in HCC patients.[12,33] Rather, it must be noted that patients with chronic alcoholism, another high-risk cohort for HCC, often show nonspecific DCP elevation, reportedly in 5–8% of patients.[34,35] The higher DCP cutoff value adopted by Marrero et al. in their study (125 mAU/mL) may be partially ascribed to the fact that their cohort included a considerable proportion of alcoholic patients (5%).[20]

In the present study, no correlation was found between the levels of AFP and DCP. This observation is consistent with previous reports.[11–17,21] These results strongly suggest that these markers are complementary to each other and that, although DCP might be superior to AFP as a single marker, the two should be evaluated in combination in clinical practice.

Although the association of tumor markers with various clinicopathological variables has been evaluated in many studies, the majority of these works assessed the associations solely with variables of interest and/or exclusively for AFP or DCP. Bearing this in mind, we investigated these associations in a comprehensive manner. While serum DCP values increased with increasing tumor size, no similar association was found for AFP (Table 2). This result is consistent with the results of previous

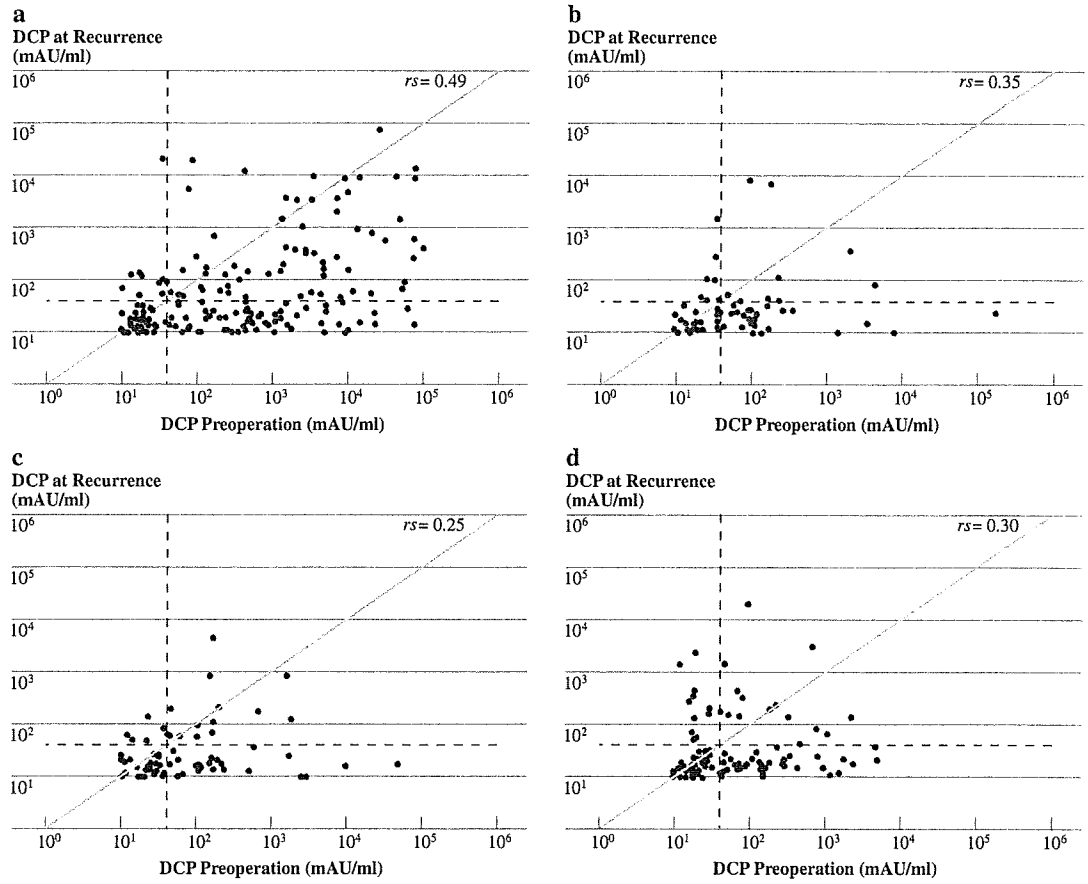


**FIG. 4** Correlations between preoperative DCP values and DCP values at recurrence stratified according to period of recurrence: (**a**) recurrence ≤6 months ($n$ = 190), (**b**) recurrence from 7 to 12 months ($n$ = 70), (**c**) recurrence from 13 to 24 months ($n$ = 70), and (**d**) recurrence >2 years ($n$ = 114). The dotted lines represent 40 mAU/ml

studies.[8,13,14,16,17,26,36] These findings suggest that the interindividual variations in the capacity of the tumor cells to synthesize AFP far exceed the elevation in the marker values with increasing tumor cell number.

While serum AFP levels tended to increase with increasing tumor number, this association was not observed for plasma DCP (Table 2). This finding is consistent with those of Kasahara et al. and Carr et al., who found a significant relationship between AFP and tumor number.[13,22] Considering that tumor number is thought to be a variable representing the degree of carcinogenicity in the background liver, the finding of the association for AFP but not for DCP is most probably explained by the elevation of AFP with advancing severity of background liver disease.[3,36,37]

In the present cohort ($n$ = 714), both increased AFP and DCP values were related to presence of indices of tumor invasiveness, such as vascular invasion, and intrahepatic metastases. To date, several studies with 72–161 patients have investigated the association of AFP and/or DCP with these indices, three of which assessed these pathological variables on surgically resected specimens.[14,21,24] A closer and/or specific relationship between these indices and DCP has been reported. Thus, the results of the present and former studies were partially contradictory. In our study, the AFP and DCP values were associated to a similar extent with the tumor cell differentiation grade (Table 2). Again, this observation is partially contradictory to the results of previous studies with 56–354 patients that claimed a specific close association with AFP or DCP.[24,26,27] The results of the present large cohort strongly suggests that both increased levels of AFP and DCP indicate the overall presence of pathological indices representing tumor invasiveness and/or increased malignant potential; however, they do not necessarily signify the presence of any specific entity.

Elevated preoperative AFP and/or DCP levels were correlated with early postoperative recurrence (≤6 months), and recurrence in the early phase was characterized by high serum levels of tumor markers. These results can most reasonably be interpreted as follows: high tumor marker levels signify an increased malignant