Measurement of Serum and Tissue *Des*-γ-carboxyprothrombin in Resectable Hepatocellular Carcinoma

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Abstract. *Background: The total des-γ-carboxyprothrombin (DCP) produced in hepatocellular carcinoma (HCC) and the surrounding non-cancer liver tissue was estimated and the correlation between tissue DCP and serum DCP levels was examined. Patients and Methods: Forty patients with a single primary HCC nodule served as the subjects. The DCP was quantified by electrochemiluminescence immunoassay and the tissue volumes were estimated by computed tomography (CT). Results: The mean tissue DCP in the cancer tissue, non-cancer tissue and the whole HCC specimen was 147,400, 20,200 and 190,000 mAU, respectively, which correlated positively with the serum DCP level (r^2=0.657, 0.452, and 0.684, respectively). The DCP expression levels in the cancer and non-cancer liver tissue were also confirmed by immunohistochemical detection of DCP. Conclusion: The elevation of serum DCP levels in HCC patients is influenced by DCP production not only in the cancer tissue, but also in the surrounding non-cancer liver tissue.*

Hepatocellular carcinoma (HCC) is one of the most common malignant diseases in Asian countries. It ranks third in men and fifth in women as a cause of death from malignant neoplasm and more than 30,000 patients die from HCC every year in Japan (1). At present, the clinical diagnosis of HCC is based not only on characteristic features observed from imaging diagnostic procedures such as computed tomography (CT), but also on elevated levels of serological tumor markers such as α-fetoprotein (AFP) and *des*-γ-carboxyprothrombin (DCP) (2).

DCP, also known as protein induced by vitamin K absence or antagonist (PIVKA-II), is an abnormal prothrombin. DCP possesses a structure similar to prothrombin except that 10 glutamic acid residues in the amino-terminal Gla domain are not completely γ-carboxylated and are therefore functionally inactive (3, 4). The clinical application of DCP as a tumor marker for HCC began when Liebman *et al*. reported an abnormally high level of DCP in the serum of 90% of patients with primary HCC and that the DCP titer was reduced after tumor resection or chemotherapy (5). The elevation of the serum DCP level is useful not only for diagnosis (6), but also for the prediction of tumor spread (7-9). In addition, unlike AFP, DCP rarely increases with cirrhosis or chronic hepatitis (10), meaning that DCP has higher specificity in differentiating HCC from nonmalignant chronic liver diseases. Despite the accumulating data on the clinical significance of the serum DCP level, the mechanism by which serum DCP is elevated in HCC patients is still not clearly elucidated. In early studies, a significantly raised level of abnormal prothrombin precursor was detected in cancer tissues and it was assumed that cancer tissue was the source of elevated serum DCP (11-13). However, recent immunohistochemical studies have proven that DCP is expressed not only in cancer tissue, but also in the surrounding non-cancer liver tissue of HCC patients (14-16). Therefore, it is reasonable to deduce that the level of serum DCP may be dependent on the total amount of DCP expressed in both the cancer and the surrounding non-cancer

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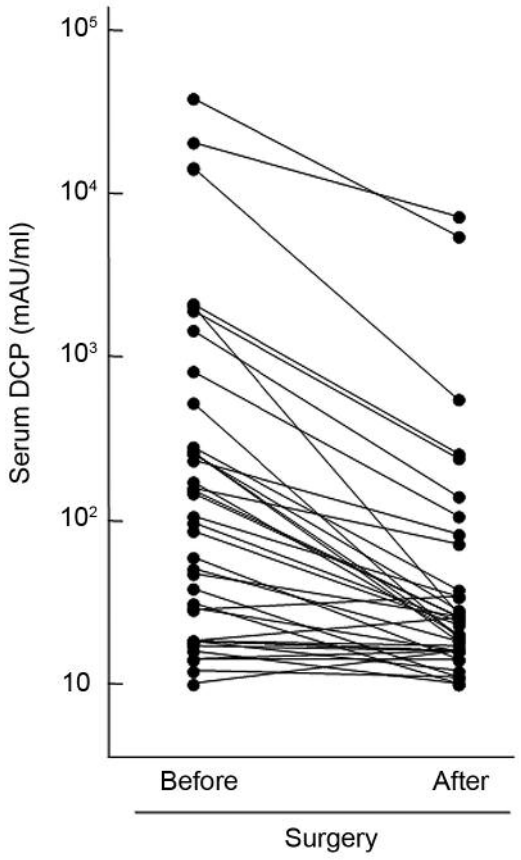

Figure 1. *Serum DCP levels before and after surgery.*

Table I. *Estimated DCP amounts in cancer and non-cancer tissue and the whole operative specimen obtained from HCC patients.*

Tissues	Weight (g)	DCP amount (mAU/0.1 g)	Total DCP (mAU)
Cancer tissue	31.4 (0.8-4,170)	506.0 (0.7-2.10 ×10^5)	147,000 (50-2.16 ×10^8)
Non-cancer tissue	100.0 (3.0-444)	19.6 (0-2.33 ×10^3)	20,200 (0-3.47 ×10^6)
Whole specimen	139.0 (19.0-4,290)	n.d.	190,000 (50-2.16 ×10^8)

Data are represented as median values (n=40). The ranges are noted in parentheses. n.d., Not determined.

Table II. *DCP amounts in the whole operative specimen in groups classified according to immunohistochemical expression pattern.*

Patient group	n	Total DCP amount in specimen (mAU)
$C^{high}N^{high}$	15	1.4×10^7 (4,400-2.16 ×10^8)*
$C^{high}N^{low}$	13	1.7×10^7 (50-1.78 ×10^8)*
$C^{low}N^{high}$	4	1.3×10^7 (1.28×10^5-4.91 ×10^7)*
$C^{low}N^{low}$	8	1.5×10^6 (1,000-1.07 ×10^7)

Patients were divided according to immunohistochemical staining characteristics (low/high) for DCP in cancer (C) and non-cancer (N) tissues. Data are indicated as mean values (n=40). The ranges are noted in parentheses. *$p<0.01$ *vs.* $C^{low}N^{low}$ group.

tissue. To clarify this assumption, we previously measured DCP in extracts of HCC tissue and the surrounding non-cancer tissue and found that the serum DCP level was positively correlated with the DCP per unit weight of both tissue types (17). However, considering the significant disparity in volume between cancer and non-cancer liver tissue, the DCP in the whole tissue rather than per unit weight of tissue should be a more accurate parameter reflecting the relationship between tissue and serum DCP levels.

In the present study, not only the quantity of tissue DCP in extracts obtained from the cancer and non-cancer liver tissue was determined, but also the volume of the corresponding parts of the liver and the total DCP in both types of tissue were thereby estimated.

Patients and Methods

Patients. Forty patients with a single primary HCC nodule who had undergone radical surgical resection at the Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, the University of Tokyo, between January 2000 and December 2001, were included in this study. There were 32 men and 8 women aged 63.0±10.3 years (mean±SD). Twenty-two patients (55%) were found to have underlying cirrhosis and 18 (45%) had chronic hepatitis. Thirty-eight patients (95%) had serological evidence of viral infection among whom 8 were positive for hepatitis B surface antigen (HBsAg), 26 were positive for hepatitis C virus antibody (HCVAb) and 4 were positive for both markers. The remaining 2 patients were negative for both hepatitis markers. The 40 tumors comprised 3 well-differentiated, 33 moderately differentiated and 4 poorly differentiated HCCs. Vascular invasion was found in 18 (45%) and intrahepatic metastatic lesions in 13 (32.5%) cases. The clinicopathological characteristics were evaluated according to the General Rules for the Clinical and Pathological study of Primary Liver Cancer (18). The International Union Against Cancer TNM system was used for tumor staging (19).

Measurement of serum DCP levels. The sera were obtained within one week before surgery and within one week after surgery. Serum DCP levels were measured by eletrochemiluminescence immunoassay (ECLIA) using a highly sensitive DCP determination kit (ED0306; Eisai, Tokyo, Japan) according to the manufacturer's instructions. The cut-off level was set to >40 mAU/ml (17) and levels above this were considered elevated.

Estimation of cancer and non-cancer tissue volumes. All the patients underwent CT before surgery. Dynamic transverse CT taken at 1.0 cm intervals (including the segment between the dome of the liver and the

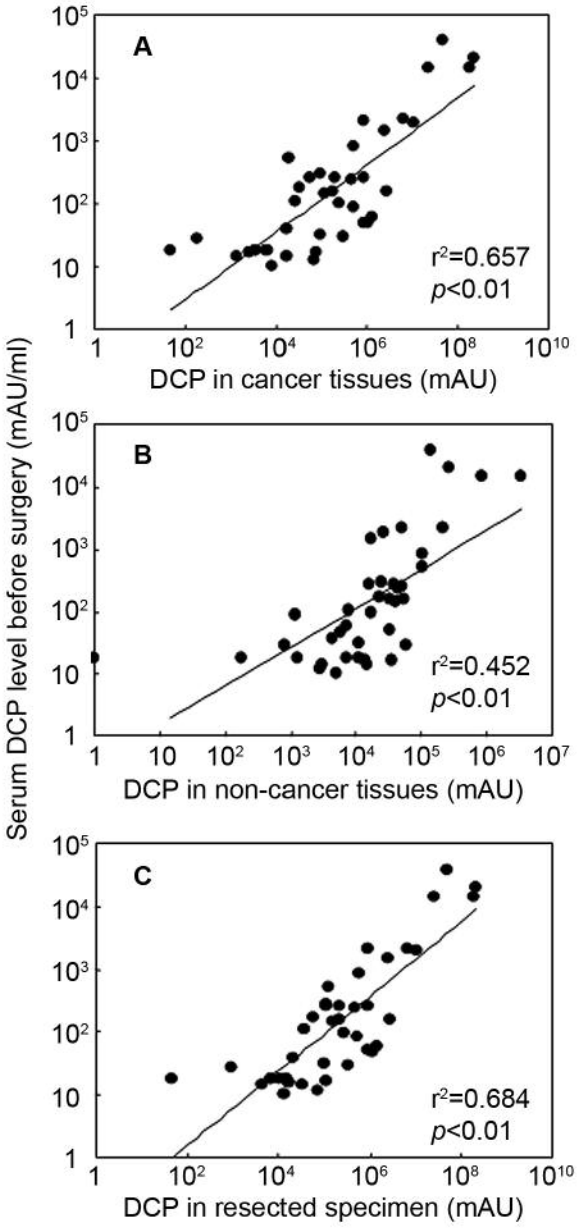

Figure 2. *Relationships between tissue DCP and serum DCP level. Correlations with serum DCP level were examined for (A) total DCP in cancer tissue, (B) that in non-cancer tissue, and (C) that in the whole specimen.*

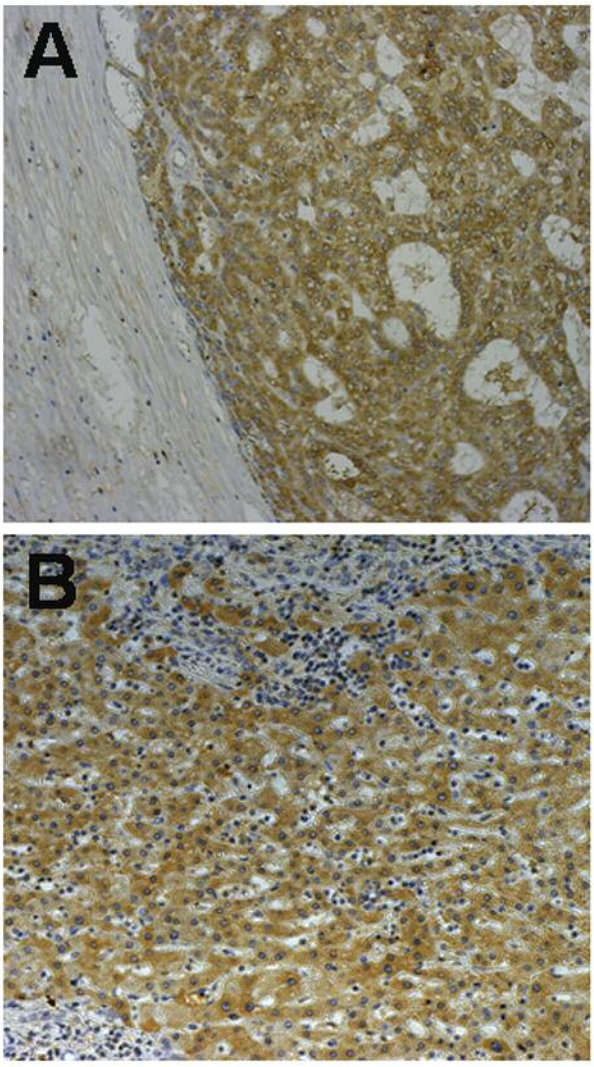

Figure 3. *Typical immunohistochemical staining for DCP in hepatocellular carcinoma (A) and the surrounding non-cancer liver tissue (B) (×200).*

most inferior part of the organ) was used to calculate the whole liver volume (LV) by the simple and accurate method of Heymsfield *et al.* (20) with minor modifications. The perimeter of the whole liver was outlined on each slice and the enclosed area was measured by a computer system. The LV was calculated through integrating values obtained by multiplying the liver area with the slide width. Similarly, the perimeter of the cancer tissue was also outlined and the cancer weight was estimated (21). The surgically resected liver specimen was weighed after removing the attached ligaments and gallbladder. The weight of the non-cancer part of the specimen was calculated by subtracting the weight of the tumor from that of the resected specimen. Similarly, the weight of the remaining liver after surgery was calculated by subtracting the weight of the resected specimen from that of the whole liver before surgery.

Measurement of tissue DCP. The tissue DCP level was measured in tissue extracts prepared from HCC and non-cancer liver tissue (about 0.05 g each) (17). Briefly, both cancer and non-cancer tissue samples were obtained from 3 randomly selected regions of the resected specimen. The tissues were then separately homogenized with 20 mM Tris-HCl, pH 8.0, containing 1% Triton® X-100 at 4˚C. After centrifugation at 105,000×g for 60 min at 4˚C, the supernatant was dialyzed against 20 mM Tris-HCl, pH 8.0. The DCP in the extracts was then quantified by ECLIA with an anti-DCP monoclonal antibody MU-3 (Eisai) according to the manufacturer's instructions. The DCP per unit weight of tissue was

determined as mAU/0.1 g tissue. The total tissue DCP was estimated from the values of mAU/0.1 g, determined by ECLIA, and the weights of the corresponding tissues were obtained by CT and by weighing of resected specimens as described above.

Immunohistochemical staining of tissue DCP. Immunohistochemistry was performed by the biotin-avidin peroxidase complex method. Sections were cut from archival formalin-fixed paraffin-embedded tissue blocks at a thickness of 4 μm they were then deparaffinized and dehydrated using a graded series of ethanol solutions. Endogenous peroxidase activity was halted by the administration of 3% hydrogen peroxide/methanol for 30 min. After a brief rinse in distilled water, the tissue sections were processed in 0.01 M citrate buffer, pH 6.0, in a glass container. The sections were then irradiated in a domestic microwave oven for 10 min at maximum power (15). After blocking with normal goat serum at room temperature for 30 min, the sections were incubated with MU-3 (1:900 dilution) for 60 min at room temperature. After washing with PBS, the sections were incubated with biotinylated anti-mouse IgG antibody and then developed using a commercial biotin-streptavidin-peroxidase complex kit (Histofine SAB-PO kit; Nichirei, Tokyo, Japan) followed by 3,3'-diaminobenzidine as the chromogen and hematoxylin as a counterstain. The evaluation of DCP staining was performed by observing 10 random fields (or all fields, if the sample comprised fewer than 10 fields) in the cancer and non-cancer tissue under microscopy (magnification, ×400). The tissues in which more than 15% of the cells were stained were considered to have high expression of DCP (15).

Statistical analysis. The difference in serum DCP levels before and after surgery, and that in tissue DCP between patient groups, divided according to immunological staining profiles, were evaluated using the Mann-Whitney *U*-test. Fisher's exact test was used to compare the categorical data. Spearman's rank correlation analysis was used to assess the correlation between serum DCP levels and tissue DCP. Statview 5.0J (Abacus Concepts, Berkeley, CA, USA) statistical software was used for data analyses. A *p*-value less than 0.05 was considered statistically significant.

Results

Relationship between serum DCP level and clinicopathological features. Twenty-six patients (65%) had elevated serum DCP levels (>40 mAU/ml). The median DCP levels decreased dramatically from before surgery to within a week after surgery (102 mAU/ml, range 10–38,600 mAU/ml *vs*. 23 mAU/ml, range 10–7,180 mAU/ml; $p<0.01$; Figure 1).

Clinicopathological parameters such as age, gender, liver cirrhosis, chronic hepatitis, growth type, capsule formation and capsule infiltration of the tumor were not significantly associated with serum DCP levels (data not shown). However, elevated serum DCP levels were significantly more frequent when the tumor diameter exceeded 2.5 cm (≤2.5 cm *vs*. >2.5 cm: 17.5% *vs*. 82.5%, $p=0.044$) and when portal invasion was present (absent *vs*. present: 37.5% *vs*. 62.5%, $p<0.01$) (data not shown).

Estimation of tissue DCP. The data are summarized in Table I. The DCP in both cancer and non-cancer tissue varied greatly among patients. The ratio of median DCP in 0.1 g cancer tissue to that in 0.1 g non-cancer tissue was 25.8:1, suggesting that DCP was expressed to a much greater degree in cancer tissue than in non-cancer tissue. However, the ratio of median total DCP in cancer tissue to that in non-cancer tissue was 7.3:1, reflecting the higher weight of non-cancer tissue. This suggests that a significant amount of DCP was expressed in the non-cancer liver tissue of the HCC patients.

Relationship between tissue DCP and serum DCP levels. As shown in Figure 2, the serum DCP level was found to be more strongly correlated with the DCP in the cancer tissue ($r^2=0.657$, $p<0.01$) and with that in the whole resected specimen ($r^2=0.684$, $p<0.01$) than with that in the non-cancer tissue ($r^2=0.452$, $p<0.01$). The highest correlation coefficient value was obtained for the whole resected specimen that included both cancer and non-cancer tissue (Figure 2C).

Detection of tissue DCP by immunohistochemistry. High expression of DCP (more than 15% of cells stained positively) (Figure 3) was noted in 70% (28/40) of the cancer tissues and 47.5% (19/40) of the non-cancer tissues (data not shown). According to the staining characteristics of the cancer (C) and non-cancer (N) tissue, the patients were divided into the following four groups: $C^{high}N^{high}$ (n=15), $C^{high}N^{low}$ (n=13), $C^{low}N^{high}$ (n=4), and $C^{low}N^{low}$ (n=8). Table II summarizes the total DCP in resected specimens of these four patient groups. The DCP level in the $C^{low}N^{low}$ group, in which immunohistochemical staining was low in both cancer and non-cancer tissue, was significantly lower than in the other three groups ($p<0.01$).

Discussion

Serum DCP has been validated as a useful tumor marker for HCC and has been applied clinically, particularly in Japan (22-27). In accordance with previous studies, the present study also revealed that serum DCP levels in HCC patients are related to clinicopathological parameters such as tumor size and portal vein invasion, supporting the role of serum DCP as a useful biomarker for HCC.

Previous studies have reported that high levels of abnormal prothrombin precursors were detected in HCC tissue (12) and that γ-glutamyl carboxylase activity and the vitamin K level were significantly decreased in cancer tissue compared to the surrounding normal tissue (12, 13, 28, 29). Therefore, it appears reasonable that DCP is synthesized by HCC cells and then released into the bloodstream and that a larger HCC will produce more DCP, leading to a higher serum level.

Since serum DCP levels drop quickly and significantly after tumor resection (Figure 1), it is reasonable to deduce that most of the DCP contributing to the elevated serum DCP level is derived from the specimen. As expected, in the present study, the total DCP in the cancer tissue was significantly correlated with the serum DCP levels (Figure 2A). However, in

agreement with other reports (13-15), significant amounts of DCP were also detected in the non-cancer tissue, which tends to account for a larger proportion of the liver, and these also showed a comparative correlation with serum DCP levels (Figure 2B). Patients with low immunohistochemical DCP staining in both cancer and non-cancer tissue had significantly less DCP in the whole specimen when compared to patients who exhibited high DCP expression in one or both of these tissue types (Table II). The highest correlation coefficient was found between the serum DCP level and quantity of DCP in the whole specimen. Although the amount of DCP produced in the tumor undoubtedly makes the greater contribution to the serum DCP level, the surrounding non-cancer tissue may play an important role as a source of serum DCP. The expression of DCP in non-cancer tissue, especially that surrounding the cancer tissue, may be somehow induced by neighboring cancer tissue. However, the present data for non-cancer tissue were obtained from surgically resected liver specimens rather than from the whole liver. Therefore, it was difficult to estimate the exact extent of non-cancer tissue producing DCP and was almost impossible to measure the level of DCP in normal liver tissue that was not removed during surgery. Further investigation will be necessary to determine the distribution of DCP-producing cells in non-cancer liver tissue.

In conclusion, the elevation of serum DCP levels in HCC patients is influenced by DCP production not only in cancer tissue, but also in the surrounding non-cancer liver tissue.

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