A novel des-γ-carboxy prothrombin in serum for the diagnosis of hepatocellular carcinoma.

A short running title: Novel DCP for diagnosis of HCC

Takahiro Tanaka,* Tatsuya Taniguchi,* Katsutaka Sannomiya,* Hidetaka Takenaka,* Tetsu Tomonari,* Koichi Okamoto,* Shinji Kitamura,* Toshiya Okahisa,* Katsuyoshi Tamaki,* Hiroaki Mikasa, † Sadao Suzuki‡ and Tetsuji Takayama*

* Department of Gastroenterology and Oncology, Institutes of Health Biosciences, University of Tokushima Graduate School, 3-18-15, Kuramoto-cho, Tokushima 770-8503, Japan.
†Medical Education Support Center, Faculty of Medicine, Tokushima University.
‡Department of Public Health, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan.

Corresponding author: Tetsuji Takayama, MD, PhD, Department of Gastroenterology and Oncology, Institute of Health Biosciences, University of Tokushima Graduate School, 3-18-15, Kuramoto-cho, Tokushima 770-8503, Japan.
E-mail; takayama@clin.med.tokushima-u.ac.jp
TEL; +81-88-633-7122, FAX; +81-88-633-9235

number of table, 1; number of figures, 5.

Abstract

Background and Aim: We meassured serum des-γ-carboxy prothrombin (DCP) levels using a newly developed electrochemiluminescence immunoassay (ECLIA, NX-DCP) and investigated the utility of NX-DCP and DCP/NX-DCP ratio for the diagnosis of hepatocellular carcinoma (HCC). We also elucidated antigenic differences in DCP between HCC and non-HCC patients.

Methods: The subjects included 170 patients with HCC, 61 with benign liver disease, 12 with obstructive jaundice, and 10 warfarin users. NX-DCP was quantitated by sandwich ECLIA employing novel anti-DCP monoclonal antibodies, P11 and P16. Conventional DCP was quantitated by standard ECLIA. DCP extracted from serum by affinity-chromatography was analyzed by western blotting.

Results: Conventional serum DCP levels were high in patients with HCC and obstructive jaundice and in warfarin users, consistent with previous reports. Serum NX-DCP levels were high only in warfarin users and obstructive jaundice patients (vitamin K-defficient patients), but not in HCC patients. The DCP/NX-DCP ratio was significantly higher in the HCC group than in the benign liver disease, obstructive jaundice, and warfarin groups ($P<0.001$). Receiver-operating characteristic analysis showed significant superiority of the DCP/NX-DCP ratio over conventional DCP as a marker for HCC diagnosis ($P<0.05$). Western blot analysis showed that P11 and P16 reacted strongly with DCP from a warfarin user and an obstructive jaundice patient, but very faintly with DCP from an HCC patient. Immunohistochemistry on HCC samples and autopsied normal liver tissues from warfarin users showed similar results.

Conclusions: The DCP/NX-DCP ratio is very useful for diagnosing HCC. DCP in HCC patients is distinct from that in vitamin K-deficient patients.

Keywords: des-γ-carboxyl prothrombin, hepatocellular carcinoma, warfarin, obstructive jaundice.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common tumor type and the third most common cause of cancer-related death worldwide.[1] It is well recognized that serological markers including α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP) are very important for the diagnosis of HCC, as are imaging modalities such as computed tomography (CT) and ultrasound.

DCP is a well-known tumor marker for diagnosis, screening, and therapeutic monitoring of HCC.[2-6] It has been reported that DCP has a sensitivity of 39% to 81% and specificity of 68% to 97% for the detection of HCC.[2,5-9] In comparison, DCP has sensitiity similar to that of AFP but is more specific. Moreover, tumor recurrence and metastasis are more frequent in HCC patients who are DCP positive than in HCC patients who are DCP negative, indicating that DCP is a prognostic factor for HCC.[4,10,11] It has also been reported that DCP is an autologous growth factor for HCC, binding directly to the hepatocyte growth factor receptor (c-MET), and that DCP promotes vascular endothelial cell proliferation and migration.[12,13] In this context, DCP is a very useful marker for prognostic prediction as well as for diagnosis and surveillance of HCC.

DCP is an aberrant prothrombin that lacks the ability to interact with other coagulation factors. The prothrombin molecule has 10 γ-carboxylated glutamic acid (Gla) residues in the Gla domain of the N-terminal region. All of the 10 glutamic acid (Glu) residues in prothrombin precursor need to be γ-carboxylated by the vitamin K-dependent enzymatic reaction of γ-glutamylcarboxylase through post-translational modification.[14] DCP is an incomplete prothrombin in which some Glu residues do not undergo γ-carboxylation.[15] Therefore, in patients receiving warfarin, a vitamin K antagonist, serum DCP levels are abnormally high.[16,17] Similarly, in patients with obstructive jaundice or jaundice due to liver failure, the serum DCP often shows abnormally high levels due to a lack of vitamin K even in the absence of HCC.[18] Thus, the major drawback of DCP as a tumor marker is that it cannot be used to determine the diagnosis and prognosis of HCC in these patients. Likewise, serum DCP levels do not reflect the tumor size in patients with HCC and concomitant jaundice.

Currently, the most commonly used kit for measuring serum DCP concentrations is the picolumi PIVKA-II (EIDIA Co., Ltd, Tokyo, Japan), in which anti-DCP monoclonal antibody MU-3 recognizing acarboxy 19,20-Glu DCP and rabbit anti-human prothrombin polyclonal antibody are used.[11,19,20] Studies on DCP heterogeneity in HCC patients suggest that the number of Gla residues in DCP can vary.[16,21] However, to our knowledge, differences in DCP between HCC patients and vitamin K-deficient patients have been analyzed in only one study, by Uehara and colleagues.[16] In that study, an analysis of DCP carboxylation status in a single warfarin user, none of the Glu residues were carboxylated. Recently, Toyoda and associates measured a novel DCP (NX-DCP) in serum using a newly developed sandwich ECLIA with new anti-DCP monoclonal antibodies p11 and p16 and reported preliminary data from only 20 HCC patients.[22] They showed that the DCP/NX-DCP ratio may be useful for the diagnosis of HCC among warfarin users. However, no studies to date have assessed NX-DCP in a large number of HCC patients, nor has NX-DCP been compared among HCC, liver cirrhosis (LC)/chronic hepatitis (CH), obstructive jaundice, and warfarin-treated patients. Therefore, in this study, we prospectively evaluated the serum NX-DCP level and DCP/NX-DCP ratio using a novel ECLIA method in patients with HCC, LC/CH, or obstructive jaundice, and in warfarin users for the diagnosis of HCC. We also analyzed the reactivity of DCP to P11, P16, and MU-3 to identify structural differences in DCP from HCC patients and patients with vitamin K deficiency due to obstructive jaundice and warfarin use.

Methods

Patients. This study was approved by the institutional review board of Tokushima University Hospital, Tokushima, Japan. During the period from April 2010 to August 2012, a total of 176 patients with HCC, 13 patients with LC, 48 patients with CH, 12 patients with obstructive jaundice (total bilirubin ≥ 5.0 mg/dl) due to advanced gastrointestinal cancer (ie, gastric cancer, pancreatic cancer), and 10 patients receiving warfarin for cardiac diseases were prospectively enrolled after obtaining written informed consent. HCC was diagnosed based on the following

classic imaging manifestations: (i) early enhancement at the arterial phase and hypoattenuation at the portal venous phase or at the equilibrium phase on contrast-enhanced dynamic CT; and (ii) hyperattenuation on CT during hepatic arteriography and hypoattenuation on CT during arterialportography.[23,24] The WHO clasification of HCC was used for HCC staging. A 2-mL blood sample was drawn from each patient for validation of the new ECLIA method for measuring serum DCP concentrations. Furthermore, additional 10-mL blood samples were drawn from 3 of the patients (a patient with HCC, a warfarin user, and an obstructive jaundice patient) for western blot analysis of DCP. All the blood samples were centrifuged to obtain serum. Lastly, 3 HCC tissue samples were obtained surgically from patients with HCC and 2 normal liver tissue samples were obtained at autopsy from 2 patients who had received warfarin. These tissues were used for immunohistochemical analysis of DCP.

NX-DCP and DCP quantitation by sandwich ECLIA. The serum NX-DCP concentration was measured using a new sandwich ECLIA method (kindly provided by EIDIA Co, Ltd., Tokyo, Japan). The method employs the novel mouse anti-human DCP monoclonal antibodies p11 and p16 which were generated by immunization with DCP from a warfarin user (Sekisui Medical Co., Ltd. Tokyo, Japan). The conventional serum DCP concentration was measured using the picolumi PIVKA-II (cutoff value 40 mAU/ml) method.[19] The method employed mouse anti-human DCP antibody MU-3 and rabbit anti-human prothrombin polyclonal antibody.[20]

Affinity chromatography of DCP. All steps were performed at 4°C. DCP and prothrombin were separated on a Sepharose 4B column coupled with mouse anti-human prothrombin F2 monoclonal antibody, and the column was then washed with Tris-buffered saline (TBS, pH=7.8). Bound protein was eluted with 2 M guanidine-HCl then immediately pooled and dialyzed in TBS.

Western blot analysis. The purified protein and prothrombin (Abcam plc, Cambridge, UK) as a control were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane.

The membrane was then blocked with 5% bovine serum albumin in TBS with 0.1% Tween 20 (TBS-T) and incubated with MU-3, P11, P16, or rabbit anti-human prothrombin antibody (Abcam plc) overnight at 4°C. The membrane was incubated with HRP-conjugated secondary antibody and developed using an ECL Prime western blotting detection system (GE Healthcare UK Ltd., Buckinghamshire, UK).

Immunohistochemistry. Immunohistochemical staining was performed using the streptavidin-biotin-peroxidase method with labeled streptavidin-biotin (CSA-II, Dako, Kyoto, Japan), according to the manufacturer's instructions. Briefly, 3-μm thick sections were cut from formalin-fixed paraffin-embedded tissues, deparaffinized in xylene and hydrated in phosphate-buffered saline (PBS). The endogenous peroxidase was inactivated by incubation with 0.3% H_2O_2-MeOH. They were blocked with Protein Block (Dako) and incubated with MU-3, P11, or P16, respectively, as the primary antibody. After washing with PBS, the slides were incubated with biotinylated secondary antibody, followed by incubation with HR-streptavidin and visualization with DAB chromogen (3', 3-diaminobenzidine, Dako). Finally, the sections were counterstained with Mayer's hematoxylin.

Statistical analysis. All data were analyzed using STATA version 8 software (Stata Corp., Texas). Scheffe's test was used for comparisons of age, serum alanine aminotransferase (ALT) level, and prothrombin time (%) among groups. Mantel-Haenzel test for linear association was used to assess differences in positivity rates for each serum marker in stage I - IV. Chi-square test and Fisher's exact test were used to compare positivity rates among each group. Bonferroni correction was performed for multiple comparisons. Survival curves were compared by log-rank test. Receiver operating characteristic (ROC) curves were compared using the DeLong mathematical model.[25]

Results

Patient characteristics. Baseline characteristics of the 259 patients (HCC, LC, CH, and obstructive jaundice groups and warfarin users) are listed in Table 1. There were 29 stage I, 47 stage II, 67 stage III, and 33 stage IV HCC patients. Patients with LC included 8 Child-Pugh A, 3 Child-Pugh B, and 2 Child-Pugh C cases. Of the CH patients, 15 had hepatitis C infection, 8 had hepatitis B infection, 9 had alcoholic hepatitis, 13 had autoimmune hepatitis, 1 had drug-induced hepatitis, and 2 had nonalcoholic steatohepatitis. The obstructive jaundice group consisted of 6 patients with pancreatic cancer, 5 with cholangiocellular carcinoma, and 1 with gastric cancer. There were 10 warfarin users who did not have any liver dysfunction. There were no statistically significant differences in the sex ratios, ages and ALT levels among the 5 patient groups. The percent prothrombin time (PT%) was significantly lower in warfarin users than in HCC, LC and CH groups. It was also significantly lower in obstructive jaundice group than in HCC and CH groups.

Serum DCP and NX-DCP levels in patients with liver diseases and in warfarin users. Conventional serum DCP levels were measured by DCP ECLIA with MU-3 in HCC, LC/CH, and obstructive jaundice patients, and in warfarin users (Fig.1A). The serum DCP levels were positive in 71.0% (125/176) of HCC patients, 29.5% (18/61) of LC/CH patients, 58.0% (7/12) of obstructive jaundice patients, and 100% (10/10) of warfarin users. Although the DCP-positive rate in the HCC group was significantly higher than that of the LC/CH group as well as in comparison with normal subjects ($P<0.02$, chi-square test), there were no significant differences between HCC and obstructive jaundice patients or between HCC patients and warfarin users (chi-square test and Fisher's exact test), which is consistent with previous reports,[17,18] indicating that the serum DCP level is a useful marker for the diagnosis of HCC in patients with LC/CH, but cannot be used for patients with jaundice or warfarin users.

We next measured serum NX-DCP levels using the new sandwich ECLIA with the novel DCP monoclonal antibodies p11 and p16. Based on data for healthy volunteers, the cut off value was set at 90 mAU/ml (mean + 2 SD). The serum NX-DCP levels were positive in 66.7% (8/12)

of patients with obstructive jaundice and in 100% (10/10) of warfarin users, but in only 36.4% (64/176) of patients with HCC, suggesting that NX-DCP was a dominant form in patients with vitamin K-deficiency, such as those with jaundice and warfarin users (Fig. 1B). In this context, we calculated the DCP/NX-DCP ratio for each disease group in order to investigate the value of this marker for differentiating HCC patients from warfarin users and obstructive jaundice patients (Fig. 1C). When we set a cutoff value of 1.4 based on ROC analysis, the DCP/NX-DCP ratio was positive in 59.7% (105/176) of HCC patients, 6.6% (4/61) of CH/LC patients, 8.3% (1/12) of obstructive jaundice patients, and 0% (0/10) of warfarin users ($P<0.01$). The positivity rate of HCC patients was significantly higher than that of CH/LC patients, obstructive jaundice patients, and warfarin users ($P<0.001$, chi-square test or Fisher's exact test). The sensitivity and specificity of the DCP/NX-DCP ratio were 59.7% (105/176) and 91.7% (88/96), respectively. In the ROC analysis, the AUC for the DCP/NX-DCP ratio was significantly higher than that for conventional DCP (Fig. 1D). Thus, the DCP/NX-DCP ratio was a superior diagnostic marker for HCC and was able to exclude non-HCC cases with high serum DCP levels due to vitamin K deficiency.

Serum DCP and NX-DCP levels and the DCP/NX-DCP ratio at each stage of HCC. The serum DCP and NX-DCP levels were evaluated for each stage of HCC. The serum DCP levels were positive in 48.3% (14/29) of stage I, 57.4% (27/47) of stage II, 79.1% (53/67) of stage III, and 93.9% (31/33) of stage IV patients, showing a significant stepwise increment of the positivity rate from stage I to IV by Mantel-Haenzel test ($P<0.05$) (Fig. 2A). There was no significant difference in the positivity rate among stages. The rate of NX-DCP positivity was very low (17.2% - 32.4%) among patients with stage I to III disease, as expected, but surprisnlgy, the rate was very high (81.8%) in patients with stage IV disease. The positivity rate in stage IV was significantly higher than that in stage III by chi-square test ($P<0.001$) (Fig. 2B). In the analysis of the DCP/NX-DCP ratios, a ratio equal to or higher than 1.4 was found in 41.4% (12/29) of stage I, 57.4% (27/47) of stage II, 55.2% (37/67) of stage III, and 87.9% (29/33) of stage IV patients,

showing a significant stepwise increment of positivity rate from stage I to IV (P<0.05) (Fig. 2C). There was no significant difference in the ratio among the groups.

Since the positivity of NX-DCP was very high in stage IV HCC (27/33, 81.8%), we next investigated clinical parameters in these patients and compared them between NX-DCP (+) and NX-DCP (-) groups in stage IV. There was no significant difference in the background liver disease, liver functional reserve (Child-Pugh score), prevalence of ascites, serum ALT level, serum total bilirubin level and prothrombin time (%) between the two groups (Fig.3A). However, patients with vascular invasion showed a significantly higher rate of NX-DCP postivity. (P<0.05) (Fig.3B). Moreover, the overall survival time of the NX-DCP (+) group was significantly lower than that of the NX-DCP (-) group (p<0.05) (Fig.3C). These results suggested that NX-DCP may serve as a biomarker for poor prognosis in association with vascular invasion although the number of stage IV patients was not yet many enough.

Western blot analysis of DCP from patients with HCC and warfarin users using MU-3, p16, or P11. To study the antigenicity of DCP from HCC and obstructive jaundice patients and warfarin users to various antibodies, DCP was extracted from sera by prothrombin (II portion)-affinity chromatographic methods, and western blot analysis was performed using the anti-DCP monoclonal antibodies MU-3, P16 or P11 (Fig.4). As expected, MU-3 strongly reacted with DCP from a warfarin user, an HCC patient, and an obstructive jaundice patient (Fig. 4A), but not with prothrombin. In contrast, P11 strongly reacted with DCP from a warfarin user and an obstructive patient, but very faintly with DCP from a patient with HCC (Fig.4B). Similarly, P16 reacted with DCP from a warfarin user and an obstructive jaundice patient, but very faintly with DCP from an HCC patient (Fig.4C). P11 and P16 did not react with prothrombin. These results clearly indicate that the antigenicity of DCP in HCC patients is distinct from that in vitamin K-deficient patients such those who are warfarin users or who have obstructive jaundice.

Immunohistochemistry for DCP. In order to investigate the expression of DCP in HCC tissues

and autopsied normal liver tissues in warfarin users, immunohistochemical staining was performed using MU-3, P11, or P16 in 3 surgically-obtained HCC tissue samples and 2 normal liver tissue samples from warfarin users. Representative staining patterns are shown in Figure 5. The cancer cells in the HCC tissue from an HCC patient were strongly stained by MU-3 although non-cancerous lesions were not. A pre-incubation test using excess amount of DCP protein showed no apparent staning signal. In contrast, the cancer cells were stained very faintly by P11 and P16 (Fig. 5A). The serum DCP and NX-DCP levels in this patient were 4981 mAU/ml and 581 mAU/ml, respectively. On the other hand, normal liver cells in the liver tissue from an autopsied warfarin user were intensely stained by P11 and P16 in addition to MU-3 (Fig. 5B). Pre-incubation testing showed no aparent staining signal. The serum DCP and NX-DCP levels in this patient were 20597 mAU/ml and 17823 mAU/ml, respectively. Essentially, similar results were obtained in the remaining 2 HCC samples and 1 normal liver sample from warfarin user. The serum DCP and NX-DCP levels in one of these HCC patients was 3153 and 354 mAU/ml, respectively, and in the other, 4591 and 646 mAU/ml respectively, whereas the levels were 18696 and 17085 mAU/ml respectively, in the warfarin user. These results of immunohistochemistry were consistent with the data for serum DCP and NX-DCP concentrations in HCC patients and warfarin users.

Discussion

The present study demonstrated that NX-DCP was predomoinantly increased in the serum of patients with vitamin K-deficiency such as warfarin users and obstructive jaundice patients, but essentially not in HCC patients. When the DCP/NX-DCP ratio was evaluated (cutoff 1.4), the positivity rate in HCC patients (59.7%) was significantly higher than in LC/CH patients (6.6%), obstructive jaundice patients (8.3%), and warfarin users (0%). Moreover, the ROC analysis revealed that the AUC of the DCP/NX-DCP ratio was significantly higher than that of conventional DCP, suggesting superiority of the former for the diagnosis of HCC. Thus, it was evident that the DCP/NX-DCP ratio is very useful for the diagnosis of HCC. In the ROC analysis

of DCP/NX-DCP ratio, the sensitivity for the diagnosis of HCC was slightly lower than that of conventional DCP at the cuttoff level of 1.4; however, the specificity (91.7%) was much higher than that of conventional DCP (61.5%). The advantage of using the DCP/NX-DCP ratio is that it makes it possible to make differentially diagnose HCC from vitamin K-deficiency with very high specificity. While, a potential disadvantage may be slightly lower sensitivity compared with that of conventional DCP. Therefore, for the screening of HCC in patients at high risk for HCC, such as those with LC/CH, it would be useful to evalutate both the conventional DCP and the DCP/NX-DCP ratio as markers; i.e., to pick up HCC-suspected patients by using the DCP value, and to exclude non-HCC through use of the DCP/NX-DCP ratio. However, it would be better to assess the DCP/NX-DCP ratio in a greater number of patients in future.

Since the DCP/NX-DCP ratio gradually increased as the stage of HCC advanced, and was highly positive (87.9%) in stage IV patients, the ratio would also be useful for the diagnosis of advanced HCC, particularly in stage IV patients. Serum NX-DCP was negative in most HCC patients at stage I to III, but highly positive in stage IV patients, and in particular, those with far advanced HCC with vascular (portal vein) invasion. These results demonstrate that NX-DCP, which appeared in patients with vitamin K-deficiency, was also detected in far advanced HCC patients with vascular invasion, and was associated with a poor prognosis. Recently it was reported that sorafenib induces marked elevation of the serum DCP level in patients with HCC. It was surmised that sorafenib caused vascular regression and subsequently induced DCP production through hypoxia in the HCC tissue.[26,27] We also found that sorafenib produced an apparent elevation in serum DCP levels in patients with HCC, and that the elevated DCP in these patients predominantly consisted of NX-DCP (data not shown). In this context, in the far advanced patients with stage IV HCC, the vascular invasion induced ischemia in the HCC tissue and promoted NX-DCP production, which was closely associated with a poor prognosis. Thus, it was indicated that NX-DCP is a good marker for a poor prognosis in HCC patients.

We evaluated not only the DCP/NX-DCP ratio but also the difference between DCP and NX-DCP (DCP – NX-DCP) for the diagnosis of HCC (data not shown). The ROC analysis revealed

that the AUC of the difference was significantly higher than that for conventional DCP, but was smaller than that for the DCP-NX/DCP ratio. Therefore, we performed subsequent analyses using the DCP/NX-DCP ratio.

Western blot analysis and immunohistochemistry revealed that the antigenicity of DCP in warfarin users differed from that in HCC patients. Since MU-3 recognizes acarboxy 19, 20-Glu residues of the DCP molecule, our results indicate that DCPs from HCC and warfarin users had in common an acarboxy 19, 20-Glu residues. On the other hand, the peptide epitope analysis revealed that P11 recognizes amino acid residues 1 – 5 of the Gla domain (unpublished data). Accordingly, it is surmised that the different reactivity of DCPs between HCC patients and vitamin K-deficient patients is ascribed to the degree of carboxylation of DCP.

In conclusion, the DCP/NX-DCP ratio is very useful for the diagnosis of HCC. In particular, it was useful for discriminating false positive cases, such as obstructive jaundice patients and warfarin users, from HCC patients. Serum NX-DCP levels frequently increased in stage IV HCC patients, particularly those with vascular invasion, and it served as a reliable biomarker for a poor prognosis. DCP from HCC patients was distinct from DCP in vitamin K-deficient patients in terms of antigenicity.

Acknowlegement

We thank Drs Keisuke Watanabe and Tomohide Asai (EIDIA, Co., Ltd.) for their technical assistance. This study was partly supported by the cooperative research program of University of Tokushima graduate school and EIDIA Co, Ltd (Tokyo, Japan).

References

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics 2010. *CA Cancer J Clin* 2010; **60**: 43-66.
2. Liebman HA, Furie BC, Tong MJ, et al. Des-gamma-carboxy (abnormal) prothrombinas a serum marker of primary hepatocellular carcinoma. *N Engl J Med* 1984; **310**: 1427-31.
3. Marrero JA, Su GL, Wei W, et al. Des-gamma carboxyprothrombin can differentiate hepatocellular carcinoma from nonmalignant chronic liver disease in american patients. *Hepatology* 2003; **37**: 1114-21.
4. Yamamoto K, Imamura H, Matsuyama Y, et al. AFP, AFP-L3, DCP, and GP73 as markers for monitoring treatment response and recurrence and as surrogate markers of clinicopathological variables of HCC. *J Gastroenterol* 2010; **45**: 1272-82.
5. Lok AS, Sterling RK, Everhart JE, et al. Des-gamma-carboxyprothrombin and alpha-fetoprotein as biomarkers for the early detection of hepatocellular carcinoma. *Gastroenterology* 2010; **138**: 493-502.
6. Soulier JP, Gozin D, Lefrere JJ. A new method to assay des-gamma-carboxyprothrombin. Results obtained in 75 cases of hepatocellular carcinoma. *Gastroenterology* 1986; **91**: 1258-62.
7. Nakamura S, Nouso K, Sakaguchi K, et al. Sensitivity and specificity of des-gamma-carboxyprothrombin for diagnosis of patients with hepatocellular carcinomas varies according to tumor size. *Am J Gastroenterol* 2006; **101**: 2038-43.
8. Sterling RK, Jeffers L, Gordon F, et al. Utility of Lens culinaris agglutinin-reactive fraction of alpha-fetoprotein and des-gamma-carboxyprothrombin, alone or in combination, as biomarkers for hepatocellular carcinoma. *ClinGastroenterolHepatol* 2009; **7**: 104-13.
9. Baek YH, Lee JH, Jang JS, et al. Diagnostic role and correlation with staging systems of PIVKA-II compared with AFP. *Hepatogastroenterology* 2009; **56**: 763-7.
10. Imamura H, Matsuyama Y, Miyagawa Y, et al. Prognostic significance of anatomical resection and des-gamma-carboxyprothrombin in patients with hepatocellular carcinoma. *Br J Surg* 1999; **86**: 1032-8.
11. Kobayashi M, Ikeda K, Kawamura Y, et al. High serum des-gamma-carboxyprothrombin level predicts poor prognosis after radiofrequency ablation of hepatocellular carcinoma. *Cancer* 2009; **115**: 571-80.
12. Suzuki M, Shiraha H, Fujikawa T, et al. Des-gamma-carboxyprothrombin is a potential autologous growth factor for hepatocellular carcinoma. *J BiolChem* 2005; **280**: 6409-15.
13. Fujikawa T, Shiraha H, Ueda N, et al. Des-gamma-carboxyl prothrombin-promoted vascular endothelial cell proliferation and migration. *J Biol Chem* 2007; **282**: 8741-8.

14. Furie B, Furie BC. Molecular basis of vitamin K-dependent gamma-carboxylation. *Blood* 1990; **75**: 1753-62.
15. Suttie JW. Vitamin K-dependent carboxylase. *Annu Rev Biochem* 1985; **54**: 459-77.
16. Uehara S, Gotoh K, Handa H, Honjo K, Hirayama A. Process of carboxylation of glutamic acid residues in the gla domain of human des-gamma-carboxyprothrombin. *ClinChimActa* 1999; **289**: 33-44.
17. Lee W, Chung HJ, Kim S, Jang S, et al. PIVKA-II is a candidate marker for monitoring the effects of the oral anticoagulant warfarin. *ClinBiochem* 2010; **43**: 1177-9.
18. Nakao A, Suzuki Y, Isshiki K, et al. Clinical evaluation of plasma abnormal prothrombin (des-gamma-carboxyprothrombin) in hepatobiliary malignancies and other diseases. *Am J Gastroenterol* 1991; **86**: 62-6.
19. Okuda H, Nakanishi T, Takatsu K, et al. Measurement of serum levels of des-gamma-carboxyprothrombin in patients with hepatocellular carcinoma by a revised enzyme immunoassay kit with increased sensitivity. *Cancer* 1999; **85**: 812-8.
20. Naraki T, Kohno N, Saito H, et al. γ-Carboxyglutamic acid content of hepatocellular carcinoma-associated des-gamma-carboxy prothrombin. *Biochim Biophys Acta* 2002; **1586**: 287-98.
21. Uehara S, Gotoh K, Handa H, Tomita H, Senshuu M. Distribution of the heterogeneity of des-gamma-carboxyprothrombin in patients with hepatocellular carcinoma. *J Gastroenterol Hepatol* 2005; **20**: 1545-52.
22. Toyoda H, Kumada T, Osaki Y, et al. Novel method to measure serum levels of des-gamma-carboxyprothrombin for hepatocellular carcinoma in patients taking warfarin: A preliminary report. *Cancer Sci* 2012; **103**: 921-5.
23. Matsui O, Ueda K, Kobayashi S, et al. Intra- and perinodular hemodynamics of hepatocellular carcinoma: CT observation during intra-arterial contrast injection. *Abdom Imaging* 2002; **27**: 147-56.
24. Araki T, Itai Y, Furui S, Tasaka A. Dynamic CT densitometry of hepatic tumors. *AJR Am J Roentgenol* 1980; **135**: 1037-43.
25. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics* 1988; **44**: 837–45.
26. Ueshima K, Kudo M, Takita M, et al. Des-γ-carboxyprothrombin may be a promising biomarker to determine the therapeutic efficacy of sorafenib for hepatocellular carcinoma. *Dig Dis* 2011; **29**: 321-5.
27. Nakazawa T, Hidaka H, Shibuya A, Koizumi W. Rapid regression of advanced hepatocellular carcinoma associated with elevation of des-gamma-carboxyprothrombin

after short-term treatment with sorafenib - a report of two cases. *Case Rep Oncol* 2010; **3**: 298-303.

Figure legends

Figure 1. Serum DCP and NX-DCP levels and DCP/NX-DCP ratio in patients with hepatocellular cancer (HCC) and non-HCC, and the ROC curve for DCP and the DCP/NX-DCP ratio. (A) The positivity rates for conventional serum DCP were high in HCC and obstructive jaundice patients, and in warfarin users. (B) The positivity rates for serum NX-DCP were high in obstructive jaundice patients and warfarin users, but relatively low in HCC patients. (C) The incidence of a DCP/NX-DCP ratio ≥1.4 in HCC patients was significantly higher than the incidence in any of the other groups (P<0.001, chi-square test or Fisher's exact test). (D) ROC analysis showed that the AUC of the DCP/NX-DCP ratio was significantly higher than the AUC for DCP, indicating superiority of the former as a diagnostic marker. * P<0.001, ** P≤0.003, NS denotes not statistically significant.

Figure 2. Serum DCP and NX-DCP levels, the DCP/NX-DCP ratio in each stage of hepatocellular cancer. (A) The positivity rates for serum DCP showed a significant stepwise increment from stage I to IV (Mantel-Haenzel test, P<0.05). However, there was no significant difference among stages. (B) The positivity rates for serum NX-DCP were very low in stage I to III, but very high in stage IV. There were significant differences between stage IV and the other stages (chi-square test, P<0.001). (C) The rate of patients with a DCP/NX-DCP ratio ≥1.4 showed a significant stepwise increment from stage I to IV (P<0.05). However, there was no sigficant difference among stages.

Figure 3. NX-DCP positivity, clinical parameters, and overall survival of HCC stage IV patients. (A) There was a significant correlation between NX-DCP positivity and vascular invasion in stage IV patients with HCC. (B) The serum NX-DCP levels in patients with vascular invasion were significantly higher than those without vascular invasion. (C) The overall survival time of the NX-DCP (+) group was significantly lower than that of the NX-DCP (-) group in stage IV (log-rank test, P=0.035). * P<0.001. NS denotes not statistically significant.

Figure 4. Western blot analysis of DCP from a hepatocellular cancer patient, a warfarin user, and an obstructive jaundice patient. Western blot analysis was performed as described in Materials and Methods. (A) The anti-DCP monoclonal antibody MU-3 detected strong bands at 60 kDa, corresponding to DCP protein, but no band for prothrombin protein. (B) P11 detected a strong band at 60 kDa in the warfarin user and obstructive jaundice patient, but a very faint band for DCP protein in the HCC case, and no band for prothrombin protein. (C) Similarly, P16 detected strong bands at 60 kDa in the warfarin user and obstructive jaundice patient, but a very faint band from the HCC patient, and no band for prothrombin protein. The bands at the bottom of each figure were detected by rabbit anti-human prothrombin antibody.

Figure 5. Immunohistochemistry for DCP in HCC tissue and autopsied normal liver tissue from warfarin user using the anti-DCP monoclonal antibody MU-3, P11, or P16. (A) The cancer cells in the HCC tissue were strongly stained by MU-3 but very faintly by P11 and P16. The pre-incubation test using excess amount of DCP protein showed no apparent staining signal. (B) The normal liver cells in the warfarin user were intensely stained by P11 and P16 as well as MU-3. The pre-incubation test showed no apparent staining signal.

Table 1. Characteristics of Subjetcts

	No. of Subjects	Sex (M/F)	Age (Mean ± SD)	ALT (IU/L) (Mean ± SD)	PT(%) (Mean ± SD)
HCC	**176**	**122/54**	**71.2 ± 9.0**	**44.9 ± 37.1**	**96.6 ± 14.4**
Stage I	29	20/9	72.2 ± 7.6	46.5 ± 34.2	90.2 ± 22.6
Stage II	47	28/19	70.6 ± 9.8	42.3 ± 32.4	98.0 ± 14.9
Stage III	67	51/16	71.9 ± 8.9	37.7 ± 20.3	95.4 ± 12.8
Stage IV	33	23/10	69.6 ± 9.1	56.2 ± 55.7	84.9 ± 24.6
Liver cirrhosis	**13**	**7/6**	**64.9 ± 7.6**	**46.1 ± 29.0**	**87.2 ± 16.0**
Child-Pugh A	8	4/4	64.9 ± 8.4	43.8 ± 18.3	96.2 ± 8.3
Child-Pugh B	3	2/1	63.7 ± 3.2	26.7 ± 3.21	82.6 ± 0.57
Child-Pugh C	2	1/1	67.0 ± 12.7	35.5 ± 9.19	59.6 ± 2.83
Chronic hepatitis	**48**	**23/25**	**60.1 ± 13.0**	**38.5 ± 16.1**	**100.8 ± 22.6**
HCV	15	7/8	66.6 ± 7.4	33.4 ± 21.4	102.9 ± 13.8
HBV	8	4/4	51.5 ± 15.8	25.1 ± 92.1	106.0 ± 12.8
Alcoholic	9	7/2	54.4 ± 15.7	39.3 ± 32.5	85.0 ± 38.2
Autoimmune	13	3/10	61.4 ± 10.6	39.0 ± 41.2	100.5 ± 21.6
Drug induced	1	1/0	62.2 ± 7.2	137.0	128.5
NASH	2	1/1	61.0 ± 28.3	24.0 ± 5.66	117.9 ± 15.1
Obstructive jaundice	**12**	**9/3**	**61.4 ± 16.1**	**51.7 ± 47.3**	**72.3 ± 26.1†**
Pancreatic cancer	6	3/3	73.8 ± 4.3	58.8 ± 66.1	65.8 ± 13.1
CCC	5	5/0	71.0 ± 10.9	35.0 ± 25.1	66.1 ± 22.7
Gastric cancer	1	0/1	84.0	77.0	136
Patients receiving warfarin	**10**	**3/7**	**68.3 ± 10.8**	**23.0 ± 12.0**	**47.7 ± 23.7‡**
Healthy volumteers	**30**	**19/11**	**66.6 ± 11.2**	**21.1 ± 11.2**	**116.0 ± 13.8**

†Significantly lower in obstructive jaundice group than in HCC and chronic hepatitis groups ($P<0.05$).
‡Significantly lower in warfarin users than in HCC, liver cirrhosis and chronic hepatitis groups ($P<0.05$).
NASH, nonalcoholic steatohepatitis; CCC, cholangiocellular carcinoma; ALT, alanine aminotransferase; PT, prothrombin time.

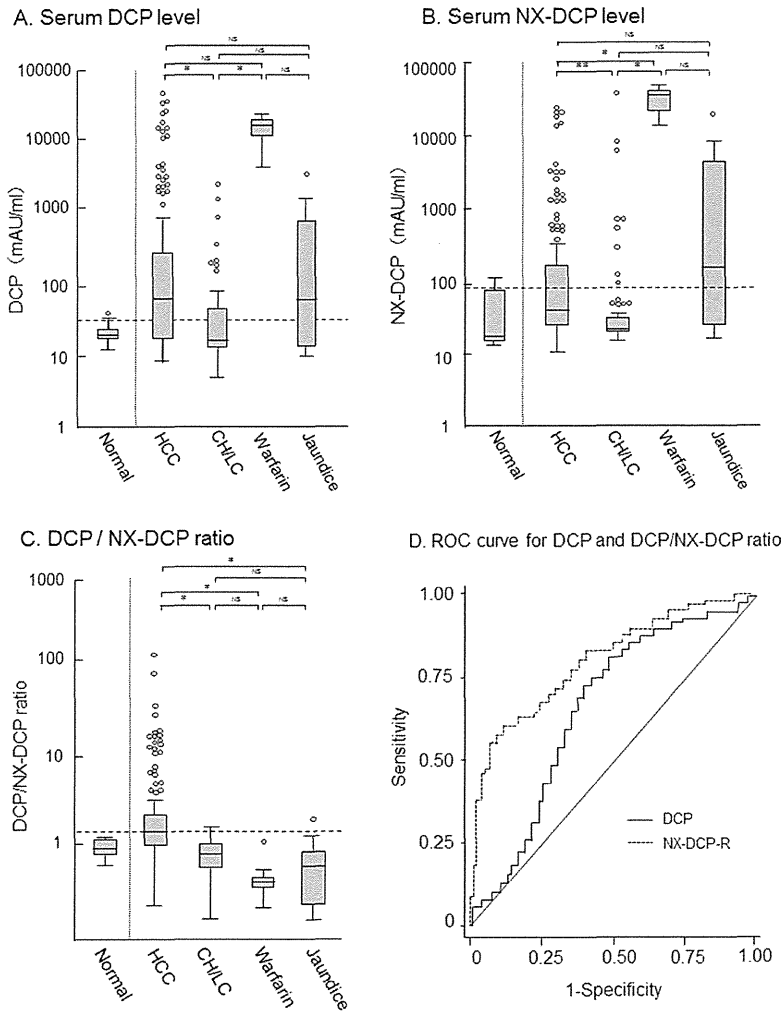

Figure 1.

190x254mm (96 x 96 DPI)