Brief Report

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Des-γ-carboxy prothrombin and c-Met were concurrently and extensively expressed in hepatocellular carcinoma and associated with tumor recurrence

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Summary **The aim of this study was to investigate co-expression of des-γ-carboxy prothrombin (DCP) and c-Met in hepatocellular carcinoma (HCC) and its significance in predicting tumor recurrence after surgical resection. Immunohistochemical techniques were used to examine DCP and c-Met expression in HCC samples collected from 153 patients. DCP and c-Met staining were observed in tumor areas in 63.4% (97/153) and 66.7% (102/153) of patients, respectively, and these figures are markedly higher than the rates at which adjacent nontumorous areas tested positive of 13.1% (20/154) and 28.8% (44/153). Furthermore, DCP and c-Met were consistently present or absent in HCC regions in 51.0% (78/153) and 20.9% (32/153) of patients, in adjacent nontumorous regions in 7.2% (11/153) and 65.4% (100/153) of patients, and in whole regions including HCC and adjacent nontumorous regions in 58.2% (89/153) and 19.6% (30/153) of patients. These results indicate that DCP and c-Met usually appeared or disappeared in HCC in a parallel manner. c-Met was found to be related to tumor recurrence in patients with HCC. When combined with DCP, c-Met is more effective at predicting non-recurrence of HCC than c-Met alone. Expression of neither DCP nor c-Met in HCC regions and adjacent regions signified a low rate of tumor recurrence after surgical resection. Results of the current study suggested that DCP and c-Met are commonly and concurrently expressed in HCC and their absence is associated with a low risk of tumor recurrence.**

Keywords: Des-γ-carboxy prothrombin (DCP), hepatocellular carcinoma (HCC), c-Met, histochemical expression, DCP/c-Met signal pathway, recurrence

1. Introduction

Hepatocellular carcinoma (HCC) is a severe condition that is found worldwide. Eastern Asian countries such as Japan and China are areas where HCC is highly prevalent due to serious hepatitis C virus (HCV)/ hepatitis B virus (HBV) infection in the population (*1,2*). Therapeutic regimens including liver transplantation, surgical resection, and local-regional therapy such as transarterial chemoembolization (TACE) play a major role in HCC management. Although these treatments as well as diagnostic modalities and clinical screening have made great progress in recent years, HCC continues to have a dismal prognosis, with 5-year recurrence at 40-70% due to invasion and metastasis prior to treatment (*3*). Therefore, a key step would be to explore prognostic factors to help distinguish patients with a high or low risk of recurrence and then adopt an appropriate approach for surveillance and treatment of this disease.

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The *c-Met* proto-oncogene was originally identified as a transforming gene activated after *in vitro* treatment of a human osteosarcoma cell line with a chemical carcinogen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (*4*). Molecular cloning studies revealed that it encodes a protein tyrosine kinase that is now known as the cell surface receptor for hepatocyte growth factor (HGF) (*5*). Activation of the c-Met signaling pathway is involved in diverse cellular responses such as mitogenesis, motogenesis, or morphogenesis depending on the particular cell type and the microenvironment (*6*). Expression of c-Met in HCC was reported to be linked to an unfavorable clinicopathologic status, including a high proliferation index, low degree of differentiation, and vascular invasion and metastasis (*3,7-11*). In addition, a high level of c-Met is correlated with a poor prognosis, including risk of tumor recurrence and short survival time (*3,12-14*). These studies suggest that c-Met might serve as a prognostic factor for HCC. That said, previous studies found that HGF, the natural ligand of c-Met, was not expressed or activated in normal liver tissue and was often absent in human HCC tissues (*8,9,15,16*). HCC tissues that are positive for HGF have a rather homogeneous HGF signal that is not correlated with the histological grade or other morphological features (*8*). These results seem to suggest that activation of the c-Met signal transduction pathway seems to depend on factors other than HGF in HCC pathology. Thus far, the potential factors that trigger c-Met signal transduction and contribute to the initiation and progression of HCC are, at least in part, largely unknown.

Recent studies by the current authors and other researchers found that des-γ-carboxy prothrombin (DCP), an abnormal cytokine secreted by HCC cells, was involved in the activation and regulation of downstream pathways of the c-Met signaling system *in vitro* and in animal studies (*17-20*). Thus, whether or not DCP and c-Met are co-expressed in human HCC and whether their co-expression is correlated with tumor recurrence are questions of great interest. The present study focuses on the expression profiles of DCP and c-Met in human HCC tissues and explores their clinical prognostic value in predicting tumor recurrence.

2. Materials and Methods

2.1. *Patients*

Liver tissue samples were collected from 153 patients (123 males and 30 females; median age of 63 years; range of 19-81 years) with a single primary HCC nodule. These patients underwent surgical resection at the Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, The University of Tokyo, between January 2005 and December 2007. The study was approved by the Ethics Committee of The University of Tokyo.

2.2. *Immunohistochemical staining*

Sections (4-μm thick) were obtained from archival formalin-fixed, paraffin-embedded tissue blocks, deparaffinized with a xylene solution and dehydrated through a graded series of ethanol solutions. Endogenous peroxidase was inactivated through administration of 0.3% hydrogen peroxide/methanol for 30 min. After microwave irradiation, the slides were incubated with blocking serum at room temperature for 30 min. The sections were then incubated with the primary anti-DCP monoclonal antibody (MU-3, 1:900 dilution; Eisai, Tokyo, Japan) for 60 min at room temperature. After the sections were incubated with biotinylated secondary antibody for 60 min, detection of DCP was achieved by the biotin streptavidin-peroxidase complex method using a commercial kit (Histofine SAB-PO kit; Nichirei, Tokyo, Japan). 3,3'-diaminobenzidine was used as the chromogen and hematoxylin was used as a counterstain. Sections that had not been subjected to primary antibody incubation served as a negative control to monitor background staining. The percentage of stained cancer cells out of the total number of cancer cells was determined in 10 random microscopic fields of each tissue sample, or using the entire area, if the tissue sample comprised fewer than 10 fields. Samples in which more than 10% of carcinoma cells were stained were defined as positive (*21,22*).

2.3. *Statistical analysis*

Statview 5.0J (Abacus Concepts, Berkeley, CA, USA) statistical software was used for data analysis. A χ^2 test was used to evaluate the relationship between DCP/c-Met expression and tumor recurrence. $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. *DCP and c-Met expression profiles in HCC and surrounding non-cancerous liver tissues*

DCP and c-Met expression in HCC and adjacent nontumorous liver tissues were determined using immunohistochemistry. Immunohistochemical staining for DCP indicated that 97 patients (97/153, 63.4%) were positive for DCP staining in cancerous tissues (Table 1). Of these 97 patients, 82 were positive for DCP staining only in HCC tissues and 15 were positive for DCP staining in both tumorous tissues and adjacent nontumorous tissues. DCP expression was detected in adjacent noncancerous tissues in 20 patients (20/153, 13.1%). A significant difference ($p < 0.01$) in DCP positivity was noted in HCC tissues compared to adjacent nontumorous tissues. The c-Met expression profile was also observed in HCC

specimens collected in the present study (Table 1). In total, 102 patients (102/153, 66.7%) were found to have c-Met-staining tumors; c-Met was detected in HCC tissues alone in 66 patients and in both HCC and surrounding nontumorous liver tissues in 36. Expression of c-Met in adjacent nontumorous tissues was noted in a markedly smaller number of patients (44/153, 28.8%) compared to that in tumorous tissues ($p < 0.01$). The expression profiles of DCP and c-Met noted in the present study indicated that HCC tissues are prone to express DCP and c-Met and implied that abnormally elevated expression of these two molecules in HCC tissues may play a role in HCC development and progression.

3.2. *Co-existence of DCP and c-Met in HCC*

One aim of the current study was to investigate whether DCP and c-Met co-existed in the liver tissues of patients with HCC. Specifically, the correlation between the presence of DCP and the presence of c-Met was analyzed in three matched groups: *i*) cancerous tissue producing DCP and cancerous tissue expressing c-Met; *ii*) adjacent non-cancerous tissue producing DCP and adjacent noncancerous tissue expressing c-Met; and *iii*) whole tissue (either cancerous or adjacent noncancerous tissue) producing DCP and whole tissue expressing c-Met. In each matched group, the patients were divided into four categories according to the presence or absence of DCP and c-Met, *i.e.* DCP positive and c-Met-positive patients, DCP-negative and c-Met-negative patients, DCP-positive and c-Met-negative patients, and DCP-negative and c-Met-positive patients.

For the first matched group, 78 patients (78/153, 51.0%) had co-existence of DCP and c-Met while 43 patients (43/153, 28.1%) had expression of only one of the two molecules, either DCP or c-Met, in HCC regions (Table 2). Of the 97 patients with cancerous tissues that were stained with DCP antibody, 78 (78/97, 80.3%) were found to have c-Met expression in tumor regions. Similarly, 76.5% of patients had DCP expression in HCC regions among the patients that were found positive for c-Met in tumor areas. These results suggested that the existence of DCP was usually accompanied by the presence of c-Met in tumorous tissue. The percentage of consistent expression of DCP and c-Met (71.9%) was markedly higher than that of inconsistent expression (28.1%) ($p < 0.0001$). In the second matched group, *i.e.* DCP and c-Met in adjacent noncancerous liver tissues, 100 patients (accounting for 65.4% of all patients) displayed no staining for the two molecules. In the group with noncancerous tissues that were not stained with DCP antibody, c-Met expression was not observed in the same area in 75.1% of specimens. Coincidentally, the group with no c-Met staining in adjacent noncancerous tissues had a negative rate of DCP staining in noncancerous areas of 91.7%. A parallel relationship between DCP and c-Met was also apparent in the adjacent nontumorous liver tissues of patients with HCC ($p = 0.0054$). Finally, co-expression of DCP and c-Met was analyzed in whole tissue, including cancerous and adjacent non-cancerous areas. Eighty-nine specimens (89/153, 58.2%) concurrently expressed DCP and c-Met while thirty (30/153, 19.6%) expressed neither DCP nor c-Met. Collectively, consistent expression of DCP and c-Met in whole tissues existed in 77.8% of patients, revealing a relationship between the existence of DCP and c-Met in patients with HCC ($p < 0.0001$). All of these results indicated that the existence of DCP and c-Met was parallel in tumor tissues, adjacent nontumorous tissues, and whole tissues of HCC samples investigated in this study (Figure 1).

Table 1. DCP and c-Met expression in cancerous tissues and adjacent noncancerous tissues

Sub-group	DCP *n* (percent)	c-Met *n* (percent)
C(+)NC(+)	15 (9.8%)	36 (23.5%)
C(+)NC(–)	82 (53.6%)	66 (43.1%)
C(–)NC(+)	5 (3.3%)	8 (5.2%)
C(–)NC(–)	51 (33.3%)	43 (28.1%)
Total cases	153	153
C(+)	97 (63.4%)	102 (66.7%)
NC (+)	20 (13.1%)	44 (28.8%)

C: cancerous tissues; NC: adjacent noncancerous tissues.

Table 2. Correlation analysis of DCP and c-Met expression in HCC specimens

Sub-group	DCP_c↔$c\text{-}Met_c$	DCP_{nc}↔$c\text{-}Met_{nc}$	DCP_w↔$c\text{-}Met_w$
DCP(+)c-Met(+) cases	78 (51.0%)	11 (7.2%)	89 (58.2%)
DCP(–)c-Met(–) cases	32 (20.9%)	100 (65.4%)	30 (19.6%)
DCP(+)c-Met(–) cases	19 (12.4%)	9 (5.9%)	13 (8.5%)
DCP(–)c-Met(+) cases	24 (15.7%)	33 (21.6%)	21 (13.7%)
Total cases	153	153	153
p value	< 0.0001	0.0054	< 0.0001

DCP_c: DCP in cancerous tissue; $c\text{-}Met_c$: c-Met in cancerous tisse; DCP_{nc}: DCP in adjacent noncancerous tissue; $c\text{-}Met_{nc}$: c-Met in adjacent noncancerous tissue; DCP_w: DCP in whole tissue, including cancerous tissue and adjacent noncancerous issue; $c\text{-}Met_w$: c-Met in whole tissue, including cancerous tissue and adjacent noncancerous issue.

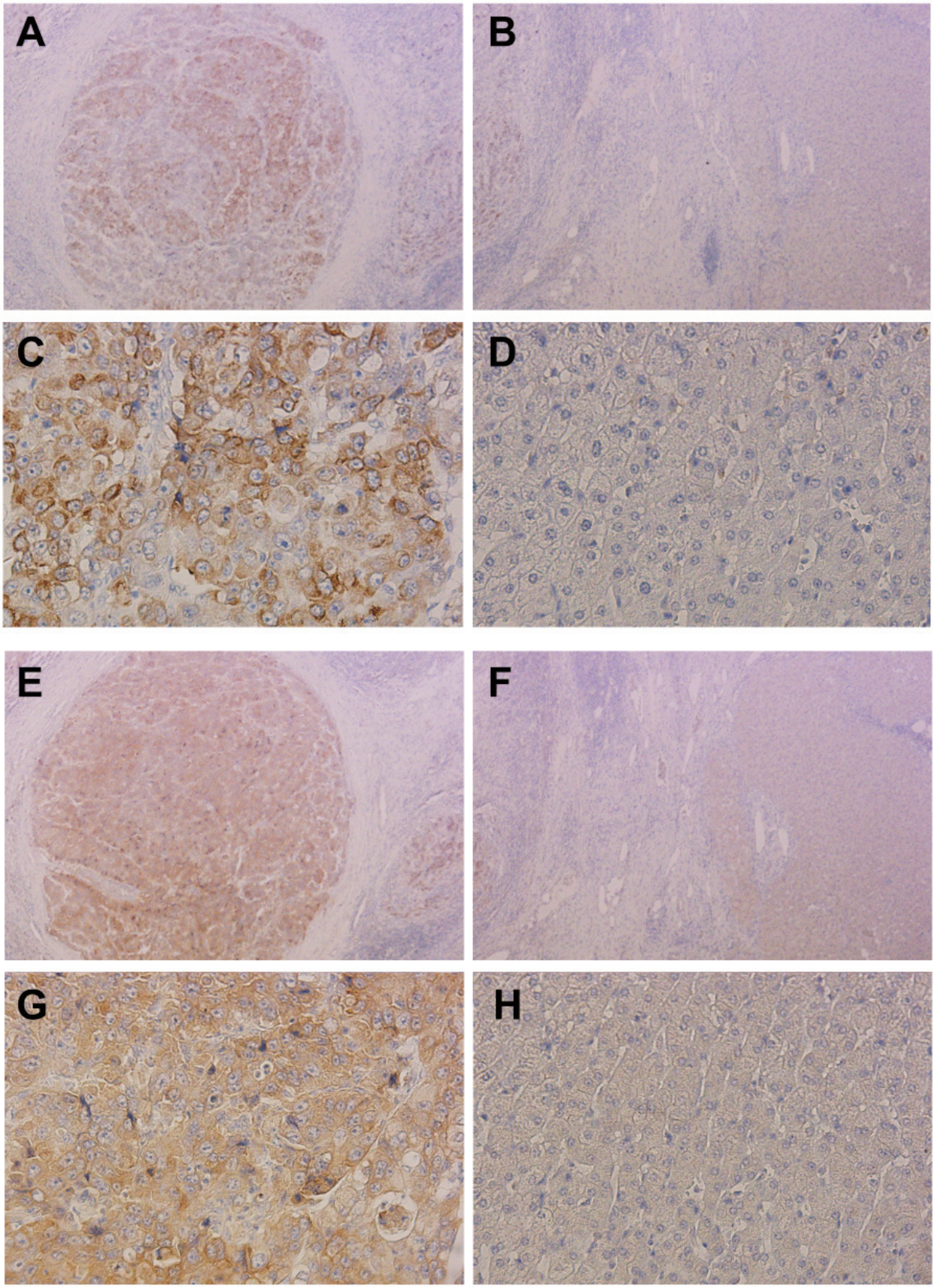

Figure 1. A representative case of DCP and c-Met staining in HCC and adjacent noncancerous tissue. HCC specimens from one patient were examined for DCP (**A**-**D**) and c-Met (**E**-**F**) expression, respectively. HCC nodule displayed immunoreactions for both DCP (**A** and **C**) and c-Met (**E** and **G**) while adjacent nontumorous liver tissue (the right area of HCC nodule) showed neither DCP (**B** and **D**) nor c-Met (**F** and **H**) immunoreactivity. Staining of DCP and c-Met was localized to both cytoplasm and cell membrane, as shown in **C** and **G**. **A**, **B**, **E**, and **F**, original magnification ×40; **C**, **D**, **G**, and **H**, original magnification ×200.

3.3. *Influence of DCP/c-Met expression on HCC recurrence*

As recurrence is a common behavior of HCC after treatment with surgical excision, the influence of DCP and/or c-Met expression on HCC recurrence was examined. The role of c-Met alone in predicting HCC recurrence was first examined. HCC recurrence was noted in 49 of 102 patients (48.0%) when c-Met was detected in tumors, whereas the group without c-Met in tumor areas had a clearly lower recurrence rate of 27.5% (14/51) (p = 0.0147) (Figure 2). When the patients were grouped according to whether c-Met was expressed in whole tissue, *i.e.* either tumorous tissue or adjacent nontumorous tissue, significant differences in recurrence rates were noted in the two groups. Patients in the c-Met-positive group were more susceptible to recurrence, at a rate of 46.4% (51/110), compared to the c-Met-negative group with a recurrence rate of 27.9% (12/43) (p = 0.0371) (Figure 2). Next, the combined role of c-Met and DCP in differentiating HCC recurrence was examined. Patients with c-Met and/or DCP in HCC and adjacent nontumorous tissues were found to have a recurrence rate of 47.2% (58/123), which is similar to that in the group positive for c-Met alone as noted above. However, patients with neither c-Met nor DCP in tumors and adjacent nontumorous tissues had a lower recurrence rate of 16.7% than patients without no c-Met (Figure 2). These results indicated that the combination of c-Met and DCP is more efficient than c-Met alone in distinguishing a low risk of recurrence in patients with HCC. Absence of c-Met and DCP in tumors and adjacent nontumorous tissues was associated with a low HCC recurrence.

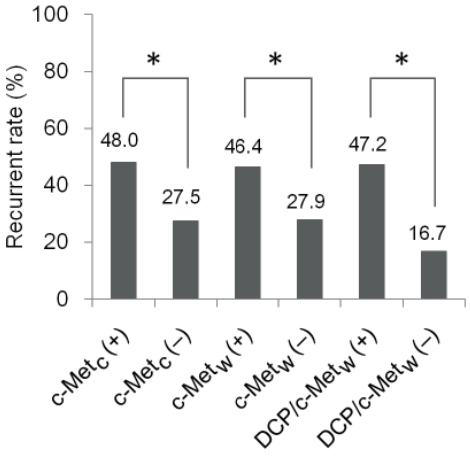

Figure 2. Tumor recurrence rates in different groups in relation to DCP/c-Met presence or absence. c-Met$_c$: c-Met in cancerous tissue; c-Met$_w$: c-Met in whole tissue, including cancerous and adjacent noncancerous tissue; DCP/c-Met$_w$: DCP and/or c-Met in whole tissue, including cancerous and adjacent noncancerous tissue. * $p < 0.05$.

The current study determined the expression of DCP and c-Met in human HCC samples and investigated their clinical value in predicting tumor recurrence. Results revealed that DCP and c-Met were expressed in HCC tissues in 63.4% and 66.7% of patients but were only observed in adjacent nontumorous tissues in 13.1% and 28.8% of patients, respectively. In addition to their extensive presence in HCC tissues, DCP and c-Met were usually concurrently expressed. This form of expression of DCP and c-Met was found to be related to tumor recurrence. Absence of DCP and c-Met in HCC and adjacent nontumorous tissues indicated a low risk of HCC recurrence. These results suggest that abnormal expression of DCP and c-Met in the liver tissues of patients with HCC are associated with tumor behavior.

DCP is an abnormal prothrombin that lacks the ability to interact with other coagulation factors (*23,24*). Its production was found to be related to decreased activity of γ-glutamyl carboxylase in hepatic cells, abnormal vitamin K metabolism, and overexpression of prothrombin precursor in hepatic cells (*25-27*). Currently, serous DCP is used as a diagnostic marker for HCC in Japan, South Korea, and Indonesia (*28-30*). Recent molecular biological studies of DCP have revealed the usefulness of this molecule as a diagnostic marker as well as its significant role in cancer progression. DCP has two kringle domains similar to those in HGF and both are considered necessary for HGF to bind with c-Met (*31*). Research has proven that DCP can bind with the surface receptor c-Met and result in c-Met phosphorylation (*31*). The current authors previously found that DCP can activate c-Met and induce matrix metalloproteinase activity in HCC cells, thus promoting the migration and invasion of HCC cells *in vitro* (*32*). The possible interaction between DCP and c-Met in human HCC was evident in the results of the present study, *i.e.* they existed extensively and concurrently in HCC tissues. Thus, DCP may trigger the c-Met signal transduction pathway, promoting HCC cells invasion and metastasis that always lead to tumor recurrence. This speculation may explain why absence of DCP and c-Met in HCC signifies a low risk of tumor recurrence as was noted in the present study.

In conclusion, this study provided evidence that DCP and c-Met usually coexist in HCC and their absence was associated with a low risk of tumor recurrence. Further studies are still needed to clarify their relationship to clinicopathological features of HCC and the prognosis for patients with HCC.

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