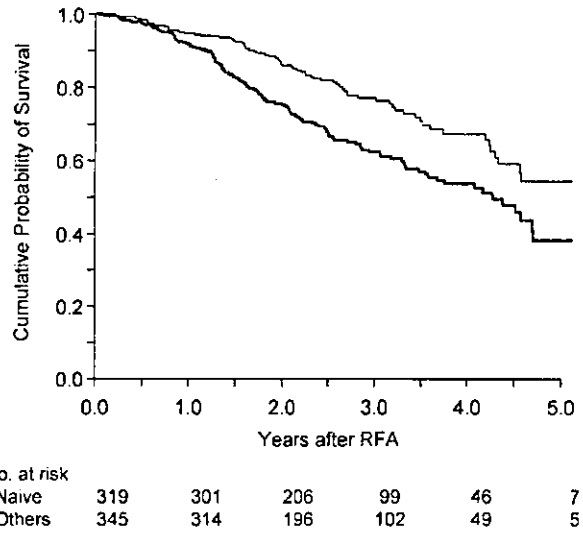

FIGURE 1. The cumulative survival rates of naive (thin line) and nonnaive (thick line) patients who received radiofrequency ablation (RFA). The cumulative survival rates estimated by the Kaplan–Meier method at 1, 2, 3, 4, and 5 years were 94.7%, 86.1%, 77.7%, 67.4%, and 54.3% for naive patients and 91.8%, 75.6%, 62.4%, 53.7%, and 38.2% for nonnaive patients, respectively.

not included. As for major complications, intraperitoneal hemorrhage was observed in four patients. In one patient, bleeding was detected during RF exposure, and arterial hemorrhage was identified by color Doppler ultrasonography. We reablated the needle track to stop the bleeding. The remaining three patients recovered after transfusion under careful observation. Hepatic infarction, defined as an increase in serum aspartate aminotransferase levels > 1000 IU/mL and/or an appearance of a wedge-shaped hypoattenuated area no smaller than a subsegment by dynamic CT scan, occurred in 2 patients. Both recovered spontaneously, although they suffered from high-grade fever lasting for 1–2 weeks. Hepatic abscess formation was found in seven patients and percutaneous drainage was performed in all. Two of them were complicated with bronchobiliary fistula and recovered after endoscopic biliary drainage. Intestinal perforation/penetration (duodenum, stomach, or colon) was observed after ablation in three patients. One patient with duodenal perforation was complicated with peritonitis and treated by surgical intervention. Colonic penetration occurred in one patient after hepatic abscess. The abscess was treated by percutaneous drainage and the anastomosis to the colon was closed by colonoscopic procedure. The patient with gastric penetration recovered with total parenteral nutrition without surgical intervention. In 1 patient, bile peritonitis occurred approximately 12 hours after RFA, requiring surgical and endoscopic biliary drainage. The patient showed intrahepatic bile duct dilatation before RFA, probably due to bile duct damage by previous PMCT.

When the 1000 treatments were divided into 2 groups as the earlier 500 and the later 500, there was a trend toward a decrease in the rates of immediate and periprocedual major complications (from 3.4% to 1.6% per treatment, $P = 0.105$ by chi-square test).

As a late complication, carcinoma seeding was identified in 15 patients with a median occult period of 17 months after the last ablation (range, 2–28 months). The area of tumor seeding was localized in nine patients and was surgically resected. Microwave coagulation was performed under thoracoscopic guidance in one patient with dissemination to the pleura. One patient with localized spread of tumor received radiotherapy. Two patients with intraperitoneal tumor spread were treated by systemic chemotherapy. The remaining two patients received best supportive care, as their liver function was impaired severely.

Long-Term Outcomes

Of the 664 patients included in the current series, 203 had died by March 31, 2004. Eight patients (1.2%) were lost to follow-up. The median observation period was 2.3 years (range, 0.17–5.1 years). Causes of death were cancer progression in 136 patients, hepatic failure in 36 patients, upper gastrointestinal bleeding in 8 patients, and liver-unrelated causes in 23 patients. The cumulative survival rates estimated by the Kaplan–Meier method at 1, 2, 3, 4, and 5 years were 94.7%, 86.1%, 77.7%, 67.4%, and 54.3% for naive patients whereas the respective rates were 91.8%, 75.6%, 62.4%, 53.7%, and 38.2% for nonnaive patients, respectively (Fig. 1). Significant differences were observed in subgroups divided by Child–Pugh class ($P = 0.000388$), tumor size ($P = 0.000234$), AFP level ($P = 0.0103$), and DCP ($P = 0.00000473$; Fig. 2 and Table 4). There was no significant difference in subgroups divided by age ($P = 0.378$), gender ($P = 0.921$), etiology of background liver disease ($P = 0.909$), number of tumors ($P = 0.464$) or pathologic grade ($P = 0.111$).

Tumor recurrence was identified in 165 of 306 patients. The median observation period for tumor recurrence was 1.59 years (range, 0.12–4.98 years). Of 165 patients with tumor recurrence, 142 (86.1%) had ≤ 3 recurrent nodules. Among these 142 patients, 137 were treated by second RFA, 4 by ethanol injection, and 1 by hepatic resection. Most of the remaining 23 patients with multiple recurrent nodules (> 3) were treated by TAE. Cumulative probabilities of tumor recurrence at 1, 2, 3, and 4 years were 20.4%, 43.4%, 59.8%, and 65.9%, respectively. Cumulative probabilities of death without tumor recurrence at 1, 2, 3, and 4 years were 3.9%, 7.0%, 8.7%, and 10.9%, respectively

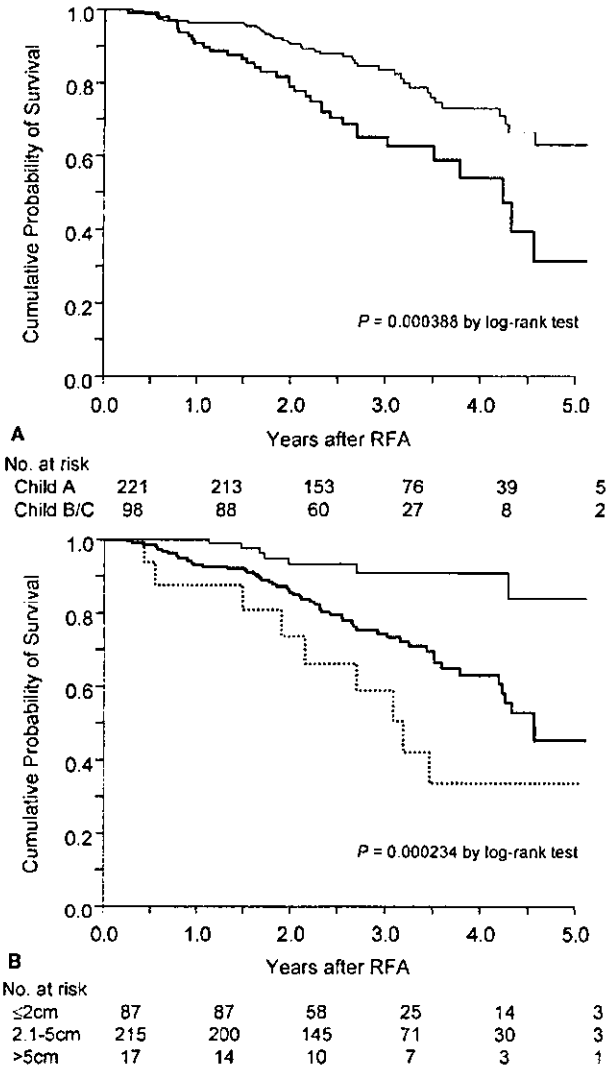

FIGURE 2. Cumulative survival rates of naive patients divided by (A) Child–Pugh Class and (B) tumor size. (A) The cumulative survival rates at 1, 2, 3, 4, and 5 years were 96.4%, 90.4%, 83.4%, 72.9%, and 63.1% for Child–Pugh A patients (thin line) and 90.7%, 79.0%, 65.0%, 53.9%, and 31.4% for Child–Pugh B/C patients (thick line), respectively. (B) The cumulative survival rates at 1, 2, 3, 4, and 5 years were 100%, 93.2%, 90.8%, 90.8%, and 83.8% for patients with tumors ≤ 2 cm (thin line), 93.0%, 85.4%, 74.3%, 63.0%, and 45.2% for patients with tumors 2.1–5.0 cm (thick line), and 87.5%, 73.4%, 58.7%, 33.6%, and 33.6% for patients with tumors > 5.0 cm (dashed line), respectively. RFA: radiofrequency ablation.

(Fig. 3A). When the patients were divided by Child–Pugh class, there was no significant difference in overall recurrence rate between Class A and Class B/C patients ($P = 0.38$ by Gray's test), whereas a significant difference in death without tumor recurrence was observed between the 2 groups ($P = 0.0026$; Fig. 3B). Cumulative probabilities of local tumor progression at 1, 2, 3, and 4 years were 1.3%, 2.4%, 2.4%, and 2.4%, respectively. (Fig. 4). No extrahepatic metastasis was observed in these 306 patients except for 2 patients with neoplastic seeding.

TABLE 4
Three-Year Survival of Patients with HCC Treated by RFA

Parameter	No. of patients	3-Year survival rate (95% CI)	*P* value
Overall	319	77.7 (72.4–83.2)	
Age (yrs)			
>68	159	76.0 (68.5–84.2)	0.378
≤68	160	79.2 (72.1–87.1)	
Gender			0.921
Male	212	77.7 (71.5–84.5)	
Female	127	77.1 (67.8–87.8)	
Etiology			0.909
HBs-Ag positive only	30	80.5 (66.3–97.8)	
HCV-Ab positive only	252	78.3 (72.5–84.6)	
Both negative	33	70.8 (54.1–92.6)	
Child–Pugh class			0.000388
A	221	83.4 (77.7–89.5)	
B/C	98	65.0 (54.9–76.3)	
Tumor size (cm)			0.000234
≤2.0	87	90.8 (83.8–98.4)	
2.1–5.0	215	74.3 (67.7–81.5)	
>5.1	17	58.7 (38.0–90.8)	
Tumor no.			0.464
Single	193	77.3 (70.1–85.2)	
2–3	105	77.5 (69.2–86.9)	
>3	21	67.5 (48.6–93.6)	
Edmondson grade			0.111
1	61	83.7 (72.6–96.7)	
2	214	76.9 (70.5–93.9)	
3	27	66.0 (48.9–89.1)	
AFP (ng/mL)			0.0103
≤100	238	82.3 (76.9–88.1)	
101–400	47	72.2 (58.6–88.8)	
>400	34	47.6 (28.6–79.4)	
DCP (mAU/mL)			0.00000473
≤40	225	86.3 (81.4–91.5)	
41–100	36	62.4 (46.6–83.4)	
>100	58	56.1 (42.4–74.4)	

HCC: hepatocellular carcinoma; RFA: radiofrequency ablation; HBsAg: hepatitis B surface antigen; HCV-Ab: hepatitis C virus antibody; AFP: α-fetoprotein; DCP: des-gamma carboxyprothrombin.

DISCUSSION

In the current study, we have shown that RFA is a safe and effective strategy for the treatment of HCC. The incidence of major complications within 30 days after the procedure was satisfactorily low (2.5% per treatment), and there were no treatment-related deaths.

As an immediate complication, intraperitoneal bleeding is a potentially fatal condition. Thus, a careful estimation of the underlying coagulopathy is important to avoid hemorrhage. We set the minimum of prothrombin time and platelet count for the indica-

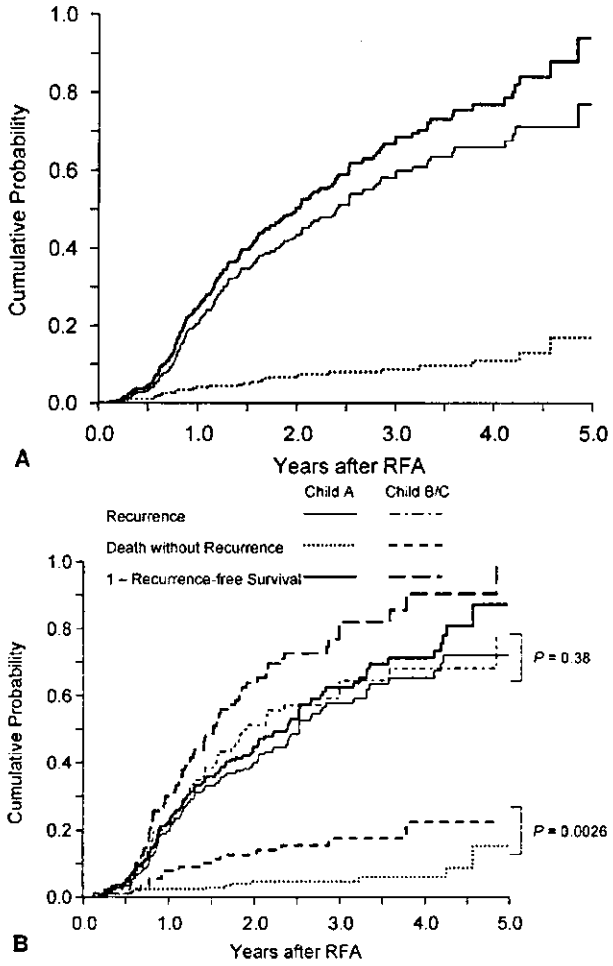

FIGURE 3. (A) Cumulative probabilities of tumor recurrence (thin line) and death without tumor recurrence (dotted line) of patients who received radiofrequency ablation (RFA) as initial treatment for hepatocellular carcinoma (HCC). The sum of the 2 probabilities is equivalent to 1 – tumor recurrence-free survival (thick line). (B) Cumulative probabilities of tumor recurrence and death without tumor recurrence of patients who received RFA as initial treatment for HCC divided by Child–Pugh class. There were significant differences between the cumulative probabilities of death without tumor recurrence of Child–Pugh A and Child–Pugh B/C patients, whereas no significant difference was observed between the cumulative tumor recurrence probabilities.

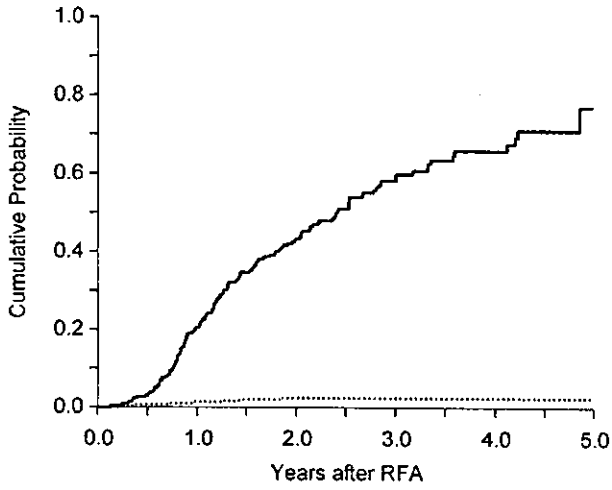

FIGURE 4. Cumulative probabilities of overall tumor recurrence (solid line) and local tumor progression (dotted line). RFA: radiofrequency ablation.

tion of RFA at higher levels (> 50% and > 5.0 × 10^4/mm^3) than those for PEIT.[12] The observed frequency of peritoneal bleeding (0.4% per treatment) was similar to that previously reported (0.3–0.5%).[22,23,30] Gastrointestinal perforation, another potentially fatal complication, can occur when the target nodule is located adjacent to the intestine, especially when the patients have a previous history of gastrointestinal surgery.[22,23] The observed frequency of gastrointestinal perforation/penetration (0.3% per treatment) was the same as previously reported, although none of those patients had a history of surgery. We recently introduced an intraperitoneal infusion technique, by which 500–1000 mL of 5% glucose solution is injected before and during the ablation for the purpose of creating space between the lesion and the intestine. The efficacy of this procedure is yet to be assessed fully.

It is well understood that the risk of complications can be reduced by proficiency in technique and refinement in pretreatment assessments. We observed a trend toward a decrease in the rates of immediate and periprocedual complications (from 3.4% to 1.6% per treatment). Conversely, we encountered an increase in the number of patients with neoplastic seeding. The main reason for that is the prolonged follow-up period because the majority of those patients were identified > 17 months after the last ablation. The rate of neoplastic seeding (1.5%) was higher than that previously reported (0.2–0.5%), although it was still very low compared with the value (12.5%) reported by Llovet et al.[31] The high incidence of seeding was likely to be related directly to the finding that tract cauterization was not performed.[23] Another possible reason is the tumor biopsy we performed in most of the patients to obtain the pathologic confirmation.[32]

The overall tumor recurrence rate for RFA in the current study was compatible with that of PEIT and hepatectomy.[33–35] The local tumor progression rate was 2.4% during a median of 19 months of follow-up, a very low rate compared with that reported elsewhere. Local tumor progression after RFA occurs mainly at the surrounding tissue.[36,37] To completely ablate a tumor ≥ 2 cm in diameter with a sufficient safety margin, multiple electrode insertion is neces-

sary by the 3-cm–exposed electrode. Thus, we decided to perform TAE with Lipiodol to tumors > 2 cm to delineate the border of the tumors at the CT scan for treatment evaluation after ablation. We tried to make sure that the entire tumor was surrounded as completely as possible by a nonenhanced area on an evaluation CT scan. It is known that occlusion of arterial flow during RFA significantly enlarges the zone of coagulation.[38,39] Our results for local tumor progression rate may be contributed by the effect although the exact impact of this confounding factor is precisely unknown when RFA is performed > 7 days after embolization (as we did) compared with when it is performed immediately (within 3 days) after embolization.

Louha et al.[40,41] reported on the likelihood of spreading cancer cells into peripheral blood by liver resection, ethanol injection, and transarterial embolization. However, the risk of hematogenous dissemination of HCC cells is negligible, because most patients had three or fewer nodules at HCC recurrence, and extrahepatic metastasis was rare in our patient series.

RFA can be repeated in patients with tumor recurrence as long as liver function is relatively intact. The finding that the majority of patients with tumor recurrence underwent RFA indicates that the initial RFA was not so invasive as to seriously damage liver function. As a locoregional therapy, RFA does not prevent de novo carcinogenesis in the remnant liver with chronic viral infection. Another strategy including viral eradication is needed to prevent overall tumor recurrence.[42]

The prognosis of patients with HCC is dependent mainly on tumor characteristics and liver function also in case of RFA. Patients with severely impaired liver function may not be eligible for RFA. The poorer survival among Child-Pugh Class B and C patients indicates that other strategies including OLT should be considered for longer survival.

RFA is a safe and effective method with satisfactory curability at least locally, and it can be repeated against tumor recurrence. The 5-year survival rate may be better than that previously reported for ethanol injection[11,43] and is obviously better than that of natural course.[44,45] Thus, RFA can be the first choice in the treatment of small HCC, although further follow-up will be necessary for the assessment of longer survival.

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Des-γ-carboxy Prothrombin Is a Potential Autologous Growth Factor for Hepatocellular Carcinoma*

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Des-γ-carboxyl prothrombin (DCP) is a well recognized tumor marker for hepatocellular carcinoma (HCC). In the present study, we demonstrate that DCP has a mitogenic effect on HCC cell lines. Purified DCP stimulated DNA synthesis of Hep3B and SK-Hep-1 cells in a dose-dependent manner. DCP was found to bind with cell surface receptor Met causing Met autophosphorylation and also to activate STAT3 signaling pathway through Janus kinase 1. Luciferase gene reporter analysis showed that DCP induced STAT3-related transcription. Small interfering RNAs against both STAT3 and Met abrogated DCP-induced cell proliferation. DCP did not affect the mitogen-activated protein kinase pathway, Myc signaling pathway, or phosphoinositide 3-kinase/Akt pathway. Based on these results, we believe that DCP acts as an autologous mitogen for HCC cell lines. The Met-Janus kinase 1-STAT3 signaling pathway may be a major signaling pathway for DCP-induced cell proliferation.

Des-γ-carboxyl prothrombin (DCP)[1] is a well recognized tumor marker for its high sensitivity and specificity in the screening and diagnosis of hepatocellular carcinoma (HCC). Forty-four to eighty-one percent of HCC patients have elevated serum DCP levels (1–5). DCP is a prothrombin precursor with no coagulation activity. Prothrombin is synthesized in the liver depending on the presence of vitamin K-dependent γ-glutamyl carboxylase. The prothrombin precursor has 10 Glu residues in the N terminus that are converted into γ-carboxy-glutamic acid (Gla) residues by γ-glutamyl carboxylase. All of these Glu residues must be converted into Gla residues before prothrombin can obtain coagulation activity. In DCP, not all of the 10 Gla residues are transformed. Instead, some remain as Glu residues (6). Reportedly, the vitamin K concentration and enzymatic activity of γ-glutamyl carboxylase are decreased significantly in HCC tissue (7–9); however, the precise mechanism underlying DCP production is still controversial.

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 (10–14), serum DCP level is used as a clinical parameter for the development of portal venous invasion of HCC (15), and cell proliferation markers have been seen to correlate with tissue DCP expression in clinical pathological studies of HCC (10, 16).

In exploring the structure of DCP, there are two kringle domains similar to those of hepatocyte growth factor (HGF), which was originally identified as a potent mitogen for mature hepatocytes (17, 18). Kringle domains are mandatory for HGF to bind with Met, and their presence implies that DCP interacts with Met. We hypothesize that DCP stimulates HCC cell proliferation through Met. Met is a membrane-spanning receptor tyrosine kinase that mediates biological responses to various tissues including cell scattering, growth stimulation, and the branching morphogenesis of cells in various tissues (19–22). Recent studies revealed that the coupling between HGF and Met integrates biological processes, such as the invasive and metastasis progression to cancer cells (23, 24).

In this study, we found that DCP stimulated the proliferation of HCC cell lines. During our investigation of the receptor for DCP, DCP was found to bind with Met. The transductional apparatus of DCP was identified to activate Janus kinase 1 (JAK1)/signal transducers and activators of the transcription 3 (STAT3) signaling pathway during cancer proliferation. These findings provide a description of a novel autocrine/paracrine growth stimulatory mechanism behind the development of HCC.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—Two human HCC cell lines, Hep3B (ATCC, Manassas, VA) and PLC/PRF/5 (ATCC) were maintained in Dulbecco's modified Eagle's medium (Invitrogen). Another human HCC cell line, SK-Hep-1 (ATCC), was maintained in Eagle's minimum essential medium (Sigma). The human colon cancer cell line HT-29 (ATCC) was cultured in McCoy's 5A medium (Sigma). All of the media were supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 1% nonessential amino acid (Sigma), 1% sodium pyruvate (Sigma), and 1% penicillin/streptomycin solution (Sigma). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Determination of DCP Levels Produced by HCC Cell Lines—DCP levels produced by HCC cell lines were determined by an electrochemiluminescence immunoassay (Picolumi PIVKA-II™; Eisai Co., Ltd., Tokyo, Japan). The electrochemiluminescence immunoassay method uses a mouse monoclonal anti-DCP antibody coated on solid phase beads and a rabbit polyclonal anti-prothrombin that has been ruthenylated. An electrochemically triggered light reaction was quantified by an electrochemiluminescence detection system.

Purification of DCP—The DCP-producing cell line PLC/PRF/5 was cultured in the presence of warfarin sodium (10 μg/ml) to enhance DCP production. DCP was purified from the conditioned media by affinity chromatography with an anti-prothrombin antibody (Eisai). The purified DCP was then separated by SDS-PAGE and transferred to an

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[1] The abbreviations used are: DCP, Des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma; STAT, signal transducers and activators of transcription; JAK1, Janus kinase 1; Gla, γ-carboxy-glutamic acid; HGF, hepatocyte growth factor; TBS-T, Tris-buffered saline with Tween 20; PI3K, phosphoinositide 3-kinase; PY-20, phosphotyrosine 20; siRNA, small interfering RNA; HPLC, high performance liquid chromatography; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase.

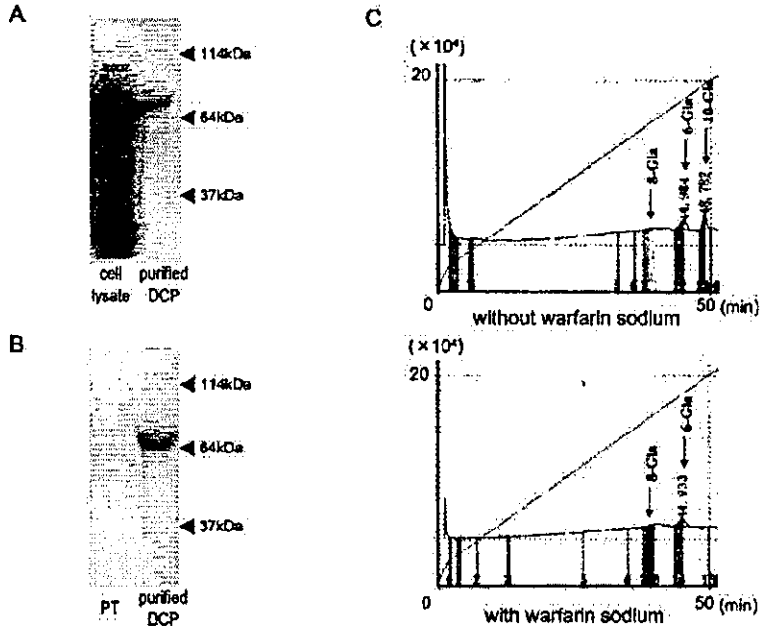

FIG. 1. **Purity of DCP extracted from PLC/PRF/5 cells.** PLC/PRF/5 cells were cultured in the tissue culture chamber with warfarin sodium (10 μg/ml). DCP was purified from the conditioned media by affinity chromatography with an anti-prothrombin antibody (Eisai). *A*, purified sample and cell lysate were analyzed by 10% SDS-PAGE and stained with Blot-FastStain™ protein staining kit (Chemicon International, Inc.). *B*, purified sample and prothrombin were analyzed by 10% SDS-PAGE and immunoblotted with anti-DCP antibody (Eisai). *C*, purified sample were purified from conditioned media of PLC/PRF/5 cells in the presence (*lower panel*) and absence (*upper panel*) of warfarin sodium (10 μg/ml). HPLC analysis was performed using a column (TSKgel; DDS-80TS, Tosoh).

Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was stained with Blot-FastStain™ protein-staining reagent (Chemicon International, Inc., Temecula, CA) according to the manufacturer's protocol. Purified DCP sample was developed by SDS-PAGE and blotted with anti-DCP antibody (Eisai). DCP could be distinguished from normal prothrombin by high performance liquid chromatography (HPLC) analysis (6). HPLC analysis was performed using a column (TSKgel; DDS-80TS, Tosoh Bioscience, Tokyo, Japan).

Cell Proliferation Assay—The cells were grown to confluence in 12-well plastic tissue culture plates. The cells were kept inactive for 24 h and then treated with DCP, prothrombin (Sigma), and apolipoprotein A (Calbiochem, San Diego, CA) at the indicated concentrations. After a 12-h incubation, 5 μCi of [^{3}H]thymidine (Amersham Biosciences) was added to each well and maintained for 8 h. The cells were treated with 5% trichloroacetic acid for 30 min at 4 °C. The cells were then harvested, and we used a liquid scintillation counter (Beckman Coulter, Fullerton, CA) to analyze the samples.

Western Blot Analysis—The cells were plated into 6-well plates and grown to confluence. After 24 h of quiescence, the cells were treated with DCP, prothrombin, HGF (PeproTech, Rocky Hill, NJ), or epidermal growth factor (Sigma) at the indicated concentrations for 15 min at 37 °C. Cell lysates were resolved by SDS-PAGE and transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore). The membranes were blocked using Tris-buffered saline with Tween 20 (Sigma) (TBS-T) buffer containing 5% bovine serum albumin for 1 h. The membranes were then incubated with anti-phospho-Met ($Tyr^{1234/1235}$) antibody (Cell Signaling Technology, Beverley, MA), anti-phospho-Met (Tyr^{1349}) antibody (Cell Signaling Technology), anti-phospho-JAK1 ($Tyr^{1022/1023}$) antibody (Sigma), anti-phospho-STAT3 (Ser^{727}) antibody (Sigma), anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) antibody (Cell Signaling Technology), anti-phospho-Raf (Ser^{259}) antibody (Cell Signaling Technology), anti-phospho-c-Myc (Thr^{58}/Ser^{62}) antibody (Cell Signaling Technology), anti-phospho-p85 phosphoinositide 3-kinase (PI3K)-binding motif antibody (Cell Signaling Technology), and anti-phospho-Akt antibody (Cell Signaling Technology) overnight at 4 °C. The membranes were washed three times with TBS-T and probed with horseradish peroxidase-conjugated secondary antibody before being developed with an ECL Western blotting detection system (Amersham Biosciences) using enhanced chemiluminescence.

Luciferase Gene Reporter Assay—The reporter plasmids, 30 μg of pFA2-Elk1, 30 μg of pFR-Luc (Path Detect™ Trans-Reporting System, Stratagene, La Jolla, CA), and 7 μg of pRL-TK control *Renilla* luciferase plasmid (Promega, Madison, WI) per 10^6 cells, were co-transfected by electroporation (voltage, 300 V; capacitor, 950 microfarad; cuvette gap, 4 mm) and plated into 6-well plates. For the STAT3 activity reporter assay, 30 μg of pSTAT3-Luc (Clontech laboratories, Palo Alto, CA) and 7 μg of pRL-TK control *Renilla* luciferase plasmid (Promega) were co-transfected per 10^6 cells. After 36 h, the media were changed just before the following treatment to avoid the effect of self-produced DCP. The cells were stimulated with DCP (2–200 ng/ml), prothrombin (2–200 ng/ml), and/or epidermal growth factor (10 nM), and HGF (50 ng/ml) and incubated for 12 h. The cells were then lysed, and the activities of firefly and *Renilla* luciferases were measured sequentially with a luminometer (Berthold Technologies, Bad Wildbad, Germany).

Far Western Blot Analysis—DCP protein was biotinylated using biotinamido hexanoic acid 3-sulfo-*N*-hydroxysuccinimide ester with an ECL biotinylation module (Sigma). The cell lysates were developed on SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membranes were then blocked with 5% bovine serum albumin in TBS-T and left to react overnight with biotin-labeled DCP in TBS-T containing 1% bovine serum albumin at 4 °C. Excess amounts of the probe were removed by four washes in TBS-T. The membranes were incubated with ExtrAvidin™ Peroxidase (Sigma) for detection. After three times washing with TBS-T, the membrane was developed using an ECL detection system (Amersham Biosciences).

Immunoprecipitation—Cell lysates containing the same amount of proteins were immunoprecipitated with anti-Met antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The precipitates were analyzed using 7.5% SDS-PAGE and immunoblotted with an anti-DCP antibody (Eisai), anti-phosphotyrosine 20 (PY-20) antibody (BD Transduction Laboratories, Lexington, KY), and anti-Met antibody.

Inhibitors for Met-JAK-STAT Signaling Pathway—A function-blocking anti-Met antibody (R & D Systems, Minneapolis, MN) was utilized for function blocking assay against Met signaling. The cells were pretreated with function-blocking anti-Met antibody (1 μg/ml) for 1 h before DCP stimulation. JAK inhibitor AG490 and STAT3 inhibitor peptide were purchased from Calbiochem.

Gene Silencing with Small Interfering RNAs—siRNA duplexes targeting STAT3 and Met sequences were obtained from Dharmacon SMART™ pool technology (Dharmacon Research, Lafayette, CO). Control (nonsilencing, 5′-AATTCTCCGAACGTGTCACGT-3′) siRNA duplexes were obtained from Sigma. siRNAs were transfected into cells using the RNAiFect™ transfection reagent (Qiagen). The cells were incubated for 12 h and utilized for the following analysis.

Total RNA was purified from siRNA transfected cells with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Lamin A/C mRNA expression level was determined by real time PCR using QuantiTect™ gene expression assay system (Qiagen) and Light-Cycler™ (Roche Applied Science) to verify that nonspecific suppression did not occur by siRNA transfection.

The interferences of STAT3 and Met protein expression were confirmed by Western blot analysis using an anti-STAT3 antibody (Cell Signaling Technology) and anti-Met antibody (Santa Cruz Biotechnology). Luciferase gene reporter assays for STAT3 activation and a cell proliferation assay were then performed as described above.

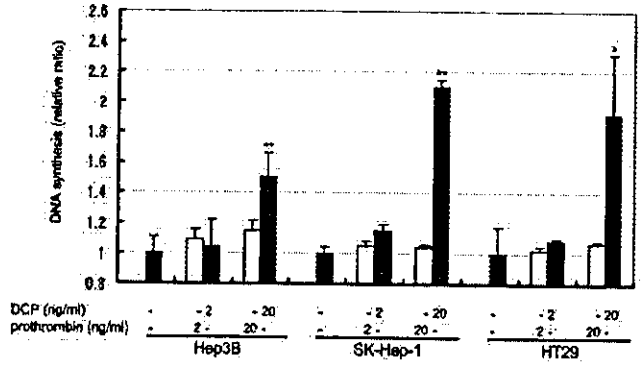

FIG. 2. **Effect of DCP on DNA synthesis in Hep3B, SK-Hep-1, and HT-29 cells.** The cells were grown to confluence in 12-well dishes and kept quiescent for 24 h. The cells were then treated with DCP or prothrombin for 20 h. A cell proliferation assay was performed as described under "Experimental Procedures" using [^{3}H]thymidine. The data are shown as ratios to untreated control cells and are the means ± S.E. of more than three independent studies. *, $p < 0.05$; **, $p < 0.01$ (*versus* nontreatment of DCP); Student's *t* test.

RESULTS

Purification of DCP from PLC/PRF/5—The purified DCP was analyzed by SDS-PAGE and HPLC analyses. More than 99% was DCP in SDS-PAGE and densitometry analysis (Fig. 1*A*). The purified DCP was analyzed by Western blot with anti-DCP antibody to demonstrate that the DCP was uncontaminated (Fig. 1*B*). Purified DCP from warfarin sodium-treated PLC/PRF/5 cells contained no detectable level of normal prothrombin in HPLC analysis (Fig. 1*C*, *lower panel*). HPLC analysis showed that DCP produced by PLC/PRF/5 cell has 6–8 Gla residues. This result is compatible with a former report (6).

Self-production of DCP by HCC Cell Lines—The DCP produced by each cell line was determined using an electrochemiluminescence immunoassay. Hep3B and PLC/PRF/5 produced DCP at rates of 0.49 ± 0.04 and 2.33 ± 0.04 (ng/ml/day/10^6 cells), respectively. SK-Hep-1 and HT-29 did not produce a detectable DCP level.

DCP Stimulates the Proliferation of HCC Cell Lines—To investigate the mitogenic effect of DCP, we incorporated [^{3}H]thymidine into DNA as an index of proliferation. As shown in Fig. 2, increasing concentrations of DCP led to increased incorporation of [^{3}H]thymidine in each cell line. DCP stimulated cell proliferation 1.50 ± 0.16-fold at a concentration of 20 ng/ml in the DCP self-producing Hep3B cell, whereas normal prothrombin stimulated cell proliferation 1.15 ± 0.07-fold at the same concentration. This effect reached a plateau with a DCP stimulus of 200 ng/ml (data not shown). In SK-Hep-1 non-DCP-producing cells, DCP showed a significant potential mitogenic effect of 2.10 ± 0.05-fold at a concentration of 20 ng/ml. Normal prothrombin hardly affected proliferation of the SK-Hep-1 cell line. In fact, cell proliferation in SK-Hep-1 cells was not even induced with 100 μg/ml of prothrombin, the physiological plasma concentration (data not shown). Moreover, we assessed the effect of DCP in colon cancer cell line HT-29, which does not produce DCP but expresses Met. DCP stimulated cell proliferation 1.91 ± 0.40-fold at a concentration of 20 ng/ml in HT-29 cells, but prothrombin had little effect. Moreover, apolipoprotein A was employed as a control serum protein that contains kringle domains (25). Apolipoprotein A did not affect cell proliferative activity at its physiological serum concentration (280 ng/ml) in HCC cells (1.13 ± 0.17-, 0.90 ± 0.04-, and 0.96 ± 0.03-fold for Hep3B, SK-Hep1, and HT-29, respectively; not significant).

DCP Binds to HGF Receptor Met and Phosphorylates Tyrosine Residues of Met—Met expression in Hep3B, SK-Hep-1, and HT-29 cells was confirmed by Western blot analysis. Far Western blot analysis using a biotin-labeled DCP probe showed that DCP interacted with Met. In all of the cell lysates, a 145-kDa protein was detected by this probe and identified as Met (Fig. 3*A*). The extracts of Hep3B, SK-Hep-1, and HT-29 cells were immunoprecipitated with anti-Met antibodies and blotted with

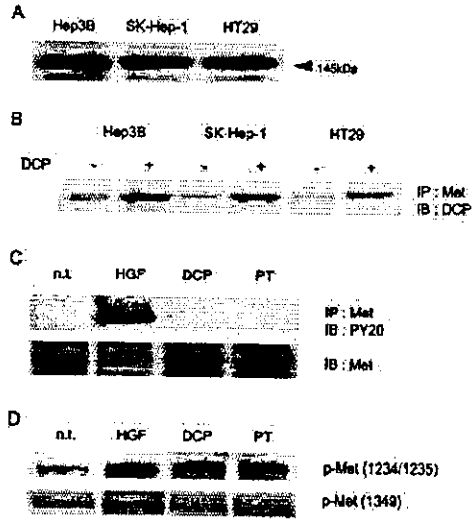

FIG. 3. **Far Western blot analysis (*A*) and co-immunoprecipitation analysis (*B*) for Met and analysis for phosphorylation of Met (*C* and *D*).** *A*, cells were grown to confluence in a 10-cm tissue culture dish and lysed with buffer containing sodium vanadate and sodium pyrophosphate. Cell lysates were developed on SDS-PAGE with nonreduced condition and transferred to a polyvinylidene difluoride membrane. The membrane was probed with biotin-labeled DCP, and the Far Western blot was developed with streptavidin-conjugated alkaline phosphatase. Shown is the representative blot of two independent studies. *B*, cells were grown to confluence in a 10-cm dish and kept quiescent for 24 h. The cells were then stimulated with DCP (20 ng/ml) for 15 min. Met protein was immunoprecipitated with anti-Met antibody (Santa Cruz Biotechnology) from total cell lysates. The immunoprecipitates were analyzed by Western blot using anti-DCP antibody. Shown is the representative blot of two independent studies. *IP*, immunoprecipitation; *IB*, immunoblot. *C*, Hep3B cells were grown to confluence in a 10-cm dish and kept quiescent for 24 h. The cells were stimulated with HGF (50 ng/ml), DCP (20 ng/ml), and prothrombin (20 ng/ml) for 15 min. Met protein was immunoprecipitated from total cell lysates. The precipitates were then analyzed by SDS-PAGE and immunoblotted with anti-PY-20 antibody and anti-Met antibody. Shown are the representative blot of two independent studies. *D*, Western blots demonstrating the effects of DCP on tyrosine phosphorylation of Met in the Hep3B cell line. The cells were kept quiescent for 24 h and then stimulated with HGF (50 ng/ml), DCP (20 ng/ml), and prothrombin (20 ng/ml) for 15 min. The cell extracts were analyzed using Western blot and anti-phospho-specific Met $Tyr^{1234/1235}$ and Tyr^{1349} antibodies. Shown are the representative blots of three independent studies. *n.t.*, nontreated control.

an anti-DCP antibody. When anti-Met antibody was used for immunoprecipitation, DCP could be visualized with an anti-DCP antibody (Fig. 3*B*). When Met is activated by its ligand HGF, specific tyrosine residues are reported to be autophosphorylated (26, 27). HGF treatment induced the autophosphorylation of Met as detected by the anti-PY-20 antibody in

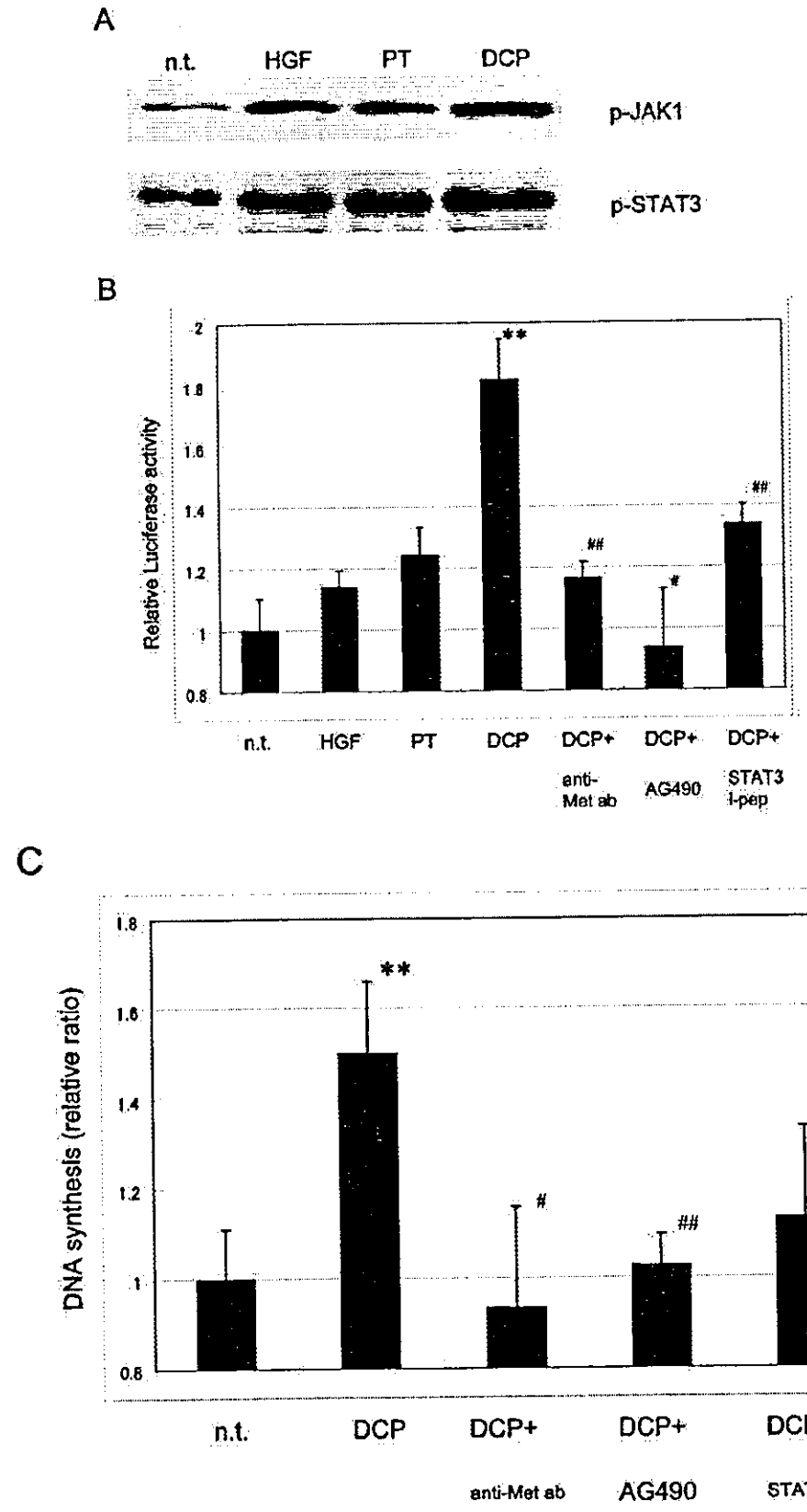

FIG. 4. **JAK1-STAT3 signals induced by DCP stimulation.** *A*, Hep3B cells were kept quiescent for 24 h and then stimulated with HGF (50 ng/ml), prothrombin (*PT*, 20 ng/ml), and DCP (20 ng/ml) for 15 min. The cell extracts were analyzed using Western blot with an anti-phospho-specific JAK1 antibody (*upper panel*) and an anti-phospho-specific STAT3 antibody (*lower panel*). Shown are the representative blots of more than three independent studies. *B*, STAT3 activation was determined using the luciferase reporter assay. Hep3B cells were transiently transfected with pSTAT3-Luc reporter plasmid and pRL-TK control *Renilla* luciferase plasmid. After 36-h of quiescence, the cells were treated with HGF (50 ng/ml),

Hep3B cells (Fig. 3*C*). In the phosphorylation analysis using the anti-PY-20 antibody, DCP did not induce phosphorylation of Met as did HGF. Therefore, we conducted further analysis of Met phosphorylation. Activation of the Met receptor results in the autophosphorylation of tyrosines 1234 and 1235 of the tyrosine kinase domain and tyrosines 1349 and 1356 of the multifunctional docking site (27–29). In this study, DCP phosphorylated only those tyrosine residues located in the kinase activation loop ($Tyr^{1234/1235}$) and not the C-terminal tail (Tyr^{1349}), whereas HGF phosphorylated residues in both locations in Hep3B (Fig. 3*D*). Similar results were obtained with SK-Hep-1 cells (data not shown).

DCP Stimulates the JAK-STAT Pathway through Met—To identify the signaling pathway following DCP stimulation, cell lines were treated with DCP, and JAK1 phosphorylation was examined using Western blot analysis. Following exposure of cells with DCP, JAK1 ($Tyr^{1022/1023}$) was phosphorylated in Hep3B (Fig. 4*A*). JAK is known as the mediator of phosphorylation of STATs (30–32). We observed that DCP phosphorylated STAT3 in Hep3B by Western blot analysis (Fig. 4*A*). Similar results were obtained with SK-Hep-1 cells (data not shown). A luciferase reporter assay was conducted to determine whether DCP induced activation of the STAT3-responsive element. As shown in Fig. 4*B*, DCP induced a 1.82 ± 0.08-fold increase in luciferase activity in Hep3B cells. Prothrombin phosphorylated STAT3 but induced only a 1.24 ± 0.05-fold increase in luciferase activity in the STAT3-responsive element.

To determine whether the biological effect of DCP is brought out through the Met-JAK-STAT3 signaling pathway, a functional blocking anti-Met antibody (R & D Systems), JAK inhibitor AG490, and STAT3 inhibitor peptide were utilized. DCP-induced STAT3 activation was reduced by 80, 108, and 58% by function-blocking anti-Met antibody, AG490, and STAT3 inhibitor peptide, respectively (Fig. 4*B*). DCP-induced incorporation of [^{3}H]thymidine was also decreased by 113, 95, and 74% by function-blocking anti-Met antibody, AG490, and STAT3 inhibitor peptide, respectively (Fig. 4*C*).

Gene Silencing Using Met siRNA and STAT3 siRNA Abrogates the Mitogenic Effect of DCP—To determine whether Met-STAT3 signaling pathway is a major signaling pathway for DCP-induced mitogenesis, HCC cells were treated with Met siRNA and STAT3 siRNA. Western blot analysis showed that Met protein expressions were reduced by 67% in Hep3B cells and 89% in SK-Hep-1. Lamin A/C mRNA remained constant in both cells (78 ± 13 and 88 ± 19% as ratio to no siRNA transfected cells for Hep3B and SK-Hep-1 cells, respectively). STAT3 siRNA inhibited STAT3 protein expression by 50% in Hep3B cells and 55% in SK-Hep-1 cells. Lamin A/C mRNA remained constant (86 ± 15 and 103 ± 24% as ratio to control siRNA transfected cells for Hep3B and SK-Hep-1 cells, respectively). DCP did not cause the phosphorylation of STAT3 in the Met-silenced Hep3B cells (data not shown). Met siRNA inhibited DCP-induced STAT3 luciferase activity by 67.4% in Hep3B cells and 91.4% in SK-Hep-1 cells (Fig. 5*A*). RNA interference gene silencing against STAT3 also abrogated DCP-induced luciferase activity in the STAT3-responsive element by 80.3% in Hep3B cells and 100% in SK-Hep-1 cells (Fig. 5*A*).

When cell proliferative activities were analyzed by [^{3}H]thymidine incorporation assay, Met siRNA transfection decreased DCP-induced incorporation of [^{3}H]thymidine by 39.3% in the Hep3B cell line and 66.6% in the SK-Hep-1 cell line (Fig. 5*B*). Moreover, STAT3 siRNA transfection decreased DCP-induced incorporation of [^{3}H]thymidine by 56.8% in the Hep3B cell line and by 100% in the SK-Hep-1 cell line (Fig. 5*B*).

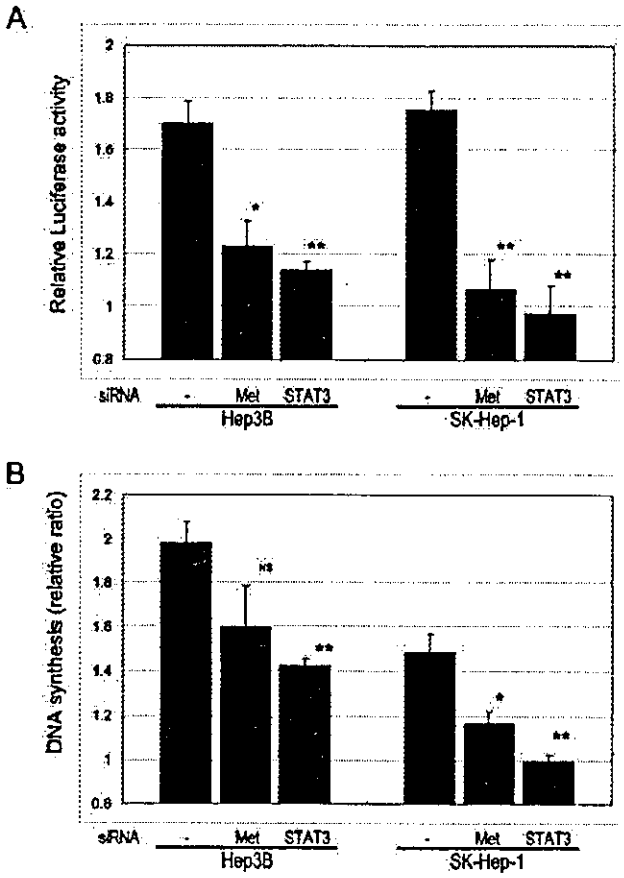

FIG. 5. **The effect of Met and STAT3 gene silencing on DCP-induced STAT3 activation (*A*) and cell proliferation (*B*).** *A*, STAT3 activation was determined using a luciferase reporter assay. Met siRNA duplexes (100 nM), STAT3 siRNA duplexes (100 nM), and control (nonsilencing) siRNA duplexes (100 nM) were transfected into cells and incubated for 12 h. The cells were transiently transfected with pSTAT3-Luc reporter plasmid and pRL-TK control *Renilla* luciferase plasmid. After 36-h of quiescence, the cells were treated with DCP (20 ng/ml) for 12 h. Cellular extracts were collected, and luciferase activities were measured. The STAT3 activity, depending on luciferase activities, was normalized to the constitutive active *Renilla* luciferase activity. *B*, after treatment with siRNA duplexes, a cell proliferation assay was performed as described under "Experimental Procedures" using [^{3}H]thymidine. The data are shown as the ratios to DCP untreated control cells and are the means ± S.E. of more than three independent studies. *NS*, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$ (*versus* control nonsilencing siRNA); Student's *t* test.

DCP Did Not Affect the Raf-MEK ERK-MAPK Pathway—HCC cell lines were treated with DCP for the indicated period. Raf and ERK-MAPK phosphorylation was analyzed by Western blot analysis. DCP did not phosphorylate either Raf (data not shown) or ERK-MAPK (Fig. 6). In addition, we performed luciferase reporter assays for Elk activation and found that DCP did not raise Elk-dependent luciferase activity in Hep3B cells (Fig. 6). We further investigated whether DCP stimulates

prothrombin (20 ng/ml), DCP (20 ng/ml), AG490 (80 μM), function-blocking anti-Met antibody (R&D Systems) (1 μg/ml), and/or STAT3 inhibitor peptide (100 μM) for 12 h. Cellular extracts were collected, and luciferase activities were measured. *C*, a cell proliferation assay was performed as described under "Experimental Procedures" with DCP (20 ng/ml), function-blocking anti-Met antibody (1 μg/ml), AG490 (80 μM), and/or STAT3 inhibitor peptide (100 μM). The data are shown as the ratios to untreated control cells and are the means ± S.E. of more than three independent studies. **, $p < 0.01$ (*versus* nontreatment of DCP); *NS*, $p > 0.05$; #, $p < 0.05$; ##, $p < 0.01$ (*versus* DCP-stimulated cells); Student's *t* test. *n.t.*, nontreated control.

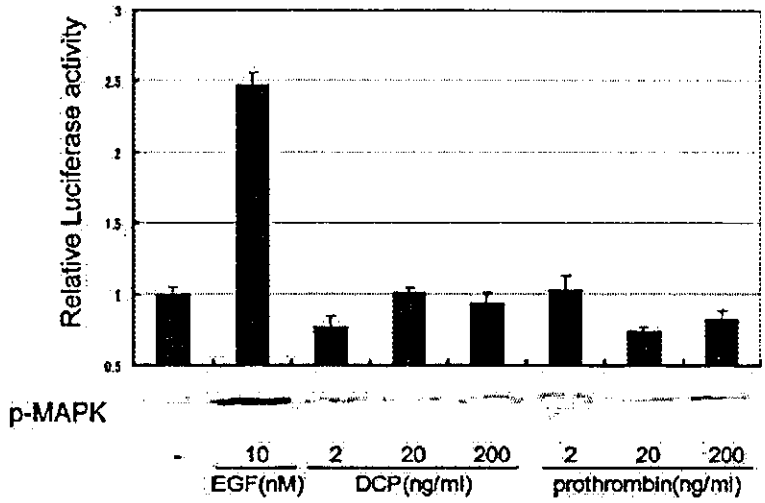

FIG. 6. **The effect of DCP on Raf-MAPK signaling.** Hep3B cells were grown to confluence in a 6-well tissue culture dish and kept quiescent for 24 h. The cells were treated with epidermal growth factor (10 nM), DCP (2–200 ng/ml), and prothrombin (2–200 ng/ml) for 15 min. The cell extracts were analyzed by Western blot using a phospho-specific p44/42 MAPK (Thr^{202}/Tyr^{204}) antibody. MAPK-related transcriptional factor Elk activities were analyzed using a luciferase reporter assay. The cells were transiently transfected with Elk reporter plasmid and then treated with epidermal growth factor, DCP, and prothrombin for 12 h. The cellular extracts were collected, and luciferase activities were measured. The data are shown as the ratios to untreated control cells and are the means ± S.E. of more than three independent studies.

the c-Myc and PI3K-Akt signaling pathway by Western blot analysis. DCP did not affect the phosphorylation of c-Myc, p85-PI3K, and Akt (data not shown). Moreover, other Met signaling pathways were screened using an antibody array (MaxArray™ RTK Antibody Array) (Zymed Laboratories Inc. Laboratories, South San Francisco, CA), which contains antibodies for proteins related to receptor tyrosine kinases. Other signaling pathways were not affected significantly by DCP (data not shown). Similar results were obtained with SK-Hep-1 cells (data not shown).

DISCUSSION

In the current study, we present DCP as a novel autocrine/paracrine mitogen for HCC cell lines. Clinical studies show a significant correlation between serum DCP level and the clinical malignancy of HCC (11–15, 33); however, the molecular basis for this correlation is still poorly understood. Herein, DCP was demonstrated to stimulate the Met-JAK-STAT signaling pathway in HCC cell lines. To the best of our knowledge, this is the first study that clearly demonstrates a mitogenic effect of DCP in HCC cell lines.

Proto-oncogene c-Met, which encodes the cell surface tyrosine kinase receptor for HGF, is overexpressed in a significant proportion of HCC cases as well as in other human cancers (19, 24, 34–39). Met ligand HGF is known to be produced in various cancer cells (40, 41), and extensive studies suggest that autologous Met activation causes cancer cell growth and invasion (42–44). ERK-MAPK, STAT, and the PI3K-Akt signaling pathway are elicited by Met activation (45). Through ERK-MAPK, HGF stimulates proliferative activity of the HCC cells (46). In contrast, some studies have shown that HGF has an inhibitory effect or no effect on HCC cell growth (47, 48). In keeping with these studies, we found that HGF stimulated neither cell proliferation nor the ERK-MAPK signaling pathway (data not shown) in Hep3B and SK-Hep1 cells. Both DCP and HGF bound to Met and caused its autophosphorylation. HGF induced full phosphorylation of the Met autophosphorylation site, including $Tyr^{1234/1235}$ and Try^{1349}, whereas DCP only stimulated $Tyr^{1234/1235}$. This partial phosphorylation may cause a different biological effect on cell proliferation. Although the biological response through a single receptor that binds to different ligands is usually indistinguishable, some receptors reportedly can discriminate between ligands and induce different biological responses (49, 50). We found, however, that DCP did not activate the ERK-MAPK signaling pathway. Thus, further study is needed to elucidate ligand discrimination in the autophosphorylation and biological response of the Met receptor.

In searching for the downstream signaling pathway of DCP, the JAK-STAT signaling pathway was found to be a promising candidate. A wide variety of extracellular signals activate the STAT class of transcription factors. Many cytokines, lymphokines, and growth factors signal through a related superfamily of cell surface receptor tyrosine kinases that are associated with the JAK-STAT signaling pathway. It was previously reported that HCC cell proliferation is activated by STAT3 signals (51), and STAT3 activation has been reported in various cancer tissues (52, 53). The HBx protein from the hepatitis B virus (54) and hepatitis C virus core protein may activate STAT3 (55). This is important because STAT3 signaling is generally considered to be involved in carcinogenesis and cancer development, including HCC. We also show in the present study that STAT3 played a major role in DCP-induced cell proliferation. Both prothrombin and HGF induced phosphorylation of STAT3 in Western blot analysis, but they did not activate STAT3-induced transcription or cell proliferation. This discrepancy may be caused by a Ser^{727}-independent STAT3 regulatory mechanism. Although two phosphorylation sites (Tyr^{705} and Ser^{727}) are reported to play a major role in STAT3 activation (56–59), other studies show that Ser^{727} phosphorylation causes an inhibitory effect on STAT3-induced transcription (60–62). The effect of Ser^{727} phosphorylation of STAT3 is varied depending on the status of other cells (63, 64). RNA interference for STAT3 and STAT3 inhibitor peptide were utilized to confirm whether STAT3 was the key molecule in the DCP signaling pathway. Both STAT3 inhibitor peptide and siRNA against STAT3 abrogated DCP-induced cell proliferation as well as STAT3-induced transcription (Figs. 4 and 5). Thus, the JAK1-STAT3-pathway is considered to be a major signaling pathway for DCP stimulation.

Other Met-related signaling pathways were not affected by DCP stimulation. PI3K-Akt signaling is known to promote survival under apoptotic stress (65), but DCP did not significantly affect this signaling pathway. Furthermore, neither the expression nor phosphorylation of c-Myc was affected by DCP stimulation.

In the current study we demonstrate a novel autocrine/paracrine mechanism of DCP-positive HCC *in vitro* in which the DCP-Met-STAT3 signaling pathway becomes a capable therapeutic target to inhibit the development of HCC. Further *in vivo* studies are necessary to validate this mechanism.

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Cytokine gene polymorphisms in Japanese patients with hepatitis B virus infection—association between TGF-β1 polymorphisms and hepatocellular carcinoma

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***Background/Aims*: In this study, we determined the frequencies of the genotypes associated with the polymorphism of the cytokines genes, and investigated their association with the risk of hepatocellular carcinoma (HCC) in hepatitis B virus (HBV) carriers.**

***Methods*: Genetic polymorphism in the cytokines TNF-α, IFN-γ, TGF-β1, IL-6, and IL-10 were studied in 236 Japanese patients with HBV infection. The genetic polymorphisms of these cytokines were analyzed by polymerase chain reaction-sequence-specific primer (SSP).**

***Results*: There was no statistically significant difference in the genetic polymorphisms of TNF-α, IFN-γ, and IL-10 genes between HBV carriers with HCC and those without HCC. However, the TGF-β1+29 (codon 10) C/C genotype was lower in HBV carriers with HCC than in those without HCC (HCC 14.6% vs non-HCC 31.9%). The association of HCC was significantly lower in HBV carriers with C/C genotype than in those with T/C or T/T genotype in position + 29 of the TGF-β1 gene.**

***Conclusions*: Our findings suggest that the genetic polymorphism in codon 10 of the TGF-β1 gene may play a role in HCC development in patients with chronic HBV infection.**

***Keywords*: Cytokines; Hepatitis B virus; Hepatocellular carcinoma; Polymorphism; Transforming growth factor-β1**

1. Introduction

Hepatitis B virus (HBV) infection is a major cause of progressive liver disease such as chronic hepatitis and liver cirrhosis in most industrialized countries [1]. An association between HBV infection and hepatocellular carcinoma (HCC) has also been established [2]. The factors involved in the progression of chronic HBV infection to HCC require investigation. The risk for developing HCC increases with severity of inflammation and fibrosis [3]. However, the host genetic factors that affect the HCC association remain unclear. A strong genetic component determining the outcome of HBV infection has been suggested in family studies [4]. Cytokines, as the product of host responses to inflammation, play an important role in the defense against viral infections and carcinogenesis [5]. An individuals' capacity for cytokine production has a major genetic component, and the variation among individuals can be striking [6,7]. This variation has been considered to be associated with polymorphisms within the promoter lesion or signal sequence of cytokine genes [8]. For example, the promoter of the IL-10 gene contains three biallelic polymorphisms at positions −1082, −819, and −592 from the transcription start site, and these influence the capacity to produce IL-10 [9]. These genetic polymorphisms may

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affect the development of inflammation, fibrosis, and carcinogenesis. The aim of the present study was to characterize cytokine gene polymorphisms in chronic HBV infection and their associations with HCC in a Japanese population.

2. Patients and methods

2.1. Patients

Of consecutive Japanese patients with chronic HBV infection who consulted the outpatient clinic of the National Nagasaki Medical Center and Nagasaki University Hospital between 2000 and 2004, we studied 236 patients. They were regularly followed with measurements of serum ALT and HBV markers such as HBsAg, HBeAg, and anti-HBeAb using commercially available radioimmunoassay kits (Dainabot, Tokyo, Japan) every month, and ultrasonography or computed tomography of the liver every 3 months. All patients were positive for HBsAg and did not have any other types of liver diseases such as chronic hepatitis C, alcoholic liver diseases, autoimmune liver diseases, or metabolic liver diseases. Total 236 patients were divided into two groups; patients with HCC and without HCC. The following clinical parameters of patients were obtained at the time of whole blood collection; age, gender, serum alanine aminotransferase (ALT) levels, and platelet counts. The diagnosis of HCC was made by several imaging modalities and confirmed histologically by sonography-guided fine-needle biopsy specimens in all patients.

The study protocol was approved by the Ethics Committees of both National Nagasaki Medical Center and Nagasaki University Hospital and informed consent was obtained from each individual.

2.2. DNA extraction

Genomic DNA was isolated from whole blood using the QIAamp DNA blood protocol according to the manufacturer's instruction (Qiagen Ltd, UK).

2.3. PCR sequence-specific primer typing

Single nucleotide mutations were analyzed in five different cytokines, leading to the genotype and phenotype assignment (Table 1)

In PCR sequence-specific primer (SSP) typing, oligonucleotide primers were designed to obtain amplification of specific alleles or groups of alleles. This typing method is based on the principle that a completely matched primer will be used more efficiently in the PCR reaction than with a primer with one or more mismatches. This means that the specificity of the typing system is a part of the PCR reaction. Assignment of alleles is then based on the presence or absence of amplified product detected by agarose gel electrophoresis. In this study, PCR amplification of selected TNF-α, TGF-β1, IL-10, IL-6, and IFN-γ alleles and an internal control, the human β-globulin gene were carried out according to the manufacturer's instruction (One Lambda, VH Bio Ltd, Gateshead, Tyne and Wear, UK). Briefly, after addition of the appropriate primer pairs, salts, buffer, and Taq polymerase, the samples were subjected to PCR in a 9600 Perkin-Elmer Thermocycler. The sequences of the primer pair have been described previously [9]. Amplification conditions were 1 cycle of 130 s at 96 °C dropping at 62 °C for an additional 60 s, nine cycles of 10 s at 96 °C and 60 s at 63 °C, and then the final 20 cycles included a three-temperature ramp annealing for 10 s at 96 °C, 50 s at 59 °C and finally 30 s at 72 °C. The amplified products were then separated by agarose gel electrophoresis and visualized by staining with ethidium bromide and exposure to ultraviolet light. Interpretation of PCR results was based on the presence of the internal control band together with the presence or absence of a specific amplified fragment.

2.4. Statistical analysis

Results are expressed as mean ± SD. Comparisons were made by Student's *t* test, Fisher's extract probability test, and the χ^2 test. All *P* values were two-tailed, and *P* values <0.05 were considered to indicate statistical significance.

3. Results

3.1. Patient characteristics

As shown in Table 2, there was no significant difference in gender and serum ALT levels between HBV carriers with HCC and those without HCC. In HBV carriers with HCC, age and proportion of cirrhosis were higher and platelet counts were lower than those without HCC.

Table 1
Characteristics of the cytokine gene polymorphisms

Gene	Posltion of the polymorphism	Allele	Haplotype	Phenotype	Reference
TNF-α	Promoter −308	A,G	A/A	High	[9]
			G/A	High	
			G/G	Low	
TGF-β	Codon 25	C,G	G/G	High	[9,10,11]
			G/C	Intermediate	
			C/C	Low	
IL-10	Promoters −1082; −819 and −592	A,G;T,C and A,C	GCC/GCC	High	[9]
			GCC/ACC	Intermediated	
			GCC/ATA	Intermediate	
			ACC/ACC	Low	
			ACC/ATA	Low	
			ATA/ATA	Low	
IL-6	Promoter −174	C,G	G/G	High	[12]
			G/C	High	
			C/C	Low	
IFN-γ	Intron +874	T,A	T/T	High	[9]
			T/A	Intermediate	
			A/A	Low	

TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; IL-10, interleukin-10;IL-6, interleukin-6; INF-γ, interferon-γ.

Table 2
Baseline characteristics of HBV carriers

	Total HBV ($n=236$)	Without HCC ($n=188$)	With HCC ($n=48$)	Statistical analysis
Mean age (yr)	53.7±15.2	51.5±15.6	62.5±8.9	$P<0.001$. Student's t-test
Sex (M/F)	160/76	127/67	39/9	NS, χ^2
Mean ALT (U/L)	70.4±104.2	73.6±112.2	57.8±63.8	NS, Student's t-test
Mean PLT ($10^3/\mu l$)	158.2±71.8	169.7±63.7	119.2±83.8	$P<0.001$. Student's t-test
Mean albumin (g/dl)	4.3±.6	4.4±0.5	3.6±0.6	$P<0.001$. Student's t-test
Cirrhosis (%)	45.8	34.6	91.7	$P<0.001$. χ^2

HCC, hepatocellular carcinoma; ALT, alanine aminotransferse; PLT, platelet; NS, not significant.

3.2. Polymorphism in the TNF-α and IFN-γ genes

There was no polymorphism at the position +74 (codon 25) of the TGF-β1 gene and the position of -174 of the IL-6 gene in Japanese population studied. In the genotype frequencies at position −308 of the TNF-α genes and position of +874 of the IFN-γ gene, no statistically significant difference was found between HBV carriers with HCC and those without HCC (Table 3).

3.3. IL-10 gene promoter polymorphisms in hepatitis B virus carriers

We examined the three biallelic polymorphisms in the IL-10 gene promoter, at positions −1082, −819, and −592 from the transcription start site, respectively, which produce three different haplotypes: GCC, ACC, and ATA. The genotype frequencies are shown in Table 3, and the haplotype frequencies in Table 4. The frequencies of the ACC haplotype were increased in HBV carriers with HCC (33.3%) compared to those without HCC (30.3%), though the difference was not statistically significant.

3.4. TGF-β1 gene codon 10 polymorphisms

The distributions of the genotype of the polymorphism at the position +29 (codon 10) in HBV carriers with or without HCC are shown in Table 3. The genotype distributions were different in HBV carriers with HCC and those without HCC. In HBV carriers without HCC, the genotype frequencies were 18.6% for T/T, 49.5% for T/C, and 31.9% for C/C. In HBV carriers with HCC, on the other hand, the genotype frequencies were 22.9% for T/T, 62.5% for T/C, and 14.6% for C/C. As shown in Table 5, the association with HCC was significantly lower in HBV carriers with C/C genotype than in those with T/T or T/C genotype ($P=0.028$, odds=0.36, 95% CI; 0.154–0.859). Associations between cytokine gene polymorphism and the development of virus hepatitis were also reported. Ben-Air et al. reported that A/A genotype in the position +874 of IFN-γ gene was associated the development of HBV infection [13]. Gewaltig et al., reported that the presence of proline at TGF-β1gene codon 10 (C/C or C/T genotype) was associated with higher stage of fibrosis in HCV-infected patients [11]. Therefore, we assessed the genetic polymorphism of TGF-β1gene codon10 and the position +874 of IFN-γ gene in HBV carriers with or without liver cirrhosis (LC). As shown in Table 6 and 7, there was no significant association between these genes polymorphisms and the presence of LC.

Table 3
Genotype frequencies in patients with HBV carriers

Variables	Patients with HBV		
	Total ($n=236$) (%)	Without HCC ($n=188$) (%)	With HCC ($n=48$) (%)
TNF-α			
G/G	230(97.5)	183(97.3)	47(97.9)
G/A	6(2.5)	5(2.7)	1(2.1)
A/A	0	0	0
TGF-β1-10			
C/C	67(28.4)	60(31.9)	7(14.6)
T/C	123(52.1)	93(49.5)	30(62.5)
T/T	46(19.5)	35(18.6)	11(22.9)
TGF-β1-25			
G/G	236(100)	188(100)	48(100)
G/C	0	0	0
C/C	0	0	0
IL-10			
ATA/ATA	102(43.2)	85(45.2)	17(35.4)
ACC/ATA	91(38.6)	72(38.3)	19(39.6)
ACC/ACC	25(10.6)	19(10.1)	6(12.5)
GCC/ATA	10(4.2)	6(3.2)	4(8.3)
GCC/ACC	5(2.1)	4(2.1)	1(2.1)
GCC/GCC	3(1.3)	2(1.1)	1(2.1)
IL-6			
G/G	236(100)	188(100)	48(100)
G/C	0	0	0
C/C	0	0	0
IFN-γ			
A/A	198(83.9)	157(83.5)	41(85.4)
T/A	38(16.1)	31(16.5)	7(14.6)
T/T	0	0	0

Note. The genotypes are shown as frequency (percentage). Abbreviations: HBV; hepatitis B virus, HCC; hepatocellular carcinoma, TNF-α; tumor necrosis factor-α, TGF-β; transforming growth factor-β, IL-6; Interleukin-6, IL-10; interleukin-10, IFN-γ; interferon-γ.

Table 4
Haplotype frequency distributions of IL-10 gene promoter in HBV carriers

Haplotype (−1082/−819/−592)	Without HCC	With HCC
ATA	0.660	0.594
ACC	0.303	0.333
GCC	0.037	0.073

4. Discussion

In this study, we investigated the cytokine genes polymorphisms and determined whether these genetic factors are related to the occurrence of HCC in a Japanese population infected with HBV. Our results showed that the risk of HCC was significantly lower in HBV carriers with TGF-β1 codon 10 C/C genotype than in those with T/C or T/T genotype. A previous study demonstrated that the TGF-β1 polymorphism at codon 25 is associated with the progression of fibrosis in chronic HCV infection [14]. However, no polymorphism was found at codon 25 of the TGF-β1 gene in a Japanese population. These findings raise the possibility that the polymorphism of codon 10 in the TGF-β1 gene may play a role in determining the susceptibility to HCC of HBV-infected patients.

TGF-β1 is a pluriopotent cytokine that is potentially linked with fibrosis and neoplasm in the liver [15]. It is well established that this cytokine promotes hepatic fibrosis by stimulating the synthesis of the extracellular matrix [16]. TGF-β1 induces the activation of hepatic stellate cells to myofibroblasts, which is considered to be a crucial biological step in liver fibrogenesis [17]. TGF-β1 is also implicated in carcinogenesis. In normal cells, TGF-β1 acts as a tumor suppressor by inhibiting cellular proliferation or by promoting cellular differentiation and apoptosis [18]. In contrast, the expression of TGF-β1 appears to be increased in cancer cells [19]. Its impact on the initiation or progression of neoplasm is controversial. This is due to the large parts of the multiple actions of TGF-β [20]. In vitro studies have shown that the increased activity in the TGF-β1 pathway leads to tumor inhibition in most mammary cell lines [21]. Transgenic mice with a single gene deletion of TGF-β1 are more susceptible to liver tumors induced by carcinogens [22].

Table 5
Differential distribution of TGF-β1 genotype in HBV carriers

Locus	Genotype	Without HCC	With HCC	OR (95% CI)	P
TGF-β1 codon 10	T/C, T/T	128 (68.1%)	41 (85.4%)	–	
	C/C	60 (31.9%)	7 (14.6%)	0.36 (0.154–0.859)	0.028

OR, odds ratio; 95% CI, 95% confidential interval.

Table 6
Distribution of TGF-β1 genotype in HBV carriers with or without LC

Locus	Genotype	Without LC	With LC	OR (95% CI)	P
TGF-β1 codon 10	T/T	19 (14.8%)	27 (25.0%)	–	
	T/C, C/C	109 (85.2%)	81 (75.0%)	0.52 (0.27–100)	0.072

OR, odds ratio; 95% CI, 95% confidential interval.

The T to C transition at position +29 the TGF-β1 gene results in a change from leucine to proline at codon 10. The presence of proline rather than leucine in the hydrophobic region of the signal sequence is thought to affect the export efficiency of the newly synthesized protein [23]. In fact, the C/C genotype at position +29 in a Japanese population was found to be associated with higher serum levels of TGF-β1 than T/T or T/C genotype [24]. Clinical studies indicated that the C/C genotype at the +29 position of the TGF-β1 gene is associated with a reduced risk of breast cancer [25]. These findings indicate that the TGF-β1-induced suppression of oncogenesis could be augmented by the increased TGF-β1 levels resulting from these genetic factors. Our data suggest that the increased serum levels of TGF-β1 in subjects with the C/C genotype in codon 10 may contribute to the suppression of hepatic tumorigenesis and lead to the lower risk of HCC. Originally, TGF-β1 gene polymorphism at codon 10 was reported to be associated with the progression of liver fibrosis. Gewaltig et al., demonstrated that the presence of proline at TGF-β1 gene codon 10 (C/C or C/T genotype) was associated with higher stage of fibrosis in HCV-infected patients [11]. However, there was no statistically significant association between this TGF-β1 gene polymorphism and the presence of LC in our data. Our data are consistent to those of Powell et al. [14] demonstrating no association between TGF-β1 gene codon 10 polymorphism and the stage of fibrosis in HCV-infected populations. Also, this discrepancy could be attributable to the differential genetic background of investigated populations and the difference of HCV and HBV infection. More recently, Kim et al. reported that the risk of HCC was lower in Korean HVB carriers with C/C or T/C genotypes at the +29 position of the TGF-β1 gene than those with T/T genotype [26]. In our study, the presence of C/C genotype was associated with

Table 7
Distribution of IFN-γ1 genotype in HBV carriers with or without LC

		Without LC	With LC	OR (95% CI)	P
IFN-γ+ 874	T/A	18 (14.1%)	20 (18.5%)	–	
	A/A	110 (85.9%)	88 (81.5%)	0.72 (0.35–1.44)	0.35

OR, odds ratio; 95% CI, 95% confidential interval.

a reduced risk with HCC, however, the presence of C/C or T/C genotypes was not associated with the risk of HCC significantly. This discrepancy may be due to the different ethnic populations studied.

Previous results in cohort studies demonstrated that advanced age and liver function impairment have been associated with a higher HCC incidence in patient with virus hepatitis [27,28]. Consistent with these findings, the age and the presence of LC were significantly higher in HCC groups compared to those of non-HCC groups in our study. Therefore, TGF-β1 genotype could be one of the factors influencing the development of HCC in addition to these major factors.

IL-10, produced mainly by macrophages, is a potent immunosuppressive cytokine that down-regulates the Th1 cytokines [29]. The greater susceptible effects of IL-10 haplotype on chronic hepatitis B progression were demonstrated. We previously reported that the frequencies of ACC haplotype of IL-10 were higher in progressive HBV carriers than in asymptomatic carriers [30]. Shin et al. reported that the IL-10-ACC haplotype showed a strong association with the occurrence of HCC [31]. We found that the frequency of the ACC haplotype appears to be increased in HCC patients, though the statistical difference, compared with that in non-HCC patients, was not significant. Further studies are needed to determine the association between the IL-10 haplotype and the HCC occurrence.

In summary, we found that the presence of the TGF-β1 C/C genotype at codon 10 was associated with a reduced risk of HCC occurrence in patients with HBV infection. These data suggest that the TGF-β1 polymorphism is one of the genetic factors affecting hepatic carcinogenesis in patients with HBV infection.

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