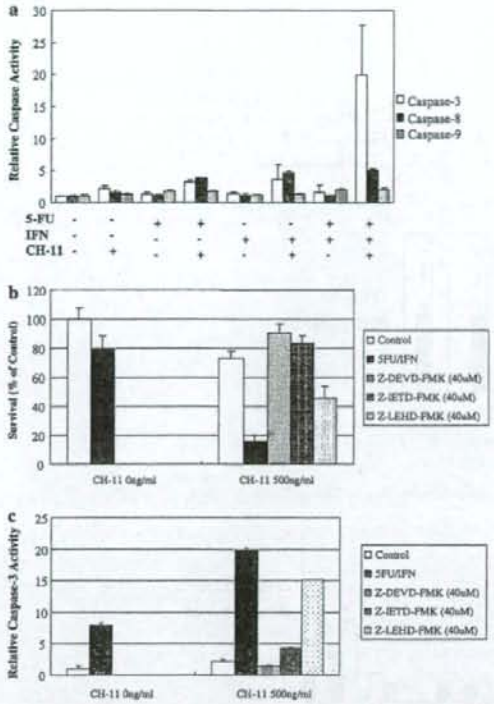

Fig. 5. (a) Activation of caspase-3, -8 and -9 assessed using the Caspase Colorimetric Protease Assay Kit. Data are given relative to results for untreated HepG2 cells. The activity of caspase-3 after 12 h CH-11 (500 ng/ml) treatment was significantly increased by IFNα (500 U/ml) and 5-FU (0.5 μg/ml) alone, but the maximal effect of the CH-11 antibody was observed with the combination of IFNα and 5-FU. (b) Apoptosis blocking effect of specific caspase inhibitors (40 μM) was examined by 48 h MTT assay. Z-DEVD-FMK and Z-IETD-FMK almost totally blocked CH-11 induced apoptosis enhanced by IFNα/5-FU. (c) Colorimetric caspase assay using specific caspase inhibitors (40 μM) was performed. Z-DEVD-FMK inhibited caspase-3 activation induced by CH-11 and IFNα/5-FU. Experiments were performed three times independently. Results represent mean values of three experiments ± SD.

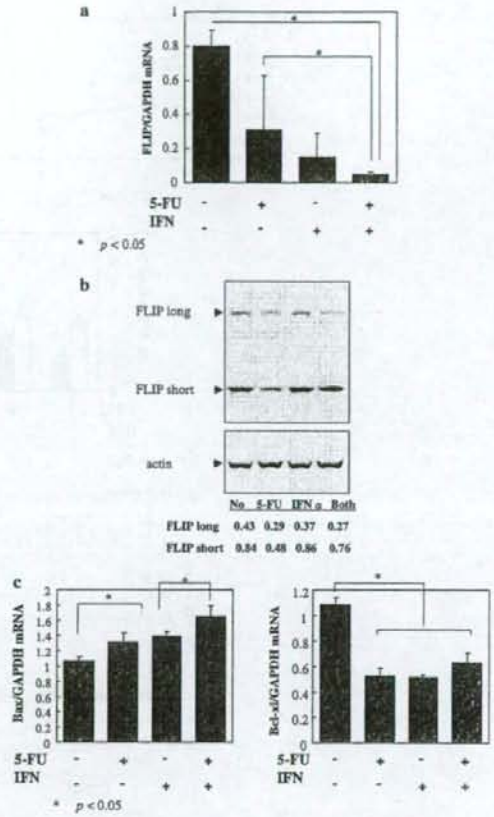

Fig. 6. (a) Expression of FLIP mRNA by RT-PCR. Combination of IFNα (500 U/ml) and 5-FU (0.5 μg/ml) significantly decreased the expression of FLIP mRNA. (b) Western blot analysis of FLIP. Expression of FLIP was decreased by IFNα (500 U/ml) and 5-FU (0.5 μg/ml) in the protein level. (c) Expression of apoptotic factors at mRNA level. Results represent mean values of three experiments ± SD.

and saw an increase in released ^{51}Cr and maximum interaction between effecter cells and target cells. This enhancement was markedly blocked by ZB4 (Fig. 7e).

3.9. Influences of IFNα/5-FU to the normal hepatocyte

MTT assay and TUNEL assay were performed using non-tumorigenic SV40-immortalized human liver epithelial cell line (THLE-2). CH-11 inhibited the cell growth of THLE-2 in the dose-dependent manner (Fig. 8a). But IFNα/5-FU did not affect this growth inhibiting effect (Fig. 8b); and TUNEL assay showed IFNα/5-FU did not increase the CH-11 related apoptosis at all (Fig. 8c). These results suggested that THLE-2 has the sensitivity to Fas/FasL-mediated apoptosis, IFNα/5-FU did not enhance that effect. Concerning these results, TUNEL staining of resected liver specimens was performed. The patients of these specimens received IFNα and 5-FU therapy before the surgery. In the normal tissue, TUNEL-positive cells were very few (Fig. 8, i and iii); there were many TUNEL-positive cells in the tumor site (Fig. 8, ii and iv), as shown in Fig. 8.

4. Discussion

Fas (CD95/Apo-1) belongs to the tumor necrosis factor receptor family of proteins that are expressed at the cell surface in various normal and neoplastic cells [24–

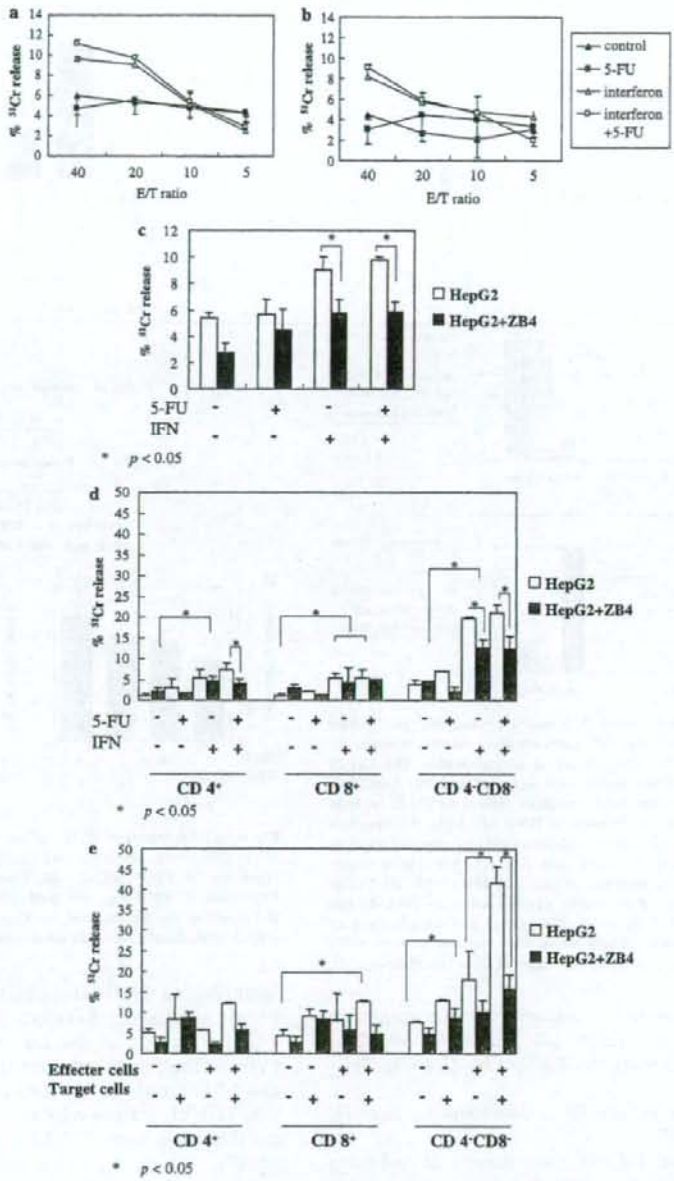

Fig. 7. Cytotoxicity of PBMCs assessed by the 8-h ^{51}Cr-release assay. (a) HepG2 cells were used as target cells. PBMCs stimulated with IFNα (500 U/ml) and/or 5-FU (0.5 μg/ml) for 24 h were used as effecter cells at *E/T* ratios as indicated, without neutralizing anti-Fas antibody, ZB4. (b) The target cells were pretreated with ZB4 (500 ng/ml). (c) Results with an *E/T* ratio of 20. Open bar, without ZB4. Solid bar, with ZB4. (d) $CD4^+$ cells, $CD8^+$ cells, and $CD4^-CD8^-$ cells purified from PBMCs using magnetic sorting were used as effecter cells at an *E/T* ratio of 20. (e) Effecter or target cells or both were pretreated with IFNα (500 U/ml) and 5-FU (0.5 μg/ml) for 24 h and an 8-h ^{51}Cr-release assay was performed. All data represent means ± SD values of at least triplicate samples. Similar results were observed in three independent experiments.

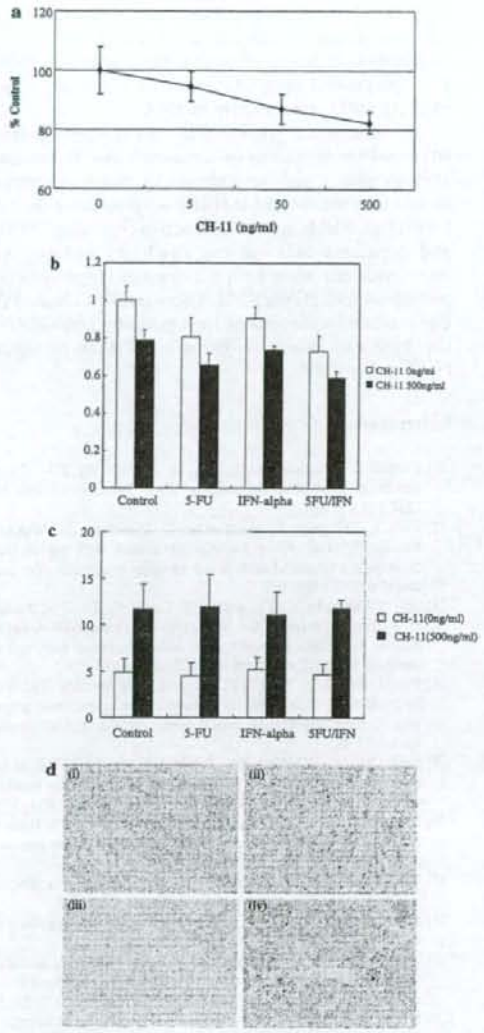

Fig. 8. Hepatotoxicity of IFNα/5-FU was examined using normal hepatocyte cell line THLE-2 and resected human samples. (a) THLE-2 showed sensitivity to CH-11 in the dose-dependent manner in 48 h MTT assay. (b) THLE-2 were incubated with IFNα (500 U/ml) and/or 5-FU (0.5 μg/ml) and with agonistic anti-Fas monoclonal antibody CH-11 (500 ng/ml) (□) or without CH-11 (■) for 48 h. THLE-2 showed sensitivity to IFNα and/or 5-FU or CH-11, but no synergistic effect was observed. (c) TUNEL assay was performed in the same condition with MTT assay. Apoptotic cells were not increased with the stimulation of IFNα and/or 5-FU (d) TUNEL staining of resected samples who received IFNα/5-FU therapy preoperatively (original magnification 100). (i) Normal liver tissues; (ii) tumor site of the specimen; (iii and iv) normal and tumor site of the other patient. TUNEL-positive apoptotic cells were only seen in the tumor. There were very few apoptotic cells in the normal liver. [This figure appears in colour on the web.]

26]. Fas is a 45-kDa type I membrane protein receptor that induces apoptosis by triggering a cascade of caspases following ligation with the Fas Ligand (FasL) on the cell surface of T-cells and NK cells, and plays a major role in T-cell-mediated cytotoxicity [27,28]. Fas/FasL signaling also acts to enhance the effect of many chemotherapeutic drugs against various neoplastic cells, including bleomycin, cisplatin, methotrexate, adriamycin, carboplatin and 5-FU [29–33], and Jiang et al. [34] showed that 5-FU increased Fas/FasL pathway-mediated apoptosis in HCC. However, the mechanism by which apoptosis is induced or enhanced by chemotherapeutic agents remains unclear. In the present study, we confirm the apoptotic effect induced synergistically by IFNα and 5-FU via the Fas/FasL pathway in HCC cells in vitro.

In the first step of this study, we investigated the cell growth response of HCC cells treated with IFNα and/or 5-FU. MTT and annexin-V assays showed that the combination of IFNα and 5-FU significantly increased the sensitivity to Fas-mediated apoptosis in three Fas-positive cell lines, and to a greater extent than either drug used alone. This synergy was not mechanistically related to the level of cell surface Fas expression after treatment, although there was good correlation between the level of constitutive cell surface Fas expression and the extent of Fas-mediated apoptosis. The suggestions of Yano et al. [35] may provide an explanation for these results: (i) protective proteins inhibit apoptosis; (ii) intracellular Fas signaling activation does not happen; and, (iii) regulatory mechanisms exist to enhance Fas-mediated apoptosis other than Fas upregulation [35]. Activated caspase-3 is either partially or totally responsible for cleaving the non-caspase death substrates, which eventually leads to cellular and nuclear morphological changes and ultimately to cell death [25,26]. We speculate here that IFNα and 5-FU affected some apoptotic factors thereby regulating caspase-3 and -8. In addition, these caspases are activated by FLIP, a potent inhibitor of Fas-mediated apoptosis, and regulator of the proteolytic cleavage of caspase-8, based on our results [21,22,36]. Our data also indicated that the increase in caspase-3 expression was more profound than that of caspase-8. This difference might be explained by the regulation of IAPs (inhibitors of apoptosis proteins). IAPs are known to block apoptosis induced by a wide spectrum of triggers, and recent studies have shown that IFNα downregulates IAPs [36–40]. Moreover, PCR array analysis using clinical HCC samples revealed that the BIRC4 gene, which encodes IAP, was significant in predicting the response to IFNα/5-FU therapy [41]. These findings therefore suggest that caspase-8, caspase-3 and FLIP might interact to induce Fas-mediated apoptosis after IFNα/5-FU treatment.

The ^{51}Cr-release assay demonstrated that IFNα markedly induced cytotoxicity of PBMCs against the

HCC cells, and particularly the $CD4^-CD8^-$ cells. Our previous report revealed that $CD56^+$ NK cells and $CD14^+$ monocytes were the major effecter cells involved in the IFNα-induced cytotoxicity of PBMCs against HCC cells in this immunological process [16]. IFNα increases FasL expression on PBMCs and upregulates the immune response [42], and several investigators have reported FasL on the surface of $CD4^+$ cells, $CD8^+$ cells and NK cells. In fact, IFNα induced greater cytotoxicity in the $CD4^-CD8^-$ cells than in either the $CD4^+$ cells or $CD8^+$ cells. The data also indicated that both $CD4^+$ cells and $CD8^+$ cells reacted 2-fold to IFNα stimulation. In addition, pretreatment of HCC cells increased their sensitivity to Fas-mediated apoptosis, suggesting that (i) Fas-mediated cytotoxicity is enhanced by IFNα in innate immune effecter cells, particularly NK cells and monocytes, and (ii) Fas sensitivity of HCC cells regulated by IFNα/5-FU leads to increased cytotoxic interaction compared to regulation by either drug alone.

As described above, Fas/FasL may contribute to the anti-cancer effect of IFNα/5-FU via a tumor immune response, but we consider that such a mechanism only partially explains the anti-tumor activity of combination therapy. This is because the Fas-negative cell lines (HuH7, PLC/PRF/5 and Hep3B) showed no response to CH-11 induction, and IFNα/5-FU did not affect the Fas-mediated apoptosis. Our previous report showed some Fas-negative cell lines respond in other ways to IFNα/5-FU treatment [15–17,43]. In PLC/PRF/5, which express a high level of IFN receptor, IFNα/5-FU acted mainly through the direct cell arrest effect of IFNα [15,43]. Also, HLE, a responder to Fas-mediated apoptosis, also underwent cell death via a TRAIL/TRAIL-R pathway after IFNα/5-FU stimulation [16]. Thus, the therapeutic effect of IFNα/5-FU against HCC might be mediated via various pathways according to the specific characteristics of the cancer cells. This might also explain why the response rate to IFNα/5-FU is not complete, being almost 50% in HCC treatment [10,11,13].

Finally, the influence of IFNα/5-FU combined therapy to normal hepatocytes was evaluated in terms of apoptosis mediated by Fas/FasL system. Several investigators have reported that normal hepatocytes express Fas on their surface and that is cause of fulminant hepatitis with administration of agonistic anti-Fas antibody [35,44–47]. Although the results of in vitro assays revealed that normal hepatocyte has sensitivity to agonistic anti-Fas antibody in the present study, no enhancement was induced by the IFNα/5-FU about apoptosis in Fas/FasL system. On the other hand, the apoptotic effect of FasL was significant in the all Fas-positive HCC cells. In addition, TUNEL staining of resected human samples who received IFNα/5-FU therapy before operation also showed that apoptotic cells were only counted in the tumor site; no apoptotic cells in normal liver. These results suggested that IFN/5-FU did not enhance the cell death because of the uncertain mechanism of the escape from the apoptotic system of Fas/FasL pathway in normal hepatocyte and IFN/5-FU upregulated apoptotic effect of Fas/FasL system, showing anti-tumor activity in HCC.

In conclusion, IFNα and 5-FU synergistically enhanced the sensitivity of hepatoma cell to Fas-mediated apoptosis with an increase in caspase-3 activity. In addition, we showed that IFNα upregulates the cytotoxicity of PBMCs, and interactions between PBMCs and hepatoma cells via the Fas/FasL pathway were most enhanced when both effecter and target cells were pretreated with IFNα/5-FU. These results indicated that Fas-mediated apoptosis at least partially contributes to the beneficial effect of IFNα/5-FU therapy against HCC in the clinic.

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Patterns and clinicopathologic features of extrahepatic recurrence of hepatocellular carcinoma after curative resection

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Background. *Little is known about the metastatic pattern in patients with extrahepatic metastasis after the removal of primary hepatocellular carcinoma (HCC). The aim of the present study was to determine the clinicopathologic characteristics and prognosis of patients with extrahepatic metastasis from HCC according to the recurrence pattern.*

Methods. *Among the patients who underwent hepatic resection for HCC between 1981 and 2001, 80 patients had no recurrence; 221 patients had intrahepatic recurrence, and 47 patients experienced extrahepatic metastasis within a mean follow-up period of 4.8 ± 3.7 years (±SD; range, 2-15 years). The pattern of extrahepatic metastasis after hepatic resection was divided into pattern I (first recurrence in the liver and then spread outside the liver after repetitive intrahepatic recurrences and repetitive locoregional treatments), pattern II (simultaneous recognition of intrahepatic and extrahepatic recurrences), and pattern III (extrahepatic, but no intrahepatic, lesions at first recurrence).*

Results. *There were significant differences in proportions of patients with invasion of the portal vein, hepatic vein, or inferior vena cava, intrahepatic metastases, and tumor stage between patients with intra- and extrahepatic metastases. The disease-free survival and extrahepatic metastasis-free survival in pattern I were better than pattern II. Survival after extrahepatic metastasis did not correlate with the 3 patterns.*

Conclusion. *Although long-term overall survival was better in patients with pattern I of extrahepatic recurrences, prognosis was poor in all patterns once extrahepatic metastasis developed.*
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HEPATOCELLULAR CARCINOMA (HCC) IS A common malignancy worldwide and is now the third major cause of cancer-related death in Japan.[1] With the recent progress in diagnostic modalities, pre- and postoperative treatment, and operative techniques, more patients have become suitable candidates for hepatic resection.[2-6] Consequently, the results of operative treatment of HCC have been improving steadily. The long-term outcome of patients with HCC, however, is still poor because of the high incidence of recurrence even after curative resection.[7-9] Although intrahepatic recurrence predominates, probably because of the early spread of neoplasm and metachronous multicentric carcinogenesis, several effective therapeutic modalities can control recurrent disease (such as repeated hepatectomy, transcatheter arterial embolization (TAE), percutaneous ethanol injection therapy, microwave coagulation therapy, and radiofrequency ablation).[10-15] Compared with the frequent intrahe-

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patic recurrence, the incidence of distant metastases is relatively low in HCC.[10,16,17] Recently, with progress in the control of intrahepatic lesions, the survival rate after hepatectomy for HCC has improved steadily.[18-22] Longer survival, however, may lead to an increased incidence of extrahepatic metastasis. Because of this possibility and potentially to improve the prognosis after hepatectomy for HCC, we studied the characteristic features and predictors and the potential strategy to deal with extrahepatic metastases. In contrast, once distant metastases occur, effective treatment is limited, except for some selected situations,[8,10] although prolonged survival is sometimes noted. It is important to identify these patients and to define the clinicopathologic characteristics of extrahepatic metastasis.

In the present study, we compared the clinicopathologic features of patients with HCC who experienced extrahepatic metastatic lesions after hepatic resection, with patients who were free of such complications or with only intrahepatic recurrence. We divided extrahepatic recurrences into 3 patterns according to the clinical patterns of recurrence after hepatic resection and attempted to clarify the features of extrahepatic recurrence in each pattern.

PATIENTS AND METHODS

Study design. Three hundred forty-eight patients underwent curative hepatectomies in the Department of Surgery, Osaka University Hospital, between 1981 and 2001. A *curative hepatectomy* was defined as an operation in which all neoplasms were removed macroscopically, and there was no residual neoplasm in the remnant liver or abdominal cavity after clinical and ultrasonographic examination. All patients were followed closely after discharge. Serum alpha-fetoprotein (AFP) and the concentration of protein-induced vitamin K absence II[23] were measured monthly; ultrasonography, computed tomography with contrast medium, or both were performed at least every 3 months. When recurrence was suspected, the patient was readmitted for angiography, magnetic resonance imaging, computed tomography–arteriography, and/or computed tomography–arterial portography. Patients were divided into 3 groups: no recurrence, intrahepatic recurrence, and extrahepatic recurrence. The third group was further subdivided into 3 patterns according to the process of HCC recurrence after resection: Pattern I represented patients whose first recurrence was in the liver and in whom extrahepatic metastasis developed later after repetitive locoregional treatments; pattern II included patients in whom both intrahepatic and extrahepatic recurrences were evident when recurrence was identified; and pattern III included patients who had only extrahepatic metastasis without intrahepatic metastases at the time of diagnosis of metastasis.

Clinicopathologic factors. The tumor factors included AFP, protein-induced vitamin K absence II, tumor size, macroscopic classification, formation of capsule, infiltration that involved the capsule, invasion of the portal vein, invasion of the hepatic vein or inferior vena cava, invasion of the bile duct, intrahepatic metastases, tumor staging by tumor node metastasis classification, and Edmondson histologic classification. The host factors were sex, age, hepatic damage, viral status (hepatitis B or C virus), and hepatic cirrhosis.

Survival and treatment. Treatment after extrahepatic metastasis for the 3 patterns was compared. According to the condition of the patients and the sites of metastatic foci, attempts at operative resection, TAE, radiation therapy, chemotherapy, or nontreatment were adopted. The indication for operative intervention was that the patients had a resectable neoplasm without intrahepatic recurrence or that the intrahepatic neoplasm had been well-controlled by TAE, percutaneous ethanol injection, or by other methods. Disease-free survival, extrahepatic metastasis-free survival, overall survival, and survival after the first recurrence and after extrahepatic metastasis were determined.

Statistical analysis. The cumulative survival was compared across the 3 groups. For the extrahepatic recurrence group, the disease-free survival, extrahepatic metastasis-free survival, survival after first recurrence, and survival after extrahepatic metastasis were compared for patterns I, II, and III. The survival rate was calculated by the product limit method of Kaplan-Meier, and the differences in survival rate between the groups were compared with the use of the log rank test. Data are expressed as mean ± SD. A probability value of $<.05$ was considered significant.

RESULTS

Incidence of recurrence. After the initial hepatic resection, 348 patients (men, 286; women, 62; age range, 34-81 years) were followed from 2 to 15 years (mean, 4.8 ± 3.7 years). Among them, 221 patients (64%) experienced intrahepatic recurrences. Extrahepatic metastases were detected in 47 patients (14%), and included lung (31 patients), bone (16 patients), brain (7 patients), adrenal gland (7 cases), and bladder, skin, and lymph node (1 patient each) metastases. The remaining 80 patients

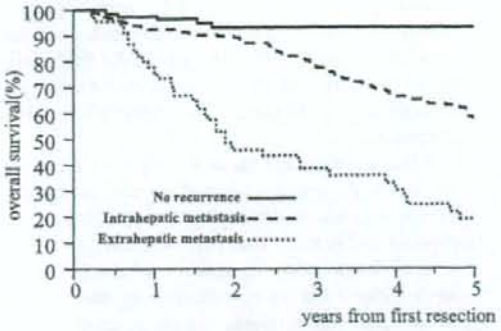

Fig 1. Comparison of overall survival rates after the first resection among the nonrecurrence group, intrahepatic recurrence group, and extrahepatic recurrence group. The overall survival rate of the latter group was worse than those of the other 2 groups ($P < .0001$ each).

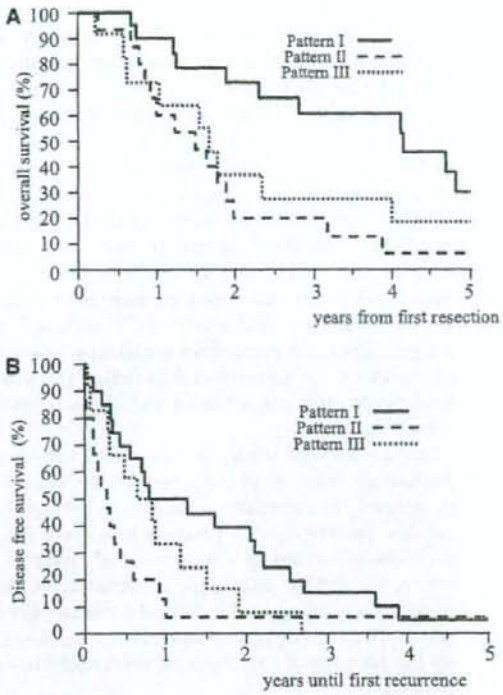

Fig 2. Comparison of (**A**) overall survival rates and (**B**) disease-free survival rates after the first resection among patients with patterns I, II and III. The overall survival rate and disease-free survival rate of pattern I was greater than that of pattern II ($P < .05$ each). There were no significant differences between patterns I and III or between patterns II and III.

(23%) were confirmed to be without recurrence. For the extrahepatic recurrence group, 20 patients were pattern I; 15 patients were pattern II, and 12 patients were pattern III.

Clinicopathologic features of HCC at the initial hepatic resection. The host factors and tumor factors of all patients with HCC in this study were compared . Clinical staging was determined according to the tumor node metastasis classification proposed by The Liver Cancer Study Group of Japan.[24] The host and tumor factors at the time of initial hepatic resection were compared in patients with intra- and extrahepatic recurrence. Metastases were more common in patients with invasion of the portal vein, invasion of the hepatic vein, intrahepatic metastases (microscopic and macroscopic), and advanced tumor stage between the 2 groups. In patients with extrahepatic recurrences, the clinicopathologic factors of patterns I, II, and III were nearly the same, except that the percentages of stages 3 and 4 and the prevalence of intrahepatic metastases were greater among patients with pattern II than with pattern I or pattern III.

Treatment and survival. Only 6 patients with extrahepatic recurrence received locoregional therapies such as operative resection (3 patients; 6%) or TAE (3 patients; 6%). In the present study, 3 patterns of extrahepatic metastasis were evident; the conditions of 3 patients (6.4%) were suitable for operative resection, all of whom had adrenal metastases (pattern I, 1 patient; pattern III, 2 patients). Three other patients with adrenal metastases were treated by TAE. Chemotherapy and radiation therapy were applied in 9 patients (19%) and 6 patients (13%), respectively. More than one half the patients (26 [55%]) could not tolerate any of the therapies that have been mentioned because of their poor condition, and supportive therapy was the only choice.

The overall cumulative survival rate at 5 years was 93% for the group without recurrence, 61% for the intrahepatic recurrence group, and 19% for the extrahepatic recurrence group ($P < .0001$; Fig 1). In the extrahepatic recurrence group, the overall cumulative survival rates at 1 year postoperatively were 90%, 65%, and 73% for patterns I, II, and III, respectively, and then gradually decreased and showed significant differences between patterns I and II (Fig 2, *A*). In the extrahepatic recurrence group, the disease-free survival times were 2.1 ± 0.8, 0.8 ± 0.5, and 0.9 ± 0.2 years, and the extrahepatic metastasis-free survivals were 3.2 ± 0.8, 0.8 ± 0.5, and 0.9 ± 0.2 years for patterns I, II and III, respectively (Figs 2, *B*, and Fig 3). Compared

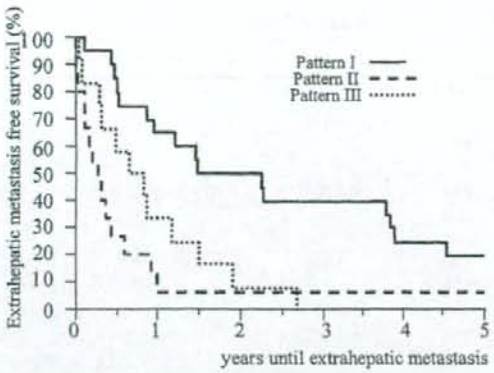

Fig 3. Comparison of extrahepatic metastasis-free survival rates among patterns I, II, and III. The interval until extrahepatic metastasis in pattern I was longer than those of patterns II and III ($P < .05$ each).

with patients with patterns II and III, patients with pattern I had a longer extrahepatic metastasis-free interval. The disease-free survival interval in patients with pattern I was also longer than that of pattern II. There was no significant difference among these 3 patterns with regard to survival after extrahepatic metastasis, except that the survival after the first recurrence in pattern I was greater than that of pattern II. The therapies that were used for patients with extrahepatic metastases, however, did influence outcome. Patients who received locoregional therapy, such as operative resection or TAE, had a longer survival than those who received chemotherapy, radiation therapy, or supportive therapy (nontreatment; 3.6 ± 1.4 years vs 0.9 ± 0.1 years, respectively). There was no difference between the chemotherapy treated and the nontreatment groups. Radiation therapy did not increase survival time, although it relieved pain in patients with bone metastases.

Prognostic clinicopathologic factors. The factors at the time of initial hepatectomy that influenced prognosis after hepatic resection were AFP <400 IU/L ($P < .0001$), absence of portal vein invasion ($P < .0001$), and absence of intrahepatic metastasis ($P < .0001$). These factors were subjected to a multivariate Cox proportional hazard model, and AFP <400 IU/L ($P = .01$), absence of portal vein invasion macroscopically ($P < .01$), and absence of intrahepatic metastasis (both macro- and microscopically; $P = .018$) were independent prognostic factors that influenced overall survival after hepatectomy (Table I).

DISCUSSION

Treatment of extrahepatic metastasis after hepatic resection of HCC has not been pursued actively, because HCC is believed to be an aggressive neoplasm[25]; some investigators consider any extrahepatic metastasis of HCC to be a contraindication for further treatment.[8,9] Relatively few studies discuss the efficacy of treatments for extrahepatic metastasis.[8,26-28] Sasaki et al[26] presented the first report of a patient who underwent successful operative resection for pulmonary and adrenal metastases of HCC. Lam et al[8] reported that resection was effective in 9 patients with lung metastasis. Taniai et al[28] reported several successful patients after resection of adrenal metastases. In selected patients with extrahepatic metastatic lesions, operative resection was effective in controlling the extrahepatic disease and offered the only chance of long-term survival.[8,9] In our series, the 3 patients with adrenal metastases who underwent resection of the adrenal metastasis survived for 5.3 ± 2.9 years. In this context, patients with resectable extrahepatic metastasis were treated aggressively.[8,16,29,30] Unfortunately, most extrahepatic metastases of HCC are multiple and are not amenable to operative resection; only a small number of patients can undergo operative resection. Therefore, further investigation such as finding recurrence at an early stage, establishing a comprehensive follow-up system, and selecting patients who may benefit from a resection of these metastases should be performed to elucidate this issue.

Subsequent intrahepatic recurrences developed after the initial resection of the HCC, which suggests that multicentric carcinogenesis or local intrahepatic metastasis may occur.[31] Although it is difficult to distinguish the precise recurrent pattern because of multicentric HCC as opposed to intrahepatic metastases, the extrahepatic metastases appeared to occur from the intrahepatic metastases and not from multicentric carcinogenesis. Therefore, in the treatment of extrahepatic metastases for any of the patterns, apart from locoregional therapy, prophylactic systemic chemotherapy is recommended. Some reports, however, have shown that systemic adjuvant chemotherapy offers no additional benefit.[32-34] Moreover, our series indicated that the effect of chemotherapy on metastasis was difficult to predict and showed no significant difference when compared with the nontreatment group, which is consistent with other reports.[35] Reports of randomized trials suggested that outcomes could be improved after operative resection of HCC by the use of various modalities including

Table I. Relative risk of overall survival with Cox's proportional Hazard Model

Variable	*Univariant*		*Odds ratio (95% CI)*	P *value*
Tumor size (cm)	N	*P* value		
<3	163	.0842		
>3	181			
AFP (ng/mL)				
<400	253	<.0001	0.65 (0.467-0.904)	.0105
>400	86			
Protein-induced vitamin K absence–II (mAU/mL)				
<400	150	.1500		
>400	79			
Formation of capsule				
+	289	.6926		
−	54			
Infiltration of capsule				
+	151	.1165		
−	177			
Infiltration of the portal vein macroscopically				
+	30	<.0001	0.359 (0.206-0.625)	.0003
−	316			
Infiltration of the hepatic vein macroscopically				
+	5	.0046	0.493 (0.178-1.365)	.1736
−	341			
Infiltration of the bile duct macroscopically				
+	8	.1589		
−	338			
Intrahepatic metastasis macroscopically				
+	96	<.0001	0.586 (0.377-0.91)	.0173
−	248			
Formation of capsule macroscopically				
+	284	.4355		
−	54			
Infiltration of capsule microscopically				
+	208	.0281	1.405 (1.03-1.917)	.0318
−	128			
Infiltration of the portal vein microscopically				
+	86	<.0001	0.913 (0.606-1.375)	.6617
−	260			
Infiltration of the hepatic vein microscopically				
+	5	.0045		
−	340			
Infiltration of bile duct microscopically				
+	10	.1609		
−	336			
Intrahepatic metastasis microscopically				
+	113	<.0001	0.839 (0.535-1.314)	.4423
−	233			

The plus (+) represents yes; the minus (−) represents no.

retinoids, immunologically based approaches, radiotherapy, and combinations.[32] As more is learned about HCC, cytokine networks, and tumor angiogenesis, agents that affect these pathways will also warrant investigation,[34] such as antivascular endothelial growth factor or antiangiopoietin antibodies.

We divided the extrahepatic metastasis into 3 patterns according to the patterns of recurrence after the initial hepatic resection and attempted to define the features of each pattern. Pattern I showed a remarkable disease-free survival and extrahepatic metastasis-free survival compared with pattern II, although no differences were observed in tumor factors and other host factors, except tumor stage and intrahepatic metastasis. Lo et al[27] reported that a long, disease-free interval predicts a

more favorable outcome after the resection of extrahepatic recurrence. In this regard, to improve the survival of the patients with extrahepatic metastasis, it is necessary to achieve a long disease-free interval after initial hepatic resection.

The survival rates after the diagnosis of extrahepatic metastasis were not different across the 3 patterns of extrahepatic metastases, and all patterns of recurrence had poor survival. We found that, in pattern III (extrahepatic metastasis), the invasion of the portal vein, hepatic vein or inferior vena cava, intrahepatic metastasis, and tumor stage were more severe than those in pattern I (intrahepatic metastasis) by comparing the clinicopathologic factors with the use of univariate and multivariate analyses, which shows that these factors can be important prognostically. In pattern I, portal vein invasion predominated, whereas in pattern III hepatic vein invasion took the main role. In addition to the tumor factors mentioned earlier, we found that the metastatic site and treatment for extrahepatic metastasis foci were also important in the prognosis of HCC. All these patterns may benefit by the development of effective adjuvant therapy after the initial hepatectomy.

In conclusion, although long-term overall survival was better in patients with pattern I extrahepatic recurrences, prognosis was poor in all patterns once extrahepatic metastasis developed. To achieve long survival intervals after the extrahepatic metastases, new promising modalities, such as a noble systemic chemotherapy and molecular-targeting treatment, will be necessary.

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Original Article

Interferon alpha receptors are important for antiproliferative effect of interferon-α against human hepatocellular carcinoma cells

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Aim: Interferon (IFN)-α is a promising drug for the prevention and treatment of hepatocellular carcinoma (HCC). We reported that responders to IFN-α/5-fluorouracil combination therapy expressed higher IFN alpha receptor (IFNAR)2 in tumor. Herein we studied involvement of IFNARs in response to IFN-α in HCC cells.

Methods: IFN-α sensitivity and expression of IFNARs were studied in six HCC cell lines (HuH7, PLC/PRF/5, HLE, HLF, HepG2, Hep3B) using growth-inhibitory and RT-PCR, Western blot assays. Short interfering RNAs (SiRNAs) against IFNAR1 and 2 were used to analyze the role of the IFNARs in IFN-α's effect and signal transduction.

Results: The expressions of IFNAR1 and 2c mRNAs were higher in PLC/PRF/5 cells than those in other cell lines, and PLC/PRF/5 cells expressed abundant IFNAR2c on their cell membrane. When we examined the sensitivity of the HCC cell lines to the growth-inhibitory effect of IFN-α, PLC/PRF/5 exhibited a significant response, while the other cells were much more resistant. Knockdown of either IFNAR1 or 2 using siRNAs suppressed the IFN-α's signal transduction (2.5-fold), and decreased the growth-inhibitory effect (down by 69.9% and 67.3%).

Conclusion: The results suggest that the expression of IFNAR1 and IFNAR2c independently are important for the antiproliferative effect of IFN-α in HCC cells.

Key words: antiproliferative effect, hepatocellular carcinoma, interferon alpha receptor, interferon, short interfering RNA

INTRODUCTION

RECENT CLINICAL STUDIES suggested that combination chemotherapies with interferon (IFN), especially the combination of 5-fluorouracil (5-FU) and IFN-α, might be effective for patients with advanced hepatocellular carcinoma (HCC).[1–4] We reported that a combination of 5-FU and IFN-α therapy expanded the median overall survival period to 11.8 months in patients with highly advanced HCCs, and especially it reached 24.4 months in responders to the therapy (with a response rate in 43.6% of the patients).[5] At this same stage cancer patients die within 2.7 months with symptomatic therapy.[6] However, the effectiveness of IFN-α monotherapy for treatment of HCC is controversial.[7,8]

It has emerged that IFN-α exerts its antitumor effect by both a direct antiproliferative effect (through cell cycle arrest and induction of apoptosis) and indirectly (by immunomodulation or inhibition of tumor angiogenesis). This antiproliferative action is perhaps mediated by the interaction of IFN with multisubunit receptors – IFN alpha receptor (IFNAR) (including IFNAR1 and IFNAR2a [soluble subunit], 2b [short], 2c [long]) on the cell surface and further activation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, which induces transcription of various genes.[9–11]

We reported that responders to IFN-α/5-FU combination therapy expressed higher IFNAR2 in HCC than non-responders.[5] Alternatively, Yano *et al.* reported that IFNAR2 expression does not correlate with responsiveness of HCC cell lines to IFN-α.[12] In this study we further investigated the roles of IFNAR1 and IFNAR2 in

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the direct growth-inhibitory effect of IFN-α, conducting a mechanistic study on HCC cell lines.

METHODS

Cell lines and specific reagents

THE HUMAN HCC cell lines HuH7, PLC/PRF/5, HLE, HLF and HepG2 were purchased from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and the Hep3B cell line was obtained from the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). They were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified incubator with 5% CO_2 in air.

Human natural IFN-α (OIF) was obtained from Otsuka Pharmaceutical Co. (Tokyo, Japan). The IFN was prepared and assessed for experiments as described previously.[13] The concentration of the IFN was based on that used in previous studies.[13–16] Specific rabbit antihuman IFNAR2 (Otsuka Pharmaceutical), phosphorylated STAT (pSTAT)1 (Tyr701) (Cell Signaling Technology, Beverly, MA), pSTAT2 (Tyr689) (Upstate Biotechnology, Lake Placid, NY), pSTAT3 (Tyr705) (Cell Signaling Technology), actin (Sigma, St. Louis, MO, USA), and donkey antirabbit coupled to horseradish peroxidase (Amersham Biosciences, Buckinghamshire, UK) antibodies were used for Western blotting. Four different siRNAs predesigned to either human IFNAR1 or IFNAR2, and control non-silencing siRNA were purchased from Qiagen K.K. (Tokyo, Japan).

RNA extraction and RT-PCR analysis

Total RNA extraction was performed with a single-step method using Trizol Reagent (Invitrogen, Carlsbad, CA, USA), and complementary DNA (cDNA) was generated with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA) as described previously.[17]

Conventional and quantitative real-time PCR (qRT-PCR) assays were performed using Genamp PCR System 9600 (Perkin-Elmer, Foster City, CA, USA) and the Light Cycler (Roche Diagnostics, Mannheim, Germany), as described in references.[17,18] The primer pair sequences were obtained from published sequences of IFNAR1, 2[12] and β-actin,[18] and synthesized from commercial sources. Conventional PCR conditions were the same as published previously.[12,18] qRT-PCR conditions for IFNAR1, 2 and β-actin were as follows: one cycle of denaturing at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 35 s, and the final extension at 72°C for 10 min (or annealing at 58°C for β-actin). In IFNAR2 qRT-PCR, we set one more additional step in each cycle with acquisition at 85°C for specific detection of the fluorescence of 481 bp product (IFNAR2c).

Cell preparation and Western blot analysis

For separation of the membrane fraction for detection of IFNAR2 by Western blot, we used the method described previously with minor modifications.[19] Briefly, the cells were solubilized with 1.5% Triton X-114 (Sigma) in TBS on ice for 15 min. The extract was separated by centrifugation, and the supernatant was warmed for 2 min at 37°C and centrifuged at 12 000 r.p.m. at 22°C. The membrane fraction (lower, hydrophobic phase) was diluted with RIPA buffer (25 mM Tris [pH 7.5], 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% SDS, 1 mM phenylmethylsulfonyl fluoride and 500 KIE/mL 'Trasylol' proteinase-inhibitor [Bayer Leverkusen, Germany]) and assayed for Western blotting as described in our previous studies.[15,17]

For study of pSTAT proteins, the cells were cultured in a medium containing 0.5% FBS for two days, then in FBS-free medium for 2 h. They were then incubated in medium with or without IFN-α for 20 min, and the cells were lyzed in RIPA buffer with 1 mM sodium orthovanadate and probed on Western blotting.

Cell growth curves

The cells were uniformly seeded in 12-well dishes (7×10^3 cells/well for HLE, HLF; 2×10^4 cells/well for HuH7, HepG2, Hep3B; 4×10^4 cells/well for PLC/PRF/5). From the following and every other day, the medium was replaced with fresh medium with or without IFN-α (500 IU/mL), and/or adherent cells were counted using the Celltac semiautomatic analyzer (Nihon Kohden, Tokyo).[17]

Knockdown of IFNARs with siRNA

The subconfluent (50–80%) growing PLC/PRF/5 cells were transiently transfected with 5 nM siRNAs using HiPerFect Reagent (Qiagen K.K.) according to the manufacturer's instructions. Knockdown efficacy for IFNAR1 or IFNAR2 were examined for 24 h or 48 h after transfection, respectively, using qRT-PCR or Western blot analyses as described above. The suitable siRNAs were selected and used for further experiments based on the highest knockdown efficacy for each IFNAR (data not shown). For the cell proliferation experiment the medium with or without IFN-α was applied 48 h after

siRNA transfection, and the cell numbers were counted after 48 h with the Celltac semiautomatic analyzer. This observation time (48 h) was chosen based on cell confluency. In all experiments the non-silencing siRNA was used as a control.

RESULTS

Expression of IFNARs in HCC cell lines

WE EXAMINED THE expressions of IFNARs in the cell lines at transcriptional level. Although the IFNAR1 mRNA was similar in all cells by conventional PCR (Fig. 1A upper), it was higher in the PLC/PRF/5 cell line by qRT-PCR (lower table). As shown in Figure 1B, the cells expressed clear bands of full length IFNAR2c subunit (481 bp) and faint bands of short subunit – IFNAR2b (713 bp), while there is no expression of the 350 bp band that corresponds to IFNAR2a (soluble subunit). IFNAR2c expression was slightly higher in PLC/PRF/5 compared to other cell lines, which was confirmed by qRT-PCR (Fig. 1B, lower table). Because the cell surface IFNAR2c subunit is suggested to be important in the binding of IFN-α,[20,21] we examined the IFNAR2c protein on cell membrane. As shown in Figure 1C, only PLC/PRF/5 cells displayed prominent expression of the IFNAR2c in their membrane fraction.

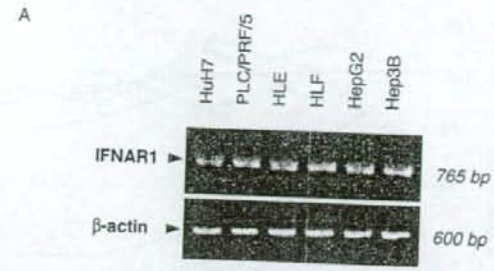
Growth-inhibitory effect of IFN-α on HCC cells

Next, we examined the sensitivity of HCC cells to IFN-α. As shown in Figure 2, IFN-α (500 U/mL) showed strong growth-inhibition in PLC/PRF/5 cells (closed squire curve, 90.8% growth-inhibition at 8th day), while it did not show a significant effect in other cell lines (growth-inhibition ranged between 7.6 and 35.8%). As reported in our previous study with a dose escalation experiment, the 50% growth-inhibitory concentration (IC50) of IFN-α was 6600 IU/mL for PLC/PRF/5, and higher than 25 000 IU/mL for the other cell lines[13] (data not shown).

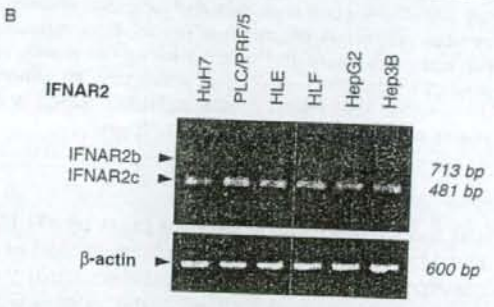
Impact of IFNAR1 and 2 on IFN signaling and antiproliferative effect

To clarify the role of IFNARs in the IFN-α growth-inhibitory effect, we performed knockdown of the receptors with siRNAs designed to IFNAR1 or 2, and studied the IFN-α's signaling and antiproliferative effect on the PLC/PRF/5 cell line, which expresses the IFNARs and exhibits a response to IFN-α (Figs 1,2).

As shown in Figure 3A, the selected siRNAs suppressed the expression of the correspondent IFNAR1



	HuH7	PLC/PRF/5	HLE	HLF	HepG2	Hep3B
IFNAR1 / β-actin	0.51±0.06	1.81±0.35	0.59±0.10	0.69±0.19	1.10±0.41	1.25±0.26



	HuH7	PLC/PRF/5	HLE	HLF	HepG2	Hep3B
IFNAR2c / β-actin	1.52±1.05	2.33±0.92	0.64±0.38	0.63±0.41	0.98±0.50	0.81±0.50

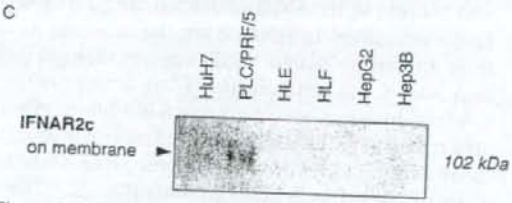


Figure 1 Expressions of IFNAR1 and 2 in HCC cell lines. (A) By conventional PCR assay, the IFNAR1 was expressed in all cell lines in similar levels; while with qRT-PCR, the expression of the IFNAR1 (shown in table) was higher in PLC/PRF/5 than those of the other cells. The IFNAR2's expression was examined in transcriptional (B) and protein (C) levels. PLC/PRF/5 cells expressed higher IFNAR2c (full length subunit) mRNA and displayed a prominent band of the IFNAR2c in their cell membrane. Protein load in each lane was equal by Ponso-S staining (data not shown). Figures are representative of similar results in repeat experiments.

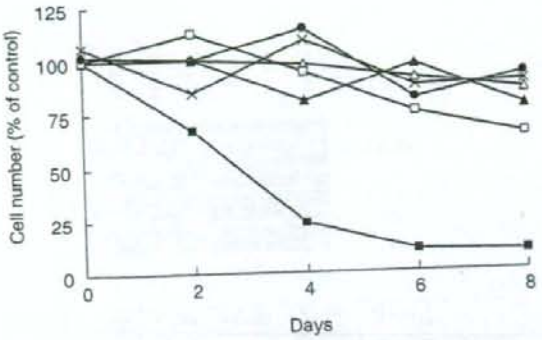


Figure 2 Growth-inhibitory effects of IFN-α in HCC cell lines. The curves were drawn for cells treated or untreated with 500 IU/mL IFN-α over eight days, and are shown as mean cell number percentages of untreated control from three independent experiments. IFN induced strong cell growth suppression in PLC/PRF/5 cells (closed squire curve, ■). However, the other cell lines (HuH7 *, HLE △, HLF □, HepG2 ▲ and Hep3B ●) were much more resistant to IFN-α.

and 2 down to 19.9% (upper right panel, by qRT-PCR) and 32.9% (lower right panel, by Western blot) of the corresponding controls. Since the IFN-α's signal transduction involves tyrosine phosphorylation of STAT1, 2, 3, we examined activation of the STATs with IFN-α after the knockdown. Inductions of pSTAT1 and 3, but not that of pSTAT2, were decreased with the receptor knockdown (Fig. 3B). pSTAT1 and 3 were suppressed 2.8-fold and 2.4-fold in IFNAR1 knockdown, and 2.3- and 2.4-fold, respectively, in IFNAR2 knockdown than in controls. Expression of base STATs was not changed by the knockdown and IFN induction (data not shown).

When we examined the growth-inhibitory effect of IFN-α 48 h after the IFNARs knockdowns, the effect was decreased in knockdown cells compared with non-silencing siRNA transfected controls (Fig. 3C). The viabilities of IFN-treated control cells were 60.7% and 62.1%, while they were 88.2% and 87.6%, respectively, in IFNAR1 and IFNAR2 knockdown cells. This means that the IFNAR1 and 2 siRNAs suppressed IFN-α's effect by 69.9% and 67.3%, respectively.

DISCUSSION

IFN-α EXERTS ITS effect through specific cell surface receptors (IFNARs), which subsequently activate the JAK/STAT pathway and result in the transcription of various genes realizing multiple effects.[9–11] Previously Yano *et al.* reported that IFNAR expression at the transcriptional and protein levels, which were detected with conventional PCR and flow cytometry methods, does not correlate with responsiveness of HCC cell lines to IFN-α.[12] Consistently, when we employed conventional PCR analysis, the IFNAR mRNAs were expressed at similar levels in our studied cell lines. However, when we quantified the expressions of IFNAR1 mRNA using qRT-PCR, as well as when we examined the expression of cell membrane IFNAR2c subunit using Western blot analysis, the expressions were significantly higher in IFN-sensitive PLC/PRF/5 cell line (Fig. 1). The subunits of IFNAR2 appear to have different functions, and cell surface full length IFNAR2 (IFNAR2c) is reported as functional and involved in IFN binding and signal transduction.[20] Furthermore, IFNAR2 expressions in mRNA and protein levels had some variation, suggesting its possible regulation in post-transcriptional or membrane presentation levels. Correspondingly, the relationship between the antiproliferative effect of IFN-α and mRNA expression levels of IFNARs was not clear in 'less sensitive' cell lines (HuH7, HLE, HLF, HepG2 and Hep3B), perhaps due to insufficient expression of IFNARs, especially the absence of IFNAR2c protein on their cell surface. Furthermore, IFN signal transduction (phosphorylation of STAT1, 3) was also greater in IFN-sensitive cell line (data not shown). Examination of IFNAR1 expression in protein level was unfeasible due to the lack of suitable antibody.

Generally, IFN-α exerts its growth-inhibitory effect by induction of cell cycle arrest and apoptosis in cancer cells.[9,10] The mechanism of strong antiproliferative effect of IFN-α in PLC/PRF/5 cells was studied previously. The studies showed an increase of S phase population of PLC/PRF/5 cells treated with IFN-α for 24 h.[14,22] Moreover, the proportion of apoptosis induced by IFN-α treatment for 3–10 days was 4–6.1% (vs 1–1.3% in untreated control cells).[14,15]

To verify the role of the IFNARs in IFN-α's antitumor effect, we reduced the receptors with siRNAs against IFNAR1 or 2 and examined the signal transduction and growth-inhibitory effect. From the results, knockdown of either IFNAR1 or 2 significantly decreased the signaling as well as antiproliferative effect of IFN-α (Fig. 3). Although we could perform only partial knockdown of the receptors, the decrease of the signaling and growth-inhibitory effect was almost parallel to the knockdown level. Alternatively, when we performed the neutralizing experiments using an anti-IFNAR2 antibody binding to the intracellular part of the receptor, the IFN-α's effect and signal transduction were suppressed, confirming

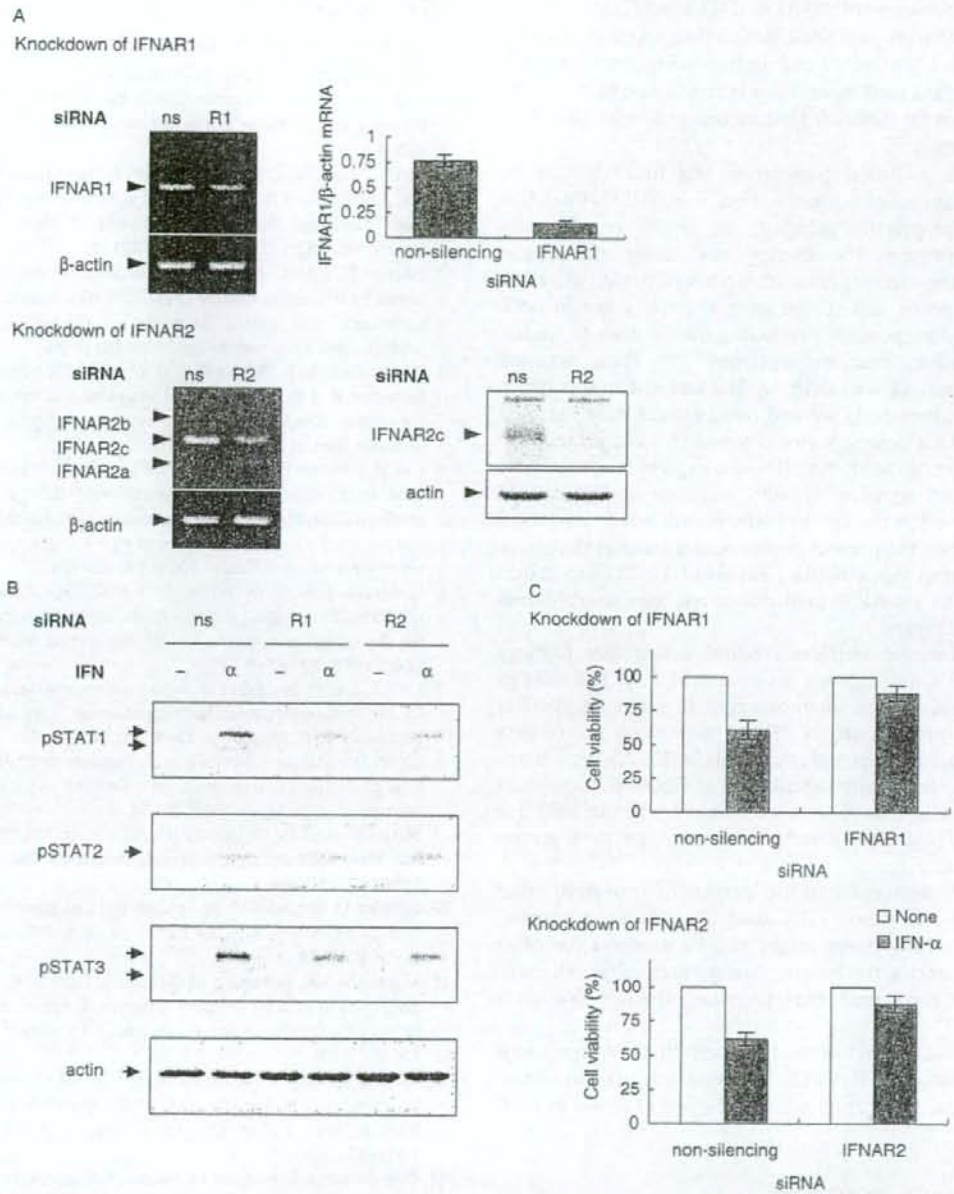


Figure 3 Knockdown of IFNAR1 and IFNAR2 using siRNAs. (A) The siRNAs suppressed the expressions of the correspondent IFNAR1 (left) and 2 (right) down to 19.9% and 32.9% of the controls. The expression of the internal control – β-actin – was the same in either non-silencing or IFNAR siRNA transfectants. (B) IFN-α signal transduction (inductions of pSTAT1 and 3, but not that of pSTAT2) was decreased with the receptor knockdown. (C) IFN-α's antiproliferative effect was decreased with IFNAR knockdown compared with non-silencing siRNA transfected controls, and it was suppressed by 69.9% and 67.3% of the primary antiproliferative effect on control cells by IFNAR1 and 2 siRNA. Data are representative of similar results in repeat experiments or mean of three independent experiments. ns, non-silencing; R1, IFNAR1; R2, IFNAR2.

the importance of IFNAR2 in IFN's effect (Damdinsuren *et al.*, 2006, unpublished data). Taken together, the existence of high IFNARs may induce subsequent intensive signal transduction, and this is considered to be one of the essential factors for the success of the IFN-α antitumor action.

From a clinical perspective, our findings may be important for two reasons. First, it is vital that patients be appropriately selected for IFN-α combination chemotherapy. The therapy may cause side-effects, including neutropenia, thrombocytopenia or renal insufficiency, and crucial survival time is lost in non-responders, possibly precluding their chance to undertake other treatment options.[1–3,23,24] Thus, accurate prediction of sensitivity to first-line chemotherapy is critical. Previously we and other groups have reported that HCCs express varied levels of IFNAR2 protein.[25,26] Also, we showed that IFNAR2 expression in tumors correlates significantly with response to IFN-α/5-FU combination therapy in patients with advanced HCC.[5] Therefore, the present *in vitro* results support the above suggestion that IFNAR2 (and also IFNAR1) expression might be a suitable predictor of response to IFN-based chemotherapy.

The second important point is that our findings suggest a new strategy for increasing response rates to IFN combination chemotherapy. It might be possible with upregulation of IFNAR expression in cancers lacking the receptor, for example by IFNAR gene transfer. We previously described that IFNAR2c-transfected HCC cells treated with combination IFN-α/5-FU (or with IFN-α alone) underwent apoptosis to a greater extent than control non-transfected cells.[15] Wagner *et al.* recently demonstrated the increase of antitumor effect of IFN-α in cancer cells using IFNAR2c gene transfection.[27] This strategy might also be practical for other malignancies, for instance melanomas, renal cell carcinomas and lymphomas, for which the IFN therapy is useful.[28]

In conclusion, our results suggest that the expressions of IFNAR1 and IFNAR2c independently play an important role in the antiproliferative effect of IFN-α in HCC cells.

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Overexpression of MT3-MMP in Hepatocellular Carcinoma Correlates with Capsular Invasion

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ABSTRACT

Background/Aims: Extracellular matrix-degrading matrix metalloproteinases (MMPs) are invariably up-regulated in epithelial cancers and are key agonists of angiogenesis, invasion and metastasis. Recent studies have shown high levels of various MMPs, including MT1-MMP, MMP-1, MMP-2 and MMP-9, and their involvement in tumor progression in human hepatocellular carcinoma (HCC). However, the expression and role of MT3-MMP in HCC remains unclear.

Methodology: We examined the immunohistochemical expression of MT3-MMP in surgically resected HCCs (n=58), hepatitis C virus (HCV) and hepatitis B virus (HBV)-related chronic hepatitis (n=34) and cirrhosis (n=24).

Results: MT3-MMP expression was observed in all non-cancerous liver tissues. In HCCs, 52% (30/58) of patients showed high MT3-MMP expression while the remaining 48% (28/58) of patients showed low expression. A clinicopathological survey demonstrated a significant correlation between high MT3-MMP expression and capsular invasion of carcinoma ($p=0.034$) although there was no correlation between high MT3-MMP expression in HCC and overall survival or disease-free survival.

Conclusions: MT3-MMP was expressed not only in chronic hepatitis and liver cirrhosis, but also in HCC, and high MT3-MMP expression correlated significantly with capsular invasion of carcinoma.

KEY WORDS:
MT3-MMP; HCC; Capsular invasion; Immunohistochemistry

ABBREVIATIONS:
Disease-Free Survival (DFS); Dulbecco's Modified Eagle Medium (DMEM); Extracellular Matrix (ECM); Fetal Bovine Serum (FBS); Hepatitis B Virus (HBV); Hepatocellular Carcinoma (HCC); Hepatitis C Virus (HCV); Matrix Metalloproteinases (MMPs); Overall Survival (OS)

INTRODUCTION

Hepatocellular carcinoma (HCC) ranks as the fifth most common cancer in the world and in Southeast Asia and Africa the incidence of this cancer is about 30 per 100,000 males per year (1). The prognosis of patients with HCC is poor, because most HCC patients develop tumor recurrence, which occurs at a rate of 20-40% within 1 year, and about 80% within 5 years after therapy, even if curative treatment is performed (2-5).

In HCCs, it is well known that infiltration into the tumor capsule and portal involvement are important histopathological characteristics predicting worse clinical outcomes of HCC (2-5). Portal involvement, a crucial route of intrahepatic metastasis, requires destruction of the basement membrane because approximately 80% of HCCs are encapsulated by fibrous tissue and nearly all HCCs arise from extracellular matrix (ECM)-rich diseased liver (6,7). Thus, ECM degradation might play a major role in HCC invasion and progression to metastasis.

Proteinase plays a major role in ECM degradation. Four types of proteinase have been identified, including asparagine proteinase, cysteine proteinase, serine proteinase and matrix metalloproteinases (MMPs) (8). MMPs are of great significance for degradation of the ECM in cancers that have extensive decomposing activity (8). Recent studies have shown increased levels of MMPs and implications for tumor progression in human HCC including MT1-MMP, MMP-1, MMP-2 and MMP-9 (9-13). However, little is known about the expression and role of MT3-MMP in HCC. The present study was designed to determine the expression of MT3-MMP in human HCC cell lines and in human HCC, and to define the relationship between its expression and clinicopathological features.

METHODOLOGY

Epithelial Cancer Cell Lines

Seven HCC cell lines (HLE, HLF, HuH7, PLC/PRF/5, HepG2, SKHep1, and PLL), five colorectal carcinoma cell lines (Lovo, HCT116, HT29, DLD and SW48), five gastric carcinoma cell lines (MKN-1, MKN-28, MKN-74, MKN-45 and KATO-III), four esophageal carcinoma cell lines (TE-2R, TE-2S, TE-8 and TT), and five pancreatic carcinoma cell lines (PANC1, PSN-1,

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