surgical removal of the tumor performed with the right timing; and (4) the use of an appropriate drug delivery system into the liver through an intraarterial catheter. Mainly, we think that the chemotherapeutic effects of IFN-α/5-FU prevented intrahepatic metastasis and improved the prognosis. Futhermore, in this patient, the elimination of HCV by the IFN therapy may have been a contributing factor in suppressing the occurrence of multicentric HCC.

Most chemotherapy regimens for HCC, such as those including doxorubicin or cisplatin, cause severe leucopenia or thrombocytopenia, leading to discontinuation of the therapy. On the other hand, there are few reported adverse myelosupressive effects of the IFN-α/5-FU combination therapy that would lead to its discontinuation. However, an adverse effect of IFN-α/5-FU combination therapy is catheter trouble, because 5-FU is administered through the catheter intraarterially. Catheter troubles – such as a subcutaneous seroma or hematoma forming around the implanted reservoir; gastrointestinal ulcer, or inflammation due to dislocation of the catheter; and pseudoaneurism in the femoral or subclavian artery – often occur and lead to discontinuation of the therapy. In the present patient, the catheter dropped out to the common hepatic artery and it caused a gastric ulcer as 5-FU flowed into the gastric mucosa via the right gastric artery. Fortunately, the dislocation of the catheter was easily corrected, and the patient was able to continue the therapy.

In conclusion, IFN-α/5-FU combination therapy is a useful treatment for HCC in patients who have multiple intrahepatic metastases and portal vein thrombosis. In addition to this therapy, combined modality therapy, including, for example, surgical resection, can sometimes have a dramatic therapeutic effect, leading to tumor markers dropping to normal levels. Randomized controlled trials would be need to clarify the efficacy of this therapy, not only as the treatment for advanced HCC but also as the adjuvant therapy after curative resection.

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# Role of the Fas/FasL pathway in combination therapy with interferon-α and fluorouracil against hepatocellular carcinoma in vitro ☆,☆☆

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***Background/Aims*: Several studies have reported the efficacy of combination therapy of interferon (IFN) α and 5-fluorouracil (5-FU) for hepatocellular carcinoma (HCC). However, the mechanism underlying the clinical anti-tumor effects of this treatment is not well understood. The aim of this study was to determine the role of Fas/FasL signaling in the anti-tumor effect of this combination therapy.**

***Methods and Results*: We used six human hepatoma cell lines, three of which are known Fas-expressing cells. Growth of Fas-positive hepatoma cell lines was inhibited by an agonistic anti-Fas antibody in a dose-dependent manner, and these effects were enhanced by IFNα or 5-FU alone, but even more so by combination therapy using both agents. Annexin-V assay implicated apoptosis as the main mechanism underlying these growth inhibitory effects, although changes in Fas expression regulated by IFNα and/or 5-FU did not correlate with increased apoptosis. Caspase-3 activation was exclusively increased by IFNα/5-FU combination treatment, which was compatible with enhancement of the synergistic apoptotic effect, and other caspases and apoptotic factors (FLIP, BCL-xl, and Bax) were also regulated by IFNα/5-FU. $^{51}$Cr-release assay revealed that pretreatment with IFN activated cytotoxicity of peripheral blood mononuclear cells (PBMCs) against HCC cells. The largest interaction was observed when both PBMC and HCC cells were pretreated with the combination of IFNα/5-FU. These cytotoxicities were markedly inhibited by a neutralizing anti-Fas antibody.**

***Conclusions*: Our results indicated that IFNα/5-FU combination treatment enhances the induction of apoptosis and the cytotoxic effect of PBMCs via the Fas/FasL pathway. The Fas/FasL pathway seems, at least in part, to contribute to the anti-tumor effects of IFNα/5-FU against HCCs.**



***Keywords*: Hepatocellular carcinoma; Combination therapy; Interferon-α; 5-Fluorouracil; Apoptosis; Fas/FasL pathway; Caspase-3**

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## 1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common solid tumors [1]. The prognosis for patients with HCC remains poor and most die within several months after diagnosis, particularly in advanced cases with tumor thrombosis in the major portal vein (Vp3-4) [2–4]. Chemotherapy is the traditional first choice

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for the treatment of unresectable solid tumors; however, these drugs are not effective in promoting tumor regression and prolonging survival in HCC [5,6]. In addition, conventional therapeutic modalities such as transcatheter arterial embolization, radiofrequency ablation and microwave coagulation therapy are not recommended when portal vein tumor thrombosis (PVTT) is present because of low efficacy and potential complications [7,8]. Therefore, a new effective modality is needed to treat advanced HCC, especially in those cases with portal vein involvement.

Interferon (IFN) has a variety of biological properties including immunomodulation and anti-tumor activity. The anti-tumor effect of IFN against HCC was tested in several studies. From a randomized controlled trial, Llovet et al. [9] concluded that IFN used alone provides no clinical benefit for HCC patients with respect to tumor progression rate and survival. However, several other investigators reported a strong anti-tumor activity for IFN in HCC, when used in combination with some other chemotherapeutic agents. Urabe et al. [10] found that treatment with a combination of subcutaneous IFNα injection and intra-arterial infusion of 5-fluorouracil (FU), cisplatin and methotrexate for HCC with PVTT achieved a response rate of 46.7%. In addition, Patt et al. [11] reported that combination treatment with FU and IFN promoted anti-tumor has activity in HCC and could be tolerated even by cirrhotic patients. We also previously reported the beneficial results of subcutaneous IFNα injection and intra-arterial 5-FU infusion against HCC with PVTT [12–14]. This therapy showed an anti-tumor effect with a response rate approaching 50%, including several complete remissions of the tumor and prolonged survival without major adverse effects. From these results, we proposed that the combination chemotherapy of IFNα and 5-FU should become a standard therapy for advanced HCC.

We have already reported the synergistic effects of IFNα and 5-FU in influencing cell-cycle progression into the S phase via $p27^{Kip1}$, inducing apoptosis by downregulating Bcl-xl, and modulating the immune response via the TRAIL/TRAIL-receptor pathway [15–17]. The present study is an extension of this previous work, to investigate the role of the Fas/FasL pathway in the IFNα/5-FU treatment effect. Fas/FasL signaling participates in an apoptosis-inducing mechanism related to cytotoxic T Lymphocytes (CTL) and natural killer (NK) cells, which was implicated as a major pathway of T-cell-mediated cytotoxicity and a mediator of apoptosis via an IFN-stimulated gene [18]. In addition, we also investigated the mechanism underlying the apoptosis-enhancing effect of IFNα/5-FU that acts via the Fas/FasL pathway.

## 2. Materials and methods

### 2.1. Cells

Human HCC cell lines (HuH7, PLC/PRF/5, HLE, HLF and HepG2) were obtained from the Japan Cancer Research Resources Bank (JCRB) (Osaka, Japan) and the human HCC cell line, Hep3B, was obtained from the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin at 37 °C in a humidified incubator with 5% $CO_2$ in air. A non-tumorigenic SV40-immortalized human liver epithelial cell line (THLE-2) was obtained from American Type Culture Collection (Manassas, VA). THLE-2 cells were maintained as an adherent monolayer in Bronchial epithelial medium (BEGM bullet kit, Combrex, NJ) from which remove the gentamicin/amphotericin and epinephrine and to which add extra 5 ng/ml EGF, 70 ng/ml phosphoethanolamine and 10% fetal bovine serum (FBS).

### 2.2. Reagents

Purified human IFNα was obtained from Otsuka Pharmaceutical Co. (Tokushima, Japan), and 5-FU was kindly provided by Kyowa Hakko Co. (Tokyo, Japan). Antibodies against Fas (UB-2, CH-11 and ZB4) were obtained from Medical and Biological Laboratories (Nagoya, Japan). Caspase-3-specific inhibitor (Z-DEVD-FMK), caspase-8-specific inhibitor (Z-IETD-FMK) and caspase-9-specific inhibitor (Z-LEHD-FMK) were purchased from Calbiochem (San Diego, CA).

### 2.3. Flow cytometric analysis of Fas expression

HCC cells were characterized for their surface expression of Fas receptors by flow cytometry. Cells ($1 \times 10^6$) were incubated with 2.5 μg/ml of anti-Fas antibody (IgG, UB-2) for 30 min at 4 °C. After washing with PBS, the cells were analyzed on a FACScan (BD Transduction Laboratories, Lexington, KY), and data were processed using Cell Quest™ software (BD Transduction Laboratories).

### 2.4. Cell growth assay

Cell growth was assessed by the 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, $3 \times 10^4$ cells were seeded on a 96-well plate in 100 μl of medium and left overnight to adhere. Several concentrations of the test drugs in 100 μl volumes were added, and the cells were incubated for 48 h. After treatment, 10 μl of MTT solution was added to each well and incubated for another 4 h at 37 °C. Then 100 μl of acid–isopropanol was added, and after 24 h at 4 °C, reduced MTT was measured spectrophotometrically in a dual-beam microtiter plate reader at 570 nm with a 650 nm reference.

### 2.5. Flow cytometric analysis of annexin V-FITC binding

The binding of annexin V-FITC was used as a sensitive method for measuring apoptosis, according to a modification of a previously described method [19]. Briefly, after treatment with IFNα/5-FU and/or anti-Fas antibody CH-11, the cultured cells ($1 \times 10^6$) were incubated with binding buffer (10 mM Hepes, 140 mM NaCl and 2.5 mM $CaCl_2$, pH 7.4) containing saturating concentrations of annexin V-FITC (BioVision Research Products, Mountain View, CA) and propidium iodide (PI) for 15 min at room temperature. After incubation, the cells were pelleted and analyzed on a FACScan (BD), and data were processed using Cell Quest™ software (BD).

### 2.6. Caspase activity

After treatment with the test drugs and/or anti-Fas antibody, cytosolic extracts were prepared using lysis buffer. The caspase activity in the cell cytosol was measured using a Caspase Colorimetric Protease Assay Kit (MBL) as per the instructions provided by the manufacturer. This assay is based on the spectrophotometric detection of the chromophore, *p*-nitroanilide after cleavage from the labeled substrate. Caspase-3, -8 and -9 assay kits were used in this study.

### 2.7. Real-time PCR

The LightCycler PCR and detection system (Roche Diagnostics, Mannheim, Germany) was used for amplification and quantification. For detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the amplification products of some apoptotic factors, the LightCycler DNA Master SYBR Green I (Boehringer Mannheim, Mannheim, Germany) was used. Real-time PCRs were performed in a sample mixture containing each primer (final concentration, 0.2 μM), 1× LC-DNA Master SYBR Green I, 4 mM of $MgCl_2$, and 2 μl of cDNA as a template using the following primers: human GAPDH (forward: 5′-CAACTACATGGTTTACATGTTC-3′, reverse: 5′-GCCAGTGGACTCCACGAC-3′); Bcl-xl (forward: 5′-GTAAACTGGGGTCGCATTGT-3′, reverse: 5′-TGGATCCAAGGCTCTAG GTG-3′), and Bax (forward: 5′-CCAGCTGCCTTG GACTGT-3′, reverse: 5′-ACCCCCTCAAGACCACTCTT-3′) yielding products of 182, 146 and 135 bp, respectively [20]. The GAPDH PCR cycle conditions were set up as follows: one cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 10 s and 72 °C for 20 s. Fluorescence was acquired at the end of every 72 °C extension phase. The annealing temperatures for Bcl-xl and Bax were 60 and 61 °C, respectively. Quantitative analysis of data was performed using the LightCycler™ analysis software (Roche Diagnostics).

### 2.8. Western blot analysis

The sub-confluent growing cells were washed with PBS (Sigma) and lysed in an ice-cold 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)]. Total protein concentration was determined using the Bradford protein assay (Bio-Rad, Hercules, CA) and Western blot analysis was performed as described in our previous study [17]. The antibodies were used in dilutions of 1:100 for FLIPS/L (sc-5276; Santa Cruz Biotechnology, Santa Cruz, CA), 1:1000 for actin (A-2066; Sigma), and 1:2000 for secondary donkey anti-rabbit (NA934V; Amersham Biosciences, Buckinghamshire, UK) antibodies. The expression of proteins was evaluated by measuring the optical densities of protein bands, using the National Institute of Health Image analysis software version 1.61 and the expression value was calculated relative to that of actin.

### 2.9. Cytotoxicity assay

Target cells ($1 \times 10^6$) were labeled with 40 μCi $Na^{51}CrO_4$ for 45 min at 37 °C. $^{51}Cr$-labeled target cells ($1 \times 10^4$) and effector cells (peripheral blood mononuclear cells, PBMCs) were mixed in U-bottomed wells of a 96-well microplate at the indicated *E/T* ratios. After 8 h of incubation, the cell-free supernatants were collected and counted on a gamma counter. The percent-specific cytotoxicity was calculated using the formula: [100 × (experimental release − spontaneous release)/(total release − spontaneous release)]. Total or spontaneous release was determined in the presence of 1% NP-40 or medium alone. For blocking, anti-Fas mAb ZB-4 was added at a final concentration of 500 ng/ml before the cytotoxicity assay for 1 h in accordance with the manufacturer's instructions.

### 2.10. Magnetic sorting

PBMCs obtained from a healthy volunteer were prepared by Ficoll–Hypaque centrifugation. $CD4^+$ T cells, $CD8^+$ T cells, and $CD4^-CD8^-$ cells were isolated from PBMCs by using anti-CD4 and anti-CD8 immunomagnetic beads and a Magnetic Cell Sorter (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of each subset was estimated at >95% by flow cytometry.

### 2.11. TUNEL assay

To detect apoptosis, we used the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method, using the Apop Tag in situ apoptosis detection Kit (Chemicon International, Inc., Temecula, CA) as described previously [17]. This method can detect fragmented DNA ends of apoptotic cells. Briefly, the paraffin-embedded sections were deparaffinized in xylene and rehydrated in a graded series of ethanol baths. The sections were treated with 20 μg/ml of proteinase K in distilled water for 10 min at room temperature. The adherent cultured THLE-2 cells were fixed in 1% paraformaldehyde for 10 min. To block endogenous peroxidase, the slides were incubated in methanol containing 0.3% hydrogen peroxide for 20 min. The remaining procedures were performed according to the instructions provided by the manufacturer. For quantification of apoptosis, five microscopic fields were randomly selected at high power magnification (200×) and the average counts of TUNEL-positive cells were calculated.

### 2.12. Statistical analysis

Statistical analysis was performed using the StatView J-5.0 program (Abacus Concepts, Inc., Berkeley, CA). Data are expressed as means ± SD. Differences between groups were examined for statistical significance using the Dunnett method and Student's *t*-test. $P < 0.05$ denotes a statistically significant difference.

## 3. Results

### 3.1. Fas expression in human hepatoma cell lines

Flow cytometry using an anti-Fas antibody (UB-2) revealed expression of Fas receptor on the cell surface in three of the six cell lines (HLE, HLF and HepG2), but not on HuH7, PLC/PRF/5 and Hep3B (Fig. 1).

### 3.2. Response to agonistic anti-Fas antibody with dose escalation

We confirmed the response described above using the agonistic anti-Fas monoclonal antibody, CH-11, which is used widely to replace FasL in vitro. The 48-h MTT assay showed that CH-11 treatment inhibited the growth of three Fas-positive hepatoma cell lines (HLE, HLF and HepG2) in a dose-dependent manner. In contrast, no Fas-negative cell lines (HuH7, PLC/PRF/5 and Hep3B) were growth-responsive to CH-11 (Fig. 2a). Dose dependency of IFNα and 5-FU was examined with various CH-11 concentrations. This effect was synergistic and observed in the combination of CH-11 and 5-FU in the doses of 0–0.5 μg/ml of 5-FU. There was seen little difference between 0.5 and 1 μg/ml of 5-FU (Fig. 2b).

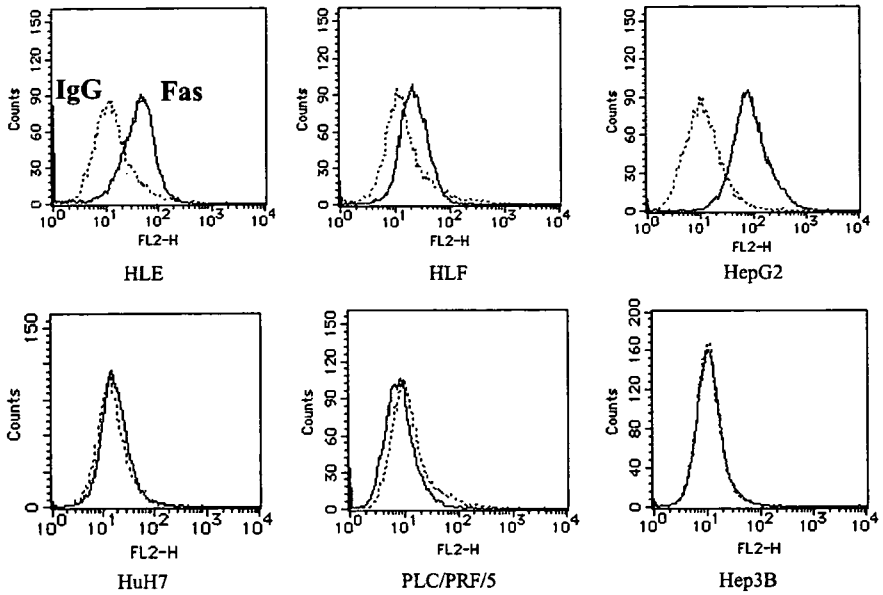


**Fig. 1. Fas expression in six hepatoma cell lines assessed using flow cytometry with an anti-human Fas antibody (IgG, clone UB2). Histogram with dotted line represents cells stained with secondary antibody alone; histogram with solid line is those cells stained with anti-Fas antibody. Fas was expressed on the surface of three of the six cell lines (HLE, HLF and HepG2). All experiments were performed three times independently.**

### *3.3. Influence of IFNα and/or 5-FU on apoptosis induced by agonistic anti-Fas antibody*

We next evaluated the effects of IFNα, 5-FU and combination treatments on growth inhibition induced by CH-11 using the MTT assay. In Fas-expressing cell lines, the inhibitory effect of CH-11 was enhanced with IFNα or 5-FU alone, but the maximum effect was observed with a combination treatment of both agents (Fig. 3a). In the HepG2 cells, the anti-proliferating effect of CH-11 alone was 26.7 ± 1.8%, and the effect of either IFNα or 5-FU used alone was 18.0 ± 4.7%, which did represent a significant enhancement. However, the combination treatment (CH-11 + IFNα/5-FU) yielded a markedly increased effect of 83.8 ± 6.3% ($P = 0.01$). Without CH-11, none of the agents, whether used alone or in combination, had any anti-proliferative effects. Similar results were obtained in the other Fas-positive cell lines, HLE and HLF.

To confirm these results, we performed an annexin-V assay to detect Fas-mediated apoptosis. Results with the HepG2 cells were comparable with those from the MTT assay (Fig. 3b, Table 1), in that an increase in apoptotic cell numbers induced by CH-11 was found with stimulation by IFNα alone, 5-FU alone, and particularly strongly with the combination treatment. In contrast, the effects of CH-11 and the influence of IFNα/5-FU were not observed with the three Fas-negative cell lines (HuH7, PLC/PRF/5 and Hep3B) in both the MTT and annexin-V assay (Fig. 3a; MTT assay, data not shown; annexin-V assay).

### *3.4. Regulation of Fas expression by IFNα and/or 5-FU*

To investigate the mechanism underlying the upregulation of Fas-mediated apoptosis, we analyzed the relationship between the change in Fas expression and the regulation of apoptosis. Out of the six cell lines, 5-FU increased Fas expression in the HepG2 cells only, while IFNα also increased Fas in the HuH7 and PLC/PRF/5 cells (Fig. 4). No additional effects were seen with the combination of IFNα and 5-FU compared with each drug used alone. In the other three hepatoma cell lines (HLE, HLF and Hep3B), neither IFNα nor 5-FU affected the level of cell surface Fas.

### *3.5. Caspase activation after stimulation with agonistic anti-Fas antibody and/or IFNα/5-FU*

Results indicated that the change in Fas expression seen in this study would not be related to the CH-11 and IFNα/5-FU-mediated effects on apoptosis. Therefore, we next tested for variations in caspase activity in the HepG2 cells, as the representative Fas-positive cell line, using a caspase colorimetric protease assay kit (Fig. 5a). Caspase-3 activity (downstream of caspase cascades) was increased after 12 h of CH-11 stimulation. IFNα and 5-FU alone enhanced this upregulation, with the combination treatment having a further effect (Fig. 5a); 5-FU alone, IFNα alone and combination therapy did not affect caspase-3 activity without CH-11. Caspase-8 activity also

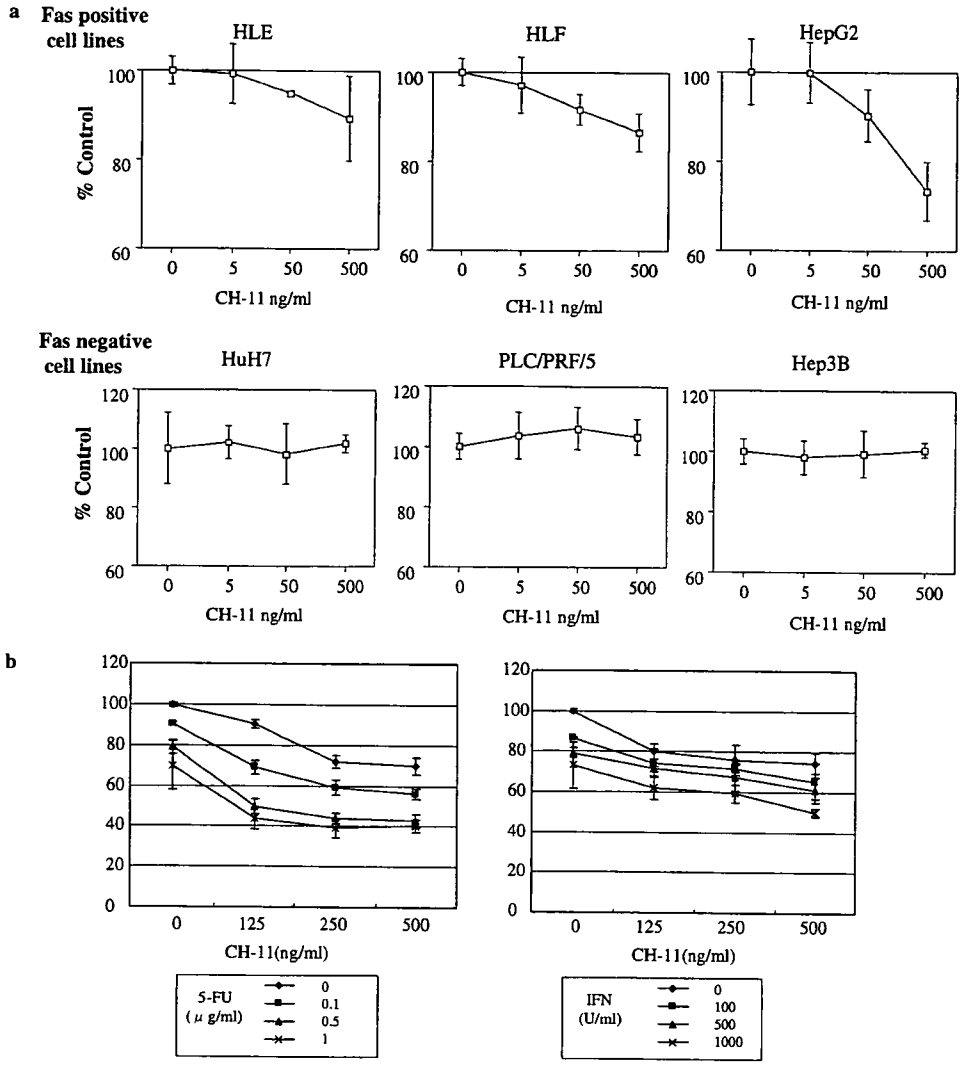


**Fig. 2. Susceptibility of six hepatoma cell lines to agonistic anti-Fas monoclonal antibody CH-11-mediated apoptosis was measured by MTT assay. (a) Each cell line was incubated with CH-11 at various concentrations for 48 h. Three Fas-positive cell lines (HLE, HLF and HepG2) were naive to CH-11 in a dose–response manner. Fas-negative cell lines (HuH7, PLC/PRF/5 and Hep3B) were resistant to Fas-mediated apoptosis. (b) Dose dependency of combination treatment was examined. CH-11 and 5-FU or IFNα were added to HepG2 cells with indicated doses. Results are expressed as percent of cell growth of each untreated cell. Data represent means ± SD of at least triplicate samples. Similar results were observed in three independent experiments.**

increased with CH-11 stimulation compared with the control and further increased with CH-11 + IFNα/5-FU; there was no significant difference between stimulation of CH-11 + IFNα and the combination. On the other hand, caspase-9 activity showed a different tendency from the data for caspase-3 and -8. Stimulation with CH-11+5-FU or CH-11 + IFNα/5-FU slightly increased the caspase-9 activity, but the effects were much less pronounced than for caspase-3 and caspase-8, and they were not significant (Fig. 5a). To confirm the significance of caspase activities in the apoptotic effect in Fas/FasL system, MTT assay using specific caspase inhibitors was performed. All specific caspase inhibitors blocked the apoptotic effect of CH-11 with IFNα/5-FU totally or partially in the dose-dependent manner (data not shown). Caspase-3-specific inhibitor (Z-DEVD-FMK) and caspase-8-specific inhibitor (Z-IETD-FMK) almost completely blocked CH-11 induced apoptosis enhanced by IFNα/5-FU (Fig. 5b). Caspase-9-specific inhibitor (Z-LEHD-FMK) showed only partial blocking effect. These results were compatible to the results of caspase assay (Fig. 5a). Colorimetric caspase assay using specific caspase inhibitors showed Z-DEVD-FMK blocked caspase-3 activation induced by CH-11 and IFNα/5-FU (Fig. 5c).

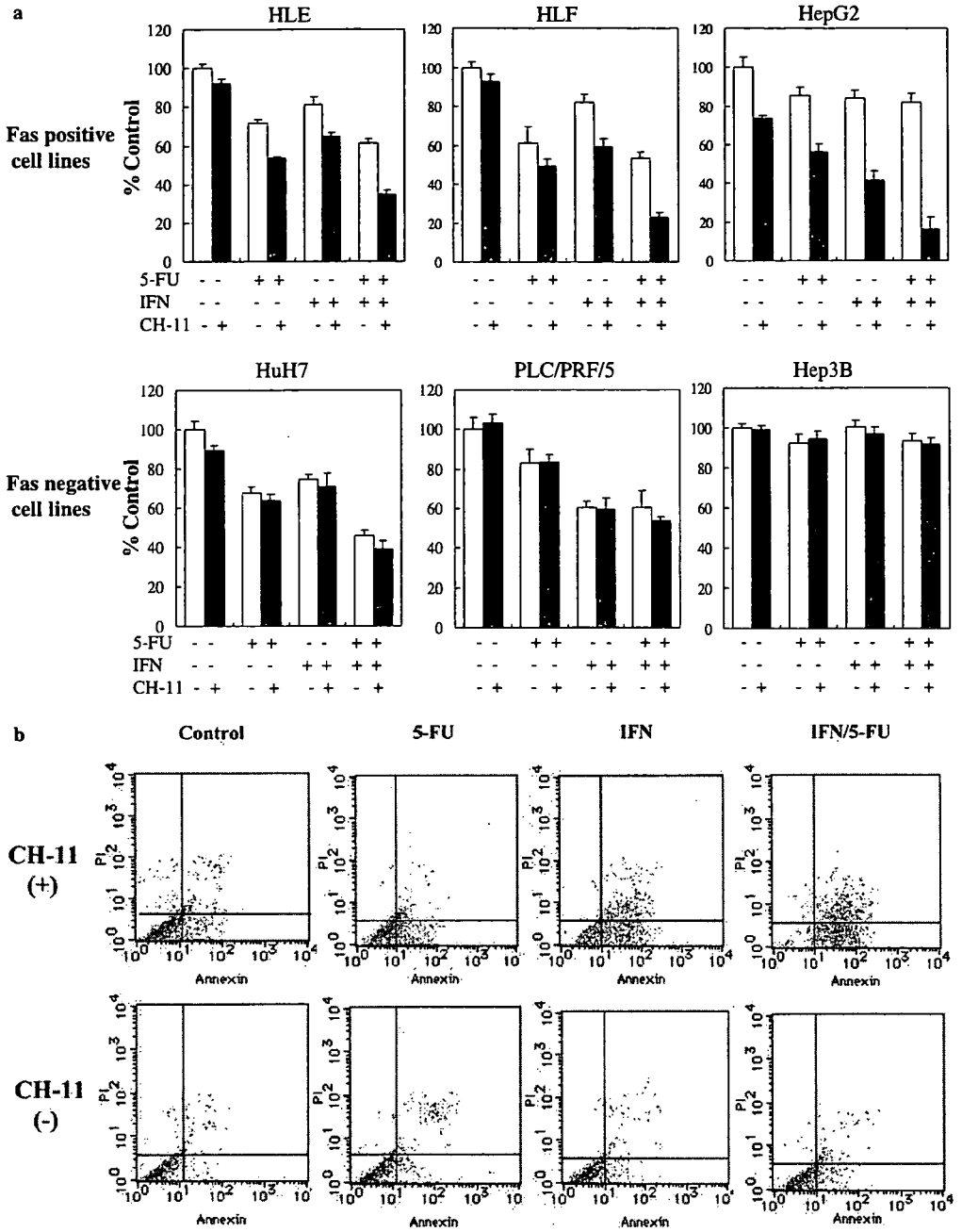


**Fig. 3. (a) Effects of IFNα and/or 5-FU on Fas-mediated apoptosis in six hepatoma cell lines measured by MTT assay. All cells were incubated with IFNα (500 U/ml) and/or 5-FU (0.5 μg/ml) and with agonistic anti-Fas monoclonal antibody CH-11 (500 ng/ml) (□) or without CH-11 (■) for 48 h. The susceptibility of Fas-positive hepatoma cells to Fas-mediated apoptosis was significantly enhanced by IFNα or 5-FU alone, and further so in the combination treatment. Results were expressed as percent of cell viability of untreated cells. Data represent means ± SD values of at least triplicate samples. Similar results were observed in three independent experiments. (b) Apoptotic cells were determined using the annexin-V assay (HepG2). [This figure appears in colour on the web.]**

### *3.6. Regulation of FLIP*

Several factors involved in apoptosis were next examined at the mRNA expression level. The expression of FLIP (FLICE/caspase-8 inhibitory protein), which is an inhibitor of caspase-8 [21,22], was markedly decreased by treatment with IFNα or 5-FU, as shown by the FLIP/GAPDH ratio, compared with untreated

**Table 1**
**Comparison of results of MTT and annexin-V assays**

| | Cell toxicity (%) | Annexin (+) cells (%) |
|---|---|---|
| Control | 0.0 | 5.8 |
| 5-FU | 14.4 | 9.5 |
| IFN | 16.2 | 10.0 |
| IFN + 5FU | 18.0 | 19.7 |
| CH-11 | 26.7 | 27.8 |
| CH-11 + 5FU | 43.8 | 37.6 |
| CH-11 + IFN | 58.0 | 62.4 |
| CH-11 + IFN + 5FU | 83.8 | 93.5 |

The two independent assays showed similar tendency. Annexin-V assay indicated that the growth inhibition effect noted in MTT assay was caused by apoptosis.

cells with the combination treatment again providing the most significant decrease (Fig. 6a). Western blot analysis was performed to examine the change of FLIP at protein level. Expression of FLIP long was decreased by the combination treatment (Fig. 6b).

### *3.7. Regulation of apoptotic factors*

Caspase-9 acts through mitochondria and is regulated by certain apoptotic factors [23]. Although the levels of caspase-9 were not increased, we checked the expression of the apoptotic factors Bax and Bcl-xl (Fig. 6c). The IFNα/5-FU combination significantly increased Bax expression, although the effect was not dramatic. Each of the above treatments in turn reduced Bcl-xl expression, although in this case the IFNα/5-FU combination produced no enhancement.

### *3.8. Involvement of the Fas/FasL pathway in IFNα/5-FU-induced PBMC cytotoxicity against HCC cells*

We performed $^{51}$Cr-release assay to evaluate the interaction between PBMC and hepatoma cell lines via the Fas/FasL pathway by which IFNα/5-FU appear to exert their influence (Fig. 7). In the Fas-positive HepG2 cells, we first established the optimal *E/T* ratio, which is directly proportional to the increase in released $^{51}$Cr (Fig. 7a). The blocking effect of neutralizing anti-Fas antibody, ZB-4, is shown in Fig. 7b. Based on these data, an *E/T* ratio of 20 was chosen, to produce the most distinct difference in the presence and absence of ZB4 (Fig. 7c). Fig. 7c shows that IFNα increased the released $^{51}$Cr and that this enhanced cytotoxicity was blocked by ZB4. Next, we tried to identify the main proponent of the cytotoxic effect using a magnetic sorting technique. $CD4^+$ cells, $CD8^+$ cells, and $CD4^-CD8^-$ cells were isolated from PBMCs and used as effecter cells in the $^{51}$Cr-release assay after pretreatment with IFNα and/or 5-FU (Fig. 7d). The HepG2 target cells received no pretreatment. The results show that the $CD4^-CD8^-$ cells were the most cytotoxic and that IFNα enhanced this effect more than 5-fold. This IFNα-induced cytotoxicity was markedly inhibited by ZB4. Lastly, we pretreated both effecter and target cells with IFNα/5-FU

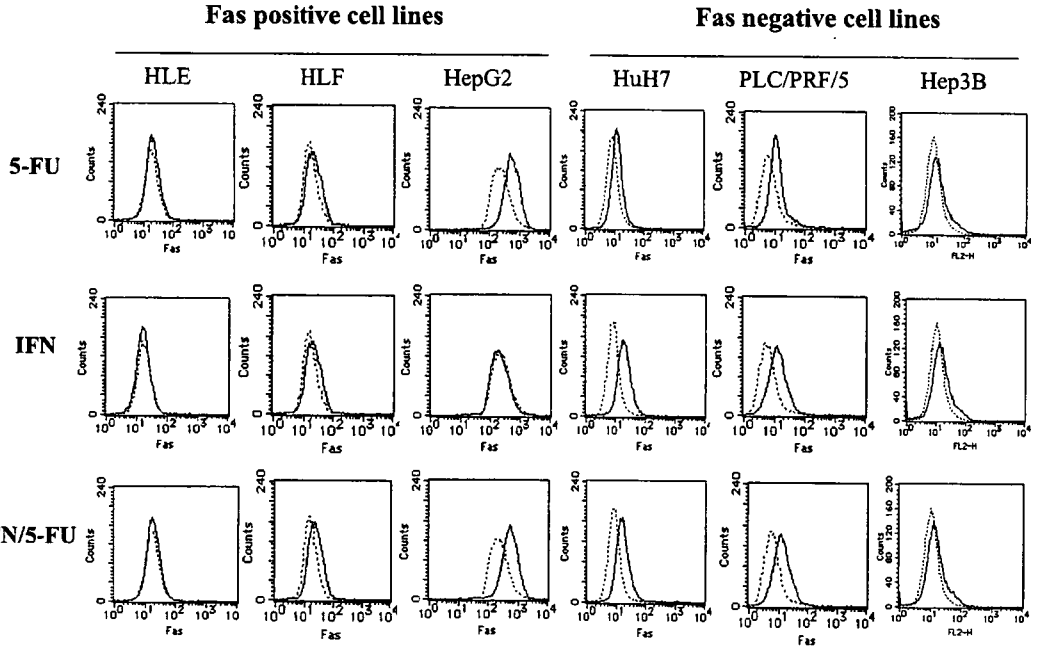


**Fig. 4. Regulation of Fas expression induced by IFNα and/or 5-FU. Adherent cells were incubated with IFNα (500 U/ml) and/or 5-FU (0.5 μg/ml) for 24 h. Cell surface Fas was detected by flow cytometry using a mouse monoclonal anti-human Fas IgG (UB2). Histogram with dotted line shows untreated; histogram with solid line represents the drug-treated cells.**

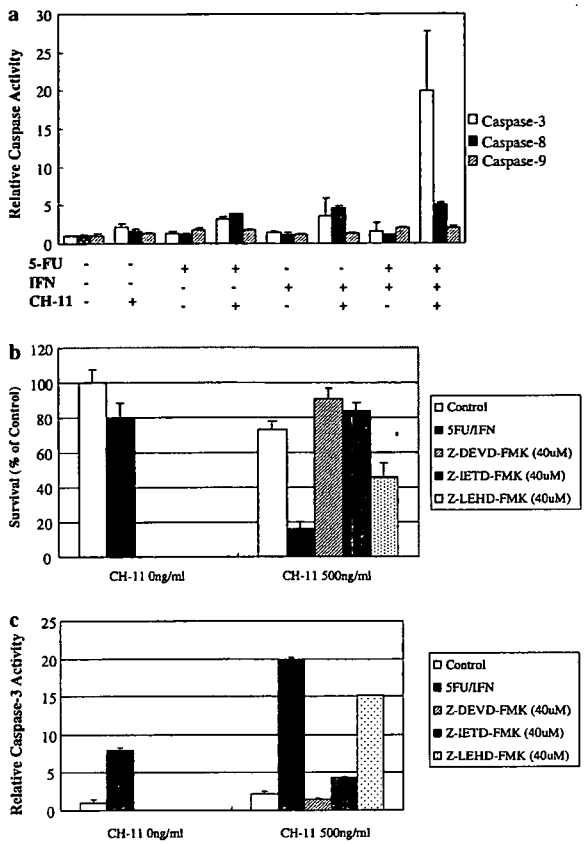


**Fig. 5. (a) Activation of caspase-3, -8 and -9 assessed using the Caspase Colorimetric Protease Assay Kit. Data are given relative to results for untreated HepG2 cells. The activity of caspase-3 after 12 h CH-11 (500 ng/ml) treatment was significantly increased by IFNα (500 U/ml) and 5-FU (0.5 μg/ml) 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.**

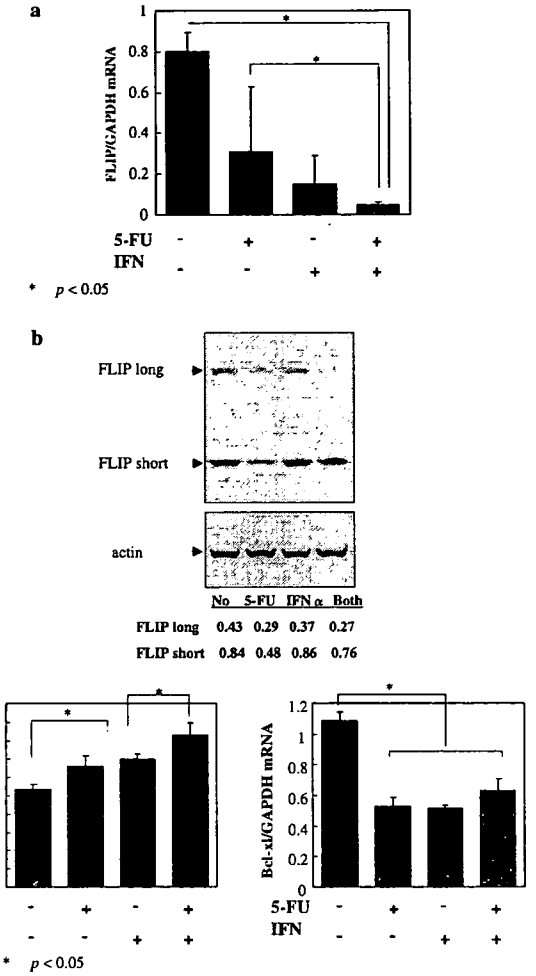


**Fig. 6. (a) Expression of FLIP mRNA by RT-PCR. Combination of IFNα (500 U/ml) and 5-FU (0.5 μg/ml) significantly decreased the expression of FLIP mRNA. (b) Western blot analysis of FLIP. Expression of FLIP was decreased by IFNα (500 U/ml) and 5-FU (0.5 μg/ml) in the protein level. (c) Expression of 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–

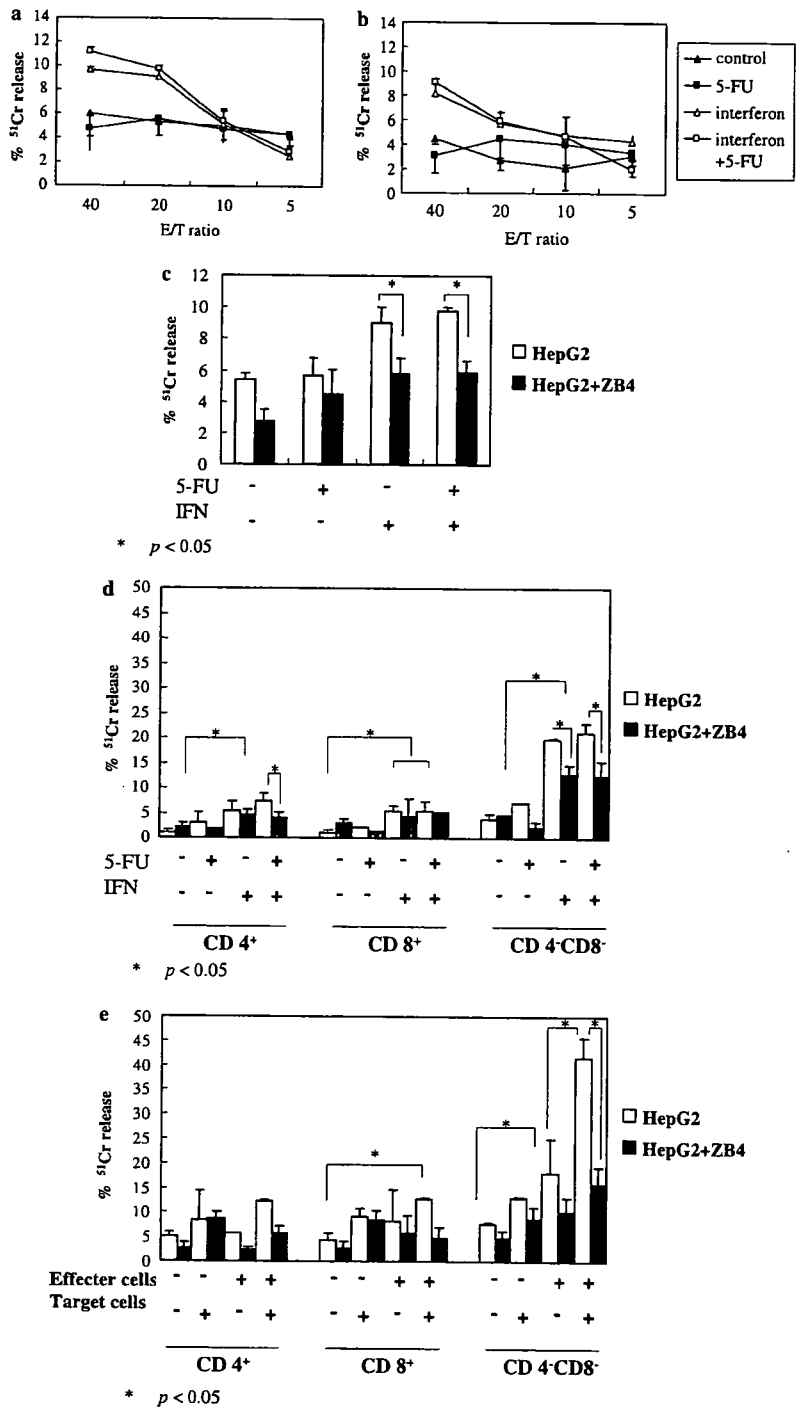


Fig. 7. Cytotoxicity of PBMCs assessed by the 8-h $^{51}$Cr-release assay. (a) HepG2 cells were used as target cells. PBMCs stimulated with IFNα (500 U/ml) and/or 5-FU (0.5 μg/ml) for 24 h were used as effecter cells at *E/T* ratios as indicated, without neutralizing anti-Fas antibody, ZB4. (b) The target 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.

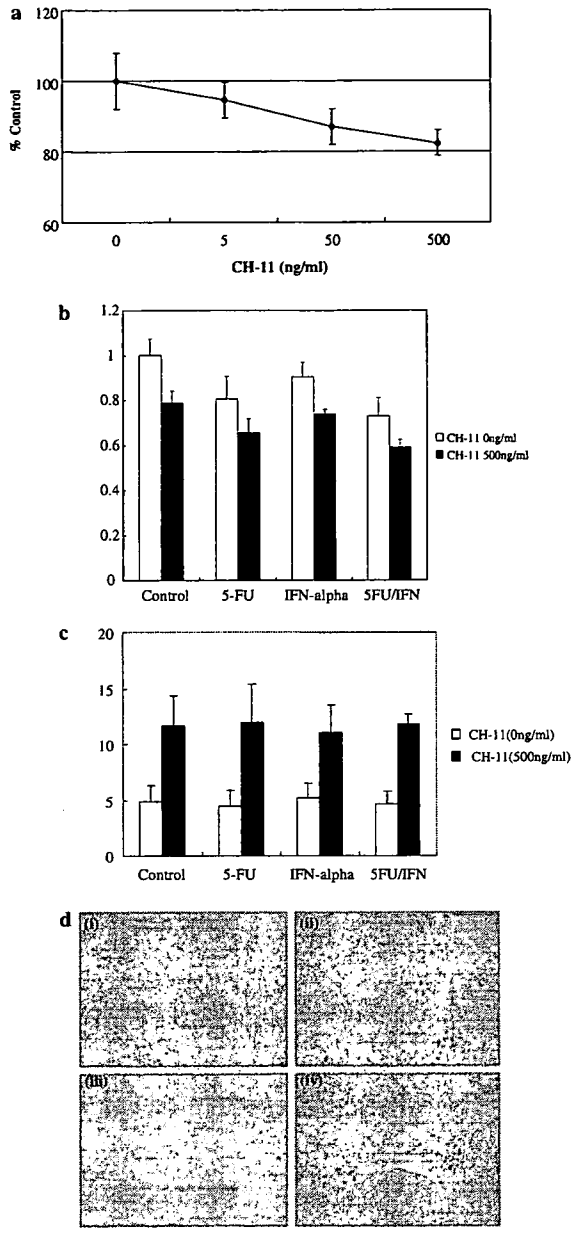


**Fig. 8. Hepatotoxicity of IFNα/5-FU was examined using normal hepatocyte cell line THLE-2 and resected human samples. (a) THLE-2 showed sensitivity to CH-11 in the dose-dependent manner in 48 h MTT assay. (b) THLE-2 were incubated with IFNα (500 U/ml) and/or 5-FU (0.5 μg/ml) and with agonistic anti-Fas 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

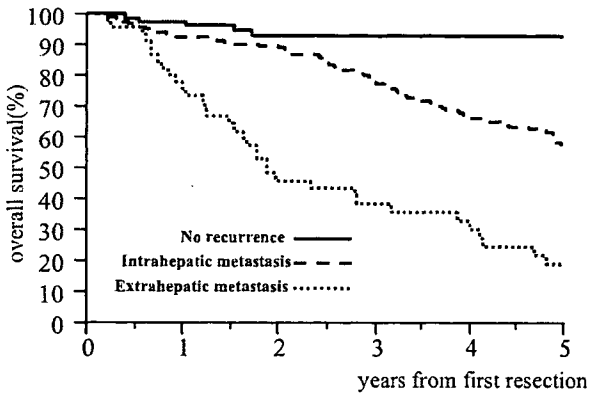


**Fig 1.** Comparison of overall survival rates after the first resection among the nonrecurrence group, intrahepatic recurrence group, and extrahepatic recurrence group. The overall survival rate of the latter group was worse than those of the other 2 groups ($P < .0001$ each).

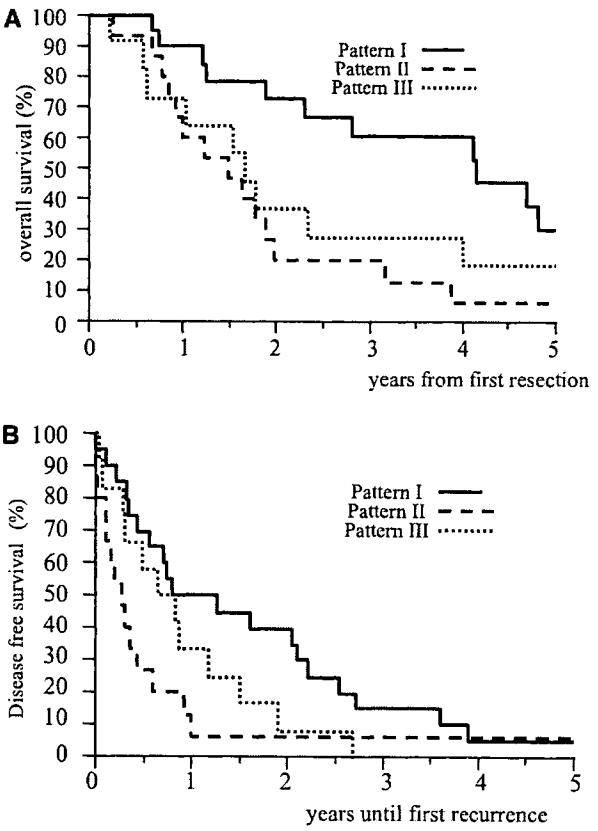


**Fig 2.** Comparison of (**A**) overall survival rates and (**B**) disease-free survival rates after the first resection among patients with patterns I, II and III. The overall survival rate and disease-free survival rate of pattern I was greater than that of pattern II ($P < .05$ each). There were 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

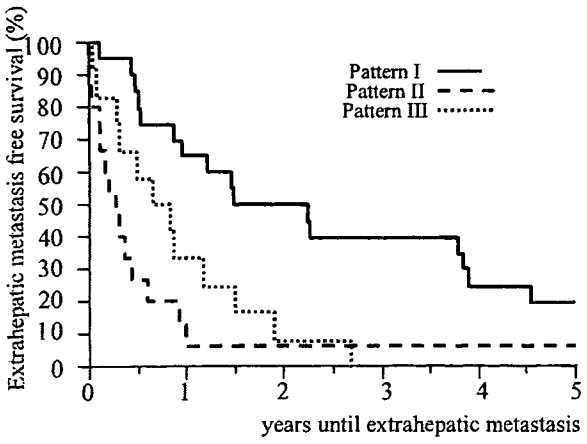


**Fig 3.** Comparison of extrahepatic metastasis-free survival rates among patterns I, II, and III. The interval until extrahepatic metastasis in pattern I was longer than those of patterns II and III ($P < .05$ each).

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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