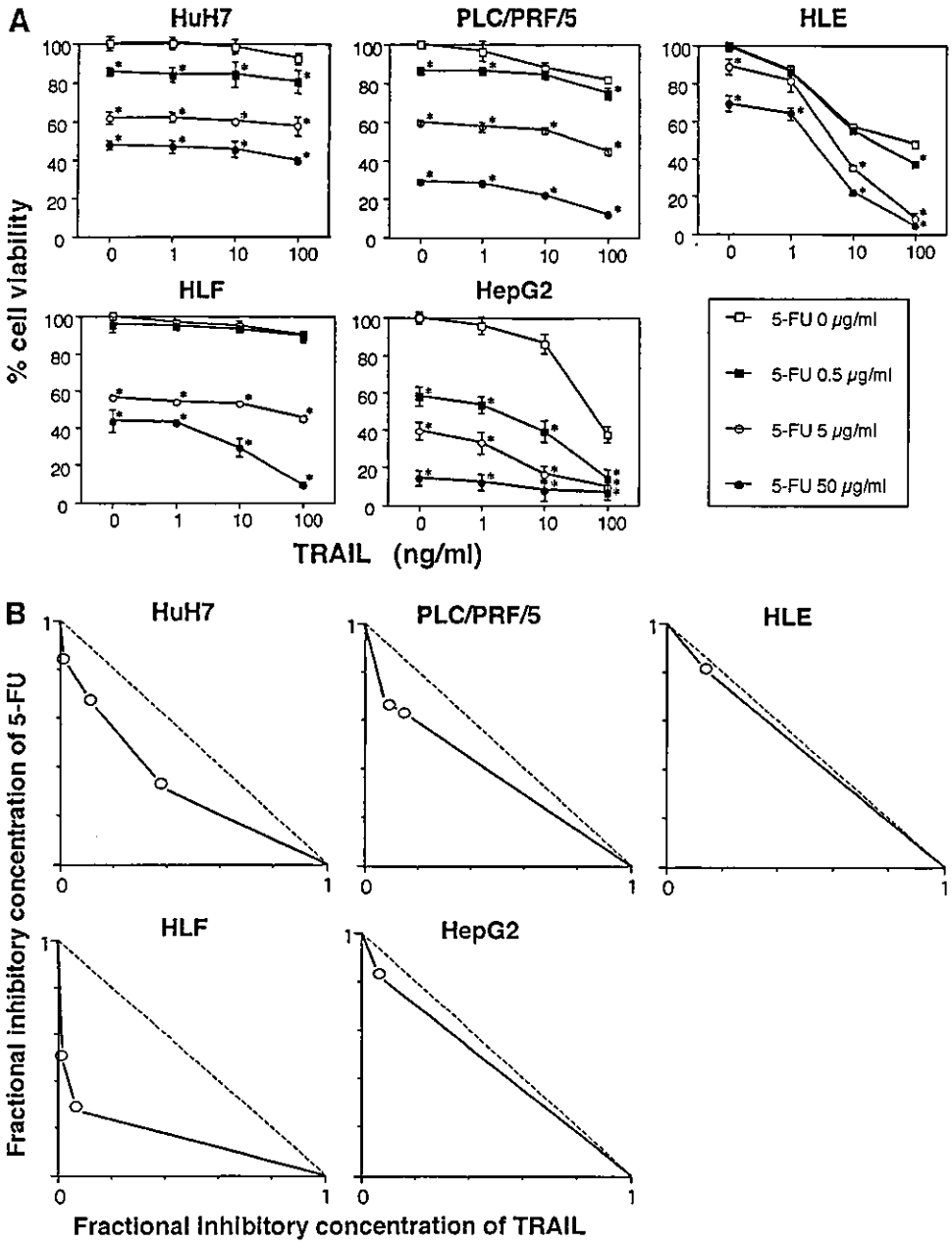

Fig. 1 Effects of 5-FU on TRAIL-induced cytotoxicity in HCC cell lines. *A*, HuH7, PLC/PRF/5, HLE, HLF, and HepG2 cells were treated simultaneously with the indicated concentrations of soluble human TRAIL and 5-FU for 48 hours. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was done to evaluate cell viability. Data are represented as mean ± SD of triplicate samples. Similar results were obtained in three independent experiments. Differences in the percentage of cell viability between TRAIL alone and TRAIL plus 5-FU were determined by the Dunnett post hoc procedure. *, $P < 0.05$. *B*, synergy was estimated by isobolographic analysis.

TRAIL receptor. The effect of combination of 5-FU and IFNα was nearly equal to that of 5-FU alone. As summarized in Fig. 2*C*, the effects of 5-FU and/or IFNα on the expression of TRAIL receptors on other HCC cell lines were similar to the HepG2 case, except for HLE cells, which showed no changes in response to either 5-FU or IFNα. These results indicate that 5-FU, but not IFNα, can significantly increase the expression of TRAIL-R1 and R2 on some HCC cell lines.

Effect of 5-FU and/or IFNα on TRAIL Expression on PBMC. We next examined the effects of 5-FU and/or IFNα on TRAIL expression on PBMC subpopulations (Fig. 3*A*). TRAIL expression was not detected on freshly prepared PBMC by flow cytometry. Incubation with IFNα (500 units/mL) for 24 hours markedly induced TRAIL expression on $CD14^+$ monocytes and $CD56^+$ NK cells. $CD4^+$ T cells, but not $CD8^+$ T cells, also expressed TRAIL at a low level after IFNα stimulation. On the other hand, 5-FU (0.5 μg/mL) did not induce TRAIL expression alone or altered the effect of IFNα on TRAIL expression. IFNα treatment resulted in overexpression of TRAIL mRNA in $CD14^+$ monocytes and $CD56^+$ NK cells. In contrast, no such increase was noted in $CD19^+$ B cells (Fig. 3*B*).

Involvement of TRAIL in IFNα-Stimulated PBMC Cytotoxicity against 5-FU-Treated HCC Cells. We did the ^{51}Cr release assay to investigate the involvement of TRAIL in

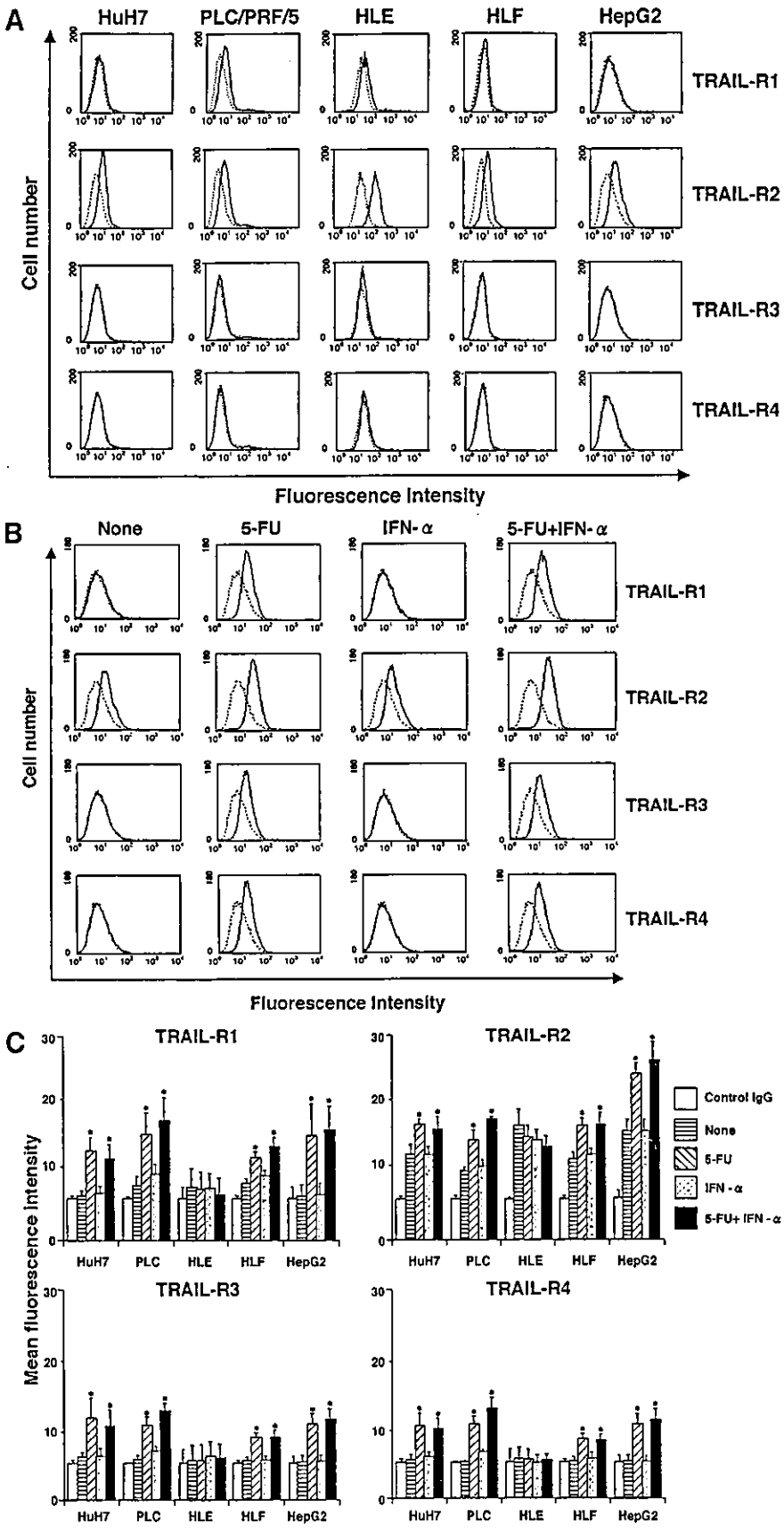

Fig. 2 *A*, Expression of TRAIL receptors on the surface of HCC cell lines. *Dotted lines*, staining with biotinylated control IgG; *solid lines*, staining with biotinylated mAb against the indicated TRAIL receptors. *B* and *C*, effects of 5-FU and/or IFNα on TRAIL receptor expression on HCC cell lines. HepG2 cells (*B*) and the indicated HCC cells (*C*) were treated with 5-FU (0.5 μg/mL) and/or IFNα (500 units/mL) for 48 hours. *B*, *dotted lines*, staining with biotinylated control IgG; *solid lines*, staining with biotinylated mAb against the indicated TRAIL receptors. Similar results were obtained in three independent experiments. *C*, *open bars*, the mean fluorescence intensity of staining with biotinylated control IgG; *other bars*, the mean fluorescence intensity of staining with biotinylated mAbs against the indicated TRAIL receptors. Data are mean ± SD of three independent experiments. *, $P < 0.05$ compared with untreated cells.

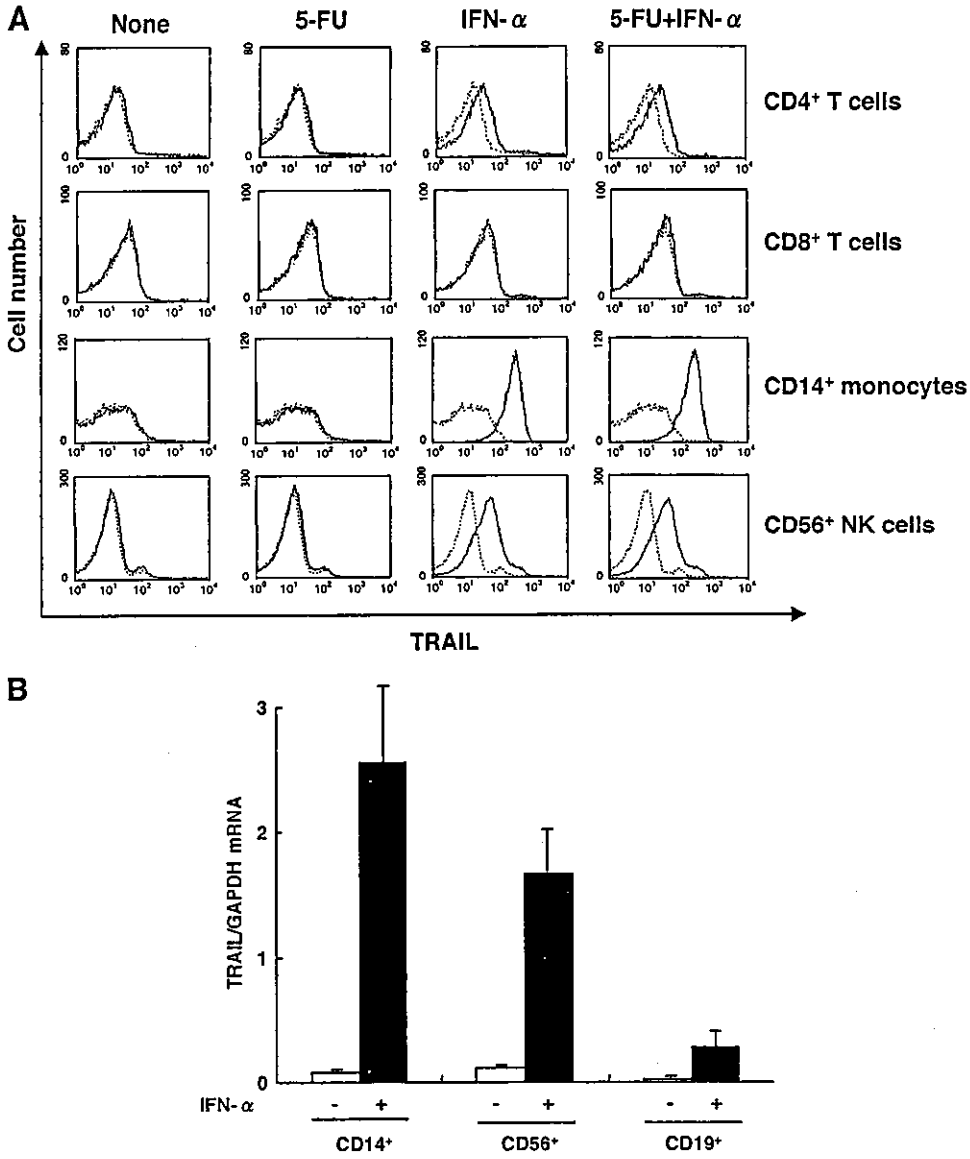

Fig. 3 *A*, regulation of TRAIL expression on $CD4^+$ T cells, $CD8^+$ T cells, $CD14^+$ monocytes, and $CD56^+$ NK cells in PBMC by 5-FU and/or IFNα. PBMC from healthy subjects were cultured in the presence or absence of 5-FU (0.5 μg/mL) and/or IFNα (500 units/mL) for 24 hours and then stained with biotinylated anti-TRAIL mAb followed by phycoerythrin-labeled avidin and FITC-labeled antihuman CD4, CD8, CD14, or CD56 mAb. *Dotted lines*, staining with biotinylated control IgG; *solid lines*, staining with biotinylated anti-TRAIL mAb. Similar results were obtained in three independent experiments. *B*, TRAIL mRNA expression in $CD14^+$ monocytes, $CD56^+$ NK cells, and $CD19^+$ B cells. Cells were isolated from PBMC by using anti-CD14, anti-CD56, and anti-CD19 immunomagnetic beads and Magnetic Cell Sorting and cultured in the presence (*closed bars*) or absence (*open bars*) of IFNα (500 units/mL) for 24 hours. LightCycler PCR and detection system were used to do quantitative RT-PCR for TRAIL, and expression of TRAIL mRNA is indicated relative to GAPDH. Data are mean ± SD of three independent experiments.

IFNα-stimulated PBMC cytotoxicity against untreated or 5-FU–treated HCC cell lines for which 5-FU and soluble TRAIL exhibited synergistic (HLF) or additive (HepG2) effect. We first used $CD4^+$ T cells, $CD8^+$ T cells, and $CD4^-CD8^-$ cells isolated from PBMC as the effector cells after incubation with or without IFNα for 24 hours. As shown in Fig. 4*A*, $CD4^-CD8^-$ cells, but not $CD4^+$ or $CD8^+$ T cells, were the main effector cells that exhibited substantial cytotoxicity against HLF and HepG2. When $CD4^-CD8^-$ cells were stimulated by IFNα, the cytotoxicity was significantly increased from 3.6 to 18% against HLF and from 43 to 63% against HepG2 at an E:T ratio of 20. Moreover, when the target cells were pretreated with 5-FU, IFNα enhanced the cytotoxicity of $CD4^-CD8^-$ cells from 19 to 54% against HLF and from 48 to 66% against HepG2. These results indicate a synergistic effect against HLF and an additive effect against HepG2 of the combination of IFNα and 5-FU on $CD4^-CD8^-$ cell-mediated cytotoxicity.

To investigate which subset of $CD4^-CD8^-$ cells was responsible for the IFNα-induced cytotoxicity, we prepared effector cells that were depleted of $CD14^+$ monocytes and/or $CD56^+$ NK cells from $CD4^-CD8^-$ cells. As shown in Fig. 4*B*, $CD4^-CD8^-CD14^-$ cells (mainly composed of NK cells and B cells) and $CD4^-CD8^-CD56^-$ cells (mainly composed of monocytes and B cells) exhibited higher cytotoxicity against HLF cells than $CD4^-CD8^-CD14^-CD56^-$ cells (mainly composed of B cells). IFNα significantly enhanced the cytotoxicity of $CD4^-CD8^-CD14^-$ cells, and pretreatment of the target cells with 5-FU significantly enhanced the cytotoxicity by both $CD4^-CD8^-CD14^-$ cells and $CD4^-CD8^-CD56^-$ cells. These results suggested that both $CD56^+$ NK cells and $CD14^+$ monocytes were the major effector cells that largely contributed to the IFNα-induced cytotoxicity of PBMC against 5-FU–treated HCC cells.

Finally, we examined the contribution of TRAIL by using

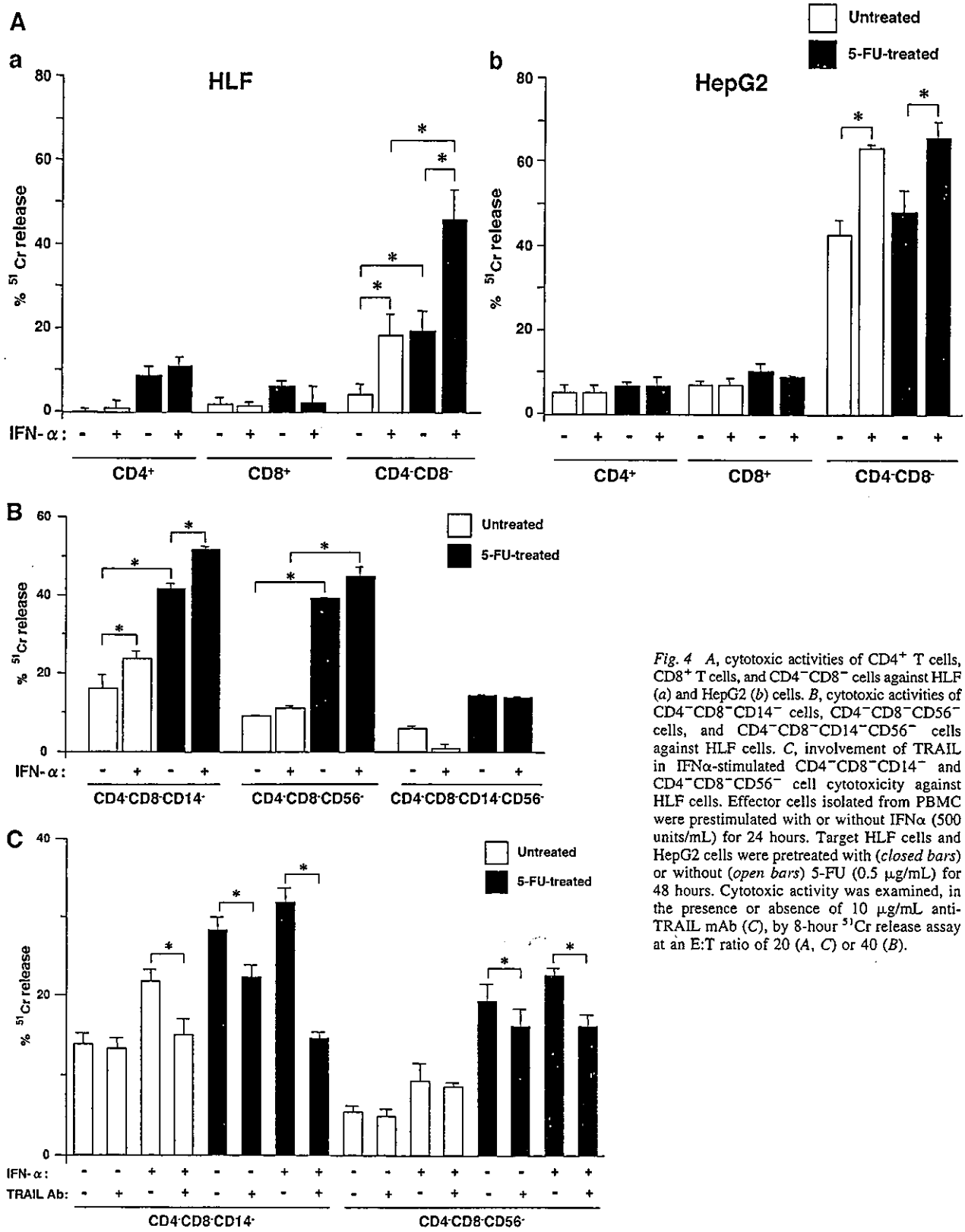

Fig. 4 *A*, cytotoxic activities of $CD4^+$ T cells, $CD8^+$ T cells, and $CD4^-CD8^-$ cells against HLF (*a*) and HepG2 (*b*) cells. *B*, cytotoxic activities of $CD4^-CD8^-CD14^-$ cells, $CD4^-CD8^-CD56^-$ cells, and $CD4^-CD8^-CD14^-CD56^-$ cells against HLF cells. *C*, involvement of TRAIL in IFNα-stimulated $CD4^-CD8^-CD14^-$ and $CD4^-CD8^-CD56^-$ cell cytotoxicity against HLF cells. Effector cells isolated from PBMC were prestimulated with or without IFNα (500 units/mL) for 24 hours. Target HLF cells and HepG2 cells were pretreated with (*closed bars*) or without (*open bars*) 5-FU (0.5 μg/mL) for 48 hours. Cytotoxic activity was examined, in the presence or absence of 10 μg/mL anti-TRAIL mAb (*C*), by 8-hour ^{51}Cr release assay at an E:T ratio of 20 (*A*, *C*) or 40 (*B*).

neutralizing anti-TRAIL mAb (RIK-2). As shown in Fig. 4*C*, IFNα-induced cytotoxicity of $CD4^-CD8^-CD14^-$ cells was almost completely abrogated by anti-TRAIL mAb, irrespective of pretreatment of HLF target cells with 5-FU. In addition, the enhanced cytotoxicity of $CD4^-CD8^-CD56^-$ cells against 5-FU-treated HLF cells was also significantly inhibited by anti-TRAIL mAb. These results suggest that IFNα-induced cytotoxicity of $CD56^+$ NK cells is predominantly mediated by TRAIL and that 5-FU–enhanced cytotoxicity of $CD14^+$ monocytes is at least partly mediated by TRAIL.

Increased Expression of TRAIL mRNA in PBMC of HCC Patients Who Responded to IFNα/5-FU Therapy. The above *in vitro* results suggest the possible contribution of TRAIL to IFNα/5-FU combination therapy. In the next step, we addressed the clinical relevance of the *in vitro* studies by using clinical samples. PBMC from 12 patients with unresectable HCC associated with multiple intrahepatic tumors and Vp3–4, who were treated with IFNα/5-FU combination therapy, were collected 48 hours after the last subcutaneous injection of IFNα. The characteristics of these 12 patients are shown in Table 1. Before starting the therapy, blood samples were also collected as control from all patients. Six patients (responders) showed clinical benefits (complete response [CR] in one of six and partial response [PR] in five of six) whereas the other six (nonresponders) did not (progressive disease [PD] in six of six) according to the Eastern Cooperative Oncology Group criteria. The age of the patients was from 32 to 80. The mean age of the responders was higher (56.8) than that of nonresponders (46.6). All 12 patients had chronic hepatitis that progressed to liver cirrhosis except cases 12. Five patients had hepatitis B virus infection, and seven patients had hepatitis C. Nine of the 12 patients were classified as Child-Pugh class B, whereas two patients were class C, and one patient was class A. In all patients, HCCs were diagnosed as unresectable because of multiple intrahepatic metastasis and the presence of tumor thrombi in the major branch (Vp3) or trunk (Vp4) of the portal vein. Moreover, two patients had tumors that invaded the bile duct. Extrahepatic metastases were not found, and the stage of the disease was IVA ($T_4N_0M_0$) in all 12 patients. Five of the six responders had received >4 treatment cycles, which was associated with prolongation of prognosis (from 6 to 23 months), whereas all six nonresponders could not be treated for >2 cycles and died within 6 months. There were no apparent technical side effects resulting from catheter insertion and subcutaneous implantation of the reservoir. Grade 3 leukopenia was observed in three patients (cases 3, 4, and 11) and was well managed by the administration of granulocyte colony-stimulating factor. Grade 3 thrombocytopenia was observed in one patient (case 10) and temporarily necessitated discontinuation of therapy. Three patients reported diarrhea, and it was managed by binding medicine, but was not the reason for interruption of therapy. Case 11 developed gastric ulcer during therapy in addition to diarrhea and leukopenia, and the patient decided to stop the therapy. In contrast, 6 of the 12 patients showed no remarkable adverse effects. Fever was commonly observed but easily controlled by nonsteroidal anti-inflammatory drugs.

The expression of TRAIL mRNA in peripheral blood of these patients was measured by real-time RT-PCR. Although the expression levels of TRAIL mRNA in PBMC varied from one individual to another, the average TRAIL mRNA level increased by 1.5- to 2.5-fold after initiation of therapy in the patients who clinically showed a complete response or partial response(Fig. 5*A*). In contrast, no such increase was observed in the patients who clinically showed PD. We also measured the fold index of TRAIL/β-actin, and the result was consistent (data not shown). These results suggest that induction of TRAIL in PBMC correlates with the clinical response to IFNα/5-FU combination therapy.

Expression of TRAIL Receptor and TRAIL in HCC Tissues. To confirm the involvement of TRAIL/TRAIL receptor system in the liver of IFNα/5-FU–treated patients, we further investigated the expression of TRAIL-R1, R2, and TRAIL in HCC tissues of the three patients who exhibited

Table 1 Characteristics of the patients with unresectable hepatocellular carcinoma associated with multiple intrahepatic tumors and Vp3–4, who were treated with IFNα/5-FU combination therapy

Case	Age	Gender	Hepatitis	Child-Pugh classification	Tumor pathology	Stage of disease	Treatment cycles	Response	Side effects *	Outcome (mos)
1	55	M	HBV	B	Vp4, multiple	$T_4N_0M_0$ Stage IVA	6	PR	Diarrhea	23, dead
2	70	M	HCV	B	Vp3, multiple	$T_4N_0M_0$ Stage IVA	2	PR	-	17, dead
3	65	F	HCV	B	Vp3, multiple	$T_4N_0M_0$ Stage IVA	5	PR	Leukopenia	13, dead, lung metastasis
4	54	M	HBV	B	Vp3, multiple	$T_4N_0M_0$ Stage IVA	6	PR	Leukopenia	13, dead
5	65	F	HCV	C	Vp3, multiple	$T_4N_0M_0$ Stage IVA	4	PR	-	9, dead
6	32	M	HBV	C	Vp3, multiple, B (+)	$T_4N_0M_0$ Stage IVA	4	CR	-	6, dead
7	51	M	HBV	B	Vp4, multiple	$T_4N_0M_0$ Stage IVA	2	PD	-	5, dead
8	57	M	HCV	B	Vp3, multiple	$T_4N_0M_0$ Stage IVA	2	PD	-	4, dead
9	43	M	HBV	B	Vp4, multiple	$T_4N_0M_0$ Stage IVA	3	PD	-	4, dead
10	49	F	HBV	B	Vp3, multiple	$T_4N_0M_0$ Stage IVA	2	PD	Thrombocytopenia	4, dead
11	80	F	HCV	B	Vp3, multiple, B (+)	$T_4N_0M_0$ Stage IVA	1	PD	Diarrhea, gastric ulcer leukopenia	3, dead
12	70	M	HCV	A	Vp3, multiple	$T_4N_0M_0$ Stage IVA	1	PD	Diarrhea	2, dead

Abbreviations: Vp4, tumor thrombosis in the trunk of the portal vein; Vp3, tumor thrombosis in the major branches of the portal vein; B (+), bile duct invasion; CR, complete response; PR, partial response; PD, progressive disease.

* Thrombocytopenia and leukopenia represent platelet and leukocytecounts $<0.4 \times 10^5/\mu L$ and $2{,}000/\mu L$, respectively.

partial response to IFNα/5-FU combination therapy (responders) and one patient who exhibited PD (nonresponder). Both TRAIL-R1 and R2 were expressed in HCC cells of all IFNα/5-FU–treated patients regardless of their clinical benefits. Expression of TRAIL-R1 and R2 was located mainly in the cytoplasm and plasma membrane. Representative specimens are shown in Fig. 5*B-a* and 5*B-b*. Immunohistologic studies also showed TRAIL expression in tumor-infiltrating mononuclear

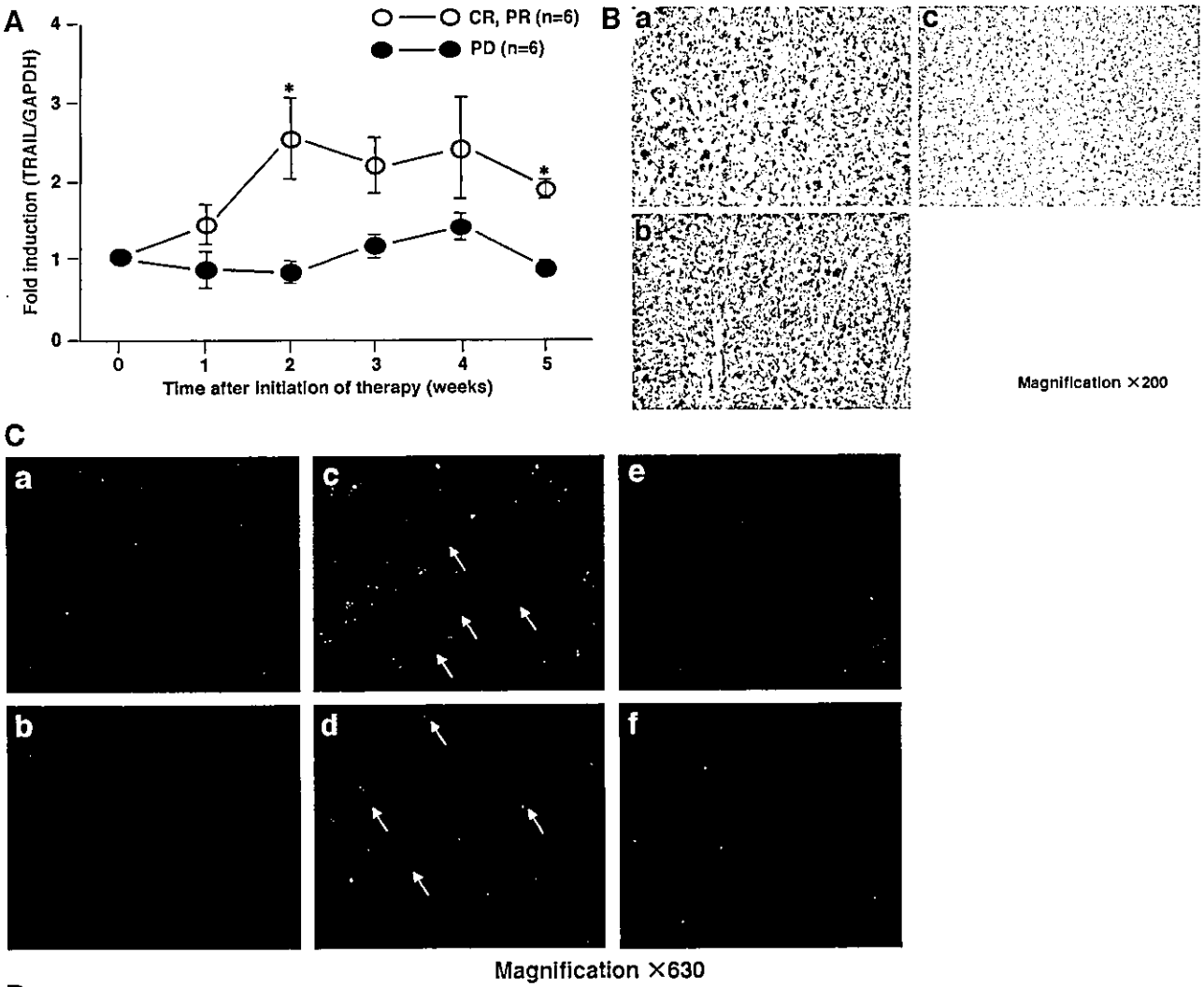

Fig. 5 *A*, TRAIL mRNA expression in PBMC from patients with HCC who received the IFNα/5-FU combination therapy. *Open circles*, patients who showed a clinical response [complete response (CR) or partial response (PR)]; *closed circles*, patients who showed no clinical response [progressive disease (PD)]. Data are mean ± SEM of six patients in each group. *, $P < 0.05$ compared with the PD groups. *B*, immunohistochemical staining of liver specimens with (*a*) antihuman TRAIL-R1 antibody, (*b*) antihuman TRAIL-R2 antibody, and (*c*) normal rabbit IgG (original magnification ×200). TRAIL-R1 and TRAIL-R2 were expressed in all HCC tissues of all four patients. *C*, expression of TRAIL in tumor-infiltrating mononuclear cells. For immunofluorescent staining, the tissue was stained with antihuman TRAIL antibody and Alexa 568-labeled streptavidin. TRAIL-positive cells appear *red* in color (*arrows*; original magnification ×630). Nontumor area of the liver tissue from the nonresponder (*a*), tumor site of the liver tissues from the nonresponder (*b*), nontumor site of liver tissue from a representative responder (*c*), tumor site of liver tissue from a representative responder (*d*), normal liver tissue (*e*), negative control staining with the immunizing peptide in the TRAIL-antibody (*f*). *D*, TRAIL-positive cell counts in liver tissues. NT, nontumor site; T, tumor site. Data are mean ± SD of more than six 360 magnification fields. *, $P < 0.05$ compared with the normal liver.

cells of three responders. Representative specimens are shown in Fig. 5*C-c* and 5*C-d*. In contrast, TRAIL-expressing cells were not detected in the nonresponder or in the normal liver (Fig. 5*C-a, b, e*). Quantitative analysis revealed significantly higher numbers of TRAIL-positive mononuclear cells in the tumor site and nontumor site of liver tissues of responders compared with those of nonresponder and normal liver tissue (Fig. 5*D*). These results suggest the possible contribution of TRAIL at the tumor site.

DISCUSSION

HCC often invades major adjacent vessels such as the portal vein, causes intra-hepatic metastasis, and finally becomes uncontrollable despite repetitive and multimodal therapy (1). We have previously reported the efficacy of IFNα and 5-FU combination therapy against advanced HCC based on its high responsive rate and low incidence of side effects (8). Although some reports have characterized the synergistic action of IFNα and 5-FU against colon carcinoma cells (9, 10), the reasons for IFNα enhancement of the antitumor effect of 5-FU against HCC has not yet been elucidated. In the present study, we showed in our *in vitro* experiments that TRAIL receptors on HCC cells were up-regulated by 5-FU and that IFNα induced TRAIL on $CD4^+$ T cells, $CD14^+$ monocytes, and $CD56^+$ NK cells. Furthermore, the cytotoxic action of $CD14^+$ monocytes and $CD56^+$ NK cells against HCC cells was enhanced through the TRAIL-mediated pathway after treatment of effector cells by IFNα and target HCC cells by 5-FU. These *in vitro* results suggest the possible contribution of TRAIL/TRAIL receptor-mediated antitumor effector mechanism by host innate immune cells to the antitumor effect of IFNα/5-FU combination therapy. Consistent with this notion, a positive correlation between the clinical response and expression of TRAIL in PBMC was observed in patients with HCC who received the combination therapy. Moreover, TRAIL-R1 and R2 were expressed in HCC cells of all IFNα/5-FU–treated patients, and TRAIL-expressing tumor infiltrating cells were found in those patients who responded to the combination therapy.

It is conceivable that the TRAIL-mediated antitumor activity requires the expression of death-inducing TRAIL receptors, TRAIL-R1 and TRAIL-R2, on tumor cells (29, 30). However, it is still not clear whether the basal expression level of TRAIL-R1 or TRAIL-R2 correlates with sensitivity to TRAIL. Previous studies suggested a positive correlation between TRAIL sensitivity and TRAIL-R1 or TRAIL-R2 expression in melanomas, renal cell carcinomas, and Jurkat clones (29–31), whereas other studies suggested no correlation in breast carcinomas and multiple myelomas (32, 33). In HCC cell lines, it has been reported that their TRAIL sensitivity did not correlate with the basal expression level of TRAIL receptors (34, 35). In the present study, three of five HCC cell lines (HuH7, PLC/PRF/5, and HLF) were rather resistant and two of five HCC cell lines (HLE and HepG2) were highly sensitive to soluble TRAIL. Compared with TRAIL-resistant HCC cell lines, the TRAIL-sensitive HCC cell lines expressed higher basal levels of TRAIL-R2. In contrast, the basal expression of TRAIL-R1 did not correlate with TRAIL sensitivity. All five HCC cell lines showed negative basal expression of TRAIL-R3 and R4, despite different TRAIL sensitivity. These results indicate that the basal expression level of TRAIL-R2 correlates positively with TRAIL sensitivity of HCC cells.

Treatment of HCC cells with TRAIL and 5-FU showed a variable sensitivity to TRAIL among the five HCC cell lines. A synergistic effect was observed against one cell line (HLF) and an additive effect was observed against the other four cell lines. It has been reported that chemotherapeutic agents, such as doxorubicin, camptothecin, and cisplatin, also synergized with TRAIL to induce apoptosis in HCC cell lines (34, 35). These results are consistent with the report that coadministration of recombinant TRAIL and 5-FU exhibited a synergistic antitumor effect in a mouse model (36). Concerning the modulation of TRAIL receptor expression by 5-FU, all TRAIL receptors, including TRAIL-R1 and TRAIL-R2, were significantly up-regulated in four of five HCC cell lines. A similar up-regulation of TRAIL-R2 was observed previously with DNA-damaging chemotherapeutic drugs such as doxorubicin, cisplatin, and etoposide (33, 37, 38). Previous studies suggested that the synergistic effect of TRAIL and chemotherapeutic agents might be induced through the induction of TRAIL-R2 expression (29, 33, 38). Our result also showed that 5-FU synergistically enhanced TRAIL-mediated cytotoxicity through up-regulation of TRAIL-R1 and R2 in HLF cells. Although up-regulation of TRAIL-R1 and R2 by 5-FU was observed, 5-FU additively enhanced TRAIL-mediated cytotoxicity in HuH7, PLC/PRF/5, and HepG2 cells. In addition, neither up-regulation of TRAIL-receptors nor synergy of 5-FU and TRAIL were observed in HLE cells. These results imply that alteration of TRAIL-receptor expression might depend on differences between HCC cell lines. Previous studies suggested that the alternation of other intracellular factors determines the TRAIL sensitivity (31, 39). It has been shown that TRAIL-induced apoptosis could be inhibited by FLIP and Bcl-xL (40, 41). Recent reports showed the importance of Bax and Bak, which regulate the release of cytochrome *c* and Smac/DIABLO from the mitochondria during TRAIL-induced apoptosis (42, 43). Additional studies are needed to address the possible effect of 5-FU on these intracellular molecules in HCC cells.

We showed that IFNα markedly induced TRAIL expression on $CD14^+$ monocytes and $CD56^+$ NK cells in PBMC. These results are consistent with previous reports (16, 44). 5-FU did not affect the induction of TRAIL by IFNα. We showed that $CD14^+$ monocytes and $CD56^+$ NK cells were the major effector cells that kill target HCC cells *in vitro*, and their cytotoxicity was enhanced by IFNα. This enhancement was predominantly mediated by TRAIL, because neutralizing anti-TRAIL mAb mostly abrogated it. Moreover, when target HCC cells were pretreated with 5-FU, the cytotoxicity by IFNα-stimulated $CD14^+$ monocytes and $CD56^+$ NK cells was markedly enhanced, which was also at least partly mediated by TRAIL. These results suggested that the induction of TRAIL by IFNα in innate immune effector cells, such as NK cells and monocytes, and the modulation of TRAIL sensitivity of HCC target cells may be involved in the beneficial effect of IFNα/5-FU combination therapy. Because FasL and perforin, in addition to TRAIL, are key cytotoxic effector molecules used by IFNα-

activated NK cells (45, 46), additional studies are needed to clarify the contribution of these molecules.

Among the HCC patients who received IFNα/5-FU combination therapy, the expression of TRAIL mRNA in PBMC was significantly higher in clinical responders than in nonresponders. This suggests that up-regulation of TRAIL mRNA in PBMC may be a predictor of the clinical response to the combination therapy. Consistently, Lancaster *et al.* (47) reported recently that overexpression of TRAIL mRNA was associated with favorable survival after chemotherapy in ovarian cancers. In addition, we showed the infiltration of TRAIL-expressing mononuclear cells into the TRAIL-R1– and TRAIL-R2–expressing HCC tissue in responders to the combination therapy. These results support the possible contribution of TRAIL to the clinical response.

Despite the development of various treatment modalities, the prognosis of patients with advanced HCC associated with Vp3 remains extremely poor (6). One of the major obstacles is the low sensitivity of this type of HCC to chemotherapeutic drugs (7). Therefore, the development of combination therapy with various agents exerting differential antitumor effects is urgent and mandatory. In this respect, IFNα-induced TRAIL-mediated cytotoxicity by immune effector cells may be promising, because TRAIL exerts antitumor activity without significant toxicity against normal tissues (37, 48, 49). Consistent with this notion, no measurable hepatotoxicity has been observed in patients receiving IFNα/5-FU combination therapy.

In conclusion, the results of our *in vitro* and *in vivo* studies suggest that modulation of the TRAIL/TRAIL receptor-mediated cytotoxic pathway might partially contribute to the antitumor effects of IFNα and 5-FU combination therapy against HCC. Strategies aimed at further increasing TRAIL expression in immune effector cells and further sensitizing tumor cells to TRAIL are expected to augment the therapeutic effect of such treatment.

REFERENCES

1. Farmer DG, Rosove MH, Shaked A, Busuttil RW. Current treatment modalities for hepatocellular carcinoma. Ann Surg 1994;219:236–47.
2. Befeler AS, Di Bisceglie AM. Hepatocellular carcinoma: diagnosis and treatment. Gastroenterology 2002;122:1609–19.
3. Barnett CC Jr, Curley S. A. Ablative techniques for hepatocellular carcinoma. Semin Oncol 2001;28:487–96.
4. Fujii T, Takayasu K, Muramatsu Y, et al. Hepatocellular carcinoma with portal tumor thrombus: analysis of factors determining prognosis. Jpn J Clin Oncol 1993;23:105–9.
5. Ikai I, Yamaoka Y, Yamamoto Y, et al. Surgical intervention for patients with stage IV-A hepatocellular carcinoma without lymph node metastasis: proposal as a standard therapy. Ann Surg 1998;227:433–9.
6. Fan J, Wu ZQ, Tang ZY, et al. Multimodality treatment in hepatocellular carcinoma patients with tumor thrombi in portal vein. World J Gastroenterol 2001;7:28–32.
7. Shen DW, Lu YG, Chin KV, Pastan I, Gottesman MM. Human hepatocellular carcinoma cell lines exhibit multidrug resistance unrelated to MRD1 gene expression. J Cell Sci 1991;98:317–22.
8. Sakon M, Nagano H, Dono K, et al. Combined intraarterial 5-fluorouracil and subcutaneous interferon-alpha therapy for advanced hepatocellular carcinoma with tumor thrombi in the major portal branches. Cancer (Phila) 2002;94:435–42.
9. Schwartz EL, Hoffman M, O'Connor CJ, Wadler S. Stimulation of 5-fluorouracil metabolic activation by interferon-alpha in human colon carcinoma cells. Biochem Biophys Res Commun 1992;182:1232–9.
10. van der Wilt CL, Smid K, Aherne GW, Noordhuis P, Peters GJ. Biochemical mechanisms of interferon modulation of 5-fluorouracil activity in colon cancer cells. Eur J Cancer 1997;33:471–8.
11. Eguchi H, Nagano H, Yamamoto H, et al. Augmentation of antitumor activity of 5-fluorouracil by interferon alpha is associated with up-regulation of p27Kip1 in human hepatocellular carcinoma cells. Clin Cancer Res 2000;6:2881–90.
12. Brassard DL, Grace MJ, Bordens RW. Interferon-alpha as an immunotherapeutic protein. J Leukoc Biol 2002;71:565–81.
13. Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. Annu Rev Biochem 1998;67:227–64.
14. Kayagaki N, Yamaguchi N, Nakayama M, Eto H, Okumura K, Yagita H. Type I interferons (IFNs) regulate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on human T cells: a novel mechanism for the antitumor effects of type I IFNs. J Exp Med 1999;189:1451–60.
15. Sato K, Hida S, Takayanagi H, et al. Antiviral response by natural killer cells through TRAIL gene induction by IFN-alpha/beta. Eur J Immunol 2001;31:3138–46.
16. Griffith TS, Wiley SR, Kubin MZ, Sedger LM, Maliszewski CR, Fanger NA. Monocyte-mediated tumoricidal activity via the tumor necrosis factor-related cytokine, TRAIL. J Exp Med 1999;189:1343–54.
17. Wiley SR, Schooley K, Smolak PJ, et al. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 1995;3:673–82.
18. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J Biol Chem 1996;271: 12687–90.
19. Pan G, O'Rourke K, Chinnaiyan AM, et al. The receptor for the cytotoxic ligand TRAIL. Science (Wash DC) 1997;276:111–3.
20. Walczak H, Degli-Esposti MA, Johnson RS, et al. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. EMBO J 1997;16: 5386–97.
21. Pan G, Ni J, Wei YF, Yu G, Gentz R, Dixit VM. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science (Wash DC) 1997;277:815–8.
22. Sheridan JP, Marsters SA, Pitti RM, et al. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science (Wash DC) 1997;277:818–21.
23. Degli-Esposti MA, Dougall WC, Smolak PJ, Waugh JY, Smith CA, Goodwin RG. The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. Immunity 1997;7:813–820.
24. Shipman CM, Croucher PI. Osteoprotegerin is a soluble decoy receptor for tumor necrosis factor-related apoptosis-inducing ligand/Apo2 ligand and can function as a paracrine survival factor for human myeloma cells. Cancer Res 2003;63:912–6.
25. Smyth MJ, Takeda K, Hayakawa Y, Peschon JJ, van den Brink MR, Yagita H. Nature's TRAIL-on a path to cancer immunotherapy. Immunity 2003;18:1–6.
26. Kayagaki N, Yamaguchi N, Nakayama M, et al. Involvement of TNF-related apoptosis-inducing ligand in human CD4+ T cell-mediated cytotoxicity. J Immunol 1999;162:2639–47.
27. Phillips TA, Ni J, Pan G, et al. TRAIL (Apo-2L) and TRAIL receptors in human placentas: implications for immune privilege. J Immunol 1999;162:6053–9.
28. Berenbaum MC. Criteria for analyzing interactions between biologically active agents. Adv Cancer Res 1981;35:269–335.
29. Griffith TS, Fialkov JM, Scott DL, et al. Induction and regulation of tumor necrosis factor-related apoptosis-inducing ligand/Apo-2 ligand-mediated apoptosis in renal cell carcinoma. Cancer Res 2002;62: 3093–9.

30. Nguyen T, Zhang XD, Hersey P. Relative resistance of fresh isolates of melanoma to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. Clin Cancer Res 2001;7:966s–973s.

31. Jang YJ, Park KS, Chung HY, Kim HI. Analysis of the phenotypes of Jurkat clones with different TRAIL-sensitivities. Cancer Lett 2003; 194:107–17.

32. Keane MM, Ettenberg SA, Nau MM, Russell EK, Lipkowitz S. Chemotherapy augments TRAIL-induced apoptosis in breast cell lines. Cancer Res 1999;59:734–41.

33. Mitsiades CS, Treon SP, Mitsiades N, et al. TRAIL/Apo2L ligand selectively induces apoptosis and overcomes drug resistance in multiple myeloma: therapeutic applications. Blood 2001;98:795–804.

34. Yamanaka T, Shiraki K, Sugimoto K, et al. Chemotherapeutic agents augment TRAIL-induced apoptosis in human hepatocellular carcinoma cell lines. Hepatology 2000;32:482–90.

35. Shin EC, Seong YR, Kim CH, et al. Human hepatocellular carcinoma cells resist to TRAIL-induced apoptosis, and the resistance is abolished by cisplatin. Exp Mol Med 2002;34:114–22.

36. Ashkenazi A, Pai RC, Fong S, et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Investig 1999;104:155–62.

37. Wu GS, Burns TF, McDonald ER 3rd, et al. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. Nat Genet 1997;17:141–3.

38. Nagane M, Pan G, Weddle JJ, Dixit VM, Cavenee WK, Huang HJ. Increased death receptor 5 expression by chemotherapeutic agents in human gliomas causes synergistic cytotoxicity with tumor necrosis factor-related apoptosis-inducing ligand in vitro and in vivo. Cancer Res 2000;60:847–53.

39. Lacour S, Hammann A, Wotawa A, Corcos L, Solary E, Dimanche-Boitrel MT. Anticancer agents sensitize tumor cells to tumor necrosis factor-related apoptosis-inducing ligand-mediated caspase-8 activation and apoptosis. Cancer Res 2001;61:1645–51.

40. Okano H, Shiraki K, Inoue H, et al. Cellular FLICE/caspase-8-inhibitory protein as a principal regulator of cell death and survival in human hepatocellular carcinoma. Lab Investig 2003;83:1033–43.

41. Kim IK, Jung YK, Noh DY, et al. Functional screening of genes suppressing TRAIL-induced apoptosis: distinct inhibitory activities of Bcl-XL and Bcl-2. Br J Cancer 2003;88:910–7.

42. Kandasamy K, Srinivasula SM, Alnemri ES, et al. Involvement of proapoptotic molecules Bax and Bak in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced mitochondrial disruption and apoptosis: differential regulation of cytochrome c and Smac/DIABLO release. Cancer Res 2003;63:1712–21.

43. Kim JY, Kim YH, Chang I, et al. Resistance of mitochondrial DNA-deficient cells to TRAIL: role of Bax in TRAIL-induced apoptosis. Oncogene 2002;21:3139–48.

44. Takeda K, Hayakawa Y, Smyth MJ, et al. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. Nat Med 2001;7:94–100.

45. Kirou KA, Vakkalanka RK, Butler MJ, Crow MK. Induction of Fas ligand-mediated apoptosis by interferon-alpha. Clin Immunol 2000;95: 218–26.

46. Kaser A, Enrich B, Ludwiczek O, Vogel W, Tilg H. Interferon-alpha (IFN-alpha) enhances cytotoxicity in healthy volunteers and chronic hepatitis C infection mainly by the perforin pathway. Clin Exp Immunol 1999;118:71–7.

47. Lancaster JM, Sayer R, Blanchette C, et al. High expression of tumor necrosis factor-related apoptosis-inducing ligand is associated with favorable ovarian cancer survival. Clin Cancer Res 2003;9:762–6.

48. Walczak H, Miller RE, Ariail K, et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat Med 1999;5:157–63.

49. Lawrence D, Shahrokh Z, Marsters S, et al. Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. Nat Med 2001;7:383–5.

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Journal of Hepatology 39 (2003) 1004–1012

Journal of Hepatology

www.elsevier.com/locate/jhep

Molecular features of non-B, non-C hepatocellular carcinoma: a PCR-array gene expression profiling study

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***Background/Aims*: Hepatocellular carcinoma (HCC) usually develops following chronic liver inflammation caused by hepatitis C or B virus. Through expression profiling in a rare type of HCC, for which the causes are unknown, we sought to find key genes responsible for each step of hepatocarcinogenesis in the absence of viral influence.**

***Methods*: We used 68 non-B, non-C liver tissues (20 HCC, 17 non-tumor, 31 normal liver) for expression profiling with PCR-array carrying 3072 genes known to be expressed in liver tissues. To select the differentially expressed genes, we performed random permutation testing. A weighted voting classification algorithm was used to confirm the reliability of gene selection. We then compared these genes with the results of previous expression profiling studies.**

***Results*: A total of 220 differentially expressed genes were selected by random permutation tests. The classification accuracies using these genes were 91.8, 92.0 and 100.0% by a leave-one-out cross-validation, an additional PCR-array dataset and a Stanford DNA microarray dataset, respectively. By comparing our results with previous reports on virus-infected HCC, four genes (ALB, A2M, ECHS1 and IGFBP3) were commonly selected in some studies.**

***Conclusions*: The 220 differentially expressed genes selected by PCR-array are potentially responsible for hepatocarcinogenesis in the absence of viral influence.**

Keywords: **Hepatocellular carcinoma; DNA microarray; Expression profile; Cryptogenic cirrhosis; IGFBP3**

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. HCC usually develops following the chronic liver inflammation caused by hepatitis virus (HCV, HBV). In East Asia and Africa, where HCC morbidity is the highest in the world, exposure to aflatoxin B1 is an additional major causative factor [1]. The specific mechanism of carcinogenesis is thought to depend upon the type of risk factor involved. Although structural alterations in many cancer-related genes have been reported in HCC [2], the high number of genes involved suggests that different etiologic factors may affect different genes subsets within hepatocytes. Thus, distinct, but related, genetic pathways may be altered during hepatocarcinogenesis, possibly due to different initiators and promoters. Multiple studies linking hepatitis viruses and chemical carcinogens to hepatocarcinogenesis have provided clues for understanding tumorigenesis in this system [3,4], but have not uncovered the genetic events that occur common to the development of HCC. Such results have differed, depending upon the etiologic factors examined; studies on cases of HCC without such backgrounds have been lacking. Biological analysis of this rare type of HCC, in which the carcinogenic causes are unknown, may allow the determination of common genetic events and pathways involved in hepatocarcinogenesis. The molecular features of this disease should represent the key features common to all types of HCC.

Received 23 May 2003; received in revised form 18 August 2003; accepted 26 August 2003

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0168-8278/$30.00
doi:10.1016/S0168-8278(03)00473-2

Gene expression profiling is a powerful molecular technique, wherein the expression levels of an entire mRNA population of a tissue may be characterized. This method is extremely useful for understanding the molecular abnormalities underlying cancer; in fact, HCC has been previously studied using this approach [5–9]. Most previous studies, however, only analyzed cases of HCC stemming from HCV or HBV infection. Here, we sought to examine through an expression profiling study using the rare cases of HCC for which the underlying risk factors are unknown. Although DNA microarray have contributed to the expression profiling studies to some degree, DNA microarrays detect only a fraction of the changes in gene expression which can be detected by RT-PCR [10]. We have therefore prepared a novel technology, a PCR-array system, which is a high throughput quantitative PCR method based on adaptor-tagged competitive PCR (ATAC-PCR) [11]. Using adequate standards, such experiments can be easily controlled. In addition, the assay requires smaller amounts of RNA. Such a PCR-based assay of selected genes costs far less than DNA microarrays, which are likely to require at least several hundred spotted genes for diagnosis. The strength of this system for cancer research has been established in previous work on breast and colorectal cancers [12,13].

To find key genes responsible for each step of hepatocarcinogenesis in the absence of viral influence, we compared the gene expression profiles of three tissue types, tumorous, non-tumorous and normal liver. Strict statistical analysis allowed us to identify 220 genes differentially expressed among the three tissues. Further comparison with previous studies suggested that misexpression of four specific genes is common to HCC, independent of hepatitis virus infection status.

2. Materials and methods

2.1. Tissues and patients

We obtained 37 non-B, non-C liver tissues (20 HCC, 17 non-tumor liver) from 20 patients, with informed written consent, who underwent hepatic resection for HCC at Osaka University Hospital between 1997 and 2001. Tumor (T) and non-tumor (NT) tissue samples were enucleated separately. Serologically, none of the cases were hepatitis B surface antigen- or HCV antibody-positive. Additionally, to rule out possible cryptic HBV or HCV infection, the absence of viral genomes was confirmed by RT-PCR as reported [14]. In brief, to detect HBV RNA, we performed RT-PCR using three independent primer pairs set within the S, C or X region of the HBV mRNA. HCV RNA was also examined by RT-PCR as described [15]. Patients had no history of exposure to aflatoxin B1. As determined pathologically, there were no cases of hemochromatosis, alcoholic hepatitis, or non-alcoholic steatohepatitis (NASH). The clinicopathological characteristics of these 20 cases were shown in Table 1. We also prepared 31 normal liver (N) tissues from patients with other malignancies who underwent hepatic resection for metastatic liver cancer during the same time period. By serological and biological examination, we confirmed the absence of HCV or HBV infection for each of these normal liver specimens. All aspects of our study protocol were performed according to the ethical guidelines set by the committee of the three Ministries of the Japanese Government.

2.2. PCR-array system

To select only genes actually expressed in liver tissues, we constructed cDNA libraries from three sources: a mixture of HCC and non-tumor livers, normal livers, and metastatic liver cancers, as described [16]. A mixture of the total RNAs from seven HCC and six non-tumor samples were used for the HCC library, and 2673 unique sequences were obtained from 9600 clones. We designed PCR primers for the ATAC-PCR reaction for a total of 2384 genes from this EST collection. We also prepared 130 primers from the normal liver library and 260 primers from the metastatic liver cancer library. Altogether, we prepared 3072 primers for ATAC-PCR, which also includes primers for an additional 298 genes known to be expressed in liver from previous literature. The specificity of this gene selection provides an advantage over more universal sets, which include genes not detected in liver tissues. The ATAC-PCR experimental procedure was performed as previously described [17]; a detailed protocol is also available at our web site (http://love2.aist-nara.ac.jp/laboratory/index_frame.html).

2.3. Analysis of PCR-array data

Relative expression levels were calculated by calibration using a common standard, a mixture of 15 HCC tissues and eight non-tumor tissues, as previously described [12,13]. Expression levels below 0.05 were regarded as missing values, possibly due to noise. Genes with missing values in more than 25% of cases were excluded from further analysis. Following conversion to a logarithmic scale, the data matrix was normalized by standardizing each sample to a mean of 0. Hierarchical cluster analysis by neighbor joining with the Pearson correlation coefficient was performed using GeneMaths 2.0 software.

2.4. Random permutation test

Permutation testing, which involves randomly permuting class labels to determine gene-class correlations, was used to determine statistical significance [18–22]. We selected genes differentially expressed between T and NT and between NT and N tissues and performed the permutation test as follows:

When the labels (class A or B) were not permuted, we defined μ_A as the mean of the expression levels of class A samples and μ_B as that of class B samples. The original score, $So = |\mu_A - \mu_B|$, of each gene was then calculated. The labels of all samples were randomly permuted, and scores were re-calculated between two classes consisting of the new members. Repetition of this permutation 100,000 times provides a score number (No) larger than the original score (So). For each gene, the permutation P value was determined as $P = No/100{,}000$.

2.5. Weighted voting algorithm

As a classification method between two classes, we adopted a weighted voting (WV) algorithm, generally used in gene expression profiling [19–23]. In brief, we calculated the signal-to-noise ratio, $S_i = (\mu_A - \mu_B)/(\sigma_A + \sigma_B)$, where μ and σ represent the mean and standard deviation of expression for each class (A and B), respectively. The magnitude of such a 'vote' (v_i) reflects the deviation of the expression level in the test sample (X_i) from the average of the two classes: $v_i = S_i \times (X_i - (\mu_A + \mu_B)/2)$. We summed the votes to obtain total votes for class A (V_A) and class B (V_B). The prediction strength (PS) is $(V_A - |V_B|)/(V_A + |V_B|)$, classifying the class of the test sample; $PS > 0$ means class A, $PS < 0$ means class B. This model can be evaluated by leave-one-out cross-validation, whereby one sample is randomly withheld, a model is trained on the remaining samples, and the model is then used to predict the class of the withheld sample [19–24]. The prediction process was repeated until all samples were tested, and the cumulative accuracy was then recorded.

3. Results

3.1. Expression features of non-B, non-C HCC

We analyzed the expression patterns of 3072 liver-expressed genes in 68 non-B, non-C liver samples,

Table 1
Clinicopathological characteristics of 20 non-B, non-C HCC

Case	Age	Sex	AFP level[a]	Diff. degree[b]	Tumor size[c]	Tumor multiplicity	Vascular invasion[d]	TNM stage[e]	Non-tumor liver
1	67	M	Low	Wel	Small	Solitary	−	I	Normal
2	73	M	Low	Mod	Large	Solitary	−	II	Normal
3	69	M	Low	Por	Large	Solitary	−	II	Normal
4	64	M	Low	Mod	Large	Solitary	−	II	Chronic hepatitis
5	81	F	Low	Mod	Large	Solitary	−	II	Cirrhosis
6	59	M	Low	Mod	Large	Solitary	+	IIIA	Chronic hepatitis
7	59	M	Low	Mod	Small	Solitary	+	II	Chronic hepatitis
8	74	M	High	Por	Large	Solitary	+	IIIA	Normal
9	56	M	Low	Por	Large	Multiple	−	IIIA	Normal
10	64	M	Low	Mod	Large	Multiple	+	IIIA	Chronic hepatitis
11	51	F	High	Por	Large	Multiple	++	IVA	Normal
12	51	F	High	Por	Large	Multiple	++	IVA	Normal
13	70	M	Low	Wel	Small	Solitary	−	I	Cirrhosis
14	59	M	Low	Mod	Large	Multiple	−	IVA	Cirrhosis
15	76	M	Low	Por	Large	Solitary	−	II	Normal
16	64	M	Low	Mod	Small	Solitary	−	I	Cirrhosis
17	60	M	Low	Por	Large	Solitary	++	IVA	Cirrhosis
18	63	M	High	Por	Small	Solitary	−	I	Cirrhosis
19	58	M	low	Mod	Large	Solitary	−	II	Chronic hepatitis
20	74	M	high	Por	Large	Solitary	−	II	Chronic hepatitis

[a] Serum AFP level was divided into either 'low' (< 100 ng/ml) or 'high' (≥ 100 ng/ml).
[b] Histological degree of HCC; wel: well differentiated, mod: moderately differentiated, por: poorly differentiated.
[c] Tumor size was divided into either 'small' (≤ 2 cm) or 'large' (> 2 cm).
[d] Degree of pathological vascular invasion; −: tumor without vascular invasion, +: tumor with vascular invasion, ++: tumor involving a major branch of the portal or hepatic vein.
[e] TNM stage was according to the Union Internationale Contre Le Cancer (UICC) classification (5th version).

including 20 T and 17 NT samples, that were obtained from non-B, non-C HCC patients, and 31 N samples from metastatic liver cancer patients. We selected 1812 genes which included fewer missing values, and performed a hierarchical cluster analysis of samples. When the clinical samples were sorted on the basis of similarity in the expression of these 1812 genes, two major clusters of samples were obtained (Fig. 1). Most cases of the smaller cluster (yellow-cluster) were belonged to T tissues. On the other hand, the larger cluster was consisted of two subclusters (blue- and pink-cluster). Most cases of blue-cluster were belonged to NT tissues, and most pink-cluster cases were N tissues.

3.2. Selection of genes characterizing tissue types

To focus on genes which especially characterize these tissue types, we used the random permutation test as mentioned above. Using $P < 0.01$ as a measure of significance, a total of 61 genes were selected as differentially expressed between T and NT samples (Fig. 2A; Table 2). Similarly, we identified 159 genes which were differentially expressed between NT and N tissues (Fig. 2B; Table 3). The numbers of genes selected by this test were much greater than would be expected by chance (18 genes in both trials).

3.3. Expression of known cancer-related genes

We additionally examined the expression of 12 cancer-related genes, p53, p73, RB1, cyclin D1, M6P/IGF2R, PTEN, IGFBP3, beta-catenin, K-ras, c-myc, c-fos, and p21, which have all been reported to be associated with HCC (Fig. 3) [25–27]. Among these 12 genes, the expression of IGFBP3, c-fos and p21 were significantly different ($P < 0.01$) between T and NT tissues by t test. In addition, p53 and cyclin D1 were found to be differentially expressed between NT and N samples.

3.4. Validation with classification algorithms

To estimate the reliability of gene selection, we utilized a classification system for tissue types based on the WV algorithm, which was evaluated by leave-one-out cross-validation as mentioned above. Using the 61 or 159 differentially expressed genes, the accuracies of classification were 91.9 and 91.7%, respectively. When 20 independent non-B, non-C samples (5 T, 5 NT, and 10 N) were tested, the accuracies were as high as for the original 68 samples (T vs NT: 90.0%, NT vs N: 93.3%). We next sought to re-confirm our findings using results from a different technology, DNA microarrays, which were applied to a Stanford dataset of 24 non-B, non-C samples (10 T and 14 NT) (http://genome-www.stanford.edu/hcc/supplement.shtml) [5]. Of the 61 genes differentially expressed between T and NT tissue, 25 were

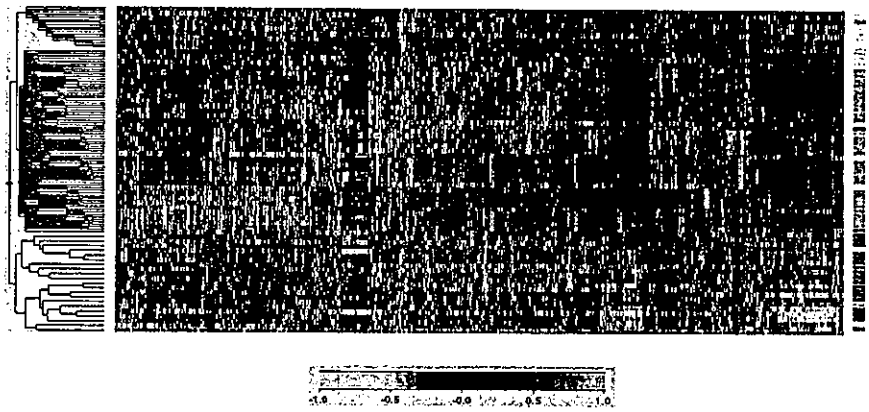

Fig. 1. Hierarchical clustering of 1812 genes in 68 non-B, non-C liver samples (20 HCC, 17 non-tumor, and 31 normal). Each column represents a single gene, while each row represents a patient sample. The tissue type of each sample is designated by a right bar, with red, yellow, and green bars indicating HCC, non-tumor and normal liver samples, respectively. The color scale at the bottom indicates the relative expression levels in terms of standard deviations from the mean.

represented in the Stanford dataset. At the expression level, 21 of the 25 genes displayed significant differences ($P < 0.01$) between the T and NT samples, as determined by t test (data not shown). When the expression data of these 25 genes was applied to the WV algorithms, all 24 cases were correctly classified as either T or NT without exception.

4. Discussion

As it shows striking differences according to geographic location, HCC may be one of the many tumors whose origins differ based on the milieu of epidemiological, clinical, and pathologic risk factors under which it develops. The incidence of HCC is sharply on the rise, most likely due to the spread of HCV, particularly in Japan, Spain and Italy [28]. HBV also represents a major risk factor in East Asia, where the prevalence of this infection is high [28]. In South

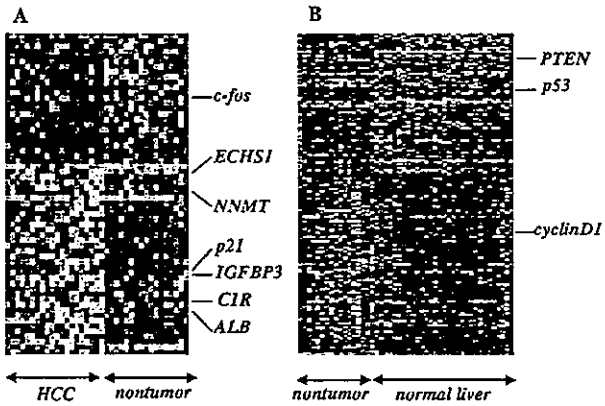

Fig. 2. (A) The expression pattern of the 61 genes differentially expressed between HCC and non-tumor liver. (B) The expression pattern of 159 genes differentially expressed between non-tumor and normal liver. Each column represents a patient sample, while each row represents a single gene. All genes are rearranged according to ranking by signal-to-noise ratio, and only a few selected gene names are shown due to limited space.

Africa and China, moreover, it is well known that continuous exposure to aflatoxin B1 leads to an increased likelihood of HCC occurrence [29]. Of course, alcoholic abuse often leads to HCC as a result of alcoholic hepatitis, even in the absence of viral infections or chemical exposures [30]. In recent years, NASH has considered to be one of risk factors of HCC [31]. Like fibrolamellar HCC, a variant of HCC, there are cases whose etiological causes are still unknown. To date, HCC has been studied for association with such risk factors, and many efforts have been made to investigate whether phenotypic differences between tumors of the same cellular origin are associated with distinct genetic changes [32]. Some differences according to the risk factor have been discovered. For example, the HBX protein of HBV has been shown to possess transactivating activity, and the onocogenic potential of HBX has been reported [3]. In addition, exposure to aflatoxin B1 has been shown to cause a specific mutation in the p53 tumor-suppressor gene [4]. However, no clear specific oncogenic mechanism can be attributed to other HCC risk factors, and the entire molecular pathogenesis of HCC is still poorly understood. Moreover, very few studies, clinical or biological, regarding HCC cases with no known risk factors have been reported until now. Studying this type of case with the correct methodology should help to find key genes responsible for each step of hepatocarcinogenesis in the absence of viral influence, and this was the major goal of our effort.

We first applied all non-B, non-C tissue samples to unsupervised hierarchical clustering, which revealed distinct expression patterns of the three tissue types tested, T, NT, and N. In a previous report on human HCC samples from HCV- or HBV-positive individuals, there was an obvious difference between T and NT by hierarchical cluster analysis [5]. Our study indicated that non-B, non-C HCC also displayed a profile different from non-tumor tissue. Moreover, the NT expression patterns from HCC patients were readily distinguished

Table 2
The 61 genes differentially expressed between HCC and non-tumor liver tissue

Upregulated genes[a]					Downregulated genes[a]				
Rank[b]	*P* value[c]	GenBank ID	Locus	Gene name	Rank[b]	*P* value[c]	GenBank ID	Locus	Gene name
1	0.00030	AK026920	17q11–q12	*MYO1D*	26	0.00950	NM_018947	7p21.2	*HCS*
2	0.00112	BE467071	Unknown	*EST*	27	0.00430	NM_014645	4q11	*KIAA0635*
3	0.00248	X02811	22q13.1	*PDGF-B*	28	0.00752	NM_004092	10q26.2–q26.3	*ECHS1*
4	0.00196	NM_138425	12p13.32	*LOC113246*	29	0.00805	NM_004169	17p11.2	*SHMT1*
5	0.00262	NM_003127	9q33–q34	*SPTAN1*	30	0.00820	NM_006169	11q23.1	*NNMT*
6	0.00626	NM_033551	5q34	*MGC19556*	31	0.00781	NM_130786	19q13.4	*A1BG*
7	0.00117	X12830	1q21	*IL6R*	32	0.00284	NM_014007	9p24.1–q22.33	*ZNF297B*
8	0.00860	NM_002766	17q24–q25	*PRPSAP1*	33	0.00645	NM_022129	10pter-q25.3	*MAWBP*
9	0.00540	AB037796	3p22.1	*PDCD6IP*	34	0.00849	NM_138578	20q11.1	*BCL2L1*
10	0.00687	M80899	11q12–q13	*AHNAK*	35	0.00745	NM_005141	4q28	*FGB*
11	0.00356	NM_138623	2q12.3	*BCL2L11*	36	0.00115	NM_004132	10q26.11	*HABP2*
12	0.00529	NM_003801	8q24.3	*GPAA1*	37	0.00550	NM_004211	11p15.2–p15.1	*SLC6A5*
13	0.00345	NM_005252	14q24.3–q31	*c-fos*	38	0.00577	NM_003966	5p15.2	*SEMA5A*
14	0.00739	NM_000617	12q13	*SLC11A2*	39	0.00464	NM_006813	6q16.1	*PROL2*
15	0.00691	NM_016456	1q41	*LOC51235*	40	0.00412	AW571941	Unknown	*EST*
16	0.00869	NM_006445	17p13.3	*PRPF8*	41	0.00724	AB051509	19q13.3	*GRLF1*
17	0.00581	NM_005505	12q24.32	*CD36L1*	42	0.00221	NM_138340	18q11.1	*LABH3*
18	0.00767	AB028978	15q24.1	*KIAA1055*	43	0.00413	NM_004925	9p13	*AQP3*
19	0.00948	NM_005463	4q13–q21	*HNRPDL*	44	0.00364	NM_003877	12q	*STAT12*
20	0.00873	NM_006096	8q24	*NDRG1*	45	0.00630	NM_078467	6p21.2	*p21*
21	0.00697	NM_004539	18q21.2–q21.3	*NARS*	46	0.00477	NM_000598	7p13–p12	*IGFBP3*
22	0.00825	NM_003819	1p32–p36	*PABPC4*	47	0.00342	NM_001443	2p11	*FABP1*
23	0.00656	NM_002904	6p21.3	*RDBP*	48	0.00235	NM_013233	2q24.3	*STK39*
24	0.00988	NM_001743	2p21	*CALM2*	49	0.00338	M13536	3q23–q25	*CP*
25	0.00955	BC028242	5pter-p15.2	*PDCD6*	50	0.00813	AF006011	1p36	*DVL1*
					51	0.00108	AK024951	12p13	*C1R*
					52	0.00674	NM_000477	4q11–q13	*ALB*
					53	0.00166	NM_005950	16q13	*MT1G*
					54	0.00864	AK055718	2q32.3–q33	*CED-6*
					55	0.00414	NM_014324	5p13.2–q11.1	*AMACR*
					56	0.00106	NM_000014	12p13.3–p12.3	*A2M*
					57	0.00151	NM_021870	4q28	*FGG*
					58	0.00159	NM_003171	10q22.1	*SUPV3L1*
					59	0.00018	AF220656	12q15	*PHLDA1*
					60	0.00005	NM_025233	17q12–q21	*NBP*
					61	0.00003	NM_021175	19q13.1	*HAMP*

[a] Upregulation or downregulation were defined as expression in HCC tissues compared to non-tumor tissues.
[b] Ranking was according to signal-to-noise ratio.
[c] *P* values were calculated by random permutation test.

from that of the N samples. Of course, since these N samples were obtained from patients with non-liver metastatic cancer, they might produce some stress responses in the host liver. However, these results may indicate a potential change in non-B, non-C HCC normal liver tissue mRNA expression patterns in the absence of HCV or HBV infection.

Among our NT samples, about a half of them pathologically revealed cirrhosis or chronic hepatitis, indicating the influence of various causes that have not yet been uncovered. For example, other virus infections such as HGV, TT virus, or SEN virus may be associated with HCC. However, extensive etiological studies have shown that these viruses do not account for the bulk of non-hepatitis induced hepatocarcinogenesis [33–38]. None of the patients in our study had a history of exposure to aflatoxin B1, and there were no cases of hemochromatosis, alcoholic hepatitis, or NASH pathologically. Although the main cause of these non-B, non-C HCC cases is still unknown, the unsupervised analyzes conducted clearly demonstrate that the molecular features of HCC, non-tumor, and normal liver were distinct from each other.

We identified a total of 220 genes which were likely to be involved in the process of hepatocarcinogenesis, as they were differentially expressed between the different tissue types studied. The reliability of this gene selection was validated with additional datasets not only based on our PCR-arrays, but on DNA microarrays. These 220 genes were then divided into two groups, the first comprised of 61 genes expressed differentially between T and NT tissues, and the second comprised of 159 genes expressed

Table 3
The 159 genes differentially expressed between non-tumor and normal liver tissue

Upregulated genes[a]					Downregulated genes[a]				
Rank[b]	*P* value[c]	GenBank ID	Locus	Gene name	Rank[b]	*P* value[c]	GenBank ID	Locus	Gene name
1	0.00001	NM_004877	19q13.2	*GMFG*	74	0.00976	NM_033326	11p15.3	*SOX6*
2	0.00085	NM_016097	Unknown	*HSPC039*	75	0.00783	NM_002218	3p21–p14	*ITIH4*
3	0.00000	NM_006356	17q25	*ATP5H*	76	0.00862	NM_004494	Xq25	*HDGF*
4	0.00018	NM_001663	7q22.1	*ARF6*	77	0.00962	AF250310	8	*SKIP1*
5	0.00004	AL833223	Unknown	*EST*	78	0.00822	NM_006744	10q23–q24	*RBP4*
6	0.00010	NM_014177	18q22.3	*HSPC154*	79	0.00588	NM_006903	4q24	*LOC92033*
7	0.00020	NM_016525	9p22–p21	*UBAP1*	80	0.00966	NM_001074	4q13	*UGT2B7*
8	0.00048	AB040891	4p12	*KIAA1458*	81	0.00830	NM_001443	2p11	*FABP1*
9	0.00027	NM_022833	9q34.13	*EST*	82	0.00889	NM_001482	15q14	*GATM*
10	0.00616	NM_004458	Xq22.3–q23	*FACL4*	83	0.00600	NM_003365	3p21.3	*UQCRC1*
11	0.00030	NM_001813	4q24–q25	*CENPE*	84	0.00302	NM_005836	8q22	*UK114*
12	0.00897	M88311	Unknown	*HBSAA*	85	0.00319	NM_000096	3q23–q25	*CP*
13	0.00109	NM_020365	1p34.1	*EIF2B3*	86	0.00834	NM_005746	7q11.23	*PBEF*
14	0.00159	NM_000314	10q23.3	*PTEN*	87	0.00736	NM_000216	Xp22.32	*KAL1*
15	0.00020	NM_139159	19p13.3	*DPP9*	88	0.00660	NM_003869	16q23.1	*CES2*
16	0.00168	NM_001748	1q41–q42	*CAPN2*	89	0.00823	NM_002085	19p13.3	*GPX4*
17	0.00249	NM_020123	10q24.2	*SMBP*	90	0.00325	NM_007120	2q37	*UGT1A4*
18	0.00237	NM_003473	10p14–p13	*STAM*	91	0.00664	NM_006443	6p12.3	*RCL*
19	0.00042	BC020909	Unknown	*EST*	92	0.00828	NM_002568	8q22.2–q23	*PABPC1*
20	0.00396	NM_018983	4q25	*NOLA1*	93	0.00748	BM477769	Unknown	*EST*
21	0.00269	NM_003375	10q22	*VDAC2*	94	0.00389	NM_000508	4q28	*FGA*
22	0.00268	NM_001537	16q24.1	*HSBP1*	95	0.00866	NM_002889	7q35	*RARRES2*
23	0.00038	NM_024638	Unknown	*FLJ12960*	96	0.00555	NM_003057	6q26	*SLC22A*
24	0.00189	NM_000593	6p21.3	*TAP1*	97	0.00373	NM_003134	15q22	*SRP14*
25	0.00406	NM_004421	1p36	*DVL1*	98	0.00241	AK024356	18	*FLJ14294*
26	0.00057	NM_005923	6q22.33	*MAP3K5*	99	0.00578	NM_053056	11q13	*cyclin D1*
27	0.00185	NM_000546	17p13.1	*P53*	100	0.00470	NM_000067	8q22	*CA2*
28	0.00104	NM_030969	6p25.1–p23	*MGC1223*	101	0.00596	NM_021101	3q28–q29	*CLDN1*
29	0.00043	AK056634	Unknown	*FLJ32072*	102	0.00568	NM_000146	19q13.3–4	*FTL*
30	0.00563	NM_014497	2p13.2–p13.1	*NP220*	103	0.00971	AL122072	1	*FLJ10004*
31	0.00205	NM_138578	20q11.1	*BCL2L1*	104	0.00420	NM_000777	7q21.1	*CYP3A5*
32	0.00918	NM_014165	6q16.3	*HSPC125*	105	0.00206	NM_015313	11q23.3	*ARHGEF12*
33	0.00097	NM_005044	Unknown	*PRKX*	106	0.00179	NM_005651	4q31–q32	*TDO2*
34	0.00702	AL117596	11	*EST*	107	0.00314	NM_004546	7q32.3	*NDUFB2*
35	0.00090	NM_002038	1p35	*G1P3*	108	0.00760	NM_003217	12q12–q13	*TEGT*
36	0.00209	NM_017925	9p21.2	*FLJ20686*	109	0.00778	NM_003124	2p14–p12	*SPR*
37	0.00245	NM_000368	9q34	*TSC1*	110	0.00361	NM_004563	14q11.2	*PCK2*
38	0.00573	AB067502	1p32.1	*KIAA1915*	111	0.00274	NM_032366	16p13.12	*MGC13114*
39	0.00513	NM_152516	2p13.3	*MURR1*	112	0.00789	NM_015415	1q24	*EST*
40	0.00428	NM_004388	1p22	*CTBS*	113	0.00455	NM_006441	15q23	*MTHFS*
41	0.00577	NM_015317	2p22–p21	*PUM2*	114	0.00100	NM_000506	11p11–q12	*F2*
42	0.00117	BC020854	19	*EST*	115	0.00282	NM_022373	7p15.3	*FLJ22313*
43	0.00012	AK024905	Unknown	*FLJ21252*	116	0.00171	NM_032012	9q31	*C9orf5*
44	0.00159	NM_014940	Unknown	*KIAA0872*	117	0.00075	NM_005666	1q31–q32.1	*HFL3*
45	0.00135	AK023586	Unknown	*SDCCAG8*	118	0.00337	NM_021126	22q13.1	*MPST*
46	0.00823	NM_012216	Xq22	*MID2*	119	0.00259	BE971379	Unknown	*EST*
47	0.00226	R40589	Unknown	*CRL2*	120	0.00587	NM_017746	9q31.1	*FLJ20287*
48	0.00591	NM_002024	Xq27.3	*FMR1*	121	0.00382	NM_006003	19q12–q13.1	*UQCRFS1*
49	0.00208	BC020116	Unknown	*FLJ12687*	122	0.00164	NM_000624	14q32.1	*SERPINA5*
50	0.00394	NM_014719	Unknown	*KIAA0738*	123	0.00085	NM_022481	5q31.3	*ARAP3*
51	0.00255	AK027219	Unknown	*FLJ23566*	124	0.00163	NM_006741	12q12	*PPP1R1A*
52	0.00218	NM_007267	17q25.3	*EVIN1*	125	0.00254	NM_018242	17p11.1	*FLJ10847*
53	0.00223	BF768652	Unknown	*EST*	126	0.00063	NM_000071	21q22.3	*CBS*
54	0.00155	NM_000177	9q33	*GSN*	127	0.00138	NM_004637	3q22.1	*RAB7*
55	0.00270	AK026459	Unknown	*FLJ22806*	128	0.00100	NM_005326	16p13.3	*HAGH*
56	0.00214	NM_019839	14q11.2–q12	*LTB4R2*	129	0.00220	NM_001133	4q11–q13	*AFM*
57	0.00703	NM_014341	6pter-p24.1	*MTCH1*	130	0.00050	AF320003	7q21.3	*PON3*
58	0.00322	AI741059	Unknown	*AD7C-NTP*	131	0.00127	NM_006841	3p21.3	*SLC38A3*
59	0.00227	NM_002496	11q13	*NDUFS8*	132	0.00136	NM_025221	4p15.31	*CALP*

(*continued on next page*)

Table 3 (*continued*)

Upregulated genes[a]					Downregulated genes[a]				
Rank[b]	*P* value[c]	GenBank ID	Locus	Gene name	Rank[b]	*P* value[c]	GenBank ID	Locus	Gene name
60	0.00940	NM_005529	1p36.1–p35	*HSPG2*	133	0.00581	NM_014504	7p13	*RABEX5*
61	0.00633	NM_004417	5q34	*DUSP1*	134	0.00042	NM_017460	7q21.1	*CYP3A4*
62	0.00584	NM_000938	4q12	*POLR2B*	135	0.00265	NM_000039	11q23–q24	*APOA1*
63	0.00363	NM_003343	Unknown	*UBE2G2*	136	0.00029	NM_032951	7q11.23	*WBSCR14*
64	0.00972	NM_005567	17q25	*LGALS3BP*	137	0.00199	AV699591	Unknown	*FLJ36984*
65	0.00352	AK095137	2	*FLJ37818*	138	0.00012	NM_080661	11q12.3	*MGC15937*
66	0.00451	AK025156	Unknown	*FLJ21503*	139	0.00062	NM_000792	1p32–p33	*DIO1*
67	0.00597	NM_014956	Unknown	*KIAA1052*	140	0.00031	NM_001737	5p14–p12	*C9*
68	0.00796	NM_018997	1	*MRPS21*	141	0.00015	NM_000143	1q42.1	*FH*
69	0.00356	NM_000088	17q21.3–q22.1	*COL1A1*	142	0.00043	NM_004264	12p12.3	*SURB7*
70	0.00552	AV737902	Unknown	*FLJ20489*	143	0.00022	NM_000767	19q13.2	*CYP2B6*
71	0.00967	NM_001908	8p22	*CTSB*	144	0.00011	NM_006215	14q31–q32.1	*SERPINA4*
72	0.00865	XM_114611	8q24.3	*KIAA1833*	145	0.00032	NM_000055	3q26.1–q26.2	*BCHE*
73	0.00868	NM_020485	1p36.11	*RHCE*	146	0.00036	NM_005488	22q13.1	*TOM1*
					147	0.00016	NM_000285	19q12–q13.2	*PEPD*
					148	0.00024	NM_005807	1q25–q31	*PRG4*
					149	0.00012	NM_003500	3p14.3	*ACOX2*
					150	0.00063	NM_003645	15q21.2	*SLC27A2*
					151	0.00008	AF058954	3q14.2	*SUCLG2*
					152	0.00017	NM_004905	1q23.3	*AOP2*
					153	0.00045	NM_017969	2q14.2	*FLJ10006*
					154	0.00000	NM_003944	1q21–q22	*SELENBP1*
					155	0.00001	NM_000186	1q32	*HFL1*
					156	0.00011	NM_031925	7p12.3	*TMPIT*
					157	0.00002	NM_016628	10	*WAC*
					158	0.00000	NM_000607	9q31–q32	*ORM1*
					159	0.00002	AF327354	14q32.33	*DMR*

[a] Upregulation or downregulation were defined as expression in non-tumor tissues compared to normal liver tissues.
[b] Ranking was according to signal-to-noise ratio.
[c] *P* values were calculated by random permutation test.

differentially between NT to N samples. Both groups included some cancer-related genes which were previously reported to be associated with HCC. As examples, the former group contains IGFBP3, c-fos, and p21, while the latter group contains cyclin D1, p53, and PTEN. IGFBP3 plays a key role in regulating cell proliferation and apoptosis, and also plays an important inhibitory role in the development and/or growth of HCC [39–41]. C-fos, the human cellular homolog of v-fos oncogene, is well-known to be involved in hepatocarcinomagenesis [26,42]. Likewise, p21, cyclin-dependent kinase inhibitor-1A, is thought to be closely related to hepatocarcinogenesis [27], and recent reports indicated a strong interaction with hepatitis B virus X protein or hepatitis C virus core protein [43,44]. Cyclin D1 plays a role in the procession from normal liver to chronic hepatitis, and from there to HCC [45]. P53 inhibits cell growth through initiation of cell cycle arrest and apoptosis, and a close correlation between p53 and cirrhotic liver has been also reported [46]. PTEN, a putative tumor suppressor gene, may function by negatively regulating cell interactions with the extracellular matrix, and it is considered to be important in hepatocarcinogenesis [47]. Thus, dysfunction of these well-known cancer-related genes may participate in hepatocarcinogenesis also in non-B, non-C HCC cases.

When compared with the results of previous gene expression profiling studies on virus-infected HCC using DNA arrays [7–9], ten genes (C1R, ALB, NNMT, A2M, ECHS1, HABP2, FGB, FGG, FABP1 and IGFBP3) were

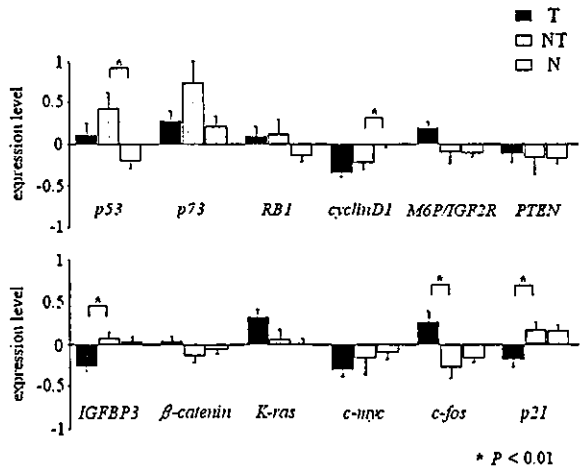

Fig. 3. Expression levels of 12 cancer-related genes in non-B, non-C HCC (T), non-tumor liver (NT), and normal liver (N). The averages and standard errors of relative expression levels of p53, p73, RB1, cyclin D1, M6P/IGF2R, PTEN, IGFBP3, beta-catenin, K-ras, c-myc, c-fos, and p21 are shown. *P* values were calculated by *t* test.

consistently selected as being differentially expressed between T and NT tissue. Moreover, four (ALB, A2M, ECHS1 and IGFBP3) of these ten genes were selected in more than two studies. As mentioned above, IGFBP3 has been thought for some time to be intimately involved in the development of HCC [39–41]. Because the previous studies did not define as rigorously the statistical significance of the identified genes, it may yet be too early to make conclusions regarding the similarities and differences between the gene expression profiles of non-B, non-C HCC and virus-infected HCC. However, we can at least conclude that misexpression of these four genes may be common to both types of HCC, independently of viral infection.

To our knowledge, this is the first gene expression profiling study to attempt to shed light on non-B, non-C HCC, and it may illustrate aspects of this disease which are not due to either HCV or HBV. A total of 220 candidate genes were identified as potentially responsible for each step of hepatocarcinogenesis, and our confidence in these candidates is bolstered by the fact that this list contains a large number of well-known cancer-related genes, many of which have been previously reported to play a role in the development of HCC. These genes, which are involved in hepatocarcinogenesis separate from the effects of hepatitis virus, may prove to be valuable targets for drug development against HCC stemming from multiple etiologic factors.

Acknowledgements

We thank Ms Chiyuri Maruyama, Ms Keiko Miyaoka, Ms Mihoko Yoshino and Ms Satoko Maki for expert technical assistance. This work was supported by Grant-in-Aid for the Development of Innovative Technology from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

[1] Stuver SO. Towards global control of liver cancer? Semin Cancer Biol 1998;8:299–306.
[2] Murakami Y, Hayashi K, Hirohashi S, Sekiya T. Aberrations of the tumor suppressor p53 and retinoblastoma genes in human hepatocellular carcinomas. Cancer Res 1991;51:5520–5525.
[3] Andrisani OM, Barnabas S. The transcriptional function of the hepatitis B virus X protein and its role in hepatocarcinogenesis (Review). Int J Oncol 1999;15:373–379.
[4] Bressac B, Kew M, Wands J, Ozturk M. Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. Nature 1991;350:429–431.
[5] Chen X, Cheung ST, So S, Fan ST, Barry C, Higgins J, et al. Gene expression patterns in human liver cancers. Mol Biol Cell 2002;13: 1929–1939.
[6] Xu XR, Huang J, Xu ZG, Qian BZ, Zhu ZD, Yan Q, et al. Insight into hepatocellular carcinogenesis at transcriptome level by comparing gene expression profiles of hepatocellular carcinoma with those of corresponding non-cancerous liver. Proc Natl Acad Sci USA 2001;98: 15089–15094.
[7] Okabe H, Satoh S, Kato T, Kitahara O, Yanagawa R, Yamaoka Y, et al. Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: identification of genes involved in viral carcinogenesis and tumor progression. Cancer Res 2001;61:2129–2137.
[8] Xu L, Hui L, Wang S, Gong J, Jin Y, Wang Y, et al. Expression profiling suggested a regulatory role of liver-enriched transcription factors in human hepatocellular carcinoma. Cancer Res 2001;61: 3176–3181.
[9] Delpuech O, Trabut JB, Carnot F, Feuillard J, Brechot C, Kremsdorf D. Identification, using cDNA macroarray analysis, of distinct gene expression profiles associated with pathological and virological features of hepatocellular carcinoma. Oncogene 2002;21:2926–2937.
[10] Holland MJ. Transcript abundance in yeast varies over six orders of magnitude. J Biol Chem 2002;277:14363–14366.
[11] Kato K. Adaptor-tagged competitive PCR: a novel method for measuring relative gene expression. Nucleic Acids Res 1997;25:4694–4696.
[12] Iwao K, Matoba R, Ueno N, Ando A, Miyoshi Y, Matsubara K, et al. Molecular classification of primary breast tumors possessing distinct prognostic properties. Hum Mol Genet 2002;11:199–206.
[13] Muro S, Takemasa I, Oba S, Matoba R, Ueno N, Maruyama C, et al. Identification of expressed genes linked to malignancy of human colorectal carcinoma by parametric clustering of quantitative expression data. Genome Biol 2003;4:R21.
[14] Yotsuyanagi H, Shintani Y, Moriya K, Fujie H, Tsutsumi T, Kato T, et al. Virologic analysis of non-B, non-C hepatocellular carcinoma in Japan: frequent involvement of hepatitis B virus. J Infect Dis 2000; 181:1920–1928.
[15] Schneeberger PM, Keur I, Vliet W, Hoek K, Boswijk H, Loon AM, et al. Hepatitis C virus infections in dialysis centers in the Netherlands: a national survey by serological and molecular methods. J Clin Microbiol 1998;36:1711–1715.
[16] Matoba R, Kato K, Saito S, Kurooka C, Maruyama C, Sakakibara Y, et al. Gene expression in mouse cerebellum during its development. Gene 2000;241:125–131.
[17] Matoba R, Kato K, Kurooka C, Maruyama C, Sakakibara Y, Matsubara K. Correlation between gene functions and developmental expression patterns in the mouse cerebellum. Eur J Neurosci 2002;12: 1357–1371.
[18] Ramaswamy S, Golub TR. DNA microarrays in clinical oncology. J Clin Oncol 2002;20:1932–1941.
[19] Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek M, Mesirov JP, et al. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 1999;286: 531–537.
[20] MacDonald TJ, Brown KM, LaFleur B, Peterson K, Lawlor C, Chen Y, et al. Expression profiling of medulloblastoma: PDGFRA and the RAS/MAPK pathway as therapeutic targets for metastatic disease. Nat Genet 2001;29:143–152.
[21] Pomeroy SL, Tamayo P, Gaasenbeek M, Sturla LM, Angelo M, McLaughlin ME, et al. Prediction of central nervous system embryonal tumour outcome based on gene expression. Nature 2002;415:436–442.
[22] Armstrong SA, Staunton JE, Silverman LB, Pieters R, den Boer ML, Minden MD, et al. MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. Nat Genet 2002;30:41–47.
[23] Ramaswamy S, Ross KN, Lander ES, Golub TR. A molecular signature of metastasis in primary solid tumors. Nat Genet 2003;33: 49–54.
[24] van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, et al. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 2002;347:1999–2009.
[25] Tannapfel A, Wittekind C. Genes involved in hepatocellular carcinoma: deregulation in cell cycling and apoptosis. Virchows Arch 2002;440:345–352.

[26] Arbuthnot P, Kew M, Fitschen W. C-fos and c-myc oncoprotein expression in human hepatocellular carcinomas. Anticancer Res 1991; 11:921–924.
[27] Kobayashi S, Matsushita K, Saigo K, Urashima T, Asano T, Hayashi H, et al. P21WAF1/CIP1 messenger RNA expression in hepatitis B, C virus-infected human hepatocellular carcinoma tissues. Cancer 2001; 91:2096–2103.
[28] Montalto G, Cervello M, Giannitrapani L, Dantona F, Terranova A, Castagnetta LA. Epidemiology, risk factors, and natural history of hepatocellular carcinoma. Ann N Y Acad Sci 2002;963:13–20.
[29] Wogan GN. Aflatoxins as risk factors for hepatocellular carcinoma in humans. Cancer Res 1992;52:2114s–2118s.
[30] Mohamed AE, Kew MC, Groeneveld HT. Alcohol consumption as a risk factor for hepatocellular carcinoma in urban southern African blacks. Int J Cancer 1992;51:537–541.
[31] Bugianesi E, Leone N, Vanni E, Marchesini G, Brunello F, Carucci P, et al. Expanding the natural history of non-alcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. Gastroenterology 2002;123:134–140.
[32] Unsal H, Yakicier C, Marcais C, Kew M, Volkmann M, Zentgraf H, et al. Genetic heterogeneity of hepatocellular carcinoma. Proc Natl Acad Sci USA 1994;91:822–826.
[33] Alter HJ, Nakatsuji Y, Melpolder J, Wages J, Wesley R, Shih JW, et al. The incidence of transfusion-associated hepatitis G virus infection and its relation to liver disease. N Engl J Med 1997;336: 747–754.
[34] Kubo S, Nishiguchi S, Kuroki T, Hirohashi K, Tanaka H, Tsukamoto T, et al. Poor association of GBV-C viremia with hepatocellular carcinoma. J Hepatol 1997;27:91–95.
[35] Takahashi K, Hoshino H, Ohta Y, Yoshida N, Mishiro S. Very high prevalence of TT virus (TTV) infection in general population of Japan revealed by a new set of PCR primers. Hepatol Res 1998;12: 233–239.
[36] Matsumoto A, Yeo AE, Shih JW, Tanaka E, Kiyosawa K, Alter HJ. Transfusion-associated TT virus infection and its relationship to liver disease. Hepatology 1999;30:283–288.
[37] Mikuni M, Moriyama M, Tanaka N, Abe K, Arakawa Y. SEN virus infection does not affect the progression of non-A to -E liver disease. J Med Virol 2002;67:624–629.
[38] Yoshida H, Kato N, Shiratori Y, Shao R, Wang Y, Shiina S, et al. Weak association between SEN virus viremia and liver disease. J Clin Microbiol 2002;40:3140–3145.
[39] Gong Y, Cui L, Minuk GY. The expression of insulin-like growth factor binding proteins in human hepatocellular carcinoma. Mol Cell Biochem 2000;207:101–104.
[40] Huynh H, Chow PK, Ooi LL, Soo KC. Apossible role for insulin-like growth factor-binding protein-3 autocrine/paracrine loops in controlling hepatocellular carcinoma cell proliferation. Cell Growth Differ 2002;13:115–122.
[41] Hanafusa T, Yumoto Y, Nouso K, Nakatsukasa H, Onishi T, Fujikawa T, et al. Reduced expression of insulin-like growth factor binding protein-3 and its promoter hypermethylation in human hepatocellular carcinoma. Cancer Lett 2002;176:149–158.
[42] Yuen MF, Wu PC, Lai VC, Lau JY, Lai CL. Expression of c-Myc, c-Fos, and c-jun in hepatocellular carcinoma. Cancer 2001;91:106–112.
[43] Han HJ, Jung EY, Lee WJ, Jang KL. Cooperative repression of cyclin-dependent kinase inhibitor p21 gene expression by hepatitis B virus X protein and hepatitis C virus core protein. FEBS Lett 2002;518: 169–172.
[44] Lee MN, Jung EY, Kwun HJ, Jun HK, Yu DY, Choi YH, et al. Hepatitis C virus core protein represses the p21 promoter through inhibition of a TGF-beta pathway. J Gen Virol 2002;83:2145–2151.
[45] Masaki T, Shiratori Y, Rengifo W, Igarashi K, Matsumoto K, Nishioka M, et al. Hepatocellular carcinoma cell cycle: study of Long-Evans cinnamon rats. Hepatology 2000;32:711–720.
[46] Minouchi K, Kaneko S, Kobayashi K. Mutation of p53 gene in regenerative nodules in cirrhotic liver. J Hepatol 2002;37:231–239.
[47] Rahman MA, Kyriazanos ID, Ono T, Yamanoi A, Kohno H, Tsuchiya M, et al. Impact of PTEN expression on the outcome of hepatitis C virus-positive cirrhotic hepatocellular carcinoma patients: possible relationship with COX II and inducible nitric oxide synthase. Int J Cancer 2002;100:152–157.

消化器病セミナー97

進行肝癌に対する治療法の進歩

インターフェロン併用動注化学療法

FU arterial infusion and interferon therapy (FAIT)

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消化器病セミナー・97：109～118，2004. 12.

へるす出版

はじめに

肝細胞癌に対する治療における初発肝内病巣の制御に対する進歩は目覚ましく，肝切除，化学塞栓療法（transcatheter arterial embolization：TAE），経皮的エタノール注入療法（percutaneous ethanol injection：PEI），マイクロ波凝固療法（microwave coagulation therapy：MCT），ラジオ波焼灼療法（radiofrequency ablation：RFA）などにより，治療効果を上げている。しかし肝細胞癌の場合，仮に初発病巣については十分に治療されたとしても，高率に残肝再発を来すため，再度局所療法が必要になる。このように残肝再発に対する肝内局所治療の反復のなかで，最終的に門脈内腫瘍栓などの脈管侵襲，び漫性結節，遠隔転移などのいわゆる治療抵抗性の病巣となり，いわゆる既存の局所治療は全く効果を示さず，最終的には原病死に至る症例は少なくない。したがって，肝細胞癌治療におけるさらなる予後改善のためには，このような難治性進行肝細胞癌に対する新しい治療法の開発こそが，急務であると考えられる。

最近われわれは，抗癌剤（フルオロウラシル：以下5-FU）の肝動脈内化学療法にインターフェロン（interferon：以下IFN）を併用することにより，Vp3以上の肉眼的門脈内腫瘍栓を伴う高度進行肝細胞癌に対して良好な成績をあげてきた[1]。本稿においては，このIFNと5-FUを用いたIFN併用動注化学療法（FU Arterial infusion and Interferon Therapy，以下FAIT）について，最近の治療成績とともにその研究成果について概説したい。

I　悪性腫瘍に対するIFN併用化学療法

IFNはその多彩な生理活性により生体に対してさまざまな作用をもたらすが，近年，大腸癌をはじめとした固形癌に対して，5-FUなどの抗腫瘍剤とIFNを併用することによって，その抗腫瘍効果が増強されるとの報告がなされている。Wadlerら[2]の手術不能の大腸癌患者にIFNと5-FUの併用療法を試み，76%の有効率を認めたとの報告以降，さまざまな臓器で本療法が試みられてきた。胃癌，食道癌などでも本療法の臨床応用がなされており[3][4]，効果についてはさまざまな報告があるものの有効との報告もあり，治療不能な悪性疾患に対する選択肢としての今後が期待されている。

肝細胞癌に対しても同様に，AFP値の低い肝癌症例で31%の有効性を認めたとの報告[5]や，進行肝細胞癌症例に5-FUとIFNに加えcisplatin，methotrexateを併用し46.7%の有効性を認めたとの報告[6]，cisplatin，doxorubicinを併用し，36例の手術不能患者のうち9人が手術可能となり術後再発もなかったとの報告[7]などがある。さらに最近では，5-FUの全身投与に加えて，IFNの皮下投与が有効であったとのphase II臨床試験の報告もある[8]。

これらの知見をふまえ，既存の治療法では十分な治療効果の期待できない高度進行肝細胞癌に対して，われわれはIFN-αと5-FUを併用した治療法を1997年より行い，きわめて良好な結果を得，報告してきた[9][10]。