TABLE II. Relationship of HIF-1α to VEGF and IGF-2 Expression

	HIF-1α (−) (n = 77)	HIF-1α (+) (n = 49)	*P*-value
VEGF			
Negative	58	28	0.03
Positive	19	21	
IGF-2			
Negative	53	28	0.181
Positive	24	21	

TABLE III. Relationship of MVD to HIF-1α, VEGF, and IGF-2

HIF-1α	HIF-1α (−) (n = 77)	HIF-1α (+) (n = 49)	*P*-value
MVD	57.5 ± 29.6	79.5 ± 30.9	<0.005
VEGF	VEGF (−) (n = 87)	VEGF (+) (n = 39)	*P*-value
MVD	66.1 ± 33.1	66.1 ± 29.3	0.996
IGF-2	IGF-2 (−) (n = 81)	IGF-2 (+) (n = 45)	*P*-value
MVD	66.1 ± 33.4	76.8 ± 30.0	<0.005

from 126 patients, 49 (38.9%) were positive for HIF-1α immunoreactivity. The relationships between HIF-1α expression and clinicopathological features of the tumors are shown in Table I. HIF-1α expression was positively correlated with tumor size ($P < 0.005$) and with depth of invasion ($P = 0.018$) and was more frequent in cases of tumors with lymphatic invasion and undifferentiated adenocarcinomas. Tumors with HIF-1α expression at the invading edge were significantly larger than tumors lacking this expression pattern ($P = 0.03$). VEGF expression was negatively correlated with lymph node metastasis ($P = 0.041$) and lymphatic invasion ($P = 0.015$). IGF-2 expression also correlated with increased tumor size, depth of invasion, lymph node metastasis, lymphatic and venous invasion ($P < 0.005$ in all comparisons).

VEGF expression was significantly correlated to HIF-1α expression, being detected in 24.7% of HIF-1α negative and 42.9% of HIF-1α positive (Table II). Additionally, cytoplasmic staining of IGF-2 was observed more frequently in HIF-1α positive tumors (46.7%) than in HIF-1α negative tumors (31.2%). Expression of HIF-1α, VGEF, and IGF-2 proteins was observed in serial sections of several specimens (Fig. 2a–c). The intra-tumor MVD, determined using anti-CD34 antibodies, was significantly higher (79.5 ± 30.9, mean ± SD) in tumors from HIF-1α positive patients than in HIF-1α negative sections (57.5 ± 29.6, mean ± SD) (Table III). Tumor expressing IGF-2 showed a significant increase in MVD, similar to those expressing HIF-1α.

The prognosis of the 126 Japanese cancer patients in this study was monitored for a median follow-up time of 55.4 months. During the observation period, 36 patients died from gastric carcinoma and the 5-year survival rate was 58.4% for HIF-1α positive patients and 81.5% for HIF-1α negative patients. The cumulative overall survival rates for these two populations were determined (Fig. 3) and the prognosis for HIF-1α positive patients was significantly poorer than that of HIF-1α negative patients ($P = 0.009$). A multivariate analysis of all clinicopathological factors and all three markers for all 126 patients showed that depth of invasion, lymph node metastasis, and HIF-1α expression were independent prognostic factors (Table IV).

DISCUSSION

In surgically resected specimens of gastric carcinoma, HIF-1α expression was more frequent in larger, deeply invasive tumors. Gastric carcinomas having either a large size or deep invasiveness grew expansively and contained necrotic regions and deep ulcerations within the tumor tissue. In typical cases, nuclear staining of HIF-1α was detected around the invading edges and necrotic regions, including deep ulcerations. A similar pattern of HIF-1α expression is prominent in glioblastoma multiforme, an extremely aggressive tumor [14]. A tumor with invasive properties can rapidly grow beyond the capacity of available blood supplies,

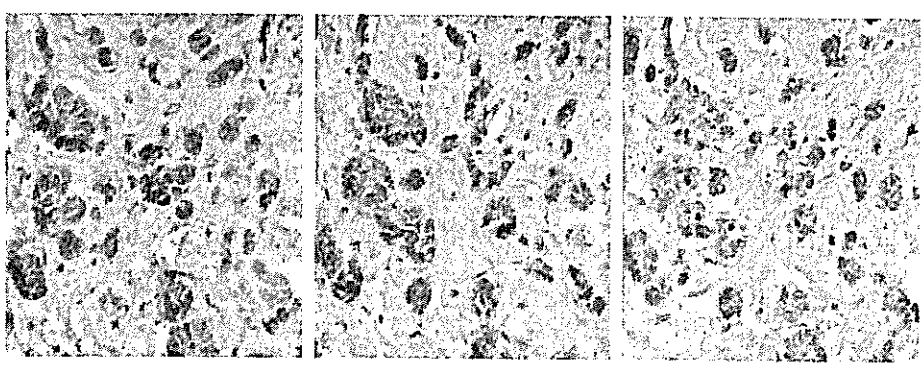

Fig. 2. Immunohistochemical analysis using serial sections showing colocalization of (a) HIF-1α, (b) VEGF, and (c) IGF-2 expression (400×).

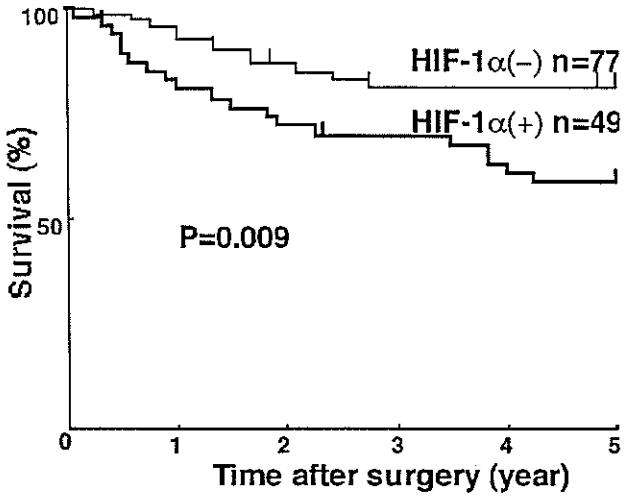

Fig. 3. Cumulative overall survival of two populations, with and without HIF-1α overexpression, respectively, was determined using the Kaplan–Meier method. Differences in survival were statistically significant ($P = 0.009$). (Bold line) HIF-1α positive patients. (Thin line) HIF-1α negative patients.

which results in hypoxia. Since most tumor cells that are distant from blood vessels are exposed to reduced oxygen pressure and nutrients, HIF-1α expression is induced. Conversely, we observed some tumor tissues containing necrotic regions that did not express HIF-1α. Increasing hypoxia may reduce further proliferation in tumors that cannot induce adaptive responses to hypoxic microenvironments. Tumors with necrotic regions or deep ulcerations that were HIF-1α negative tended to correlate with a more favorable prognosis for the patient.

In human gastric cancer models, inhibition of HIF-1α function is associated with the inhibition of gastric tumor growth and angiogenesis [15]. In the current study, HIF-1α expression in gastric carcinomas correlated with VEGF expression and increased intra-tumor MVD. Tumor angiogenesis and neovascularization requires the expression of VEGF and the binding of HIF-1 to HRE in the VEGF promoter is a major pathway leading to induction of VEGF expression under hypoxic conditions [16]. Our findings suggest that in gastric carcinoma HIF-1α induces VEGF and this event leads to formation of vascular networks which supply oxygen and nutrients. Pancreatic cancer cell lines with high constitutive levels of HIF-1α protein produce higher basal levels of VEGF [17]. An aggressive tumor may therefore contain a high vascular density with the concomitant expression of HIF-1α and VEGF during continued growth.

Several studies have shown that IGF-2 is over-expressed in tissues from patients with gastric carcinoma, compared to normal gastric tissues [18,19]. IGF-2 may therefore play an important role in the development and growth of gastric carcinoma and we examined the pattern of IGF-2 expression in gastric carcinomas and compared it to the HIF-1α expression profiles. Although there was no statistically significant correlation, coincident expression of HIF-1α, VGEF, and IGF-2 was observed in serial sections in tumor tissue from several subjects. This finding may indicate that HIF-1α functioned as a transcription factor to upregulate expression of various downstream genes in these regions.

In our study, aggressive gastric carcinomas were suggested to have hypoxic regions due to elevated levels of oxygen consumption, and to induce HIF-1α as an adaptation to hypoxic conditions. HIF-1α then activates transcription of VEGF, which mediates angiogenesis when secreted by the tumor cells. Thus, enhancement of angiogenesis by hypoxia is a prerequisite for progressive growth of gastric carcinomas, and the level of intra-tumor MVD has been previously reported to correlate with patient outcome in subjects with gastric carcinoma [20]. Significant associations between HIF-1α overexpression and patient outcome have been shown in many human carcinomas, including gastrointestinal tumors of the stomach [21]. Our data also suggest that increasing HIF-1α expression plays an important role in tumor progression of gastric carcinomas. Although it is a point of some controversy as to whether the HIF-1α-induced genes can in fact promote malignant tumor growth, disruption of the HIF-1 pathway by genetic or pharmacological means may potentially have anti-tumor effects [22,23].

In summary, HIF-1α overexpression in gastric carcinoma correlates with VEGF expression and increased

TABLE IV. Association of Various Factors With Overall Survival Determined by the Cox Proportional Hazard Model

	Regression coefficient	Standard error	Relative risk (95% CI)	*P*-value
Depth of invasion	3.13	1.05	22.9 (2.96–178)	0.005
Lymph node metastasis	1.77	0.552	5.83 (1.98–17.2)	0.005
HIF-1 expression	0.738	0.348	2.09 (1.08–4.06)	0.032

CI, confidence interval.
The covariates included age, gender, depth of invasion, histology, tumor size, lymph node metastasis, lymphatic invasion, venous invasion, HIF-1α, VEGF expression, IGF-2 expression, MVD.

intra-tumor MVD and was shown to be an independent prognostic factor. Therefore, gastric carcinomas that express HIF-1α may continue to grow by various adaptive responses, especially VEGF-mediated angiogenesis which has a negative impact on the prognosis of patients with gastric carcinoma.

ACKNOWLEDGMENTS

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High expression of insulin-like growth factor binding protein-3 is correlated with lower portal invasion and better prognosis in human hepatocellular carcinoma

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Insulin-like growth factor binding protein-3 (IGFBP-3) modulates cell proliferation of various cancer cell types. However, it remains unclear how IGF–IGFBP-3-signaling is involved in growth and progression of hepatocellular carcinoma (HCC). The aim of the present study was to evaluate the role of IGFBP-3 in HCC. Type 1 receptor for IGF (IGF-1R) was expressed at various levels in the seven lines examined, but IGF-2R was not expressed. Of the seven lines, the growth of HAK-1B, KIM-1, KYN-2 and HepG2 cells was stimulated in a dose-dependent manner by the exogenous addition of IGF-I or IGF-II, but the HAK-1A, KYN-1 and KYN-3 cell lines showed no growth. Exogenous addition of IGFBP-3 markedly blocked IGF-I and IGF-II-stimulated cell growth of KYN-2 and HepG2 cells, and moderately stimulated that of KIM-1 and HAK-1B cells, but no growth of the KYN-1, KYN-3 and HAK-1A cell lines was observed. IGF-I enhanced the phosphorylation of IGF-1R, Akt and Erk1/2 in KYN-2 cells, and coadministration of IGFBP-3 blocked all types of activation by IGF-I investigated here. In contrast, no such activation by IGF-I was detected in KYN-3 cells. IGFBP-3 also suppressed IGF-I-induced cell invasion by KYN-2 cells. Moreover, we were able to observe the apparent expression of IGFBP-3 in KYN-3 cells, but not in the other six cell lines. Furthermore reduced expression of IGFBP-3, but not that of IGF-1R, was significantly correlated with tumor size, histological differentiation, capsular invasion and portal venous invasion. Low expression of IGFBP-3 was independently associated with poor survival. IGFBP-3 could be a molecular target of intrinsic importance for further development of novel therapeutic strategy against HCC. (*Cancer Sci* 2006; 97: 1182–1190)

Hepatocellular carcinoma (HCC) is one of the most common and aggressive malignant tumors worldwide. The long-term prognosis of HCC patients has remained unsatisfactory due to the high incidence of intrahepatic recurrence, which depends on portal venous invasion and the high incidence of intrahepatic metastasis, as well as multicentric development of new tumors.[1,2] Moreover, our understanding of the molecular mechanisms underlying the progression of HCC and the development of effective therapeutic targets remain to be studied in further detail.

One candidate among the many growth factors that are closely associated with growth of HCC cells is insulin-like growth factor (IGF).[3] The biological effects of IGF are mediated via type 1 IGF receptor (IGF-1R), which leads to activation of the mitogen-activated protein kinase (MAPK) signaling pathway involved in cell growth and metabolism.[4,5] Mutation of another type 2 receptor (M6P/IGF-2R) and upregulation of IGF-II are expected to be responsible for the early stages of human hepatocarcinogenesis.[6,7] However, it remains unclear how the IGF system is involved in the progression of HCC. IGF are known to bind to IGF binding proteins (IGFBP), which regulate activity and function of IGF.[8] IGFBP-3 is the most abundant IGFBP that is present in non-cancerous liver tissues and serves as a negative regulator of cell proliferation in human HCC.[9–12] IGFBP-3 is also known to regulate cell growth independently of its effects on IGF-stimulated growth in other types of malignancy.[13–16]

In our present study, we investigated how IGFBP-3 exerts its antiproliferative effects on HCC cell lines in culture, and using immunohistochemical analyses we further examined whether or not the expression of IGFBP-3 in human clinical samples is associated with the clinicopathological characteristics of HCC. We discuss plausible roles of IGFBP-3 in the IGF-dependent and -independent cell proliferation of HCC, and also the clinical significance of IGFBP-3 in patients with HCC.

Materials and Methods

Cell culture and reagents. HAK-1A, HAK-1B, KIM-1, KYN-1, KYN-2 and KYN-3 were established at Kurume University (Kurume, Japan) as described previously.[17–21] HepG2 was purchased from American Type Culture Collection (Manassas, VA, USA). These cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Human IGF-I (hIGF-I), hIGF-II and hIGFBP-3 were purchased from R&D systems (Minneapolis, MN, USA). Anti-IGF-1Rα, antiphospho-IGF-1R, anti-IRS-1, anti-PKB/Akt, antiphospho-PKB/Akt, anti-Erk and antiphospho-Erk were obtained from Cell Signaling (Beverly, MA, USA). Anti-IGFBP-3 was from Santa Cruz (Santa Cruz, CA, USA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from TREVIGEN (Gaithersburg, MD, USA).

Western blotting. Western blotting was carried out as described previously.[22] Briefly, cells were lysed in a lysis buffer (pH 7.4) containing 20 mM Tris-HCl, 1% Triton X-100, 50 mM each of NaCl and NaF, 5 mM ethylenediaminetetraacetic acid, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonylfluoride and 10 μg/mL each of aprotinin and leupeptin. The lysates were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis, and then transferred to a nitrocellulose membrane.

Small interfering RNA transfection. The small interfering RNA (siRNA) corresponding to nucleotide sequences of IGF-1R (5′-GCAUCGAACUCCUCUUCUCAGUUAA-3′) and IGFBP-3 (5′-AAUCAUCAUCAAGAAAGGGCAUU-3′)[23] were purchased

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from Invitrogen (Carlsbad, CA, USA) and QIAGEN (Valencia, CA, USA), respectively. A negative control siRNA was obtained from Invitrogen. siRNA duplexes were transfected using Lipofectamine 2000 and Opti-MEM medium (Invitrogen) according to the manufacturer's recommendations.

Real-time quantitative polymerase chain reaction. The extraction of total RNA was carried out using TRIsol solution (Life Technologies, Grand Island, NY, USA). Real-time quantitative polymerase chain reaction (PCR) was carried out using the Real-Time PCR system 7300 (Applied Biosystems, Foster City, CA, USA) as described previously.[24] In brief, the PCR amplification reaction mixtures (20 μL) contained cDNA, primer pairs, dual-labeled fluorogenic probe and TaqMan Universal PCR Master Mix. The primer pairs and probe were obtained from Applied Biosystems. The relative gene expression for each sample was determined using the formula:

$$2^{-\Delta Ct} = 2^{Ct(GAPDH)-Ct(IGF\text{-}1R)},$$

which reflected the IGF-1R gene expression normalized to GAPDH levels.

Cell proliferation assay. Aliquots of medium containing 3.0×10^3 cells were seeded into a 96-well plate. The following day, the medium was replaced with serum-free DMEM medium with or without IGF. The plate was then treated for 72 h before the addition of WST-8 (2-[2-methocy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tertrazolium) and absorbance recorded at 450 nm.

Quantification of IGFBP-3 in conditioned medium. The concentration of IGFBP-3 in conditioned medium from the HCC cell lines was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D systems). The cells were suspended at a density of 3.0×10^3 cells/mL in 48-well plates and the suspensions were then cultured for 16 h. The supernatant was replaced with fresh medium after 24 h.

Matrigel invasion assay. This assay carried out as described previously.[25] In brief, BD BioCoat Matrigel Invasion Chambers (BD Bioscience, Bedford, MA, USA) were used according to the manufacturer's instructions. KYN-2 cells (1×10^5) in serum-free DMEM containing 0.1% bovine serum albumin were seeded onto Matrigel-coated filters in the upper chambers. In the lower chambers, DMEM medium with or without IGF-I was added as a chemoattractant. After 24 h of incubation, cells on the upper surface of the filters were removed with a cotton swab, and the filters were fixed with 100% methanol and stained with Giemsa dye. The cells that had invaded to the lower side of the filters were viewed under a microscope and counted in five fields of view. The invasive ability of the cancer cells was expressed as the mean number of cells in five fields. The assay was carried out as three independent experiments.

Patients and samples. We reviewed the clinical data and surgically resected tissue from 87 consecutive patients who underwent hepatectomy for primary HCC without preoperative treatment between 1992 and 2000. Written informed consent was obtained from each patient before tissue acquisition. The study was approved by the Human Investigation Committee at the Kyushu University School of Medicine (Fukuoka, Japan). All specimens were obtained from files at the Department of Anatomic Pathology at Kyushu University. All tumors were defined as HCC, and the pathological features were determined histologically based on the classification of the Liver Cancer Study Group of Japan.[26]

For measurement of serum IGF-I and IGFBP-3 levels, 92 subjects with HCC were included (age, 45–83 years; median, 67.6 years; male/female, 64/28; hepatitis B virus antigen (HBsAg) positive, 16; hepatitis C virus antibody (HCV-Ab) positive, 68; and HBsAg/HCV-Ab negative, 8). The diagnosis was based on ultrasonography, contrast-enhanced computed tomography, magnetic resonance imaging angiography and histological findings. All patients underwent hepatectomy for primary HCC without preoperative treatment at the Kurume University Hospital.

Immunohistochemistry. The tissue sections (4 μm) were stained with anti-IGFBP-3, anti-IGF-1Rα or antiphospho-IGF-1R. A biotinylated secondary antibody was then applied and incubated with peroxidase-conjugated streptavidin, chromogenized by diaminobenzidine. The staining was evaluated semiquantitatively in the selected HCC components containing a predominant histological grade and the results were compared with those in adjacent, non-neoplastic hepatocytes. The staining for IGFBP-3 was divided into low (less than 10% of tumor cells are positive) and high (more than 10% of tumor cells are positive) expression groups according to the percentage of immunoreactive cells. The evaluation for IGF-1R was dependent on its intensity when IGFBP-3 was evaluated by counting immunoreactive cells.

Statistical analysis. Differences in cell number, the levels of proteins and serum IGF-I and IGFBP-3 levels being examined were analyzed using the Mann–Whitney *U*-test. The correlation between serum IGF-I and IGFBP-3 levels and degree of HCC progression, and between immunohistochemical results and clinicopathological factors, were evaluated using the χ^2-test, Fisher's exact test and Mann–Whitney *U*-test. Overall survival was measured from the time of surgery until death with disease, or until the end of follow up. Patients who died of causes unrelated to the disease were censored at the time of death ($n = 3$). One patient was lost from follow up at 2 months after surgery. Survival curves were calculated using the Kaplan–Meier method, and the differences between the curves were analyzed using the log-rank test. Cox's proportional hazard model with a stepwise procedure was used for the multivariate survival analysis. The results were considered significant if the *P*-value was less than 0.05.

Results

IGF-dependent cell growth in some HCC cell lines. IGF-1R, IRS-1, Akt and Erk were expressed at various levels among the seven cell lines, although no IGF-2R expression was detected (Fig. 1A). Both IGF-I and IGF-II stimulated cell proliferation of HAK-1B, KIM-1, KYN-2 and HepG2 in a dose-dependent manner. Moreover, IGF-I and IGF-II stimulated only slight, if any, cell growth among HAK-1A, KYN-1 and KYN-3 (Fig. 1B). IGF-I effectively stimulated cell proliferation of KYN-2 much more than IGF-II did, but both factors showed similar stimulatory effects on growth of HAK-1B, KIM-1 and HepG2 cells.

We next examined whether or not IGF-dependent cell growth of HCC cells is mediated through IGF-1R. The transfection of IGF-1R siRNA in KYN-2 cells led to a downregulation of IGF-1R expression in a dose-dependent manner (Fig. 1C). The growth of KYN-2 cells increased to approximately 1.5-fold the size of untreated controls when treated with IGF, and this IGF-dependent stimulation of growth was almost completely blocked by 40–100 nM IGF-1R siRNA (Fig. 1D).

Effect of IGFBP-3 on IGF-induced cell growth. The effect of IGFBP-3 on IGF-induced cell growth was examined. The IGF-dependent cell growth of KYN-2 and HepG2 was almost completely blocked by treatment with IGFBP-3 at 1–10 μg/mL, whereas that of HAK-1B and KIM-1 was significantly blocked by treatment with 10 μg/mL IGFBP-3. In contrast, the growth of HAK-1 A, KYN-1 and KYN-3 cells was not at all inhibited by treatment with IGFBP-3 (Fig. 2A). Next we carried out an ELISA assay in order to determine whether or not HCC cell lines produce IGFBP-3. Of the seven cell lines examined here, we were able to observe unequivocal production of higher amounts of IGFBP-3 in the KYN-3 cells, but this was not the case in the other cell lines (Fig. 2B).

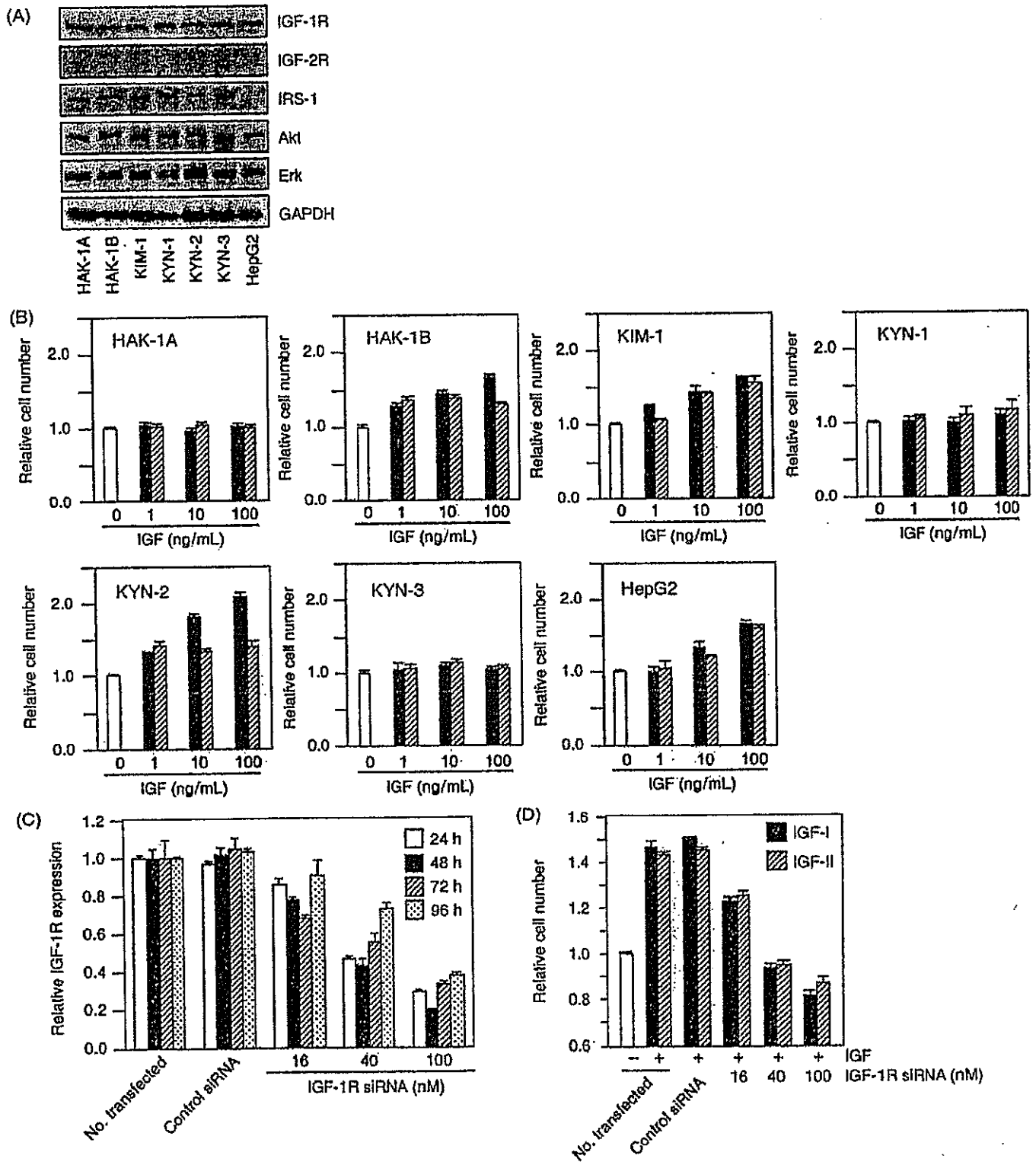

Fig. 1. (A) Expression of type 1 insulin-like growth factor (IGF) receptor (IGF-1R), IGF-2R, IRS-1, Akt and Erk was determined by immunoblotting conducted on protein lysates extracted from these cell lines. The detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a loading control. (B) Effects of IGF-I and IGF-II on the proliferation of seven hepatocellular carcinoma cell lines. The cells were treated with or without IGF at concentrations of 1, 10 or 100 ng/mL for 72 h in serum-free media, and then colorimetric WST assays were carried out. Each bar represents IGF-I (closed bar) or IGF-II (hatched bar). The data are expressed as the mean ± SD. (C) Inhibition by IGF-1R small interfering RNA (siRNA) treatment of IGF-1R gene expression of KYN-2 cells. KYN-2 cells were transfected with IGF-1R siRNA at concentrations of 0, 16, 40 and 100 nM, and the cells were incubated for the periods of time indicated. After incubation, total RNA was extracted and gene silencing was analyzed by real-time quantitative polymerase chain reaction. (D) Effect of IGF-1R siRNA on the proliferation of IGF-I (closed bar) or IGF-II (hatched bar)-stimulated KYN-2 cells. The cells were stimulated with 100 ng/mL of IGF-I or IGF-II after 24 h of siRNA treatment, and a WST assay was carried out 72 h after IGF stimulation. The data are expressed as the mean ± SD.

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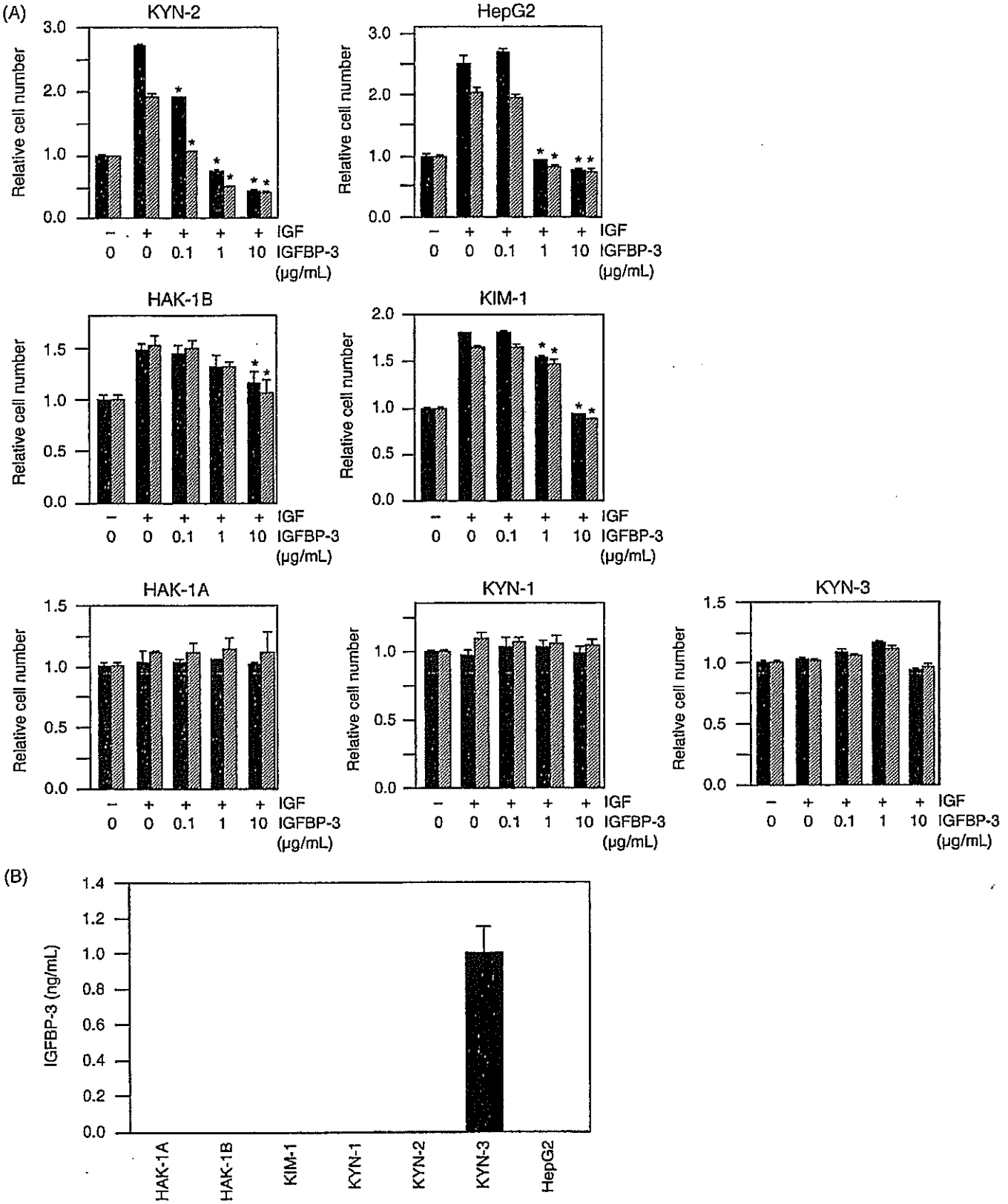

Fig. 2. (A) The effects of insulin-like growth factor (IGF) binding protein (IGFBP)-3 on IGF-I- or IGF-II-dependent cell proliferation of seven hepatocellular carcinoma cell lines. The cells were incubated with either serum-free medium, 100 ng/mL of IGF-I (closed bar) or 100 ng/mL of IGF-II (hatched bar) in the presence of various concentrations of IGFBP-3 for 72 h. After incubation, colorimetric WST assays were carried out. *Significant differences ($P < 0.01$) compared with treatment with IGF alone in the absence of IGFBP-3. The data are expressed as the mean ± SD. (B) Cellular production of IGFBP-3 in KYN-3 cells. IGFBP-3 protein levels in the culture medium with the seven cell lines examined here were assayed quantitatively with enzyme-linked immunosorbent assay systems. The data are the average of triplicate wells ± SD.

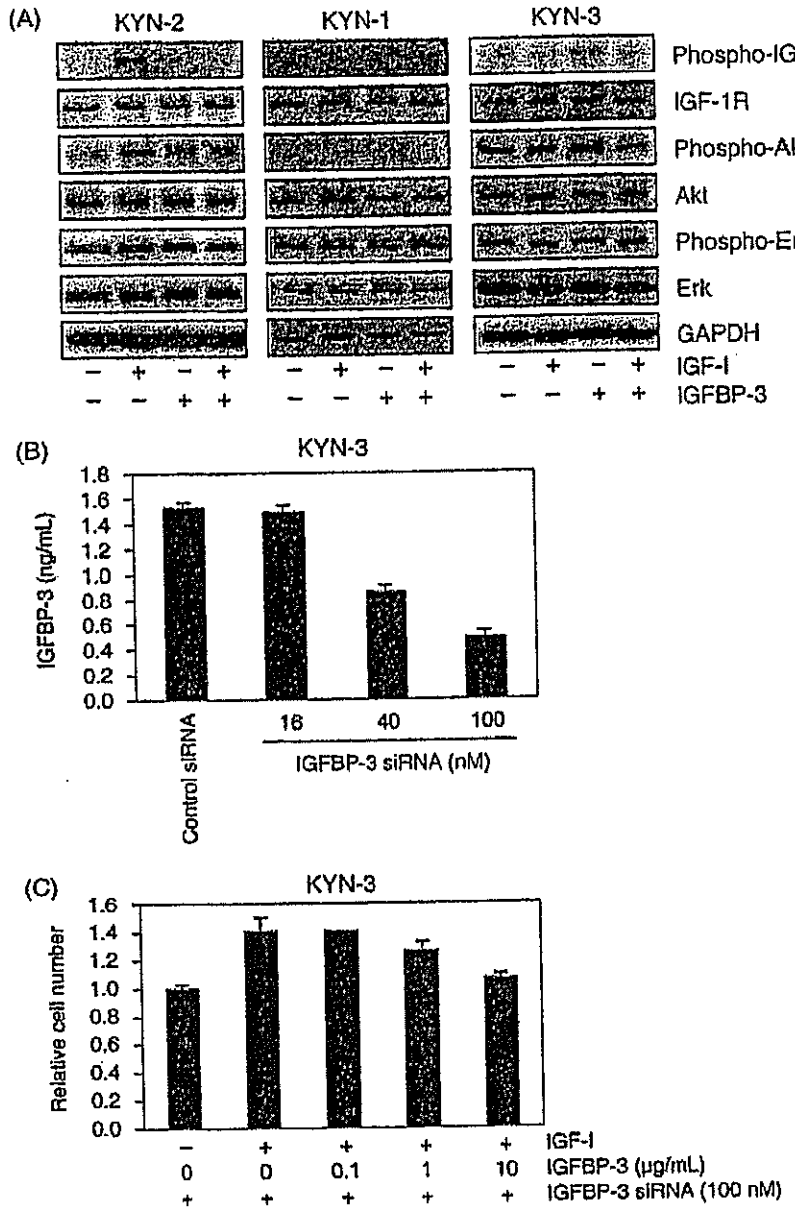

Fig. 3. (A) Effects of insulin-like growth factor (IGF)-I and IGF binding protein (IGFBP)-3 on the phosphorylation of type 1 IGF receptor (IGF-1R), Akt and Erk in KYN-2, KYN-1 and KYN-3 cells. Serum-deprived cells were treated with 100 ng/mL IGF-I and/or with 10 μg/mL IGFBP-3 for 10 min. Cell lysates were blotted with the antibodies indicated. The detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a loading control. (B) Inhibition by IGFBP-3 small interfering RNA (siRNA) treatment of IGFBP-3 expression in KYN-3 cells. KYN-3 cells were transfected with IGFBP-3 siRNA at concentrations of 0, 16, 40 and 100 nM, and the cells were incubated for 24 h. After incubation, IGFBP-3 protein levels in the culture medium were assayed quantitatively with enzyme-linked immunosorbent assay systems. The data are the average of triplicate wells ± SD. (C) Restoration of IGF-I-stimulated proliferation of KYN-3 cells by IGFBP-3 siRNA. KYN-3 cells were treated for 24 h with 100 nM IGFBP-3 siRNA and further incubation with 100 ng/mL of IGF-I in the absence or presence of various doses of IGFBP-3 for 72 h. The data are expressed as the mean ± SD.

Effect of IGFBP-3 on IGF-dependent signaling, cell growth and invasion. We further examined whether the activation of IGF-1R and its downstream signaling were modulated by IGFBP-3 in three cell lines: KYN-2 with IGF-dependent cell growth, and KYN-1 and KYN-3 with IGF-independent cell growth. Treatment with IGF-I markedly enhanced phosphorylation of IGF-1R, with concomitant phosphorylation of both Akt and Erk in KYN-2 cells, but such effects were not observed in KYN-1 and KYN-3 cells (Fig. 3A). The IGF-I-induced phosphorylation of IGF-1R, Akt and Erk was almost completely inhibited in KYN-2 by coadministration of IGFBP-3.

Cell growth of KYN-3 was not stimulated by IGF and not blocked by coadministration of IGFBP-3: KYN-3 cells produced a significant amount of IGFBP-3 (Fig. 2). We next examined whether production of IGFBP-3 by KYN-3 cells could be responsible for their reduced response to IGF. The transfection of IGFBP-3 siRNA in KYN-3 cells led to a knock-down of IGFBP-3 expression in a concentration-dependent manner (Fig. 3B). Cell growth of KYN-3 was found to be increased to approximately 1.4-fold over the untreated controls by IGF-I when treated with 100 nM IGFBP-3 siRNA (Fig. 3C). This IGF-I-dependent growth stimulation was significantly inhibited by 10 μg/mL IGFBP-3 in IGFBP-3 siRNA-treated KYN-3 cells (Fig. 3A,C).

Insulin-like growth factor-I plays an important role in invasion and migration of various malignant cell types including melanoma and pancreatic carcinoma.[27,28] We next examined whether IGFBP-3 could affect the invasive ability of IGF-stimulated KYN-2 cells by Matrigel invasion assay. Invaded cells were increased to approximately 9.0-fold of untreated controls when treated with 1000 ng/mL IGF-I (Fig. 4). In addition, the IGF-I-induced cell invasion was significantly suppressed by 52% by treatment with 100 μg/mL IGFBP-3 (Fig. 4). Thus, IGFBP-3 could inhibit both invasion and cell proliferation induced by IGF in HCC.

Expression of IGFBP-3 and IGF-1R and their clinicopathological implications in clinical HCC. The expression of IGF-1R and IGFBP-3 was determined in human HCC samples by

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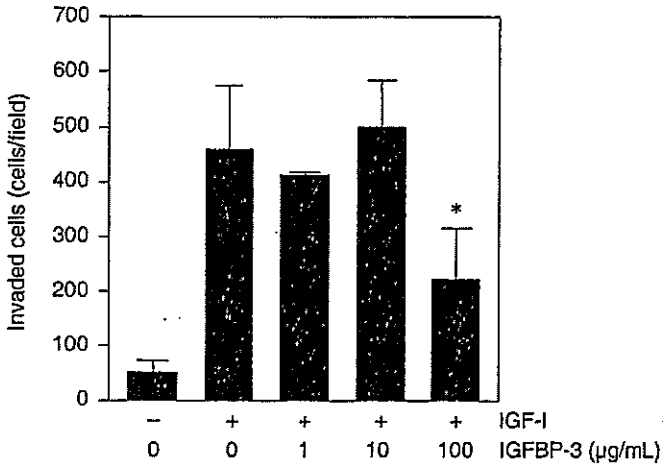


Fig. 4. Effect of insulin-like growth factor (IGF) binding protein (IGFBP)-3 on invasion by IGF-stimulated KYN-2 cells in Matrigel invasion assay. Serum-deprived KYN-2 cells (1×10^5) were seeded onto Matrigel-coated filters in the upper chambers, and Dulbecco's modified Eagle's medium with or without IGF-I (1000 ng/mL) in the presence of various concentrations of IGFBP-3. The cell invasiveness was quantified as the mean cell number in five fields of view per filter. Columns, mean of three independent experiments; bars, ±SD.

immunohistochemical analysis. The clinicopathological characteristics of 87 HCC patients, from whom the clinical samples were derived, are shown in Table 1. A representative immunohistochemical data of case 1 of well-differentiated HCC is shown in Fig. 5A: the expression of both IGFBP-3 and IGF-1R protein was higher in carcinoma tissue than in adjacent hepatocytes (Fig. 5). In contrast, a representative case of poorly differentiated HCC (case 2 in Fig. 5A) exhibited reduced expression of both IGFBP-3 and IGF-1R in carcinoma cells, compared with that in adjacent hepatocytes (Fig. 5A,d–f). In non-cancerous tissue of case 2, IGF-1R and IGFBP-3 were found to be expressed in hepatocytes, but not in stromal cells such as inflammatory cells, Kupffer cells and endothelial cells (data not shown). Table 2 gives a summary of the immunohistochemical analysis of IGFBP-3 and IGF-1R expression in 87 clinical specimens. The immunohistochemical analysis of IGF-1R and IGFBP-3 expression in HCC samples revealed that the intensity of staining in carcinoma was similar to or stronger than that of adjacent hepatocytes in 57 cases of IGFBP-3 and 58 cases of IGF-1R (Table 2).

We then determined whether or not the expression of IGFBP-3 and IGF-1R was associated with clinicopathological features of clinical HCC samples. However, we were unable to obtain any statistically significant results regarding IGF-1R expression and clinicopathological parameters. In contrast, a close association between IGFBP-3 expression and certain clinicopathological characteristics was observed (Table 2). Low expression of IGFBP-3 was observed in cases with larger tumor size, poorly differentiated histology, capsular invasion and portal venous invasion. Moreover, IGF-1R expression was found to correlate positively with IGFBP-3 expression.

Univariate and multivariate survival analysis. The overall survival of patients with low IGFBP-3 expression was significantly worse than those with high IGFBP-3 expression (Fig. 5B). In the univariate postoperative survival analysis, tumor size, histological differentiation, portal vein invasion, TNM classification and intrahepatic metastasis were also associated with poor survival (data not shown). The multivariate survival analysis revealed that TNM classification (stage 3/4), low IGFBP-3 expression and larger tumor size (>4 cm) were independent prognostic factors (Table 3).

Table 1. Clinicopathological characteristics of 87 patients

Characteristic	*n*
Sex	
Male	67
Female	20
Age (years)	36–83 (mean: 62.3)
Tumor size (cm)	0.8–14.5 (mean: 3.9)
Virus marker	
HBV*	13 (14.9%)
HCV*	60 (69.0%)
HBV* and HCV*	4 (0.5%)
Liver cirrhosis	37 (42.5%)
TNM stage	
I	12
II	31
III	19
IV	15
Histological differentiation	
Well	11
Moderate	54
Poor	22
Capsular invasion	56 (64.4%)
Portal venous invasion	40 (46.0%)
Intrahepatic metastasis	29 (33.3%)

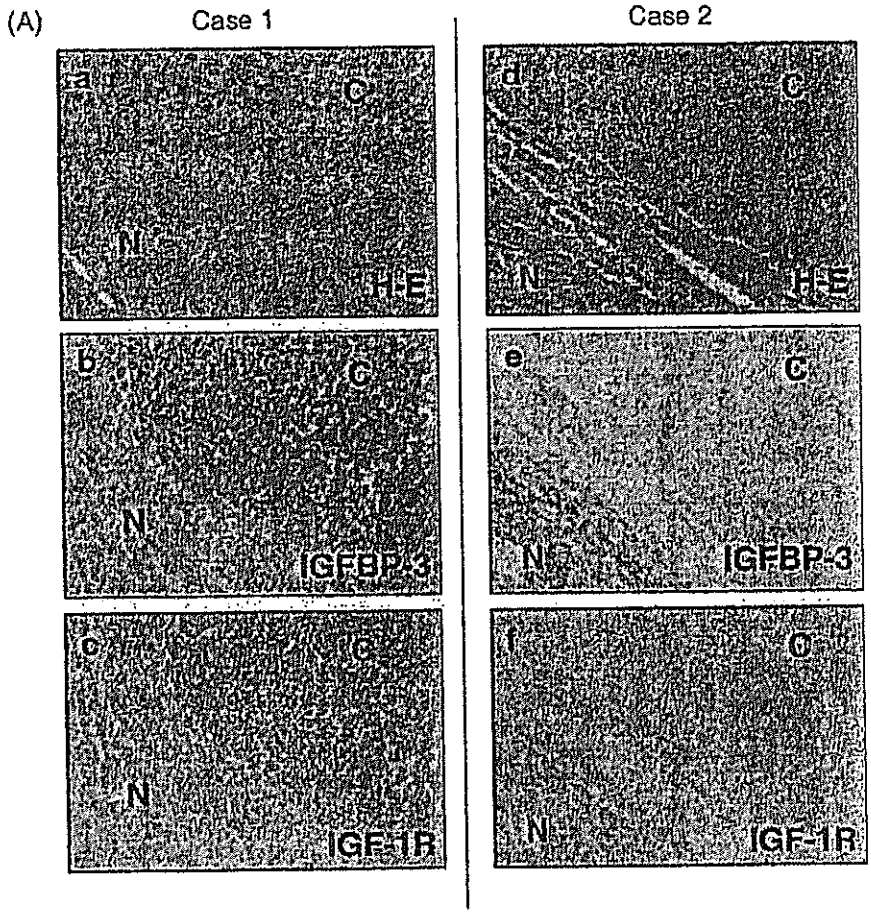
HBV*, positive for hepatitis B virus antigen; HCV*, positive for hepatitis C virus antibody.

Serum IGF-I and IGFBP-3 levels in patients with HCC. Finally, we examined whether serum levels of IGFBP-3 or IGF-I were associated with HCC staging in 92 patients. Serum mean levels of IGF-I and IGFBP-3 in the stage I, II, III and IV groups were 92.1 ± 35.2 and 1.36 ± 0.34, 99.6 ± 37.4 and 1.30 ± 0.35, 74.6 ± 27.1 and 1.02 ± 0.30, and 91.7 ± 58.0 and 1.35 ± 1.10, respectively. There were negative correlations between staging of HCC and serum IGF-I levels (Fig. 6A) or serum IGFBP-3 levels (Fig. 6B). However, no statistically significant difference was observed in the correlation between HCC staging and serum IGF-I levels or serum IGFBP-3 levels.

Discussion

In the present study, we classified seven HCC cell lines into two groups that exhibited IGF-dependent (HAK-1B, KIM-1, KYN-2 and HepG2) and independent (HAK-1A, KYN-1 and KYN-3) growth, whereby all seven cell lines expressed various levels of IGF-1R. The growth of the former four cell lines was susceptible to the inhibitory effects of IGFBP-3, whereas the growth of the latter three cells lines did not show such susceptibility. IGF-1R, Akt and Erk were markedly activated in response to IGF-I in KYN-2 cells with IGF-dependent growth and invasion, and their activities were almost completely blocked by coadministration of IGFBP-3. In contrast, IGF-I did not activate IGF-1R, Akt or Erk in KYN-3 cells exhibiting IGF-independent growth, and IGFBP-3 was unable to block phosphorylation of these molecules. Relevant studies have shown that the growth of HepG2 cells was inhibited by coadministration of IGFBP-3,[9,12] which is consistent with our present data regarding HepG2 cells. These findings suggest that IGFBP-3 inhibits both growth and invasion of HCC cells through its interaction with IGF.

Of these seven cell lines, only KYN-3 produced significant amounts of IGFBP-3. The cellular levels of IGF-1R in the KYN-3 cells were similar to those in KYN-2 cells, which were also highly sensitive to IGFBP-3-induced growth inhibition. The constitutive expression of IGFBP-3 might interfere



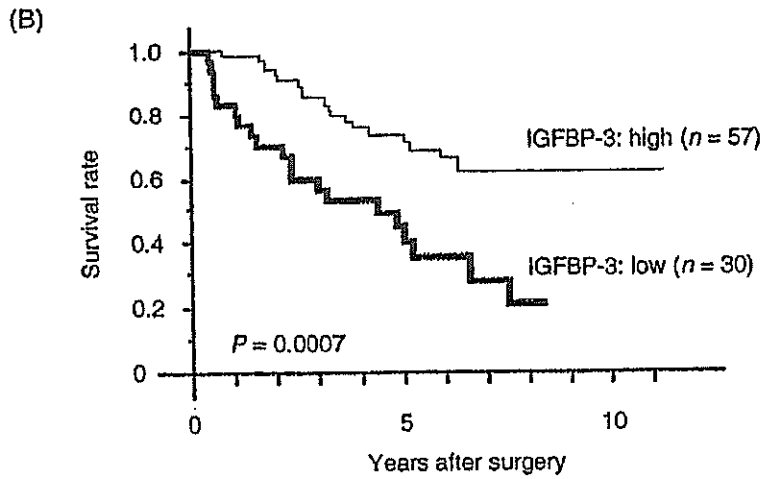


Fig. 5. (A) Immunohistochemical expression of insulin-like growth factor (IGF) binding protein (IGFBP)-3 in hepatocellular carcinoma (HCC). Two representative cases of well-differentiated HCC (case 1) and poorly differentiated HCC (case 2). The expression of both IGFBP-3 and type 1 IGF receptor (IGF-1R) was stronger in the HCC than in the adjacent liver in the cases of well-differentiated HCC (b,c). In contrast, cases of poorly differentiated HCC showed reduced expression of IGFBP-3 and IGF-1R in the HCC tissue compared with that of the adjacent liver tissue (e,f). (a,d) Hematoxylin–eosin (H–E) staining. C, cancerous region; N, non-cancerous region. (B) The overall survival of patients with low IGFBP-3 expression was significantly worse than that of patients with high IGFBP-3 expression ($P = 0.0007$).

with IGF-1R-dependent growth signaling in KYN-3 cells, resulting in lowered susceptibility to growth inhibition by the exogenous addition of IGFBP-3. Reduced expression of IGFBP-3 by treatment with siRNA could restore IGF-I-dependent cell growth in KYN-3 cells. The IGF-1R in KYN-3 cells might be occupied with endogenous IGFBP-3, thus resulting in a lack of further inhibition by exogenous IGFBP-3.

However, the growth of KYN-1 and HAK-1A was not stimulated by exogenous addition of these IGF, and was also insensitive to growth inhibition by IGFBP-3 when both cell lines expressed cellular levels of IGF-1R comparable to those in KYN-3 and KYN-2. Unlike the KYN-3 cells, neither the KYN-1 nor the HAK-1A cells produced significant amounts of IGFBP-3. It remains unclear why IGFBP-3 was unable to block the proliferation of these two cell lines, HAK-1 A and KYN-1, both of which express IGF-1R. Although IGF can activate IGF-1R as well as Akt and Erk, in KYN-2, KIM-1, HAK-1B and HepG2, all of which exhibit IGF-dependent cell growth, these IGF were unable to activate IGF-1R and its downstream signaling pathways in HAK-1A and KYN-1 cells, and in KYN-3 cells, all of which exhibit IGF-independent cell growth. It is possible that IGF-1R in the HAK-1A and KYN-1 cell lines

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Table 2. Correlation of clinicopathological features and type 1 insulin-like growth factor (IGF) receptor (IGF-1R) or IGF binding protein (IGFBP)-3 protein expression in hepatocellular carcinoma

Variable	IGF-1R			IGFBP-3		
	High (n = 58)	Low (n = 29)	P-value	High (n = 57)	Low (n = 30)	P-value
Mean age (years)	62.5	61.9	0.7397	63.2	60.6	0.1827
Male : female	45 : 13	22 : 7	0.8570	45 : 12	22 : 8	0.5542
Mean tumor size (cm)	3.6	4.6	0.1337	3.0	5.7	<0.0001
Liver cirrhosis	25 (43.1%)	12 (41.4%)	0.8781	26 (45.6%)	11 (36.7%)	0.4223
Stage						
I/II	31	12	0.2885	31	12	0.2021
III/IV	27	17		26	18	
Histological differentiation						
Well/moderate/poor	9/38/10	2/16/12	0.0557	11/38/8	0/16/14	0.0007
Capsular invasion	37 (63.8%)	19 (65.5%)	0.8742	31 (54.3%)	25 (83.3%)	0.0074
Portal venous invasion	26 (44.8%)	14 (48.3%)	0.7610	17 (29.8%)	23 (76.7%)	<0.0001
Intrahepatic metastasis	19 (32.8%)	10 (34.5%)	0.8722	19 (33.3%)	10 (33.3%)	>0.9999
High IGF-1R expression	–	–		45 (77.6%)	13 (43.3%)	0.0008

Table 3. Significant variables determined by multivariable survival analysis

Variables	Coefficient	SE	Coefficient/SE	P-value
Stage III/IV	0.988	0.353	2.797	0.0052
IGFBP-3	0.841	0.329	2.558	0.0105
Tumor size (>4 cm)	0.670	0.332	2.015	0.0440

IGFBP, insulin-like growth factor binding protein.

might be functionally inactive. There appeared to be no mutations in either the transmembrane domain or in the tyrosine kinase domain of IGF-1R in any of the HCC cell lines used in the present study (data not shown). Further studies should be carried out in order to gain a better understanding of the mechanism by which IGFBP-3 exerts its inhibitory effects on the growth of these HCC cell lines.

Thirty-nine percent of HCC clinical samples have been found to exhibit lower levels of IGF-1R than does non-neoplastic adjacent liver tissue.[12] Consistent with the results of that previous study, our immunohistochemical analysis of 87 HCC samples demonstrated lower levels of IGF-1R expression in approximately 40% of the human HCC tissue samples investigated, compared with matched, non-tumorous tissue samples. The expression of IGF-1R in HCC was not found to be significantly associated with any of a number of clinicopathological characteristics. However, to our surprise, high levels of IGF-1R expression were significantly associated with high levels of IGFBP-3 expression in HCC. Higher levels of IGF-1R expression might modulate the expression of IGFBP-3 in cases of HCC, but this possibility will require further investigation.

Immunohistochemical analysis of the expression of IGFBP-3 in cancerous and non-cancerous lesions in cases of HCC further demonstrated a close correlation of high or low IGFBP-3 expression with tumor size, histological differentiation and portal venous invasion, but not with intrahepatic metastasis. The close association of tumor size and differentiation in HCC with the expression of IGFBP-3 suggests that IGFBP-3 might play a key role in tumor growth and progression in HCC. The malignant characteristics of HCC are partly due to their metastatic potential via intrahepatic metastasis and portal venous invasion. Of these two types of metastatic potential in cases of HCC, the expression of IGFBP-3 was found to be more specifically correlated with portal venous invasion than with intrahepatic metastasis, thus suggesting the role of IGFBP-3 in portal venous invasion. However, there appeared no significant correlation of HCC staging with serum levels of IGFBP-3 or serum levels of IGF-I when 92 HCC patients were analyzed. Our present clinical data with determination of serum IGFBP-3 levels are also consistent with previous relevant studies.[11,29] Further study should be carried out in order to determine the potential role of IGFBP-3 in the above-mentioned malignant characteristics associated with HCC.

In conclusion, the absence or presence of IGFBP-3 expression and a functional IGF-1R could affect the state of IGF-dependent cell growth in HCC cell lines in culture. The expression of

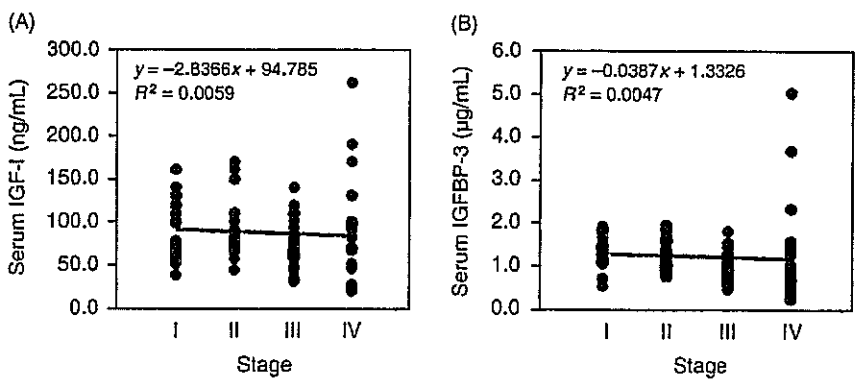

Fig. 6. Correlation between hepatocellular carcinoma (HCC) staging and serum insulin-like growth factor (IGF)-I levels or serum IGF binding protein (IGFBP)-3 levels in patients with HCC (n = 92). (A) Correlation between HCC staging and serum IGF-I levels. (B) correlation between HCC staging and serum IGFBP-3 levels. Patients of stage I (n = 22), II (n = 17), III (n = 31) and IV (n = 22).

IGFBP-3 was also significantly correlated with histological differentiation, tumor size, portal venous invasion and prognosis, but not with intrahepatic metastasis in the HCC studied here. As IGFBP-3 plays a pivotal role in tumor enlargement and metastasis in HCC, it should be considered as a possible molecular target for the development of novel therapeutic strategies used in the treatment of HCC.

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消化器癌の網羅的遺伝子解析に基づいた分子標的治療の開発

—遺伝子解析と抗癌剤感受性—

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はじめに

癌の治療には，手術療法のみならず，化学療法，放射線療法などの補助療法を加えた集学的な治療が必要である．しかし，化学療法，放射線療法はすべての患者へ効果が一定しているわけではなく，実際の治療，特に化学療法には副作用が生じることがある．そこで，症例毎に応じたレジメンの決定や，薬剤の選択，新しい分子標的の探索を目的とした，いわゆる個別化医療の必要性が提唱されるようになってきた．当教室では，2003年から2年間にわたり，九州大学のP&P(九州大学教育研究プログラム・研究拠点形成プロジェクト)より研究支援を頂き，「消化器癌の網羅的遺伝子解析に基づいた分子標的治療の開発」に関する研究を行なった．

この研究では，まず，DNAマイクロアレイを用いて，網羅的な遺伝子解析を行ない，抗癌剤に耐性な細胞において，どのような遺伝子が高発現しているのか，また，転移しやすい細胞においてどのような遺伝子が高発現しているのかについて検討した．それにより，膵臓癌細胞株におけるゲムシタビン耐性に関わる遺伝子や[1]，胆管癌細胞株における腹膜播種に関わる遺伝子なども同定された．その他にも，新しい分子標的を集約的に探索するため，抗癌剤によるシグナル伝達機構や，抗癌剤とDNAミスマッチ修復機構についての研究も行なった(図1)．例えば，新しい分子標的治療薬の標的分子としても米国などで注目を集めているシグナル伝達分子AKTが，抗癌剤による細胞死の抑制に重要な役割を果たしていること，また胃癌や乳癌では，そのシグナル伝達経路が持続的に活性化していることも明らかにした[2][3]．

1．網羅的遺伝子解析と癌治療

まず，網羅的遺伝子解析に基づいた抗癌剤感受性予測の現状について概説する．

A) 網羅的遺伝子解析と抗癌剤感受性予測

癌という疾患の多様性を考えると，特定の遺伝子の発現状況のみで感受性・耐性を予測することは困難であることが容易に想像できる．近年になりDNAマイクロアレイという手法が開発され，一度に数千あるいは数万という大量の遺伝子発現が解析できるようになった．この技術を用いて，網羅的遺伝子発現解析を行ない，薬剤感受性・耐性の予測を行なう試みが世界的に行なわれてきた．だが，術後補助療法において効果予測因子を同定するには，生存率に代わる有効な評価法がないため，多くの症例と長い追跡期間を要する．一方，術前化学療法では，薬剤の感受性の評価が容易であり，効果予測因子に関する研究に適している．そこで，術前化学療法開始前に採取した組織で網羅的に遺伝子発現パターンを解析し，化学療法の効果予測因子を同定しようとする試みが急速に進んだ．まず2003年Changらにより，マイクロアレイ技術を用いて抗癌剤感受性を規定する遺伝子発現パターンを，臨床試験において示した最初の論文が発表された[4]．本研究では，ドセタキセルによる術前化学療法のPhase II studyに参加した局所進行乳癌24症例を対象に，針生検により採取した乳癌組織からRNAを抽出し，DNAチップを用いて発現解析を行い，治

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Development of a Molecular Target Therapy on the Basis of Global Gene Analyses of Gastrointestinal Carcinoma

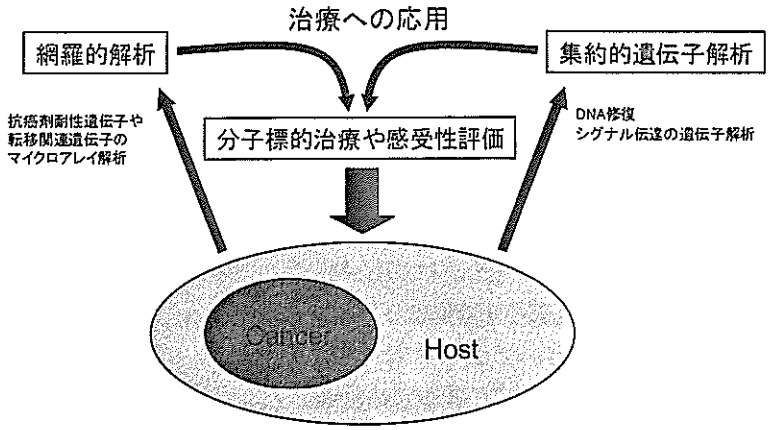

図1　網羅的，集約的遺伝子解析に基づいた，分子標的治療，薬剤感受性評価法の開発．癌や宿主の遺伝子を網羅的，集約的に解析することにより新しい分子標的の候補を同定し，臨床応用を目指す．

療効果との関連を解析した．ここで使用したDNAチップでは12625遺伝子の発現解析が可能で，これらの遺伝子から選択された1628遺伝子サブセットでさらに解析を行い，その中の92遺伝子の発現パターンの違いでドセタキセル感受性を予測できることが示された．検証試験の結果，特異度90%，感度85%，正診率は88%で，これらの92遺伝子を予測因子として用いた場合の陽性適中率および陰性適中率はそれぞれ92%と83%であった．本論文では感受性群と耐性群の判定を，各群に同程度の数が含まれるように化学療法後の残存腫瘍量25%で分けている．しかし，術前化学療法において最も予後に相関するのは組織学的奏効度，特にpCR（pathological complete response）であることはいうまでもなく，肉眼的に評価した残存腫瘍の割合が，真のドセタキセル感受性を表しているのか不明である．また，ここで選ばれた92遺伝子の中に，従来の研究でタキサン系抗癌剤の感受性に関与することが示されてきた遺伝子が含まれていないことも，この感受性・耐性群の定義や評価法に問題があるためかもしれない．一方，Ayersらはdoxorubicin，cyclophosphamide，5-FU併用療法に続いてpaclitaxelを投与する治療法（T/FAC）の治療効果を，cDNAマイクロアレイを用いた遺伝子解析で検討した[5]．この研究では，治療前にfine-needle吸引で得られたサンプルからRNAを抽出し，30,721の遺伝子配列を有するcDNAマイクロアレイを用いて解析している．この研究ではpCR（非浸潤成分の残存も含む）を治療効果の指標として，効果予測の指標となる74個の遺伝子セットを同定した．この遺伝子セットの信頼性を検証したところ，正診率は78%（14/18），pCRの陽性的中率は100%（3/3），陰性的中率は73%（11/15），感度43%（3/7），特異度100%であり，この方法での治療効果予測の有用性を報告している．最近になり国内の多くの施設でも，化学療法前の腫瘍組織での遺伝子発現プロファイルを用いて治療効果を予測する試みが行なわれるようになっているが，施設や報告者により遺伝子解析の方法，解析対象の遺伝子数，治療のプロトコール，治療効果の判定基準が異なっている．治療効果は最も臨床的意義のある因子で評価すべきであり，真に臨床的意義のあるマーカーは，いかなる解析法をもってしても同じ結果をもたらすものでなければならない．

私たちの今回の研究では，細胞株やヌードマウスを用いて，薬剤感受性や治療の標的となり得る分子をまずin vitroで網羅的に解析することを試みた．

B）ヒト膵癌細胞におけるゲムシタビン耐性予測因子

ゲムシタビンは膵癌の治療に使用されている新しい標準的抗癌剤であるが，その感受性におけるメカニズムには不明な点が多い．本研究においてはこのゲムシタビン感受性を調整するメカニズムを解明するために，ゲムシタビン感受性膵癌細胞株KLM1を用い，ゲムシタビンを10 μg/mlの濃度で培養液中に添加

した状態で1週間培養．その後，細胞はゲムシタビンを除いた培養液にて細胞密度が回復するまで2週間培養を続けた．この操作を4回繰り替えし，50%の細胞増殖阻害を示すIC50において20倍の耐性を有する株KLM1-Rを作製した．このKLM1とKLM1-Rとの間の遺伝子発現変化についてマイクロアレイによる遺伝子解析を行った（図2A）．5倍以上の発現変化比を示した25遺伝子のうち5遺伝子はKLM1-Rにおいて発現亢進をしており，20遺伝子が発現低下していた．今回施行したマイクロアレイにおいてセレノプロテインPがKLM1-Rにて9.699倍と最も発現が亢進していた（図2B）．実際にRT-PCRでも確認したところ，KLM1-Rにて発現が亢進していた．セレノプロテインPはヒト血漿内のセレン含有細胞外蛋白であるセレノプロテインファミリーの一つであり[6]，セレノプロテインファミリーは抗酸化作用に関わっていることが報告されている[7][8]．一方，我々の行ったマイクロアレイ解析において，一般的に薬剤耐性に関わるとされるbcl-2関連遺伝子（*bcl-2*，*bfl-1*，*bag-1*，*bad*，*bak*，*bcl-xL*）および，多剤耐性遺伝子（*MDR1*，*MDR3*，*MRP5*）はゲムシタビン耐性株KLM1-Rにおいて遺伝子発現変化は認められなかった．セレノプロテインPが膵癌細胞をゲムシタビンの細胞毒性から防御するどうかを評価するため，ゲムシタビン投与後の細胞生着率を検討したところ，セレノプロテインP 1 μg/mlを培養液中に添加することにより，ゲムシタビン72時間暴露後の細胞の生着率は回復した．また，ゲムシタビンによりKLM1細胞の細胞内活性酸素種（ROS）レベルが上昇する一方，セレノプロテインPはゲムシタビンにより誘導されるこのROSの上昇を抑制した．さらにIFN-γはセレノプロテインPのmRNAの発現を抑制し，その結果KLM1-Rにおけるゲムシタビン感受性を改善した．これらの結果はセレノプロテインPが細胞内ROSレベルを低下させることによりゲムシタビンに対する感受性を低下させるというメカニズムを示唆していた．

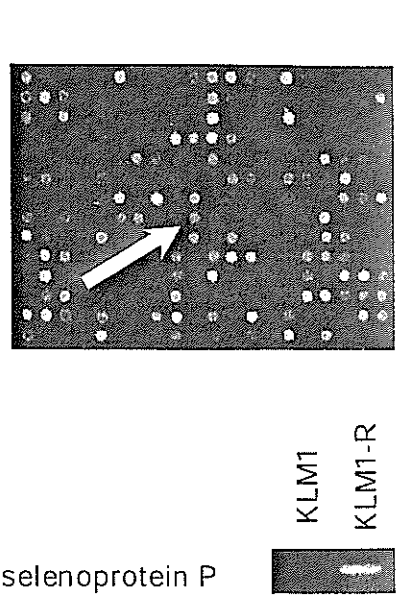

図2　A. KLM1とKLM1-Rとの間の遺伝子発現変化を12,814クローンのcDNA　マイクロアレイを用いて解析した．矢印はセレノプロテインPであり，KLM1-Rにおいて9.699倍と最も発現が亢進していた．B. セレノプロテイン のmRNAの発現を評価するためにKLM1細胞株及び，KLM1-R細胞株においてRT-PCRを行った．セレノプロテインPのmRNAはKLM1-R細胞株において明らかに発現の亢進を認めた．

C）胆管細胞癌細胞株における網羅的遺伝子解析による分子標的の探索

胆管細胞癌は，高頻度で腹膜播種をする予後の悪い腫瘍の一つである．胆管細胞癌株HUCCT-1をヌードマウスに移植し，出現した腹膜転移，リンパ節転移，肺転移をそれぞれ，再び別のヌードマウスに移植する方法を繰り返すことで，高腹膜播種株，リンパ節転移株，肺転移株を樹立した．各臓器への高転移株についてcDNAマイクロアレイを用いて親株との比較で網羅的遺伝子解析を行った結果，共通して発現が変化し，あるいは転移臓器別に特異的に変化を示す遺伝子群が判明した．高腹膜播種株には，IL-1βが共通して発現が増強していた．そこで，レトロウイルスベクターを用い，IL-1βのStable transfectantを樹立し，その機能を解析した．するとIL-1βのStable transfectantはin vitroで浸潤能が亢進し，さらに血管新生や浸潤に関連するIL-8やMMP-1の発現を亢進させていた．in vivoでも，腹膜播種を高頻度に形成した．その他，肺転移株において高発現を認めたDickopff-1は肺転移株細胞質に高発現しており，臨床検体を用いた免疫組織化学染色での検討では，予後不良因子であることが明らかとなった．これらの遺伝子を分子標的にすることで，転移臓器特異的な治療が開発される可能性がある．

2．新しい分子標的探索のための集約的な遺伝子解析

前述のような網羅的遺伝子解析に対して，本研究では薬剤感受性に関わるとされる遺伝子の集約的な解析も行なった．特に，PTEN/AKT/PI3K シグナル経路に対する研究および DNA 修復と消化器癌の薬物治療に関する研究の二つを重点的に行なっている．ともに抗癌剤の感受性を規定する因子として注目されている．

A）抗癌剤によるシグナル伝達機構に関する研究

今回，消化器癌や乳癌における PI3K/AKT シグナル伝達経路の異常を詳細に観察することで，新しい分子標的治療の開発の布石となり得る基礎的データを得た．IGF-1R，HER2/Neu，VEGF-R，PDGF-R などのチロシンキナーゼレセプターからのシグナルは，PI3K およびセカンドメッセンジャーである PIP3 を介して AKT に集約され，その結果 AKT は，その下流の血管新生やアポトーシスの抑制などに関与する NF- kappaB,mTOR,Forkhead,Bad,GSK-3,MDM-2 などのリン酸化を調節することが知られている（図 3）[9][10]．また，癌抑制遺伝子の一つとして最近知られるようになった PTEN は AKT/PI3K 系のリン酸化経路のフォスファターゼであり，癌における PTEN の異常は AKT や TOR などの PI3K 系シグナルの持続的活性化につながる．私達は，胃癌における PTEN/AKT/PI3K シグナル伝達系をターゲットとした遺伝子解析の意義について検討を加えた．まず，119 症例の胃癌を対象とし，DNA を抽出後，2 つのマイクロサテライトマーカーを用いて PTEN の LOH を解析した．メチル化の検討は MSP 法で行ない，免疫染色で蛋白の発現解析も行なった．AKT のリン酸化の評価は特異抗体を用いた免疫染色で行った．その結果，PTEN の LOH は 76 例中 13 例(17.1%)で認められ，pAKT(リン酸化 AKT)は 76 例中 22 例(28.9%)で陽性であった．PTEN が正常な症例では 20.5%のみに pAKT が観察されたのに対し，PTEN の LOH の症例では，77.8%が pAKT 陽性であり，明らかに PTEN が LOH の症例で AKT がリン酸化されている症例が多かった．PTEN のメチル化や免疫染色は pAKT と有意な関係は認めなかった．また，pAKT 陽性の癌では，抗癌剤に耐性である傾向を認めた（図 4）[2]．乳癌で解析すると，HER2 陽性例で AKT のリン酸化されている症例が多く（$p<0.001$），HER2 陽性で PTEN も LOH を示す症例では AKT のリン酸

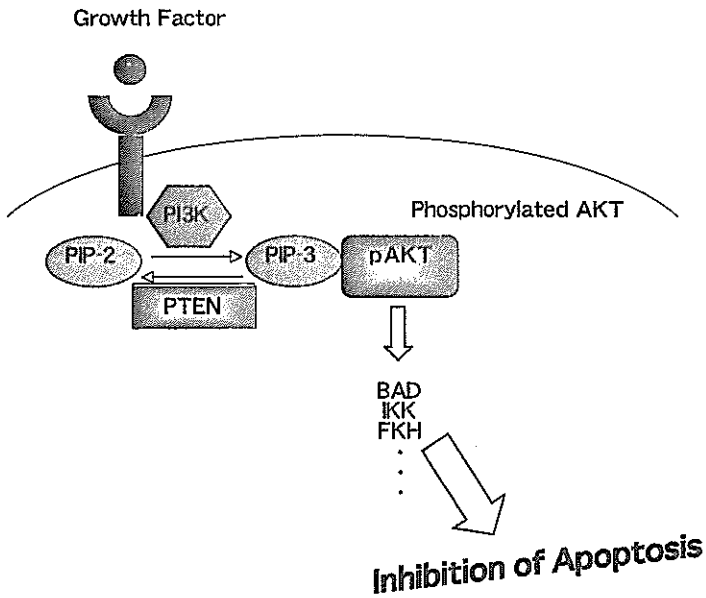

図 3　AKT/PI3K シグナル伝達経路によるアポトーシス抑制機構．癌では癌抑制遺伝子の一つとも考えられる PTEN の異常により，この経路が持続的に活性化され，アポトーシスを抑制し，抗癌剤感受性を低下させている可能性がある．

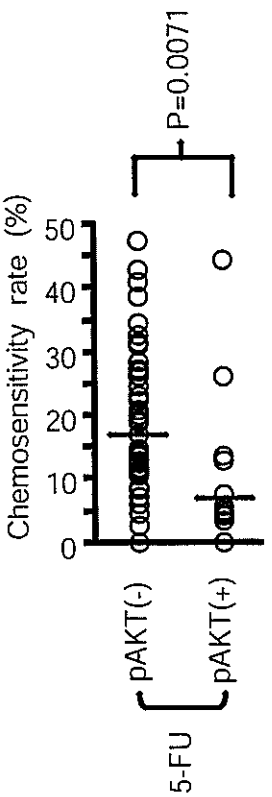

図4 新鮮手術標本からMTT assayを行ない，5-FUの感受性とAKTのリン酸化の状態を比較した．AKTがリン酸化されている腫瘍で抗癌剤が低感受性である傾向を認めた．

化されている症例が多かった．さらにHER2陽性でPTENもLOHを示す症例ではProgesterone receptor（PR）が認められず，AKT/PI3Kシグナル伝達系の異常とPRの発現に何らかの関係があることが示唆された[3]．欧米では既にこの経路を阻害するmTOR阻害剤を用いた臨床試験が開始されている．我々の研究は，固形癌において，このPTENの異常とAKTの活性化が従来の抗癌剤の抵抗性やホルモン感受性と関係していることを世界で初めて具体的に明らかにしており，今後の新しい分子標的治療法の開発に繋がる可能性がある．

B）抗癌剤感受性とDNAミスマッチ修復機構

ごく最近になって，DNA修復が薬物の感受性因子として注目されるようになってきた．抗癌剤感受性因子として，細胞のDNA修復活性が極めて重要である事実を初めて報告したのは，P. Karranらのグループである[11]．彼らが注目したのは，DNA複製の精度を保証するための修復機構のひとつ，DNAミスマッチ修復（DNA mismatch repair，MMR）であった．彼らは細胞の1個アルキル化剤に対する感受性は，このMMR活性によって劇的に変化することを明らかにした．今では，このMMRはフッ化ピリミジンやプラチナ製剤，トポイソメラーゼ阻害剤などの多様な抗癌剤の感受性を左右する重要な感受性因子とされている[12][13]．ところで，真核生物のゲノム上には，マイクロサテライトと呼ばれるリピート配列が存在する．MMR遺伝子の変異が存在する場合，このマイクロサテライト配列の不安定化，すなわちマイクロサテライト不安定性（microsatellite instability，MSI）が生じることが知られている．われわれの研究グループは，オートシーケンサーと蛍光プライマーを用いてMSIを詳細に解析する手法を以前より確立している[14]．MSIの陽性判定には，通常曖昧な判定法が用いられることが多いが，我々はミスマッチ修復遺伝子を欠く細胞株を多数サブクローニングし，マイクロサテライト配列を増幅した後シーケンサーから得られる波形（ゲル上のバンドパターン）を詳細に解析し，独自のMSI判定基準を確立した[15][16]．

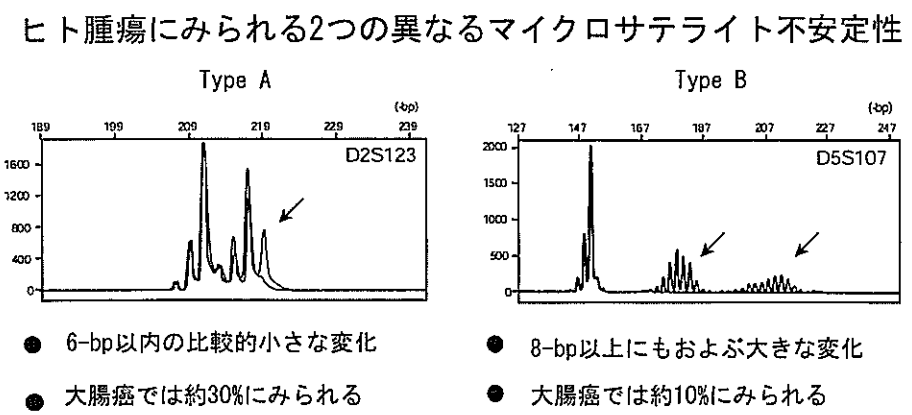

図5 Type Aの定義は，変化の幅が6-bp以内であること．大腸癌では，このType Aは1つか2つのマーカーにのみ観察されることが多い．一方，Type Bは8-bp以上にもおよぶような大きな変化を呈するもの．あたかも新しいアレルが出現したかのような所見を呈するのが特徴．Type Bは観察したそのほとんどのマーカーで観察される傾向がある．大腸癌では，Type Aはその約30%に，Type Bは約10%に観察される．

この方法を用いて，ミスマッチ修復遺伝子欠損マウスに生じた腫瘍や，胃癌，大腸癌を中心とする2000例に及ぶ臨床検体の解析を行った．我々の解析からMSIは，マイクロサテライト配列の変化が6 bp以内のType Aと，マイクロサテライト配列のPCR増幅によって，オリジナルとは全く異なる長さを持った多数のバンドが出現するType Bという，マイクロサテライトlocus毎の個別判定が可能であることが明らかとなった（図5）．実際にそれぞれのカテゴリーの症例でMMR遺伝子群およびp53のシーケンスを行い，ミスマッチ修復遺伝子欠損マウスの解析結果も総合すると，変化が軽微なType Aが，本来のミスマッチ修復異常のカテゴリーであることが明らかになった[17]．また．大腸癌では，P53の変異がType Aに多いことや（表1），癌の発生部位によりカテゴリーが異なる（近位側はType A，遠位側はType B）ことが明らかとなり，部位別の発癌の分子背景の違いが認められた．MSIは少なくとも2つのカテゴリーに分類され，それぞれ異なる分子機序を基盤としていると考えられる．今後はこの考え方に基づいた治療感受性に関する研究が行なわれるであろう．

表1　*p53*遺伝子変異とType A，Type B MSI

		MSI			Subtotal
		Type A	Type B	Negative	
p53	Wild Type	13	14	29	56
	Mutant	12	0	11	23
	Subtotal	25	14	40	79

P=0.006

まとめ

九州大学のP&P（九州大学教育研究プログラム・研究拠点形成プロジェクト）より研究支援を頂いた，「消化器癌の網羅的遺伝子解析に基づいた分子標的治療の開発」に関する研究結果について概説した．前述のように，実際には網羅的な遺伝子解析だけでなく，薬物代謝に焦点を絞った遺伝子解析も同時に行なってきた．これまでの研究で，癌の治療効果に関わるいくつかの重要な分子が分子標的治療の候補として同定されている．最終的な目標は新規分子標的治療の開発であり，今後は工学部などと連携しtranslational researchとしてインパクトの高い独創的研究を行ないたい．

文　献

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(参考文献のうち，数字がゴシック体で表示されているものについては，著者により重要なものと指定された分です.)

プロフィール

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◆略歴 昭和52年九州大学医学部卒業．昭和58年九州大学大学院医学系研究科外科系修了．昭和58年九州大学生体防御医学研究所生化学部門助手．昭和60年九州大学医学部附属病院助手．平成3年九州大学医学部附属病院講師．平成7年米国ハーバード大学客員助教授．平成11年九州大学大学院消化器・総合外科（第二外科）助教授．平成14年より九州大学大学院消化器・総合外科（第二外科）教授．現在に至る．

◆研究テーマと抱負 専門領域としては，消化器（食道・胃・大腸，肝・胆・膵，門亢症），肺，乳腺，血管，移植と多岐にわたりますが，研究者の使命として，外科的疾患の本質に迫り，常に新しい治療法の開発へ向けて研究を進めたいと考えています．また，教室・関連施設共同の臨床試験を通してエビデンスを創出し，世界へ向けて標準治療を発信すること，Translational Research として，Molecular Biology と Medical Technology による最新の成果を診療へ展開することを目標としています．

◆趣味 犬の散歩，ガーデニング