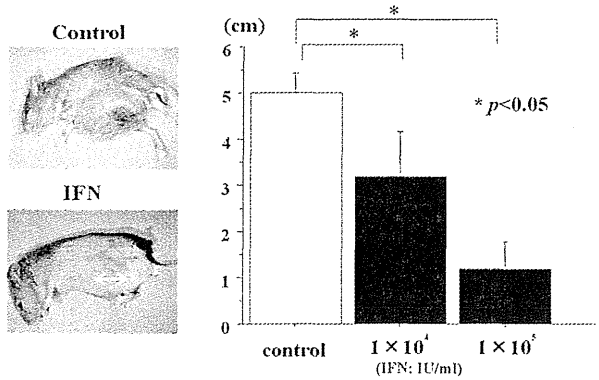

FIG. 2. Dose-dependent change in tumor diameter of subcutaneously injection model. The mice received a subcutaneous injection of 1×10^4 and 1×10^5 IU/mL PEG-IFN-α2b. PEG-IFN-α2b significantly suppressed tumor growth ($P < 0.05$).

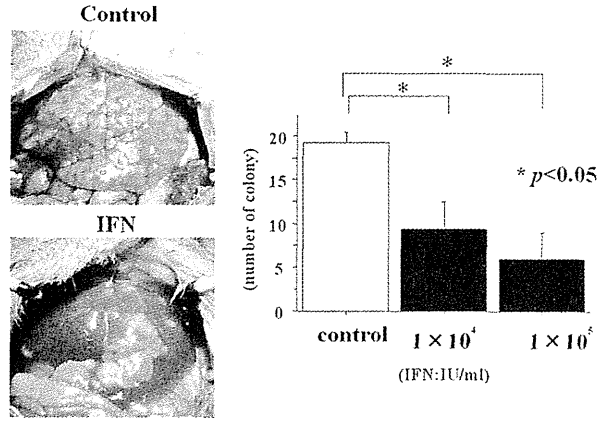

FIG. 3. Intrahepatic metastasis *via* portal vein model showed number of liver metastasis nodule seven days after operation. The mice received a subcutaneous injection of 1×10^4 and 1×10^5 IU/mL PEG-IFN-α2b. PEG-IFN-α2b significantly decreased number of liver metastasis ($P < 0.05$).

decreased the number of liver metastases (19.3 *versus* 6.0, $P < 0.05$, Fig. 3).

Tumor Angiogenesis (Microvessel Density)

The number of blood vessels was quantified by counting the stained regions in five high-power fields (×400). PEG-IFN-α2b significantly suppressed angiogenesis compared with control mice ($P < 0.05$, Fig. 4).

Expressions of VCAM and ICAM Protein

These pictures show non-tumorous area of mouse liver. Immunohistochemically, they showed weak positive reaction in the entire field, so both the VCAM and ICAM expressions showed no difference between the metastatic tumors or the non-tumorous liver tissues (Fig. 5).

DISCUSSION

The metastasis of cancer cells has a multi-step and key-molecule so-called 'seed and soil' theory [18]. The steps of metastasis are characterized by cells that lose their cell–cell contact (E-cadherin, β-catenin), cross basement membrane [matrixmetaloprotease (MMP) family], invade stroma (MMP family), spread across blood vessels, adhere vascular endothelial cells (Sialyl-Lewis X, integrin, ICAM, VCAM) and form new neoplastic tissue and angiogenesis (integrin, VEGF, bFGF, angiopoietin 2) in sites other than that of the original tumor.

IFN has been shown to reduce the incidence of preneoplastic foci and cancer in HCC model [19, 20]. IFN has been already reported to inhibit the growth of a variety of cancer cells, including multiple myeloma, ovarian cancer, and liver cancer cells [21]. In HCC, IFNα is reported to up-regulate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in T cells, NK cells and monocytes [3], and Fas/Fas ligand pathway [5]. It also exerts immunomodulatory effects by stimulating T cells, NK cells, and monocytes [3].

In this study, we use the mouse cell lines. The effect of human IFNs on murine tumor cells *in vivo* has already been reported [22]. First, we demonstrate that PEG-IFN-α2b can inhibit the invasion of floating MH134 HCC cells. Second, we can demonstrate the anti-metastatic effect of PEG-IFN-α2b with *in vivo* intrahepatic and portal vein metastasis model showing the reduction of the liver metastases. So these results can cause the speculation about the anti-metastatic effect of PEG-IFN-α2b. However, few studies have reported the anti-metastatic effects of IFNα, and the mechanisms of these effects are still unclear. The invasiveness

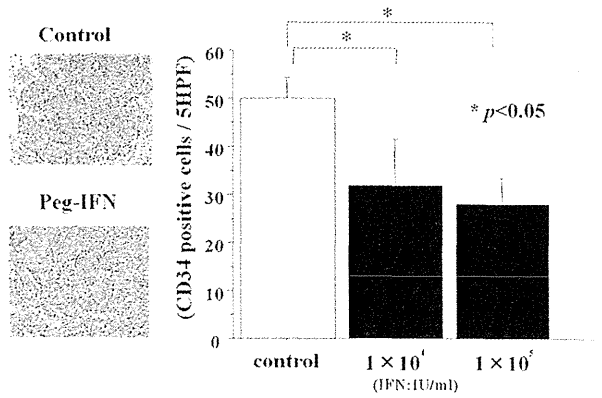

FIG. 4. Microvessel density in metastatic liver tumor. Detection of CD34 positive cells by immunohistochemical staining (×400). Any brown-staining cell cluster distinct from adjacent microvessels, tumor cells, or other stromal cells was considered a single countable microvessel, and the number of CD34 positive cells was significantly lower compared with the control group ($P < 0.05$).

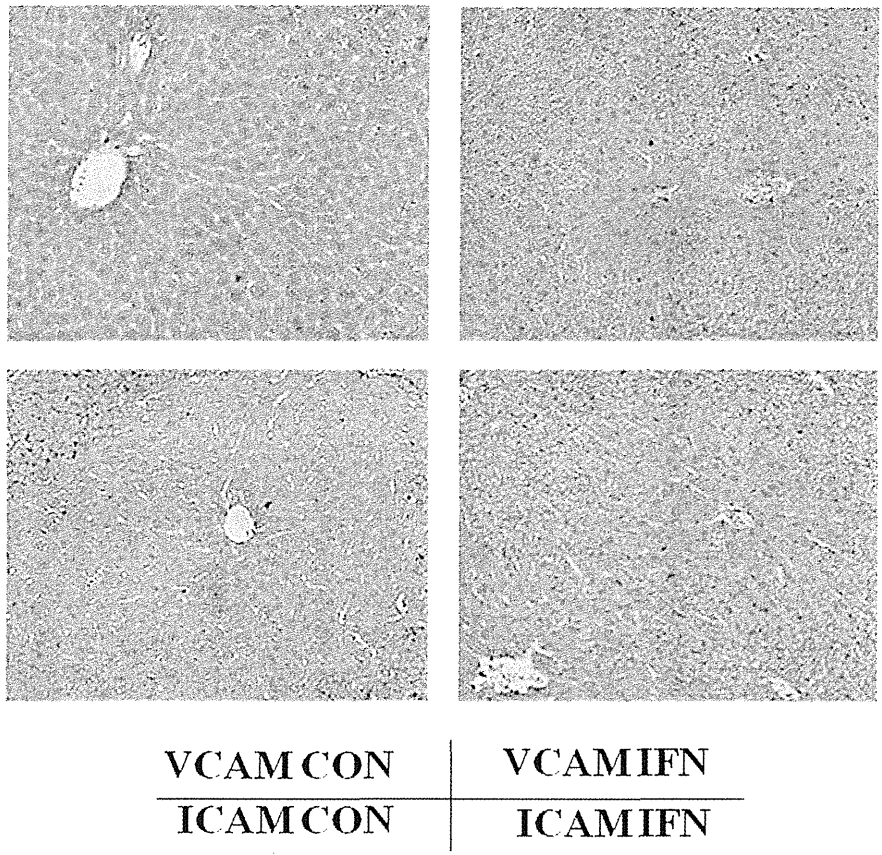

FIG. 5. Detection of VCAM and ICAM proteins in liver tissue by immunohistochemical staining (×400). There was no difference compared with the control group. Positive expression of VCAM and ICAM was almost not found at liver.

of tumor cells represents one of several important properties that are necessary for the formation of metastasis [23]. The cell invasion kit is created in an effort to accelerate the screening process for compounds that influence cell migration through extracellular matrices, which is a fundamental function of cellular processes such as angiogenesis, embryonic development, immune response, and metastasis of cancer cells [14].

On the other hand, angiogenesis is essential for cancers to metastasize. We have demonstrated that PEG-IFN-α2b inhibits the mRNA expression levels of VEGF in mouse HCC cells. These MH134 cells have been shown to produce endogenous VEGF [24]. IFN has been shown to down-regulate the expressions of the major stimulatory molecules, such as bFGF, VEGF, interlukin-8, MMP-2, and MMP-9, and to inhibit angiogenesis in most malignant tumors [25]. Continuous contact with exposure to 1000 IU/mL PEG-IFN-α2b induces strong antitumor effects in HCC cells [13]. The concentrations of PEG-IFN-α2b used in this study are almost identical to those of another study showing the inhibitory effects of 1000–3000 IU/mL IFNα on the VEGF mRNA expression in the human HCC cell line [25]. Tumor stromal cells might also provide angiogenic signaling, such as MMPs, which interact with tumor cells to stimulate angiogenesis [26]. In response to angiogenic signaling, tumor cells secrete a group of pro-angiogenetic polypeptides, known as angiogenic factors, which trigger the formation of neovasculature from the host vessels. VEGF is one of the first isolated angiogenic peptides and the most studied angiogenic factor so far. It has a specific mitogenic effect on endothelial cells, and it also increases vascular permeability and promotes extravasation of proteins from tumor vessels, leading to the formation of a fibrin matrix that supports the growth of endothelial cells and allows invasion stromal cells into the developing tumor [27]. The effects of VEGF are mediated *via* its receptors, VEGF-1 (Flt-1) and VEGF-2 (KDR), in endothelial cells [28]. VEGF appears to play a significant role in the early stage of hepatocarcinogenesis. Its expression increases gradually from low-grade dysplastic nodules to high-grade dysplastic nodules to early HCC [29]. FGF is a family of heparin-binding growth factor that includes at least 22 structurally related members, of which bFGF is the best known member. Tumor cell-derived bFGF that acts as a paracrine endothelial mitogen, and endothelial cells themselves, produce and release bFGF, which acts in an autocrine fashion

independently. The bFGF appears to act synergistically with VEGF in the induction of angiogenesis [30, 31]. However, in the current study, the mRNA expression of bFGF dose not change in several different dosages (data not shown). In fact, there is no report demonstrating that IFN inhibits bFGF expression.

The intratumor micro vessel density (MVD) in the IFN group is lower than in the control group, possibly resulting from the inhibition of the VEGF expression by IFN. The intratumor MVD is a direct reflection of tumor angiogenesis. It can be visualized by immunohistochemical staining with antibodies to anti-CD34 and α-smooth muscle actin [32]. MVD levels have a close relationship with intrahepatic recurrence, disease-free survival, and could be a predictive marker for disease-free survival [33].

Cell adhesion is also an important process of metastasis, and several adhesion factors, E-cadherin, vascular cell adhesion molecule-1 (VCAM), intercellular adhesion molecule-1 (ICAM), integrin family are related with cell adhesion. The expression level of E-cadherin invasively correlates with HCC histologic grade and prognosis. E-cadherin underexpression might have some contribution to the early recurrence of HCC [34]. It has been reported that focal adhesion kinase and E-cadherin appear to be significantly up-regulated after exposure to retinoic acid with IFN on SCC [35]. However, it is not shown in the primary tumor but in metastatic tumor in this study. ICAM and VCAM are important for tumor cells first adhesion to the endothelium as 'soil' factor and, therefore, recurrence or metastasis [36]. Serum concentration of intercellular adhesion molecule-1 (sICAM-1) in patients with HCC is a marker for disease progression and prognosis. Higher sICAM levels are more frequently observed in the patients with multiple lesions and intrahepatic metastasis, and have a poor prognosis. Detecting sICAM-1 is of important value in predicting tumor recurrence after surgery [37]. However, ICAM and VCAM are not significantly expressed in the liver as 'soil' factor in this study. Additional experiments might be required regarding other cell adhesion molecules, such as MMP, Sialyl-Lewis X, and integrin.

In conclusion, IFN in itself has remarkable antimetastatic effects. These findings suggest a mechanism by which IFN inhibits angiogenesis and cell invasion in HCC.

ACKNOWLEDGMENTS

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ORIGINAL ARTICLE

Expression of hypoxia-inducible factor-1 alpha (HIF-1α) in patients with the gallbladder carcinoma

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Abstract
Background Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that plays a central role in biologic processes under hypoxic conditions, especially concerning tumor angiogenesis. Vascular endothelial growth factor (VEGF) is a potent proangiogenic agent and a multifunctional angiogenic cytokine in many malignant tumors.
Methods This study was conducted to clarify the role of HIF-1 expression in gallbladder carcinoma. Thirty-one patients with gallbladder carcinoma underwent surgical treatment between 1994 and 2003 at the Department of Surgery, University of Tokushima, Japan. Both HIF-1α and VEGF were evaluated by immunohistochemistry, and correlations between the expression of these factors and clinicopathological features including prognosis were analyzed.
Results Among 31 specimens from patients with gallbladder carcinoma, 22 (70%) and 9 (30%) were positive for HIF-1α and VEGF expression, respectively. Expression of HIF-1α was significantly correlated with stage, tumor curability, lymph node metastasis, venous invasion, hepatic infiltration, and lymphatic invasion ($P < 0.05$). The survival rate for patients with HIF-1α positive staining was significantly lower than that for patients with HIF-1α negative staining. However, VEGF overexpression did not correlate with clinical outcomes. We demonstrated that HIF-1α expression was associated with a malignant behavior risk category in gallbladder cancer.
Conclusion Expression of HIF-1α was correlated with the poor prognostic indicators, such as lymph node metastasis and venous invasion. Therefore, HIF-1α could serve as an auxiliary parameter for predicting malignant behavior for gallbladder carcinomas.

Keywords Hypoxia-inducible factor-1 alpha · Vascular endothelial growth factor · Gallbladder carcinoma · Immunohistochemistry

Abbreviations

HIF-1	Hypoxia-inducible factor-1
VEGF	Vascular endothelial growth factor
GBC	Gallbladder carcinoma

Introduction

Gallbladder carcinoma (GBC) is the fifth most common malignancy of the digestive tract in Japan. This carcinoma is an aggressive tumor with a poor prognosis because of its inherent biology and often advanced stage at diagnosis [1, 2], despite the recent advances in diagnostic modalities [3, 4]. Curative surgical approaches remain the principal treatment associated with improvement in 5-year survival rates, but prognosis is closely correlated with the extent of tumor invasion and lymph node metastasis, suggesting survival is stage dependent (if carcinoma remains within the muscle layer and with no lymph node metastasis, the overall 5-year survival rate is about 90%) [5, 6]. Hypoxia-inducible factor-1 (HIF-1)—which consists of alpha (α)

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and beta (β) subunit, both members of the helix–loop–helix family of transcription factors [7, 8]—is a master transcriptional regulator of multiple gene expression in critical pathways involved in angiogenesis, tumor growth, and metastasis. In particular, the alpha subunit of HIF-1 is predominantly induced by hypoxic conditions and mediates a series of transcriptional responses. HIF-1α is both strongly induced and stabilized under hypoxic conditions, which can be translocated from the cytoplasm into the nucleus where its target genes promote cell proliferation, viability, angiogenesis, and metabolic adaptation to hypoxia. Induction of HIF-1α expression appears to be a critical step in the hypoxic response. It occurs via increased messenger ribonucleic acid (mRNA) expression, nuclear localization, and augmented activity of transcriptional activation domains. Nuclear accumulation of this protein can be detected immunohistochemically [9–11]. Vascular endothelial growth factor (VEGF) is known to be a potent angiogenic mitogen that plays an important role in tumor angiogenesis [12] and a multifunctional cytokine that stimulates angiogenesis and increases microvascular permeability through binding to specific receptors expressed on vascular endothelial cells. Although VEGF is produced by several tumors and hypoxic tissue, its receptors are expressed primarily through endothelial cells [13, 14]. The aim of this study was to investigate the clinicopathological role, including prognosis, of HIF-1α expression and its correlation with VEGF expression in GBC.

Materials and methods

Patients

This study included 31 patients with GBC who underwent surgical treatment between 1994 and 2003 at the Department of Surgery, Institute of Health Biosciences, University of Tokushima Graduate School, Japan. The pathological examination of surgical specimens was undertaken using hematoxylin- and eosin-stained tissue preparations. This study was authorized in advanced by the Institutional Review Board of the University of Tokushima Graduate School of Medicine, and all patients provided written informed consent.

Immunohistochemical staining

Four micrometer thick sections were cut from archival formalin-fixed paraffin-embedded tissue blocks. The samples were deparaffinized and dehydrated using a graded series of ethanol solutions. Endogenous peroxidase activity was halted through the administration of 0.3% hydrogen peroxidase and methanol for 20 min. After having been rinsed in phosphate-buffered saline (PBS), the tissue sections were processed in a 0.01 M citrate buffer (pH 6.0) inside a heat-resistant plastic container. Sections were then irradiated in a domestic microwave oven for 20 min. After microwave irradiation, the slides were allowed to cool at room temperature. The sections required a primary mouse monoclonal antibody against HIF-1α (H1alpha67) NB100-105 (Novus Biological) and a rabbit polyclonal antibody against VEGF (A-20): sc-152 overnight at 4°C. Both the mouse monoclonal antibody HIF-1α diluted at 1:500 and the rabbit polyclonal antibody VEGF diluted at 1:100 were used. After overnight rinsing, the sections were incubated using Daco REAL^{TM} Envision^{TM}/HRP, Rabbit/Mouse (ENV) for 45 min followed by three washes in PBS. After washing in PBS, peroxidase labeling was developed by incubating the section in 3.3′-diaminobenzidine tetrahydrochloride (DAB) for 5 min. Finally, nuclear counterstaining was done using Mayer's hematoxylin solution. All cell counts were performed using a Nikon digital camera DXM1200F photomicroscope at a magnification of $\times$200 ($\times$20 objectives and $\times$10 eyepiece). The area counted in each section was randomly selected from representative tumor field. For each section, 8 areas were assessed; the counts were expressed as the mean percentage of positive tumor cells out of the total number of cells and high power fields.

Assessment of HIF-1α and VEGF

A positive value was recorded if there was nuclear staining in >10% of the tumor cells; concomitant cytoplasm staining was not counted because HIF-1α protein in the nucleus determined the functional activity of the HIF-1α complex [15, 16]. Clinicopathological variables including postoperative survival rates were compared between the positive and negative expression groups. The average was used to define two groups of low-stain and high-stain VEGF reactivity. VEGF high expression was determined by counting the number of tumor cells with nuclei staining. If 25% or more of the cells were positive, it was regarded as VEGF high expression [17, 18].

Statistics

All statistical analysis was calculated through Stat View statistical software. (Stat View 5.0, SAS Institute, Cary, NC, USA). The contingency table-raw data was used to analyze correlations between the HIF-1α, VEGF, and relevant clinicopathological features. Survival curves were plotted using the Kaplan–Meier method and were compared using the log-rank test. Statistical significance was defined as $P < 0.05$.

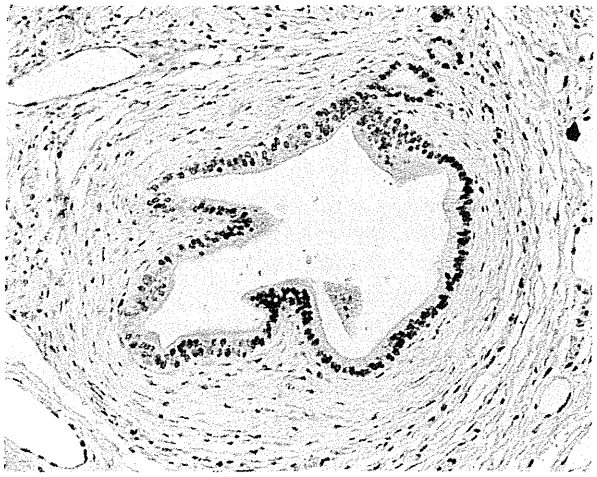

Fig. 1 Photomicrograph (×200) showing hypoxia-inducible factor-1 alpha (HIF-1α) immunohistochemical staining in gallbladder carcinoma specimen. HIF-1α-positive staining

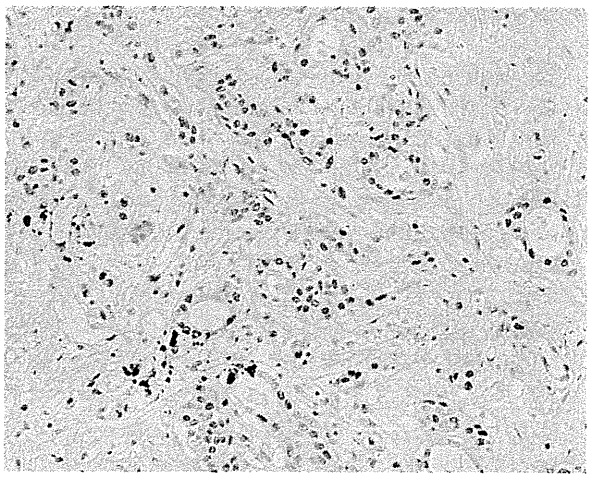

Fig. 2 Photomicrograph (×200) showing hypoxia-inducible factor-1 alpha (HIF-1α) immunohistochemical staining in gallbladder carcinoma specimen. High vascular endothelial growth factor (VEGF) staining

Results

HIF-1α and VEGF expression

The expression patterns of HIF-1α in the tumor cells were mixed nuclear/cytoplasm staining. This was not seen in normal gallbladder cells, but expression of HIF-1α in GBC tissue was significantly higher than that in the normal gallbladder tissue. HIF-1α expression through nuclear staining of positive cells was predominant at the invading tumor margin and at the periphery of necrotic region within tumors (Fig 1). The positive HIF-1α expression in cancer cells was present in 22 (70%) of 31 cases. Nine of 31 (30%) cases were then classified as negative staining for HIF-1α expression.

The high staining of VEGF was located in the cytoplasm of GBC cells (Fig 2). The high staining for VEGF was seen in 9 (29%) of the 31 cases. Twenty-two (71%) of the 31 cases were then classified as low staining for VEGF.

Correlations with clinicopathological characteristics

This study included 14 men and 17 women ranging from 45 to 85 years with a mean age of 65 years (Table 1). Expression of HIF-1α was significantly correlated with stage, curability, lymph node metastasis, venous invasion, and hepatic infiltration ($P < 0.05$). However, other clinicopathological parameters were not associated with HIF-1α expression (Table 2).

Relationship between VEGF expression and clinicopathological features is summarized in Table 3. VEGF expression was significantly correlated with lymph node metastasis, lymphatic invasion, serosal invasion and bile duct infiltration ($P < 0.05$). However, there was no relationship between VEGF expression and the other parameters, including age, gender, stage, differentiation, curability, venous invasion, hepatic infiltration, and bile duct infiltration. This study involved an investigation of the correlation between HIF-1α and VEGF protein. Against our expectations, there was no correlation between HIF-1α and VEGF expression.

Table 1 Background of patients with gallbladder carcinoma

Variables	Data
No. of patients	31
Age (years)	
Median	70
Range	45–85
Gender	
Male/female	13/18
Tumor size (cm)	1.2–6.5
Surgical stage	
1, 2	13 (42%)
3, 4	18 (58%)
Curability	
R0, 1	18 (58%)
R2	13 (42%)
N-stage (*n*)	
Negative	11 (36%)
Positive	20 (64%)
Lymph node metastasis (*n*)	20 (64%)
Hepatic infiltration (*n*)	15 (48%)
Lymphatic invasion (*n*)	19 (62%)
Venous invasion (*n*)	11 (35%)

Survival analysis

We examined the postoperative survival of the 31 patients who underwent curative surgery according to HIF-1α and VEGF expression. Survival curves according to positive or negative HIF-1α staining are shown in Fig 3. Respectively, survival rates for patients with HIF-1α-positive staining were significantly lower than for patients with HIF-1α-negative staining (overall survival $P = 0.02$) [95% confidence interval (CI) 1.125–6.885]. The 5-year overall survival rate was 23.8% in the HIF-1α-positive group. The cumulative overall survival rates between high and low VEGF expression groups were determined (Fig 4), and the prognosis for VEGF overexpression was not significantly lower (overall survival $P = 0.6$) (95% CI 0.4815–3.304). In order to search for independent prognostic factors, we carried out univariate and multivariate Cox regression analysis. In univariate analysis, the expression of stage, curability, lymph node metastasis, differentiation, lymphatic invasion, venous invasion, and HIF-1α all had significant effects. Variables that appeared significant in the univariate analysis were entered into the multivariate analysis. Curability, lymph node metastasis, and venous invasion, such as HIF-1α-related factor, were found to be prognostic factors, though HIF-1α was not an independent prognostic factor. (Table 4).

Table 2 Relationship between expressions of hypoxia-inducible factor-1 alpha (HIF-1α) with clinicopathological data in gallbladder carcinoma

Clinicopathological features	HIF-1 (neg) $n = 9$	HIF-1 (pos) $n = 22$	P value
Age	69 ± 9	66 ± 11	NS
Gender male/female	5/4	13/9	NS
Stage 1, 2/3, 4	7/2	6/16	0.009
Curability R0, 1/R2	8/1	10/12	0.01
Differentiation pap & well/other	8/1	15/7	NS
Venus invasion (−)/(+)	9/0	11/11	0.008
Hepatic infiltration (−)/(+)	7/2	9/13	0.049
Lymph node metastasis (−)/(+)	6/3	5/17	0.02
Lymphatic invasion (−)/(+)	6/3	6/16	0.04
Serosal invasion (−)/(+)	7/2	10/12	NS
Bile duct infiltration (−)/(+)	8/1	13/9	NS
VEGF low/high	5/4	17/5	NS

VEGF vascular endothelial growth factor, *NS* not significant

Table 3 Relationship between expressions of vascular endothelial growth factor (VEGF) with clinicopathological data in gallbladder carcinoma

Clinicopathological features	VEGF (low) $n = 22$	VEGF (high) $n = 9$	P value
Age	70 ± 6	65 ± 9	NS
Gender male/female	13/9	5/4	NS
Stage 1, 2/3, 4	8/14	5/4	NS
Curability R0, 1/R2	11/11	7/2	NS
Differentiation pap & well/other	17/5	6/3	NS
Venus invasion (−)/(+)	13/9	7/2	NS
Hepatic infiltration (−)/(+)	10/12	6/3	NS
Lymph node metastasis (−)/(+)	5/17	6/3	0.02
Lymphatic invasion (−)/(+)	6/16	6/3	0.04
Serosal invasion (−)/(+)	9/13	8/1	0.01
Bile duct infiltration (−)/(+)	13/9	1/8	0.01
HIF-1 neg/pos	5/17	4/5	NS

HIF-1 hypoxia-inducible factor-1, *NS* not significant

Discussion

In this study, significant correlation was found between HIF-1α expression and associated clinicopathological characteristics and prognosis. Hypoxic conditions in rapidly growing tumors allow malignant cells to become hypoxic vascular solid tumors, which are aggressive and metastatic [16, 18]. The presence of HIF-1 controls the expression of gene products that stimulate angiogenesis, such as VEGF, and promote adaptation to hypoxia, such as glycolytic enzymes, thus providing a molecular basis for involvement of HIF-1 in tumor growth and angiogenesis [19, 20]. Immunohistochemical analysis of human tumor biopsies indicate that HIF-1α is overexpressed in common cancers and that the level of expression is correlated with tumor grade and angiogenesis. In addition to intramural hypoxia, genetic alterations in tumor suppressor genes and oncogenesis induce HIF-1 activity [21–23]. On the other hand, most solid tumors developed regions of low oxygen tension because of an imbalance in oxygen supply and consumption. Hypoxia in the tumor microenvironment is

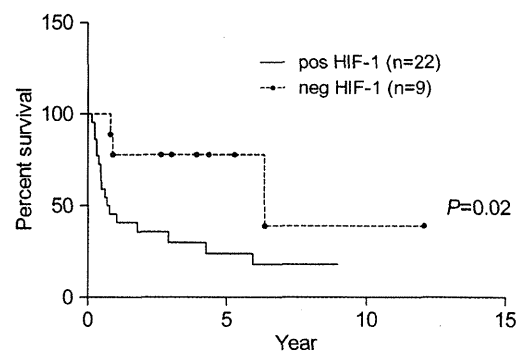

Fig. 3 Cumulative overall survival rates of two populations, with and without hypoxia-inducible factor-1 alpha (HIF-1α) overexpression, was determined using the Kaplan–Meier method. Differences in survival rates were statistically significant ($P = 0.02$)

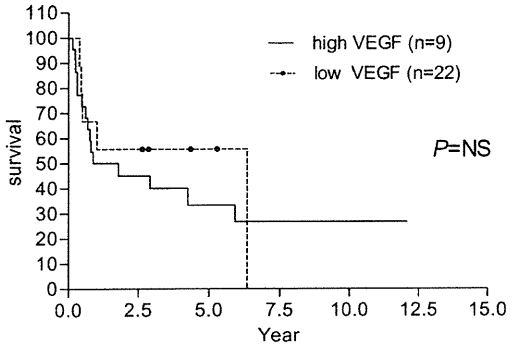

Fig. 4 Overall survival curve of patients with different expressions of vascular endothelial growth factor (VEGF)

Table 4 Univariate and multivariate analyses of prognostic factors in gallbladder carcinoma

Independent factors		Univariate analysis *P* value	Hazard ratio (HR)	Multivariate analysis *P* value
Stage	1, 2/3, 4	<0.05	8.09	0.110
Curability	R0, 1/R2	<0.05	29.41	0.001
Lymph node metastasis	(−)/(+)	<0.05	33.33	0.008
Differentiation	Pap & well/other	<0.05	2.13	0.179
Lymphatic invasion	(−)/(+)	<0.05	1.86	0.464
Venus invasion	(−)/(+)	<0.05	4.38	0.008
HIF-1	(−)/(+)	<0.05	1.14	0.878

HIF-1 hypoxia-inducible factor-1, *VEGF* vascular endothelial growth factor

sufficient to activate hypoxia-inducible-factor-dependent gene expression [24, 25]. The transcriptional factor HIF-1α plays an important role in angiogenesis by up-regulating hypoxia-induced genes, including VEGF [26, 27]. Overexpression of HIF-1α is associated with poor prognosis in patients with bladder, cervical, gastrointestinal stromal tumor, brain, and breast cancer. HIF-1α expression was associated with deeper invasion and poorer prognosis. In other malignant tumors, Mizokami et al. [25] reported that HIF-1α expression was related to angiogenesis in carcinomas of the gastric intestinal tract. In human gastric cancer models, inhibition of HIF-1α function is associated with inhibition of gastric tumor growth and angiogenesis [25, 28]. However, tumor growth rate may not always be associated with hypoxic conditions, and HIF-1α expression may be influenced by factors other than hypoxia. A major role for HIF-1α in determining gene expression, tumor angiogenesis, and tumor growth has been demonstrated in these studies [15, 23].

Angiogenesis is essential for growth, invasion, and metastasis of a tumor. The mechanism of angiogenesis has been shown to involve the release of substances from growing tumors that stimulate outgrowth of blood vessels from the host vasculature [22, 29]. Recently, studies have focused on the main outcome of angiogenesis, tumor vascularity. Tumor vascularity can be assessed by staining the blood vessel endothelia in the tissue and expressed through microvessel density.

VEGF has a strong selective mitogenetic effect on endothelial cells. It is also known as the vascular permeability factor due to its enhancement of vascular permeability. In regular cases, VEGF expression can be seen in ischemic hypoxic conditions. VEGF in malignant tumors shows remarkable overexpression during angiogenesis in tumor progressions. On the other hand, VEGF is also a major positive angiogenic factor and was seen to overexpress in various malignant tumors [17, 30]. We observed the clinicopathological role of VEGF expression and the correlation between overall survival rates related to VEGF expressions on GBCs. However, VEGF expression is significantly associated with lymph node metastasis, lymphatic invasion, and serosal invasion in GBC. VEGF can simulate the lymphatic endothelial cell as it does the vascular endothelial cell, which implicates VEGF in pathogenesis of GBC lymphatic metastasis.

In this study, HIF-1α expression was found to be associated with a malignant behavior category, including postoperative prognosis. Furthermore, HIF-1α expression was correlated with venous invasion, hepatic infiltration, and lymph node metastasis. In multivariate analysis, HIF-1α was not an independent prognostic factor; however, HIF-1α was correlated with some independent prognostic factors, which were curability, lymph node metastasis, and venous invasion. These findings suggest that HIF-1α may play an important role in tumor growth and progression of GBC. Regarding VEGF, we could not demonstrate the correlation between HIF-1α and VEGF expression in GBC. In addition, contrary to previous reports, high VEGF expression was negatively correlated with lymph node metastasis and serosal invasion. This may be due to small sample size in our study. In addition, this result may explain that there was no correlation between VEGF and HIF-1α expression.

In conclusion, this study was a comprehensive analysis of HIF-1α expression in GBC, and the results suggest that HIF-1α may have an important role in predicting prognosis for patients with GBC and indicate that identification of high-risk patients based on HIF-1α expression will improve treatment strategies and help improve patient outcomes for this lethal disease. HIF-1α expression was correlated with poor prognostic indicators, such as lymph node metastasis and venous invasion. Therefore, HIF-1α could serve as an

auxiliary parameter for predicting malignant behavior, and it is an independent prognostic factor for GBC. For a future perspective, biological agents targeting HIF-1α might be more effective at treating the earlier stages of GBC.

Conflict of interest statement The author has no conflict of interest.

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Serum evaluation of soluble interferon-α/β receptor and high-sensitivity C-reactive protein for diagnosis of the patients with gastrointestinal and hepatobiliary-pancreatic cancer

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ABSTRACT

Serum soluble interferon-α/β receptor (sIFN-α/βR) and high-sensitivity C-reactive protein (hs-CRP) levels were evaluated in the patients with gastrointestinal and hepatobiliary-pancreatic cancer. We compared the sensitivity and specificity of serum sIFN-α/βR with that of serum hs-CRP and evaluated the two diagnostic parameters in combination. Serum sIFN-α/βR levels were measured in 92 patients and 25 healthy individuals by enzyme-linked immunosorbent assay. The diagnoses were 37 cases of hepatocellular carcinoma, 17 cases of pancreatic cancer, 15 cases of colon cancer, 13 cases of biliary tract cancer, and 10 cases of gastric cancer. Serum levels of sIFN-α/βR and hs-CRP were significantly higher in the patients than in healthy individuals ($p < 0.05$). The optimal cut-off values of sIFN-α/βR and hs-CRP were 3600 pg/ml and 0.5 μg/ml, respectively. The sensitivity and specificity for these thresholds were 94.6% and 88.0%, whereas positive predictive and negative predictive values were 96.7% and 81.5%. These results suggest that a combination of serum sIFN-α/βR and hs-CRP thresholds may be more reliable diagnostic parameter for gastrointestinal and hepatobiliary-pancreatic cancer.

1. Introduction

Tumor markers are useful tests in the management and follow-up of patients with cancer [1–3], while tumor markers are generally not useful for diagnosis because of their low sensitivity and specificity. Numerous serum markers have been investigated in different tumors [4,5], however, the screening and diagnosis for patients with various malignant disorders has remained unsatisfactory. Reliable markers to identify patients with various malignant disorders at early stage would be beneficial.

Inflammation may play an important role in various neoplasms formation [6]. In fact, various kinds of inflammatory cells, cytokines, and chemokines are involved in carcinogenesis. These inflammatory constituents may result in the initiation or promotion of neoplasm. Several studies suggest that inflammatory cytokines play a key role in the development of various malignant solid tumors [7–10]. Cytokines are a large family of low molecular weight proteins [7,11]. They act in a complex network and stimulate the mitotic division, migration, growth, differentiation or apoptosis of various cancer cells. Additionally, different studies discuss the role of prognostic cytokine markers such as IL-6, IL-8, IL-10, IL-12, IL-15, IFN-γ, TNF-α, TGF-β and GM-CSF [12–16].

Similarly, soluble interferon-α/β receptor (sIFN-α/βR) and high-sensitivity C-reactive protein (hs-CRP) known to be inflammatory cytokine have produced interesting results for various neoplasms. A few studies of these serum cytokine for various malignant disorders have shown higher serum levels in patients than in healthy individuals, however, those diagnostic relevance have not been adequately investigated.

In the present study, we investigated whether serum levels of sIFN-α/βR and hs-CRP in patients with gastrointestinal and hepatobiliary-pancreatic cancer are useful as a diagnostic parameter of these malignant disorders. The diagnostic value, sensitivity, specificity, predictive values and efficiency values were calculated for each parameter and their combination.

2. Materials and methods

2.1. Patients

Twenty-five normal individuals of both genders who were ages 20–69 years were included. Ninety-two cancer patients of both

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genders who were ages 19–87 years were asked to donate a blood sample for the current study. The distribution of diseases was as follows: 37 cases of hepatocellular carcinoma, 17 cases of pancreatic cancer, 15 cases of colon cancer, 13 cases of biliary tract cancer, 10 cases of gastric cancer (Table 1).

Blood samples were collected in citrate buffer containing Vacutainer™ tubes (Becton Dickinson, San Jose, CA) or in a Vacutainer tube without any anticoagulant, kept at room temperature for 2–4 h, and centrifuged at 1500*g* for 20 min. Serum fractions separated from the blood were stored frozen at −80 °C until analyzed. The current study was approved by the Ethics Committee of Tokushima University and informed consent was obtained from the patients before the blood samples were obtained.

2.2. Assessment of serum sIFN-α/βR and hs-CRP

Serum fractions separated from the blood samples were stored at −20 °C until use. Concentrations of sIFN-α/βR in serum samples were measured with sIFN-α/βR ELISA kit (Otsuka Pharmaceutical Ltd., Tokyo, Japan) as described previously [17].

Concentrations of hs-CRP in serum samples were measured with ELISA kit (Otsuka Pharmaceutical Ltd., Tokyo, Japan). Briefly, A 96-well ELISA plate was coated with goat polyclonal anti-human CRP antibody (0805-5; NIPPON BIOTEST LABO., Tokyo, Japan). Serially 3-fold diluted recombinant human CRP (0.14–100 ng/ml) and samples were incubated at room temperature for 2 h in each well. Rabbit anti-recombinant human CRP antiserum diluted 50,000 times was applied to the plate. Horseradish peroxidase-labeled polyclonal goat anti-rabbit antibody and substrate system were used to develop the reaction. Absorbance at 492 nm was measured with a 96-well plate reader (Multiskan JX; Labsystems, Vienna, VA) and a standard curve was made. The CRP concentration of the samples was calculated from the standard curve. The detection limit of this ELISA was 0.07 μg/ml and there was no cross-reaction with other cytokines. Experiments were carried out in triplicate in duplicate plates.

2.3. Statistical analysis

Data are expressed as the mean ± SD. Analysis of variance (ANOVA) was carried out to determine sIFN-α/βR and hs-CRP levels of significance with $p < 0.05$ considered statistically significant. Linear regression lines for the relationship between were calculated using Spearman's correlation coefficient. Receiver operator characteristic (ROC) curve analysis was used to calculate the area under the curve (AUC) as a factor of discrimination between the different pair-wise comparisons and to determine cut-off values of sIFN-α/βR and hs-CRP levels for evaluation of the diagnostic specificity and sensitivity and positive and negative predictive values (PPV and NPV) [18]. Calculations were made with the commercially available software JMP version 6.0 (SAS Institute, Cary, NC, USA). A p value less than 0.05 was considered statistically significant.

Table 1
Characteristics of subjects.

	Healthy individuals	Patients
Number of subjects	25	92
(Gastric cancer)		(10)
(Colon cancer)		(15)
(Hepatocellular carcinoma)		(37)
(Pancreatic cancer)		(17)
(Biliary tract cancer)		(13)
Sex (M/F)	14/11	62/30
Age (years old)		
Median	34	64
Range	20–69	19–87
Virus		
No virus	25	60
HBV	0	9
HCV	0	23
sIFN-α/βR (pg/ml)		
<3600	23	18
>>3600	2	74
hs-CRP (μg/ml)		
<0.5	22	21
>>0.5	3	71

3. Results

3.1. Serum levels of sIFN-α/βR and hs-CRP

Serum levels of sIFN-α/βR and hs-CRP in 92 patients with various malignant disorders and 25 healthy control subjects were assessed. As shown in Fig. 1, sIFN-α/βR and hs-CRP levels of serum samples widely varied from 1401 to 12602 pg/ml and 0.09 to 10.0 μg/ml. The median values of serum sIFN-α/βR were 2992 pg/ml (range 1401–4800) in healthy individuals, 3345 pg/ml (range 2328–5552) in gastric cancer, 4658 pg/ml (range 2933–12602) in colon cancer, 5754 pg/ml (range 2674–9216) in hepatocellular carcinoma, 4258 pg/ml (range 2805–7317) in pancreatic cancer, and 4420 pg/ml (range 2674–7926) in biliary tract cancer. The median values of serum hs-CRP were 0.25 μg/ml (range 0.09–3.97) in healthy individuals, 1.59 pg/ml (range 0.12–2.83) in gastric cancer, 1.91 μg/ml (range 0.21–9.61) in colon cancer, 2.85 μg/ml (range 0.11–10.0) in hepatocellular carcinoma, 2.53 μg/ml (range 0.11–

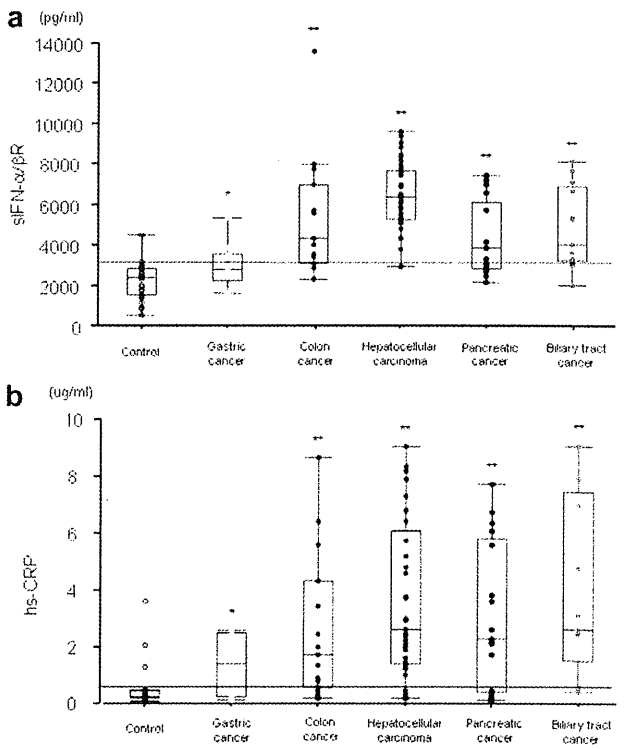

Fig. 1. Serum sIFN-α/βR and hs-CRP levels in healthy individuals and patients with various malignant disorders. Data are plotted as "box plots," in which the horizontal line represents the median value, upper and lower limits of the box correspond to 75 and 25 percentiles, respectively, and error bars indicate 10 and 90 percentiles. $^{*}p < 0.05$, $^{**}p < 0.001$ in comparison with healthy individuals. The straight line parallel to the x-axis represents the chosen cut-off point of (a) 3600 pg/ml in serum sIFN-α/βR and (b) 0.5 μg/ml in serum hs-CRP.

8.57) in pancreatic cancer, and 2.85 μg/ml (range 0.41–10.0) in biliary tract cancer. Serum levels of sIFN-α/βR and hs-CRP in patients were significantly higher than those in healthy individuals with respect to various type of malignant disorders ($p < 0.05$).

To evaluate the possible correlation between the serum sIFN-α/βR or hs-CRP level and age, Spearman correlation rank coefficients (two-tailed) were used. As shown in Fig. 2, age was correlated with the sIFN-α/βR level ($r = 0.386$, $p < 0.001$) and hs-CRP level ($r = 0.369$, $p < 0.001$). There was no significant difference between the female and male group.

3.2. Diagnostic validity of serum sIFN-α/βR and hs-CRP

ROC curve analysis was carried out to determine the diagnostic sensitivity and specificity of serum sIFN-α/βR and hs-CRP. As shown in Fig. 3, the AUC were 0.929 for serum sIFN-α/βR and 0.871 for serum hs-CRP. Optimal cut-off values, 3600 pg/ml for sIFN-α/βR and 0.5 μg/ml for hs-CRP were obtained from the ROC curve analysis.

There are two patients with low serum sIFN-α/βR (less than 3600 pg/ml). Sensitivity, specificity, positive predictive value, negative predictive value and efficiency for sIFN-α/βR were 80.4%, 92.0%, 97.4%, 56.1% and 82.9%, respectively. There are three patients with low serum hs-CRP (less than 0.5 μg/ml). Sensitivity, specificity, positive predictive value, negative predictive value and efficiency for hs-CRP were 77.2%, 88.0%, 95.9%, 51.2% and 79.5%, respectively.

Threshold values of 3600 pg/ml for sIFN-α/βR and 0.5 μg/ml for hs-CRP were combined to discriminate between healthy individuals and patients with gastrointestinal and hepatobiliary-pancreatic cancer. As shown in Fig. 4, 21 of 25 health individuals had both low

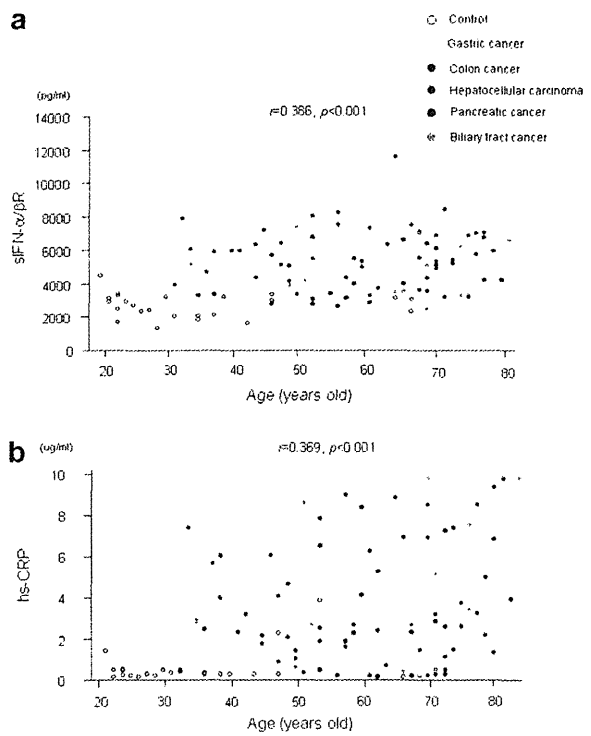

Fig. 2. Age-dependent profiles of serum sIFN-α/βR and hs-CRP levels. (a) Serum sIFN-α/βR and (b) serum hs-CRP levels of 25 healthy individuals and 97 patients with various malignant disorders. Spearman's correlation test was applied to evaluate the possible correlation between the serum sIFN-α/βR or the hs-CRP levels and age.

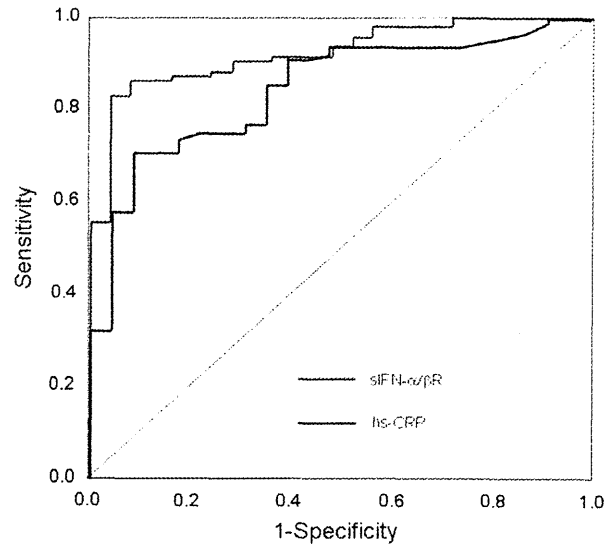

Fig. 3. Receiver operating characteristic (ROC) curves for serum soluble interferon-α/β receptor (AUC = 0.929) and hs-CRP (AUC = 0.871) in the patients with gastrointestinal and hepatobiliary-pancreatic cancer.

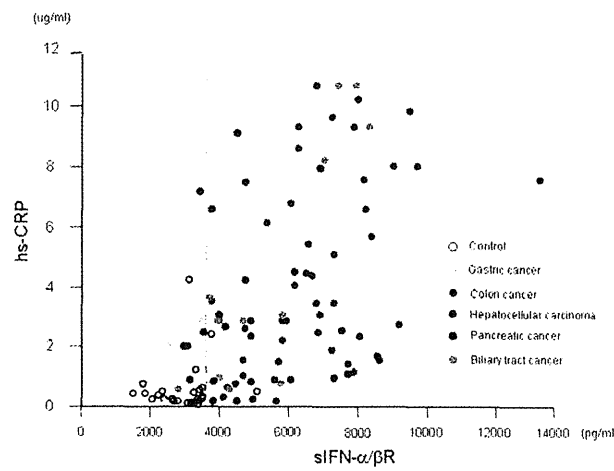

Fig. 4. Discriminatory capacity of serum levels of sIFN-α/βR or the hs-CRP with respect to diagnostic parameter of gastrointestinal and hepatobiliary-pancreatic cancer. Hepatocellular carcinoma: 37 cases, pancreatic cancer; 17 cases, colon cancer; 15 cases, biliary tract cancer; 13 cases, gastric cancer; 10 cases and healthy individuals: 25 cases. The straight line parallel to the *x*-axis represents the chosen cut-off point of 0.5 μg/ml in serum hs-CRP, parallel to the *y*-axis represents the chosen cut-off point of 3600 pg/ml in serum sIFN-α/βR.

sIFN-α/βR and low hs-CRP. Sensitivity, specificity, positive predictive value, negative predictive value and efficiency of combined sIFN-α/βR and hs-CRP thresholds were 94.6%, 88.0%, 96.7%, 81.5% and 93.2%, respectively (Table 2).

4. Discussion

Primary aim of the present study was to evaluate the serum sIFN-α/βR and hs-CRP levels and their diagnostic value in patients with gastrointestinal and hepatobiliary-pancreatic cancer. Many reports regarding the clinical significance of serum sIFN-α/βR or hs-CRP in disease states have been published. Elevated levels of serum sIFN-α/βR and hs-CRP predicts the presence of extensive

Table 2
Diagnostic relevance of serum sIFN-α/βR and hs-CRP for gastrointestinal and hepatobiliary-pancreatic cancer.

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Efficiency (%)	Cut-off point
sIFN-α/βR	80.4 (74/92)	92.0 (23/25)	97.4 (74/76)	56.1 (23/41)	82.9 (96/117)	>>3600 (pg/ml)
hs-CRP	77.2 (71/93)	88.0 (22/25)	95.9 (71/74)	51.2 (22/43)	79.5 (93/117)	>>0.5 (μg/ml)
Combined sIFN-α/βR and hs-CRP	94.6 (87/92)	88.0 (22/25)	96.7 (87/90)	81.5 (22/27)	93.2 (109/117)	

disease in acquired immunodeficiency syndrome (AIDS), chronic hepatitis C, cardiovascular diseases or various neoplasm [17,19–21]. However, as far as we know, there is no study which was conducted using these two inflammatory cytokine, sIFN-α/βR and hs-CRP to achieve a higher sensitivity, specificity, positive predictive value, negative predictive value and efficiency for diagnosis in the literature. The most striking feature of this study was the demonstration that combination of serum sIFN-α/βR and hs-CRP levels showed higher diagnostic value for gastrointestinal and hepatobiliary-pancreatic cancer.

Type I interferon (IFN) have various biological activities, including antiviral, anti-proliferative, immunomodulatory [22–24], and antiangiogenic effects [25,26], mediated by the type I IFN receptor. Type I IFN receptor has a multichain structure consisting of at least 2 subunits, IFNAR-1 and IFNAR-2 [27–30]. The IFNAR-2 subunit of 100 kDa is anchored on the outer surface of the cell membrane and mediates intracellular signal transduction. A soluble form of IFNAR-2 proteins of 40 kDa, designated sIFN-α/βR, is found in body fluids including peripheral blood and urine [31]. Of these receptor molecules, sIFN-α/βR molecules inhibited the activity of type I IFN as shown by biological assay with human WISH cells [29,31,32]. In addition, elevated serum levels of sIFN-α/βR were found in patients with AIDS [19], chronic hepatitis C [17] and various neoplasms [20].

On the one hand, C-reactive protein is well-known acute-phase indicator of inflammation in the body [33]. This marker indicates not only an acute-phase response but also a chronic low-level inflammation and is associated with the risk of cardiovascular diseases [21]. This indicator correlates well with the proinflammatory cytokines mentioned above [34]. The significance of serum elevation of CRP as an indicator of the malignant potential of the tumor and/or therefore the unfavorable outcome of the patients has been investigated in human gastrointestinal carcinomas [35–42], hepatocellular carcinoma [43], renal cell carcinoma [44], ovarian carcinoma [45], or myeloma [46]. It has also been reported that measurement of serum levels of CRP using a high-sensitivity assay can reveal subclinical inflammatory states which may reflect neoplasm formation as well as vascular inflammation [47–49].

Our results showed that serum levels of two inflammatory cytokine, sIFN-α/βR and hs-CRP for diagnostic parameter in gastrointestinal and hepatobiliary-pancreatic cancer patients. Both two parameters showed elevation in serum of cancer patients when compared to healthy individuals. Ikei et al. observed that chemotherapy for hepatocellular carcinoma changed the level of CRP and other inflammatory cytokines, whereas Tang et al. reported that pelvic radiotherapy did not change the level of CRP. Another study determined that the level of TNF-α and ceruloplasmin increased during radiotherapy for gynecologic cancer [50–52]. Since our study includes a number of patients who with chemotherapy and radiotherapy, those treatment may influence our results.

In the present study, we tried to improve the predictive power of diagnostic parameters for gastrointestinal and hepatobiliary-pancreatic cancer with the combination of serum sIFN-α/βR and hs-CRP. Serum sIFN-α/βR and hs-CRP levels showed a diagnostic value with sensitivity, specificity, positive predictive and negative predictive value showed 80.4%, 92.0%, 97.4% and 56.1% in serum sIFN-α/βR and 77.2%, 88.0%, 95.9% and 51.2% in serum hs-CRP, respectively. The high sensitivity, specificity, positive predictive and negative predictive value using combinations of sIFN-α/βR and hs-CRP were 94.6%, 88.0%, 96.7% and 81.5%. These results describe better diagnostic values than not only the results with sIFN-α/βR or hs-CRP alone but also previously reported assay for various malignant disorders [16,20,49,53–55].

Although no report regarding the relationship between the sIFN-α/βR and hs-CRP have been published, the elevated sIFN-α/βR level might be related with CRP production. While our results imply that sIFN-α/βR and CRP might be produced by tumor, the mechanism and pathological significance of sIFN-α/βR and hs-CRP remains unclear. In future, these points should be discussed more fully in the further investigation.

In conclusion, measurement of serum sIFN-α/βR or hs-CRP is a reliable diagnostic parameter for gastrointestinal and hepatobiliary-pancreatic cancer and furthermore, combining sIFN-α/βR and hs-CRP can be more valuable to improve detection of these malignant disorders. Many large-scale studies for combinations of these two cytokine is needed in this field and exciting new knowledge will ultimately emerge for its diagnostic values.

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Original Article

Role of dihydropyrimidine dehydrogenase and thymidylate synthase expression in immunohistochemistry of intrahepatic cholangiocarcinoma

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Aims: Dihydropyrimidine dehydrogenase (DPD) and thymidylate synthase (TS) are key enzymes in the metabolism of 5-fluorouracil and have been implicated as possible prognostic markers for cancer patients. However, the clinical roles of DPD and TS in intrahepatic cholangiocarcinoma (IHCC) have not been investigated. The aim of this study was to clarify the clinicopathological role of DPD and TS expressions in IHCC.

Methods: Twenty-nine patients who had undergone hepatic resection for IHCC were enrolled in this study. Expressions of DPD and TS in the resected IHCC specimens were examined using anti-DPD or anti-TS antibody. The patients were divided into positive and negative groups according to DPD/TS expressions: DPD-positive group ($n = 18$) and DPD-negative group ($n = 11$)/TS-positive group ($n = 14$) and TS-negative group ($n = 15$). Clinicopathological factors were compared between the two groups.

Results: The overall survival rate was significantly lower in the DPD-negative group than in the DPD-positive group (1-year 36.4% vs. 77.4%, 3-year 18.2% vs. 43.0%; $P < 0.05$). The disease-free survival rate in the DPD-negative group tended to be lower than that in the DPD-positive group. The overall survival rate or disease-free survival rate did not appear to be associated with the TS-expression status. The Ki-67 labeling index in the DPD-negative group was significantly higher than that in the DPD-positive group ($16.9 \pm 3.2\%$ vs. $13.2 \pm 3.3\%$; $P < 0.05$).

Conclusions: The negative DPD expression was significantly associated with the enhanced tumor cell proliferation and poorer prognosis in patients with IHCC. DPD expression is a potential prognostic indicator for IHCC.

Key words: intrahepatic cholangiocarcinoma, Ki-67 index, prognosis, recurrent pattern

INTRODUCTION

INTRAHEPATIC CHOLANGIOCARCINOMA (IHCC) accounts for five percent of primary malignant liver tumors, arising from biliary epithelium,[1] and well known to be one of the most malignant solid tumors found in the digestive organs.[1–5] This highly malignant carcinoma is associated with lymph node metastasis, intrahepatic metastasis, peritoneal dissemination, bile duct invasion, and vascular invasion.[2–5] Prognosis of IHCC is very poor with a 5-year survival rate ranging from 25% to 35%.[1–5] Therefore, it is important to elucidate tumor characteristics and prognostic factors after surgical resection for IHCC.

Dihydropyrimidine dehydrogenase (DPD) and thymidylate synthase (TS) act as key enzymes of pyrimidine cascade and 5-fluorouracil (5-FU) metabolism.[6–8] In this cascade, 5-FU is first catabolised by DPD. TS decreases the synthesis of deoxythymine monophosphate from deoxyuridine monophosphate, and exhibits antitumor effects.[8]

Expressions of DPD and TS are correlated with the antitumor effects of 5-FU and 5-FU based chemotherapy, such as tegafur-uracil, and S-1.[9–11] Recently, DPD and TS have been reported to play an important role in various kinds of cancers. Down regulation of DPD gene expressions may enhance the negative prognostic effect in colorectal tumors[12] and ovarian cancer.[13]

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Overall survival was significantly better in the TS negative patients than in the TS positive patients among resected colon cancer patients.[14] High expression of TS in tumors enhanced distant metastasis after surgery.[15]

We have previously reported that a low expression of DPD mRNA was a poor prognostic factor in hepatocellular carcinoma (HCC).[16] However, to the best of our knowledge, only one *in vitro* study examined the DPD and TS expressions in IHCC cell lines.[17]

This is the first report evaluating the association of the DPD and TS expressions with the clinicopathological variables in surgical patients with IHCC.

PATIENTS AND METHODS

Patients

TWENTY-NINE PATIENTS who had undergone surgical resection for IHCC at Tokushima University Hospital between 1992 and 2009 were included in this study. There were 20 men and 9 women, with a mean age of 66.9 years (range, 43–84 years). In 19 patients (65.5%), hepatic lobectomy was performed. Lymph node dissections of the hepatoduodenal ligament and along the common hepatic artery or more extended lymphadenectomies were performed in 14 patients (48.3%). Extrahepatic bile duct resections were performed on 11 patients (37.9%). Consequently, 22 patients (75.9%) had received R0 or R1 resections. None of the patients received prior chemotherapy or irradiation before surgical resection. Mean follow-up period was 29 months (range, 2–111 months). The clinical stages were defined according to the Classification of Primary Liver Cancer Study Group of Japan.[18]

The current study was authorized in advance by the Institutional Review Board of the University of Tokushima, and all patients provided written informed consent.

Immunohistochemistry

The expressions of DPD and TS in the resected IHCC specimens were evaluated with using immunohistochemistry as described previously.[19,20] Surgical specimens were fixed in 10% formaldehyde embedded in paraffin and cut into 4-μm thick sections. Sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. Deparaffinized sections were retrieved by microwaving for 20 min. Endogenous peroxidase activity was blocked by soaking the sections in 0.3% hydrogen peroxide in methanol for 30 min. After washing with PBS, sections were placed in normal goat serum (2% in PBS) for 30 min to reduce nonspecific staining. The sections were subsequently incubated with DPD antibody (rabbit polyclonal, dilution 1:200; Taiho Pharmaceutical, Tokushima) or TS antibody (rabbit polyclonal, dilution 1:200; Taiho Pharmaceutical), overnight at 4 °C in moist chambers. The sections were incubated with goat anti-mouse immunoglobulin for 20 min and then with horseradish peroxidase-conjugated streptavidin complex (Histofine SAB-PO Kit; Biogenex Laboratories, Tokyo). To visualize immunoreactivity, diaminobenzidine/H_2O_2 (1 mg/mL) in PBS was used as the substrate. The sections were counter stained with hematoxyline, dehydrated with ethanol, and treated with xylene.

Assessment of DPD and TS staining was expressed as the percentage of stained cells in the cytoplasms out of total number of tumor cells and devided into two groups as follows: <5%; negative expression, ≥5%; positive expression (Fig. 1).[19] The assessment of immunohistochemistry was conducted without knowledge of the results of other experiments.

Determination of the Ki-67 labeling index

The correlation between the Ki-67 labeling index and DPD or TS expression was investigated. Determination of the Ki-67 labeling index was previously reported.[16] Five hundred tumor cells were counted in each 4-μm thick section. The Ki-67 labeling index was defined as the number of Ki-67 positive nuclei divided by total number of cancer cells, and expressed as a percentage.

Statistics

All statistical analysis was calculated through Stat View statistical software (Stat View 5.0; SAS Institute, Cary, NC). Relationships between DPD or TS expression and the clinicopathological variables were analyzed with the χ^2 test and Mann–Whitney *U*-test. Survival curves were calculated using the Kaplan–Meier method and compared using the Wilcoxon test. All significant factors by univariate analysis were included in the Cox's proportional hazards model of multivariate analysis to identify independent factors influencing survival. Statistical significance was defined as $P < 0.05$.

RESULTS

Immunohistochemistry

IN DPD EXPRESSION, there were 18 (62.1%) positive and 11 (37.9%) negative cases. Regarding TS expression, there were 14 (48.3%) positive and 15 (51.7%) negative cases.

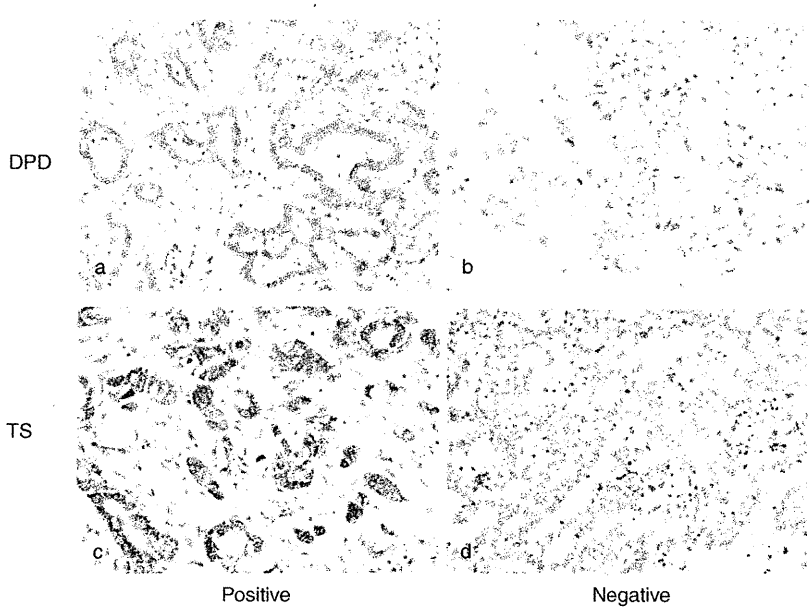

Figure 1 Dihydropyrimidine dehydrogenase (DPD) and thymidylate synthase (TS) expressions in intrahepatic cholangiocarcinoma (IHCC). The positive immunostaining of DPD or TS was recognized in cytoplasm of cancer cells (a) DPD-positive (b) DPD-negative (c) TS-positive (d) TS-negative.

Correlation between DPD/TS expressions and clinicopathological characteristics

No significant correlations were observed in any clinicopathological variables, such as staging, curability, vascular invasion, intrahepatic metastasis, and other tumor factors according to the expression levels of DPD or TS. However, in the DPD negative group, the tumor tended to be located more frequently in the hilar region (Table 1). In the TS positive group, the incidences of advanced clinical stage, non curative surgical resection, larger tumor size, vessels infiltration, and intrahepatic metastasis tended to be higher than in the TS negative group (Table 2).

Overall and disease-free survival according to DPD/TS expressions

Figure 2 shows overall and disease-free survival rates according to DPD/TS expressions. The overall survival rate was significantly lower in the DPD-negative group than in the DPD positive group (1-year 36.4% vs. 77.4%, 3-year 18.2% vs. 43.0%; $P < 0.05$) (Fig. 2A). However, there were no differences in the overall survival rate between the TS-negative and the TS-positive group (1-year 58.2% vs. 64.3%, 3-year 39.9% vs. 26.8%; Fig. 2B).

Similarly, the disease-free survival rate in the DPD-negative group tended to be lower than in the DPD-positive group (1-year 22.2% vs. 57.1%, 3-year 11.1% vs. 31.2%), although there was no statistical significance

Table 1 Clinicopathological characteristics according to dihydropyrimidine dehydrogenase (DPD) expression

Factors	DPD expression		*P*-value
	Positive ($n = 18$)	Negative ($n = 11$)	
Mean age (years)	68.3 ± 7.5	64.5 ± 13.7	0.448
Sex (Male/Female)	13/5	7/4	0.628
Virus ([-]/HBV/HCV/Combined)	9/4/4/1	10/1/0/0	0.140
Staging (I, II/III, IV)	5/13	3/8	0.976
Curability (R0, 1/2)	14/4	8/3	0.758
Location (hilar/peripheral)	4/14	5/6	0.190
Tumor diameter (<4 cm/≥4 cm)	9/9	5/6	0.812
Macroscopic type: T/T + I	9/9	4/7	0.474
Differentiation: Diff./Undiff.	7/11	4/7	0.892
LN metastasis: −/+	6/12	4/7	0.868
Vessels infiltration: −/+	7/11	6/5	0.412
Intrahepatic metastasis: −/+	12/6	9/2	0.376

Diff, differentiation; T, mass-forming type; T + I, mass-forming + periductal infiltrative type; Undiff, undifferentiation.

Table 2 Clinicopathological characteristics according to TS expression

Factors	TS expression		*P*-value
	Positive ($n = 14$)	Negative ($n = 15$)	
Age: Mean	68.3 ± 7.5	64.5 ± 13.7	0.448
Gender: Male/Female	10/4	10/5	0.782
Virus:(–)/HBV/HCV/ Combined	9/2/3/0	10/3/1/1	0.528
Staging: I, II/III, IV	2/12	6/9	0.122
Curability: R0, 1/2	9/5	13/2	0.159
Location: Hilar/ Peripheral	4/10	5/10	0.782
Tumor diameter: <4 cm/ ≥4 cm	5/9	9/6	0.191
Macroscopic type: T/T + I	5/9	8/7	0.340
Differentiation: Diff./ Undiff.	4/10	7/8	0.316
LN metastasis: –/+	9/5	10/5	0.893
Vessels infiltration: –/+	4/10	9/6	0.089
Intrahepatic metastasis: –/+	8/6	13/2	0.076

Diff, differentiation; T, mass-forming type; T + I, mass-forming + periductal infiltrative type; Undiff, undifferentiation.

(Fig. 2C). No significant difference in the disease-free survival rate was observed between the TS-negative and the TS-positive group (1-year 46.2% vs. 40.0%, 3-year 23.1% vs. 20.0%; Fig. 2D).

Univariate and multivariate analysis of prognostic factors

Table 3 shows the results of univariate and multivariate analysis of prognostic factors. Univariate analysis revealed that location, differentiation, macroscopic type, vessels infiltration and intrahepatic metastasis were not significant factors in terms of postoperative survival. In contrast, curability ($P = 0.0009$), tumor size ($P = 0.0066$), lymph nodes metastases ($P = 0.0127$), and negative expression of DPD ($P = 0.0498$) were found to be significant prognostic factors for survival after surgical resection. In multivariate analysis using the Cox's proportional hazard model, tumor size (≥4 cm) was found to be an only independent prognostic factor. Negative expression of DPD tended to be an independent prognostic factor, although there was no statistical significance ($P = 0.1293$).

Recurrent pattern

Table 4 shows the correlation between the recurrent patterns and the DPD expression status. The recurrence rate in the DPD-positive group was similar to that in the DPD-negative group. However, in the DPD-negative group, the incidence of recurrence in the liver was significantly higher ($P < 0.05$) and that of lymph node and remote organ tended to be higher compared to the DPD-positive group.

Ki-67 proliferating index

The Ki-67 labeling index in the DPD-negative group was significantly higher than the DPD-positive group (16.9 ± 3.2% vs.13.2 ± 3.3%) ($P < 0.05$). There was no difference in the Ki-67 labeling index between the TS-positive group and the TS-negative group (14.1 ± 3.8% vs.15.5 ± 3.5%).

DISCUSSION

THESE RESULTS SHOW that negative DPD expression was significantly associated with poorer prognosis, higher proliferation index, and a higher incidence of recurrence in the liver. However, TS expression was not related to patient prognosis after surgical resection.

Several other reports have documented that the expression of DPD is related to prognostic and clinicopathological factors.[12,13] In primary liver cancer, however, only a few reports are available on the role of DPD.[21,22] The DPD activity in HCC was lower than in non cancerous tissue and a gradual decrease in DPD activity was associated with liver damage.[21] We previously reported that low mRNA expression of DPD was a poor prognostic factor and significantly related to advanced clinical stage, undifferentiated histology, microscopic intrahepatic metastasis, and related to tumor proliferation in HCC.[16] Regarding biliary tract cancer, DPD concentration was higher in cancerous tissue than in noncancerous tissue, although the prognosis is not different.[23] Ajiki *et al.* reported DPD expression was not a prognostic factor in gallbladder cancer.[24]

In this study, the patients in DPD-negative group had a significantly poorer prognosis. The mechanism of such results is not fully understood at present. However, in pyrimidine cascade, negative expression of DPD leads the synthesis of both uracil and thymine. Increased synthesis of uracil and thymine may relate to the enhancement of pyrimidine nucleotide pools[25] and may cause the cell proliferation of IHCC. Johnston SJ *et al.* suggested that the down-regulation of DPD expression might create a favorable environment for tumor growth.[26] Further, it was suggested that low DPD expression was associated with an increase in the metastatic activity. DPD activity in highly malignant murine neu-

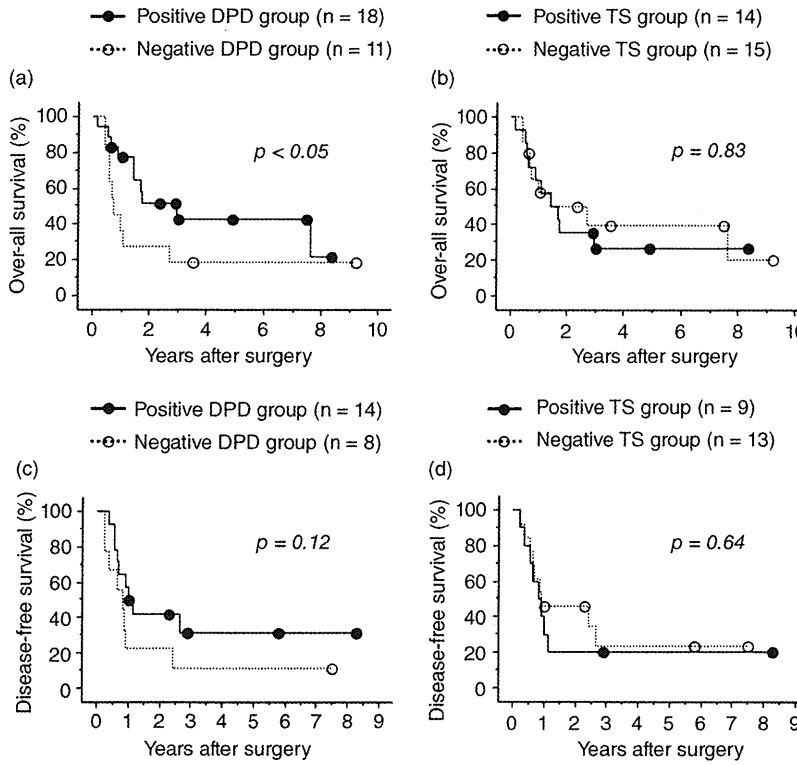

Figure 2 Overall survival rates in positive and negative expression of dihydropyrimidine dehydrogenase (DPD; a) and thymidylate synthase (TS; b). The survival rate in DPD-negative group was significantly lower than in the DPD-positive group. Disease-free survival rates in positive and negative expression of (c) DPD and (d) TS.

roblastoma cell line was lower than that in low malignant cell line.[27,28] Consistent with these reports, DPD-negative group had a significantly higher Ki-67 labeling index and higher incidence of recurrence in the liver than the DPD-positive group in our study.

Table 3 Univariate and Multivariate analysis of prognostic factors

	DP D positive ($n = 14$)	DPD negative ($n = 8$)	*P*-value
Recurrence	9	6	0.604
Recurrent pattern			
Liver ($n = 12$)	9	3	0.018
Lymph nodes ($n = 4$)	1	3	0.095
Peritoneum ($n = 1$)	0	1	0.205
Remote organ ($n = 2$)	0	2	0.063

Table 4 Correlation between DPD expression and recurrent site in patients with R0/1 resection ($n = 22$)

	Univariate *P*-value	Hazard ratio	Multivariate 95% C.I.	*P*-value
Curability: R2	0.0009	2.832	0.804–10.000	0.1052
Tumor Diameter: ≥4 cm	0.0066	4.413	1.427–7.705	0.0099
Lymph nodes metastasis: +	0.0127	2.921	0.851–10.030	0.0885
DPD expression: –	0.0498	2.132	0.801–5.672	0.1293

T + I, mass-forming + periductal infiltrative type.