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Table 4. Univariate and multivariate analyses of factors associated with post-operative multicentric recurrence in HCC patients (n=59)

Factor		Univariate analysis		Multivariate analysis	
		Risk ratio (95% C.I.)	*p* value	Risk ratio (95% C.I.)	*p* value
Age		1.0047 (0.9359-1.0823)	0.8985	-----	
Sex	Male	1			
	Female	1.0701 (0.5999-1.7781)	0.8038	-----	
Child-Pugh class*	A	1			
	B	0.0664 (0.0176-5.7947)	0.7029	-----	
Tumor size		0.9517 (0.6300-1.2943)	0.7801	-----	
Number of tumors	Single	1			
	Multiple	1.1331 (0.4469-2.1714)	0.7510	-----	
Differentiation**	Well-	1			
	Moderately/poorly	1.5198 (0.8959-2.8769)	0.1249	-----	
Growth pattern**	Expansive	1			
	Infiltrative	1.3486 (0.7124-2.2884)	0.3270	-----	
Portal vein invasion**	Absent	1			
	Present	1.2908 (0.5077-2.4730)	0.5312	-----	
Non-hypervascular	Absent	1		1	
hypointense nodules	Present	2.8436 (1.6900-4.8407)	0.0002	2.8436 (1.6900-4.8407)	0.0002

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C.I., conidence interval.

*Child-Pugh class A includes patients without cirrhosis.

**Evaluated by pathologic examination of resected specimens.

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Int J Clin Oncol
DOI 10.1007/s10147-011-0361-9

ORIGINAL ARTICLE

Clinical impact of c-Met expression and its gene amplification in hepatocellular carcinoma

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Received: 18 May 2011 / Accepted: 6 December 2011
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Abstract
Background *c-Met* is an oncogene encoding a receptor for hepatocyte growth factor and, as such, plays a key role in hepatocellular carcinomas (HCC). We evaluated c-Met protein expression and its gene amplification in order to assess whether they were related to tumor recurrence and survival rates among patients who had undergone tumor resection.
Methods We used the polymer-based method to perform an immunohistochemistry analysis of c-Met expression on 59 formalin-fixed, paraffin-embedded sections of surgical specimens. *c-Met* gene amplification was investigated with fluorescence in-situ hybridization. Kaplan–Meier methods and Cox proportional hazards models were used to investigate relationships between c-Met expression, patient characteristics, tumor recurrence, and survival.
Results c-Met expression was associated with portal vein invasion ($p = 0.006$). Recurrence-free survival rates were significantly lower in patients with high levels of c-Met expression ($p < 0.001$). However, c-Met expression levels did not significantly affect overall survival rates ($p = 0.12$). Only 1 patient was found to have *c-Met* gene amplification; 22 patients were found to have aneuploidy of chromosome 7, on which the *c-Met* gene is located. Tumors with chromosome 7 polysomy tended to have higher levels of c-Met expression than those with chromosome 7 monosomy or disomy, but this difference was not statistically significant.
Conclusion Although c-Met expression was not significantly associated with *c-Met* gene amplification, it may be a useful predictive marker of recurrence in resected HCC patients.

Keywords c-Met · Hepatocellular carcinoma · Recurrence · Amplification

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Introduction

Liver cancer is the sixth most common cancer worldwide, with an estimated 626,000 new cases occurring each year. It causes 598,000 deaths annually, making it the third most deadly cancer [1]. The most common type of primary liver cancer is hepatocellular carcinoma (HCC), and is often secondary to hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, both of which increase the risk of liver cancer 20-fold [2].

c-Met was discovered as an oncogene and encodes a tyrosine kinase-type growth factor receptor with an affinity for hepatocyte growth factor (HGF). The HGF–c-Met signaling pathway regulates multiple cellular functions,

Published online: 05 January 2012

including proliferation, motility, differentiation, tubulogenesis, and angiogenesis [3]. c-Met signaling controls cell invasion and metastasis in a variety of cancers, including HCC [4]. Downregulation of c-Met expression by RNA interference inhibits human HCC cell growth and invasion. A variety of selective c-Met inhibitors have thus been developed as cancer therapies.

In clinical practice, a number of systems have been proposed for predicting the prognosis of HCC patients; however, none of these systems has been universally adopted. These prognostic classifications variably incorporate four features that have been recognized as important determinants of survival: the severity of the underlying liver disease, the size of the tumor, extension of the tumor into adjacent structures, and the presence of metastasis. The three most commonly used systems are the TNM and Okuda staging systems and the CLIP score [5, 6]. Moreover, several molecules have been proposed as predictive markers for HCC, but these factors have not received adequate clinical testing [7, 8].

In this study, immunohistochemical (IHC) analysis was used to investigate c-Met protein expression and its gene amplification in HCC. We hypothesized that c-Met expression would be correlated with tumor invasion and that this correlation would predict recurrence and overall survival (OS).

Materials and methods

Patients

Patients qualified for enrollment in this study if they had undergone tumor resection at the National Cancer Center Hospital in Tokyo between April 2001 and May 2005, did not display apparent distant metastasis, and had not previously received any other treatment. A total of 59 consecutive patients were included in the study.

All patients were followed up every 3–6 months after tumor resection, and patients underwent follow-up examinations to identify possible tumor recurrence. Exam methods included computed tomography, magnetic resonance imaging, abdominal ultrasonography, and measurement of serum alpha-fetoprotein (AFP) levels. Although recurrence could be diagnosed by clinical, radiological, or pathological methods, the main evaluation technique was radiological (e.g., computed tomography and ultrasonography). Clinical and pathological profiles were obtained from a database of liver tumors based on the medical records of the patients.

This study was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan, and written informed consent was obtained from all patients.

Immunohistochemistry

A polymer-based method (Envision + Dual Link System-HRP; Dako, Glostrup, Denmark) was used to perform IHC analysis of c-Met expression on formalin-fixed, paraffin-embedded sections of surgical specimens, according to the manufacturer's instructions. For antigen retrieval, sections were autoclaved in 10 mM citrate buffer (pH 6.0) at 121°C for 10 min. A rabbit anti-c-Met polyclonal antibody (Immuno-Biological Laboratories Co., Ltd., Gumma, Japan) was used at a dilution of 1:500. Two pathologists (SK and HO) independently performed blind evaluations of the staining intensity. c-Met overexpression in each tumor was assessed using a 4-point scoring system of epidermal growth factor receptor 2 testing in breast cancer and was performed as described previously [9], as follows: 0 = no staining observed in invasive tumor cells; 1+ = weak, incomplete membrane staining in any proportion of the invasive tumor cells, or weak, complete circumferential membrane staining in fewer than 10% of cells; 2+ = weak but complete membrane staining in at least 10% of cells, or intense complete circumferential membrane staining in 30% or fewer of tumor cells; 3+ = intense complete circumferential membrane staining in more than 30% of tumor cells. We defined scores 0 and 1+ as c-Met^{low}, and scores 2+ and 3+ as c-Met^{high}.

Fluorescence in-situ hybridization for the *c-Met* gene locus

Fluorescence in-situ hybridization (FISH) was also performed for 44 tumor samples with IHC scores of 3+, 2+, and 1+. *c-Met* gene amplification was investigated with FISH using a *c-Met/CEP7* probe cocktail prepared with *c-Met* DNA developed in-house (RP 11-95I20 BAC clone) labeled with SpectrumRed, and *CEP7* DNA labeled with SpectrumGreen (Abbott Molecular, Abbott Park, IL, USA). The FISH assays were carried out as previously described [10].

Tissue sections, 5 μm thick, were subjected to pretreatment with 2× SSC at 75°C and digestion with proteinase K for 7–15 min each, co-denaturation with probe DNA at 85°C for 15 min, hybridization for 12–18 h, and rapid post-hybridization washes with 2× SCC/0.4 NP40.

Signals were enumerated in 20 tumor nuclei per specimen, using a fluorescence microscope with single-interference filter sets for green (FITC), red (Texas red), and blue (DAPI), as well as dual (red/green) band-pass filters. For these 20 tumor cell nuclei, the total number of *c-Met* and *CEP7* was counted, and the ratio of *c-Met* and *CEP7* (*c-MET/CEP7*) calculated. Positivity for gene amplification was defined as *c-Met/CEP7* = 2.0 or higher. In parallel, chromosome 7 aneusomies were defined as mean *CEP7* signals of 2.5 or higher per nucleus.

Statistical analyses

Differences in response rate and association with clinical characteristics were compared by Fisher's exact tests or chi-squared tests. We monitored for relapse every 3–6 months after resection with image testing and AFP. For recurrence-free survival (RFS; calculated from the time of surgical resection to the time of disease progression or last disease assessment), OS (calculated from the time of surgical resection to patient death or last contact) and survival curves were drawn using the Kaplan–Meier method. Group RFS and OS were compared using the log-rank test. Multivariate analysis was performed with a Cox proportional hazards regression model. For all analyses, statistical significance was defined as $p < 0.05$. All statistical analyses were conducted using SPSS version 18 (SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics

A total of 59 HCC specimens surgically resected from 59 patients were included in this analysis. These patients were mainly males (83%), and their tumors were classified as Child–Pugh A (93%), uninodular (70%), possessing high AFP (35%), and displaying histologically moderate differentiation (59%) (Table 1). At the analysis, 46 patients had recurrence (intrahepatic recurrence 34 patients and extrahepatic recurrence 12 patients).

Table 1 Association of c-Met protein expression with patient characteristics

Parameter	c-Met IHC		Total	*p*
	c-Met^{low}	c-Met^{high}		
Total	44	15	59	
Age (years, mean ± SD)	62 ± 11	59 ± 11		
Sex				
Male	36	13	49 (83%)	0.67
Female	8	2	10 (17%)	
Hepatitis				
HCV	7	2	9 (15%)	0.21
HBV	17	8	25 (42%)	
HBV + HCV	10	0	10 (17%)	
Others	10	5	15 (25%)	
Child–Pugh				
A	40	15	55 (93%)	0.22
B	4	0	4 (7%)	
UICC TNM stage				
I	25	2	27 (46%)	0.01
II	10	8	18 (30%)	
III	9	5	14 (24%)	
Tumor morphology				
Multinodular	15	3	18 (30%)	0.31
Uninodular	29	12	41 (69%)	
Portal vein invasion				
Positive	10	13	23 (39%)	<0.001
Negative	34	2	36 (61%)	
Size				
Over 5 cm	17	10	27 (46%)	0.06
Below 5 cm	27	5	32 (54%)	
AFP (IU/L)				
≥400	12	9	21 (36%)	0.02
<400	32	6	38 (64%)	
Histology				
Poorly differentiated	14	7	21 (36%)	0.40
Moderately differentiated	27	8	35 (59%)	
Well differentiation	3	0	3 (5%)	

c-Met protein expression results

Levels of nonspecific background staining were very low. There was also some evidence of c-Met staining within most tumor samples, predominantly localized to the cell membrane in non-neoplastic cells including endothelial cells and fibroblasts. c-Met staining was 3+ for 3 (5%) tumors, 2+ for 12 (20%) tumors, 1+ for 29 (49%) tumors, and negative for 15 (25.3%) tumors (Fig. 1). Low c-Met protein expression (c-Met^{low}) was defined as an IHC score of 0 or 1+, and high c-Met expression (c-Met^{high}) as an IHC score 2+ or 3+. Portal vein invasion was seen in 15 (34%) of the c-Met^{low} patients and 13 (87%) of the c-Met^{high} patients ($p < 0.001$). The difference between the two groups was statistically significant in AFP level ($p = 0.02$). However, the two groups were not statistically different in terms of tumor morphology, type of infection (HBV vs. HCV), size of tumor, or histology (Table 1).

Throughout the median follow-up period of 1266 days (range 84–3251 days), the median RFS across the whole population was 300 days. The median RFS time became significantly shorter as the intensity of c-Met expression increased (0: 1647 days; 1+: 493 days; 2+: 133 days; 3+: 166 days). The median RFS time among patients with c-Met^{high} tumors (166 days; 95% confidence interval [CI] 99.0–232.9) was significantly shorter than the median RFS time among those with c-Met^{low} tumors (748 days; 95% CI 355.2–1140.8) ($p < 0.001$; Fig. 2a).

There were no clear relationships between c-Met expression level and median OS time when each level of expression was considered individually (0: 1450 days, 95% CI 595.6–2304.4; 1+: 1760 days, 95% CI 473.4–2687.8;

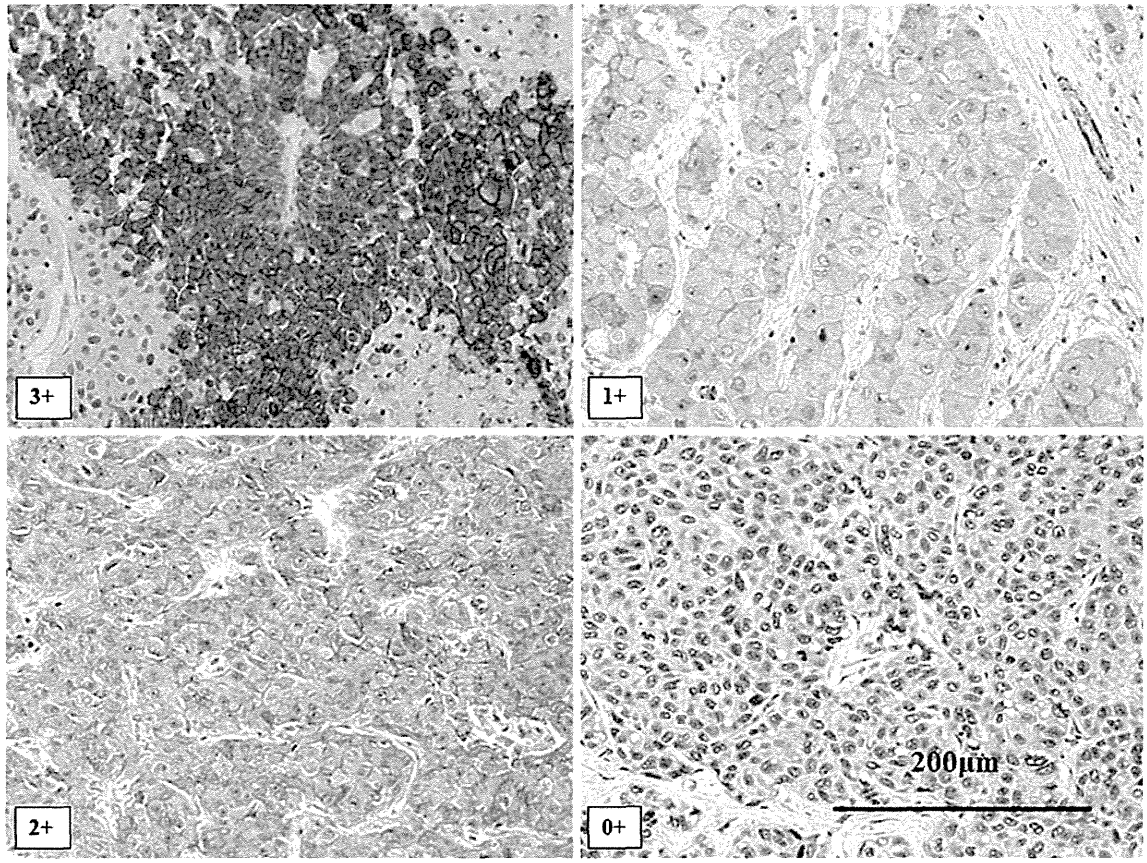

Fig. 1 Representative immunohistochemical staining scores for c-Met expression. 3+, complete membrane staining in almost all tumor cells. 2+, weak complete membrane staining in ≤20% of the cells. Although complete, membrane staining is non-uniform and weak. 1+, weak incomplete membrane staining in invasive tumor cells. 0, no staining observed in invasive tumor cells. *Scale bar* 200 μm

2+: 456 days, 95% CI 0–982.2; 3+: 784 days, 95% CI 371.1–1196.8). The median OS time was not significantly different between the c-Met^{low} (1754 days, 95% CI 1130.1–2377.9) and c-Met^{high} (740 days, 95% CI 325.9–1154.0) groups ($p = 0.12$; Fig. 2b).

Multivariate analysis indicated that recurrence was more likely in patients classified as c-Met^{high}, those who showed multinodular tumor morphology, or those who experienced portal vein invasion, than in patients classified as c-Met^{low} (HR 3.10; $p = 0.002$), or those who experienced no portal vein invasion (HR 3.21; $p = 0.006$). c-Met expression did not significantly affect OS (HR 0.96; $p = 0.91$) (Table 2).

c-Met FISH results

FISH analysis revealed *c-Met* gene amplification (4.09 copies per cell) in only 1 tumor (Fig. 3). Chromosome 7 aneusomy was detected in 25 of 44 tumors (57%) analyzed by FISH. Polysomy was detected in a total of 18 tumors (41%), 1 of which also displayed *c-Met* amplification.

Chromosome 7 monosomy without *c-Met* gene amplification was seen in 7 (16%) tumors. Among c-Met IHC-positive patients, there was only 1 patient (2% of the total) in whom *c-Met* gene amplification was detected (Table 3). IHC analysis revealed that 9 (50%) of 18 tumors from patients with chromosome 7 polysomy, with or without *c-Met* gene amplification, were c-Met^{high}. On the other hand, IHC analysis revealed that only 6 (23%) of 26 tumors from patients with chromosome 7 monosomy or disomy were c-Met^{high}. However, this difference was not statistically significant ($p = 0.17$). Moreover, chromosome 7 aneusomy was not significantly related to either RFS ($p = 0.77$) or OS ($p = 0.99$).

Discussion

In the present study, IHC analysis showed that c-Met^{high} expression in HCC was significantly correlated with pathological vascular invasion and shorter RFS, indicating that this characteristic could serve as a predictive factor for recurrence of HCC after resection.

The HGF/c-Met pathway promotes cell proliferation and inhibits apoptosis in tumor cells [11, 12] and, additionally,

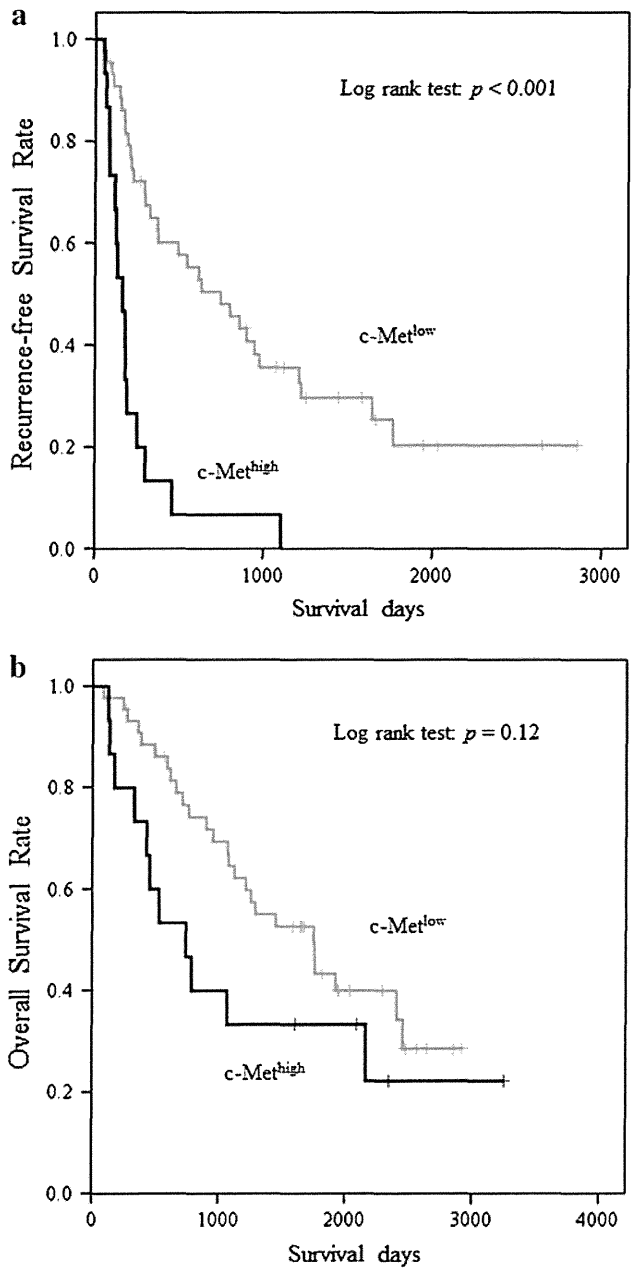

Fig. 2 **a** Kaplan–Meier estimates of recurrence-free survival (RFS) rates of patients with hepatocellular carcinoma (HCC). Median RFS time was 166 days for patients with c-Met^{high} tumor (*black line*; $n = 15$) and 748 days for patients with c-Met^{low} tumor (*gray line*; $n = 44$). The survival curves were significantly different ($p < 0.001$). **b** Kaplan–Meier estimates of overall survival (OS) rates of patients with HCC. Median OS time was 740 days for patients with c-Met^{high} tumor (*black line*; $n = 15$) and 1754 days for patients with c-Met^{low} tumor (*gray line*; $n = 44$). The curves were not significantly different ($p = 0.12$)

it has been reported to stimulate cell motility (e.g., cell dissociation, migration, and infiltration), promote changes in cell distribution, and affect morphogenesis [13]. In our study, the tendency of c-Met to promote HCC cells was poorly differentiated, and a statistical association between c-Met expression level and pathological differentiation was not shown. In HCC patients, the extracellular stromas, hypoxia and inflammatory cytokines or pro-angiogenic factors are activated by c-Met, which aggravates the intrinsic malignant properties of transformed cells by proliferative, anti-apoptotic, and migratory signals. Moreover, increases in c-Met expression have been associated with both decreased survival and increased proliferative activity of HCC cells, suggesting that c-Met could be used as a prognostic factor [14]. Previously, Ke et al. [15] reported that poor prognosis of patients with HCC resulted from an interaction between tetraspanin CD151 and c-Met, and that c-Met protein expression alone did not predict low survival rates. However, we observed high levels of c-Met expression in 25% of cases, which is similar to the rate reported by Ke et al. However, in contrast to the results of their study, we found that c-Met overexpression alone was capable of serving as a predictive factor for recurrence of HCC after surgery.

Patients with intrahepatic recurrence after surgery received additional local treatments, including surgical resection, local necrosis therapy via radiofrequency ablation, ethanol injection therapy, and hepatic arterial embolization therapy. Therefore, even if recurrence was promoted by c-Met, the use of multimodality therapy for lesion control made it difficult to determine whether c-Met expression could accurately predict prognosis. The high frequency of intrahepatic recurrence in this study may lend support to the view that c-Met expression cannot be used as a prognostic factor.

c-Met gene amplification has been reported in stomach [16], colorectal [17], and lung cancers [18], and it is thought to be correlated with metastasis and outcome in gastric and colorectal cancers. Amplification of the *c-MET* gene has been reported in lung cancer cases that were resistant to EGFR-TKI gefitinib [19]. Additionally, mRNA amplification has been reported in HCC patients [20]. However, we found that *c-Met* gene amplification was rare (1 in 44 patients), suggesting that it may not be involved in high c-Met expression in HCC.

Alterations in the *c-Met* gene were first reported in association with hereditary papillary renal carcinoma [21, 22], and were later found in children with HCC [23] and squamous cell carcinomas of the head and neck [24]. To date, there have been few reports of gene alterations in cases of adult HCC, and their frequency is not expected to be high. However, research on the *HER2* breast cancer gene showed correlations between non-*HER2*-amplification-related aberrations and a variety of breast cancer characteristics, such as nuclear histological grade and tumor stages. Furthermore, these aberrations have been associated with increased HER2 expression, as assessed by IHC analysis [25]. Thus, we examined correlations between

Table 2 Univariate and multivariate Cox regression analyses for RFS and OS

Parameter	RFS Hazard ratio (95% CI)	*p*	OS Hazard ratio (95% CI)	*p*
Univariate Cox regression analyses				
Age				
Over 70 versus under 70 years	1.50 (0.59–3.82)	0.39	1.60 (0.56–4.55)	0.38
Sex				
Male versus female	1.30 (0.64–2.63)	0.47	1.68 (0.65–4.35)	0.28
HCV versus non-HCV	1.51 (0.81–2.80)	0.19	1.61 (0.78–3.23)	0.20
HBV versus non-HBV	1.35 (0.75–2.42)	0.32	1.17 (0.60–2.27)	0.65
Child–Pugh				
B versus A	1.59 (0.38–6.58)	0.53	1.57 (0.47–5.20)	0.46
Portal vein invasion				
Positive versus negative	5.56 (2.89–10.68)	<0.001	3.78 (1.90–7.53)	<0.001
AFP				
Over 400 IU/L versus below 400 IU/L	4.59 (2.27–9.29)	<0.001	2.30 (1.19–4.47)	<0.01
Tumor morphology				
Multinodular versus uninodular	1.59 (0.86–2.93)	0.14	1.11 (0.56–2.19)	0.77
Size				
Over 5 cm versus 5 cm or less	4.85 (1.17–20.10)	0.03	1.47 (0.45–4.86)	0.53
Histology				
Poorly versus moderately + well	3.85 (2.03–7.31)	<0.001	2.66 (1.38–5.14)	<0.01
c-Met^{high} versus c-Met^{low}	4.11 (2.12–7.98)	<0.001	1.75 (0.86–3.54)	0.12
Multivariate Cox-regression analyses				
Portal vein invasion				
Positive versus negative	3.21 (1.40–10.56)	0.006	4.09 (1.56–10.76)	0.004
AFP				
Over 400 IU/L versus below 400 IU/L	2.15 (0.86–5.32)	0.10	0.82 (0.31–2.12)	0.68
Size				
Over 5 cm versus 5 cm or less	2.43 (0.56–10.56)	0.24	0.67 (1.81–2.49)	0.55
Histology				
Poorly versus moderately + well	0.98 (0.40–2.41)	0.97	1.55 (0.57–4.19)	0.39
c-Met^{high} versus c-Met^{low}	3.10 (1.49–6.46)	0.002	0.96 (0.44–2.07)	0.91

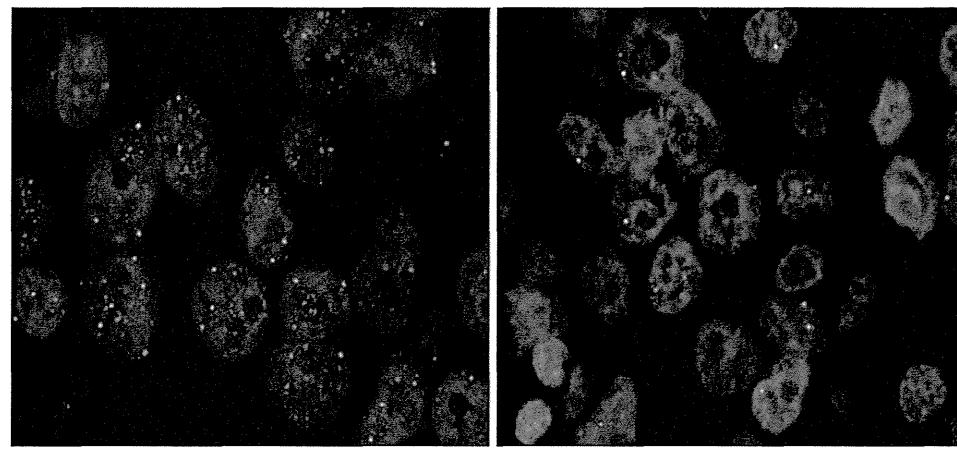

Fig. 3 Results of interphase FISH analysis of paraffin-embedded tumor tissue sections. Tumor cells with *c-Met* gene amplification (*left*), 20 or more *c-Met* gene signals (*red*), and 1–5 copies of *CEP7* (*green*) are present in each nucleus. Non-amplified tumor cells (*right*), 1–2 *c-Met* gene signals (*red*), and 1–2 *CEP7* signals (*green*) are present in each nucleus

Table 3 Association of c-Met protein expression with FISH analysis

FISH analysis	c-Met^{low}	c-Met^{high}	Total
Disomy	14	5	19/44 (43.2%)
Amplification with polysomy	0	1	1/44 (2.3%)
Polysomy without amplification	9	8	17/44 (38.6%)
Monosomy	6	1	7/44 (15.9%)

FISH fluorescence in-situ hybridization

overexpression of the c-Met protein and aneusomy (e.g., monosomy or polysomy) of chromosome 7, on which the *c-Met* gene is located. However, we found no correlation between chromosome 7 aneusomy and increased c-Met expression, although the incidence of high c-Met expression tended to be higher in tumors from patients with chromosome 7 polysomy than in those with chromosome 7 monosomy or disomy. Thus, an increased number of *c-Met* copies caused by gene amplification or chromosomal polysomies does not appear to be the mechanism behind increased levels of c-Met expression. It is possible that other mechanisms, such as autocrine or paracrine HGF, ligand-independent interactions with other receptors, and/or epigenetic expression regulation may play an important role in c-Met expression.

In conclusion, the present study demonstrates that c-Met can be used as a predictive factor for the recurrence of HCC after resection. It may also provide a useful target for future HCC treatments.

Acknowledgments We are grateful to Dr. Koh Furuta (Clinical Laboratory Division, National Cancer Center Hospital, Tokyo, Japan) for his helpful technical advice.

Conflict of interest The authors declare that they have no conflicts of interest.

References

1. Parkin DM, Bray F, Ferlay J et al (2005) Global cancer statistics, 2002. CA Cancer J Clin 55:74–108
2. Donato F, Boffetta P, Puoti M (1998) A meta-analysis of epidemiological studies on the combined effect of hepatitis B and C virus infections in causing hepatocellular carcinoma. Int J Cancer 75:347–354
3. Furge KA, Zhang YW, Vande Woude GF (2000) Met receptor tyrosine kinase: enhanced signaling through adapter proteins. Oncogene 19:5582–5589
4. Kaposi-Novak P, Lee JS, Gomez-Quiroz L et al (2006) Met-regulated expression signature defines a subset of human hepatocellular carcinomas with poor prognosis and aggressive phenotype. J Clin Invest 116:1582–1595
5. Okuda K, Ohtsuki T, Obata H et al (1985) Natural history of hepatocellular carcinoma and prognosis in relation to treatment. Study of 850 patients. Cancer 56:918–928
6. Chevret S, Trinchet JC, Mathieu D et al (1999) A new prognostic classification for predicting survival in patients with hepatocellular carcinoma. Groupe d'Etude et de Traitement du Carcinome Hepatocellulaire. J Hepatol 31:133–141
7. Iizuka N, Oka M, Yamada-Okabe H et al (2003) Oligonucleotide microarray for prediction of early intrahepatic recurrence of hepatocellular carcinoma after curative resection. Lancet 361:923–929
8. Yoshioka S, Takemasa I, Nagano H et al (2009) Molecular prediction of early recurrence after resection of hepatocellular carcinoma. Eur J Cancer 45:881–889
9. Miyamoto M, Ojima H, Iwasaki M et al (2011) Prognostic significance of overexpression of c-Met oncoprotein in cholangiocarcinoma. Br J Cancer 105:131–138
10. Cappuzzo F, Janne PA, Skokan M et al (2009) MET increased gene copy number and primary resistance to gefitinib therapy in non-small-cell lung cancer patients. Ann Oncol 20:298–304
11. Birchmeier C, Birchmeier W, Gherardi E et al (2003) Met, metastasis, motility and more. Nat Rev Mol Cell Biol 4:915–925
12. Liu X, Yao W, Newton RC et al (2008) Targeting the c-MET signaling pathway for cancer therapy. Expert Opin Investig Drugs 17:997–1011
13. Comoglio PM, Giordano S, Trusolino L (2008) Drug development of MET inhibitors: targeting oncogene addiction and expedience. Nat Rev Drug Discov 7:504–516
14. Ueki T, Fujimoto J, Suzuki T et al (1997) Expression of hepatocyte growth factor and its receptor, the c-met proto-oncogene, in hepatocellular carcinoma. Hepatology 25:619–623
15. Ke AW, Shi GM, Zhou J et al (2009) Role of overexpression of CD151 and/or c-Met in predicting prognosis of hepatocellular carcinoma. Hepatology 49:491–503
16. Nakajima M, Sawada H, Yamada Y et al (1999) The prognostic significance of amplification and overexpression of c-met and c-erb B-2 in human gastric carcinomas. Cancer 85:1894–1902
17. Di Renzo MF, Olivero M, Giacomini A et al (1995) Overexpression and amplification of the met/HGF receptor gene during the progression of colorectal cancer. Clin Cancer Res 1:147–154
18. Cappuzzo F, Marchetti A, Skokan M et al (2009) Increased MET gene copy number negatively affects survival of surgically resected non-small-cell lung cancer patients. J Clin Oncol 27:1667–1674
19. Engelman JA, Zejnullahu K, Mitsudomi T et al (2007) MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 316:1039–1043
20. Boix L, Rosa JL, Ventura F et al (1994) c-met mRNA overexpression in human hepatocellular carcinoma. Hepatology 19:88–91
21. Schmidt L, Duh FM, Chen F et al (1997) Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. Nat Genet 16:68–73
22. Schmidt L, Junker K, Nakaigawa N et al (1999) Novel mutations of the MET proto-oncogene in papillary renal carcinomas. Oncogene 18:2343–2350
23. Park WS, Dong SM, Kim SY et al (1999) Somatic mutations in the kinase domain of the Met/hepatocyte growth factor receptor gene in childhood hepatocellular carcinomas. Cancer Res 59:307–310
24. Di Renzo MF, Olivero M, Martone T et al (2000) Somatic mutations of the MET oncogene are selected during metastatic spread of human HNSC carcinomas. Oncogene 19:1547–1555
25. Krishnamurti U, Hammers JL, Atem FD et al (2009) Poor prognostic significance of unamplified chromosome 17 polysomy in invasive breast carcinoma. Mod Pathol 22:1044–1048

Phase I/II Study of Radiologic Hepatic Arterial Infusion of Fluorouracil Plus Systemic Irinotecan for Unresectable Hepatic Metastases from Colorectal Cancer: Japan Clinical Oncology Group Trial 0208-DI

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ABSTRACT

Purpose: Treatment of patients who have metastatic colorectal cancer (CRC) by using a combination of hepatic arterial infusion chemotherapy (HAIC) and systemic chemotherapy has resulted in promising clinical outcomes. Additionally, image-guided HAIC is reported to be less invasive and distribute drugs more accurately than surgical HAIC. The purpose of this study was to assess the combination of image-guided delivery of fluorouracil through HAIC and systemic irinotecan in a multicenter phase I/II study.

Materials and Methods: Twenty-five patients with unresectable liver metastases from CRC were fitted with hepatic arterial catheter and port systems by using image-guided methods. Intraarterial fluorouracil (1,000 mg/m^2) was administered on days 1, 8, and 15 of each treatment cycle. The dose of systemic irinotecan on days 1 and 15 was escalated from 75 mg/m^2.

Results: No dose-limiting toxicity was encountered during phase I, and the recommended dose of irinotecan was set at 150 mg/m^2. Grade 3 or higher adverse events included hyperglycemia (15%), elevated γ-glutamyl transpeptidase levels (15%), and neutropenia (9%). The response rate and median survival time were 72% and 49.8 months (95% CI, 27.5–78.1 mo), respectively.

Conclusions: The combination of image-guided delivery of fluorouracil through HAIC and systemic irinotecan yielded favorable safety, response rate, and survival results. This combination should be evaluated in a large study.

ABBREVIATIONS

AE = adverse event, CRC = colorectal cancer, DLT = dose-limiting toxicity, DSA = digital subtraction angiography, HAIC = hepatic arterial infusion chemotherapy, MTD = maximum tolerated dose, OS = overall survival, RD = recommended dose, WBC = white blood cell

Modern chemotherapy with the use of active agents, such as irinotecan, oxaliplatin, and molecular-targeted therapies, has significantly prolonged the survival of patients with metastatic colorectal cancer (CRC) (1,2). However, achieving complete response and long-term survival is still rare, even with intensive therapy with combinations of these agents.

Although hepatic arterial infusion chemotherapy (HAIC) with fluorinated pyrimidines has demonstrated high local response rates for CRC liver metastases, 10 of 11 randomized

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This study was funded by the Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan.

None of the authors have identified a conflict of interest.

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J Vasc Interv Radiol 2012; 23:1261–1267

http://dx.doi.org/10.1016/j.jvir.2012.06.031

Table 1. Eligibility Criteria

Inclusion criteria

1. Histologically documented colorectal cancer
2. Unresectable liver-limited metastases as determined by imaging studies
3. Previous resection of primary tumor with D1 or D2 lymph node dissection
4. No previous chemotherapy except adjuvant chemotherapy with fluoropyrimidines completed > 3 mo before study
5. At least one measurable tumor in the liver per RECIST (version 1.0)
6. Between 20 and 70 y of age
7. ECOG performance status of 0–2
8. Adequate hematological, hepatic, renal, and cardiac functions
9. Written informed consent

Exclusion criteria

1. Massive ascites or pleural effusion
2. Active gastrointestinal bleeding
3. Active infection
4. Watery diarrhea
5. Severe comorbid conditions
6. Other untreated cancers
7. Previous abdominal radiotherapy
8. Positive serum hepatitis B antigen or hepatitis C antibody
9. Allergy to iodinated contrast material
10. Severe mental disorder
11. Previous catheter placement into the hepatic artery
12. Pregnancy or nursing

ECOG = Eastern Cooperative Oncology Group, RECIST = Response Criteria in Solid Tumors.

controlled trials published before 2006 did not find any survival benefit of HAIC greater than that of systemic chemotherapy (3–13). Metaanalyses of HAIC studies have also demonstrated that HAIC does not improve survival in patients with CRC (14–16). Consequently, HAIC is not generally considered a first-line treatment or a component of standard treatment regimens.

Laparotomy was employed for HAIC catheter and pump placement in all previous randomized controlled trials of HAIC in Western countries. In Japan, on the contrary, a percutaneous technique for hepatic arterial catheter and port placement was developed in the 1980s and was established in the 1990s as an image-guided interventional radiologic procedure, with drug distribution evaluated by using contrast-enhanced computed tomography (CT) via the indwelling catheter–port system (17–20). The advantages of this technique are that it is minimally invasive and provides accurate periodic evaluation of drug delivery. In addition, HAIC treatment outcomes with this technique are favorable; phase II studies (17,21–23) of intermittent HAIC with fluorouracil in patients with CRC liver metastases with or without extrahepatic metastasis had median survival times of 18.6–26 months. HAIC treatment success requires monitoring of drug distribution to ensure that the administered drug is delivered directly to all liver tumors without reaching extrahepatic organs (20).

Kemeny et al (24) reported a phase I study of HAIC with floxuridine and dexamethasone combined with systemic irinotecan that was or was not followed with cryosurgery. The study demonstrated a response rate of 74% and a median survival time of 17 months in patients who did not undergo cryosurgery. In their study, however, surgical laparotomy was used for implantation instead of a radiologic intervention (24), and the drug and administration schedules were different from those of Japanese phase II studies. Thus, we conducted a multicenter phase I/II study to assess the feasibility, safety, and preliminary efficacy of image-guided delivery of fluorouracil through HAIC combined with systemic irinotecan.

MATERIALS AND METHODS

Patients

Inclusion and exclusion criteria are listed in **Table 1**. A Consolidated Standards of Reporting Trials diagram of this study is shown in **Figure 1**. The study protocol was approved by the institutional review boards of all participating institutions. All patients provided written informed consent. This study was registered to UMIN-CTR (UMIN C000000051, 2005/08/08).

Treatment

Placement of Intraarterial Catheter and Port System. A catheter and port system was implanted within 2 weeks of enrollment in the study. Details of the procedure are described elsewhere (19,25). In brief, percutaneous implantation of a catheter and port system was performed under local anesthesia by using an interventional radiologic technique. Before each cycle of chemotherapy, drug delivery was evaluated by digital subtraction angiography (DSA) and CT angiography through the implanted catheter and port system (**Fig 2**).

Chemotherapy Administration. After implantation of the catheter and port system, chemotherapy was started when the patient's laboratory values were as follows: white blood cell (WBC) count of at least 4,000/mm^3 and no greater than 12,000/mm^3, platelet count of at least 100,000/mm^3, aspartate aminotransferase and alanine aminotransferase levels no greater than three times the upper limit of normal, bilirubin level no greater than 1.5 mg/dL, and serum creatinine level no greater than 1.5 mg/dL. Patients received concurrent systemic chemotherapy and HAIC in 4-week cycles, and the treatment protocol was considered to be complete after five cycles of this regimen. In each cycle, 1,000 mg/m^2 of fluorouracil in saline solution plus 100 mg of hydrocortisone were administered on days 1, 8,

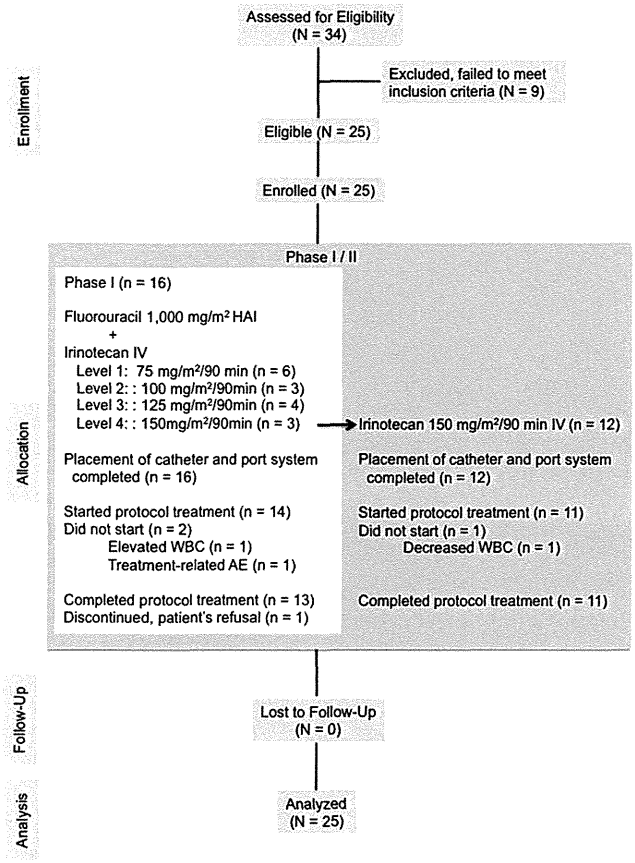

Figure 1. Consolidated Standards of Reporting Trials diagram. HAI = hepatic arterial infusion. (Available in color online at *www.jvir.org.*)

and 15 by continuous 5-hour infusion via a disposable balloon pump system. This dose was determined based on a previous phase I/II study of HAIC with fluorouracil (23). On days 1 and 15, following HAIC, irinotecan diluted in 5% glucose was administered via a 90-minute intravenous drip. The irinotecan doses planned for phase I of the trial were 75, 100, 125, and 150 mg/m^2. After the maximum tolerated dose (MTD) was determined, the study was advanced to phase II.

Patient and Tumor Evaluations

Pretreatment evaluations included medical history, physical examination, and laboratory examinations. Laboratory examinations included evaluation of complete blood counts, total bilirubin, alkaline phosphatase, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, and carcinoembryonic antigen. Baseline evaluation of tumors was performed by contrast-enhanced CT scans of the chest and abdomen. During the course of treatment, each patient was assessed weekly for toxicity, including laboratory determination of complete blood counts, and blood chemistry. CT examination was planned before treatment and after one, three, and five cycles of treatment. Patient responses to treatment were evaluated by three radiologists based on Response Evaluation Criteria In Solid Tumors, version 1.0. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria, version 2.0.

Study Design and Statistical Analysis

This trial was designed as a phase I/II study. The primary endpoints of phase I were to identify dose-limiting toxicities (DLTs), MTD, and the recommended dose (RD) of systemic irinotecan when combined with HAIC that uses a fixed dose of fluorouracil. DLTs in phase I were defined as any grade 4 neutropenia or thrombocytopenia or any nonhematologic toxicity of grade 3 or more severe. We treated patients in cohorts of three to six. The first cohort received the lowest dose (ie, dose level 1) of irinotecan, and doses were escalated in a stepwise fashion. If DLTs were observed in less than one third of the cohort members, subsequent patients were treated at the next dose level. If more than one third of cohort members developed DLTs, the preceding dose level was identified as the MTD.

Based on the results of previous studies, 12 patients were needed in this study with a null proportion of 30%–45% and an alternative proportion of 74% to achieve 80% power, given that the one-sided significance level was 10% (24).

Secondary endpoints of the study included HAIC initiation rate, overall response rate, response rate in the liver, and toxicity. Survival analysis was performed by using the Kaplan–Meier method. Demographics and baseline variables were summarized by using descriptive statistics. Statistical significance was set at 0.05, and differences between groups were examined by using two-tailed *t* tests. We used SPSS software (version 17; SPSS, Chicago, Illinois) to perform all statistical analyses.

RESULTS

Patient Demographics

Twenty-five patients from five participating institutions were enrolled between November 2003 and March 2008. Patient characteristics are listed in **Table 2**. Synchronous liver metastases were seen in 84% of the patients, and 92% of the patients had not received previous adjuvant chemotherapy.

Initiation of HAIC and Systemic Chemotherapy

A catheter and port system was successfully placed in all 25 patients. Catheters were inserted via the left subclavian artery in all patients. Treatment consisting of HAIC and systemic chemotherapy was initiated according to the study protocol in 22 patients (88%; **Fig 1**). Treatment was not started in three patients as a result of elevated WBC count (n = 1), decreased WBC count (n = 1), and cerebral infarction that was presumably caused by catheter placement (n = 1). The elevated WBC count observed in one patient at dose level 1 and the decreased WBC count

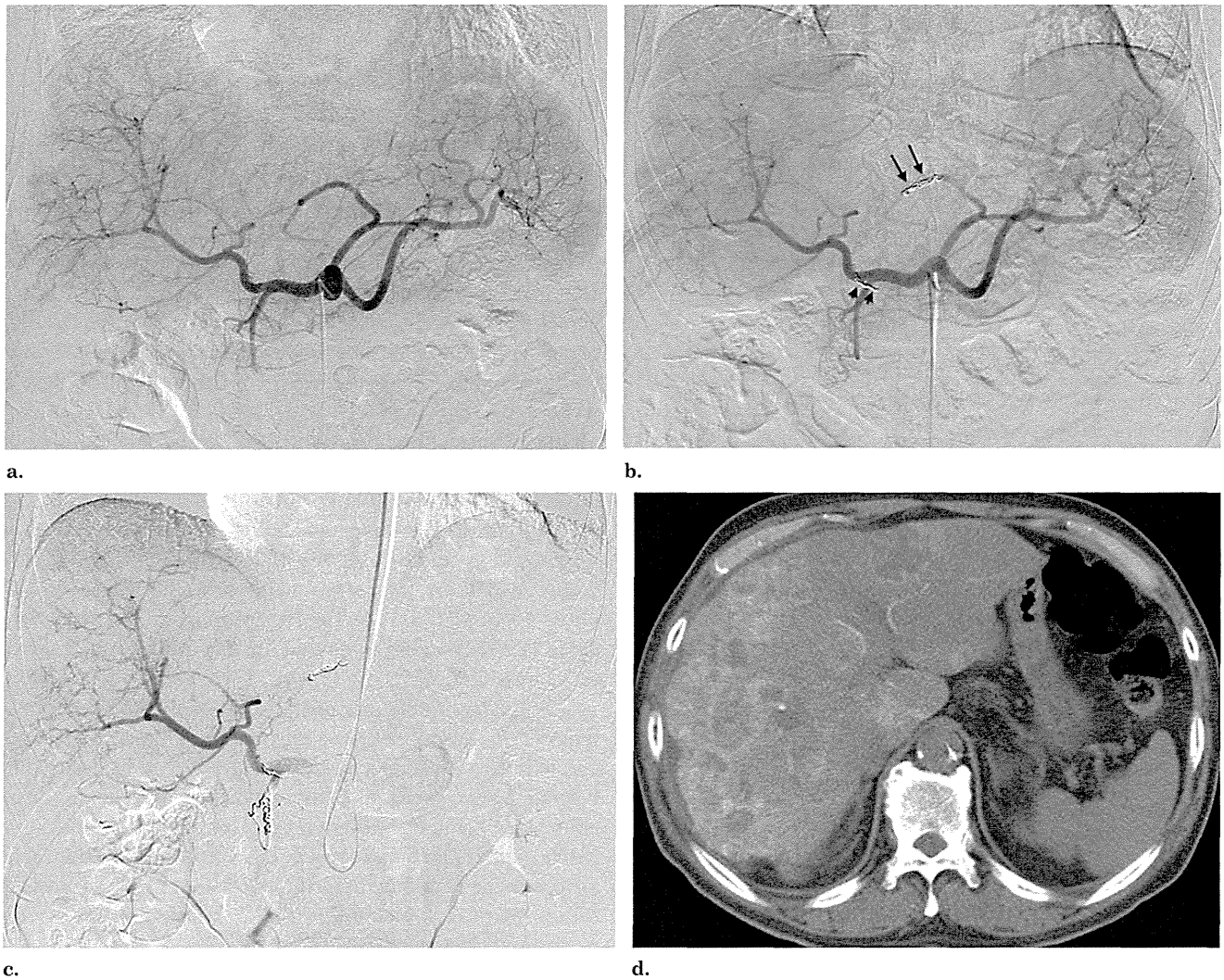

Figure 2. Image-guided insertion of catheter and port system for HAIC. **(a)** DSA of the celiac artery. The left hepatic artery is arising from left gastric artery (ie, replaced left hepatic artery). **(b)** DSA after embolization of the replaced left hepatic artery (long arrows) and right gastric artery (short arrows). The left hepatic artery is visualized from the collateral vessels. **(c)** DSA via the implanted port. The indwelling catheter is implanted via the left subclavian artery. **(d)** CT angiography via the implanted port. Adequate drug distribution is confirmed with enhancement of hepatic metastases by contrast material injected through the implanted port.

observed in one patient at dose level 4 were likely related to the primary disease process, because no clinical findings of infection were found. These two patients were removed from the study and treated by HAIC alone. Details of the patient who developed cerebral infarction are described in the Safety section. The HAIC initiation rate was 96%, including the two patients who were later removed from the study.

Dose-escalation Findings

In phase I, one of six patients developed DLT at dose level 1 (**Fig 1**). Of the 14 patients who started the treatment protocol, 13 patients completed five cycles. Because no DLT was encountered at dose levels 2–4, we were unable to determine the MTD of irinotecan. Dose level 4 (150 mg/m^2) was selected as the RD for phase II of the study.

Safety

There were no treatment-related deaths in this study. The incidence of grade 2 or higher adverse events (AEs) occurring during chemotherapy is shown in **Table 3**. In 106 cycles of protocol treatment, the following grade 3 or higher AEs occurred: leukopenia (2%), neutropenia (9%), elevated γ-glutamyl transpeptidase level (15%), hyperglycemia (15%), and hypokalemia (1%). The only grade 4 AE was neutropenia (2%).

Before the initiation of chemotherapy, one patient (4%) developed central nervous system ischemia. One day after placement of the catheter and port system, the patient developed hemiparesis, and magnetic resonance imaging confirmed multiple cerebral infarctions. The patient subsequently had moderate hemiparesis, but no other neurologic deficits. The indwelling catheter was thought to have caused the cerebral infarctions.

Table 2. Patient Characteristics (N = 25)

Characteristic	Value
Age (y)	
Median	63
Range	45–70
Sex	
Male	21 (84)
Female	4 (16)
ECOG performance status	
0	24 (96)
1	1 (4)
Location of primary tumor	
Colon	13 (52)
Rectum	12 (48)
Differentiation	
Well	7 (28)
Moderate	15 (60)
Poor	3 (12)
Synchronous	
Yes	21 (84)
No	4 (16)
Liver involvement	
< 30%	22 (88)
30%–60%	3 (12)
> 60%	0
Previous adjuvant chemotherapy	
Yes	2 (8)
No	23 (92)

Values in parentheses are percentages. ECOG = Eastern Cooperative Oncology Group.

Table 3. Per-patient Incidence of Grade 2 or Higher Adverse Events in All Cycles of Chemotherapy (N = 106)

Adverse Event	Grade 2	Grade 3	Grade 4
Nausea	3 (3)	0	0
Diarrhea	8 (8)	0	0
Stomatitis	1 (1)	0	0
Fatigue	3 (3)	0	0
Alopecia	12 (11)	0	0
Vertigo	0	1 (1)	0
Glycosuria	2 (2)	0	0
Cystitis	2 (2)	0	0
Leukopenia	21 (20)	2 (2)	0
Neutropenia	15 (14)	7 (7)	2 (2)
Anemia	10 (9)	0	0
Thrombocytopenia	2 (2)	0	0
Hyperbilirubinemia	1 (1)	0	0
GGT	11 (10)	16 (15)	0
ALP	3 (3)	0	0
Hyperglycemia	26 (25)	16 (15)	0
Hypokalemia	0	1 (1)	0

Values in parentheses are percentages. ALP = alkaline phosphatase, GGT = γ-glutamyl transpeptidase.

Response

A total of 25 patients were included in the response analyses. The overall response in the liver was 72%, and included four complete responses (16%) and 14 partial responses (56%). Four patients (16%) exhibited stable disease in the liver, and the responses of three patients (12%) could not be evaluated because the treatment protocol was not initiated. During the course of treatment, no patients developed any observable extrahepatic metastases. Therefore, the overall response rate was 72%.

Survival

Survival analysis was conducted based on all 25 patients (**Fig 3**). With a median follow-up period of 55.0 months (range, 22.8–87.7 mo), the median overall survival (OS) time was 49.8 months (95% CI, 27.5–78.1 mo).

DISCUSSION

The present study is a prospective trial to evaluate image-guided HAIC combined with systemic chemotherapy for patients with unresectable hepatic metastases from CRC. Our results demonstrate that this treatment may be effective and safe as an initial therapy, as 23 of the 25 patients in the study had not undergone previous chemotherapy. Twenty-one of these 23 patients were enrolled in the study after surgery to remove the primary tumor. Other noteworthy characteristics of the patients in this trial include good performance status (24 patients with a performance status of 0) and moderate tumor involvement in the liver (22 patients with < 30% involvement). To summarize, the characteristics of this patient cohort were resectable primary tumor, synchronous and unresectable liver metastases of moderate intrahepatic extent, good performance status, and limited previous chemotherapy.

We determined the feasibility and the safety of image-guided HAIC combined with systemic irinotecan. Generally, AEs caused by fluorouracil are well tolerated, and bone marrow suppression is not significant. Given that systemic irinotecan has a different toxicity profile than fluorouracil, and the intraarterial effects of fluorouracil were minimal in the present study, it could be surmised that the RD of irinotecan in this study should be the same as the standard RD of 150 mg/m^2 used in Japanese patients. However, modification of the usual treatment schedule of weekly fluorouracil HAIC to a schedule that included a 1-week treatment-free interval in the fourth week of each treatment cycle may account for the minimal hematologic toxicity we observed and the undetermined MTD (23). During phase II of this trial, grade 3 AEs were observed in 21 of 60 treatment cycles (35%), and no grade 4 AEs occurred. Furthermore, all patients, except one who did not meet the criteria for the initiation of chemotherapy, completed the planned five cycles of treatment. Therefore, this

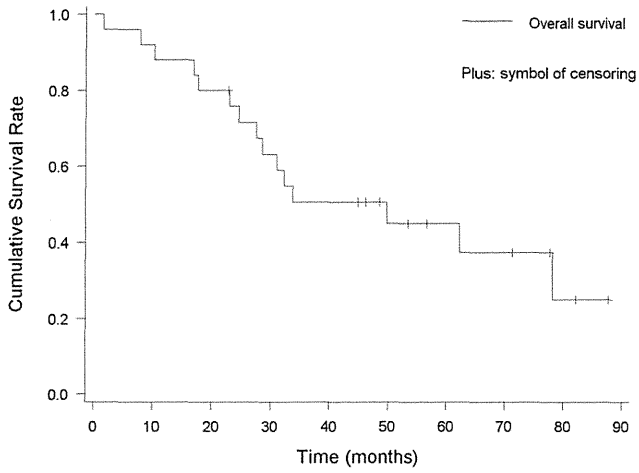

Figure 3. Graph of OS. The median OS time is 49.8 months (95% CI, 27.5–78.1 mo).

study demonstrated that image-guided HAIC with fluorouracil combined with systemic irinotecan (150 mg/m^2) is feasible and safe.

Hyperglycemia was a notable AE in this study. Hyperglycemia occurred in 56% of the 106 treatment cycles. The incidence of hyperglycemia in phase I was almost the same as in phase II. As there have been no reports of hyperglycemia as a result of irinotecan therapy, and intraarterial hydrocortisone (100 mg) was administered with fluorouracil on days 1, 8, and 15 to reduce vascular endothelial injury, this intraarterial hydrocortisone may have influenced the occurrence of hyperglycemia. There is some possibility that intraarterial direct administration of fluorouracil to the liver leads to deterioration of the glucose tolerance of the liver. Hyperglycemia does not directly alter the short-term patient prognosis, but it may become more important if longer survival is achieved with this treatment.

The HAIC initiation rate in the present study is comparable to rates seen in previous studies of image-guided HAIC. Tanaka et al (26) reported a technical success rate of 99.8% among 426 patients undergoing image-guided HAIC. Deschamps et al (27) reported a technical success rate of 94% among 93 patients. Moreover, Ganeshan et al (25) mentioned in their review of HAIC that interventional radiology played a vital role in establishing vascular access and assessing outcomes. On the contrary, the technical success rates of surgical HAIC, a technique widely employed in published randomized controlled trials, are not included in the reports of these trials or result in lower HAIC initiation rates than seen with image-guided HAIC. Kerr et al (10) reported an HAIC initiation rate of 68% following the surgical procedure used in their randomized study comparing HAIC with systemic chemotherapy. In the present small, prospective study, the HAIC initiation rate was 96%. This suggests that image-guided catheter placement is suitable for the initiation of HAIC.

In the present study, one patient developed cerebral infarction after catheter implantation. There have been several reports of cerebral ischemia as a complication of catheter implantation via the subclavian artery, and the incidence of this complication is approximately 5% (28,29). This complication should therefore be recognized as a severe AE caused by radiologic catheter placement via the subclavian artery, and other access routes such as the femoral or hypoepigastric artery should be considered.

The liver response rate of 72% we observed is similar to those of other studies of image-guided deliver of fluorouracil with the same infusion protocol through HAIC (17,21,22). This indicates that the addition of systemic irinotecan might not affect tumor response in the liver. However, previous studies have demonstrated an incidence of extrahepatic metastases of approximately 70% when patients were treated with HAIC alone. In the present study, no extrahepatic metastases were observed during the study. Therefore, systemic irinotecan may have reduced the occurrence of extrahepatic metastases. Because more than 90% of the fluorouracil administered via the hepatic artery is reported to pass through the liver and enter systemic circulation (30), irinotecan combined with fluorouracil may have prevented extrahepatic metastases in the present study.

It is notable that the median OS of the present study exceeded 4 years. The large proportion of patients with good PS may be a prominent factor in this result. Standard first- and second-line systemic chemotherapies have demonstrated a median survival of 18–24 months (31–34). Concerning the combination of HAIC with systemic chemotherapy, Kemeny et al (35) reported an OS of 36 months with fluorodeoxyuridine HAIC plus systemic oxaliplatin and irinotecan, and an OS of 22 months with fluorodeoxyuridine HAIC plus systemic oxaliplatin, fluorouracil, and leucovorin. Ducreux et al (36) reported an OS of 27 months with HAIC of oxaliplatin plus systemic irinotecan and fluorouracil. Therefore, HAIC combined with systemic chemotherapy may prolong the survival of patients with unresectable liver metastases from CRC.

The present study has several limitations. First, it was a phase I/II study involving a small number of patients. Second, we did not record postprotocol treatment. Therefore, OS may have been influenced by modern systemic chemotherapy with fluorouracil, leucovorin, oxaliplatin, irinotecan, and molecular-targeting agents. However, the OS observed in the present study is still a promising result. Thus, accurate HAIC that uses CT angiography for appropriate drug distribution in combination with systemic chemotherapy may lead to improved patient outcomes.

The present study demonstrates the feasibility of HAIC as an interventional procedure and that HAIC of fluorouracil combined with systemic irinotecan at 150 mg/m^2 is well tolerated. Also, the OS exceeding 4 years was a promising result, although it may have been affected by the treatments after the protocol. In conclusion, this combination chemotherapy should be evaluated in a larger study.

ACKNOWLEDGMENTS

The authors thank Haruhiko Fukuda, MD, for contributions to the study design.

REFERENCES

1. Masi G, Vasile E, Loupakis F, et al. Triplet combination of fluoropyrimidines, oxaliplatin, and irinotecan in the first-line treatment of metastatic colorectal cancer. Clin Colorectal Cancer 2008; 7:7–14.
2. Souglakos J, Mavroudis D, Kakolyris S, et al. Triplet combination with irinotecan plus oxaliplatin plus continuous-infusion fluorouracil and leucovorin as first-line treatment in metastatic colorectal cancer: a multicenter phase II trial. J Clin Oncol 2002; 20:2651–2657.
3. Hohn DC, Stagg RJ, Friedman MA, et al. A randomized trial of continuous intravenous versus hepatic intraarterial floxuridine in patients with colorectal cancer metastatic to the liver: the Northern California Oncology Group trial. J Clin Oncol 1989; 7:1646–1654.
4. Kemeny N, Daly J, Reichman B, Geller N, Botet J, Oderman P. Intrahepatic or systemic infusion of fluorodeoxyuridine in patients with liver metastases from colorectal carcinoma. A randomized trial. Ann Intern Med 1987; 107:459–465.
5. Martin JK Jr, O'Connell MJ, Wieand HS, et al. Intra-arterial floxuridine vs systemic fluorouracil for hepatic metastases from colorectal cancer. A randomized trial. Arch Surg 1990; 125:1022–1027.
6. Chang AE, Schneider PD, Sugarbaker PH, Simpson C, Culnane M, Steinberg SM. A prospective randomized trial of regional versus systemic continuous 5-fluorodeoxyuridine chemotherapy in the treatment of colorectal liver metastases. Ann Surg 1987; 206:685–693.
7. Kemeny MM, Goldberg D, Beatty JD, et al. Results of a prospective randomized trial of continuous regional chemotherapy and hepatic resection as treatment of hepatic metastases from colorectal primaries. Cancer 1986; 57:492–498.
8. Rougier P, Laplanche A, Huguier M, et al. Hepatic arterial infusion of floxuridine in patients with liver metastases from colorectal carcinoma: long-term results of a prospective randomized trial. J Clin Oncol 1992; 10:1112–1118.
9. Allen-Mersh TG, Earlam S, Fordy C, Abrams K, Houghton J. Quality of life and survival with continuous hepatic-artery floxuridine infusion for colorectal liver metastases. Lancet 1994; 344:1255–1260.
10. Kerr DJ, McArdle CS, Ledermann J, et al. Intrahepatic arterial versus intravenous fluorouracil and folinic acid for colorectal cancer liver metastases: a multicentre randomised trial. Lancet 2003; 361:368–373.
11. Kemeny NE, Niedzwiecki D, Hollis DR, et al. Hepatic arterial infusion versus systemic therapy for hepatic metastases from colorectal cancer: a randomized trial of efficacy, quality of life, and molecular markers (CALGB 9481). J Clin Oncol 2006; 24:1395–1403.
12. Lorenz M, Muller HH. Randomized, multicenter trial of fluorouracil plus leucovorin administered either via hepatic arterial or intravenous infusion versus fluorodeoxyuridine administered via hepatic arterial infusion in patients with nonresectable liver metastases from colorectal carcinoma. J Clin Oncol 2000; 18:243–254.
13. Allen-Mersh TG, Glover C, Fordy C, Mathur P, Quinn H. Randomized trial of regional plus systemic fluorinated pyrimidine compared with systemic fluorinated pyrimidine in treatment of colorectal liver metastases. Eur J Surg Oncol 2000; 26:468–473.
14. Reappraisal of hepatic arterial infusion in the treatment of nonresectable liver metastases from colorectal cancer. Meta-Analysis Group in Cancer. J Natl Cancer Inst 1996; 88:252–258.
15. Harmantas A, Rotstein LE, Langer B. Regional versus systemic chemotherapy in the treatment of colorectal carcinoma metastatic to the liver. Is there a survival difference? Meta-analysis of the published literature. Cancer 1996; 78:1639–1645.
16. Mocellin S, Pilati P, Lise M, Nitti D. Meta-analysis of hepatic arterial infusion for unresectable liver metastases from colorectal cancer: the end of an era? J Clin Oncol 2007; 25:5649–5654.
17. Arai Y, Sone Y, Inaba Y, Ariyoshi Y, Kido C. Hepatic arterial infusion chemotherapy for liver metastases from breast cancer. Cancer Chemother Pharmacol 1994; 33(suppl):S142–S144.
18. Arai Y, Inaba Y, Takeuchi Y. Interventional techniques for hepatic arterial infusion chemotherapy. In: Castaneda-Zuniga WR, Tadavarthy SM, eds. Interventional radiology, 3rd ed. Baltimore: Williams and Wilkins, 1997;192–205.
19. Arai Y, Takeuchi Y, Inaba Y, et al. Percutaneous catheter placement for hepatic arterial infusion chemotherapy. Tech Vasc Interv Radiol 2007; 10:30–37.
20. Seki H, Kimura M, Kamura T, Miura T, Yoshimura N, Sakai K. Hepatic perfusion abnormalities during treatment with hepatic arterial infusion chemotherapy: value of CT arteriography using an implantable port system. J Comput Assist Tomogr 1996; 20:343–348.
21. Arai Y, Inaba Y, Matsueda K, Ariyoshi Y. Weekly 5 hour hepatic arterial infusion of high dose 5FU for unresectable liver metastases from colorectal cancer in patients without extra-hepatic lesions [ASCO abstract 1098]. Presented at the American Society of Clinical Oncology 34th Annual Meeting, May 17–19, 1998, Los Angeles, California, p. 285a.
22. Arai Y, Takeuchi Y, Inaba Y, et al. Hepatic arterial infusion chemotherapy for liver metastases from digestive cancer. Gan To Kagaku Ryoho 2006; 33:1231–1235.
23. Arai Y, Inaba Y, Takeuchi Y, Ariyoshi Y. Intermittent hepatic arterial infusion of high-dose 5FU on a weekly schedule for liver metastases from colorectal cancer. Cancer Chemother Pharmacol 1997; 40:526–530.
24. Kemeny N, Gonen M, Sullivan D, et al. Phase I study of hepatic arterial infusion of floxuridine and dexamethasone with systemic irinotecan for unresectable hepatic metastases from colorectal cancer. J Clin Oncol 2001; 19:2687–2695.
25. Ganeshan A, Upponi S, Hon LQ, Warakaulle D, Uberoi R. Hepatic arterial infusion of chemotherapy: the role of diagnostic and interventional radiology. Ann Oncol 2008; 19:847–851.
26. Tanaka T, Arai Y, Inaba Y, et al. Radiologic placement of side-hole catheter with tip fixation for hepatic arterial infusion chemotherapy. J Vasc Interv Radiol 2003; 14:63–68.
27. Deschamps F, Elias D, Goere D, et al. Intra-arterial hepatic chemotherapy: a comparison of percutaneous versus surgical implantation of port-catheters. Cardiovasc Intervent Radiol 2011; 34:973–979.
28. Hirota T, Yamagami T, Tanaka O, Iida S, Kato T, Nishimura T. Catheter redundancy in the aortic arch increases the risk of stroke in left subclavian arterial port-catheter systems. J Vasc Interv Radiol 2005; 16:471–476.
29. Hirota T, Yamagami T, Tanaka O, et al. Brain infarction after percutaneous implantation of port-catheter system via the left subclavian artery. Br J Radiol 2002; 75:799–804.
30. Collins JM. Pharmacologic rationale for regional drug delivery. J Clin Oncol 1984; 2:498–504.
31. Douillard JY, Cunningham D, Roth AD, et al. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. Lancet 2000; 355:1041–1047.
32. Giacchetti S, Perpoint B, Zidani R, et al. Phase III multicenter randomized trial of oxaliplatin added to chronomodulated fluorouracil-leucovorin as first-line treatment of metastatic colorectal cancer. J Clin Oncol 2000; 18:136–147.
33. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 2004; 350:2335–2342.
34. Cascinu S, Berardi R, Salvagni S, et al. A combination of gefitinib and FOLFOX-4 as first-line treatment in advanced colorectal cancer patients. A GISCAD multicentre phase II study including a biological analysis of EGFR overexpression, amplification and NF-kB activation. Br J Cancer 2008; 98:71–76.
35. Kemeny N, Jarnagin W, Paty P, et al. Phase I trial of systemic oxaliplatin combination chemotherapy with hepatic arterial infusion in patients with unresectable liver metastases from colorectal cancer. J Clin Oncol 2005; 23:4888–4896.
36. Ducreux M, Ychou M, Laplanche A, et al. Hepatic arterial oxaliplatin infusion plus intravenous chemotherapy in colorectal cancer with inoperable hepatic metastases: a trial of the gastrointestinal group of the Federation Nationale des Centres de Lutte Contre le Cancer. J Clin Oncol 2005; 23:4881–4887.

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IGFBP7 downregulation is associated with tumor progression and clinical outcome in hepatocellular carcinoma

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Insulin-like growth factor-binding protein 7 (IGFBP7) functions in several cellular processes including proliferation, senescence and apoptosis. This study analyzed IGFBP7 function in hepatocellular carcinoma (HCC) cells by gene manipulation and investigated the prognostic significance of IGFBP7 expression in clinical HCC samples. In this study, we investigated changes in malignant potential such as cell growth and invasiveness in an HCC cell line, PLC/PRF/5, after transfection with shRNA against *IGFBP7*. The extent of apoptosis and cell cycle progression were examined after the transfection. The correlation between immunohistochemically determined IGFBP7 expression and long-term postoperative prognosis after curative resection was also investigated in clinical HCC specimens obtained from 104 patients. PLC/PRF/5 cells transfected with shRNA against *IGFBP7* showed significantly more rapid growth and stronger invasiveness than control cells. Annexin V assays showed that the IGFBP7-depleted cells were significantly more resistant to apoptosis than the control cells, and showed decreased expression of cleaved caspase-3 and PARP. Cell cycle progression was more rapid in the IGFBP7-suppressed cells. In clinical HCC specimens, IGFBP7 expression was judged as positive in 67 patients (64.4%) and negative in the remaining 37 patients (35.6%). The IGFBP7 downregulation correlated significantly with poor postoperative prognosis, and IGFBP7 status was identified as an independent significant prognostic factor. Our results indicated that IGFBP7 expression correlated significantly with the malignant potential in HCC cells, suggesting that the expression could be a useful prognostic marker for HCC.

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Hepatocellular carcinoma (HCC) is a common malignancy worldwide, but especially in Japan and other East Asian countries.[1,2] Although surgery plays a major role in the treatment of HCC, less than 30% of patients with HCC are surgical candidates owing to limiting factors such as severe impairment of reserve hepatic function, bilobar tumor distribution and extrahepatic metastasis. Additionally, no effective chemotherapy regimens have been established for treating HCC.[3] Thus, no effective therapy can be offered in many cases of HCC. Such dismal prognosis is not always predicted by conventional prognostic indicators such as vascular invasion, tumor multiplicity and tumor size.[4–6] New indicators are thus clearly needed.

Insulin-like growth factor binding protein 7 (IGFBP7), which is also known as IGFBP-rP1 and MAC25, has been implicated in several cellular processes such as proliferation, senescence and apoptosis. IGFBP7 also shows tumor suppressive activity through the induction of apoptosis and it is downregulated in some cancers.[7–13] In addition, several studies found a significant association between IGFBP7 and not only apoptosis, but also prognosis, in some kinds of cancers including colorectal and breast cancer.[14,15] However, the functional significance of IGFBP7 in HCC remains unclear.

This study analyzed the function of IGFBP7 in HCC cells in gene manipulation experiments, and investigated the prognostic significance of IGFBP7 expression in clinical HCC samples by immunohistochemical analysis of resected specimens.

Key words: hepatocellular carcinoma (HCC), insulin-like growth factor binding protein 7 (IGFBP7), apoptosis

Abbreviations: 95% CI: 95% confidence interval; AFP: alpha-fetoprotein; Anti-HCV Ab: anti-hepatic C virus antibody; DFS: disease-free survival; HBs-Ag: hepatitis B surface antigen; HCC: hepatocellular carcinoma; IGFBP7: insulin-like growth factor binding protein 7; OR: odds ratio; OS: overall survival; PBGD: porphobilinogen deaminase; pERK: phoshorylated ERK; PI: propidium iodide; PIVKA-II: protein induced by vitamin K absence or antagonists-II; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; shRNA: short hairpin RNA

DOI: 10.1002/ijc.25994

History: Received 6 Sep 2010; Accepted 2 Feb 2011; Online 16 Feb 2011

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Material and Methods

HCC cell lines and clinical tissue specimens

Four human HCC cell lines, PLC/PRF/5, HuH7, HLE and HepG2 were obtained from the Japan Cancer Research

Resources Bank (Tokyo, Japan). These cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified incubator with 5% CO_2 in air.

Surgical specimens were obtained from 104 patients with HCC who underwent curative hepatic resection in the Osaka University Hospital from 2000 to 2007 after informed consent in accordance with the institutional ethical guidelines of Osaka University. Curative resection was defined as complete removal of all macroscopically evident tumors. Patients who underwent transarterial chemoembolization preoperatively were excluded from this study. After hepatic resection, the patients were followed up at regular intervals of 3–4 months with physical examination, assaying of tumor markers including alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonists-II (PIVKA-II), liver biochemistry testing, abdominal ultrasonography and abdominal computed tomography. The median duration of clinical follow-up after the initial hepatectomy was 3.5 ± 2.3 years.

Drugs and reagents

A polyclonal goat anti-human IGFBP7 antibody and polyclonal rabbit anti-human IGFBP7 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used for immunohistochemistry and western blot analysis, respectively. Antibodies to caspase-3, cleaved caspase-3, PARP, cleaved PARP, ERK, phoshorylated ERK (pERK), cyclin D1 and p27 were purchased from Cell Signaling Technology (Beverly, MA), antibodies to cyclin E and p21 were purchased from Santa Cruz Biotechnology, and an antibody to actin was purchased from Sigma-Aldrich Co. (Louis, MO).

Plasmids and transfection

Plasmid coding for short hairpin RNA (shRNA) against *IGFBP7* and *IGFBP7* expression plasmid were purchased from OriGene Technologies (Rockville, MD) and used to transfect HCC cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the instructions provided by the manufacturer. After transfection of the shRNA plasmid and the *IGFBP7* expression plasmid into the HCC cells for 24 hr, stable transfectants were selected and maintained in 1.0 μg/ml of puromycin (Sigma-Aldrich, St. Louis, MO) and 600 μg/ml of G418 (Gibco-BRL, Grand Island, NY), respectively. The control vector plasmid expressing non-effective shRNA was similarly introduced into cells to establish negative control cells for the shRNA plasmid experiments. Empty vector plasmid was also similarly used to establish negative control cells for the *IGFBP7* expression plasmid for the *IGFBP7* expression plasmid experiments.

Cell proliferation assay

Cells were uniformly seeded (4×10^4/well for PLC/PRF/5 and 2×10^4/well for HuH7) in triplicates into 24-well dishes (Day 0). Cells were counted using a CellTac kit (Nihon Koden, Tokyo, Japan) on Days 1–5.

Real-time quantitative reverse transcription-polymerase chain reaction

Total RNA isolated from cells was prepared using TRIzol reagent (Invitrogen), and reverse transcription was performed with SuperScript II (Invitrogen) based on the protocols supplied by the manufacturer. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using the Light Cycler (Roche Diagnostics, Mannheim, Germany), and the amount of target gene expression was calculated. The expression of the target gene was normalized relative to the expression of *porphobilinogen deaminase* (*PBGD*), which was used as an internal control. The designed PCR primers were as follows; *IGFBP7* forward primer; 5′-CTGGGTGCTGGTATCTCCTC-3′; *IGFBP7* reverse primer; 5′-TATAGCTCGGCACCTTCACC-3′; *SMARCB1* forward primer; 5′-TCTGGATTTGAACCCGCTGA-3′; *SMARCB1* reverse primer; 5′-TGCTGTATGCGATGGTGGTG-3′; *BNIP3L* forward primer; 5′-CGGACTCGGCTTGTTGTGTT-3′; *BNIP3L* reverse primer; 5′-ATGGGTAGCTCCACCCAGGA-3′; *PBGD* forward primer; 5′-TGTCTGGTAACGGCAATGCGGCTGCAAC-3′; *PBGD* reverse primer; 5′-TCAATGTTGCCACCACACTGTCCGTCT-3′

Western blot analysis

Cells grown to semiconfluence were washed and collected with a rubber scraper. After centrifugation, the cell pellets were resuspended, and the extracts were centrifuged and the supernatant fraction was collected. Western blot analysis was carried out as described previously.[16,17] The expression of the target protein was evaluated by comparison to the expression of actin.

Annexin V assay

The binding of annexin V was used here as a sensitive assessment of apoptosis, as described previously.[17,18] Cells were stained by Annexin V-APC and propidium iodide (PI) (BD Biosciences, Franklin Lakes, NJ), and analyzed on a FACS Aria (BD Biosciences).

Invasion assay

The invasion assay was performed using transwell culture chambers (BD Biosciences) according to the instructions provided by the manufacturer. The upper chamber was loaded with cell suspension and the lower chamber was loaded with 10% FBS. After incubation (48 hr for PLC/PRF/5 and 24 hr for HuH7), cells that had invaded the undersurface of the membrane were counted under a microscope. Four microscopic fields were randomly selected for cell counting.

Cell cycle analysis

Cell cycle analysis was performed based on flow cytometric analysis, as described previously.[19] Briefly, cells were washed and fixed. PI and RNase (Sigma-Aldrich) were then added, and data were acquired on the FACS Calibur (BD Biosciences). The cell cycle analysis was carried out using ModFIT software (BD Biosciences).

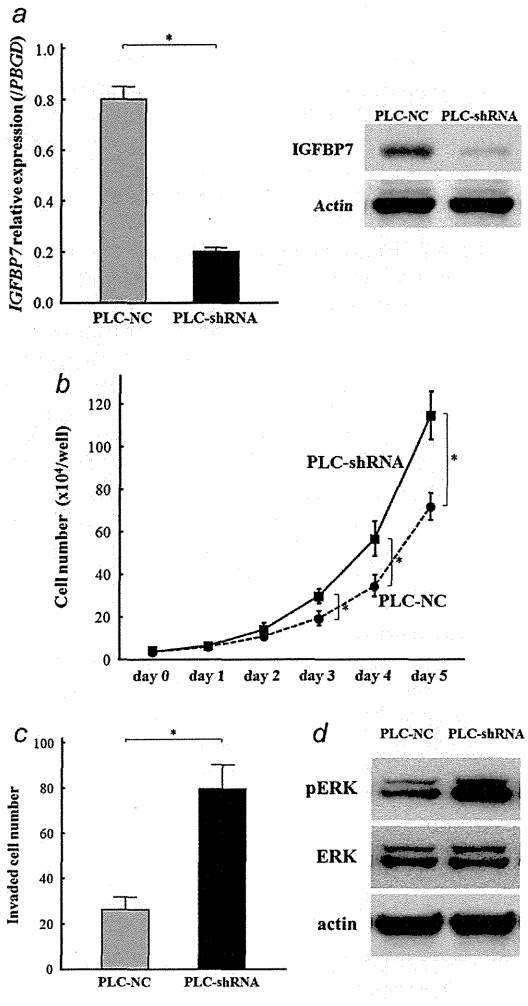

Figure 1. Characteristics of PLC/PRF/5 transfected with shRNA against *IGFBP7*. (*a*) qRT-PCR (Left panel) and western blot analysis (Right panel) indicated a significant decrease in IGFBP7 in cells transfected with shRNA compared to control cells (*$p < 0.05$). (*b*) Proliferation assays showed significantly quicker growth in the IGFBP7-suppressed cells compared with the control cells (*$p < 0.05$). (*c*) The invasion assay showed that the invasive ability of the IGFBP7-suppressed cells was significantly greater than that of the control cells (*$p < 0.05$). (*d*) Western blot analysis demonstrated significantly increased pERK expression in the IGFBP7-supperessed cells compared to the control cells. Data are mean ± SD of 3 experiments.

Immunohistochemical staining

Resected tissue samples were fixed in 10% buffered formalin and finally embedded in paraffin. Immunohistochemical staining for IGFBP7 in the same samples was performed as described previously.[17,20] Briefly, after deparaffinization and blocking, the sections were incubated overnight at 4°C with polyclonal goat anti-human IGFBP7 antibody, and then counterstained with Mayer's hematoxylin. IGFBP7 expression was defined as the presence of specific staining in the cytoplasm of cancer cells. IGFBP7 expression was evaluated as positive or negative, as previously prescribed.[17]

Statistical analysis

Data are expressed as mean ± SD. Differences between groups were assessed using the χ^2-test, and continuous variables were compared using Student's *t*-test. Survival rates were calculated according to the Kaplan-Meier method and compared using the log-rank test. Statistical analysis was performed using StatView (version 5.0; SAS Institute, Cary, NC). A p value < 0.05 was considered statistically significant.

Results

In vitro studies

IGFBP7 downregulation promotes proliferation and invasive activity. First, *IGFBP7* expression was examined by qRT-PCR in 4 HCC cell lines, PLC/PRF/5, HuH7, HLE and HepG2. The *IGFBP7* expression levels in PLC/PRF/5 and HuH7 were the highest and lowest of the 4 cell lines, respectively. A plasmid coding for shRNA against *IGFBP7* was then transfected into PLC/PRF/5, whose IGFBP7 expression level was the highest in the 4 cell lines. The IGFBP7 expression was suppressed by the transfection, as confirmed by qRT-PCR and western blot analysis (Fig. 1*a*). The proliferation assay showed significantly more rapid growth in the IGFBP7-suppressed cells compared to control cells (Fig. 1*b*). In addition, the invasive ability of the IGFBP7-suppressed cells was significantly greater than that of the control cells (Fig. 1*c*). Based on previous studies that IGFBP7 downregulation promotes cell proliferation through ERK signaling, we analyzed the levels of total ERK and pERK in our cells.[10,21] pERK expression was significantly increased in the IGFBP7-supperessed cells, while total ERK expression was not changed, which coincided with previous reports (Fig. 1*d*). On the other hand, as we previously reported, there were no significant differences in the expression of total Akt or phoshorylated Akt between the IGFBP7-supperessed cells and the control cells.[17]

Downregulation of IGFBP7 attenuates apoptosis. The extent of apoptosis of these cells was examined by the Annexin V assay. The percentages of early apoptotic cells and late apoptotic cells defined by Annexin V-positive/PI-negative cells and Annexin V-positive/PI-positive cells respectively were significantly lower in the IGFBP7-suppressed cells than those in the control cells (Fig. 2*a*).[22] This significant difference of the extent of apoptosis was also confirmed under the condition where apoptosis is induced by some agents, which was reported previously.[17] Next, the expression of proteins related to apoptosis was examined. The result showed that cleaved caspase-3 and cleaved PARP are significantly decreased in

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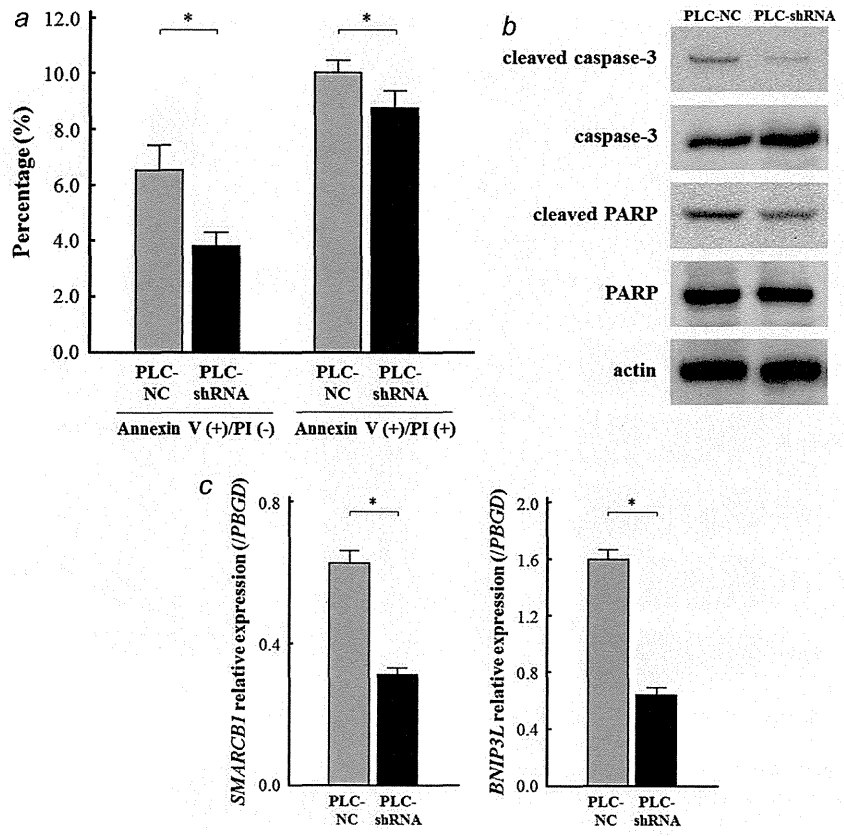

Figure 2. The extent of apoptosis evaluated by the amount of apoptotic cells and the expression of apoptosis-related molecules. (*a*) The Annexin V assay showed the percentage of cells in early apoptosis and in late apoptosis defined by Annexin V-positive/PI-negative cells and Annexin V-positive/PI-positive cells respectively were significantly lower in the IGFBP7-suppressed cells than the control cells (*$p < 0.05$). (*b*) Cleaved caspase-3 and cleaved PARP were decreased in the IGFBP7-suppressed cells. (*c*) qRT-PCR indicated that *SMARCB1* (Left panel) and *BNIP3L* (Right panel) expression levels were significantly decreased in the IGFBP7-supressed cells than the control cells (*$p < 0.05$). Data are mean ± SD of 3 experiments.

the IGFBP7-suppressed cells, while total caspase-3 and PARP expressions were not changed (Fig. 2*b*). In addition, since apoptosis induced by IGFBP7 was reported to occur *via* SMARCB1 and BNIP3L upregulation, we also evaluated the expression levels of *SMARCB1* and *BNIP3L* by qRT-PCR.[10] The results showed that *SMARCB1* and *BNIP3L* expressions were significantly decreased in the IGFBP7-supressed cells compared with the control cells (Fig. 2*c*).

Downregulation of IGFBP7 promotes cell cycle progression. The influence of IGFBP7 on cell cycle was examined by flow cytometric analysis. Prior to the examination, cells were synchronized in the G_0/G_1 phase by serum starvation for 72 hr, and then put back in the regular medium with 10% fetal bovine serum. Dynamic changes in percentage between G_0/G_1 phase and S phase are shown in Figure 3*a*. The proportion of G_0/G_1 phase and S phase on the end of the starvation (0 hr) was almost comparable between the IGFBP7-suppressed cells and the control cells. As shown in Figure 4, the time with minimum percentage of G_0/G_1 phase and maximum percentage of S phase was 24 hr in the control cells, while the time was 12 hr in the IGFBP7-suppressed cells, which suggests that the cell cycle progression was more rapid in the IGFBP7-suppressed cells than that in the control cells. Furthermore, we found that cyclin D1 and cyclin E were increased and p27 was decreased in the IGFBP7-suppressed cells than those in the control cells, and that there was no significant difference in p21 expression between the 2 cells, which was agreement with more rapid cell cycle progression in the IGFBP7-suppressed cells (Fig. 3*b*).

Transfection of IGFBP7 attenuates proliferation and invasive activity. Next, the *IGFBP7* expression plasmid was transfected into HuH7, whose IGFBP7 expression level was the lowest in the 4 HCC cell lines. The IGFBP7 expression was increased by