Table 2. Comparison of US with conventional CT

	(*n*)	US	Conventional CT	*p* value
Tumor detection rate				
Total		98% (96/98)	79% (77/98)	<0.001
T-stage				
Tis	7	100% (7/7)	57% (4/7)	0.192
T1	19	89% (17/19)	26% (5/19)	<0.001
T2	7	100% (7/7)	100% (7/7)	0.999
T3	46	100% (46/46)	93% (43/46)	0.240
T4	19	100% (19/19)	100% (19/19)	0.999
Tumor size				
≤30 mm	33	94% (31/33)	48% (16/33)	<0.001
>30 mm	65	100% (65/65)	97% (63/65)	0.990
Accuracy of tumor infiltration depth				
T3	46	67% (31/46)	72% (33/46)	0.650
T4	19	47% (9/19)	42% (8/19)	0.742

Table 3. Agreement in T-stage (three-tier method)

US-stage	Staged by histopathology			
	Tis/T1	T2	T3/T4	Total (*n*)
Tis/T1	**21**	2	1	24
T2	3	**4**	4	11
T3/T4	0	1	**60**	61
Total (*n*)	24	7	65	96
Sensitivity	88%	57%	92%	
Specificity	96%	92%	97%	
PPV	88%	36%	98%	
NPV	96%	97%	86%	
Accuracy	94%	90%	94%	

Bold values indicate the agreement between US and pathologic diagnosis
Diagnostic agreement: 89% (85/96), $\kappa = 0.77$ (0.64–0.90), $p < 0.001$

diagnosed by colonoscopy appeared quite adequate. However, if US were utilized for screening purposes, blinding sonographers to findings by colonoscopy or CT, the tumor detection rate would be lower.

Compared with conventional CT, US achieved a higher rate of tumor detection and comparable accuracy in diagnosing stage T3 and T4 lesions. However, CT examinations were routinely performed to evaluate metastasis (nodal and distant) and to differentiate stages T3 and T4, not to detect tumors of small size or to assign T-stage by evaluating layers of colonic wall. In fact, recent studies underscore the high performance level of multidetector CT in assessing T-stage of colorectal cancer, showing diagnostic accuracies of 70–96% at ≤T2 level, 80–97% at T3 level, and 89–100% at T4 level [15–17]. Hence, the superiority of US in detecting colonic tumors and determining T-stage is not proven by this study. Nevertheless, our findings do suggest that the potential for detecting colonic tumors by US is greater than heretofore appreciated and is at least non-inferior to conventional CT. Consequently, the role of US in preoperative investigation of colon cancer should be reconsidered.

This prospective study demonstrated a somewhat unimpressive 64% (61/96) rate of agreement in US and histopathology T-stage tumor assessments, indicating moderate reproducibility ($\kappa = 0.48$). Compared with tumors below or within MP, where diagnostic accuracy was >90%, the diagnostic accuracy of US for stage T3 and T4 tumors was <80%. The latter was attributed to an ill-defined subserosal fat layer and poorly demarcated serosal border, which presently may be the limiting factors in diagnosing stage T3 and T4 colon cancer by transabdominal US.

Evaluating colon cancer by US relies on locating the relatively thick and thus recognizable MP layer, as shown in Figs. 2, 3, 4, 5 and 6. To render preoperative US staging of colon cancer more practical and convenient, we introduced three strata: below, within, and beyond MP. In Japan, D2 or D3 lymph node dissection is recommended for tumor within MP, whereas D3 lymph node dissection is usually done for tumors beyond MP (per domestic guidelines) [13]. In addition, tumor invasion beyond MP is predictive of a poor prognosis in patients with colorectal cancer [1], including a considerably higher risk of lymph node metastasis (from 21% at stage T2 to >40% at stage T3 and beyond) [13]. Thus, a precise diagnosis, hinged upon involvement of the MP layer, is warranted prior to surgery. Extension of tumor beyond serosa has little impact on surgical planning, provided other organs are not invaded. Our modified approach yielded better diagnostic agreement, increasing

Table 4. Diagnostic accuracy of US at various tumor locations

	(*n*)	Diagnostic agreement	
		Standard	Three-tier
Cecum	9	4 (44%)	7 (78%)
Ascending colon	30	18 (60%)	28 (93%)
Transverse colon	17	13 (76%)	17 (100%)
Descending colon	7	5 (71%)	6 (86%)
Sigmoid colon	33	21 (64%)	27 (81%)

Table 5. Univariate analysis of factors impacting T-stage determination

Variable	(*n*)	Standard method			Three-tier method		
		Agreement (*n* = 61)	Disagreement (*n* = 35)	*p* value	Agreement (*n* = 85)	Disagreement (*n* = 11)	*p* value
Gender							
Male	58	40	18	0.173	51	7	0.924
Female	38	21	17		34	4	
Age (years)							
<70	53	35	18	0.573	49	4	0.311
≥70	43	26	17		36	7	
Body mass index (kg/m^2)							
<25	74	46	28	0.607	64	10	0.436
≥25	22	15	7		21	1	
Hemoglobin (mg/dL)							
<10	22	13	9	0.621	19	3	0.987
≥10	74	48	26		66	8	
Albumin (mg/dL)							
<3.5	11	5	6	0.321	9	2	0.810
≥3.5	85	56	29		76	9	
C-reactive protein (mg/dL)							
<0.2	58	40	18	0.173	53	5	0.453
≥0.2	38	21	17		32	6	
Tumor size (cm)							
<5	61	42	19	0.154	52	9	0.315
≥5	35	19	16		33	2	
Mural thickness (mm)							
<10	37	25	12	0.516	32	5	0.864
≥10	59	36	23		53	6	
Tumor differentiation							
tub1	31	19	12	0.901	26	5	0.251
tub2	55	36	19		49	6	
others	10	6	4		10	0	
Distant metastasis							
Yes	20	11	9	0.372	20	0	0.157
No	76	50	26		65	11	

tub1, Well-differentiated; tub2, moderately differentiated

the accuracy of diagnosing tumor invasion beyond MP (stages T3 and T4) to 94% (90/96), with improved sensitivity (92%, 60/65) and specificity (97%, 30/31), as shown in Table 3. These findings suggest that this stratification is more appropriate and should be implemented. Unfortunately, we have no data to illustrate how preoperative US assessment impacts surgical planning in actual practice, so any related clinical benefits must await future investigations.

In early colorectal cancer, endoscopic ultrasonography is pivotal in determining treatment strategy (i.e., endoscopic mucosal resection and submucosal dissection or surgery), owing to its high accuracy (>80%) [18–22]. Herein, diagnostic agreement in tumors extending below MP was relatively high (88%; 23/26); but our sample population was small, and sensitivity was only about 70% at stages Tis and T1. With this in mind, it seems premature and speculative to choose a treatment strategy (endoscopic or surgical) accordingly. With respect to T2 staging, only seven patients were studied, so more data is clearly needed. It is our hope that future studies, enlisting a larger number of patients, will validate the instrumental role of US in determining appropriate treatment options for colon cancer.

In this study, tumors at various locations in the colon showed significant diagnostic agreement by both methods utilized (Table 4), with exception of cecum and sigmoid colon. Diameter of the cecum is usually larger than elsewhere in the colon, with copious feces and gas present. This anatomic disadvantage and the high echoic

quality attached may hamper any assessment of infiltrated layers. In the sigmoid colon, the relatively poor diagnostic accuracy seemed to result from individual variations in length and location and any co-existence of small intestine in the pelvic cavity. Appropriate attention must be paid when interpreting lesions in these locations. None of the tumor characteristics and patient demographics analyzed (Table 5), including BMI (with an analytic cutpoint of 25) seemed to influence the accuracy of T-stage determinations. However, this study was confined to Japanese subjects, with typically lower BMIs than Europeans and Americans. Indeed, only four patients with BMIs $>$ 30 were included. Higher values should be addressed to better determine if BMI is a factor in the accuracy of diagnostic US.

Another problem related to preoperative US assessment is the high level of technical skills that are required. In fact, this study was conducted under the guidance of a seasoned expert who reviewed movie clips and provided coaching in real time; and because US is not often used in investigations of colorectum, more experienced operators are needed. Structured educational programs that help sonographers gain experience and expertise in colorectal examinations may help overcome the technical hurdles to widespread use of US for assessments of this nature.

Conclusions

Use of US to preoperatively evaluate T-stage in patients with colon cancer is in developmental stages but should be promoted to improve surgical outcomes and limit the discomfort of patients in this setting.

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References

1. Smith NJ, Bees N, Barbachano Y, et al. (2007) Preoperative computed tomography staging of nonmetastatic colon cancer predicts outcome: implications for clinical trials. Br J Cancer 96:1030–1036
2. O'Malley ME, Wilson SR (2003) US of gastrointestinal tract abnormalities with CT correlation. Radiographics 23:59–72
3. Maconi G, Radice E, Bareggi E, Porro GB (2009) Hydrosonography of the gastrointestinal tract. AJR Am J Roentgenol 193:700–708
4. Shirahama M, Koga T, Ishibashi H, Uchida S, Ohta Y (1994) Sonographic features of colon carcinoma seen with high-frequency transabdominal ultrasound. J Clin Ultrasound 22:359–365
5. Fujii Y, Taniguchi N, Koibuchi H, Yasuda Y, Nagai H (2008) Compressibility of gastrointestinal tract tumors during transabdominal sonographic examination: a clue to the diagnosis of gastrointestinal lymphoma. J Clin Ultrasound 36:59–62
6. Martinez-Ares D, Alonso-Aguirre PA, Souto-Ruzo J, et al. (2009) Ultrasonography is an accurate technique for the diagnosis of gastrointestinal tumors in patients without localizing symptoms. Rev Esp Enferm Dig 101:773–786
7. Tomizawa M, Shinozaki F, Hasegawa R, et al. (2013) Screening ultrasonography is useful for the diagnosis of gastric and colorectal cancer. Hepatogastroenterology 60:517–521
8. Lim JH, Ko YT, Lee DH (1994) Transabdominal US staging of gastric cancer. Abdom Imaging 19:527–531
9. Segura JM, Olveira A, Conde P, Erdozain JC, Suarez J (1999) Hydrogastric sonography in the preoperative staging of gastric cancer. J Clin Ultrasound 27:499–504
10. Liao SR, Dai Y, Huo L, et al. (2004) Transabdominal ultrasonography in preoperative staging of gastric cancer. World J Gastroenterol 10:3399–3404
11. Ishigami S, Yoshinaka H, Sakamoto F, et al. (2004) Preoperative assessment of the depth of early gastric cancer invasion by transabdominal ultrasound sonography (TUS): a comparison with endoscopic ultrasound sonography (EUS). Hepatogastroenterology 51:1202–1205
12. Chaubal N, Dighe M, Shah M, Chaubal J (2006) Sonography of the gastrointestinal tract. J Ultrasound Med 25:87–97
13. Watanabe T, Itabashi M, Shimada Y, et al. (2012) Japanese Society for Cancer of the Colon and Rectum (JSCCR) guidelines 2010 for the treatment of colorectal cancer. Int J Clin Oncol 17:1–29
14. Acunas B, Rozanes I, Acunas G, et al. (1990) Preoperative CT staging of colon carcinoma (excluding the recto-sigmoid region). Eur J Radiol 11:150–153
15. Tezcan D, Turkvatan A, Turkoglu MA, et al. (2013) Preoperative staging of colorectal cancer: accuracy of single portal venous phase multidetector computed tomography. Clin Imaging 37:1048–1053
16. Sinha R, Verma R, Rajesh A, et al. (2006) Diagnostic value of multidetector row CT in rectal cancer staging: comparison of multiplanar and axial images with histopathology. Clin Radiol 61:924–931
17. Mainenti PP, Cirillo LC, Camera L, et al. (2006) Accuracy of single phase contrast enhanced multidetector CT colonography in the preoperative staging of colo-rectal cancer. Eur J Radiol 60:453–459
18. Lin S, Luo G, Gao X, et al. (2011) Application of endoscopic sonography in preoperative staging of rectal cancer: six-year experience. J Ultrasound Med 30:1051–1057
19. Santoro GA, Gizzi G, Pellegrini L, Battistella G, Di Falco G (2009) The value of high-resolution three-dimensional endorectal ultrasonography in the management of submucosal invasive rectal tumors. Dis Colon Rectum 52:1837–1843
20. Harewood GC (2005) Assessment of publication bias in the reporting of EUS performance in staging rectal cancer. Am J Gastroenterol 100:808–816
21. Nesbakken A, Lovig T, Lunde OC, Nygaard K (2003) Staging of rectal carcinoma with transrectal ultrasonography. Scand J Surg 92:125–129
22. Garcia-Aguilar J, Pollack J, Lee SH, et al. (2002) Accuracy of endorectal ultrasonography in preoperative staging of rectal tumors. Dis Colon Rectum 45:10–15

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Original Article

Multiplication of alpha-fetoprotein and protein induced by vitamin K absence-II is a powerful predictor of prognosis and recurrence in hepatocellular carcinoma patients after a hepatectomy

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Aim: To evaluate the oncological implications of multiplication of α-fetoprotein (AFP) and protein induced by vitamin K absence or antagonists-II (PIVKA-II) in patients with hepatocellular carcinoma (HCC).

Methods: Data were prospectively collected from 516 consecutive patients who underwent a curative primary hepatectomy for HCC between 1998 and 2010. The AP-factor (AFP × PIVKA-II) was evaluated in relation to 2-year survival outcomes by receiver–operator curve analysis to determine the cut-off values. Patient survival, recurrence-free survival and risk factors were analyzed in accordance with the preoperative AP-factor.

Results: The AP-factor was categorized into three groups depending on the serum concentrations of AFP and PIVKA-II as follows: AP1 (n = 206; AFP < 200 ng/mL and PIVKA-II < 100 mAU/mL), AP2 (n = 152; AFP × PIVKA-II < 10^5) and AP3 (n = 158; AFP × PIVKA-II ≥ 10^5). The AP-factor was found to be significantly related to pathological factors such as differentiation, portal vein invasion, hepatic vein invasion and intrahepatic metastasis. Multivariate analysis was performed to identify the risk factors for survival and recurrence. Albumin, AP-factor and pathological factors including portal vein invasion, hepatic vein invasion and intrahepatic metastasis are independent risk factors for survival. Tumor number, AP-factor, and a non-cancerous liver were determinants of recurrence.

Conclusion: The AP-factor is closely related to differentiation and microscopic vascular invasion, and was selected by multivariate analysis as an independent factor for survival and recurrence, in HCC. Patients hopeful of obtaining good outcomes after a hepatectomy could be selected by the AP-factor evaluation.

Key words: alpha-fetoprotein, hepatocellular carcinoma, hepatectomy, protein induced by vitamin K absence or antagonists-II, prognosis, recurrence

INTRODUCTION

LIVER RESECTION HAS the highest capacity for local control of hepatocellular carcinoma (HCC) among all local treatment options and results in a good survival rate.[1] However, the recurrence rates of HCC continue to remain high even after curative hepatectomy.[2] Many factors related to the prognosis and recurrence of HCC have been reported with vascular invasion to the portal and/or hepatic vein identified as the most important factor that influences the outcome of hepatic resection.[3] Macroscopic vascular invasion is detectable by ultrasonography (US), computed tomography (CT) and magnetic resonance imaging (MRI). However, microscopic invasion can only be detected by performing a pathological examination just after hepatectomy and cannot be diagnosed preoperatively. Although tumor differentiation is reported to be an independent predictor of a poor outcome,[4] it also cannot be evaluated preoperatively. Hence, the serum levels of α-fetoprotein (AFP) and protein induced by vitamin K absence or antagonism factor-II (PIVKA-II), and the HCC tumor

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size and number are regarded as surrogate markers of microvascular invasion and tumor differentiation.[5,6]

α-Fetoprotein and PIVKA-II have shown utility as tumor markers of HCC and are associated with a poor prognosis after hepatectomy.[7] AFP is related to tumor differentiation,[8] whereas PIVKA-II is related to vascular invasion.[9] Individually, the presence of these tumor markers has less serious implications than microvascular invasion,[10] the latter being the most influential determinant of recurrence and survival in HCC patients undergoing a hepatectomy.[11] However, the oncological implications of determining a numerical value that would account for interaction of both AFP and PIVKA-II, namely, multiplication of the serum levels of AFP and PIVKA-II, have not yet been evaluated.

To further identify factors related to the prognosis and recurrence of HCC, we herein analyze the survival and recurrence outcomes in 516 consecutive patients who underwent a primary curative hepatectomy for HCC at our center. Specifically, we seek to evaluate the oncological implications of numerically determining the serum levels of AFP and PIVKA-II.

METHODS

Patients

BETWEEN JANUARY 1998 and December 2010, 516 consecutive adult patients underwent a hepatectomy for HCC at our center. The remaining patients were classified according to their preoperative serum levels of AFP and PIVKA-II. The mean age of these patients was 61.8 years and the age range was 18–88 years. Of the 516 HCC patients, 425 (82.4%) were male, 222 (43.0%) were hepatitis B virus surface antigen positive, 189 (36.6%) were hepatitis C virus antibody positive and 178 (34.5%) had cirrhosis. The preoperative serum AFP and PIVKA-II levels were simultaneously measured using standard methods at least 2 weeks before hepatectomy, when imaging studies were also performed. AFP was measured using an immune enzymometric assay with a commercially available kit (ST AIA-PACK AFP; TOSOH, Tokyo, Japan). PIVKA-II was measured by chemiluminescent immunoassay using a sensitive des-γ-carboxyprothrombin (DCP) antibody (Picolumi PIVKA-II; Eisai, Tokyo, Japan).

The patient subjects were divided into three groups according to their AFP levels (AFP low, ≤200 ng/mL; AFP mid, 200–1000 ng/mL; and AFP high: >1000 ng/mL). The patients were also divided into three groups according to their PIVKA-II levels (PII low, ≤100 mAU/mL; PII mid, 100–1000 mAU/mL; and PII high, >1000 mAU/mL). We evaluated multiplication of AFP and PIVKA-II to build a model that incorporates interaction effects of covariates of these two tumor markers in multivariate analysis of the Cox proportional hazards model. We evaluated the AP-factor, which was a tumor-related factor, the same as microvascular invasion, based on 2-year survival outcome, because the importance of microvascular invasion in regard to tumor recurrence and early death within 2 years after liver resection was reported in patients with small HCC.[12,13] Receiver–operator curve (ROC) analysis of the AP-factor (a product of the serum levels of AFP and PIVKA-II), AFP and PIVKA-II to evaluate the cut-off values for 2-year survival confirmed that the area under the curve (AUC) of the AP-factor (AUC = 0.74607) is significantly higher than that of AFP (AUC = 0.69804, $P = 0.0271$) and PIVKA-II (AUC = 0.69130, $P = 0.0065$) (Fig. 1). The patients were then classified into three groups in accordance with an AP-factor cut-off value of 10^5 (AUC = 0.74607, sensitivity = 63.27% specificity = 77.41%) as follows: AP1 (AFP < 200 ng/mL and PIVKA-II < 100 mAU/mL), AP2 (AFP × PIVKA-II < 10^5) and AP3 (AFP × PIVKA-II ≥ 10^5). AP1 (AFP < 200 ng/mL and PIVKA-II < 100 mAU/mL) was set accordingly because the 5-year patient survival (PS) rates for AFP low (AFP < 200 ng/mL) and PII low (PIVKA-II < 100 mAU/mL) were 76.4% and 81.3% and significantly higher than the other groups. The clinicopathological characteristics of these groups are summarized in Table 1. Among the 516 HCC patients in our cohort, 499 (96.7%) were categorized as Child–Pugh class A. These patients were followed up for a median of 107.7 months (range, 24.7–185.0). All the analyses in this study were performed in accordance with the ethical guidelines of Hokkaido University Hospital. This study was approved by the institutional review board of Hokkaido University.

Hepatectomy

Anatomical resection is defined as a resection in which lesion(s) are completely removed anatomically on the basis of Couinaud's classification (segmentectomy, sectionectomy and hemihepatectomy or extended hemihepatectomy) in patients with sufficient functional reserve. Non-anatomical partial but complete resection was achieved in our HCC patients. In all patients, R0 resections were performed, and the resection surface was found to be histologically free of HCC. An indocyanine green retention rate at 15 min (ICG-R15) was measured

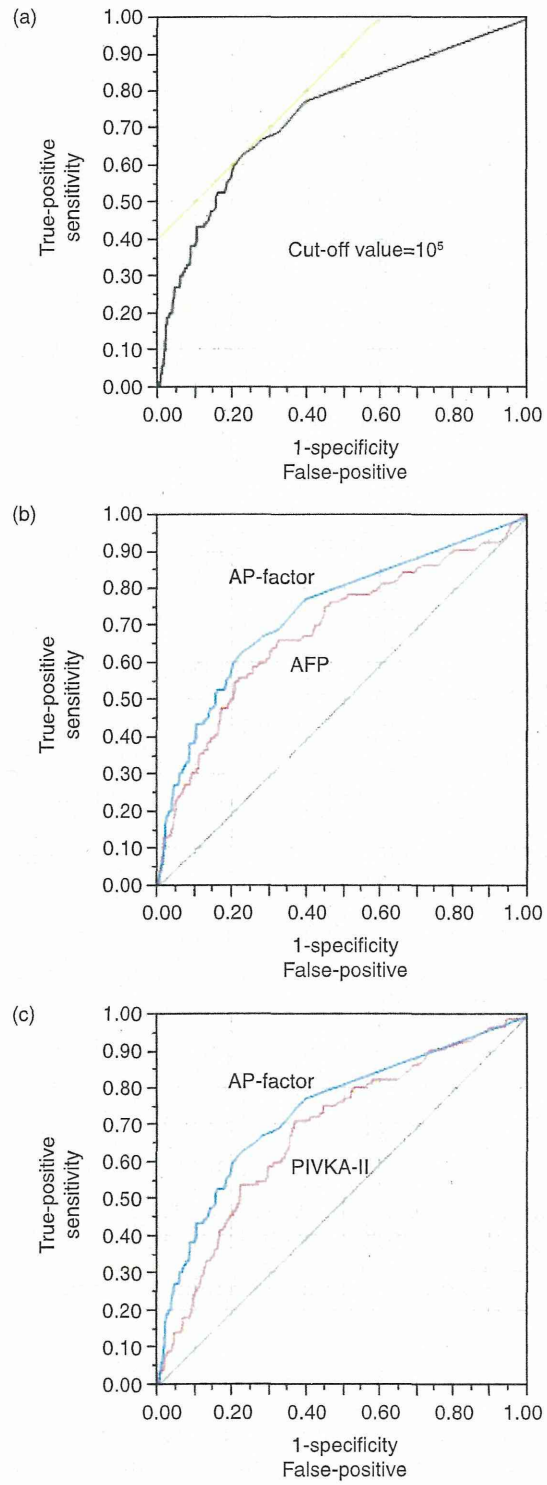

Figure 1 (a) The AP-factor – a product of the serum levels of AFP and PIVKA-II – was evaluated with respect to 2-year survival outcomes by ROC analysis which determined a cut-off value of 10^5 (AUC = 0.74607, sensitivity = 63.27% specificity = 77.41%). (b) ROC analysis of the AP-factor and AFP to evaluate the cut-off values for 2-year survival confirmed that the AUC of the AP-factor (AUC = 0.74607) is significantly higher than that of AFP (AUC = 0.69804, $P = 0.0271$). (c) ROC analysis of the AP-factor and PIVKA-II to evaluate the cut-off values for 2-year survival confirmed that the AUC of the AP-factor (AUC = 0.74607) is significantly higher than that of PIVKA-II (AUC = 0.69130, $P = 0.0065$). AFP, α-fetoprotein; AUC, area under the curve; PIVKA-II, protein induced by vitamin K absence or antagonist-II; ROC, receiver–operator curve.

for the evaluation of the liver function reserve, regardless of the presence or absence of cirrhosis.

HCC recurrence

For the first 2 years after hepatectomy, the patients underwent follow-up evaluations every 3 months comprising liver function tests, measurements of the tumor marker AFP and PIVKA-II, US and dynamic CT. After 2 years, routine CT was performed once in every 4 months. If recurrence was suspected, CT and MRI were performed, with CT during angiography and bone scintigraphy also performed if necessary. This enabled the precise diagnoses of the site, number, size and extent of invasiveness of the recurrent HCC lesions.

Statistical analysis

Patient survival and recurrence-free survival (RFS) rates were determined using the Kaplan–Meier method and compared between groups by the log–rank test. Univariate analysis of variables was also performed, but only significant variables were analyzed using the Cox proportional hazard model for multivariate analysis. Statistical analyses were performed by using standard tests (χ^2-test, Student's *t*-test) where appropriate. Significance was defined by *P*-values of less than 0.05. Statistical ROC analyses were performed using JMP version 10 for Windows (SAS Institute, Cary, NC, USA).

RESULTS

Clinicopathological characteristics and operative variables for the HCC patients

PATIENT CHARACTERISTICS, TUMOR-RELATED factors and perioperative outcomes are listed in Table 1. In the AP1, AP2 and AP3 groups, there were

Table 1 Clinicopathological characteristics of HCC patients classified according to AP-factor level

		AP-factor			*P*
		AP1 $n = 206$	AP2 $n = 152$	AP3 $n = 158$	
Sex	Male	177	121	127	0.2209
	Female	29	31	31	
Age (years)	<60	75	63	72	0.2060
	≥60	131	89	86	
HBsAg	Positive	72	82	68	0.0047
	Negative	134	76	84	
HCV	Positive	94	47	48	0.0024
	Negative	112	111	104	
Albumin (g/dL)	<4	75	57	73	0.1334
	≥4	131	95	85	
Total bilirubin (mg/dL)	<0.8	93	66	85	0.0740
	≥0.8	113	86	73	
ICG-R15 (%)	<15	93	92	98	0.0014
	≥15	113	60	60	
Tumor number	1	152	105	83	<0.0001
	2/3	50	33	45	
	≥4	4	14	30	
Tumor size (cm)	≤2	44	17	4	<0.0001
	3–4	128	71	43	
	≥5	34	64	111	
Macroscopic vascular invasion (portal vein, hepatic vein)	Absent	196	136	110	<0.0001
	Present	10	16	48	
Anatomical resection	Yes	124	120	136	<0.0001
	No	82	32	22	
Differentiation	Well	27	11	4	<0.0001
	Moderate	132	89	62	
	Poor	40	49	92	
	Unknown	7	3	0	
Microscopic portal vein invasion	Absent	188	81	114	<0.0001
	Present	18	71	44	
Microscopic hepatic vein invasion	Absent	201	139	127	<0.0001
	Present	5	13	31	
Microscopic intrahepatic metastasis	Absent	162	103	78	<0.0001
	Present	44	49	80	
Non-cancerous liver	Cirrhosis	83	46	49	0.0775
	Non-cirrhosis	123	106	109	

AP-factor – a product of the serum levels of AFP and PIVKA-II – was evaluated for 2-year survival by ROC analysis which determined a cut-off value for the AP-factor of 10^5. The HCC patients were classified into three groups accordingly: AP1 (AFP < 200 ng/mL and PIVKA-II < 100 mAU/mL), AP2 (AFP × PIVKA-II < 10^5) and AP3 (AFP × PIVKA-II ≥ 10^5).
AFP, α-fetoprotein; HBsAg, hepatitis B virus s antigen; HCV; anti-hepatitis C virus antibody; ICG-R15, indocyanine green retention rate at 15 min; PIVKA-II, protein induced by vitamin K absence or antagonism factor-II.

significant differences found in a number of variables including ICG-R15, tumor number, tumor size, macroscopic vascular invasion, anatomical resection, differentiation, microscopic portal vein invasion, microscopic hepatic vein invasion and microscopic intrahepatic metastasis. The *P*-values of the AP-factor for differentiation, microvascular portal invasion, microvascular hepatic vein invasion and intrahepatic metastasis were lower than or equal to those for AFP and PIVKA-II individually.

PS and RFS outcomes

The PS rates for the AP1, AP2 and AP3 groups at 5 years were 82.7%, 78.8% and 41.3%, respectively. The PS of the AP1 and AP2 patients was significantly higher than that of the AP3 cases ($P < 0.0001$ and <0.0001, respectively; Fig. 2a). The RFS outcomes for the AP1, AP2 and AP3 groups at 5 years were 34.0%, 40.7% and 17.1%, respectively. The RFS of AP3 was significantly lower than that of either AP1 or AP2 ($P < 0.0001$ and <0.0001, respectively; Fig. 2b).

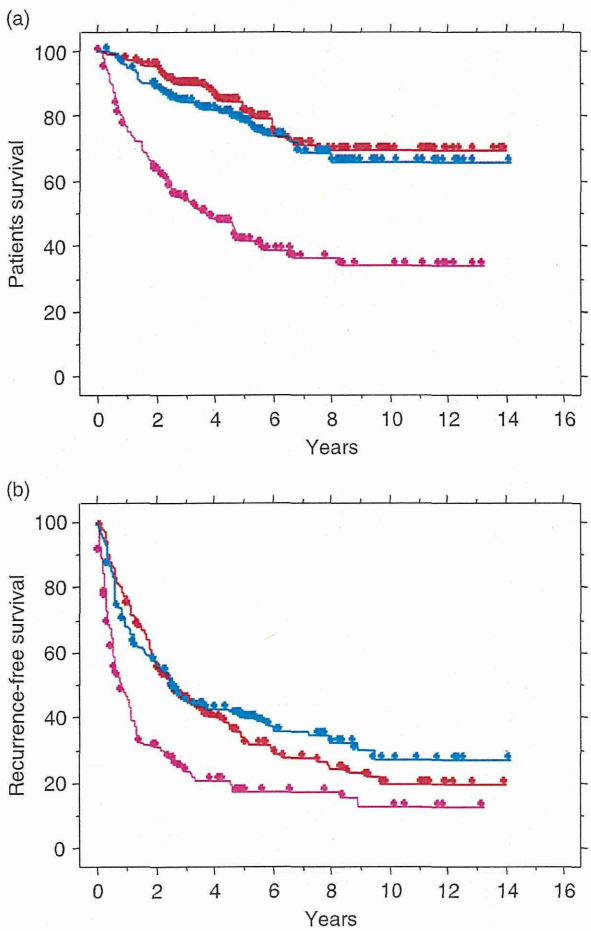

Figure 2 (a) Patient survival in accordance with the AP-factor classification. The PS rate for the AP1 group was significantly higher than those of the AP2 and AP3 groups (both $P < 0.0001$). (b) Patient recurrence-free in accordance with the AP-factor classification. The RFS rate of the AP3 group was significantly lower than that of the AP1 and AP2 patients (both $P < 0.0001$). —, AP1 ($n = 206$); —, AP2 ($n = 152$); —, AP3 ($n = 158$). PS, patient survival; RFS, recurrence-free survival.

Causes of death

There were 209 deaths among our 516 HCC patients (40.5%) due to HCC recurrence ($n = 164$; 78.5%), liver failure ($n = 17$; 8.1%) and other causes ($n = 28$; 13.4%).

Recurrent sites of HCC during follow up

Of the 139 cases of HCC recurrence in group AP1, 112 patients (80.6%) had a recurrence only in the liver and 27 (19.4%) in extrahepatic sites, including or excluding the liver. Of the 94 cases of recurrence in group AP2, 68 (72.3%) had recurrence only in the liver and 26 (27.7%) in extrahepatic sites, including or excluding the liver. Of the 122 cases of recurrence in group AP3, 63 (51.6%) had recurrence only in the liver and 59 (48.4%) in extrahepatic sites, including or excluding the liver. Importantly, recurrence in AP1 patients tended to occur only in the liver, whereas in AP3 patients it tended to occur in extrahepatic sites, including or excluding the liver ($P < 0.0001$).

Univariate and multivariate analyses of overall survival and RFS

When univariate analysis was performed to identify survival factors, serum albumin levels, tumor number, tumor size, macroscopic vascular invasion, AFP, PIVKA-II, AP-factor, differentiation, microscopic portal vein invasion, microscopic hepatic vein invasion and microscopic intrahepatic metastasis were found to be significant risk factors for survival outcomes (Table 2). When univariate analysis was also performed to identify the risk factors for recurrence, the serum albumin level, ICG-R15, tumor number, tumor size, macroscopic vascular invasion, AFP, PIVKA-II, AP-factor, differentiation, microscopic portal vein invasion, microscopic hepatic vein invasion, microscopic intrahepatic metastasis and a non-cancerous liver were identified as significant risk factors (Table 2).

The significant factors identified by univariate analysis for survival and HCC recurrence were included in multivariate analysis which showed that serum albumin levels ($P = 0.0056$), AP-factor ($P = 0.0062$), microscopic portal vein invasion ($P = 0.0027$), microscopic hepatic vein invasion ($P = 0.0056$) and microscopic intrahepatic metastasis ($P = 0.005$) are independent risk factors for survival (Table 3), and that tumor number ($P < 0.0001$), AP-factor ($P = 0.0161$) and non-cancerous liver ($P = 0.012$) are independent risk factors for recurrence (Table 3). ROC analysis of the AP-factor, AFP and PIVKA-II to evaluate the cut-off values for 2-year survival confirmed that the AUC of the AP-factor

Table 2 Univariate analysis of predictive values (clinical and tumor-associated factors) for patient survival and recurrence free survival

		n	*P* Survival	*P* Recurrence
Sex	Male	425	0.5111	0.3435
	Female	91		
Age (years)	<60	210	0.7956	0.5780
	≥60	306		
HBsAg	Positive	222	0.2211	0.2528
	Negative	294		
HCV	Positive	189	0.7128	0.7939
	Negative	327		
Albumin (g/dL)	<4	205	0.0001	<0.0001
	≥4	311		
Total bilirubin (mg/dL)	<0.8	264	0.6859	0.5098
	≥0.8	252		
ICG-R15 (%)	<15	283	0.7407	0.0084
	≥15	233		
Tumor number	1	340	<0.0001	<0.0001
	2/3	128		
	≥4	48		
Tumor size (cm)	≤2	65	<0.0001	<0.0001
	3–4	242		
	≥5 cm	209		
Macroscopic vascular invasion (portal vein, hepatic vein)	Absent	442	<0.0001	<0.0001
	Present	74		
Anatomical resection	Yes	380	0.7212	0.0756
	No	136		
AFP (ng/mL)	≤200	376	<0.0001	0.0021
	201–1000	42		
	>1000	98		
PIVKA-II (mAU/mL)	≤100	254	<0.0001	<0.0001
	101–1000	111		
	>1000	151		
AP-factor	AP1	206	<0.0001	<0.0001
	AP2	152		
	AP3	158		
Differentiation	Well	42	0.0021	<0.0001
	Moderate	283		
	Poor	181		
	Unknown	10		
Microscopic portal vein invasion	Absent	379	<0.0001	<0.0001
	Present	130		
Microscopic hepatic vein invasion	Absent	463	<0.0001	<0.0001
	Present	46		
Microscopic intrahepatic metastasis	Absent	340	<0.0001	<0.0001
	Present	170		
Non-cancerous liver	Cirrhosis	178	0.0656	0.0003
	Non-cirrhosis	338		

AFP, α-fetoprotein; AP-factor, a product of the serum levels of AFP and PIVKA-II; HBsAg, hepatitis B virus s antigen; HCV, anti-hepatitis C virus antibody; ICG-R15, indocyanine green retention rate at 15 min; PIVKA-II, protein induced by vitamin K absence or antagonism factor II.

Table 3 Multivariate analysis of values that are predictive for HCC patient survival and recurrence

		P	Risk ratio	95% CI
Survival				
Albumin (g/dL)	<4	0.0056	1.601	1.147–2.233
	≥4		1	
Tumor number		0.1855		
	1	0.0681	0.613	0.363–1.037
	2/3	0.2325	0.738	0.449–1.215
	≥4		1	
Tumor size (cm)		0.0776		
	≥5	0.0583	2.109	0.974–1.002
	3–4	0.3458	1.422	0.974–4.567
	≤2		1	
Macroscopic vascular invasion	Absent	0.7447	0.915	0.534–1.565
	Present		1	
AFP (ng/mL)		0.2125		
	>1000	0.2414	0.700	0.385–1.272
	≤200	0.0812	0.582	0.316–1.069
	201–1000		1	
PIVKA-II (mAU/mL)		0.3284		
	>1000	0.4571	0.840	0.531–1.329
	≤100	0.1471	0.595	0.295–1.201
	99–1000		1	
AP-factor		0.0062		
	AP1	0.0548	2.125	0.984–4.589
	AP3	0.0079	2.066	1.210–3.528
	AP2		1	
Differentiation		0.9550		
	Well	0.7066	1.488	0.188–11.789
	Moderate	0.6230	1.646	0.226–12.015
	Poor	0.6467	1.597	0.216–11.833
	Unknown		1	
Microscopic portal vein invasion	Absent	0.0027	0.517	0.336–0.796
	Present		1	
Microscopic hepatic vein invasion	Absent	0.0056	0.473	0.278–0.804
	Present		1	
Microscopic intrahepatic metastasis	Absent	0.0050	0.533	0.344–0.828
	Present		1	
Recurrence				
Albumin (g/dL)	<4	0.0667	1.239	0.985–1.557
	≥4		1	
ICG-R15 (%)	<15	0.0610	0.801	0.635–1.010
	≥15		1	
Tumor number		<0.0001		
	1	<0.0001	0.364	0.229–0.579
	2/3	0.0529	0.665	0.439–1.005
	4		1	
Tumor size (cm)		0.1443		
	≥5	0.0616	1.498	0.981–2.287
	3–4	0.3229	1.202	0.834–1.733
	≤2		1	
Macroscopic vascular invasion	Absent	0.7274	0.920	0.574–1.474
	Present		1	

Table 3 *Continued*

		P	Risk ratio	95% CI
AFP (ng/mL)		0.9638		
	>1000	0.7967	1.069	0.645–1.772
	≤200	0.9361	1.021	0.621–1.676
	201–1000		1	
PIVKA-II (mAU/mL)		0.4997		
	>1000	0.7397	1.060	0.750–1.499
	≤100	0.3008	0.766	0.463–1.269
	101–1000		1	
AP-factor		0.0161		
	AP1	0.0431	1.731	1.017–2.947
	AP3	0.0553	1.474	0.991–2.191
	AP2		1	
Differentiation		0.0760		
	Well	0.2922	2.184	0.510–9.348
	Moderate	0.1192	3.050	0.750–12.404
	Poor	0.0768	3.577	0.872–14.675
	Unknown		1	
Microscopic portal vein invasion	Absent	0.0561	0.716	0.508–1.009
	Present		1	
Microscopic hepatic vein invasion	Absent	0.2297	0.749	0.468–1.200
	Present		1	
Microscopic intrahepatic metastasis	Absent	0.2509	0.832	0.608–1.139
	Present		1	
Non-cancerous liver	Cirrhosis	0.0120	1.356	1.069–1.720
	Non-cirrhosis		1	

AFP, α-fetoprotein; AP-factor, a product of the serum levels of AFP and PIVKA-II; CI, confidence interval; HBsAg, hepatitis B virus s antigen; HCV, anti-hepatitis C virus antibody; ICG-R15, indocyanine green retention rate at 15 min; PIVKA-II, protein induced by vitamin K absence or antagonism factor-II.

(AUC = 0.74607) is significantly higher than that of AFP (AUC = 0.69804, $P = 0.0271$) and PIVKA-II (AUC = 0.69130, $P = 0.0065$).

DISCUSSION

IN OUR PRESENT study, the AP-factor was found to be closely related to both tumor differentiation and vascular invasion and was also identified as an independent factor related to PS and RFS outcomes with a *P*-value lower or equal to that of microscopic portal invasion, although AFP and PIVKA-II were not found to be independent survival factors. ROC analysis to evaluate 2-year survival in our HCC patient subjects who had received a hepatectomy confirmed that the AP-factor is a significantly superior indicator compared with AFP and PIVKA-II. Hence, the AP-factor is suggested to be a more reliable marker than other well-known indicators including AFP, PIVKA-II and microscopic portal invasion for the accurate prediction of survival and recurrence in HCC patients after a hepatectomy.

Previous reports have shown that AFP is an independent predictor of prognosis,[14] even in patients who have undergone a hepatectomy.[15] However, in our present analyses when the AP-factor was simultaneously inputted, AFP was not found by multivariate analysis to be an independent factor related to survival and recurrence in HCC. Although high levels of AFP in fully developed HCC or in the serum of the host are associated with more aggressive behavior and increased anaplasis,[16] it has been suggested that AFP regulates immune responses and induces either stimulatory or inhibitory growth activity.[17] On the other hand, it is well established that the AFP levels may increase in some patients with acute and chronic hepatitis without HCC,[18] and that the elevation of AFP levels correlates with the inflammation of background disease and hepatocyte regeneration.[19] Hence, because AFP does not always directly reflect tumor malignancy, its levels did not influence survival and recurrence in HCC cases according to multivariate analysis in our current study.

Protein induced by vitamin K absence or antagonists-II is also known as DCP. The specificity of PIVKA-II is approximately 95%, which is higher than that of AFP.[20] Recently, a highly sensitive assay for PIVKA-II was developed.[21] While sensitivity is still at approximately 50% for most small HCC,[22] the frequency of HCC patients in our present study with a lower than 40-mAU/mL PIVKA-II level was 36.6%. It is reported that the elevation of PIVKA-II correlates with the presence of vascular invasion.[9,23] DCP is reportedly an indicator of portal vein invasion of HCC,[24] as well as an independent prognostic indictor of recurrence and survival after hepatectomy.[7,10] However, in our present study, when we simultaneously inputted the AP-factor into our multivariate analysis, the results suggested that PIVKA-II is not an independent factor related to survival and recurrence. In previous studies that have assessed the value of DCP in predicting recurrence and survival after hepatectomy, the assays used were not highly sensitive. Hence, most of the cases that tested positive in these earlier studies had widespread or advanced HCC, and the biological nature of PIVKA-II positivity might have been overstated. Moreover, PIVKA-II may not reflect all of the factors related to the malignancy of HCC as it mainly indicates vascular invasion and not differentiation. PIVKA-II was therefore not selected an independent factor for HCC patient outcomes after hepatectomy in our current study.

The AP-factor – a product of the serum levels of AFP and PIVKA-II – was found in our current analyses to be significantly associated with all of the pathological factors tested including differentiation, microvascular portal invasion, microvascular hepatic vein invasion and intrahepatic metastasis (all $P < 0.0001$). From these results, we revealed that the AP-factor may have a duality in its relationship with AFP and PIVKA-II. It was previously reported that AFP has prognostic limitations in the case of microvascular hepatic vein invasion, as does PIVKA-II in the case of differentiation, in HCC.[8–10,23,25] The AP-factor overcomes these limitations because its *P*-value in relation to microvascular hepatic vein invasion was found to be very low ($P < 0.0001$). Because the AP-factor may represent the dual characteristics of both AFP and PIVKA-II, it may be a surrogate marker of both tumor differentiation and vascular invasion and more directly reflect tumor malignancy than either AFP or PIVKA-II individually. These findings may involve the fact that recurrence in AP1 patients tended to occur only in the liver, whereas in AP3 patients it tended to occur in extrahepatic sites, including or excluding the liver. Therefore, we identified the AP-factor as an independent factor very closely related to survival following microscopic vascular invasion, and closely related to recurrence in cases of increased tumor number.

Shimada *et al.* have reported that the positivity of both DCP and AFP is an independent indicator of a poor prognosis in HCC in terms of disease-free survival and PS.[7] For this reason, these authors suggested that both DCP and AFP produced by the HCC itself promote either tumor growth or tumor metastasis in an autocrine and/or paracrine fashion. Kaibori *et al.* have also reported that a positive status for both AFP and DCP at recurrence is an important prognostic indicator for HCC recurrence after hepatic resection.[26] However, our current patients were classified mainly by their AP-factor (AFP × PIVKA-II) levels because we hypothesized that this factor may be a surrogate marker of both tumor differentiation and vascular invasion and will more directly reflect tumor malignancy than either AFP or PIVKA-II individually. Moreover, ROC analysis of 2-year survival outcomes in our patients showed a significant superiority of the AP-factor over AFP and PIVKA-II as a prognostic indicator. For these reasons, the AP-factor may be a more reliable prognostic marker of PS and RFS of patients with HCC. Moreover, the classification of AP1, AP2 and AP3 is meaningful because it was possible to determine that AP2 was also equal to AP1, which was hoped to have the best outcome, and AP3 had the worst outcome in these three groups. Kiriyama *et al.* reported that triple positive tumor markers for HCC showed poor prognosis and invasive characteristics in pathological findings.[27] However, in this paper it was described that most of the patients in this study had less than the minimum detectable limit for *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3). Therefore, we evaluated the malignancy from AFP and PIVKA-II without AFP-L3.

Transplantation is considered to be the treatment of choice even for resectable small HCC in Child–Pugh class A patients.[28] Because the overall survival rates after hepatectomy for small HCC are shown to be equal to those after liver transplantation, hepatectomy before transplantation should be first performed for respectable HCC in patients with preserved liver function.[29] Moreover, because Poon *et al.* reported no differences in the cumulative survival curves of patients without microscopic venous invasion in resection and transplantation groups,[30] it is proposed that patients without microscopic portal invasion according to the Milan criteria should first be treated by hepatectomy. On the other hand, if HCC patients show microscopic portal invasion, the outcomes of liver transplantations are also

unfavorable[31] and patients who may be rendered transplantable after hepatectomy may be selected. However, microscopic portal invasion cannot be diagnosed preoperatively and a pathological examination is required to evaluate this factor. From our current data, the AP-factor was shown to be very closely related to both tumor differentiation and vascular invasion and was selected as an independent factor related to survival with an equal *P*-value to microscopic portal invasion, and an independent factor related to recurrence with a lower *P*-value. Hence, the AP-factor is suggested to be a critical HCC marker with an accuracy that equals microscopic portal invasion at preoperatively predicting tumor malignancy. Hence, HCC patients in whom the same outcomes can be expected for hepatectomy as with transplantation or who may be rendered transplantable by hepatectomy could be selected by measuring their AP-factor.

REFERENCES

1 Arii S, Yamaoka Y, Futagawa S *et al.* Results of surgical and nonsurgical treatment for small-sized hepatocellular carcinomas: a retrospective and nationwide survey in Japan. The Liver Cancer Study Group of Japan. *Hepatology* 2000; **32** (6): 1224–9.

2 Kamiyama T, Nakanishi K, Yokoo H *et al.* Recurrence patterns after hepatectomy of hepatocellular carcinoma: implication of Milan criteria utilization. *Ann Surg Oncol* 2009; **16** (6): 1560–71.

3 Ikai I, Arii S, Kojiro M *et al.* Reevaluation of prognostic factors for survival after liver resection in patients with hepatocellular carcinoma in a Japanese nationwide survey. *Cancer* 2004; **101** (4): 796–802.

4 Sasaki Y, Yamada T, Tanaka H *et al.* Risk of recurrence in a long-term follow-up after surgery in 417 patients with hepatitis B- or hepatitis C-related hepatocellular carcinoma. *Ann Surg* 2006; **244** (5): 771–80.

5 Jonas S, Bechstein WO, Steinmuller T *et al.* Vascular invasion and histopathologic grading determine outcome after liver transplantation for hepatocellular carcinoma in cirrhosis. *Hepatology* 2001; **33** (5): 1080–6.

6 Shirabe K, Itoh S, Yoshizumi T *et al.* The predictors of microvascular invasion in candidates for liver transplantation with hepatocellular carcinoma-with special reference to the serum levels of des-gamma-carboxy prothrombin. *J Surg Oncol* 2007; **95** (3): 235–40.

7 Shimada M, Takenaka K, Fujiwara Y *et al.* Des-gamma-carboxy prothrombin and alpha-fetoprotein positive status as a new prognostic indicator after hepatic resection for hepatocellular carcinoma. *Cancer* 1996; **78** (10): 2094–100.

8 Tamura S, Kato T, Berho M *et al.* Impact of histological grade of hepatocellular carcinoma on the outcome of liver transplantation. *Arch Surg* 2001; **136** (1): 25–30.

9 Toyoda H, Kumada T, Kiriyama S *et al.* Prognostic significance of simultaneous measurement of three tumor markers in patients with hepatocellular carcinoma. *Clin Gastroenterol Hepatol* 2006; **4** (1): 111–7.

10 Imamura H, Matsuyama Y, Tanaka E *et al.* Risk factors contributing to early and late phase intrahepatic recurrence of hepatocellular carcinoma after hepatectomy. *J Hepatol* 2003; **38** (2): 200–7.

11 Izumi R, Shimizu K, Ii T *et al.* Prognostic factors of hepatocellular carcinoma in patients undergoing hepatic resection. *Gastroenterology* 1994; **106** (3): 720–7.

12 Kondo K, Chijiiwa K, Makino I *et al.* Risk factors for early death after liver resection in patients with solitary hepatocellular carcinoma. *J Hepatobiliary Pancreat Surg* 2005; **12** (5): 399–404.

13 Shirabe K, Kanematsu T, Matsumata T, Adachi E, Akazawa K, Sugimachi K. Factors linked to early recurrence of small hepatocellular carcinoma after hepatectomy: univariate and multivariate analyses. *Hepatology* 1991; **14** (5): 802–5.

14 Nomura F, Ohnishi K, Tanabe Y. Clinical features and prognosis of hepatocellular carcinoma with reference to serum alpha-fetoprotein levels. Analysis of 606 patients. *Cancer* 1989; **64** (8): 1700–7.

15 Hanazaki K, Kajikawa S, Koide N, Adachi W, Amano J. Prognostic factors after hepatic resection for hepatocellular carcinoma with hepatitis C viral infection: univariate and multivariate analysis. *Am J Gastroenterol* 2001; **96** (4): 1243–50.

16 Matsumoto Y, Suzuki T, Asada I, Ozawa K, Tobe T, Honjo I. Clinical classification of hepatoma in Japan according to serial changes in serum alpha-fetoprotein levels. *Cancer* 1982; **49** (2): 354–60.

17 Mizejewski GJ. Alpha-fetoprotein structure and function: relevance to isoforms, epitopes, and conformational variants. *Exp Biol Med (Maywood)* 2001; **226** (5): 377–408.

18 Silver HK, Gold P, Shuster J, Javitt NB, Freedman SO, Finlayson ND. Alpha(1)-fetoprotein in chronic liver disease. *N Engl J Med* 1974; **291** (10): 506–8.

19 Fujiyama S, Tanaka M, Maeda S, Ashihara H, Hirata R, Tomita K. Tumor markers in early diagnosis, follow-up and management of patients with hepatocellular carcinoma. *Oncology* 2002; **62** (Suppl 1): 57–63.

20 Kasahara A, Hayashi N, Fusamoto H *et al.* Clinical evaluation of plasma des-gamma-carboxy prothrombin as a marker protein of hepatocellular carcinoma in patients with tumors of various sizes. *Dig Dis Sci* 1993; **38** (12): 2170–6.

21 Ikoma J, Kaito M, Ishihara T *et al.* Early diagnosis of hepatocellular carcinoma using a sensitive assay for serum des-gamma-carboxy prothrombin: a prospective study. *Hepatogastroenterology* 2002; **49** (43): 235–8.

22 Mita Y, Aoyagi Y, Yanagi M, Suda T, Suzuki Y, Asakura H. The usefulness of determining des-gamma-carboxy prothrombin by sensitive enzyme immunoassay in the early diagnosis of patients with hepatocellular carcinoma. *Cancer* 1998; **82** (9): 1643–8.
23 Inoue S, Nakao A, Harada A, Nonami T, Takagi H. Clinical significance of abnormal prothrombin (DCP) in relation to postoperative survival and prognosis in patients with hepatocellular carcinoma. *Am J Gastroenterol* 1994; **89** (12): 2222–6.
24 Koike Y, Shiratori Y, Sato S *et al.* Des-gamma-carboxy prothrombin as a useful predisposing factor for the development of portal venous invasion in patients with hepatocellular carcinoma: a prospective analysis of 227 patients. *Cancer* 2001; **91** (3): 561–9.
25 Sumie S, Kuromatsu R, Okuda K *et al.* Microvascular invasion in patients with hepatocellular carcinoma and its predictable clinicopathological factors. *Ann Surg Oncol* 2008; **15** (5): 1375–82.
26 Kaibori M, Matsui Y, Yanagida H, Yokoigawa N, Kwon AH, Kamiyama Y. Positive status of alpha-fetoprotein and des-gamma-carboxy prothrombin: important prognostic factor for recurrent hepatocellular carcinoma. *World J Surg* 2004; **28** (7): 702–7.
27 Kiriyama S, Uchiyama K, Ueno M *et al.* Triple positive tumor markers for hepatocellular carcinoma are useful predictors of poor survival. *Ann Surg* 2011; **254** (6): 984–91.
28 Figueras J, Jaurrieta E, Valls C *et al.* Resection or transplantation for hepatocellular carcinoma in cirrhotic patients: outcomes based on indicated treatment strategy. *J Am Coll Surg* 2000; **190** (5): 580–7.
29 Otto G, Heuschen U, Hofmann WJ, Krumm G, Hinz U, Herfarth C. Survival and recurrence after liver transplantation versus liver resection for hepatocellular carcinoma: a retrospective analysis. *Ann Surg* 1998; **227** (3): 424–32.
30 Poon RT, Fan ST, Lo CM, Liu CL, Wong J. Difference in tumor invasiveness in cirrhotic patients with hepatocellular carcinoma fulfilling the Milan criteria treated by resection and transplantation: impact on long-term survival. *Ann Surg* 2007; **245** (1): 51–8.
31 Shimoda M, Ghobrial RM, Carmody IC *et al.* Predictors of survival after liver transplantation for hepatocellular carcinoma associated with Hepatitis C. *Liver Transpl* 2004; **10** (12): 1478–86.

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ORIGINAL ARTICLE

Hydrogen sulfide augments survival signals in warm ischemia and reperfusion of the mouse liver

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Abstract
Background and purpose Hydrogen sulfide (H_2S) ameliorates hepatic ischemia and reperfusion injury (IRI), but the precise mechanism remains elusive. We investigated whether sodium hydrogen sulfide (NaHS), a soluble derivative of H_2S, would ameliorate hepatic IRI, and if so, via what mechanism.
Methods Mice were subjected to partial warm ischemia for 75 min followed by reperfusion. Either NaHS or saline was administered intravenously 10 min before reperfusion. The liver and serum were collected 3, 6, and 24 h after reperfusion.
Results In the NaHS(−) group, severe IRI was apparent by the ALT leakage, tissue injury score, apoptosis, lipid peroxidation, and inflammation (higher plasma TNF-α, IL-6, IL-1β, IFN-γ, IL-23, IL-17, and CD40L), whereas IRI was significantly ameliorated in the NaHS(+) group. These effects could be explained by the augmented nuclear translocation of Nrf2, and the resulting up-regulation of HO-1 and thioredoxin-1. Phosphorylation of the PDK-1/Akt/mTOR/p70S6k axis, which is known to mediate pro-survival and anti-apoptotic signals, was significantly augmented in the NaHS(+) group, with a higher rate of PCNA-positive cells thereafter.
Conclusion NaHS ameliorated hepatic IRI by direct and indirect anti-oxidant activities by augmenting pro-survival, anti-apoptotic, and anti-inflammatory signals via mechanisms involving Nrf-2, and by accelerating hepatic regeneration via mechanisms involving Akt-p70S6k.

Keywords Hydrogen sulfide · Liver · Ischemia · Reperfusion · Mouse

Abbreviations

ALT	Alanine aminotransferase
CO	Carbon monoxide
DCD	Donation after cardiac death
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
GSH	Glutathione
4-HNE	4-hydroxy-2-nonenal
HO-1	Heme oxygenase 1
TRX-1	Thioredoxin-1
HPFs	High-power fields
H_2S	Hydrogen sulfide
HSPs	Heat shock proteins
IL-6	Interleukin 6

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I/R	Ischemia and reperfusion
IRI	Ischemia and reperfusion injury
Keap-1	Kelch-like ECH-associated protein 1
MDA	Malondialdehyde
MPT	Mitochondrial permeability transition
mTOR	Mammalian target of rapamycin
NaHS	Sodium hydrogen sulfide
NF-kappaB	Nuclear factor-kappa B
Nrf2	NF-E2-related factor 2
PDK-1	Phosphoinositide-dependent kinase 1
PI3K	Phosphoinositide 3 kinase
PKC	Protein kinase C
PNF	Primary graft non-function
PVDF	Polyvinylidene difluoride
p70s6k	70-kDa Ribosomal protein S6 kinase
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STAT3	Signal transducer and activator of transcription 3
TNF-α	Tumor necrosis factor α
TRX-1	Thioredoxin 1
TUNEL	Terminal dUTP nick end-labeling

Introduction

Warm ischemia and reperfusion injury (IRI) is a major obstacle to the safe utilization of donation after cardiac death (DCD) grafts [1]. Warm ischemia causes mitochondrial dysfunction as well as ATP depletion and reactive oxygen species (ROS) production [2] and the nuclear translocation of NF-kappaB and AP-1 [3]. These triggers during ischemia propagate oxidative injury and expression of inflammatory cytokines, leading to eventual apoptosis and necrosis [1–3]. They also inhibit protein synthesis and cellular proliferation [4]. Among the acute responses, Akt plays a major role in survival, and anti-apoptotic and proliferative signals, including mTOR-p70s6k, the Bcl2 family, Cyclin D1, and STAT3 [5–7]. A clinically applicable method to modulate these signals is needed.

Hydrogen sulfide (H_2S) can facilitate the phosphorylation of Akt and the nuclear translocation of NF-E2-related factor 2 (Nrf2) [8], leading to a reduction in IRI of the rat heart [9], kidney [10], lung [11], small intestine [12], and liver [13–16]. Although augmentation of the PI3K/Akt/p70S6k cascade in the myocardium [17] and in the small intestine [18] reduces IRI, the precise mechanism underlying this reduction is not yet fully understood. We investigated whether sodium hydrogen sulfide (NaHS), a soluble derivative of H_2S [19], ameliorates hepatic IRI in mice, focusing on the signal transduction related to acute inflammation and resulting cellular survival/death and regeneration.

Materials and methods

Chemicals and reagents

All chemicals and reagents were of the highest grade commercially available, and purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan) unless otherwise stated. The antibodies used in this study were purchased from Cell Signaling Technology (Beverly, MA) unless otherwise stated.

Animals

This study was conducted with the approval of the Hokkaido University Committee for the Care and Use of Laboratory Animals. Male C57BL/6J mice, 10–12 weeks of age and weighing 25–30 g, were purchased from Sankyo Labo Service Corporation Inc. (Tokyo, Japan). The raising conditions, including chow, were as previously described [20].

Partial hepatic warm ischemia and reperfusion (I/R)

After overnight fasting, the animals were anesthetized by inhalation of isoflurane. The median and left lateral portal branches were clamped by an atraumatic aneurysm clip as previously described [21]. After closure of the abdomen, the animals were allowed to remain awake during ischemia. After 75 min, the liver was reperfused by removing the clip. The non-ischemic lobes were not resected.

Experimental protocol

The mice were divided into three groups of six animals each. Ten min before reperfusion, either NaHS (1 mg/kg; NaHS (+) group) or saline (NaHS (−) group) was administered intravenously. Sodium hydrogen sulfide (NaHS) was dissolved in saline just before administration. A sham-operated group, without vascular occlusion, was also established (sham group). Animals were killed 3, 6, and 24 h after reperfusion (R3, R6, and R24 h, respectively).

Sample collection

The ischemic lobes of the liver were collected and stored at −80 °C until use, or fixed in 10 % buffered formalin and embedded in paraffin. Plasma was also collected and stored at −80 °C until measurement.

Histological examination

Paraffin-embedded sections of the liver at R6 h were stained with hematoxylin and eosin. Histopathological grading was performed by a single pathologist in a blinded manner

according to the grading described by Suzuki et al. [22] namely, sinusoidal congestion (0–4), vacuolization of hepatocyte cytoplasm (0–4), and parenchymal necrosis (0–4).

Plasma ALT activity

Plasma ALT activity at R6 h was evaluated by a Hitachi 7020 automatic analyzer (Hitachi, Tokyo, Japan).

Inflammatory cytokines and chemokines in plasma

The plasma levels of TNF-α, IL-6, IL-1β, IFN-γ, IL-17, IL-23, and CD40L at R3 h and R6 h were measured with a commercially available ELISA-based kit, Bioplex (Bio-Rad, Hercules, CA). Briefly, aliquots (20 μL) of the plasma were incubated with fluorescent-labeled antibodies and the fluorescence intensity was measured and expressed in pg/ml.

Western blot analysis

We minced and homogenized 50 mg of frozen tissue from R6 h in ice-cold lysis buffer containing Tris HCl 25 mM (pH 7.5), NaCl 150 mM, EDTA-2Na 5 mM, NaF 10 mM, sodium orthovanadate 10 mM, 1 % Nonidet P-40, and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The homogenate was centrifuged at 1,000×*g* for 10 min and the nuclear fraction was stored at −80 °C. The supernatant was then centrifuged at 15,000×*g* for 10 min and the resulting supernatant, being the cytosolic fraction, was stored at −80 °C. The protein concentration was measured using a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). The proteins were denatured by boiling at 95 °C for 5 min with an SDS sample buffer. Using Any-kD precast gel (Bio-Rad), 40 micrograms of protein was applied to the standard SDS polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblots were performed after transfer onto the PVDF membrane. Dilutions of 1:1000 were used for the primary antibodies: p-PDK-1, p-Akt, p-mTOR, p-p70s6k, cleaved caspase-3, HO-1 (Abcam, Cambridge, UK), TRX-1, β-actin, Nrf2 (Abcam), Lamin B1, and GAPDH. The dilution of the horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody was 1:5000. Protein bands were detected by a chemiluminescent detector Chem Doc XRS® (Bio-Rad) using a chemiluminescence reagent, West Dura (Thermo Scientific). Protein levels were normalized by β-actin or GAPDH, and further normalized by the average value in the sham-operated group.

Apoptotic index

Frozen sections of the ischemic lobe at R24 h were stained by using a fluorescent TUNEL staining kit (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, they were fixed in 95 % ethanol and 4 % paraformaldehyde, rehydrated in PBS, digested with proteinase K, washed in PBS, incubated with equilibration buffer, and incubated with rTdT at room temperature for 1 h. The slides were mounted with Prolong Gold anti-fade reagent with DAPI (Molecular Probes Inc., Eugene, OR), and then examined with a BZ-9000 fluorescence microscope (Keyence Japan, Osaka, Japan). The apoptotic index was calculated as the number of TUNEL-positive cells divided by the total number of DAPI-positive cells. Four high power fields (HPFs) were observed per sample and the average of the four values was used.

Assessment of lipid peroxidation in the liver

The level of hepatic oxidative damage at R6 h was taken as the combined amounts of MDA and 4-HNE, the stable end products of lipid peroxidation, as determined using an LPO586 kit (Oxis International, Foster City, CA) [20]. We made 10 % (%w/v) homogenate of the liver with ice-cold Tris–HCl (20 mM) containing butylated hydroxytoluene (0.05 %). The MDA and 4-HNE contents were measured according to the manufacturer's instructions. Data are expressed as the nmol 4-HNE equivalent per mg of wet tissue weight.

Immunohistochemistry of the liver

The frozen sections at R6 h were fixed in 95 % ethanol and 1 % formalin, rehydrated, permeabilized, and blocked in 3 % bovine serum albumin in PBS. After washing, the slide was incubated with rabbit polyclonal anti-Nrf2 antibody (1:250) (Abcam) for 1 h at room temperature, followed by Alexafluor488-conjugated goat anti-rabbit IgG secondary antibody (1:500) (Molecular Probes Inc.) for 45 min at room temperature. The slides were mounted with Prolong Gold anti-fade reagent with DAPI (Molecular Probes Inc.).

Immunohistochemistry with anti-proliferating cell nuclear antigen (PCNA) was performed according to the manufacturer's instructions. Briefly, the paraffin-embedded sections were subjected to epitope retrieval by microwave treatment. Monoclonal mouse anti-PCNA antibody (M0879; DAKO Japan, Tokyo) was used at a dilution of 1:300. For visualization, streptavidin (LSAB 2 system HRP; DAKO Japan) and DAB substrate (DAKO Japan) were used. Nuclear counterstaining was performed using hematoxylin.

The cell-positivity rate for nuclear Nrf2 staining was counted in four random high-power fields. The resulting values were divided by the total number of DAPI-positive cells. In the case of PCNA, the number of PCNA-positive hepatocytes was counted and divided by the total number of hepatocytes under four random high-power fields.

Statistical analysis

Values are expressed as the mean ± SD. The Student's *t* test or one-way ANOVA was used for evaluating statistical significance. Values of $P < 0.05$ were considered significant. Statistical analyses were performed using Stat View 5.0 for Windows (SAS Institute Inc., Cary, NC).

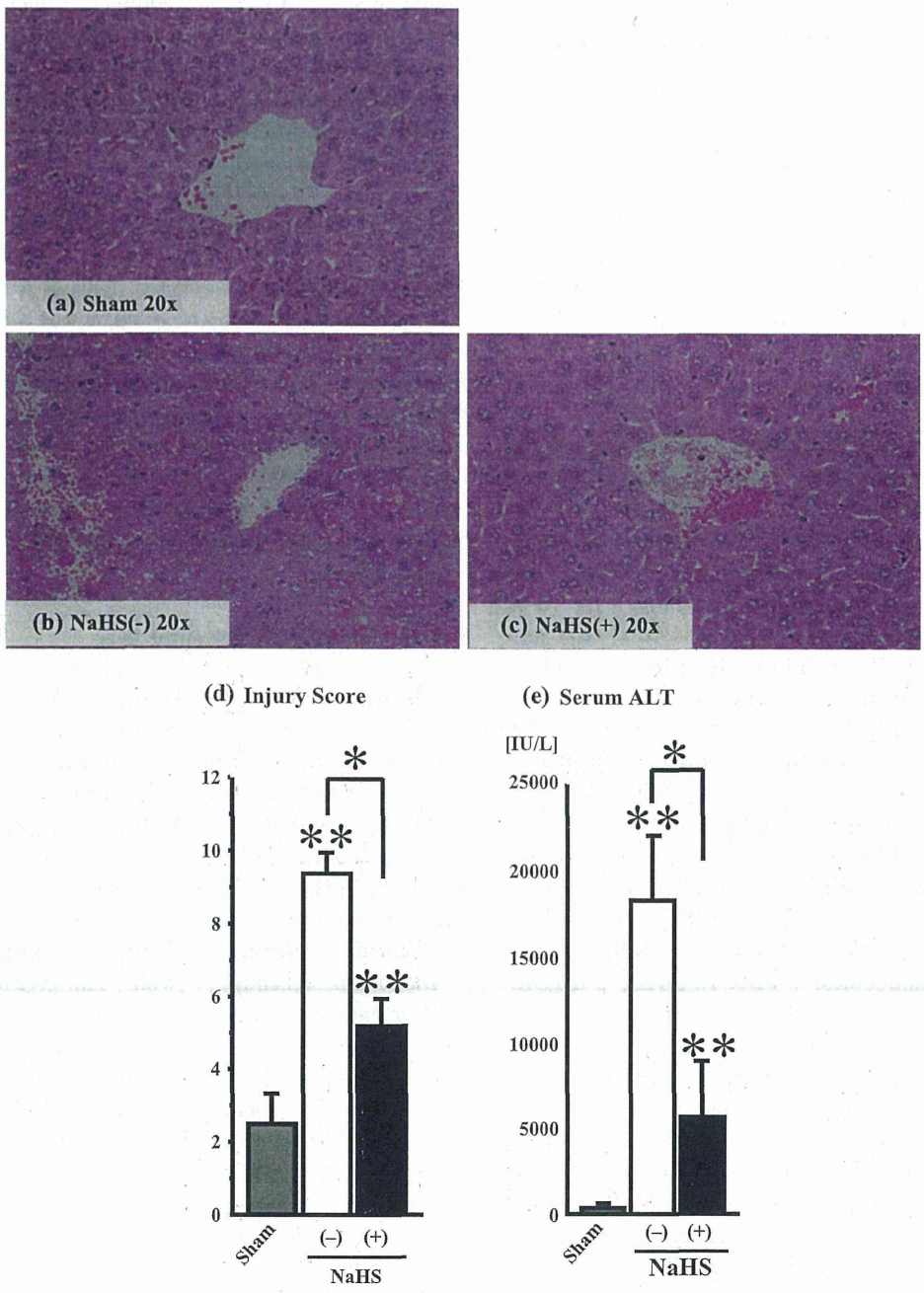

Fig. 1 Sodium hydrogen sulfide (NaHS) reduces hepatic ischemia and reperfusion injury. Mice were subjected to partial warm ischemia for 75 min and subsequent reperfusion (I/R) for 6 h. The ischemic lobe was stained with hematoxylin and eosin, and scored according to the method of Suzuki et al. Representative photographs (20× magnification) are shown. **a** Sham operation. **b** NaHS (−): I/R with vehicle treatment. **c** NaHS (+): I/R with NaHS (1.0 mg/kg) administration before reperfusion. **d** Hepatic injury score. **e** Plasma ALT activity 6 h after reperfusion. Results are expressed as the mean ± SD. $^{*}P < 0.05$, NaHS (−) vs. NaHS (+). $^{**}P < 0.05$ vs. Sham

Results

Liver injury

The liver histopathology appeared to be almost normal in the sham-operated group (Fig. 1a). Warm ischemia of the liver and reperfusion (hepatic warm I/R) caused inflammatory cell infiltration, congestion, and vacuolization with condensed nucleus at R6 h (Fig. 1b), whereas these changes were attenuated in the NaHS-treated mice (Fig. 1c). The injury score was augmented by hepatic warm I/R at R6 h in the NaHS(−) group, whereas it was significantly reduced by NaHS treatment (Fig. 1d). Plasma alanine aminotransferase (ALT) activity at R6 h was augmented in the hepatic warm I/R in NaHS(−) group, whereas it was significantly reduced by NaHS treatment (Fig. 1e).

Plasma cytokines and chemokines

Plasma levels of TNF-α (Fig. 2a), IL-6 (Fig. 2b), IL-1β (Fig. 2c), IFN-γ (Fig. 2d), IL-23 (Fig. 2e), IL-17F (Fig. 2f) and CD40L (Fig. 2g) were significantly higher in the NaHS(−) group 3 h after reperfusion (R3 h) than the respective value in the sham group, whereas the augmentation was significantly suppressed in the NaHS(+) group. By 6 h after reperfusion (R6 h), these molecules, except for CD40L, had decreased in the NaHS(−) group, and were even lower in the NaHS(+) group. Inter-group comparison

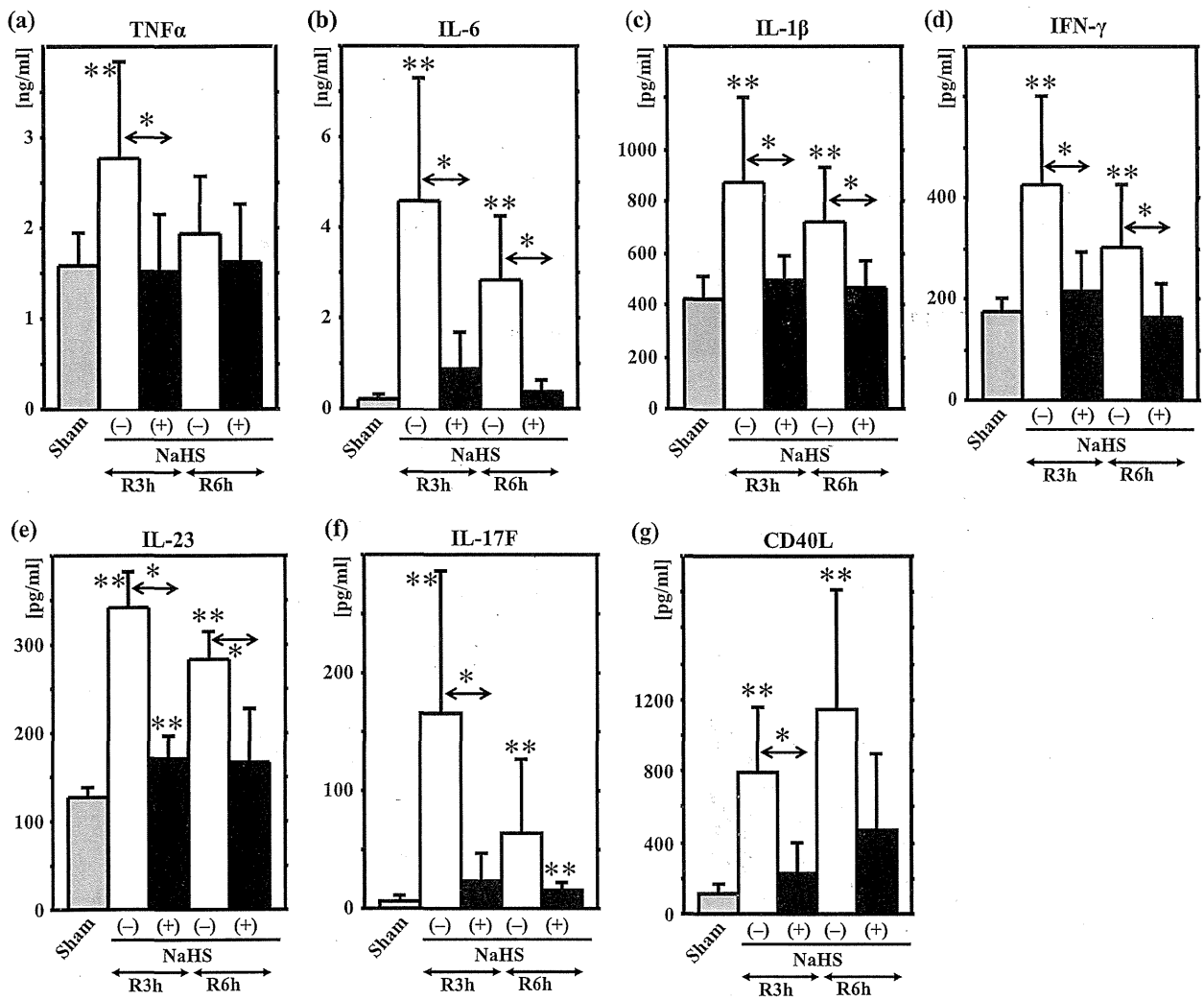

Fig. 2 Sodium hydrogen sulfide (NaHS) inhibits the expression of inflammatory cytokines and chemokines. Mice were subjected to partial warm ischemia for 75 min and subsequent reperfusion (I/R). The plasma concentrations of inflammatory cytokines and chemokines at 3 and 6 h after reperfusion were measured by an ELISA-based assay. **a** TNF-α, **b** IL-6, **c** IL-1β, **d** IFN-γ, **e** IL-23, **f** IL-17F, and **g** soluble CD40 ligand. Results are expressed as the mean ± SD. $*P < 0.05$, NaHS (−) vs. NaHS (+). $**P < 0.05$ vs. Sham

revealed significant decreases in IL-6, IL-1β, IFN-γ, and IL-23, although the decreases in TNF-α and IL17F were not significant. It noteworthy that CD40L continued to rise from R3 h to R6 h in both groups, but that NaHS treatment tended to decrease its value ($P = 0.065$).

Pro-survival signals

Pro-survival signals at R6 h were evaluated by western blots of phosphorylated PDK-1 (p-PDK1-Ser^{241}), Akt (p-Akt-Ser^{473}), mTOR (p-mTOR-Ser^{2448}), and p70S6k (p-p70S6 K-Ser^{371}). Phosphorylated PDK-1 was significantly attenuated by hepatic warm I/R, whereas the reduction was significantly less pronounced in the NaHS treatment groups (Fig. 3a). Phosphorylated Akt tended to decrease only in the NaHS(−) group, whereas in the NaHS(+) group, it was significantly higher than in the other groups (Fig. 3b). Phosphorylated mTOR and phosphorylated p70S6k were almost unchanged by hepatic warm I/R in the NaHS(−) group, whereas they were

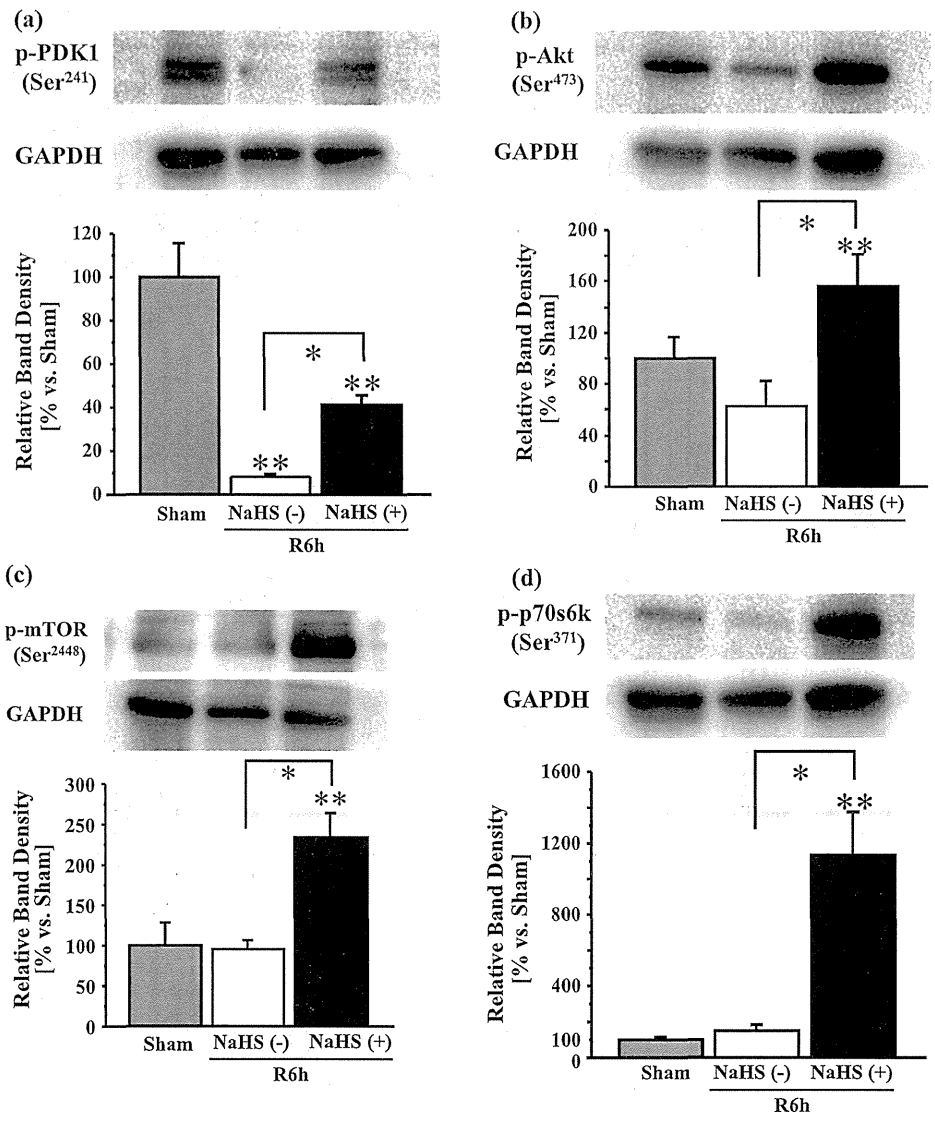

Fig. 3 Sodium hydrogen sulfide (NaHS) activates survival signals. Mice were subjected to partial warm ischemia for 75 min and subsequent reperfusion (I/R) for 6 h. Cytosolic protein in the ischemic lobe was applied to the western blot (*top*), and the relative intensity (*bottom*) is shown. *Panels* a–d show the results for **a** phosphorylated PDK-1 (Ser^{241}), **b** phosphorylated Akt (Ser^{473}), **c** phosphorylated mTOR (Ser^{2448}), and **d** phosphorylated p70s6k (Ser^{371}). Relative quantitation of each sample was performed, using GAPDH as an internal control. Each normalized value was further normalized by the mean value in the sham-operated group, and expressed as the mean ± SD. *$P < 0.05$, NaHS (−) vs. NaHS (+). **$P < 0.05$ vs. Sham