BJC

Pro-angiogenic cytokines for prediction of outcomes in patients with advanced hepatocellular carcinoma

K Miyahara[1], K Nouso*[1,2], Y Morimoto[1], Y Takeuchi[1], H Hagihara[1], K Kuwaki[1], H Onishi[1,2], F Ikeda[1,2], Y Miyake[1], S Nakamura[1], H Shiraha[1], A Takaki[1], M Honda[3], S Kaneko[3], T Sato[4], S Sato[4], S Obi[4], S Iwadou[5], Y Kobayashi[5], K Takaguchi[6], K Kariyama[7], Y Takuma[8], H Takabatake[8] and K Yamamoto[1] for the Okayama Liver Cancer Group[9]

[1]Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama-city, Okayama 700-8558, Japan; [2]Department of Molecular Hepatology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama-city, Okayama 700-8558, Japan; [3]Department of Gastroenterology, Kanazawa University Graduate School of Medicine, 13-1 Takara-Machi, Kanazawa-city, Ishikawa 920-8641, Japan; [4]Department of Hepatology, Kyoundo Hospital, 1-8 Kandasurugadai, Chiyoda-ku, Tokyo 101-0034, Japan; [5]Department of Internal Medicine, Hiroshima-city Hospital, 7-33 motomachi, naka-ku, Hiroshima-city, Hiroshima 730-8518, Japan; [6]Department of Hepatology, Kagawa Prefectural Central Hospital, 5-4-16 ban-cho, Takamatsu-city, Kagawa 760-8557, Japan; [7]Department of Liver Disease Center, Okayama city Hospital, 6-10 amase, kita-ku, Okayama-city, Okayama 700-8557, Japan and [8]Department of Gastroenterology, Kurashiki Central Hospital, 1-1-1 miwa, Kurashiki-city, Okayama 710-8602, Japan

Background: We previously reported that expressions of the pro-angiogenic cytokines angiopoietin-2 (Ang-2), follistatin, granulocyte colony-stimulating factor, hepatocyte growth factor, leptin, platelet-derived growth factor-BB, platelet endothelial cell adhesion molecule-1, and vascular endothelial growth factor were associated with the response to sorafenib in patients with advanced hepatocellular carcinoma (HCC). The aim of the present study is to examine the same relationship in a larger cohort.

Methods: In the current retrospective cohort study, we measured serum levels of the eightcytokines in 120 consecutive HCC patients who were treated with sorafenib. We evaluated the effects of increased expression of serum cytokines on progression-free survival (PFS) and overall survival (OS).

Results: Elevated expression of Ang-2 correlated both with significantly shorter PFS (hazard ratio (HR), 1.84; 95% confidence interval (CI), 1.21–2.81), and OS (HR, 1.95; 95% CI, 1.21–3.17). Patients with more than three cytokines expressed above the median similarly had significantly shorter PFS (HR, 1.98; 95% CI, 1.30–3.06) and OS (HR, 1.94; 95% CI, 1.19–3.22). Differences in OS were evident in cases with the evidence of macroscopic vascular invasion or extrahepatic metastasis.

Conclusion: High expression of Ang-2 or more than cytokines in serum is associated with poor PFS and OS in HCC patients treated with sorafenib.

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide and is associated with the second lowest 5-year survival rate of all tumour types (Jemal *et al*, 2010).

When HCC is diagnosed at an advanced stage or progresses after locoregional therapy, it is associated with a dismal prognosis for survival because of underlying liver disease and lack of effective

treatment options (Llovet *et al*, 2003; Bruix and Sherman, 2005; Llovet, 2005). Recently, sorafenib, a multikinase inhibitor, has shown promise in the treatment of HCC. Sorafenib suppresses tumour angiogenesis and proliferation by inhibiting the activity of targets such as the vascular endothelial growth factor (VEGF) receptor, platelet-derived growth factor (PDGF) receptor, mast/stem cell growth factor receptor (c-KIT), rearranged during transfection (RET), Fms-like tyrosine kinase 3 (FLT-3), and the proto-oncoprotein, c-RAF (Wilhelm *et al*, 2004; Wilhelm *et al*, 2008). The safety and efficacy of sorafenib in patients with advanced HCC were demonstrated in two phase III randomised, double-blind, placebo-controlled trials (Llovet *et al*, 2008a; Cheng *et al*, 2009), thereby establishing sorafenib as the standard systemic therapy for advanced HCC (Llovet *et al*, 2008b; Bruix *et al*, 2011). Biomarker research that predicts or monitors the efficacy of sorafenib is a growing field. Recently, Llovet *et al* (2012) reported on the results of the Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol (SHARP) trial that examined the expression of 10 molecules in the plasma of HCC patients. Although none of the biomarkers significantly predicted response to sorafenib, plasma expression levels of c-KIT and hepatocyte growth factor (HGF) were suggested as the possible predictors of response to sorafenib (Llovet *et al*, 2012).

We previously reported correlations between treatment response to sorafenib and expression of cytokines in serum, including angiopoietin-2 (Ang-2), follistatin (FST), granulocyte colony-stimulating factor (G-CSF), HGF, leptin, PDGF-BB, platelet endothelial cell adhesion molecule-1 (PECAM-1), and VEGF, in Japanese HCC patients (Miyahara *et al*, 2011). We found that responsiveness to treatment with sorafenib decreased as the number of cytokines with high-level expression in serum increased. We hypothesised that high-level expression of multiple cytokines, many of which have a role in angiogenesis, overwhelms the ability of sorafenib to adequately block tumour angiogenesis and growth. Unfortunately, the small sample size in our initial study precluded multivariate analyses of biomarkers.

In the present study, we examined the significance of high expression of these cytokines and their ability to predict the treatment efficacy of sorafenib and patient survival in subgroups and with a large sample size.

MATERIALS AND METHODS

Patient characteristics and diagnosis of HCC. Between October 2006 and June 2012, we enrolled 126 patients with advanced HCC, including 30 patients who were subjects of a prior publication (Miyahara *et al*, 2011), who were treated with sorafenib at our institute or collaborating hospitals in this retrospective cohort study. Six patients who were treated with sorafenib for < 10 days, 120 patients remained eligible for this study.

In accordance with the guidelines from the American Association for the Study of Liver Disease, we confirmed the eligibility of the diagnosis by at least two dynamic imaging modalities on the basis of typical vascular patterns. HCC was confirmed based on evidence of hyperattenuation in the arterial phase and hypoattenuation in the portal/venous phase of blood flow.

Written informed consent for taking serum and using it for future studies was obtained from all patients. The study protocol conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki and was approved by the ethics committee of the institute (approval numbers: 850 and 1452).

Treatments and follow-up. Ninety-two patients received 400 mg sorafenib twice daily. Twenty-eight patients were treated with 200 mg sorafenib twice daily owing to physician preference because of a patient's low weight or old age.

Patients were basically followed monthly by routine surveillance imaging, such as dynamic computed tomography or magnetic resonance imaging. All patients had at least one untreated target lesion that could be measured in one-dimension. In accordance with the Response Evaluation Criteria in Solid Tumors (RECIST) guidelines, version 1.1 (Eisenhauer *et al*, 2009), patients were evaluated for radiographic response in the primary and metastatic lesions within 42.2 ± 13.6 (mean ± s.d.) days after starting therapy. We evaluated the progression-free survival (PFS) and overall survival (OS) during 8.4 ± 5.9 (mean ± s.d.)-month follow-up time. In this follow-up time, 96 patients had progression of the disease and 72 patients died; observations on OS and PFS were censored in the other patients.

Data collection. Relevant demographic and clinical information was abstracted from the electronic medical record in consenting subjects. Variables included age, sex, Eastern Cooperative Oncology Group performance status (ECOG PS), viral infection, Child–Pugh grade, and serum laboratory tests, such as α-fetoprotein (AFP) and des-gamma-carboxy prothrombin (DCP). Data on HCC size, number of lesions, presence or absence of macroscopic vascular invasion (MVI), and extrahepatic spread (EHS) were collected before starting sorafenib, and they were followed up according to the method mentioned above.

Measurement of cytokines. Patient's serum was collected before starting treatment with sorafenib. The blood samples were centrifuged for 10 min at 15 000 **g**, and supernatants were frozen immediately and stored below − 30 °C until use. The samples were assayed to determine the concentration of Ang-2, FST, G-CSF, HGF, Leptin, PDGF-BB, PECAM-1, and VEGF using a BioPlex 200 System (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's protocols. The concentration of soluble (s)-c-KIT in serum was measured using BioPlex 200 System. Briefly, magnetic beads were coupled with the c-KIT monoclonal antibody (R&D Systems, Minneapolis, MN, USA; catalogue numbers MAB332) using the BioPlex amine coupling kit (Bio-Rad Laboratories), and c-KIT captured on the beads was detected using the c-KIT polyclonal antibody (R&D Systems; catalogue numbers BAF332). Samples were tested in duplicate, and the mean value was used for further analysis.

Statistical analysis. All cutoff values were defined as the median concentrations for all patients. Patients were divided into two groups according to the expression level of each cytokine and the number of cytokines that were above the median, a high angiogenic group (more than three cytokines above the median) and a low angiogenic group (three or less cytokines above the median), and the PFS and OS for each group were compared.

PFS and OS were calculated from the first day of therapy. Wilcoxon's rank sum test was used to compare continuous data. Fisher's exact test was used to compare categorical data. Cox's proportional hazards model was used to analyse hazard ratios (HRs). Factors exhibiting significance in a univariate analysis were further analysed by multivariate analysis (MVI, DCP, Ang-2, HGF, VEGF, and high angiogenic group). To avoid the effect of multicollinearity, the HRs of cytokine variables were examined separately in the multivariate analysis. Subgroup analysis for OS was performed for each variable using a multivariate Cox's proportional hazards model with the risk factors detected in all patients. For statistical analyses, $P < 0.05$ was considered significant. All statistical analyses were performed using the JMP statistical software (Version 8, SAS Institute, Inc., Cary, NC, USA).

RESULTS

Characteristics of patients and treatment response. Of the 120 patients, 105 (95.8%) were male (Table 1). The median age of all patients was 68 years. Fifty-five (45.8%) patients were positive for hepatitis C virus antibody (HCV Ab). Distant metastases were observed in 59 patients (49.2%). Fifteen patients experienced treatment interruptions, whereas 27 patients required reductions in the sorafenib dose prior to the first radiographic evaluation because of drug-related toxicity. None of the patients demonstrated a complete response (CR); seven (6%) patients had a partial response (PR), 54 (45%) had stable disease (SD), and 59 (49%) showed evidence of progressive disease (PD).

Cytokine expressions. The median concentrations of cytokine expression were as follows: 721.3 pg ml^{-1} for Ang-2, 333.9 pg ml^{-1} for FST, 22.6 pg ml^{-1} for G-CSF, 1005.8 pg ml^{-1} for HGF, 2321.8 pg ml^{-1} for leptin, 2334.7 pg ml^{-1} for PDGF-BB, 4384.3 pg ml^{-1} for PECAM-1, and 68.6 pg ml^{-1} for VEGF. According to these concentrations defined as each cutoff values, 67 and 53 patients were a high angiogenic group (more than three cytokines above the median) and a low angiogenic group (three or less cytokines above the median), respectively. No cytokine was frequently deregulated in high or low angiogenic group. The expressions of the eight cytokines were higher in the patients with PD than in those with non-PD (Figure 1). The expressions of HGF, leptin, and PECAM-1 were lower in patients with EHS than those

without EHS, whereas the expressions of Ang-2, G-CSF, HGF, and PDGF-BB were higher in patients with MVI than in those without MVI (Figure 2). The serum levels of other cytokines did not show statistical differences. The median concentration of s-c-KIT was 36.6 ng ml^{-1}, and we defined this concentration as a cutoff value.

Risk factors for treatment effect. Univariate analyses revealed that none of the clinical parameters including age, sex, ECOG PS, Child–Pugh grade, MVI, EHS, AFP, and DCP were a risk factor for PFS (data not shown). The expression of s-c-KIT was not correlated with PFS (HR, 0.99; 95% confidence interval (CI), 0.66–1.48). In contrast, high levels of Ang-2 (HR, 1.84; 95% CI, 1.21–2.81), G-CSF (HR, 1.61; 95% CI, 1.06–2.47), HGF (HR, 1.53; 95% CI, 1.01–2.31), and VEGF (HR, 2.08; 1.37–3.19) and being in the high angiogenic group (HR, 1.98; 95% CI, 1.30–3.06) were closely correlated with short PFS, as shown in Table 2.

High Ang-2, high HGF, high VEGF, and being in the high angiogenic group were also significant risk factors for OS (Table 2); however, s-c-KIT was not correlated with OS (HR, 0.98; 95% CI, 0.61–1.57). In addition to these cytokine markers, the presence of MVI and high DCP were correlated with poor OS by univariate analysis (Table 3), whereas AFP was not correlated with OS. Multivariate analyses with these two variables and high Ang-2 revealed that high levels of Ang-2 (HR, 1.83; 95% CI, 1.12–2.98) as well as the presence of MVI were the risk factors for OS (Table 4). As close correlation was observed between cytokine expressions, we used single cytokine parameter in multivariate analysis and repeated the examination to check the effect of all cytokines. In addition to high Ang-2, being in the high angiogenic group (HR, 1.76; 95% CI, 1.07–2.94) were risk factors for OS; however, HGF and VEGF were not correlated with OS.

Subgroup analysis for OS. We divided the patients into subgroups based on their clinicopathologic characteristics and compared HRs in the high angiogenic group of each subgroup (Figure 3). HRs were adjusted according to the risk factors for OS, which were MVI and DCP. HR of patients in the high angiogenic group was significantly higher when MVI and EHS were present. The differences in ECOG PS did not significantly affect the HR in the high angiogenic group. There was no difference in the characteristics between high and low angiogenic groups (Table 5).

DISCUSSION

We demonstrated that high expression of Ang-2 alone or high expression of more than three serum cytokines correlated with both inferior PFS and OS in the HCC patients treated with sorafenib. This confirmed the results of our previous publication, in which we showed that simultaneous measurement of Ang-2 and pro-angiogenic cytokines at baseline could predict the efficacy of sorafenib treatment (Miyahara *et al*, 2011). We now show that this relationship is particularly evident in cases with MVI and EHS.

Angiogenesis in cancer is mediated by various molecules released from the neoplastic cells or the supporting stroma cells (Carmeliet and Jain, 2011). With the exception of VEGF and PDGF-BB, none of the cytokines tested in this study are known molecular targets of sorafenib. The mechanisms of action of these cytokines vary. Ang-2 (Laurén *et al*, 1998; Tanaka *et al*, 1999), FST (Kozian *et al*, 1997; Glienke *et al*, 2000), HGF (Zarnegar, 1995), leptin (Sierra-Honigmann *et al*, 1998), PECAM-1 (Cao *et al*, 2009), and VEGF (Hicklin and Ellis, 2005) are known to activate endothelial cells. G-CSF (LeCouter *et al*, 2003; Shojaei *et al*, 2009) and PDGF-B (Abramsson *et al*, 2003) have been shown to recruit bone marrow-derived cells and pericytes, respectively, to promote angiogenesis. High expression of multiple cytokines suggests that tumour angiogenesis is activated by multiple pathways. This likely explains the failure of cancer treatment with angiogenesis

Table 1. Clinicopathologic characteristics of patients with hepatocellular carcinoma		
Variables		
Median age, year (range)	68	(36–91)
Sex, *n* (%)		
Male	105	(87.5)
Female	15	(12.5)
Viral infection, *n* (%)		
HBsAg-positive	35	(29.2)
HCVAb-positive	55	(45.8)
ECOG performance status, *n* (%)		
0	93	(77.5)
1–3	27	(22.5)
Child–Pugh grade, *n* (%)		
A	101	(84.2)
B	19	(15.8)
Macroscopic vascular invasion, *n* (%)	40	(33.3)
Extrahepatic spread, *n* (%)		
Lymph node	26	(21.7)
Distant metastasis	59	(49.2)
Macroscopic vascular invasion, extrahepatic spread, or both, *n* (%)	87	(72.5)
Tumour markers, median (range)		
AFP (ng ml^{-1})	236	(1.3–1 265 000)
DCP (mAU ml^{-1})	570	(10–226 930)

Abbreviations: AFP = α-fetoprotein; DCP = des-g-carboxy prothrombin; ECOG = Eastern Cooperative Oncology Group; HBsAg = hepatitis B surface antigen; HCVAb = antihepatitis C virus antibody.
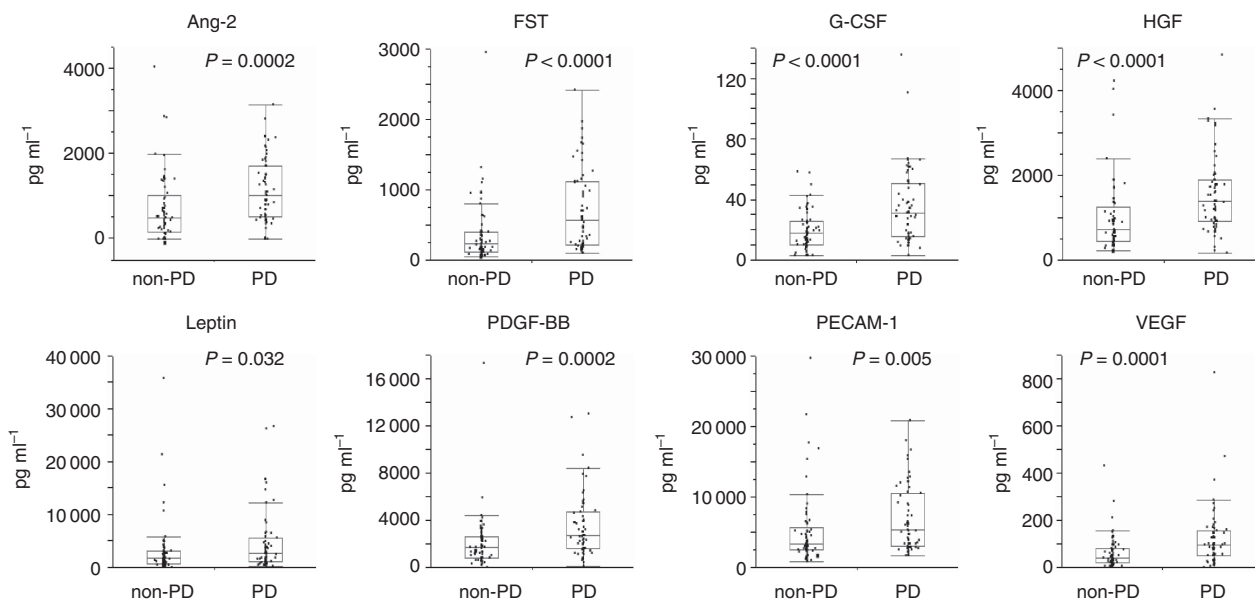
Figure 1. Serum cytokine levels in patients with progressive disease (PD) and non-PD. All cytokines were higher in PD than in non-PD. Horizontal bars in the boxes indicate the median. Abbreviations: Ang-2 = angiopoietin-2; FST = follistatin; G-CSF = granulocyte colony-stimulating factor; HGF = hepatocyte growth factor; PDGF-BB = platelet-derived growth factor-BB; PECAM-1 = platelet endothelial cell adhesion molecule-1; VEGF = vascular endothelial growth factor.
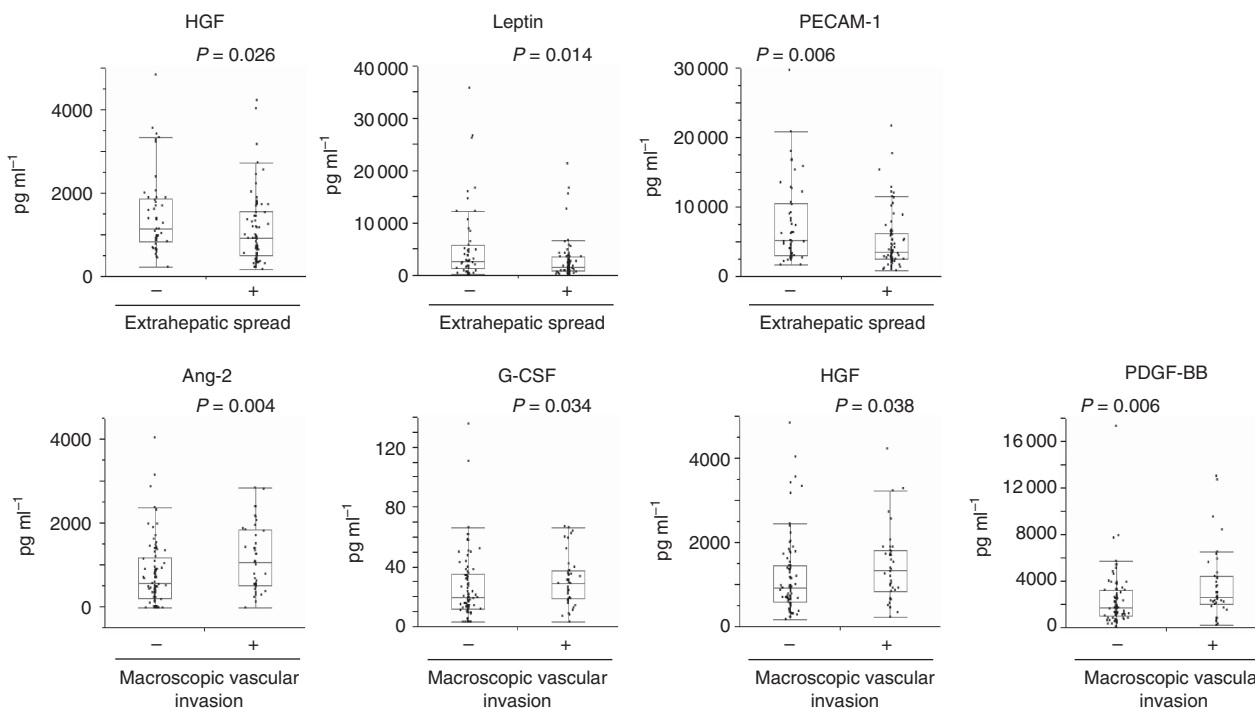

Figure 2. Serum cytokine levels in patients with and without extrahepatic spread or macroscopic vascular invasion. Only cytokines that showed significant difference were shown. Horizontal bars in the boxes indicate the median. Abbreviations: Ang-2 = angiopoietin-2; FST = follistatin; G-CSF = granulocyte colony-stimulating factor; HGF = hepatocyte growth factor; PDGF-BB = platelet-derived growth factor-BB; PECAM-1 = platelet endothelial cell adhesion molecule-1; VEGF = vascular endothelial growth factor.

inhibitors (AIs) that target one or a few signalling pathways. We quantified the expression of multiple cytokines simultaneously to assess the pro-angiogenic status of HCC patients and to demonstrate the utility of such measurements and the importance of Ang-2 expression in HCC patients who are treated with sorafenib.

HCC cells that express multiple angiogenic cytokines at high levels are considered to have high malignant potential. This is one

potential reason for the observed relationship between patients belonging to the high angiogenic group and poor treatment response to sorafenib. However, there are likely other mechanisms because we observed an association between the high angiogenic group and poor response to sorafenib treatment, even when we limited the analysis to patients with EHM or MVI, which is considered to be markers of poor differentiation. In addition, we did not observe the association between the high angiogenic group

Table 2. Univariate analyses of sorafenib-treated hepatocellular carcinoma patients to identify cytokine markers prognostic for progression-free and overall survival

Variables	Progression-free survival			Overall survival		
	Hazard ratio	95% Confidence of interval	*P*-value	Hazard ratio	95% Confidence of interval	*P*-value
Ang-2 ($>721.3\,pg\,ml^{-1}$)	1.84	1.21–2.81	0.004	1.95	1.21–3.17	0.006
FST ($>333.9\,pg\,ml^{-1}$)	1.49	0.99–2.27	0.055	1.46	0.91–2.36	0.119
G-CSF ($>22.6\,pg\,ml^{-1}$)	1.61	1.06–2.47	0.025	1.57	0.98–2.55	0.062
HGF ($>1005.8\,pg\,ml^{-1}$)	1.53	1.01–2.31	0.043	1.68	1.05–2.71	0.031
Leptin ($>2321.8\,pg/ml$)	1.22	0.81–1.84	0.339	0.82	0.51–1.30	0.390
PDGF-BB ($>2334.7\,pg\,ml^{-1}$)	1.35	0.90–2.03	0.150	1.27	0.80–2.03	0.317
PECAM-1 ($>4384.3\,pg\,ml^{-1}$)	1.44	0.95–2.17	0.084	1.16	0.73–1.85	0.538
VEGF ($>68.6\,pg\,ml^{-1}$)	2.08	1.37–3.19	0.0006	1.64	1.02–2.64	0.039
High angiogenic group	1.98	1.30–3.06	0.001	1.94	1.19–3.22	0.007

Abbreviations: Ang-2 = angiopoietin-2; FST = follistatin; G-CSF = granulocyte colony-stimulating factor; HGF = hepatocyte growth factor; PDGF-BB = platelet-derived growth factor-BB; PECAM-1 = platelet endothelial cell adhesion molecule-1; VEGF = vascular endothelial growth factor. High angiogenic group, patients with >3 serum cytokines expressed above their median values.

Table 3. Univariate analyses of sorafenib-treated hepatocellular carcinoma patients to identify clinical parameters prognostic for overall survival

Variables	Hazard ratio	95% Confidence of interval	*P*-value
Age (>68 year)	0.63	0.39–1.01	0.059
Sex (Female)	1.19	0.62–2.11	0.565
ECOG performance status (1–3)	1.80	0.99–3.10	0.052
HBVAg (positive)	1.17	0.70–1.91	0.520
HCVAb (positive)	1.08	0.68–1.72	0.727
Child–Pugh grade (B)	1.82	0.97–3.21	0.060
MVI (present)	2.48	1.50–4.04	< 0.001
EHS (present)	1.59	0.98–2.65	0.056
AFP ($>200\,ng\,ml^{-1}$)	1.31	0.82–2.12	0.258
DCP ($>500\,mAU\,ml^{-1}$)	1.64	1.02–2.65	0.039

Abbreviations: AFP = α-fetoprotein; DCP = des-gamma-carboxy prothrombin; ECOG = Eastern Cooperative Oncology Group; EHS = extrahepatic spread; HBsAg = hepatitis B surface antigen; HCVAb = antihepatitis C virus antibody; MVI = macroscopic vascular invasion.

Table 4. Multivariate analyses of sorafenib-treated hepatocellular carcinoma patients to identify prognostic factors for overall survival

Variables	Hazard ratio	95% Confidence of interval	*P*-value
Clinical parameters			
MVI (present)	2.27	1.36–3.72	0.001
DCP ($>500\,mAU\,ml^{-1}$)	1.42	0.87–2.31	0.153
Cytokine markers			
Ang-2 ($>721.3\,pg\,ml^{-1}$)	1.83	1.12–2.98	0.014
HGF ($>1005.8\,pg\,ml^{-1}$)	1.47	0.90–2.40	0.115
VEGF ($>68.6\,pg\,ml^{-1}$)	1.52	0.95–2.47	0.079
High angiogenic group	1.76	1.07–2.94	0.023

Note: The hazard ratios of cytokine markers were examined separately in the multivariate analyses. The values shown in clinical parameters were those analysed with angiopoietin-2. Abbreviations: Ang-2 = angiopoietin-2; DCP = des-gamma-carboxy prothrombin; HGF = hepatocyte growth factor; MVI = macroscopic vascular invasion; VEGF = vascular endothelial growth factor. High angiogenic group, patients with >3 serum cytokines expressed above their median values.

and poor response in patients treated with hepatic arterial infusion chemotherapy, which is not a direct inhibitor of angiogenesis (data not shown). These observations indicated that the increased expression of pro-angiogenic cytokines was not merely a consequence of the disease progression but was associated with a treatment response, especially to AIs.

Biomarkers for the prediction of efficacy of AIs have been reported. Llovet *et al* (2012) reported that plasma c-KIT and HGF are potential markers that predict response to sorafenib in HCC patients, although these results did not reach statistical significance. They also demonstrated that Ang-2 and VEGF were independent predictors of survival. Zhu *et al* (2009) reported that plasma VEGF levels may predict PFS in HCC patients treated with sunitinib. We similarly observed that high Ang-2 expression was closely related to poor PFS and OS in sorafenib-treated HCC patients. PFS was also short in HCC patients with high serum levels of VEGF. Hence, these cytokines seem to be important for
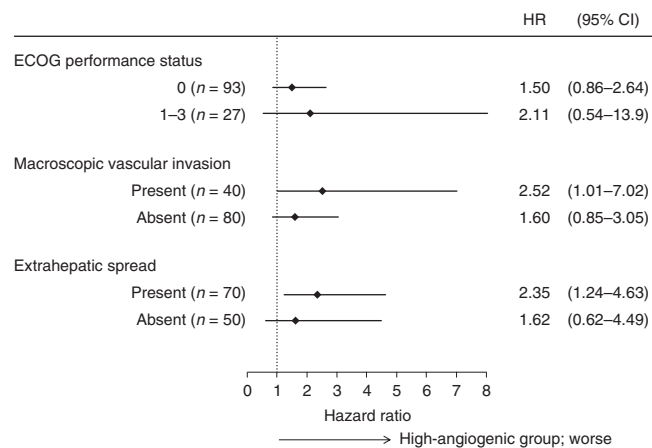

Figure 3. Subgroup analyses for overall survival. Overall survival of the high angiogenic group was significantly short when macroscopic vascular invasion or extrahepatic spread was present. ECOG denotes the Eastern Cooperative Oncology Group.

Table 5. Clinicopathologic characteristics of high- and low-angiogenic groups

Variables	High angiogenic group (n = 67)	Low angiogenic group (n = 53)	P-value
Median age, year (range)	68 (36–81)	70 (48–91)	0.254
Sex, n (%)			
Male	60 (89.6)	45 (84.9)	0.580
Female	7 (10.4)	8 (15.1)	
Viral infection, n (%)			
HBsAg-positive	16 (23.9)	19 (35.9)	0.163
HCVAb-positive	36 (53.7)	19 (35.9)	0.065
ECOG performance status, n (%)			
0	49 (73.1)	44 (83.0)	0.271
1–3	18 (26.9)	9 (17.0)	
Child–Pugh grade, n (%)			
A	54 (80.6)	47 (88.7)	0.315
B	13 (19.4)	6 (11.3)	
Macroscopic vascular invasion, n (%)	27 (40.3)	13 (24.5)	0.081
Extrahepatic spread, n (%)			
Lymph node	13 (19.4)	13 (24.5)	0.512
Distant metastasis	33 (49.3)	26 (49.1)	>0.999
Lymph node, distant metastasis, or both, n (%)	36 (53.7)	34 (64.2)	0.269
Macroscopic vascular invasion, extrahepatic spread, or both, n (%)	47 (70.2)	40 (75.5)	0.544
Tumour markers, median (range)			
AFP (ng ml)	236 (1.3–59305)	216 (2.6–1265000)	0.897
DCP (mAU ml)	688 (10–198800)	307 (10–226930)	0.597

Abbreviations: AFP = α-fetoprotein; DCP = des-g-carboxy prothrombin; ECOG = Eastern Cooperative Oncology Group; HBsAg = hepatitis B surface antigen; HCVAb = antihepatitis C virus antibody.

predicting the outcome of HCC patients treated with sorafenib, regardless of nationality or race.

On the other hand, the utility of simultaneous measurement of cytokine expression to assess the pro-angiogenic status of individuals is a new concept that has only previously been reported by our research group in our previous study. Our subgroup analysis revealed that simultaneous measurement of cytokine expression was also useful for predicting OS in HCC patients with MVI or EHS. Although sorafenib is reported to be less effective in patients with EHS, our study suggests that HCC patients with EHS and increased expression of 3 or less cytokines might represent a subgroup that would benefit from treatment with sorafenib.

We also examined the expression levels of these cytokines at 1 week after starting sorafenib treatment. Although the data were preliminary (n = 30, data not shown), most of the cytokines including Ang-2, FST, HGF, PECAM-1, and VEGF were elevated after starting sorafenib treatment; however, no correlation was observed between the changes of the cytokine levels and PFS or OS.

In this study, ECOG PS and Child–Pugh grade were not risk factors for PFS and OS, although these variables are known as prognostic factors. We treated only patients with good ECOG PS or Child–Pugh grade so that the prognostic importance of these factors might be diminished.

We confirmed the relationship between cytokine expression and the outcome of sorafenib treatment. However, we did not directly compare the utility of the biomarkers between patients treated with sorafenib or placebo. The lack of a placebo control makes it difficult to conclude whether the poor outcomes in patients with high expression of cytokines were owing to resistance to sorafenib or because HCC tumours were innately more aggressive. Another limitation is that this study is retrospective and not a randomised, placebo-controlled clinical trial.

Nevertheless, we have demonstrated that Ang-2 and simultaneous measurement of pro-angiogenic cytokines in serum predicts survival outcomes in HCC patients treated with sorafenib. Many molecular-targeted agents including anti-angiogenic agents are now under development (Kudo, 2011). The results of our study suggests that further examination is necessary to validate the clinical utility of cytokine measurement for predicting outcomes in patients treated with various AIs and chemotherapeutic agents.

REFERENCES

Abramsson A, Lindblom P, Betsholtz C (2003) Endothelial and nonendothelial sources of PDGF-B regulate pericyte recruitment and influence vascular pattern formation in tumors. *J Clin Invest* **112**: 1142–1151.

Bruix J, Sherman M. American Association for the Study of Liver Diseases (2011) Management of hepatocellular carcinoma: an update. *Hepatology* **53**: 1020–1022.

Bruix J, Sherman M (2005) Management of hepatocellular carcinoma. *Hepatology* **42**: 1208–1236.

Cao G, Fehrenbach ML, Williams JT, Finklestein JM, Zhu JX, Delisser HM (2009) Angiogenesis in platelet endothelial cell adhesion molecule-1-null mice. *Am J Pathol* **175**: 903–915.

Carmeliet P, Jain RK (2011) Molecular mechanisms and clinical applications of angiogenesis. *Nature* **473**: 298–307.

Cheng AL, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, Luo R, Feng J, Ye S, Yang TS, Xu J, Sun Y, Liang H, Liu J, Wang J, Tak WY, Pan H, Burock K, Zou J, Voliotis D, Guan Z (2009) Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol* **10**: 25–34.

Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, Rubinstein L, Shankar L, Dodd L, Kaplan R, Lacombe D, Verweij J (2009) New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* **45**: 228–247.

Glienke J, Schmitt AO, Pilarsky C, Hinzmann B, Weiss B, Rosenthal A, Thierauch KH (2000) Differential gene expression by endothelial cells in distinct angiogenic states. *Eur J Biochem* **267**: 2820–2830.

Hicklin DJ, Ellis LM (2005) Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J Clin Oncol* **23**: 1011–1027.

Jemal A, Siegel R, Xu J, Ward E (2010) Cancer statistics, 2010. *CA Cancer J Clin* **60**: 277–300.

Kozian DH, Ziche M, Augustin HG (1997) The activin-binding protein follistatin regulates autocrine endothelial cell activity and induces angiogenesis. *Lab Invest* **76**: 267–276.

Kudo M (2011) Hepatocellular carcinoma in 2011 and beyond: from the pathogenesis to molecular targeted therapy. *Oncology* **81**(Suppl 1): 1–10.

Laurén J, Gunji Y, Alitalo K (1998) Is angiopoietin-2 necessary for the initiation of tumor angiogenesis? *Am J Pathol* **153**: 1333–1339.

LeCouter J, Lin R, Tejada M, Frantz G, Peale F, Hillan KJ, Ferrara N (2003) The endocrine-gland-derived VEGF homologue Bv8 promotes angiogenesis in the testis: Localization of Bv8 receptors to endothelial cells. *Proc Natl Acad Sci USA* **100**: 2685–2690.

Llovet JM (2005) Updated treatment approach to hepatocellular carcinoma. *J Gastroenterol* **40**: 225–235.

Llovet JM, Burroughs A, Bruix J (2003) Hepatocellular carcinoma. *Lancet* **362**: 1907–1917.

Llovet JM, Di Bisceglie AM, Bruix J, Kramer BS, Lencioni R, Zhu AX, Sherman M, Schwartz M, Lotze M, Talwalkar J, Gores GJ. Panel of Experts in HCC-Design Clinical Trials (2008b) Design and endpoints of clinical trials in hepatocellular carcinoma. *J Natl Cancer Inst* **100**: 698–711.

Llovet JM, Peña CE, Lathia CD, Shan M, Meinhardt G, Bruix J. SHARP Investigators Study Group (2012) Plasma biomarkers as predictors of outcome in patients with advanced hepatocellular carcinoma. *Clin Cancer Res* **18**: 2290–2300.

Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J. SHARP Investigators Study Group (2008a) Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* **359**: 378–390.

Miyahara K, Nouso K, Tomoda T, Kobayashi S, Hagihara H, Kuwaki K, Toshimori J, Onishi H, Ikeda F, Miyake Y, Nakamura S, Shiraha H, Takaki A, Yamamoto K (2011) Predicting the treatment effect of sorafenib using serum angiogenesis markers in patients with hepatocellular carcinoma. *J Gastroenterol Hepatol* **26**: 1604–1611.

Shojaei F, Wu X, Qu X, Kowanetz M, Yu L, Tan M, Meng YG, Ferrara N (2009) G-CSF-initiated myeloid cell mobilization and angiogenesis mediate tumor refractoriness to anti-VEGF therapy in mouse models. *Proc Natl Acad Sci USA* **106**: 6742–6747.

Sierra-Honigmann MR, Nath AK, Murakami C, García-Cardeña G, Papapetropoulos A, Sessa WC, Madge LA, Schechner JS, Schwabb MB, Polverini PJ, Flores-Riveros JR (1998) Biological action of leptin as an angiogenic factor. *Science* **281**: 1683–1686.

Tanaka S, Mori M, Sakamoto Y, Makuuchi M, Sugimachi K, Wands JR (1999) Biologic significance of angiopoietin-2 expression in human hepatocellular carcinoma. *J Clin Invest* **103**: 341–345.

Wilhelm SM, Adnane L, Newell P, Villanueva A, Llovet JM, Lynch M (2008) Preclinical overview of sorafenib, a multikinase inhibitor that targets both Raf and VEGF and PDGF receptor tyrosine kinase signalling. *Mol Cancer Ther* **7**: 3129–3140.

Wilhelm SM, Carter C, Tang L, Wilkie D, McNabola A, Rong H, Chen C, Zhang X, Vincent P, McHugh M, Cao Y, Shujath J, Gawlak S, Eveleigh D, Rowley B, Liu L, Adnane L, Lynch M, Auclair D, Taylor I, Gedrich R, Voznesensky A, Riedl B, Post LE, Bollag G, Trail PA (2004) BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res* **64**: 7099–7109.

Zarnegar R (1995) Regulation of HGF and HGFR gene expression. *EXS* **74**: 33–49.

Zhu AX, Sahani DV, Duda DG, di Tomaso E, Ancukiewicz M, Catalano OA, Sindhwani V, Blaszkowsky LS, Yoon SS, Lahdenranta J, Bhargava P, Meyerhardt J, Clark JW, Kwak EL, Hezel AF, Miksad R, Abrams TA, Enzinger PC, Fuchs CS, Ryan DP, Jain RK (2009) Efficacy, safety, and potential biomarkers of sunitinib monotherapy in advanced hepatocellular carcinoma: a phase II study. *J Clin Oncol* **27**: 3027–3035.

APPENDIX

All members of the Okayama Liver Cancer Group were as follows: Chair: Kazuhide Yamamoto, Okayama University, Okayama; Members: Yuki Morimoro, Yasuto Takeuchi, Koji Miyahara, Hiroaki Hagihara, Kenji Kuwaki, Hideki Onishi, Shinichiro Nakamura, Hidenori Shiraha and Kazuhiro Nouso, Okayama University, Okayama; Yoshitaka Takuma, Hiroyuki Takabatake and Youichi Morimoto, Kurashiki Central Hospital, Okayama; Shin-ichi Fujioka and Toshiya Osawa, Okayama Saiseikai General Hospital, Okayama; Kazuya Kariyama, Okayama city Hospital, Okayama; Junichi Toshimori and Haruhiko Kobashi, Okayama Red Cross General Hospital, Okayama; Hirokazu Miyatake, Shouta Iwadou, Yoshiyuki Kobayashi, Shuji Uematsu, Ryoichi Okamoto and Yasuyuki Araki, Hiroshima City Hospital, Hiroshima; Masafumi Tatsukawa, Kazuhisa Yabushita, Toshinari Shimoe and Kohsaku Sakaguchi, Fukuyama City Hospital, Hiroshima; Tatsuro Sakata and Toshihiko Kaneyoshi, Fukuyama Medical Center, Hiroshima; Manabi Miyashita and Yasuhiro Makino, Iwakuni Clinical Center, Yamaguchi; Akio Moriya and Masaharu Ando, Mitoyo General Hospital, Kagawa; Nobuyuki Baba, Tomonori Seno, Takuya Nagano and Koichi Takaguchi, Kagawa Prefectural Central Hospital, Kagawa; Eiji Matsumoto, Sumitomo Besshi Hospital, Ehime; and Hiroki Takayama, Tsuyama Central Hospital, Okayama.