rate ranging from 24 to 27%) or pancreatic head adenocarcinomas (5-year survival rate around 15%) (2–6). However, for patients with advanced disease, an accurate prognosis cannot be made because of the lack of reliable data, with the exception of one retrospective study that examined the outcome of systemic chemotherapy in 29 patients with advanced ampullary adenocarcinoma (7). Because of the rarity of this disease, advanced adenocarcinomas are often treated using regimens designed for biliary tract adenocarcinomas or small bowel adenocarcinomas. The The National Comprehensive Cancer Network (NCCN) guidelines recommend that small bowel adenocarcinomas be treated with systemic chemotherapy according to the colon cancer guidelines which recommend folinic acid, 5-FU and oxaliplatin (FOLFOX) or folinic acid, 5-FU and irinotecan (FOLFIRI) ± bevacizumab as the initial therapy. Meanwhile, the NCCN guidelines recommend that biliary tract adenocarcinomas should be treated with gemcitabine (GEM) + cisplatin (CDDP) combination therapy. However, whether advanced ampullary adenocarcinomas should be treated as biliary tract adenocarcinomas or as small bowel adenocarcinomas remain uncertain. Additionally, ampullary adenocarcinomas can be separated into two histological phenotypes, intestinal type and pancreatobiliary type (8,9). However, no previous report has examined the outcome of systemic chemotherapy analyzed according to the histological phenotypes of advanced ampullary adenocarcinomas.

The objective of the present study was to clarify (i) the treatment outcome of systemic chemotherapy for advanced ampullary adenocarcinomas, (ii) the difference in outcomes according to the chemotherapeutic regimens, (iii) the difference in outcomes according to the disease status and (iv) the difference in outcomes according to the adenocarcinoma phenotype.

PATIENTS AND METHODS

Patients

We retrospectively reviewed the clinical data in our institution's database and extracted patients who were diagnosed as having advanced ampullary adenocarcinoma and who had received systemic chemotherapy between January 1997 and December 2010. Patients were eligible if they had a recurrent or unresectable adenocarcinoma arising from the ampulla of Vater. Written informed consent was obtained from all the patients before treatment. This study was approved by the institutional review board of the National Cancer Center Hospital (NCCH) of Japan and was performed in accordance with the Declaration of Helsinki in 1964.

The following clinical characteristics of all the patients with advanced ampullary adenocarcinoma were reviewed: age, Eastern Cooperative Oncology Group (ECOG) performance status, tumor histology and adenocarcinoma phenotype.

Classification of Chemotherapy Regimens

We examined the chemotherapy regimens and the treatment outcome of systemic chemotherapy in patients with ampullary adenocarcinomas. The responses were evaluated according to the Response Evaluation Criteria in Solid Tumors 1.0. We classified the chemotherapy regimens into two types: 5-FU based and GEM based. The chemotherapy regimens were divided into two groups because GEM is a key drug for the current treatment of biliary adenocarcinomas, while 5-fluorouracil (5-FU) has been widely used as a key drug for gastrointestinal malignancies including biliary tract adenocarcinomas, colon adenocarcinomas and small bowel adenocarcinomas. In our hospital, 5-FU-based regimens were frequently used in clinical trials (10,11) for advanced biliary tract adenocarcinomas, including ampullary adenocarcinomas, or for clinical practical use before the recognition of GEM as a key agent for the treatment of biliary tract adenocarcinomas.

Immunohistochemistry

Paraffin-embedded materials from a series of pancreaticoduodenectomy specimens ($n = 9$) and a biopsy specimen ($n = 1$) obtained at NCCH were used for the immunohistochemistry (IHC) analysis. Specimens from the other patients ($n = 16$) were not available for use at NCCH because the patients had been pathologically diagnosed as having advanced ampullary adenocarcinoma at another hospital.

For the IHC studies, the tissue sections were treated with hydrogen peroxide to inactivate endogenous peroxidases after deparaffinization in xylene and rehydration in ethanol. The slides were placed in 10 mmol/l of citrate buffer at pH 6.0, then autoclaved for antigen retrieval. The primary antibodies were incubated overnight, and a secondary antibody was used to detect protein expression using EnVision™ (Dako, Glostrup, Denmark). Diaminobenzidine was used as the chromogen, and the nuclei were counterstained with hematoxylin. The antibodies used in the analysis were as follows: MUC1 (Ma552, 1:100), MUC2 (Ccp58, 1:100), MUC5AC (CLH2, 1:100), MUC6 (CLH5, 1:100) and CD10 (56C6, 1:100) from Leica Biosystems (Newcastle Upon, Tyne, UK) and CDX2 (CDX2-88, 1:100) from Biocare medical (Concord, CA, USA).

Two independent observers without prior knowledge of the clinicopathological data scored the IHC findings; the presence of positive cancer cells at any staining intensity and accounting for $>10\%$ of the sample was considered a positive finding.

Statistical Analysis

The Fisher exact test was used to assess the hypothesis of independence between the categorical variables. For the quantitative data such as age, we used the Mann–Whiney test.

Treatment outcomes were estimated as the response rate, progression-free survival (PFS) and overall survival (OS). PFS was defined as the time from the initiation of chemotherapy to the confirmation of disease progression or death from any cause. Patients who were lost to follow-up were treated as censored observations. The OS period was defined as the time from chemotherapy until the date of death or the most recent

follow-up. Patients who were lost to follow-up were treated as censored cases. Both the PFS and the OS were estimated using the Kaplan–Meier method, and significance was determined using the log-rank test. All the statistical analyses were performed using StatView (Ver. 5.0; SAS, Inc., Tokyo, Japan).

RESULTS

Patient Characteristics

We identified 28 patients with advanced ampullary adenocarcinoma who received non-surgical treatment between March 1997 and July 2010. The treatments consisted of chemotherapy ($n = 26$) and best-supportive care ($n = 2$). Among the 26 patients who received chemotherapy, the median age of the patients was 62.0 years (range, 48–79 years) and the ECOG performance statuses were as follows: 18 patients with PS 0, 8 patients with PS 1, and 0 patients with PS 2–4. All the patients had metastatic disease. The metastatic sites were the liver ($n = 17$), lungs ($n = 7$), lymph nodes ($n = 14$), peritoneum ($n = 1$), and pleura ($n = 1$). None of the patients had locally advanced disease (Table 1). The chemotherapy regimens consisted of 5-FU + CDDP ($n = 3$), tegafur-uracil (UFT) + doxorubicin ($n = 5$), tegafur, gimeracil and oteracil potassium (S-1) ($n = 3$), GEM ($n = 10$) and GEM + CDDP ($n = 5$). The median number of cycles of first-line chemotherapy prescribed was 5-FU + CDDP in 5 (range 1–5), UFT + doxorubicin in 3 (range 1–4), S-1 in 6 (range 2–11), GEM in 3 (range 1–11) and GEM + CDDP in 3 (range 1–9).

Table 1. Patient characteristics

Variable	No. of patients (%)
Age, median (range)	62.0 (48–79)
Sex	
Male	15 (58)
Female	11 (42)
ECOG PS	
0	18 (69)
1	8 (31)
2–4	0 (0)
Stage (UICC 7th edition)	
IV	12 (46)
Recurrence	14 (54)
Metastatic sites	
Liver	17 (65)
Lymph node	14 (54)
Lungs	7 (27)
Peritoneum	1 (4)
Pleura	1 (4)

ECOG PS, Eastern Cooperative Oncology Group performance status; UICC, the Union for International Cancer Control TNM Classification of Malignant Tumors (7th edition).

Outcome of Systemic Chemotherapy for Advanced Ampullary Adenocarcinomas

The response to systemic chemotherapy was evaluated in 26 patients, and these responses are listed in Table 2. None of the patients achieved a complete response. Two patients who received 5-FU + CDDP and S-1 exhibited partial responses and 18 patients achieved stable disease. The response rate (CR + PR) was 7.7% [95% confidence interval (CI) = 0.95–25.1%], and the disease control rate (CR + PR + SD) was 76.9% (95% CI = 56.4–91.0%).

As shown in Fig. 1, the median PFS and the OS from the initiation of chemotherapy were 3.2 and 9.1 months, respectively.

Treatment Outcome According to Treatment Regimens

The chemotherapy regimens were classified into two groups: the 5-FU group, consisting of 5-FU plus CDDP ($n = 3$), UFT plus doxorubicin ($n = 5$) and S-1 alone ($n = 3$) and the GEM group, consisting of GEM alone ($n = 10$) and GEM plus CDDP ($n = 5$). In the 5-FU group, the median age of the patients was 64.0 years and the ECOG performance statuses were as follows: 8 patients (73%) with PS 0 and 3 patients (27%) with PS 1. All the patients had metastatic lesion. In the GEM group, on the other hand, the median age of the patients was 66.0 years and the ECOG performance statuses were as follows: 10 patients (67%) with PS 0 and 5 patients (33%) with PS 1. All the patients also had metastatic lesion. None of the patient characteristics were significantly different between the two treatment groups.

The responses according to the treatment groups and regimens are listed in Table 2. In the 5-FU group, two patients (18%) achieved a partial response, six (55%) remained stable and three (27%) showed progressive disease. The response

Table 2. Tumor response according to treatment groups

Regimens	RR (%)	DCR (%)	Median PFS (months)	Median OS (months)
5-FU group				
5-FU + CDDP ($n = 3$)	33	100		
UFT + doxorubicin ($n = 5$)	0	60		
S-1 ($n = 3$)	33	67		
Total ($n = 11$)	18	72.7	2.5	8.0
GEM group				
GEM ($n = 10$)	0	80		
GEM + CDDP ($n = 5$)	0	80		
Total ($n = 15$)	0	80	3.5	12.3

RR, response rate; DCR, disease control rate; PFS, progression-free survival; OS, overall survival.

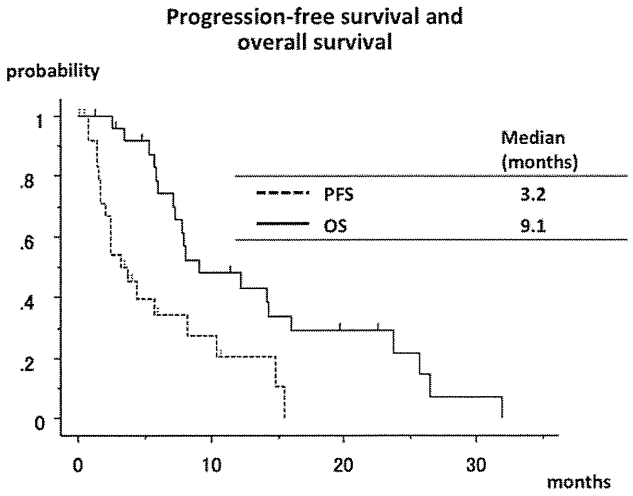

Figure 1. Progression-free survival (PFS) curve and overall survival (OS) curve calculated using the Kaplan–Meier method for all adenocarcinoma patients.

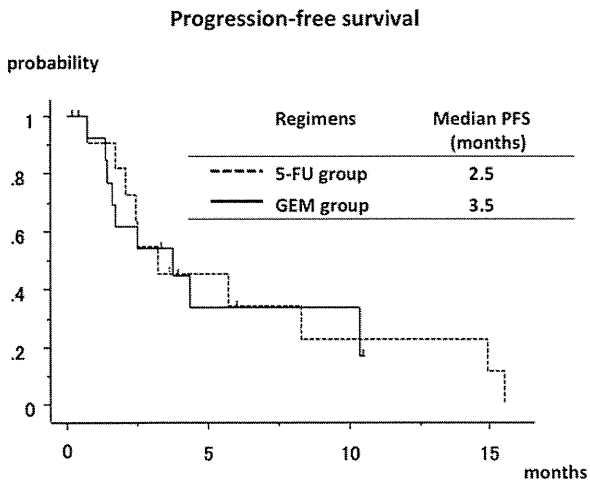

Figure 2. PFS calculated using the Kaplan–Meier method for groups classified according to the chemotherapy regimen.

rate for the 5-FU group was 18.2% (95% CI = 2.3–51.8%), and the disease control rate was 72.7% (95% CI = 39.0–94.0%). In the GEM group, on the other hand, the response rate was 0% (95% CI = 0–21.8%) and the disease control rate was 80.0% (95% CI = 51.9–95.7%). The median PFS was 2.5 and 3.5 months for the 5-FU group and the GEM group, respectively ($P = 0.79$) (Fig. 2). The median OS was 8.0 and 12.3 months, respectively ($P = 0.29$) (Fig. 3).

Three patients (27%) in the 5-FU group received second-line chemotherapy. In the GEM group, 4 (27%) patients received second-line chemotherapy.

Treatment Outcome According to Stage IV Disease or Recurrent Disease

Stage IV disease was present at the time of diagnosis in 12 patients, while recurrent disease after resection was present in 14 patients. In stage IV disease, the median age of the patients

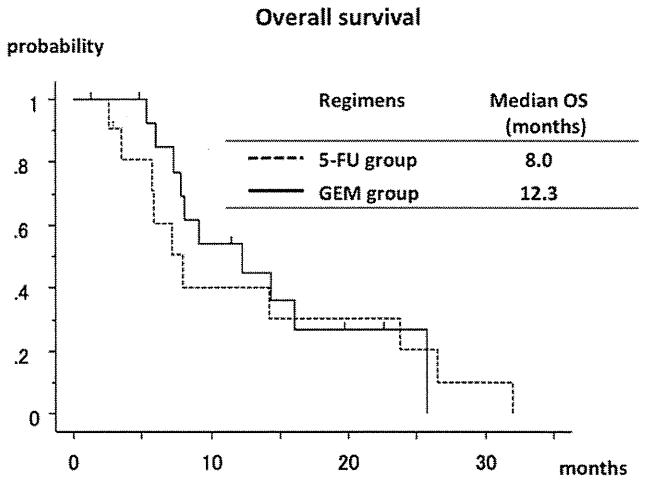

Figure 3. OS curve calculated using the Kaplan–Meier method for groups classified according to chemotherapy regimen.

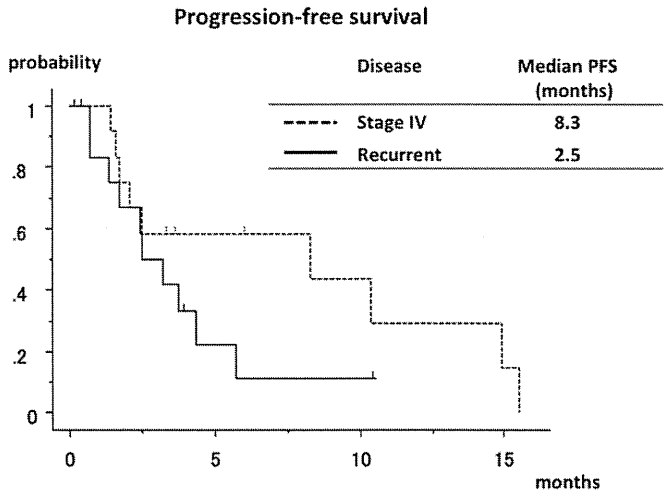

Figure 4. PFS calculated using the Kaplan–Meier method for groups classified according to stage IV disease or recurrent disease.

was 65.0 years and the ECOG performance statuses were as follows: nine patients (75%) with PS 0 and three patients (25%) with PS 1. Five of the 12 patient with stage IV disease had received 5-FU-based regimens and the remaining 7 patients received GEM-based regimens. Meanwhile, in recurrent disease, the median age of the patients was 66.0 years and the ECOG performance statuses were as follows: 9 patients (64%) with PS 0 and 5 patients (36%) with PS 1. Six of the 14 patient with recurrent disease had received 5-FU-based regimens and the remaining 8 patients received GEM-based regimens.

The response rate for stage IV disease was 8.3% (95% CI = 0.2–38.5%), and the disease control rate was 75.0% (95% CI = 42.8–94.5%). In recurrent disease, on the other hand, the response rate was 7.1% (95% CI = 0.2–33.9%) and the disease control rate was 78.6% (95% CI = 49.2–95.3%). The median PFS was 8.3 and 2.5 months for stage IV disease and recurrent disease, respectively ($P = 0.16$) (Fig. 4). The median OS was 23.8 and 7.9 months, respectively ($P = 0.02$) (Fig. 5).

Five patients (42%) in stage IV disease received second-line chemotherapy. In the recurrent disease, 2 (14%) patients received second-line chemotherapy.

Treatment Outcome According to Adenocarcinoma Phenotype

We examined 10 of the 26 ampullary adenocarcinomas to determine their phenotypes. The treatment regimens and outcomes according to the phenotypes are shown in Table 3. Eight of the 10 patients with ampullary adenocarcinoma (80%) had intestinal-type adenocarcinomas, while the remaining 2 (20%) had pancreatobiliary-type adenocarcinomas. Both patients with pancreatobiliary-type adenocarcinoma had received a GEM-based regimen, while 3 of the 8 patients with intestinal-type received 5-FU-based regimens and the remaining 5 patients received GEM-based regimens. One patient with intestinal-type adenocarcinoma who received 5-FU + CDDP responded to the treatment (PR), and the best response of the nine other patients was stable disease. The median OS was 7.9 months for the intestinal-type adenocarcinoma patients. The OS periods of the two pancreatobiliary-type adenocarcinoma patients were 12.3 months (373 days) and 14.3 months (435 days), respectively.

DISCUSSION

Ampullary adenocarcinoma is a rare disease entity, and little information regarding these tumors is available. Patients with ampullary adenocarcinomas are typically diagnosed at a relatively early stage due to the early appearance of clinical symptoms such as jaundice, and the likelihood of resectability is therefore high (12,13). On the other hand, detailed reports on advanced ampullary adenocarcinomas are extremely rare, especially regarding the treatment outcome of systemic chemotherapy for advanced stage disease.

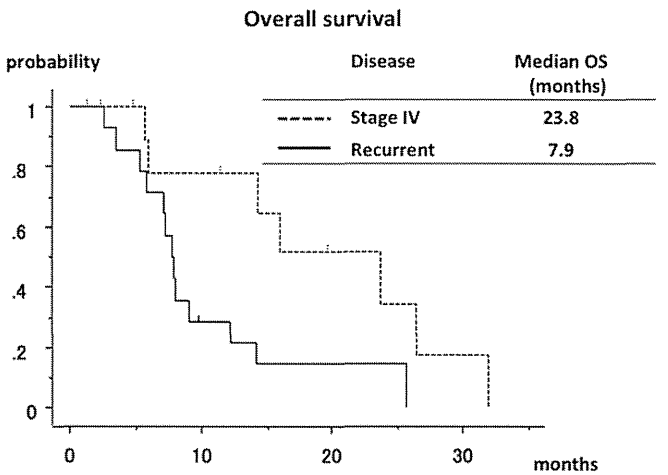

Figure 5. OS curve calculated using the Kaplan–Meier method for groups classified according to stage IV disease or recurrent disease.

A previous report discussed the efficacy of CDDP-based chemotherapy (5-FU plus CDDP or GEM plus CDDP) in 29 patients with advanced ampullary carcinoma (7). The treatment outcomes resulted in a responses rate of 27.5%, a disease control rate of 72.4%, a median time to progression of 4.9 months and a median OS period of 12.5 months, with manageable toxicities. 5-FU, CDDP and GEM were the selected agents examined in their report similar to the present study. However, in their report, the differences in the response rate, time to progression and OS were not related to the chemotherapy regimens (5-FU plus CDDP or GEM plus CDDP). In our results, the differences in the DCR, the PFS and the OS between the 5-FU group and the GEM group were also not statistically significant. Both reports indicated a modest activity for these agents against advanced ampullary adenocarcinomas; however, the optimum regimen is unknown, and patient prognosis remains dismal.

The ampulla of Vater consists of the following three distinct epithelial elements: duodenal epithelium, pancreatic ductal epithelium and biliary ductal epithelium. Because of the rarity of ampullary adenocarcinomas, they are usually regarded as biliary tract adenocarcinomas or small intestine adenocarcinomas when selecting a chemotherapeutic regimen. However, no consensus exists regarding which of these disease entities is most appropriate for the inclusion of ampullary adenocarcinomas.

In some previous reports, advanced ampullary adenocarcinomas have been included with advanced small bowel adenocarcinomas. Two prospective phase 2 studies have been performed for patients with small bowel adenocarcinoma, including ampullary adenocarcinoma. First, the combination of 5-FU, doxorubicin and mitomycin C (FAM) in 38 patients with advanced adenocarcinoma of the small bowel ($n = 34$) or ampulla of Vater ($n = 4$) resulted in a response rate of 18% and a median OS of 8 months for all the patients (14). In a subgroup analysis, the median OS of the advanced ampullary adenocarcinoma patients ($n = 4$) was 7 months, which was roughly similar to that of the small bowel adenocarcinoma patients (median OS: duodenum, 9 months; jejunum, 2 months; and ileum, 5 months). Secondly, the combination of capecitabine and oxaliplatin (CAPOX) in 30 patients with advanced adenocarcinoma of the small bowel ($n = 18$) or ampulla of Vater ($n = 12$) resulted in a response rate of 50% and a median OS of 20.4 months (15). However, in a subgroup analysis, the response rate for advanced ampullary adenocarcinoma was 33%, which was lower than the rate for small bowel adenocarcinoma (61%). This response rate was similar to that for the patients with biliary tract adenocarcinoma (16) treated with the CAPOX regimen (20%), rather than that for patients with small bowel adenocarcinoma. Although whether advanced ampullary adenocarcinomas should be treated as biliary tract adenocarcinomas or as small bowel adenocarcinomas remain uncertain, recent major recent clinical trials or retrospective studies examining the use of anticancer agents in patients with biliary tract adenocarcinoma have included ampullary adenocarcinoma as a subgroup of biliary tract

Table 3. Treatment regimens and outcomes according to phenotypes

Patient no.	Regimen	Phenotype	MUC2	CDX2	Best response	OS (months)	PFS (months)
1	S-1	Intestinal	f+	+	SD	2.6	2.1
2	UFT + Doxorubicin	Intestinal	+	−	SD	5.8	2.5
3	5-FU + CDDP	Intestinal	f+	−	PR	8.0	4.9
4	GEM + CDDP	Intestinal	−	+	SD	6.0	2.5
5	GEM + CDDP	Intestinal	−	+	NE	8.2	0.6 (c)[a]
6	GEM	Pancreatobiliary	−	−	NE	12.3	0.9 (c)[b]
7	GEM	Intestinal	f+	+	SD	7.9	1.4
8	GEM	Pancreatobiliary	−	−	SD	14.3	10.4
9	GEM	Intestinal	2+	2+	SD	7.3	4.1 (c)[b]
10	GEM	Intestinal	2+	+	SD	22.8 (c)	4.4

f+, focally positive; SD, stable disease; PR, partial response; NE, not evaluable; (c), censored case.
[a]Censored because of Grade 3 pneumonitis.
[b]Censored because of Grade 3 biliary tract infection.

cancer (17–20). The largest randomized trial examining biliary tract adenocarcinomas was the ABC-02 trial, in which the efficacy and safety of GEM alone vs. the combination of GEM plus CDDP was evaluated by British research groups (Cancer Research UK and University College of London). That study also included 20 (4.9%) patients with advanced ampullary carcinoma (21). In a subgroup analysis of the ampullary adenocarcinomas, GEM plus CDDP tended to result in a longer survival period than GEM alone, although the difference was not significant (hazard ratio 0.62: 95% confidence interval, 0.21–1.81). Although our results do not indicate whether the treatment strategy for small bowel adenocarcinomas or for biliary tract adenocarcinomas is the most suitable, the latter strategy, which recommends GEM plus CDDP, is the only evidence supported by the ABC-02 trial at present.

Previous phase II studies in patients with biliary tract adenocarcinomas demonstrated that patients with primary tumors showed worse survival than patients without primary tumors (22,23). In our analysis, there was no subject with locally advanced disease. The patients with stage IV disease had significantly longer OS than those with recurrent disease, which was different from the result of previous reports. The possible explanations for this result were the difference in the number of patients who receiving the second-line chemotherapy and the limited number of patients in this study.

Recent studies have demonstrated the importance of classifying pathological phenotypes. Ampullary adenocarcinomas can be separated into two distinct groups with significantly different survival rates for patients with resectable disease (8,9): intestinal type (50–80% of all ampullary adenocarcinomas), which has a relatively favorable prognosis and pancreatobiliary type (15–20%), which has a poor prognosis. CDX2 and MUC2 expression may be useful for distinguishing intestinal type from pancreaticobiliary type (24). In our study, 80% of the ampullary carcinomas were classified as intestinal type and 20% were classified as pancreatobiliary type using immunohistochemical examinations. These results were similar to those of previous reports. However, both of the patients with pancreatobiliary-type adenocarcinomas lived for >1 year, while the median OS for the patients with intestinal-type adenocarcinoma was only 7.9 months. This finding disagreed with existing reports on the resected ampullary adenocarcinoma (25). However, the target population of our study was advanced ampullary adenocarcinoma patients who received systemic chemotherapy; to our knowledge, this report is the first to investigate the correlation between histological phenotypes and treatment outcomes in such a population. Therefore, the reason for this discrepancy between our report and previous reports is uncertain. Possible reasons include the difference in disease stage (resectable disease vs. unresectable disease), the difference in treatment (resection vs. chemotherapy) and an insufficient sample size. The relationship between cancer phenotypes and suitable chemotherapeutic regimens is an unsolved topic of great interest. Further research such as multicenter study to investigate larger population is needed in order to obtain more detailed information.

In conclusion, advanced ampullary adenocarcinomas have an aggressive clinical course. Their sensitivity to chemotherapy is modest, and the outcomes of treatment are comparable to those of patients with other biliary tract carcinomas. GEM plus CDDP, which is the only evidence supported by the ABC-02 trial at present, is considered to be the standard therapy for advanced ampullary adenocarcinomas.

Conflicts of interest statement

None declared.

References

1. Horner MJ, Ries LAG, Krapcho M, et al. *SEER Cancer Statistics Review, 1975–2006*. Bethesda, MD: National Cancer Institute. http://seer.cancer.gov/csr/1975_2006/, based on November 2008 SEER data submission, posted to the SEER web site, 2009.
2. Chareton B, Coiffic J, Landen S, Bardaxoglou E, Campion JP, Launois B. Diagnosis and therapy for ampullary tumors: 63 cases. *World J Surg* 1996;20:707–12.
3. Miyakawa S, Ishihara S, Horiguchi A, Takada T, Miyazaki M, Nagakawa T. Biliary tract cancer treatment: 5,584 results from the biliary tract cancer statistics registry from 1998 to 2004 in Japan. *J Hepatobiliary Pancreat Surg* 2009;16:1–7.
4. Monson JR, Donohue JH, McEntee GP, et al. Radical resection for carcinoma of the ampulla of vater. *Arch Surg* 1991;126:353–7.
5. Roder JD, Schneider PM, Stein HJ, Siewert JR. Number of lymph node metastases is significantly associated with survival in patients with radically resected carcinoma of the ampulla of vater. *Br J Surg* 1995;82:1693–6.
6. Yeo CJ, Sohn TA, Cameron JL, Hruban RH, Lillemoe KD, Pitt HA. Periampullary adenocarcinoma: analysis of 5-year survivors. *Ann Surg* 1998;227:821–31.
7. Kim ST, Lee J, Lee KT, et al. The efficacy of frontline platinum-based combination chemotherapy in advanced adenocarcinoma of the ampulla of vater. *Med Oncol* 2010;27:1149–54.
8. Fischer HP, Zhou H. Pathogenesis and histomorphology of ampullary carcinomas and their precursor lesions. Review and individual findings. *Pathologe* 2003;24:196–203.
9. Kimura W, Futakawa N, Yamagata S, et al. Different clinicopathologic findings in two histologic types of carcinoma of papilla of vater. *Jpn J Cancer Res* 1994;85:161–6.
10. Ueno H, Okusaka T, Ikeda M, Takezako Y, Morizane C. Phase ii study of s-1 in patients with advanced biliary tract cancer. *Br J Cancer* 2004;91:1769–74.
11. Furuse J, Okusaka T, Funakoshi A, et al. Early phase ii study of uracil-tegafur plus doxorubicin in patients with unresectable advanced biliary tract cancer. *Jpn J Clin Oncol* 2006;36:552–6.
12. Bouvet M, Gamagami RA, Gilpin EA, et al. Factors influencing survival after resection for periampullary neoplasms. *Am J Surg* 2000;180:13–7.
13. Klempnauer J, Ridder GJ, Pichlmayr R. Prognostic factors after resection of ampullary carcinoma: multivariate survival analysis in comparison with ductal cancer of the pancreatic head. *Br J Surg* 1995;82:1686–91.
14. Gibson MK, Holcroft CA, Kvols LK, Haller D. Phase ii study of 5-fluorouracil, doxorubicin, and mitomycin c for metastatic small bowel adenocarcinoma. *Oncologist* 2005;10:132–7.
15. Overman MJ, Varadhachary GR, Kopetz S, et al. Phase ii study of capecitabine and oxaliplatin for advanced adenocarcinoma of the small bowel and ampulla of vater. *J Clin Oncol* 2009;27:2598–603.
16. Nehls O, Oettle H, Hartmann JT, et al. Capecitabine plus oxaliplatin as first-line treatment in patients with advanced biliary system adenocarcinoma: a prospective multicentre phase ii trial. *Br J Cancer* 2008;98:309–15.
17. Gruenberger B, Schueller J, Heubrandtner U, et al. Cetuximab, gemcitabine, and oxaliplatin in patients with unresectable advanced or metastatic biliary tract cancer: a phase 2 study. *Lancet Oncol* 2010;11:1142–8.
18. Lee J, Park SH, Chang HM, et al. Gemcitabine and oxaliplatin with or without erlotinib in advanced biliary-tract cancer: a multicentre, open-label, randomised, phase 3 study. *Lancet Oncol* 2012;13:181–8.
19. Iqbal S, Rankin C, Lenz HJ, et al. A phase ii trial of gemcitabine and capecitabine in patients with unresectable or metastatic gallbladder cancer or cholangiocarcinoma: Southwest Oncology Group Study s0202. *Cancer Chemother Pharmacol* 2011;68:1595–602.
20. Williams KJ, Picus J, Trinkhaus K, et al.. Gemcitabine with carboplatin for advanced biliary tract cancers: a phase ii single institution study. *HPB (Oxford)* 2010;12:418–26.
21. Valle J, Wasan H, Palmer DH, et al. Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer. *N Engl J Med* 2010;362:1273–81.
22. Okusaka T, Nakachi K, Fukutomi A, et al. Gemcitabine alone or in combination with cisplatin in patients with biliary tract cancer: a comparative multicentre study in Japan. *Br J Cancer* 2010;103:469–74.
23. Morizane C, Okusaka T, Mizusawa J, et al. Randomized phase ii study of gemcitabine plus s-1 versus s-1 in advanced biliary tract cancer: a Japan clinical oncology group trial (jcog 0805). *Cancer Sci* 2013;104:1211–6.
24. Chu PG, Schwarz RE, Lau SK, Yen Y, Weiss LM. Immunohistochemical staining in the diagnosis of pancreatobiliary and ampulla of vater adenocarcinoma: application of cdx2, ck17, muc1, and muc2. *Am J Surg Pathol* 2005;29:359–67.
25. Roh YH, Kim YH, Lee HW, et al. The clinicopathologic and immunohistochemical characteristics of ampulla of vater carcinoma: the intestinal type is associated with a better prognosis. *Hepatogastroenterology* 2007;54:1641–4.

JJCO Japanese Journal of Clinical Oncology

Jpn J Clin Oncol 2014;44(4)324–330
doi:10.1093/jjco/hyt237
Advance Access Publication 30 January 2014

Twenty-six Cases of Advanced Ampullary Adenocarcinoma Treated with Systemic Chemotherapy

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Received November 19, 2013; accepted December 27, 2013

Objective: Ampullary adenocarcinoma is a rare disease entity and little information regarding these tumors is available. The aim of the present study was to clarify the treatment outcome of systemic chemotherapy in patients with advanced ampullary adenocarcinoma.
Methods: This study consisted of a retrospective review of data obtained from patients diagnosed as having advanced ampullary adenocarcinoma who received non-surgical treatment at a single institution between 1997 and 2010.
Results: We identified 26 patients (15 men, 11 women; median age, 62.0 years) who received treatment for advanced ampullary adenocarcinoma. Twelve patients had Stage IV disease and 14 had recurrences. The chemotherapy regimens consisted of 5-fluorouracil-based regimens (5-fluorouracil + cisplatin, $n = 3$; tegafur-uracil + doxorubicin, $n = 5$ and tegafur, gimeracil and oteracil potassium, $n = 3$) and gemcitabine-based regimens (gemcitabine, $n = 10$ and gemcitabine + cisplatin, $n = 5$). The overall response rate was 7.7%. The median progression-free survival period was 3.2 months (2.5 months in the 5-fluorouracil group vs. 3.5 months in the gemcitabine group), and the median overall survival time was 9.1 months (8.0 months in the 5-fluorouracil group vs. 12.3 months in the gemcitabine group). The median overall survival was significantly longer in stage IV disease than in recurrent disease. The histological phenotype was determined in 10 of the 26 patients. Eight patients had intestinal-type adenocarcinomas and remaining two patients had pancreatobiliary-type adenocarcinomas.
Conclusions: The treatment outcome of patients with advanced ampullary adenocarcinoma was poor. Further development of novel treatments is necessary to improve the prognosis.

Key words: ampullary adenocarcinoma – chemotherapy – 5-fluorouracil – gemcitabine – histological phenotype

INTRODUCTION

Ampullary carcinoma is a particularly uncommon neoplasm. Between 1985 and 2005, the incidence of ampullary carcinoma in the USA was 0.7 cases per 10 000 males and 0.4 cases per 10 000 females (1), accounting for 0.5% of all gastrointestinal malignancies (2). The number of annual deaths because of ampullary carcinoma is only 100–200 in the USA and 800–900 in Japan (http://www.who.int/healthinfo/morttables/en/). This inconsistency in the number of annual deaths may be due to the different geographical regions.

Compared with other periampullary adenocarcinomas, ampullary adenocarcinomas is associated with a higher likelihood of resectability and a more favorable prognosis. Among patients who undergo radical resection, the overall 5-year survival rate ranges from 35 to 46%, which is better than that for patients with distal biliary adenocarcinomas (5-year survival

rate ranging from 24 to 27%) or pancreatic head adenocarcinomas (5-year survival rate around 15%) (2–6). However, for patients with advanced disease, an accurate prognosis cannot be made because of the lack of reliable data, with the exception of one retrospective study that examined the outcome of systemic chemotherapy in 29 patients with advanced ampullary adenocarcinoma (7). Because of the rarity of this disease, advanced adenocarcinomas are often treated using regimens designed for biliary tract adenocarcinomas or small bowel adenocarcinomas. The The National Comprehensive Cancer Network (NCCN) guidelines recommend that small bowel adenocarcinomas be treated with systemic chemotherapy according to the colon cancer guidelines which recommend folinic acid, 5-FU and oxaliplatin (FOLFOX) or folinic acid, 5-FU and irinotecan (FOLFIRI) ± bevacizumab as the initial therapy. Meanwhile, the NCCN guidelines recommend that biliary tract adenocarcinomas should be treated with gemcitabine (GEM) + cisplatin (CDDP) combination therapy. However, whether advanced ampullary adenocarcinomas should be treated as biliary tract adenocarcinomas or as small bowel adenocarcinomas remain uncertain. Additionally, ampullary adenocarcinomas can be separated into two histological phenotypes, intestinal type and pancreatobiliary type (8,9). However, no previous report has examined the outcome of systemic chemotherapy analyzed according to the histological phenotypes of advanced ampullary adenocarcinomas.

The objective of the present study was to clarify (i) the treatment outcome of systemic chemotherapy for advanced ampullary adenocarcinomas, (ii) the difference in outcomes according to the chemotherapeutic regimens, (iii) the difference in outcomes according to the disease status and (iv) the difference in outcomes according to the adenocarcinoma phenotype.

PATIENTS AND METHODS

Patients

We retrospectively reviewed the clinical data in our institution's database and extracted patients who were diagnosed as having advanced ampullary adenocarcinoma and who had received systemic chemotherapy between January 1997 and December 2010. Patients were eligible if they had a recurrent or unresectable adenocarcinoma arising from the ampulla of Vater. Written informed consent was obtained from all the patients before treatment. This study was approved by the institutional review board of the National Cancer Center Hospital (NCCH) of Japan and was performed in accordance with the Declaration of Helsinki in 1964.

The following clinical characteristics of all the patients with advanced ampullary adenocarcinoma were reviewed: age, Eastern Cooperative Oncology Group (ECOG) performance status, tumor histology and adenocarcinoma phenotype.

Classification of Chemotherapy Regimens

We examined the chemotherapy regimens and the treatment outcome of systemic chemotherapy in patients with ampullary adenocarcinomas. The responses were evaluated according to the Response Evaluation Criteria in Solid Tumors 1.0. We classified the chemotherapy regimens into two types: 5-FU based and GEM based. The chemotherapy regimens were divided into two groups because GEM is a key drug for the current treatment of biliary adenocarcinomas, while 5-fluorouracil (5-FU) has been widely used as a key drug for gastrointestinal malignancies including biliary tract adenocarcinomas, colon adenocarcinomas and small bowel adenocarcinomas. In our hospital, 5-FU-based regimens were frequently used in clinical trials (10,11) for advanced biliary tract adenocarcinomas, including ampullary adenocarcinomas, or for clinical practical use before the recognition of GEM as a key agent for the treatment of biliary tract adenocarcinomas.

Immunohistochemistry

Paraffin-embedded materials from a series of pancreaticoduodenectomy specimens ($n = 9$) and a biopsy specimen ($n = 1$) obtained at NCCH were used for the immunohistochemistry (IHC) analysis. Specimens from the other patients ($n = 16$) were not available for use at NCCH because the patients had been pathologically diagnosed as having advanced ampullary adenocarcinoma at another hospital.

For the IHC studies, the tissue sections were treated with hydrogen peroxide to inactivate endogenous peroxidases after deparaffinization in xylene and rehydration in ethanol. The slides were placed in 10 mmol/l of citrate buffer at pH 6.0, then autoclaved for antigen retrieval. The primary antibodies were incubated overnight, and a secondary antibody was used to detect protein expression using EnVision™ (Dako, Glostrup, Denmark). Diaminobenzidine was used as the chromogen, and the nuclei were counterstained with hematoxylin. The antibodies used in the analysis were as follows: MUC1 (Ma552, 1:100), MUC2 (Ccp58, 1:100), MUC5AC (CLH2, 1:100), MUC6 (CLH5, 1:100) and CD10 (56C6, 1:100) from Leica Biosystems (Newcastle Upon, Tyne, UK) and CDX2 (CDX2-88, 1:100) from Biocare medical (Concord, CA, USA).

Two independent observers without prior knowledge of the clinicopathological data scored the IHC findings; the presence of positive cancer cells at any staining intensity and accounting for >10% of the sample was considered a positive finding.

Statistical Analysis

The Fisher exact test was used to assess the hypothesis of independence between the categorical variables. For the quantitative data such as age, we used the Mann–Whiney test.

Treatment outcomes were estimated as the response rate, progression-free survival (PFS) and overall survival (OS). PFS was defined as the time from the initiation of chemotherapy to the confirmation of disease progression or death from any cause. Patients who were lost to follow-up were treated as censored observations. The OS period was defined as the time from chemotherapy until the date of death or the most recent

follow-up. Patients who were lost to follow-up were treated as censored cases. Both the PFS and the OS were estimated using the Kaplan–Meier method, and significance was determined using the log-rank test. All the statistical analyses were performed using StatView (Ver. 5.0; SAS, Inc., Tokyo, Japan).

RESULTS

Patient Characteristics

We identified 28 patients with advanced ampullary adenocarcinoma who received non-surgical treatment between March 1997 and July 2010. The treatments consisted of chemotherapy ($n = 26$) and best-supportive care ($n = 2$). Among the 26 patients who received chemotherapy, the median age of the patients was 62.0 years (range, 48–79 years) and the ECOG performance statuses were as follows: 18 patients with PS 0, 8 patients with PS 1, and 0 patients with PS 2–4. All the patients had metastatic disease. The metastatic sites were the liver ($n = 17$), lungs ($n = 7$), lymph nodes ($n = 14$), peritoneum ($n = 1$), and pleura ($n = 1$). None of the patients had locally advanced disease (Table 1). The chemotherapy regimens consisted of 5-FU + CDDP ($n = 3$), tegafur-uracil (UFT) + doxorubicin ($n = 5$), tegafur, gimeracil and oteracil potassium (S-1) ($n = 3$), GEM ($n = 10$) and GEM + CDDP ($n = 5$). The median number of cycles of first-line chemotherapy prescribed was 5-FU + CDDP in 5 (range 1–5), UFT + doxorubicin in 3 (range 1–4), S-1 in 6 (range 2–11), GEM in 3 (range 1–11) and GEM + CDDP in 3 (range 1–9).

Table 1. Patient characteristics

Variable	No. of patients (%)
Age, median (range)	62.0 (48–79)
Sex	
Male	15 (58)
Female	11 (42)
ECOG PS	
0	18 (69)
1	8 (31)
2–4	0 (0)
Stage (UICC 7th edition)	
IV	12 (46)
Recurrence	14 (54)
Metastatic sites	
Liver	17 (65)
Lymph node	14 (54)
Lungs	7 (27)
Peritoneum	1 (4)
Pleura	1 (4)

ECOG PS, Eastern Cooperative Oncology Group performance status; UICC, the Union for International Cancer Control TNM Classification of Malignant Tumors (7th edition).

Outcome of Systemic Chemotherapy for Advanced Ampullary Adenocarcinomas

The response to systemic chemotherapy was evaluated in 26 patients, and these responses are listed in Table 2. None of the patients achieved a complete response. Two patients who received 5-FU + CDDP and S-1 exhibited partial responses and 18 patients achieved stable disease. The response rate (CR + PR) was 7.7% [95% confidence interval (CI) = 0.95–25.1%], and the disease control rate (CR + PR + SD) was 76.9% (95% CI = 56.4–91.0%).

As shown in Fig. 1, the median PFS and the OS from the initiation of chemotherapy were 3.2 and 9.1 months, respectively.

Treatment Outcome According to Treatment Regimens

The chemotherapy regimens were classified into two groups: the 5-FU group, consisting of 5-FU plus CDDP ($n = 3$), UFT plus doxorubicin ($n = 5$) and S-1 alone ($n = 3$) and the GEM group, consisting of GEM alone ($n = 10$) and GEM plus CDDP ($n = 5$). In the 5-FU group, the median age of the patients was 64.0 years and the ECOG performance statuses were as follows: 8 patients (73%) with PS 0 and 3 patients (27%) with PS 1. All the patients had metastatic lesion. In the GEM group, on the other hand, the median age of the patients was 66.0 years and the ECOG performance statuses were as follows: 10 patients (67%) with PS 0 and 5 patients (33%) with PS 1. All the patients also had metastatic lesion. None of the patient characteristics were significantly different between the two treatment groups.

The responses according to the treatment groups and regimens are listed in Table 2. In the 5-FU group, two patients (18%) achieved a partial response, six (55%) remained stable and three (27%) showed progressive disease. The response

Table 2. Tumor response according to treatment groups

Regimens	RR (%)	DCR (%)	Median PFS (months)	Median OS (months)
5-FU group				
5-FU + CDDP ($n = 3$)	33	100		
UFT + doxorubicin ($n = 5$)	0	60		
S-1 ($n = 3$)	33	67		
Total ($n = 11$)	18	72.7	2.5	8.0
GEM group				
GEM ($n = 10$)	0	80		
GEM + CDDP ($n = 5$)	0	80		
Total ($n = 15$)	0	80	3.5	12.3

RR, response rate; DCR, disease control rate; PFS, progression-free survival; OS, overall survival.

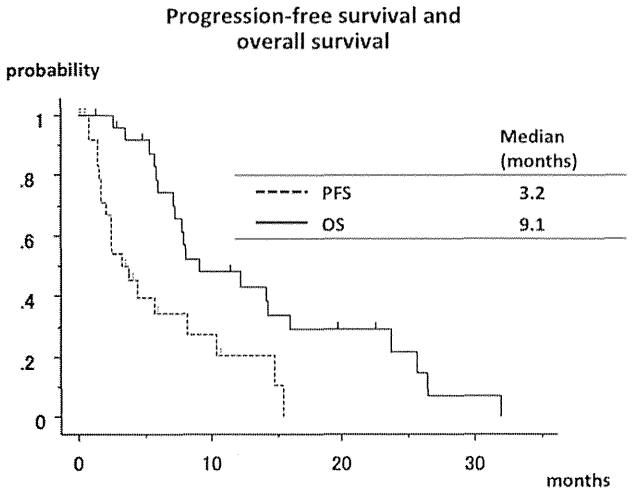

Figure 1. Progression-free survival (PFS) curve and overall survival (OS) curve calculated using the Kaplan–Meier method for all adenocarcinoma patients.

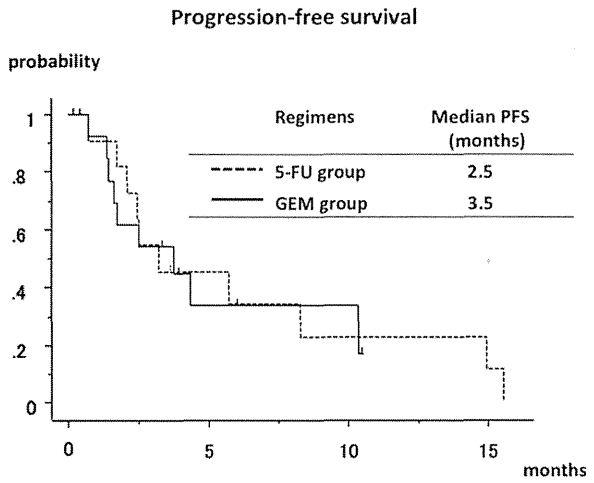

Figure 2. PFS calculated using the Kaplan–Meier method for groups classified according to the chemotherapy regimen.

rate for the 5-FU group was 18.2% (95% CI = 2.3–51.8%), and the disease control rate was 72.7% (95% CI = 39.0–94.0%). In the GEM group, on the other hand, the response rate was 0% (95% CI = 0–21.8%) and the disease control rate was 80.0% (95% CI = 51.9–95.7%). The median PFS was 2.5 and 3.5 months for the 5-FU group and the GEM group, respectively ($P = 0.79$) (Fig. 2). The median OS was 8.0 and 12.3 months, respectively ($P = 0.29$) (Fig. 3).

Three patients (27%) in the 5-FU group received second-line chemotherapy. In the GEM group, 4 (27%) patients received second-line chemotherapy.

Treatment Outcome According to Stage IV Disease or Recurrent Disease

Stage IV disease was present at the time of diagnosis in 12 patients, while recurrent disease after resection was present in 14 patients. In stage IV disease, the median age of the patients

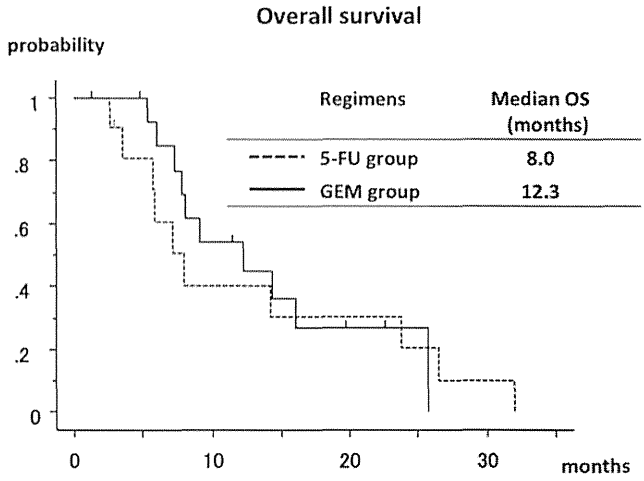

Figure 3. OS curve calculated using the Kaplan–Meier method for groups classified according to chemotherapy regimen.

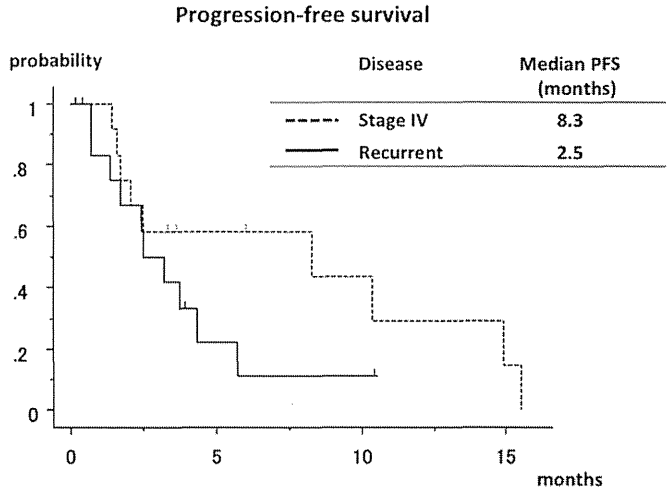

Figure 4. PFS calculated using the Kaplan–Meier method for groups classified according to stage IV disease or recurrent disease.

was 65.0 years and the ECOG performance statuses were as follows: nine patients (75%) with PS 0 and three patients (25%) with PS 1. Five of the 12 patient with stage IV disease had received 5-FU-based regimens and the remaining 7 patients received GEM-based regimens. Meanwhile, in recurrent disease, the median age of the patients was 66.0 years and the ECOG performance statuses were as follows: 9 patients (64%) with PS 0 and 5 patients (36%) with PS 1. Six of the 14 patient with recurrent disease had received 5-FU-based regimens and the remaining 8 patients received GEM-based regimens.

The response rate for stage IV disease was 8.3% (95% CI = 0.2–38.5%), and the disease control rate was 75.0% (95% CI = 42.8–94.5%). In recurrent disease, on the other hand, the response rate was 7.1% (95% CI = 0.2–33.9%) and the disease control rate was 78.6% (95% CI = 49.2–95.3%). The median PFS was 8.3 and 2.5 months for stage IV disease and recurrent disease, respectively ($P = 0.16$) (Fig. 4). The median OS was 23.8 and 7.9 months, respectively ($P = 0.02$) (Fig. 5).

Five patients (42%) in stage IV disease received second-line chemotherapy. In the recurrent disease, 2 (14%) patients received second-line chemotherapy.

Treatment Outcome According to Adenocarcinoma Phenotype

We examined 10 of the 26 ampullary adenocarcinomas to determine their phenotypes. The treatment regimens and outcomes according to the phenotypes are shown in Table 3. Eight of the 10 patients with ampullary adenocarcinoma (80%) had intestinal-type adenocarcinomas, while the remaining 2 (20%) had pancreatobiliary-type adenocarcinomas. Both patients with pancreatobiliary-type adenocarcinoma had received a GEM-based regimen, while 3 of the 8 patients with intestinal-type received 5-FU-based regimens and the remaining 5 patients received GEM-based regimens. One patient with intestinal-type adenocarcinoma who received 5-FU + CDDP responded to the treatment (PR), and the best response of the nine other patients was stable disease. The median OS was 7.9 months for the intestinal-type adenocarcinoma patients. The OS periods of the two pancreatobiliary-type adenocarcinoma patients were 12.3 months (373 days) and 14.3 months (435 days), respectively.

DISCUSSION

Ampullary adenocarcinoma is a rare disease entity, and little information regarding these tumors is available. Patients with ampullary adenocarcinomas are typically diagnosed at a relatively early stage due to the early appearance of clinical symptoms such as jaundice, and the likelihood of resectability is therefore high (12,13). On the other hand, detailed reports on advanced ampullary adenocarcinomas are extremely rare, especially regarding the treatment outcome of systemic chemotherapy for advanced stage disease.

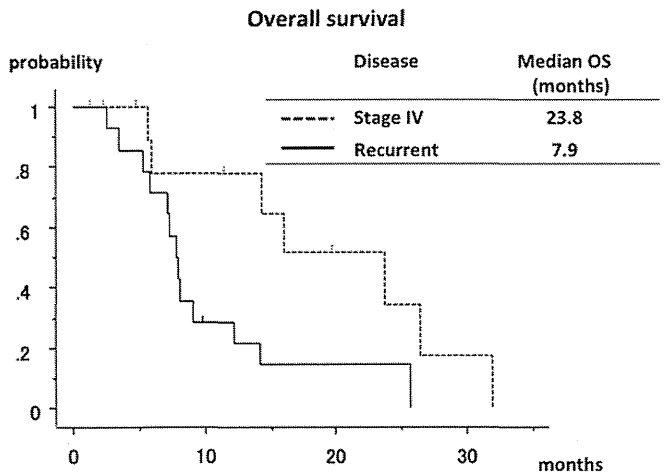

Figure 5. OS curve calculated using the Kaplan–Meier method for groups classified according to stage IV disease or recurrent disease.

A previous report discussed the efficacy of CDDP-based chemotherapy (5-FU plus CDDP or GEM plus CDDP) in 29 patients with advanced ampullary carcinoma (7). The treatment outcomes resulted in a responses rate of 27.5%, a disease control rate of 72.4%, a median time to progression of 4.9 months and a median OS period of 12.5 months, with manageable toxicities. 5-FU, CDDP and GEM were the selected agents examined in their report similar to the present study. However, in their report, the differences in the response rate, time to progression and OS were not related to the chemotherapy regimens (5-FU plus CDDP or GEM plus CDDP). In our results, the differences in the DCR, the PFS and the OS between the 5-FU group and the GEM group were also not statistically significant. Both reports indicated a modest activity for these agents against advanced ampullary adenocarcinomas; however, the optimum regimen is unknown, and patient prognosis remains dismal.

The ampulla of Vater consists of the following three distinct epithelial elements: duodenal epithelium, pancreatic ductal epithelium and biliary ductal epithelium. Because of the rarity of ampullary adenocarcinomas, they are usually regarded as biliary tract adenocarcinomas or small intestine adenocarcinomas when selecting a chemotherapeutic regimen. However, no consensus exists regarding which of these disease entities is most appropriate for the inclusion of ampullary adenocarcinomas.

In some previous reports, advanced ampullary adenocarcinomas have been included with advanced small bowel adenocarcinomas. Two prospective phase 2 studies have been performed for patients with small bowel adenocarcinoma, including ampullary adenocarcinoma. First, the combination of 5-FU, doxorubicin and mitomycin C (FAM) in 38 patients with advanced adenocarcinoma of the small bowel ($n = 34$) or ampulla of Vater ($n = 4$) resulted in a response rate of 18% and a median OS of 8 months for all the patients (14). In a subgroup analysis, the median OS of the advanced ampullary adenocarcinoma patients ($n = 4$) was 7 months, which was roughly similar to that of the small bowel adenocarcinoma patients (median OS: duodenum, 9 months; jejunum, 2 months; and ileum, 5 months). Secondly, the combination of capecitabine and oxaliplatin (CAPOX) in 30 patients with advanced adenocarcinoma of the small bowel ($n = 18$) or ampulla of Vater ($n = 12$) resulted in a response rate of 50% and a median OS of 20.4 months (15). However, in a subgroup analysis, the response rate for advanced ampullary adenocarcinoma was 33%, which was lower than the rate for small bowel adenocarcinoma (61%). This response rate was similar to that for the patients with biliary tract adenocarcinoma (16) treated with the CAPOX regimen (20%), rather than that for patients with small bowel adenocarcinoma. Although whether advanced ampullary adenocarcinomas should be treated as biliary tract adenocarcinomas or as small bowel adenocarcinomas remain uncertain, recent major recent clinical trials or retrospective studies examining the use of anticancer agents in patients with biliary tract adenocarcinoma have included ampullary adenocarcinoma as a subgroup of biliary tract

Table 3. Treatment regimens and outcomes according to phenotypes

Patient no.	Regimen	Phenotype	MUC2	CDX2	Best response	OS (months)	PFS (months)
1	S-1	Intestinal	f+	+	SD	2.6	2.1
2	UFT + Doxorubicin	Intestinal	+	−	SD	5.8	2.5
3	5-FU + CDDP	Intestinal	f+	−	PR	8.0	4.9
4	GEM + CDDP	Intestinal	−	+	SD	6.0	2.5
5	GEM + CDDP	Intestinal	−	+	NE	8.2	0.6 (c)[a]
6	GEM	Pancreatobiliary	−	−	NE	12.3	0.9 (c)[b]
7	GEM	Intestinal	f+	+	SD	7.9	1.4
8	GEM	Pancreatobiliary	−	−	SD	14.3	10.4
9	GEM	Intestinal	2+	2+	SD	7.3	4.1 (c)[b]
10	GEM	Intestinal	2+	+	SD	22.8 (c)	4.4

f+, focally positive; SD, stable disease; PR, partial response; NE, not evaluable; (c), censored case.
[a]Censored because of Grade 3 pneumonitis.
[b]Censored because of Grade 3 biliary tract infection.

cancer (17–20). The largest randomized trial examining biliary tract adenocarcinomas was the ABC-02 trial, in which the efficacy and safety of GEM alone vs. the combination of GEM plus CDDP was evaluated by British research groups (Cancer Research UK and University College of London). That study also included 20 (4.9%) patients with advanced ampullary carcinoma (21). In a subgroup analysis of the ampullary adenocarcinomas, GEM plus CDDP tended to result in a longer survival period than GEM alone, although the difference was not significant (hazard ratio 0.62: 95% confidence interval, 0.21–1.81). Although our results do not indicate whether the treatment strategy for small bowel adenocarcinomas or for biliary tract adenocarcinomas is the most suitable, the latter strategy, which recommends GEM plus CDDP, is the only evidence supported by the ABC-02 trial at present.

Previous phase II studies in patients with biliary tract adenocarcinomas demonstrated that patients with primary tumors showed worse survival than patients without primary tumors (22,23). In our analysis, there was no subject with locally advanced disease. The patients with stage IV disease had significantly longer OS than those with recurrent disease, which was different from the result of previous reports. The possible explanations for this result were the difference in the number of patients who receiving the second-line chemotherapy and the limited number of patients in this study.

Recent studies have demonstrated the importance of classifying pathological phenotypes. Ampullary adenocarcinomas can be separated into two distinct groups with significantly different survival rates for patients with resectable disease (8,9): intestinal type (50–80% of all ampullary adenocarcinomas), which has a relatively favorable prognosis and pancreatobiliary type (15–20%), which has a poor prognosis. CDX2 and MUC2 expression may be useful for distinguishing intestinal type from pancreaticobiliary type (24). In our study, 80% of the ampullary carcinomas were classified as intestinal type and 20% were classified as pancreatobiliary type using immunohistochemical examinations. These results were similar to those of previous reports. However, both of the patients with pancreatobiliary-type adenocarcinomas lived for >1 year, while the median OS for the patients with intestinal-type adenocarcinoma was only 7.9 months. This finding disagreed with existing reports on the resected ampullary adenocarcinoma (25). However, the target population of our study was advanced ampullary adenocarcinoma patients who received systemic chemotherapy; to our knowledge, this report is the first to investigate the correlation between histological phenotypes and treatment outcomes in such a population. Therefore, the reason for this discrepancy between our report and previous reports is uncertain. Possible reasons include the difference in disease stage (resectable disease vs. unresectable disease), the difference in treatment (resection vs. chemotherapy) and an insufficient sample size. The relationship between cancer phenotypes and suitable chemotherapeutic regimens is an unsolved topic of great interest. Further research such as multicenter study to investigate larger population is needed in order to obtain more detailed information.

In conclusion, advanced ampullary adenocarcinomas have an aggressive clinical course. Their sensitivity to chemotherapy is modest, and the outcomes of treatment are comparable to those of patients with other biliary tract carcinomas. GEM plus CDDP, which is the only evidence supported by the ABC-02 trial at present, is considered to be the standard therapy for advanced ampullary adenocarcinomas.

Conflicts of interest statement

None declared.

References

1. Horner MJ, Ries LAG, Krapcho M, et al. *SEER Cancer Statistics Review, 1975–2006*. Bethesda, MD: National Cancer Institute. http://seer.cancer.gov/csr/1975_2006/, based on November 2008 SEER data submission, posted to the SEER web site, 2009.
2. Chareton B, Coiffic J, Landen S, Bardaxoglou E, Campion JP, Launois B. Diagnosis and therapy for ampullary tumors: 63 cases. *World J Surg* 1996;20:707–12.
3. Miyakawa S, Ishihara S, Horiguchi A, Takada T, Miyazaki M, Nagakawa T. Biliary tract cancer treatment: 5,584 results from the biliary tract cancer statistics registry from 1998 to 2004 in Japan. *J Hepatobiliary Pancreat Surg* 2009;16:1–7.
4. Monson JR, Donohue JH, McEntee GP, et al. Radical resection for carcinoma of the ampulla of vater. *Arch Surg* 1991;126:353–7.
5. Roder JD, Schneider PM, Stein HJ, Siewert JR. Number of lymph node metastases is significantly associated with survival in patients with radically resected carcinoma of the ampulla of vater. *Br J Surg* 1995;82:1693–6.
6. Yeo CJ, Sohn TA, Cameron JL, Hruban RH, Lillemoe KD, Pitt HA. Periampullary adenocarcinoma: analysis of 5-year survivors. *Ann Surg* 1998;227:821–31.
7. Kim ST, Lee J, Lee KT, et al. The efficacy of frontline platinum-based combination chemotherapy in advanced adenocarcinoma of the ampulla of vater. *Med Oncol* 2010;27:1149–54.
8. Fischer HP, Zhou H. Pathogenesis and histomorphology of ampullary carcinomas and their precursor lesions. Review and individual findings. *Pathologe* 2003;24:196–203.
9. Kimura W, Futakawa N, Yamagata S, et al. Different clinicopathologic findings in two histologic types of carcinoma of papilla of vater. *Jpn J Cancer Res* 1994;85:161–6.
10. Ueno H, Okusaka T, Ikeda M, Takezako Y, Morizane C. Phase ii study of s-1 in patients with advanced biliary tract cancer. *Br J Cancer* 2004;91:1769–74.
11. Furuse J, Okusaka T, Funakoshi A, et al. Early phase ii study of uracil-tegafur plus doxorubicin in patients with unresectable advanced biliary tract cancer. *Jpn J Clin Oncol* 2006;36:552–6.
12. Bouvet M, Gamagami RA, Gilpin EA, et al. Factors influencing survival after resection for periampullary neoplasms. *Am J Surg* 2000;180:13–7.
13. Klempnauer J, Ridder GJ, Pichlmayr R. Prognostic factors after resection of ampullary carcinoma: multivariate survival analysis in comparison with ductal cancer of the pancreatic head. *Br J Surg* 1995;82:1686–91.
14. Gibson MK, Holcroft CA, Kvols LK, Haller D. Phase ii study of 5-fluorouracil, doxorubicin, and mitomycin c for metastatic small bowel adenocarcinoma. *Oncologist* 2005;10:132–7.
15. Overman MJ, Varadhachary GR, Kopetz S, et al. Phase ii study of capecitabine and oxaliplatin for advanced adenocarcinoma of the small bowel and ampulla of vater. *J Clin Oncol* 2009;27:2598–603.
16. Nehls O, Oettle H, Hartmann JT, et al. Capecitabine plus oxaliplatin as first-line treatment in patients with advanced biliary system adenocarcinoma: a prospective multicentre phase ii trial. *Br J Cancer* 2008;98:309–15.
17. Gruenberger B, Schueller J, Heubrandtner U, et al. Cetuximab, gemcitabine, and oxaliplatin in patients with unresectable advanced or metastatic biliary tract cancer: a phase 2 study. *Lancet Oncol* 2010;11:1142–8.
18. Lee J, Park SH, Chang HM, et al. Gemcitabine and oxaliplatin with or without erlotinib in advanced biliary-tract cancer: a multicentre, open-label, randomised, phase 3 study. *Lancet Oncol* 2012;13:181–8.
19. Iqbal S, Rankin C, Lenz HJ, et al. A phase ii trial of gemcitabine and capecitabine in patients with unresectable or metastatic gallbladder cancer or cholangiocarcinoma: Southwest Oncology Group Study s0202. *Cancer Chemother Pharmacol* 2011;68:1595–602.
20. Williams KJ, Picus J, Trinkhaus K, et al.. Gemcitabine with carboplatin for advanced biliary tract cancers: a phase ii single institution study. *HPB (Oxford)* 2010;12:418–26.
21. Valle J, Wasan H, Palmer DH, et al. Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer. *N Engl J Med* 2010;362:1273–81.
22. Okusaka T, Nakachi K, Fukutomi A, et al. Gemcitabine alone or in combination with cisplatin in patients with biliary tract cancer: a comparative multicentre study in Japan. *Br J Cancer* 2010;103:469–74.
23. Morizane C, Okusaka T, Mizusawa J, et al. Randomized phase ii study of gemcitabine plus s-1 versus s-1 in advanced biliary tract cancer: a Japan clinical oncology group trial (jcog 0805). *Cancer Sci* 2013;104:1211–6.
24. Chu PG, Schwarz RE, Lau SK, Yen Y, Weiss LM. Immunohistochemical staining in the diagnosis of pancreatobiliary and ampulla of vater adenocarcinoma: application of cdx2, ck17, muc1, and muc2. *Am J Surg Pathol* 2005;29:359–67.
25. Roh YH, Kim YH, Lee HW, et al. The clinicopathologic and immunohistochemical characteristics of ampulla of vater carcinoma: the intestinal type is associated with a better prognosis. *Hepatogastroenterology* 2007;54:1641–4.

Alternative Mammalian Target of Rapamycin (mTOR) Signal Activation in Sorafenib-resistant Hepatocellular Carcinoma Cells Revealed by Array-based Pathway Profiling*[S]

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Sorafenib is a multi-kinase inhibitor that has been proven effective for the treatment of unresectable hepatocellular carcinoma (HCC). However, its precise mechanisms of action and resistance have not been well established. We have developed high-density fluorescence reverse-phase protein arrays and used them to determine the status of 180 phosphorylation sites of signaling molecules in the 120 pathways registered in the NCI-Nature curated database in 23 HCC cell lines. Among the 180 signaling nodes, we found that the level of ribosomal protein S6 phosphorylated at serine residue 235/236 (p-RPS6 S235/236) was most significantly correlated with the resistance of HCC cells to sorafenib. The high expression of p-RPS6 S235/236 was confirmed immunohistochemically in biopsy samples obtained from HCC patients who responded poorly to sorafenib. Sorafenib-resistant HCC cells showed constitutive activation of the mammalian target of rapamycin (mTOR) pathway, but whole-exon sequencing of kinase genes revealed no evident alteration in the pathway. p-RPS6 S235/236 is a potential biomarker that predicts unresponsiveness of HCC to sorafenib. The use of mTOR inhibitors may be considered for the treatment of such tumors. ***Molecular & Cellular Proteomics 13: 10.1074/mcp.M113.033845, 1429–1438, 2014.***

Hepatocellular carcinoma (HCC)[1] is the third most common cause of cancer-related death worldwide (1). Advanced HCC often cannot be managed with local treatments (surgical resection, ethanol injection, radiofrequency ablation, chemoembolization), but no systemic chemotherapy with conventional cytotoxic agents had been shown to be effective until a landmark phase III clinical trial (the Sorafenib HCC Assessment Randomized Protocol) revealed significant survival prolongation in patients treated with sorafenib (Nexavar; Bayer Healthcare Pharmaceuticals Inc. Berlin, Germany) (2). Furthermore, it has been reported that some patients show remarkable tumor shrinkage after short-term administration of sorafenib (3). Based on these results, sorafenib monotherapy has been employed as the current standard first-line treatment for unresectable HCC. However, not all HCC patients show the desired therapeutic benefits of sorafenib. The overall survival prolongation of unselected patients in the SHARP trial was limited to 2.8 months (2), and an objective tumor response was observed only in a small proportion of patients (0.6% to 2%) (2, 4). Given the relatively high cost and occasional severe adverse events (diarrhea, hand-foot skin reaction, hypertension, and others) (2, 4), there is an urgent need to identify a predictive biomarker that could exclude advanced HCC patients who are unlikely to benefit from sorafenib therapy.

Sorafenib is a multi-kinase inhibitor that blocks tumor cell proliferation and angiogenesis through the inhibition of c-RAF and b-RAF, as well as many receptor tyrosine kinases, including vascular endothelial growth factor receptors 2 and 3, platelet-derived growth factor receptor-α, Fms-related tyrosine kinase 3, RET, and c-KIT (5). In view of this broad inhibitory spectrum, the precise mechanisms underlying the anti-

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Received August 23, 2013, and in revised form, February 2, 2014
Published, MCP Papers in Press, March 18, 2014, DOI 10.1074/mcp.M113.033845
Author contributions: M.M. and T.Y. designed research; M.M., W.C., A.M., and Y.N. performed research; M.M. and K.K. contributed new reagents or analytic tools; M.M., W.C., K.K., T.S., and C.C. analyzed data; M.M. and T.Y. wrote the paper; M.M. developed the methodology; M.O. and K.H. contributed to the conception of the study; T.Y. supervised research.

[1] The abbreviations used are: HCC, hepatocellular carcinoma; ERK, extracellular signal-regulated kinase; IC_{50}, half-maximal (50%) inhibitory concentration; p-RPS6 S235/236, ribosomal protein S6 phosphorylated at the serine 235/236 residue; MAPK, mitogen-activated protein kinase; RPPA, reverse-phase protein array; mTOR, mammalian target of rapamycin; RSK, 90-kDa ribosomal protein S6 kinase; S6K, 70-kDa ribosomal protein S6 kinase.

tumor activity remain elusive. To date, factors that have been identified as correlated with the efficacy of sorafenib include phosphorylated extracellular signal-regulated kinase 1 (p-ERK) (6), serum des-γ-carboxyprothrombin level (7), phosphorylated c-Jun protein (8), and fibroblast growth factor-3/4 gene amplification (3), but their clinical utility as predictive biomarkers has not been established.

In the present study, we developed a new technique, high-density fluorescence reverse-phase protein array (RPPA), and used it to search for a biomarker that would identify patients in whom sorafenib would be effective, employing a large library of phosphorylation-site-specific antibodies. RPPA represents an emerging technology for proteomics, and it is well suited for the profiling of phosphorylated proteins. It involves micro-format dot immunoblotting of lysates from tissues or cells (9), allowing simultaneous monitoring of the expression of a particular phosphoprotein in hundreds to thousands of samples under identical conditions in a highly quantitative manner (10). In this study we profiled the activation status of 180 key signaling nodes across a panel of 23 HCC cell lines and identified *de novo* activation of mTOR signaling in sorafenib-resistant HCC cells.

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies—Cell lines used for generating the cancer cell line RPPA are listed in supplemental Table S1 and were maintained according to their suppliers' recommendations. Recombinant EGF was obtained from R&D Systems (Minneapolis, MN). A total of 180 phosphorylation-site-specific antibodies and their dilutions used for RPPA analysis are listed in supplemental Table S2. The specificity of each antibody was verified by immunoblotting or had been previously described by other investigators.

RPPA—Cells were collected by scraping and stored at −80°C until use. Cell lysates were prepared with RIPA buffer (Thermo Scientific, Rockford, IL) supplemented with phosphatase (Thermo Scientific) and protease (Sigma, St. Louis, MO) inhibitor cocktails. Protein concentrations of lysates were determined via the Bradford method (Bio-Rad Laboratories, Hercules, CA). The lysates were serially diluted 2-fold four times and printed in quadruplicate onto ProteoChip glass slides (Proteogen, Seoul, South Korea) using a robotic spotter (Genex Arrayer, Kaken Geneqs Inc., Chiba, Japan).

The RPPA slides were incubated overnight with primary antibodies. Following tyramide signal amplification (Dako Cytomation, Glostrup, Denmark), streptavidin Alexa Fluor 647 conjugate (Invitrogen, Carlsbad, CA) was applied to the slides (11). Fluorescence images were captured by an InnoScan 700 microarray scanner (Innopsys, Carbonne, France) and quantified using Mapix software (Innopsys). After background subtraction, values relative to γ-tubulin were subjected to quantile normalization (12) to ensure a uniform distribution of values for each slide in a set of slides. Unsupervised hierarchical clustering, using the Euclidean metric and Ward's method, was conducted with R 2.13.0. The signaling components of the mTOR and MAPK pathways were selected based on KEGG pathway maps and used for clustering analyses.

Immunoblot Analysis—Immunoblot analyses were performed using the NuPAGE Bis-Tris or Tris-Acetate electrophoresis system (Invitrogen) as described previously (13). All antibodies except for an anti-p-RSK (S380) antibody (R&D Systems) were obtained from Cell Signaling Technology (Danvers, MA). Signals were detected with the ImageQuant LAS 4010 system (GE Healthcare, Giles, UK) and quantified using the ImageQuant TL software package (GE Healthcare).

Growth Inhibition Assay—Sorafenib, RAD001 (everolimus), and SL0101 were purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). CI-1040 and AZD8055 were from Selleck Chemicals (Houston, TX). Stock solutions of the chemicals were prepared in dimethyl sulfoxide and stored at −20°C until use. Cells were seeded into 96-well cell culture plates in triplicate at a density of 3000 cells per well. On the following day, serially diluted drugs were added, and 72 h later cell viability was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Fitchburg, WI). Relative cell viability was calculated as a percentage of a control treated with 0.1% dimethyl sulfoxide after background subtraction. All experiments were repeated at least three times. The data were modeled using a four-parameter log-logistic nonlinear regression curve fit with a sigmoid dose response. These curves were drawn using R 2.13.0, and IC_{50} values were calculated accordingly.

Immunohistochemistry—Formalin-fixed, paraffin-embedded sections of needle biopsy samples obtained from nine HCC patients before administration of sorafenib at Wan Fang Hospital and the Taipei Medical University Hospital were immunostained with anti-p-RPS6 Ser235/236 (#2211, Cell Signaling Technology) or anti-RPS6 (#2217, Cell Signaling Technology) antibody, as described previously (13). The stained slides were evaluated by pathologists and classified according to the percentage of positively stained cells (0 = 0%, 1 = 1% to 25%, 2 = 26% to 50%, 3 = 51% to 75%, and 4 = 76% to 100%) and the intensity of staining (0, absent; 1, weak; 2, moderate; and 3, strong). A specimen was defined as positive when either the percentage of positively stained cells or the intensity of staining was 3 or higher. The use of clinical materials was approved by the respective institutional review boards.

Kinome Sequencing—Genomic DNA was extracted from 20 HCC cell lines using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's protocol. DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Three micrograms of genomic DNA was used to construct libraries for sequencing. The quality of the constructed libraries was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). All the exon and 5′- and 3′-flanking sequences (200 bp) of 511 kinase genes were captured using a customized SureSelect Target Enrichment System (Agilent Technologies) according to the Illumina Paired-End Sequencing Platform Library Prep Protocol Version 1.0 (Agilent Technologies). Captured DNA fragments (~300 bp) were sequenced using a Genome Analyzer IIx sequencer (Illumina, San Diego, CA). Base calling was performed using the Illumina Pipeline (v1.4) with default parameters. Only paired end (2 × 75 bases) sequence reads that passed the quality control were mapped to the human reference genome build hg19 (UCSC hg19) using BWA (14) with default parameters. Sequencing artifacts were eliminated using Picard MarkDuplicates. Variants were called with SAMtools (15) and annotated using Annovar ENREF 25 (16). The final set of novel variant calls was identified using the following thresholds: SNP quality ≥ 228, coverage ≥ 20 reads, frequency ≥ 10%, and not deposited in the dbSNP database (www.ncbi.nlm.nih.gov/projects/SNP/) (version 135).

Evaluation of Synergistic Drug Combinations—The synergistic interaction of drug combinations was evaluated using the Chou–Talalay *median-dose effect method* (17) with *CompuSyn software*. AZD8055 and CI-1040 were mixed at the ratio of their IC_{50} values (1:300). The mixed solution was 2-fold serially diluted five times and added to the cells. Combination Index values were calculated at the points causing 50%, 75%, and 90% reduction of cell viability. Combination Index values equal to 1, >1, and <1 indicate additive, antagonistic, and synergistic interactions, respectively.

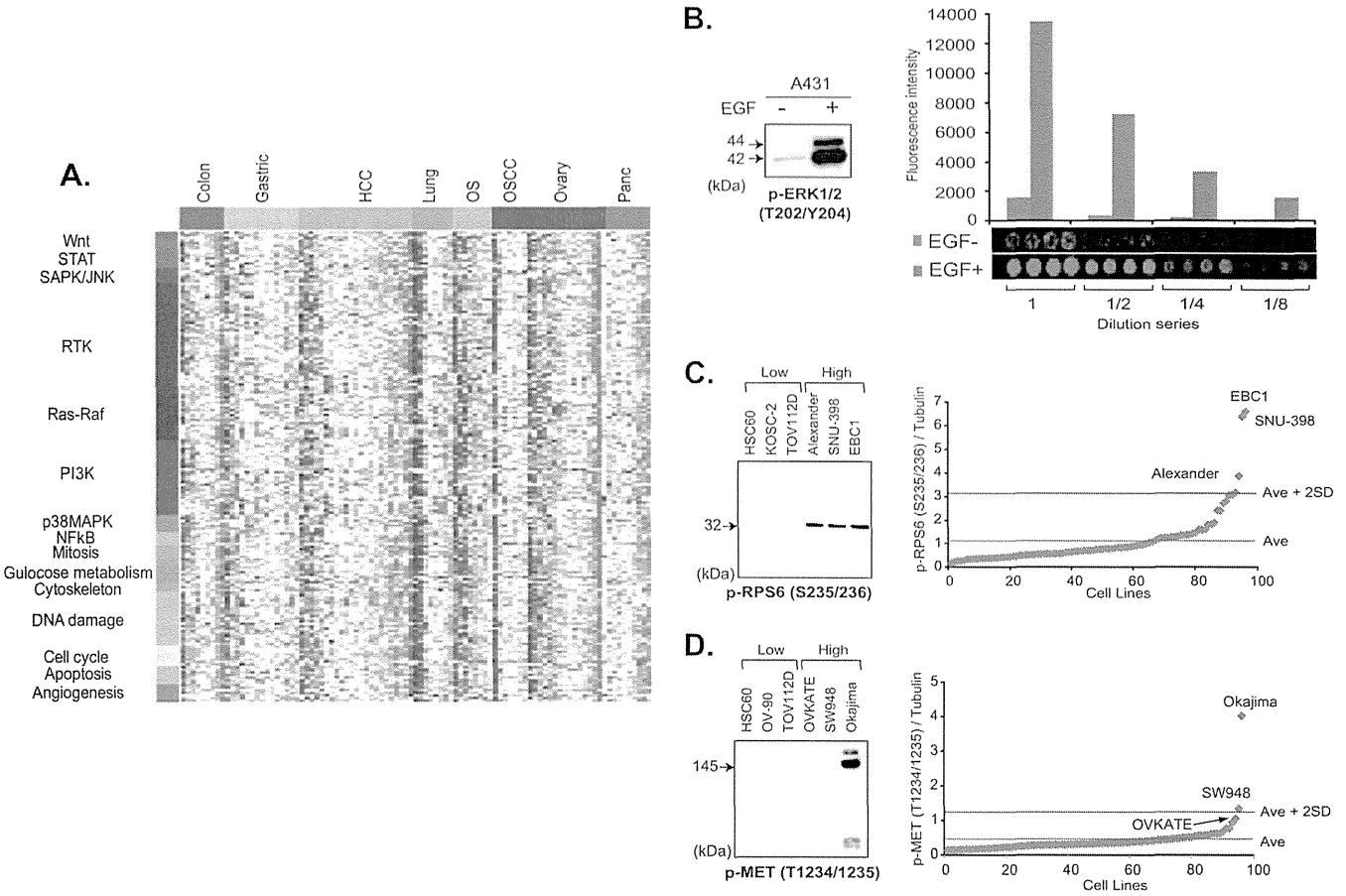


FIG. 1. **Phosphoprofiling of key signaling molecules by RPPA.** *A*, phosphorylation status of 180 signaling nodes in a panel of 95 cancer cell lines cultured in the presence of 10% FCS. Red and blue colors indicate high- and low-level phosphorylation, respectively. STAT, signal transducers and activators of transcription; SAPK/JNK, stress-activated protein kinase/c-Jun NH_2-terminal kinase; RTK, receptor tyrosine kinase; PI3K, phosphatidylinositol 3′-kinase; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor-kappaB; OS, osteosarcoma; OSCC, oral squamous cell carcinoma. *B*, immunoblot (*left*) and RPPA (*right*) analyses of A431 cells cultured without (−) and with (+) EGF for 10 min with anti-p-ERK1/2 (T202/Y204) antibody. The mean fluorescence intensity in arbitrary units (*top*) and images (*bottom*) of quadruplicate RPPA spots of lysate undiluted (1) and diluted 1:2 (1/2), 1:4 (1/4), and 1:8 (1/8) -fold are shown (*right*). *C*, *D*, relative p-RPS6 S235/236 (*C*) and p-Met T1234/1235 (*D*) expression of 95 cell lines determined via RPPA (right). Cell lines with the three highest and three lowest levels of expression were selected and subjected to immunoblotting with the same antibody (*left*). Ave, average.

RESULTS

Generation of the High-density RPPA and Phosphoprotein Profiling—We constructed an RPPA onto which lysates of 95 cell lines derived from eight different types of cancer (listed in supplemental Table S1) cultured in the presence and absence of 10% fetal calf serum (FCS) for 17 h and A431 cells untreated or treated with 200 ng/ml EGF for 10 min were randomly plotted. Each lysate was serially diluted (1:1, 1:2, 1:4, and 1:8) and spotted in quadruplicate (16 spots per lysate). This level of high-density spotting (3072 samples per array slide) was achievable because of the highly hydrophobic surface of the array slides, which prevented diffusion of the protein samples.

By applying 180 phosphorylation-site-specific antibodies (listed in supplemental Table S2), we determined the activation status of signaling proteins (Fig. 1*A*). A lysate of A431 cells treated with EGF was included as a positive internal control. A431 cells carry amplification of the *EGFR* (EGF receptor) gene. We confirmed that a >6-fold increase in the signal intensity of ERK1/2 proteins phosphorylated at the threonine 202/tyrosine 204 residue (p-ERK1/2 T202/Y204) was detectable after treatment with EGF (Fig. 1*B*).

To further verify the data obtained via RPPA, lysates of representative cell lines were electrophoresed and blotted with the same antibodies. In the RPPA analysis, EBC1, SNU-398, and Alexander cells showed a high signal intensity for anti-p-RPS6 S235/236 antibody, exceeding the average plus 2 S.D. for 96 cell lines, whereas TOV112D, KOSC-2, and HSC60 cells showed a signal intensity below the average (Fig. 1*C*, right). The results we obtained from immunoblotting were consistent (Fig. 1*C*, left).

The glass slides that we used for construction of the RPPA were free of any autofluorescence noise. The use of fluorescent dyes and original signal enhancement significantly in-

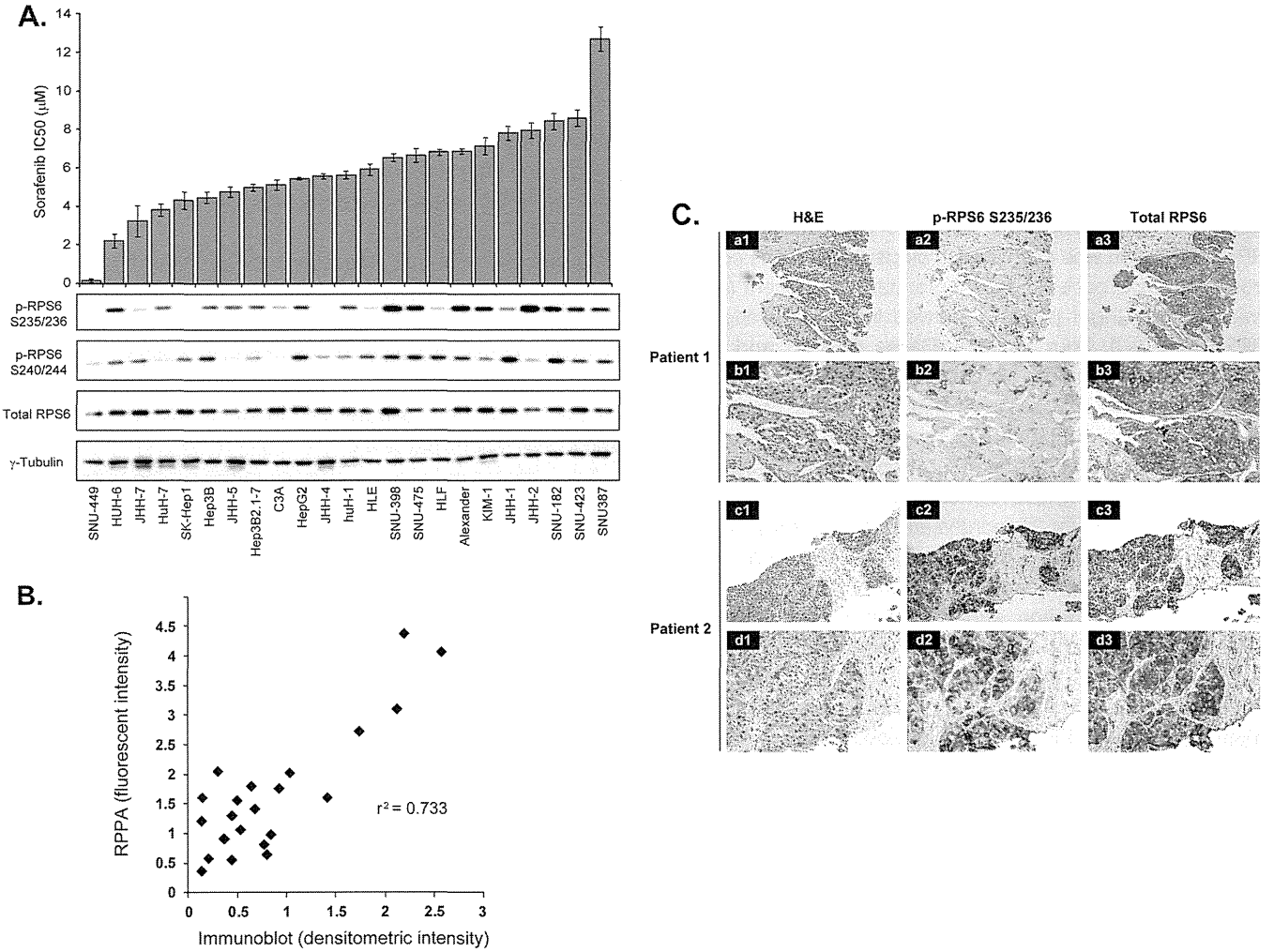


FIG. 2. **p-RPS6 S235/236 correlates with the sensitivity of HCC to sorafenib.** *A*, *upper graph*, IC_{50} values for sorafenib against 23 HCC cell lines sorted from the most sensitive (*left*) to resistant (*right*) ones. Columns and error bars represent the mean and S.D. of three independent experiments, respectively. *Lower panels*, immunoblot analysis of pRPS6 S235/236, pRPS6 S240/244, RPS6, and γ-tubulin (loading control) expression in the 23 HCC cell lines. *B*, correlation between RPPA and immunoblot analyses of p-RPS6 S235/236 expression in the 23 HCC cell lines (R^2 = 0.733). *C*, detection of p-RPS6 S235/236 in pretreatment biopsy samples. Hematoxylin and eosin (H&E) (a-d1) and immunoperoxidase staining with anti-p-RPS6 S235/236 (a-d2) and total RPS6 (a-d3) antibodies of HCC biopsy specimens obtained from a responder (patient 1 (a and b)) and a representative non-responder (patient 2 (c and d)) to sorafenib. Original magnification was ×40 (a1–3 and c1–3) and ×200 (b1–3 and d1–3).

creased the sensitivity of signal detection. In fact, MET protein with a high level of phosphorylation (p-MET T1234/1235) in Okajima cells was detectable via immunoblotting, whereas the MET protein with a relatively low level of phosphorylation in SW948 and OVKATE cells was undetectable (Fig. 1*D*).

p-RPS6 S235/236 Correlates with the Sensitivity of HCC Cells to Sorafenib—The cancer cell protein array contained 23 HCC cell lines exhibiting a wide variety of sensitivities to sorafenib (Fig. 2*A*, upper portion). SNU-449 was the most sensitive, with a half-maximal (50%) inhibitory concentration (IC_{50}) of 0.172 μM. It was ~70-fold more sensitive than the least sensitive cell line, SNU-387 (IC_{50} = 12.68 μM). We then compared the IC_{50} value of each HCC cell line with the phosphorylation level of 180 signaling nodes. Spearman's correlation coefficient analysis (supplemental Table S3) revealed that p-RPS6 S235/236 had the highest positive correlation (r = 0.58, p = 0.0044), followed by p-RPS6 at the serine 240/244 residues (p-RPS6 S240/244) (r = 0.55, p = 0.0070). 90-kDa ribosomal S6 kinase 2 (RSK2) protein phosphorylated at the serine 227 residue showed the third most significant correlation. RSK2 is one of the enzymes that phosphorylate RPS6 (18).

Consistent with the RPPA data, intense signals for p-RPS6 S235/236 were detected in the sorafenib-resistant cell lines via immunoblotting (Fig. 2*A*, lower portion). The quantified immunoblot data correlated well with those of RPPA (r^2 = 0.733), thus confirming the precision of the RPPA (Fig. 2*B*). p-RPS6 S235/236 and p-RPS6 S240/244 exhibited different

phosphorylation patterns among several cell lines (*e.g.* HLF, KIM1, JHH-1, and JHH-2) (Fig. 2*A*, lower portion), suggesting that phosphorylation of S235/236 and S240/244 residues may be mediated by distinct regulatory processes.

p-RPS6 S235/236 Is a Potential Predictor of Response to Sorafenib—We next evaluated whether high levels of p-RPS6 S235/236 were indicative of HCC resistance to sorafenib in clinical samples (supplemental Table S4). Expression of p-RPS6 S235/236 was examined in biopsy specimens collected from nine HCC patients prior to sorafenib treatment (400 mg twice a day). Eight patients showed intense staining for p-RPS6 (Fig. 2*C*). Four patients (Cases 4, 6, 7, and 9) with p-RPS6-positive tumors discontinued sorafenib treatment because of disease progression within 2.3 months. Four patients (Cases 2, 3, 5, and 8) died as a result of disease progression after starting sorafenib treatment. In contrast, the remaining patient (Case 1), whose tumor was negative for p-RPS6, received sorafenib for 24 months and survived for 27 months. In this particular patient, tumor regression was confirmed by computed tomography scans performed three months after sorafenib administration and remained stable for another three months. In addition, the α-fetoprotein level dropped from 1621 to 314 ng/ml and remained low for 10 months. These results provide preliminary evidence that that high expression of p-RPS6 S235/236 might be useful for predicting which patients are unlikely to respond to sorafenib. As biopsy is not performed routinely before sorafenib treatment, we were unable to further validate the clinical significance of p-RPS6 S235/236 by examining additional cases.

mTOR Pathway Activation in Sorafenib-resistant Cells—Given the association between p-RPS6 and sensitivity of HCC cell lines to sorafenib, we assessed the effects of sorafenib on p-RPS6 in representative sorafenib-sensitive and -resistant cell lines. The sorafenib-sensitive HUH-6 and HuH-7 cell lines demonstrated substantial dose-dependent decreases in p-RPS6 levels following treatment with sorafenib (Fig. 3*A*). Sorafenib also diminished the phosphorylation of downstream molecules in the MAPK pathway, ERK and RSK, in a dose-dependent manner (Fig. 3*A*), implying that the reduction of p-RPS6 in sorafenib-sensitive cells was likely attributable to blockade of the MAPK pathway (Fig. 3*B*).

In contrast, the phosphorylation of RPS6 S235/236 in sorafenib-resistant cell lines, especially JHH-2, SNU-423, and SNU-387 cells, was insensitive to the same sorafenib treatment (Fig. 3*C*). The level of p-RPS6 in JHH-1 and SNU-182 cells decreased to some extent after sorafenib treatment, but a high concentration (10 μM) of sorafenib was necessary in order to suppress the phosphorylation of RPS6 completely (Fig. 3*C*), reflecting that the regulation of p-RPS6 in sorafenib-resistant cell lines is different from that in sensitive cell lines. RPS6 is also known to be phosphorylated by 70-kDa ribosomal S6 kinases (S6K) downstream of mTOR (Fig. 3*B*) (19, 20). We therefore speculated that the mTOR pathway might be alternatively activated in sorafenib-resistant cell lines. In fact, we found that sorafenib-resistant cells had a high level of p-S6K1 (Fig. 3*C*), whereas p-S6K1 was barely detectable in sorafenib-sensitive cells (Fig. 3*A*).

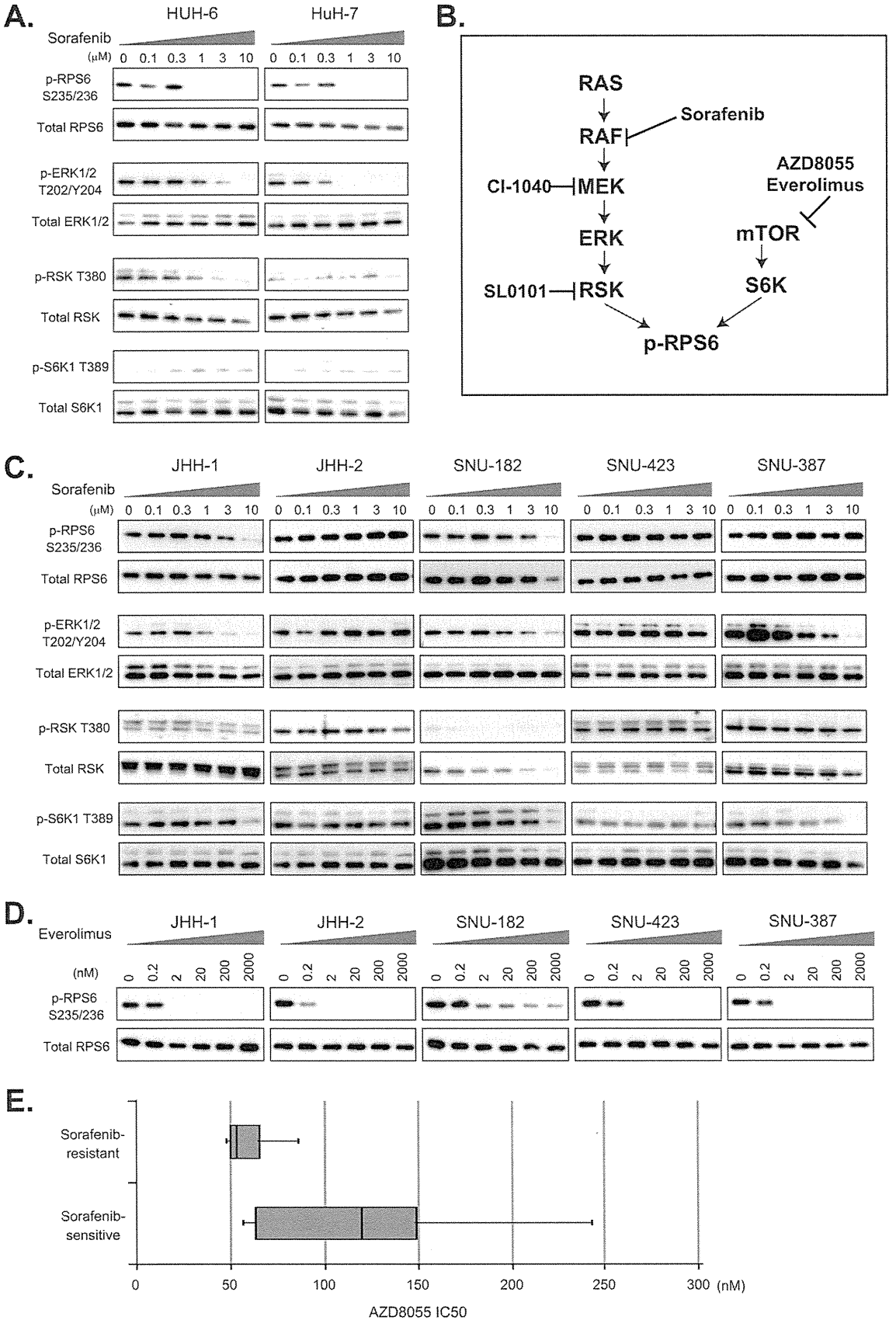
The phosphorylation of ERK in JHH-1 and SNU-182 was suppressed to some extent by sorafenib, but the low level of p-RSK and high level of p-S6K1 indicate that the main regulator of RPS6 phosphorylation was mTOR signaling rather than MAPK signaling.

Absence of Genetic Alterations in the MAPK and mTOR Pathways—RAF kinases are among the known targets of sorafenib, but sorafenib-resistant JHH2, SNU-423, and SNU-387 cells exhibited sustained activation of molecules located downstream of RAF (ERK and RSK), even in the presence of sorafenib (Fig. 3*C*), suggesting sorafenib-insensitive activation of the MAPK pathway.

To clarify the molecular mechanism driving the activation of the mTOR and MAPK pathways in sorafenib-resistant cells, we sequenced the entire exons of 511 kinases (listed in supplemental Table S5) in 20 HCC cell lines using a next-generation sequencer. Supplemental Table S6 lists all of the 322 genetic alterations that were not deposited in the dbSNP database. Due to the unavailability of normal counterparts, we were unable to determine whether these alterations were somatic. Eight kinds of DNA alterations were evident in the known mTOR and MAPK pathway genes (Supplemental Table S7). b-RAF V600E, found in SK-Hep1 cells, is a known driver mutation frequently observed in malignant melanoma (21). Two kinds of alterations were identified in the ATP-binding (S72A (JHH-7)) and AGC-kinase C-terminal (K335T (huH-1, SNU-475, and SNU-185)) domains of the RSK1 genes. Three kinds of alterations were found in the proline-rich domain (A420V (11 cell lines including SNU449) and V422I (HUH-6)) and catalytic (P267L (SK-Hep1, JHH-4, Kim1, and JHH-1)) domains of the S6K2 gene.

These eight alterations were validated using a conventional sequencing method (Supplemental Table S7), but no genetic alteration was specific to sorafenib-resistant cell lines. Infrequent alteration of the mTOR and MAPK pathway genes in HCC has been demonstrated by conventional sequencing analysis of surgical samples (22), and this was consistent with the present comprehensive sequencing data. The aberrant activation of the mTOR and MAPK pathways in sorafenib-resistant HCC cells was likely attributable to complex interplay between other oncogenic and anti-oncogenic pathways, or post-translational modifications.

mTOR Inhibitors Repress the Proliferation of Sorafenib-resistant Cells—The marked inhibition of p-RPS6 S235/236 following exposure to an mTOR inhibitor, everolimus, at a concentration as low as 2 nM (Fig. 3*D*) confirmed that activation of the mTOR pathway is responsible for the sorafenib-insensitive phosphorylation of RPS6 S235/236 in sorafenib-resistant cells. Consistently, sorafenib-insensitive cells tended to be more sensitive to another mTOR inhibitor,


A.
HUH-6
HuH-7
Sorafenib
(μM) 0 0.1 0.3 1 3 10 0 0.1 0.3 1 3 10
p-RPS6 S235/236
Total RPS6
p-ERK1/2 T202/Y204
Total ERK1/2
p-RSK T380
Total RSK
p-S6K1 T389
Total S6K1
B.
RAS
RAF
Sorafenib
CI-1040
MEK
AZD8055
Everolimus
ERK
mTOR
SL0101
RSK
S6K
p-RPS6
C.
JHH-1
JHH-2
SNU-182
SNU-423
SNU-387
Sorafenib
(μM) 0 0.1 0.3 1 3 10
p-RPS6 S235/236
Total RPS6
p-ERK1/2 T202/Y204
Total ERK1/2
p-RSK T380
Total RSK
p-S6K1 T389
Total S6K1
D.
Everolimus
(nM) 0 0.2 2 20 200 2000
p-RPS6 S235/236
Total RPS6
E.
Sorafenib-resistant
Sorafenib-sensitive
0 50 100 150 200 250 300 (nM)
AZD8055 IC50

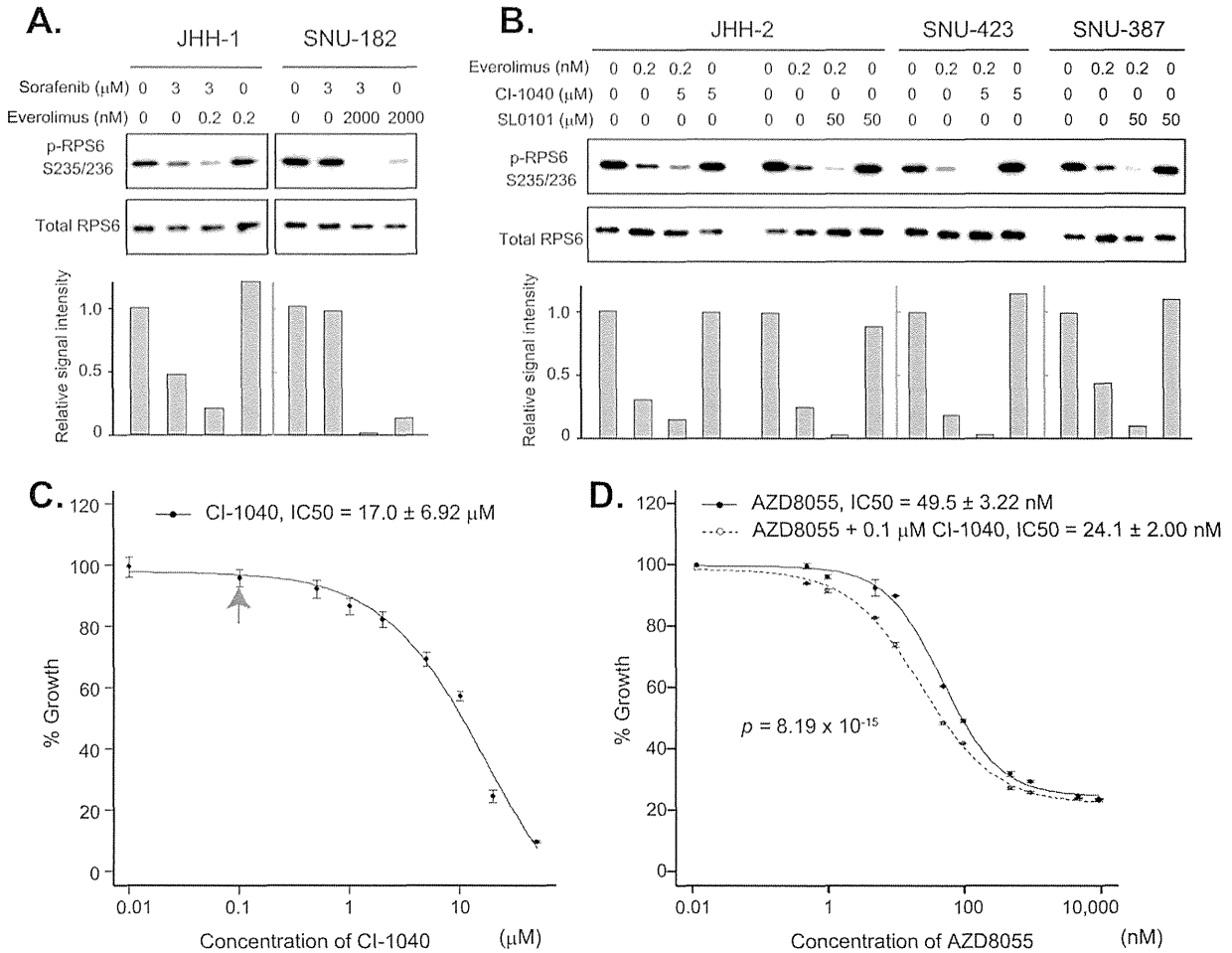


FIG. 4. **Synergy of mTOR and MAPK inhibitors.** *A*, sorafenib-resistant JHH-1 and SNU-182 cells were treated with the indicated concentrations of sorafenib and everolimus, and the expression of p-RPS6 S235/236 and total RPS6 was examined via immunoblotting (*top*). The bottom panel indicates intensity relative to control blots (no treatment). *B*, sorafenib-resistant JHH2, SNU-423 and SNU-387 cells were treated with the indicated concentrations of CI-1040, SL0101, and everolimus, and the expression of p-RPS6 S235/236 and RPS6 was examined via immunoblotting (*top*). The bottom panel indicates blot intensities relative to control blots (no drug treatment). *C*, sorafenib-resistant SNU-423 cells were treated with the indicated concentrations of CI-1040, and relative cell viability was determined 72 h later. Note that CI-1040 had no significant inhibitory effect on cell growth at 0.1 μM (indicated by a red arrow). *D*, sorafenib-resistant SNU-423 cells were treated with the indicated concentrations of AZD8055 in the presence (open circles) or absence (solid circles) of 0.1 μM CI-1040, and relative cell viability was determined 72 h later.

AZD8055, than sorafenib-sensitive cell lines (Fig. 3*E*), indicating that sorafenib-resistant cells are dependent for growth on constitutive activation of the mTOR pathway.

Synergy of mTOR and MAPK Inhibitors—Although JHH-1 and SNU-182 cells showed resistance to sorafenib, their ERK phosphorylation was dose-dependently attenuated by sorafenib (Fig. 3*C*), indicating that the MAPK pathway in these cells still retained some sensitivity to the inhibition of RAF or other unknown MAPK-pathway kinases. In fact, sorafenib augmented the down-regulation of p-RPS6 S235/236 by everolimus (Fig. 4*A*). It is noteworthy that the low level of p-RPS6 S235/236 in SNU-182 cells sustained in the presence of 2 μM everolimus was completely abrogated by the addition of 3 μM sorafenib.

FIG. 3. **Alternative mTOR signal activation in sorafenib-resistant HCC cells.** *A*, *C*, representative sorafenib-sensitive (HUH-6 and HuH-7) and -resistant (JHH-1, JHH-2, SNU-182, SNU423, and SNU-387) HCC cells were treated with the indicated concentrations of sorafenib for 3 h, and the expression of p-RPS6 S235/236, total RPS6, p-ERK1/2 T202/Y204, total ERK, p-RSK T380, total RSK, p-S6K T389, and total S6K was determined via immunoblotting. *B*, schematic representation of the mTOR and MAPK pathways and their inhibitors. *D*, representative sorafenib-resistant (JHH-1, JHH-2, SNU-182, SNU423, and SNU-387) HCC cells were treated with the indicated concentrations of everolimus for 3 h, and the expression of p-RPS6 S235/236 and total RPS6 was determined via immunoblotting. *E*, distribution of IC_{50} values of representative sorafenib-sensitive (SNU-449, HUH-6, JHH-7, HuH-7, and SK-Hep1) and -resistant (JHH-1, JHH-2, SNU-182, SNU-423, and SNU-387) HCC cells to AZ8055. Boxes indicate 25th to 75th percentiles.