Table 1 Patient demographics

	CR group (%), $n = 18$	Control group (%), $n = 1045$	P
Sex			<0.01
Male	9 (50)	840 (80)	
Female	9 (50)	205 (20)	
Age (years)			0.16
≤59	0 (0)	209 (20)	
60–69	5 (28)	320 (31)	
70–79	9 (50)	424 (41)	
≥80	4 (22)	83 (8)	
Unknown	0 (0)	9 (1)	
≤69/≥70			0.06
Bodyweight (kg)			0.13
≤29	0 (0)	1 (0.1)	
30–39	1 (6)	21 (2)	
40–49	6 (33)	127 (12)	
50–59	7 (39)	377 (36)	
60–69	3 (17)	269 (26)	
≥70	0 (0)	155 (15)	
Unknown	1 (6)	95 (9)	
≤59/ ≥ 60			0.03
Cause of hepatocellular carcinoma			0.39
Hepatitis type B	3 (17)	223 (21)	
Hepatitis type C	15 (83)	521 (50)	
Alcohol	0 (0)	93 (9)	
NASH	0 (0)	18 (2)	
Hepatitis type B + type C	0 (0)	13 (1)	
Hepatitis type B + alcohol	0 (0)	1 (0.1)	
Hepatitis type C + alcohol	0 (0)	17 (2)	
Others	0 (0)	36 (3)	
Unknown	0 (0)	123 (12)	
PS			0.79
0	14 (78)	709 (68)	
1	4 (22)	300 (29)	
2–4	0 (0)	36 (3)	
TNM stage according to Liver Cancer Study Group of Japan			0.02
I	0	10 (1)	
II	5 (28)	54 (5)	
III	3 (17)	208 (20)	
IVa	2 (11)	276 (26)	
IVb	8 (44)	487 (47)	
Unknown	0 (0)	10 (1)	
I–II/III–IV			<0.01

CR, complete response; NASH, non-alcoholic steatohepatitis; PS, performance status; TNM, tumor–node–metastasis.

bodyweight was significantly different when a cut-off of 60 kg was used to create two groups. The CR rate of patients weighing less than 60 kg was significantly greater than that of patients weighing more than 60 kg ($P = 0.03$). However, no significant differences in hepatitis-related etiology (hepatitis B surface antigen, hepatitis C virus antibody, alcohol and others) and performance status was observed between the CR and control groups.

Regarding the tumor stage according to the Liver Cancer Study Group of Japan, the percentages of stage I, II, III, IVa and IVb disease were 0%, 28%, 17%, 11% and

44% among the CR group and 1%, 5%, 20%, 26% and 47% among the control group, respectively. A significant difference in tumor stage was observed between the two groups ($P = 0.02$); the CR group had more patients with a lower disease stage than the control group ($P < 0.01$, I and II vs III and IV) (Table 1).

Although the serum aspartate aminotransferase, alanine aminotransferase and total bilirubin levels and the Child–Pugh score (albumin, bilirubin, prothrombin activity, ascites and encephalopathy) before the initiation of treatment were also surveyed and compared between the CR and control groups, no significant differences in any of these parameters were observed (Table 2).

Extrahepatic spread, such as metastases to the lymph nodes, lung and bone, was not a significant factor, and neither were the tumor marker levels (α-fetoprotein and protein induced by vitamin K absence/antagonist-II) (Table 3). Among the CR patients ($n = 18$), the number of tumors in the liver ranged from 0 to more than 11 (median, 5). The numbers of patients with 0, 1, 3, 4, 6–10 and more than 11 intrahepatic tumors were four (22%), two (11%), two (11%), one (6%), three (17%) and five (28%), respectively. The tumor number was unknown in only one patient. The tumor size ranged 0–13 cm (median, 3.2 cm in diameter). Regarding portal vein invasion, Vp 0, Vp 2, and Vp 3 were observed in 15 (83%), two (11%) and one patient (6%), respectively.

Table 2 Baseline liver functions

	CR group (%), $n = 18$	Control group (%), $n = 1045$	P
Child–Pugh score			0.93
5	8 (44)	415 (40)	
6	8 (44)	393 (38)	
7	2 (11)	140 (13)	
8–11	0 (0)	83 (8)	
Unknown	0 (0)	14 (1)	
AST (IU/L)			0.23
≤69	14 (78)	663 (63)	
70–119	1 (6)	219 (21)	
≥120	3 (17)	127 (12)	
Unknown	0 (0)	36 (3)	
ALT (IU/L)			0.59
≤69	15 (83)	866 (83)	
70–119	2 (11)	112 (11)	
≥120	1 (6)	32 (3)	
Unknown	0 (0)	35 (3)	
Bilirubin (mg/dL)			0.64
≤1.9	18 (100)	933 (89)	
≥2.0	0 (0)	75 (7)	
Unknown	0 (0)	37 (4)	

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CR, complete response.

Table 3 Baseline tumor characteristics

	CR group (%), $n = 18$	Control group (%), $n = 1045$	P
Extrahepatic metastasis	8 (44)	569 (54)	0.48
Lymph nodes	4 (22)	184 (18)	0.54
Lung	7 (39)	316 (30)	0.44
Bone	0 (0)	167 (16)	0.09
Brain	0 (0)	7 (1)	1.00
Other	0 (0)	104 (10)	0.25
AFP (ng/mL)			0.16
≤19	4 (22)	196 (19)	
20–999	4 (22)	276 (26)	
1000–9999	8 (44)	170 (16)	
≥10 000	2 (11)	162 (16)	
Unknown	0 (0)	241 (23)	
PIVKA-II (mAU/mL)			0.28
≤39	6 (33)	152 (15)	
40–299	4 (22)	169 (16)	
300–999	2 (11)	92 (9)	
≥1000	6 (33)	432 (41)	
Unknown	0 (0)	200 (19)	

AFP, α-fetoprotein; CR, complete response; PIVKA-II, protein induced by vitamin K absence/antagonist-II.

According to our data collection instrument, 89% (16/18) of all the CR population and 90% (938/1045) of the control population received prior treatments. These treatments included surgical resection, percutaneous ethanol injection, radiofrequency ablation, transcatheter arterial chemoembolization, radiotherapy and systemic chemotherapy. No significant differences in the previous treatments were observed between the two groups (Table 4).

The difference in the first dose between the two groups was statistically significant ($P < 0.01$) (Table 5). The initial dose of the CR patients was significantly lower than that for the control group. The median period from the initiation of treatment with sorafenib until the initial confirmation of a CR was 119 days (range, 35–447 days). Palmar–plantar erythrodysesthesia syndrome (PES), hypertension, diarrhea, alopecia, fatigue, nausea and anorexia were observed significantly more frequently in the CR population than in the control group ($P < 0.05$). Among these adverse events, PES ($P < 0.01$), hypertension ($P < 0.01$) and alo-

Table 4 Previous therapies†

	CR group (%), $n = 18$	Control group (%), $n = 1045$	P
Previous therapy	16 (89)	938 (90)	0.71
Surgical resection	6 (33)	372 (36)	1.00
Transplantation	0 (0)	4 (0.4)	1.00
Ethanol infusion	3 (17)	153 (15)	0.74
Radiofrequency ablation	8 (44)	360 (34)	0.45
TACE	14 (78)	696 (67)	0.45
Radiotherapy	0 (0)	105 (10)	0.24
Hepatic intra-arterial CT	4 (22)	352 (34)	0.45
Systemic CT	3 (17)	274 (26)	0.43

†Patients might have received more than one type of therapy.
CR, complete response; CT, chemotherapy; TACE, transcatheter arterial chemoembolization.

Table 5 Initial dose of sorafenib

	CR group (%), $n = 18$	Control group (%), $n = 1045$	P
Initial dose			<0.01
800 mg	7 (39)	807 (77)	
600 mg	0 (0)	6 (0.6)	
400 mg	10 (56)	210 (20)	
200 mg	1 (6)	22 (2)	
≤600 mg/800 mg			<0.01

CR, complete response.

pecia ($P < 0.01$) were more serious in the CR group than in the control group (Table 6).

DISCUSSION

THIS MULTICENTER OBSERVATIONAL study conducted in Japan revealed the characteristics of HCC patients encountered in general clinical practice who attained a CR after undergoing sorafenib treatment. To the best of our knowledge, this pilot analysis is the first to describe the characteristics of patients with HCC who attained a CR after treatment with sorafenib. These characteristics consisted of a higher sensitivity to sorafenib in women with a lower bodyweight, a smaller tumor burden, a lower initial dose of sorafenib and specific adverse events, suggesting a biological basis for this phenomenon. The latest large-sale multicenter observational study in Japan suggested female sex may be one of the independent predictors of a good response to sorafenib,[17] which may lend support to our results. Understanding the characteristics that are common to patients who achieve a CR may help to elucidate the mechanisms that underlie the response to sorafenib. These results may also lead to the ability to predict efficacy based on clinical features and adverse events, allowing predictions of whether chemotherapy will be worthwhile and improving patient prognosis.

The result that sorafenib was more effective in earlier stage patients than in advanced stage patients may be closely associated with the tumor burden. Based on the Gompertzian phenomenon and the Norton–Simon hypothesis, which are theoretical rationales for chemotherapy, mathematics can be used to advance our understanding of the complex process of tumorigenesis, and

Table 6 Adverse events

Adverse events	CR group (%), $n = 18$		Control group (%), $n = 1045$		P	
	All	Serious	All	Serious	All	Serious
Rash	6 (34)	1 (6)	206 (20)	36 (3)	0.23	0.47
PES	15 (83)	4 (22)	489 (47)	32 (3)	<0.01	<0.01
Hypertension	11 (61)	2 (11)	218 (21)	3 (0.3)	<0.01	<0.01
Diarrhea	9 (50)	1 (6)	249 (24)	21 (2)	0.02	0.31
Alopecia	10 (56)	3 (17)	102 (10)	0 (0)	<0.01	<0.01
Fatigue	11 (62)	1 (6)	69 (7)	10 (1)	<0.01	0.17
Nausea	4 (22)	0 (0)	39 (4)	2 (0.2)	<0.01	1.00
Liver dysfunction	5 (28)	2 (11)	295 (28)	151 (14)	1.00	1.00
Anorexia	9 (50)	1 (6)	150 (14)	27 (3)	<0.01	0.38
Gastrointestinal hemorrhage	1 (6)	1 (6)	53 (5)	51 (5)	0.61	0.60

CR, complete response; PES, palmar–plantar erythrodysesthesia syndrome.

the killing of tumor cells can theoretically be enhanced by using a greater chemotherapy dose rate.[18] Although subset analyses in the Asia–Pacific trial suggested that sorafenib improves patient survival irrespective of the tumor burden at baseline,[19] our data showed that a CR was achieved more frequently in patients with a small tumor burden. From the above-mentioned perspective, individuals with a lower tumor burden can easily be imagined to have a higher tendency to achieve a CR.

Why was a lower initial dose of sorafenib related to tumor shrinkage? The precise reasons were unclear, although we presumed that the attending physicians might have reduced the dose of sorafenib for patients with a lower bodyweight (who had a high CR rate) more frequently than for those with a higher bodyweight. The reasons for the difference in response may be due to differences in the plasma concentration of sorafenib and the exact inhibition of pathways regulating cancer progression of HCC. The SOFIA (Sorafenib Italian Assessment) study group indicated an increase in survival rates for patients who received a half-dose of sorafenib for a longer treatment period, compared with patients who received a full dose of sorafenib for a shorter treatment period. The differences in the treatment duration between the lesser-dosed patients and the full-dosed patients may possibly reflect differences in tolerability between the regimens, resulting in prolonged exposure to lesser dose sorafenib in less tolerant patients.[20]

In addition, our data also indicated a statistically significant correlation between adverse events (PES, diarrhea, hypertension, nausea, anorexia, fatigue and alopecia) and the efficacy of sorafenib. According to recent papers, patients who experienced skin toxicity or diarrhea have a significantly increased time-to-progression and overall survival, compared with patients without such toxicities ($P < 0.05$).[21–23] One of the main targets of sorafenib is Raf kinase, which is a downstream effector molecule of the EGFR signaling pathway. Skin toxicity commonly associated with EGF pathway inhibitors may be a predictive marker, and the onset of skin toxicity could be an optimal measure for adjusting the dose titration. Patients with documented hypertension while receiving treatment also had a significantly better overall survival (18.2 vs 4.5 months, $P = 0.016$). The development of hypertension in patients treated with sorafenib seems to be associated with a favorable efficacy.[24] In addition, nausea, anorexia, fatigue and alopecia were identified as significant adverse events in the CR group in the present study. The correlation between treatment efficacy and adverse events during sorafenib treatment can likely be explained, at least in part, by the plasma concentration of sorafenib.[25,26] The mechanisms underlying the correlation between treatment efficacy and sorafenib-induced specific adverse events should, therefore, be further investigated.

The limitations of this case–control study include a potential sampling bias, a measurement bias between the case group and the control group associated with the retrospective nature of the study, incomplete data, and heterogeneity in the data because of the individual experiences of the clinicians, because this report describes a multicenter observation a study in a Japanese population. At the same time, the number of CR patients ($n = 18$) was too small for predictive factors to be definitively identified in this study. Furthermore, such patients might have been included in the control group ($n = 1045$); in other words, the CR patients whose data was included in the nationwide survey might have been identical to the patients included in the interim report. Although the proportion of CR patients who might have been included in the control group was less than 1.7% (18/1045), we cannot deny a possible impact on the results. In a published work search, only 17 case reports contained a patient with HCC who achieved a CR with sorafenib. The clinical demographics of these patients are shown in Table 7. The patients tended to be male, to have received a full dose of sorafenib and to have had stage IV disease; these factors are different from those in the presently reported results.[27–37] Ethnic differences may be one of the possible reasons for the differences, because our data were obtained from a purely Japanese population. We also speculate that the difference in the average body surface area between Japanese females and Western males may be associated with the difference in the dose of sorafenib. Interestingly, most CR patients developed the same adverse events (e.g. PES). However, we could not obtain conclusive evidence owing to the small CR populations enrolled in the study. Further investigations are needed to clarify the differences.

In conclusion, the present study elucidated the characteristics of HCC patients who attained a CR after receiving sorafenib treatment, suggesting a specific HCC population that may be capable of achieving a CR during sorafenib treatment. The results support the possible achievement of a CR in patients with advanced HCC who are treated with sorafenib and reveal the characteristics of such "super responders". The results also suggest new lines of investigation for the identification of biomarkers capable of predicting the efficacy of sorafenib treatment and survival outcome, enabling "super responders" to be selected from amongst patients

Table 7 CR patients with HCC treated with sorafenib

No.	Age (years)	Sex	Bodyweight (kg)	Metastasis	Initial dose (mg/day)	Adverse event	Reference
1	78	Male	Unknown	Lung	800	Diarrhea G1	So[27]
2	54	Male	Unknown	Lung	800	Diarrhea, PES	Yeganeh[28]
3	74	Male	Unknown	PVTT	400	Nausea, vomiting	Wang[29]
4	68	Male	Unknown	Lung	800	Unknown	Kudo[30]
5	68	Male	Unknown	Lung Lymph nodes, adrenal glands	800	Unknown	
6	76	Female	53	Lung	400	Hepatic necrosis	Inuzuka[31]
7	69	Male	Unknown	Lymph nodes	800	PES G3 Hypertension G3 Cholecystitis G3 Diarrhea G2	Chelis[32]
8	84	Male	Unknown	PVTT	800	Unknown	Sacco[33]
9	56	Male	Unknown	Diaphragm, vena cava	800	PES G3	Curtit[34]
10	59	Male	Unknown	PVTT, omentum, lymph nodes	800	PES	Irtan[35]
11	57	Male	Unknown	PVTT	800	PES	
12	52	Male	Unknown	PVTT	800	PES G3	Poullenot[36]
13	65	Male	Unknown	Lymph nodes, peritoneum	800	Diarrhea, alopecia	
14	73	Male	Unknown	Peritoneum	800	Cerebellar stroke and others	
15	68	Male	Unknown	Liver, lung	Unknown	Unknown	
16	70	Male	Unknown	PVTT	800	paresthesia	Abbadessa[37]
17	69	Male	Unknown	PVTT	800	PES G3	

G, grade; PES, palmar–plantar erythrodysesthesia syndrome; PVTT, portal vein tumor thrombosis.

with advanced HCC population prior to the initiation of treatment. The evaluation of patients receiving sorafenib, especially in patients in whom the treatment was remarkably effective, warrants further exploration in future clinical studies.

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REFERENCES

1 Parkin DM, Bray F, Ferlay J *et al.* Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74–108.
2 Bruix J, Sherman M, Llovet JM *et al.* Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. *J Hepatol* 2001; **35**: 421–30.
3 Llovet JM, Burroughs A, Bruix A. Hepatocellular carcinoma. *Lancet* 2003; **362**: 1907–17.
4 Bruix J, Sherman M. Management of hepatocellular carcinoma. *Hepatology* 2005; **42**: 1208–36.
5 Wilhelm SM, Carter C, Tang L *et al.* 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* 2004; **64**: 7099–109.
6 Llovet JM, Ricci S, Mazzaferro V *et al.* Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378–90.
7 Cheng AL, Kang YK, Chen Z *et al.* 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* 2009; **10**: 25–34.
8 Mok TS, Wu YL, Thongprasert S *et al.* Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *N Engl J Med* 2009; **361**: 947–57.
9 Amado RG, Wolf M, Peeters M *et al.* Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol* 2008; **26**: 1626–34.
10 Cobleigh MA, Vogel CL, Tripathy D *et al.* Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J Clin Oncol* 1999; **17**: 2639–48.
11 Allgayer H, Babic R, Gruetzner KU *et al.* c-erbB-2 is of independent prognostic relevance in gastric cancer and is associated with the expression of tumor-associated protease systems. *J Clin Oncol* 2000; **18**: 2201–9.
12 Shao YY, Lin ZZ, Hsu C *et al.* Early alpha-fetoprotein response predicts treatment efficacy of antiangiogenic systemic therapy in patients with advanced hepatocellular carcinoma. *Cancer* 2010; **116**: 4590–6.
13 Ueshima K, Kudo M, Takita M *et al.* Des-gamma-carboxyprothrombin may be a promising biomarker to determine the therapeutic efficacy of sorafenib for hepatocellular carcinoma. *Dig Dis* 2011; **29**: 321–5.
14 Miyahara K, Nouso K, Tomoda T *et al.* Predicting the treatment effect of sorafenib using serum angiogenesis markers in patients with hepatocellular carcinoma. *J Gastroenterol Hepatol* 2011; **26**: 1604–11.
15 Llovet JM, Di Bisceglie AM, Bruix J *et al.* Design and endpoints of clinical trials in hepatocellular carcinoma. *J Natl Cancer Inst* 2008; **100**: 698–711.
16 Post-Marketing Surveillance Manager. *Nexavar® Tablets 200 Mg (Nonproprietary Name: Sorafenib Tosylate Tablets) Specific Drug Use Investigation Interim Report (Unresectable Hepatocellular Carcinoma)*. Osaka: Bayer Yakuhin, Ltd., 2012.
17 Takeda H, Osaki Y, Tsuchiya K *et al.* Predictive factors associated with survival and response to sorafenib therapy for unresectable hepatocellular carcinoma: a multicenter study in Japanese red cross liver study group. *7th International Liver Cancer Association Annual Conference.* 2013: abstract.
18 Norton L. Conceptual and practical implications of breast tissue geometry: toward a more effective, less toxic therapy. *Oncologist* 2005; **10**: 370–81.
19 Cheng AL, Guan Z, Chen Z *et al.* Efficacy and safety of sorafenib in patients with advanced hepatocellular carcinoma according to baseline status: subset analyses of the phase III Sorafenib Asia-Pacific trial. *Eur J Cancer* 2012; **48**: 1452–65.
20 Iavarone M, Cabibbo G, Piscaglia F *et al.* Field-practice study of sorafenib therapy for hepatocellular carcinoma: a prospective multicenter study in Italy. *Hepatology* 2011; **54**: 2055–63.

21 Strumberg D, Awada A, Hirte H *et al.* Pooled safety analysis of BAY 43-9006 (sorafenib) monotherapy in patients with advanced solid tumours: is rash associated with treatment outcome? *Eur J Cancer* 2006; **42:** 548–56.

22 Vincenzi B, Santini D, Russo A *et al.* Early skin toxicity as a predictive factor for tumor control in hepatocellular carcinoma patients treated with sorafenib. *Oncologist* 2010; **15:** 85–92.

23 Bettinger D, Schultheibeta M, Knuppel E *et al.* Diarrhea predicts a positive response to sorafenib in patients with advanced hepatocellular carcinoma. *Hepatology* 2012; **56:** 789–90.

24 Estfan B, Byrne M, Kim R. Sorafenib in Advanced Hepatocellular Carcinoma: hypertension as a potential surrogate marker for efficacy. *Am J Clin Oncol* 2013; **36:** 319–24.

25 Arrondeau J, Mir O, Boudou-Rouqette P *et al.* Sorafenib exposure decreases over time in patients with hepatocellular carcinoma. *Invest New Drugs* 2012; **30:** 2046–9.

26 Boudou-Rouquette P, Ropert S, Mir O *et al.* Variability of Sorafenib toxicity and exposure over time: a pharmacokinetic/pharmacodynamic analysis. *Oncologist* 2012; **17:** 1204–12.

27 So BJ, Bekaii-Saab T, Bloomston MA, Patel T. Complete clinical response of metastatic hepatocellular carcinoma to sorafenib in a patient with hemochromatosis: a case report. *J Hematol Oncol* 2008; **1:** 18.

28 Yeganeh M, Finn RS, Saab S. Apparent remission of a solitary metastatic pulmonary lesion in a liver transplant recipient treated with sorafenib. *Am J Transplant* 2009; **9:** 2851–4.

29 Wang SX, Byrnes A, Verma S, Pancoast JR, Rixe O. Complete remission of unresectable hepatocellular carcinoma treated with reduced dose of sorafenib: a case report. *Target Oncol* 2010; **5:** 59–63.

30 Kudo M, Ueshima K. Positioning of a molecular-targeted agent, sorafenib, in the treatment algorithm for hepatocellular carcinoma and implication of many complete remission cases in Japan. *Oncology* 2010; **78** (Suppl 1): 154–66.

31 Inuzuka T, Nishikawa H, Sekikawa A *et al.* Complete response of advanced hepatocellular carcinoma with multiple lung metastases treated with sorafenib: a case report. *Oncology* 2011; **81** (Suppl 1): 152–7.

32 Chelis L, Ntinos N, Souftas V *et al.* Complete response after sorafenib therapy for hepatocellular carcinoma in an HIV-HBV co infected patient: possible synergy with HAART? A case report. *Med Oncol* 2011; **28** (Suppl 1): S165–168.

33 Sacco R, Bargellini I, Gianluigi G *et al.* Complete response for advanced liver cancer during sorafenib therapy: case report. *BMC Gastroenterol* 2011; **11:** 4.

34 Curtit E, Thiery-Vuillemin A, Nguyen T *et al.* Complete histologic response induced by sorafenib in advanced hepatocellular carcinoma: a case report. *J Clin Oncol* 2011; **29:** e330–332.

35 Irtan S, Chopin-Laly X, Ronot M, Faivre S, Paradis V, Belghiti J. Complete regression of locally advanced hepatocellular carcinoma induced by sorafenib allowing curative resection. *Liver Int* 2011; **31:** 740–3.

36 Poullenot F, Bioulac-Sage P, Laumonier H, Saric J, Carteret T, Blanc JF. Hepatocellular carcinoma treated by sorafenib with complete radiological response according to mRECIST criteria: could we stop the treatment? About four cases and review of the literature. *Acta Oncol* 2013 (Epub ahead of print).

37 Abbadessa G, Rimassa L, Pressiani T, Carrillo-Infante C, Cucchi E, Santoro A. Optimized management of advanced hepatocellular carcinoma: four long-lasting responses to sorafenib. *World J Gastroenterol* 2011; **17:** 2450–3.

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Fibroblast Growth Factor Receptor 2 Tyrosine Kinase Fusions Define a Unique Molecular Subtype of Cholangiocarcinoma

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Cholangiocarcinoma is an intractable cancer, with limited therapeutic options, in which the molecular mechanisms underlying tumor development remain poorly understood. Identification of a novel driver oncogene and applying it to targeted therapies for molecularly defined cancers might lead to improvements in the outcome of patients. We performed massively parallel whole transcriptome sequencing in eight specimens from cholangiocarcinoma patients without *KRAS/BRAF/ROS1* alterations and identified two fusion kinase genes, *FGFR2-AHCYL1* and *FGFR2-BICC1*. In reverse-transcriptase polymerase chain reaction (RT-PCR) screening, the *FGFR2* fusion was detected in nine patients with cholangiocarcinoma (9/102), exclusively in the intrahepatic subtype (9/66, 13.6%), rarely in colorectal (1/149) and hepatocellular carcinoma (1/96), and none in gastric cancer (0/212). The rearrangements were mutually exclusive with *KRAS/BRAF* mutations. Expression of the fusion kinases in NIH3T3 cells activated MAPK and conferred anchorage-independent growth and *in vivo* tumorigenesis of subcutaneous transplanted cells in immune-compromised mice. This transforming ability was attributable to its kinase activity. Treatment with the fibroblast growth factor receptor (FGFR) kinase inhibitors BGJ398 and PD173074 effectively suppressed transformation. *Conclusion: FGFR2* fusions occur in 13.6% of intrahepatic cholangiocarcinoma. The expression pattern of these fusions in association with sensitivity to FGFR inhibitors warrant a new molecular classification of cholangiocarcinoma and suggest a new therapeutic approach to the disease. (HEPATOLOGY 2014;59:1427-1434)

Cholangiocarcinoma (CC) is a highly malignant invasive carcinoma that arises through malignant transformation of cholangiocytes.[1] It is an intractable tumor with poor prognosis, whose incidence and mortality rates are high in East Asia and have been rapidly increasing worldwide.[1,2] CC can be subdivided into intrahepatic (ICC) and extrahepatic (ECC) types, which show distinct etiological and clinical features.[2] ICC is the second most common primary hepatic malignancy after hepatocellular carcinoma, and is associated with hepatitis virus infection. Somatic mutations of *KRAS* and *BRAF* are the most common genetic alterations in CC.[3,4] Surgical resection is the only curative treatment for CC, and no standard chemotherapy regimens have been established for inoperative cases or those showing recurrence after surgical resection.[5,6]

Abbreviations: CC, cholangiocarcinoma; ECC, extrahepatic cholangiocarcinoma; FGFR, fibroblast growth factor receptor; FISH, fluorescent in situ hybridization; ICC, intrahepatic cholangiocarcinoma; TKI, tyrosine kinase inhibitor.

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A better understanding of the molecular basis of cancer would help develop targeted therapeutic agents against druggable genetic aberrations identified in cancer genomes.[7,8] Tyrosine kinase inhibitors (TKIs) that target anaplastic lymphoma kinase (ALK) are particularly effective in the treatment of a distinct subset of lung adenocarcinoma carrying *ALK* fusions.[9] *FIG-ROS1*, the first identified targetable fusion kinase in CC, has so far been reported in two patients.[10] Very recently, a novel kinase fusion, *FGFR2-BICC1*, was detected in two CC cases.[11] Thus, only a few cases harboring targetable fusion kinase genes have been reported, and the clinical characteristics of fusion-positive CC cases have not yet been described.

In the present study, we identified fibroblast growth factor receptor 2 (*FGFR2*) rearrangements including a novel *FGFR2-AHCYL1* fusion using whole transcriptome high-throughput sequencing of tumor specimens, and determined the prevalence of *FGFR2* rearrangements in CC. Our data indicate that *FGFR2*-fusions arise exclusively in ICC. *In vitro* studies suggest that *FGFR2* fusion kinase is a promising candidate for targeted therapy in CC.

Materials and Methods

Clinical Samples. Clinical specimens of cholangiocarcinoma, gastric cancer, hepatocellular carcinoma, and colorectal cancer were provided by the National Cancer Center Biobank, Japan. Total RNA was extracted from grossly dissected, snap-frozen tissue using RNAspin (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions, and RNA quality was examined using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). The study protocol was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan.

Analysis of Whole Transcriptome Sequence Data. Complementary DNA (cDNA) libraries composed of 150-200 bp inserts were prepared from 2 μg of total RNA using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA). The libraries were subjected to paired-end sequencing of 50-100 bp fragments on the HiSeq2000 instrument (Illumina) according to the manufacturer's instructions. Paired-end reads were mapped to known RNA sequences in the RefSeq, Ensembl, and LincRNA databases using the Bowtie program (v. 0.12.5) as basically described previously.[12] The detailed algorithm for fusion transcript detection is described in the Supporting Methods.

RT-PCR and Quantitative Real-Time PCR. Total RNA was reverse-transcribed to cDNA using SuperScript III (Life Technologies, Carlsbad, CA). The cDNA was subjected to PCR amplification using Ex-Taq (Takara Bio, Tokyo, Japan) with the following primers: FR2AHC-CF (GGACTCGCCAGAGATATCAACAATATAGAC) and FR2AHC-CR (GGACTG TGAGATCGAGCGAGAC) for *FGFR2-AHCYL1* fusion, FR2BIC-CF2 (GTGTTAATGTGGGAGATCT TCACTTTAGG) and FR2BIC-CR2 (CATCCATCTT CAGTGTGACTCGATTG) for *FGFR2-BICC1* fusion, FIG-e2CF1 (ACTGGTCAAAGTGCTGACTCTGGT) and ROS-e36CR2 (CAGCAAGAGACGCAGAGTCAGTTT) for *FIG-ROS1* fusion, ACTB-S (CAAGAGATGGCCACGGCTGCT) and ACTB-A (TCCTTC TGCATCCTGTCGGCA) for *β-actin*. The PCR products were directly sequenced by Sanger sequencing using the BigDye terminator kit (Life Technologies). The expression of the *FGFR2* transcript was assayed by quantitative real-time PCR (qPCR) using the LC480 thermal cycler (Roche, Penzberg, Germany). *FGFR2* expression was normalized to β-actin expression. Primers used for qPCR are as follows: FGFR2 (Fwd-GGACCCAAAATGGGAGTTTC, Rev-ACCACTTG CCCAAAGCAA), β-actin (Fwd-CCAACCGCGAGA AGATGA, Rev-CCAGAGGCGTACAGGGATAG).

Fluorescent* In Situ *Hybridization. To identify *FGFR2* rearrangements, break-apart fluorescent *in situ* hybridization (FISH) was performed on formalin-fixed, paraffin-embedded tumors using BAC clones corresponding to the 5′ (RP11-78A18) and 3′ (RP11-7P17) sequences flanking the *FGFR2* gene and labeled by nick translation in green and red, respectively.

Immunohistochemistry. Four-micrometer-thick sections from formalin-fixed paraffin-embedded block were used for immunohistochemistry. Epitope retrieval was performed with trypsin (T7168, Sigma, St. Louis,

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Additional Supporting Information may be found in the online version of this article.

MO) for 20 minutes at pH 7.7. The slides were then washed with phosphate-buffered saline (PBS) and incubated overnight with FGFR2 antibody at 4°C (1:500, ab10648, Abcam, Cambridge, UK). Immunoreactions were detected using the EnVision-FLEX system (DAKO, Glostrup, Denmark).

cDNA Cloning and Generation of Kinase-Deficient Mutants. The full-length *FGFR2-AHCYL1* and *FGFR2-BICC1* cDNAs were isolated from the corresponding tumor specimens by RT-PCR using PrimeSTAR GXL polymerase (Takara Bio) and primers FGFR2-H5F1 (ATGGTCAGCTGGGGTCGTTTCA TCTGCCTGGTCG), AHCYL-H6R1 (GTATCTGTA ATAATTAGGTTTGAATGGCCC), and BICC1-H6R1 (CCAGCGGCCACTGACACTAGCAATGTCTGA). *EZR-ROS1* cDNA was reported previously.[13] Each cDNA was subcloned into a pMXs vector (Cell Biolabs, San Diego, CA) to generate recombinant retrovirus expressing the fusion protein with a FLAG epitope tag. The kinase activity-deficient mutants were constructed by replacing tyrosine with phenylalanine at codons 568 and 569 in the *FGFR2-AHCYL1* and *FGFR2-BICC1* genes using a PrimeSTAR site-directed mutagenesis kit (Takara Bio).

Transforming Activity of FGFR2 Fusions. Mouse NIH3T3 fibroblast cells were infected with EZR-ROS1, FGFR2-AHCYL1, FGFR2-AHCYL1-KD, FGFR2-BICC1, or FGFR2-BICC1-KD-expressing retroviruses. Quantification of anchorage-independent growth was performed on day 12 in soft agar with the CytoSelect-96 kit (Cell Biolabs) in the presence or absence of FGFR inhibitors BGJ398 (#S2183, Selleck, Houston, TX) and PD173074 (#S1264, Selleck). The compound solution was added to the top layer of soft agar every 3 days.

Subcutaneous Transplantation in Immune-Compromised Mice. A total of 1×10^6 transduced NIH3T3 cells were injected subcutaneously into nude mice (BALB/c-nu/nu, CLEA Japan, Tokyo, Japan). Tumor formation was measured after 18 days. All animal procedures were performed with the approval of the Animal Ethics Committee of the National Cancer Center, Tokyo, Japan.

Immunoblot Analysis. To analyze signaling, retrovirally transduced NIH3T3 cells were serum-starved for 2 hours, after which vehicle (DMSO), BGJ398, or PD173074 was added for a further 2 hours. The culture medium was then changed to standard medium containing 10% fetal bovine serum (FBS) for 10 minutes. Whole cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to a PVDF membrane. Western blot detection was performed with the WesternBreeze Chemiluminescent Immunodetection kit (Life Technologies) using primary antibodies against FLAG tag (#1E6, Wako Chemicals, Tokyo, Japan), phospho-FGFR1-4 (Tyr653, 654) (#AF3285, R&D Systems, Minneapolis, MN), STAT3 (#610189, BD, Becton Drive, NJ), phospho-STAT3 (Tyr705) (#9138, Cell Signaling Technology, Danvers, MA), p44/42 MAPK (#4695, Cell Signaling Technology), and phospho-p44/42 MAPK (Thr202/Tyr204) (#9106, Cell Signaling Technology), AKT1 (#2967, Cell Signaling Technology), and phospho-AKT (Ser473) (#4051, Cell Signaling Technology).

Statistical Analysis. All data analyses were performed using JMP v. 8.02 (SAS Institute, Cary, NC). Fisher's exact test was used for categorical data, and the Student *t*-test was used for continuous data. Overall survival, measured from the date of surgery, was determined using the Kaplan-Meier method, and survival difference was compared using the log-rank test. Two-sided significance level was set at $P < 0.05$.

Results

Identification of a Novel FGFR2 Fusion Gene. Whole transcriptome high-throughput sequencing of tumor specimens is one of the most effective methods for the identification of fusion oncogenes. Eight primary cholangiocarcinomas without *KRAS/BRAF* mutations or *FIG-ROS1* fusion (Supporting Table 1) were analyzed to identify novel molecular alterations by massively parallel paired-end transcriptome sequencing. Aberrant paired reads that mapped to different transcription units were identified, and 17 potential fusion transcripts were predicted by our algorithm[12] (Supporting Table 2). Sequence reads spanning the junctions of eight fusion candidate transcripts indicated in-frame gene fusion (Fig. 1A-C; Supporting Table 3) and were verified by direct sequencing of RT-PCR products spanning the breakpoints. Among these, fusion transcripts of the receptor kinase gene were detected as *FGFR2-AHCYL1*, *FGFR2-BICC1*, *AHCYL1-FGFR2*, and *BICC1-FGFR2*. However, two transcripts of *AHCYL1-FGFR2* and *BICC1-FGFR2* did not encode a functional protein of relevance to cancer, and conversely *FGFR2-AHCYL1* and *FGFR2-BICC1* were predicted to form chimeric proteins carrying the kinase domain of FGFR2 (Fig. 1D). Transcriptome sequencing showed a specific increase in the expression of the fused 3′ portion of *AHCYL1* and *BICC1* (Supporting Fig. 1A,B). Therefore, the formation of *FGFR2-AHCYL1* or *FGFR2-BICC1* might play important roles in cancer transformation.

From the tumor specimens, CC64 and CC73, we obtained cDNAs corresponding to *FGFR2-AHCYL1*