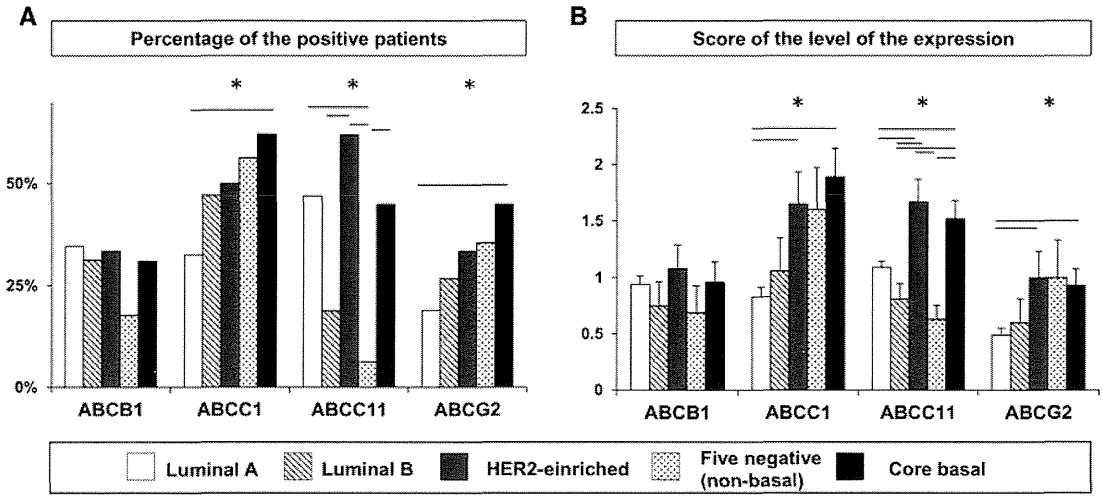

Fig. 3 Frequency (**a**) and intensity (**b**) of high ABC transporter expression classified by subtype, including luminal A (*open columns*), luminal B (*hatched columns*), HER2-enriched (*gray columns*), five-negative (*dotted columns*), and core-basal (*filled columns*). **a** Percentage of patients who showed high expression of each transporter. **b** Semi-quantification of expression level of each transporter, using a 4-point scoring system

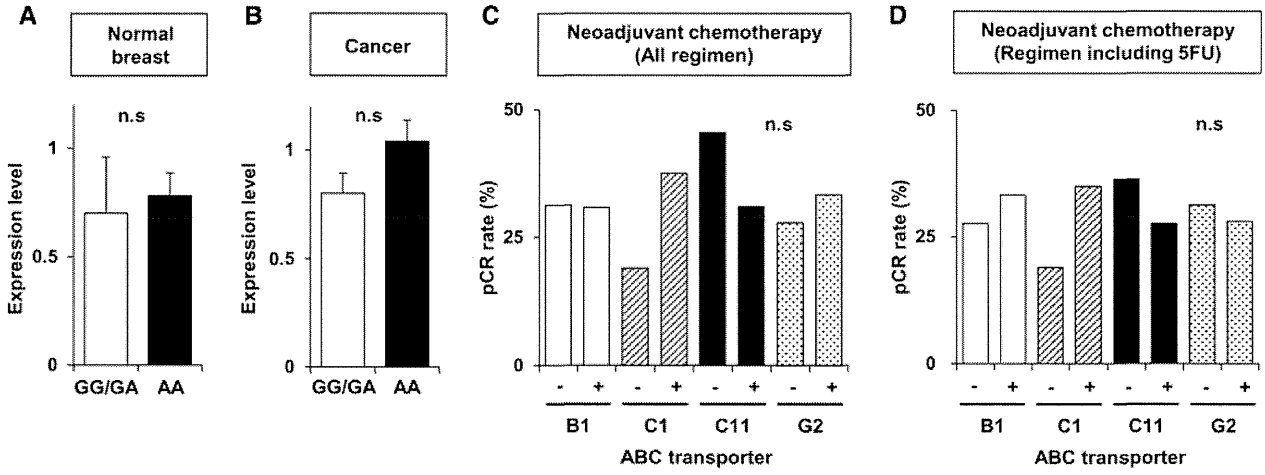

Fig. 4 Semi-quantification of ABCC11 expression levels in normal breast tissue (**a**) and cancer tissue (**b**) in patients carrying 538G/G, 538G/A (white open column, GG/GA, wet earwax phenotype), and 538A/A alleles (*black filled column* AA, dry earwax phenotype). **c**, **d** Pathological complete response ratios to neoadjuvant chemotherapy of all regimens (**c**) and regimens including 5-FU (**d**). *Bars* indicate ABCB1 (*white columns*), ABCC1 (*hatched columns*), ABCC11 (*black columns*), and ABCG2 (*dotted columns*)

cancer tissues. ABCC11 expression did not differ among the wet earwax genotype (538G/G + 538G/A) and the dry earwax genotypes (538A/A), in either normal breast tissues or breast cancer tissues.

As ABCC11 is known to efflux fluoropyrimidines (5-FU) in vitro [17], assessment of responses of ABCC11+ tumors to 5-FU-based regimens could be particularly valuable. Analysis of the association between ABC transporter expression and pathological complete response to neoadjuvant chemotherapy showed no statistically significant differences, regardless of regimen, but patients whose cancers expressed high levels of ABCC11 tended to have decreased pathological complete responses to neoadjuvant chemotherapy (Fig. 4c, d).

ABCC11+ tumors show worse prognoses among aggressive breast cancer subtypes

Because patients with ABCC1+ or ABCC11+ tumors tend to have poor prognoses, we investigated prognosis according to subtype. Patients with ABCC1+ tumors ended to have worse prognoses for luminal A tumors, but not significantly so ($P = 0.096$). Interestingly, patients with ABCC11+ tumors had significantly worse prognoses than did patients

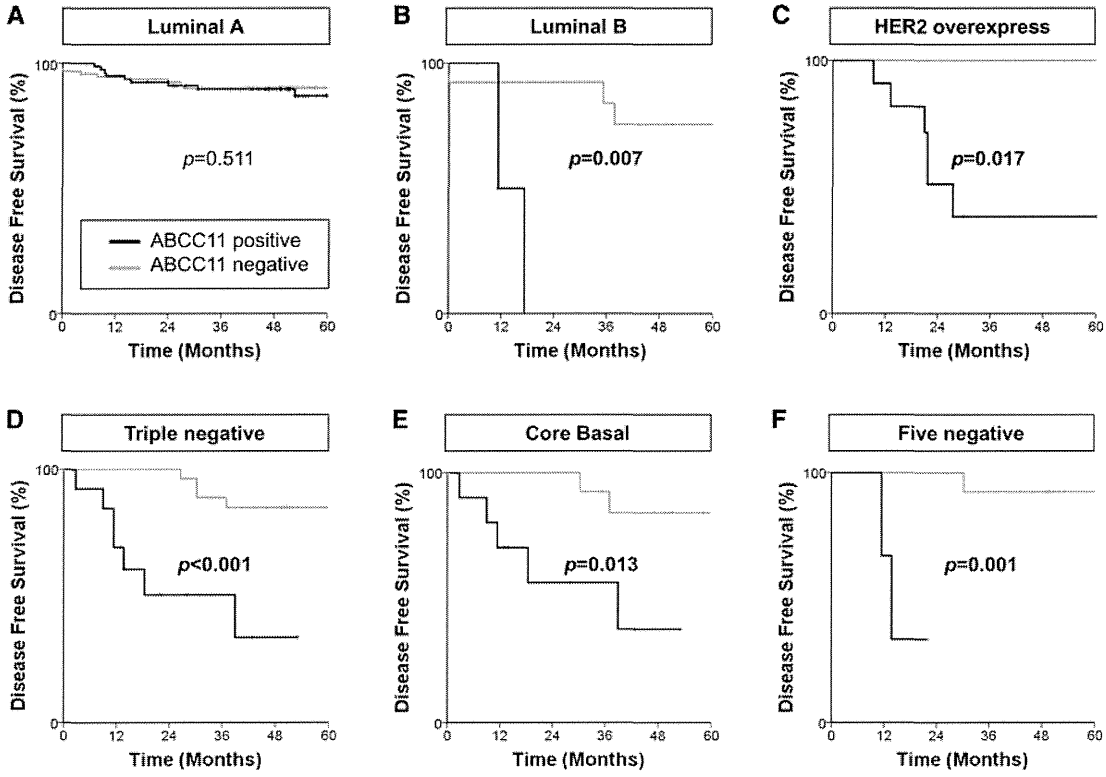

Fig. 5 Kaplan–Meier disease-free survival curve according to the subtype of breast cancer: luminal A (**a**), luminal B(B), HER2-enriched (**c**), triple-negative (**d**), core-basal (**e**), and five-negative (**f**). The *thick bold line* indicates ABCC11+, and the *light gray line* indicates ABCC11−

with ABCC11− tumors, except for the luminal A subtype, which is known to have a better prognosis than the other subtypes (Fig. 5a–f).

Discussion

Different subtypes of breast cancer have different biological behaviors, including responses to systemic and local therapies [3–5] and subsequent clinical outcomes [6, 7]. The two hormone receptor-negative subtypes, triple-negative and HER2-enriched, have poor outcomes compared with the luminal subtypes. Among the triple-negative subtypes, the core-basal subtype, which responds poorly to cytotoxic chemotherapy, has the worst prognosis. Thus, there is a particular need to elucidate drug resistance mechanisms for this subtype.

Expression of ABC transporters is reportedly related to chemoresistance [9]. Some ABC transporters, namely ABCB1, ABCC1, and ABCG2, have been identified as MDR proteins in breast cancer, which contribute to drug resistance via ATP-dependent drug efflux pumps [8]. Because ABCB1 effluxes drugs important for breast cancer—anthracyclines (doxorubicin, epirubicin, and daunomycin) and taxanes (paclitaxel, docetaxel)—ABC transporter inhibitors were the subjects of several widely anticipated clinical trials. Unfortunately, these agents proved disappointing [8, 18]. The vast majority of clinical trials targeting ABC transporters focused on ABCB1 (the most investigated ABC transporter) but data that associates patients' clinicopathological factors with ABCB1 expression tends to conflict [10]. This led us to investigate expression of multiple ABC transporters that are associated with MDR, in the context of different breast cancer subtypes. We felt that this information would be particularly relevant for the triple-negative subtype.

Patient characteristics and our tissue microarray staining data generally agree with previous reports [10, 19, 20]. The proportion of breast cancer subtypes may differ among different races or geographic populations; e.g., prevalence of the luminal A subtype may be higher, and the triple-negative subtype may be lower, in Asian women than in Western women [19]. The demographics of our tissue microarray are consistent with the prevalence among Japanese women. Leonessa et al. [10] reported that the detection rate of ABCB1 and ABCC1 in untreated tumors by immunohistochemistry was 40 % (range: 0–100 %) and 49 % (range: 20–100 %), respectively, with no clear association between ABCB1 and hormone receptors. In

agreement, our results also showed no association between ABCB1 expression and clinical features.

Among ABC transporters, ABCC11 is at relatively early stages of investigation. ABCC11 is lipophilic anion pump that can confer resistance to chemotherapeutic agents such as methotrexate and 5-FU [17]. We previously reported that a SNP in *ABCC11* is associated with the risk of developing breast cancer among Japanese women [12], although the association of *ABCC11* with breast cancer risk is unclear in Caucasian and European women [13, 14]. These reports mentioned host factors that might differ among races and thus modify the impact of this gene on breast cancer risk. *ABCC11* mRNA is reportedly over-expressed in breast tumors and breast cancer cell lines [9, 21, 22], but few studies discuss expression of the ABCC11 protein in human tumors [23]. Although the breast cancer risk conferred by the SNP in *ABCC11* is not within the scope of this study, we did not see significant differences in breast cancer prognosis by SNP genotype in our samples.

Core-basal and HER2-enriched subtypes are associated with poor clinical outcome [5]. In our series, high expressions of ABCC1 and ABCG2 were more common in aggressive subtypes such as core-basal. Strikingly, high expression of ABCC11 was more frequent and intense in both the HER2-enriched and core-basal subtypes, which implies that ABCC11 may promote the aggressive behavior of these subtypes. Indeed, ABCC11 has been shown to export not only drugs but also other factors that affect cancer biology. In agreement, our results show that patients with high tumor expression of ABCC11 have worse outcomes, particularly among the HER2-enriched and core-basal subtypes. This is the first study to show such an association.

Reportedly, ABCC11 expression is related to sensitivity and resistance to chemotherapy [17, 24–26]. In our data, only ABCC11, but not other transporters, tended to correlate with neoadjuvant chemotherapy response. Interestingly, this was true of chemotherapy regimens that both did and did not include 5-FU, which suggests that ABCC11 possesses unidentified supportive functions for drug resistance other than simple drug efflux. For example, we reported that ABCC1 and ABCG2 in breast cancer cells export sphingosine-1-phosphate [27], a bioactive lipid mediator known to affect drug resistance; we cannot exclude the possibility that ABCC11 possesses such a function. In that case, ABCC11 could become a new target in suppressing drug resistance.

Interestingly, it has been suggested that ABCB1 and ABCG2 may affect the role of cancer stem cells in drug resistance [8]. Although we do not currently have data on this relationship, it is intriguing to speculate that the worse prognosis of ABCC11-expressing tumors may be related to cancer stem cells.

Our study is limited in that it is a retrospective analysis of prospectively collected breast tumor samples, and that it shows only association of these transporters with breast cancer prognosis. To evaluate adequately the role of ABCC11 in breast cancer drug resistance, further studies of the mechanism of resistance are needed.

In conclusion, this is the first demonstration that ABCC11 expression in breast cancer is associated with aggressive subtypes and poor disease-free survival.

Acknowledgments The authors thank Dr. Harry D. Bear (Virginia Commonwealth University) for his critical review and valuable input to improve this manuscript. Kazuaki Takabe is supported by the US National Institutes of Health (R01CA160688, K12HD055881) and a Susan G. Komen for the Cure Investigator Initiated Research Grant and Career Catalyst Research Grant (KG090510).

Ethical standards This study was approved by the Institutional Review Board of Yokohama City University, Kanagawa, Japan.

Conflicts of interest The authors declare that they have no conflict of interest.

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VOLUME 31 · NUMBER 35 · DECEMBER 10 2013

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Randomized, Open-Label, Phase III Study Comparing Irinotecan With Paclitaxel in Patients With Advanced Gastric Cancer Without Severe Peritoneal Metastasis After Failure of Prior Combination Chemotherapy Using Fluoropyrimidine Plus Platinum: WJOG 4007 Trial

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Published online ahead of print at www.jco.org on November 4, 2013.

Written on behalf of the West Japan Oncology Group.

Supported by Yakult Honsha and Daiichisankyo.

Presented at the 48th Annual Meeting of the American Society of Clinical Oncology, Chicago, IL, June 1-5, 2012, and European Society of Medical Oncology Congress, Vienna, Austria, September 28-October 2, 2012.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Clinical trial information: University Hospital Medical Information Network–Clinical Trials Registry Japan No. 000001252.

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0732-183X/13/3135w-4438w/$20.00

DOI: 10.1200/JCO.2012.48.5805

ABSTRACT

Purpose
This phase III study compared treatment with weekly paclitaxel and biweekly irinotecan in patients with advanced gastric cancer refractory to treatment with fluoropyrimidine plus platinum.

Patients and Methods
Patients were randomly assigned to receive either paclitaxel (80 mg/m^2 on days 1, 8, and 15, every 4 weeks) or irinotecan (150 mg/m^2 on days 1 and 15, every 4 weeks). Primary end point was overall survival (OS), and secondary end points were progression-free survival (PFS), response rate, adverse events, and proportion of patients who received third-line chemotherapy.

Results
Of 223 patients, 219 were eligible for analysis. Median OS was 9.5 months in 108 patients allocated to the paclitaxel group and 8.4 months in 111 patients allocated to the irinotecan group (hazard ratio [HR], 1.13; 95% CI, 0.86 to 1.49; P = .38). Median PFS was 3.6 months in the paclitaxel group and 2.3 months in the irinotecan group (HR, 1.14; 95% CI, 0.88 to 1.49; P = .33). Response rate was 20.9% in the paclitaxel group and 13.6% in the irinotecan group (P = .24). Common grade 3 to 4 adverse events were neutropenia (paclitaxel group, 28.7%; irinotecan group, 39.1%), anemia (21.3%; 30.0%), and anorexia (7.4%; 17.3%). Treatment-related deaths occurred in two patients (1.8%) in the irinotecan group. Third-line chemotherapy was administered in 97 patients (89.8%) after paclitaxel treatment and in 80 patients (72.1%) after irinotecan treatment (P = .001).

Conclusion
No statistically significant difference was observed between paclitaxel and irinotecan for OS. Both are reasonable second-line treatment options for advanced gastric cancer.

J Clin Oncol 31:4438-4444. © 2013 by American Society of Clinical Oncology

INTRODUCTION

The outcomes in patients with unresectable gastric cancer are extremely poor; median survival times of 3 to 5 months have been reported with best supportive care (BSC) alone.[1-3] In randomized studies conducted in the 1990s, first-line chemotherapy for advanced gastric cancer provided survival benefit over BSC alone. After many clinical trials, at present, fluoropyrimidine plus platinum with or without epirubicin or docetaxel is regarded as standard first-line chemotherapy in the treatment of gastric cancer worldwide.[4-9]

Since S-1 was approved for treatment of advanced gastric cancer in Japan, several phase III studies have been conducted, such as the JCOG 9912 (Japan Clinical Oncology Group 9912; fluorouracil *v* S-1 *v* irinotecan plus cisplatin),[10] SPIRITS (S-1 Plus Cisplatin Versus S-1 in a Randomized Controlled Trial in the Treatment for Stomach Cancer; S-1 *v* S-1 plus cisplatin),[9] and GC0301/TOP-002 trials (Gastric Cancer 0301/Topotecin-002; S-1 *v* S-1 plus irinotecan).[11] On the basis of these study results, S-1 plus cisplatin is accepted as standard first-line chemotherapy for advanced gastric cancer

in Japan. Despite no robust evidence of survival benefit, > 70% of participants received second-line chemotherapy in these studies.[9-11]

Many phase II studies of second-line chemotherapy for advanced gastric cancer have been conducted.[12-20] In evaluations of taxanes, administration of both paclitaxel (210 mg/m^2) and docetaxel (60 mg/m^2) on a triweekly schedule resulted in high rates of grade 3 or 4 neutropenia (37% to 88%),[12-14] whereas lower rates of severe neutropenia (3% to 32%) were observed with weekly administration of paclitaxel (80 mg/m^2).[15-18] Regarding efficacy parameters, response rate (RR) and progression-free survival (PFS) were similar for patients on the triweekly and weekly schedules of paclitaxel. Two reports evaluated weekly paclitaxel as second-line chemotherapy, in which median overall survival (OS) was 5 and 6.9 months, respectively.[15,16] In other studies, combination chemotherapy including biweekly administration of irinotecan (150 mg/m^2) as second-line chemotherapy resulted in median OS of 8 to 10 months,[19,20] although toxicity seemed to be more severe than that seen with weekly paclitaxel. Thus, weekly paclitaxel has become the preferable second-line chemotherapy in Japan.

At present, taxanes and irinotecan are two main options for treatment of advanced gastric cancer refractory to fluoropyrimidine plus platinum. However, to our knowledge, no randomized study has directly compared the efficacy of these two treatments. The West Japan Oncology Group (WJOG) conducted a phase III trial (WJOG 4007) comparing paclitaxel with irinotecan in patients with advanced gastric cancer.

PATIENTS AND METHODS

Patients

Eligible patients were age 20 to 75 years with histologically confirmed metastatic or recurrent gastric adenocarcinoma. Other inclusion criteria were Eastern Cooperative Oncology Group performance status (ECOG PS) of 0 to 2; disease progression confirmed by computed tomography (CT), endoscopy, or other imaging technique during or within 1 month after last dose of first-line chemotherapy with fluoropyrimidine plus platinum; no prior chemotherapy with taxanes or irinotecan; and no severe peritoneal metastasis. Severe peritoneal metastasis was defined as ileus or subileus suggested on barium enema examination and moderate to severe ascites exceeding the pelvic cavity on spine CT scan caused by peritoneal metastasis. In case of treatment with adjuvant or neoadjuvant chemotherapy consisting of fluoropyrimidine plus platinum, patients with disease progression during treatment or within 6 months after treatment completion were eligible. Adequate bone marrow, hepatic, and renal functions were also required.

Study Design

WJOG 4007 was a prospective, multicenter, randomized, open-label, parallel-group phase III clinical trial conducted at 37 centers in Japan. The protocol was approved by the independent ethics committee or institutional review board of each participating institution. This trial was conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent before study entry. The trial was registered with the University Hospital Medical Information Network.

After checking eligibility, patients were randomly assigned at a 1:1 ratio to receive either paclitaxel or irinotecan. Random assignment was carried out centrally at the data center using minimization method with the following adjustment factors: institution, ECOG PS (0 to 1 *v* 2), and measurable lesions (presence *v* absence). Neither investigators nor patients were blinded to the allocated treatment.

Treatment

Paclitaxel (80 mg/m^2) was administered intravenously on days 1, 8, and 15, every 4 weeks. Patients were premedicated with histamine receptor-1 and -2 blockers and dexamethasone for prophylaxis of allergic reactions 30 minutes before paclitaxel administration. Irinotecan (150 mg/m^2) was administered intravenously on days 1 and 15, every 4 weeks. Dose reduction and/or cycle delays were permitted according to predefined toxicity criteria. Treatment continued until disease progression, occurrence of unacceptable serious toxicity, or patient refusal of further treatment. Subsequent chemotherapy was not specified.

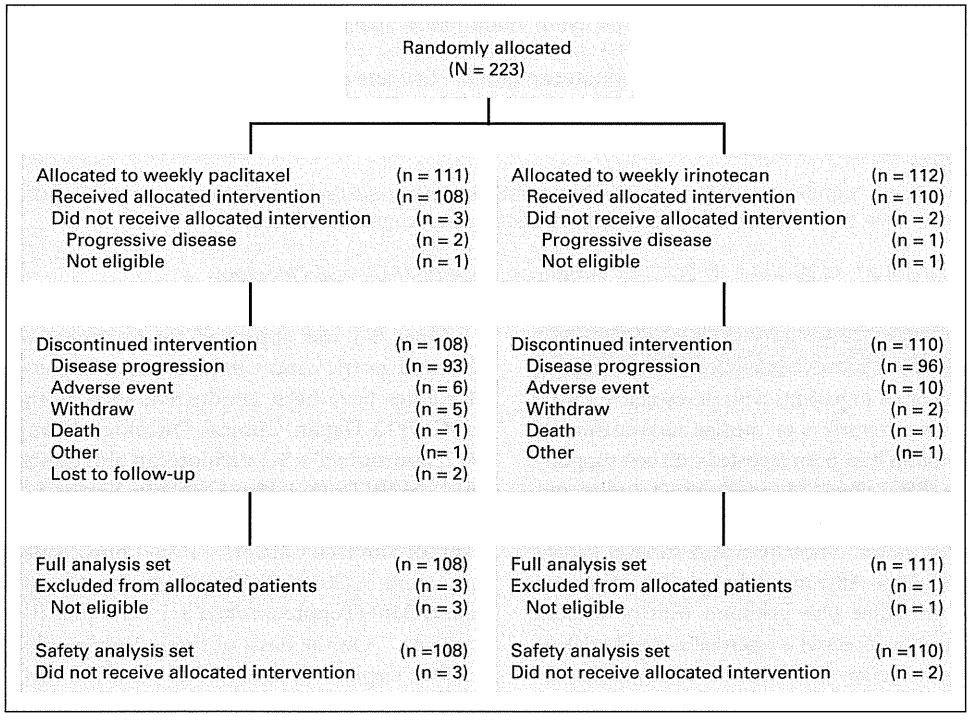

Fig 1. CONSORT diagram.

Assessments

Vital signs, ECOG PS, and laboratory tests were assessed within 7 days before study entry. Physical examinations and hematology and biochemistry tests were conducted during drug administration throughout the treatment course. Tumor assessments using CT scans of the chest, abdomen, and pelvis were performed within 28 days before study entry and repeated every 2 months after random assignment until discontinuation of protocol treatment. RECIST (version 1.0) was used to evaluate treatment responses.[21] Safety assessments were repeated every 2 weeks until initiation of subsequent chemotherapy or 6 weeks after the last protocol treatment. Severity of adverse events was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (version 3.0). The WJOG Data and Safety Monitoring Committee reviewed serious adverse events for trial safety during the protocol treatment. Investigators assessed response, progression, and toxicities in their patients; independent central assessments of response and disease progression were not performed.

Statistical Analysis

The primary end point was OS, defined as time from random assignment to death resulting from any cause. Secondary end points were PFS, defined as time from random assignment to disease progression or death resulting from any cause; RR; toxicity; and proportion of patients who received subsequent chemotherapy.

Previous single-arm studies showed median OS of 5 and 6.9 months in paclitaxel-[15,16] and 8 and 10 months in irinotecan-containing regimen.[19,20] Irinotecan was contraindicated for patients with severe peritoneal metastasis, because its biliary-excreted metabolites caused severe toxicities. In gastric cancer, peritoneal metastasis often developed along with disease progression, and we therefore speculated that subsequent irinotecan after paclitaxel would be more difficult to apply in patients compared with the reverse treatment sequence. On the basis of these previous results and our assumption, this study was designed to detect 50% improvement in median OS from 5 months in the paclitaxel group to 7.5 months in the irinotecan group (hazard ratio [HR], 0.67). Assuming accrual and follow-up periods of 36 and 12 months, respectively, and using a two-sided log-rank test with 5% α and 20% β errors, 220 patients were required for the study. No interim analyses were planned.

A full analysis set (FAS) included all randomly assigned patients who met the eligibility criteria (patients found to be ineligible after random assignment were excluded). The safety analysis set (SAS) included all randomly assigned patients who received $\geq$ one dose of study medication. OS and PFS were analyzed in the FAS and estimated using the Kaplan-Meier method. RR was assessed in patients with $\geq$ one measurable lesion at baseline. Toxicity was analyzed in the SAS.

The primary analysis was planned for 1 year after enrollment of the last patient or approximately 205 events, whichever came first. An independent statistician and data analysis center performed the primary analysis for OS with unstratified log-rank test in the FAS population. All investigators remained blinded to the data until the analysis was completed. Cox proportional hazards models were used to calculate HRs and CIs. Fisher's exact test was used to assess differences in RR, incidence of

Table 1. Baseline Patient Demographic and Clinical Characteristics

Characteristic	Weekly Paclitaxel (n = 108) No.	%	Irinotecan (n = 111) No.	%
Sex				
Male	84	77.7	87	78.4
Female	24	22.2	24	21.6
Age, years				
Median	64.5		65	
Range	37-75		38-75	
ECOG PS				
0 to 1	104	96.3	107	96.4
2	4	3.7	4	3.6
Prior gastrectomy				
Yes	37	34.3	39	35.1
No	71	65.7	72	64.9
Prior chemotherapy				
S-1 plus cisplatin	92	85.2	102	91.9
Capecitabine plus cisplatin	13	12.4	8	7.2
S-1 plus oxaliplatin	3	2.8	1	0.9
Target lesion				
Yes	91	84.3	88	79.3
No	17	15.7	23	20.7
Histology				
Intestinal	54	50.0	54	48.6
Diffuse	54	50.0	57	51.4
Peritoneal metastasis				
Yes	28	25.9	28	25.2
No	80	74.1	83	74.8
No. of metastatic sites				
One	57	52.8	64	57.7
Two or more	51	47.2	47	42.3

Abbreviation: ECOG PS, Eastern Cooperative Oncology Group performance status.

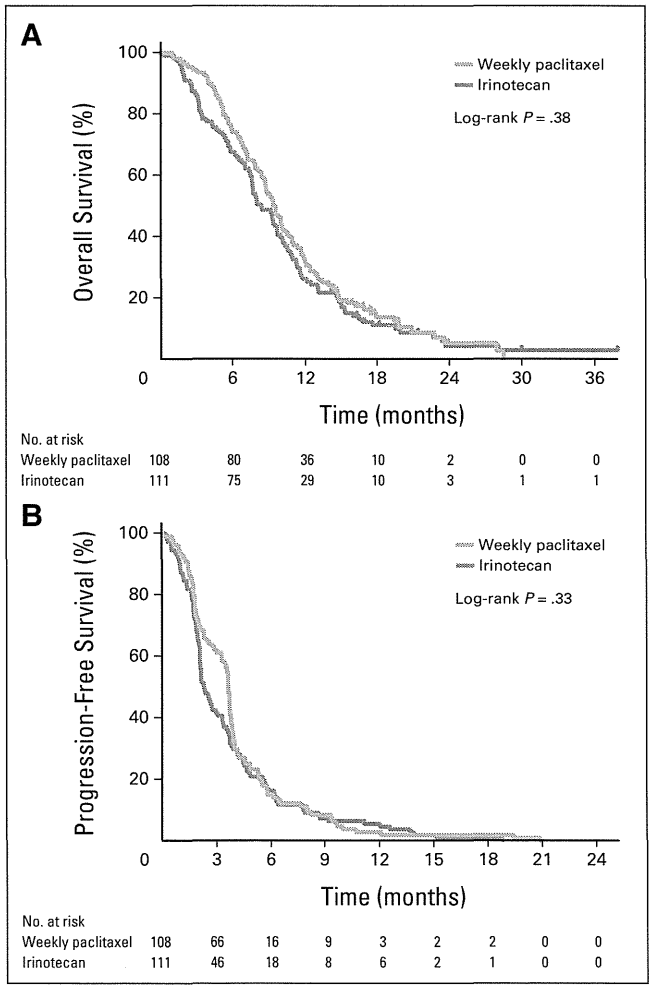

Fig 2. Kaplan-Meier curves of (A) overall and (B) progression-free survival.

adverse events, and proportion of patients who received third-line chemotherapy. Exploratory subgroup analyses of OS were performed using stratification and prognostic variables.

RESULTS

Patients

Between August 2007 and August 2010, 223 patients were enrolled from 37 centers in Japan. Of these patients, 111 were allocated to the paclitaxel group and 112 to the irinotecan group (Fig 1). Four patients, who either had received prior fluoropyrimidine monotherapy (paclitaxel group, n = 2; irinotecan group, n = 1) or had radiologically unconfirmed disease progression (paclitaxel group, n = 1), were ineligible for the study. Thus, the FAS consisted of 108 patients in the paclitaxel group and 111 patients in the irinotecan group. After random assignment, three patients in the paclitaxel group and two in the irinotecan group did not receive the protocol treatment. Thus, the SAS consisted of 108 patients in the paclitaxel group and 110 patients in the irinotecan group. Baseline characteristics of patients in the FAS were well balanced between the two treatment groups (Table 1). ECOG PS scores of 0 or 1 were found in a majority of patients. The most common first-line chemotherapy was S-1 plus cisplatin (88.6%), followed by capecitabine plus cisplatin with or without anti–epidermal growth factor receptor or anti–vascular endothelial growth factor antibodies (9.6%) and S-1 plus oxaliplatin (1.8%). One or more measurable lesions were present in approximately 80% of patients, and mild or moderate peritoneal metastasis was detected in approximately 25% of patients in both groups. Two or more metastatic sites were found in < 50% of patients.

Exposure to Chemotherapy

Median number of administrations was 11.5 (range, one to 46) in the paclitaxel group and 4.5 (range, one to 39) in the irinotecan group. Reasons for discontinuation of treatment included: disease progression (86.7%), adverse events (7.3%), withdrawal of consent (3.2%), and other reasons (2.8%). The proportion of patients in whom treatment was discontinued because of toxicity was 5.6% in the paclitaxel group and 9.1% in the irinotecan group.

Third-line chemotherapy was administered to 97 patients (89.8%) in the paclitaxel group and 80 patients (72.1%) in the irinotecan group ($P = .001$). In the paclitaxel group, third-line chemotherapy containing irinotecan was used in 81 patients (75.0%), and in the irinotecan group, a taxane-containing regimen was used in 67 patients (60.4%). Including later lines, 87 patients (80.6%) in the paclitaxel group received irinotecan, and 75 patients (67.6%) in the irinotecan group received paclitaxel.

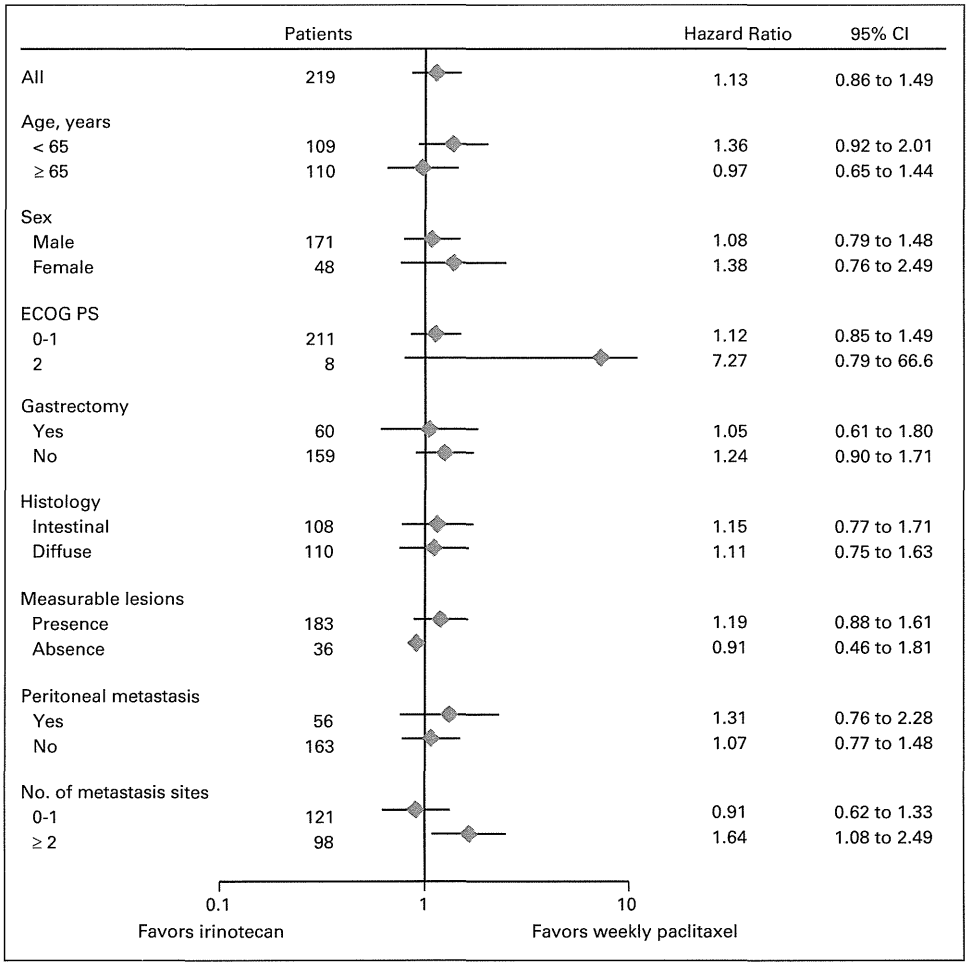

Fig 3. Forest plot of subgroup analyses. ECOG PS, Eastern Cooperative Oncology Group performance status.

Efficacy

In August 2011, after a median follow-up period of 17.6 months, 203 deaths (92.7%) were reported in the patient cohort. For the primary end point of OS, no statistically significant difference was observed between paclitaxel and irinotecan groups (HR, 1.13; 95% CI, 0.86 to 1.49; two-sided $P = .38$). Median OS was 9.5 months (95% CI, 8.4 to 10.7) in the paclitaxel group and 8.4 months (95% CI, 7.6 to 9.8) in the irinotecan group (Fig 2A). Median PFS was 3.6 months (95% CI, 3.3 to 3.8) in the paclitaxel group and 2.3 months (95% CI, 2.2 to 3.1) in the irinotecan group. This difference was not statistically significant (HR, 1.14; 95% CI, 0.88 to 1.49; two-sided $P = .33$; Fig 2B). RR was 20.9% (19 of 91 patients) in the paclitaxel group and 13.6% (12 of 88) in the irinotecan group (Fisher's exact $P = .24$).

Results of the subgroup analysis of OS are shown in Figure 3. Although treatment with weekly paclitaxel conferred a slight survival advantage in almost all subgroups, no significant interactions were observed in any subgroup. In an exploratory analysis, OS was analyzed in patients who received irinotecan and paclitaxel during second- and later-line chemotherapies. Median OS was 10.1 months in each group, and the survival curves of these two subgroups almost overlapped (HR, 0.96; 95% CI, 0.69 to 1.32; two-sided $P = .96$).

Safety

Table 2 lists adverse events and the proportion of patients experiencing adverse events during treatment in the SAS. The most common grade 3 or 4 adverse events were leukopenia (20.4%), neutropenia (28.7%), and anemia (21.3%) in the paclitaxel group. Leukopenia (19.1%), neutropenia (39.1%), anemia (30.0%), anorexia (17.3%), and hyponatremia (15.5%) were common in the irinotecan group. Grade 3 or 4 sensory neuropathy was observed in the paclitaxel group (7.4%) only. Grade 3 or 4 febrile neutropenia was more prevalent in the irinotecan group (9.1%) than in the paclitaxel group (2.8%). Three (2.7%) and four deaths (3.6%) resulting from any cause occurred within 30 days after the last administration in the paclitaxel and irinotecan groups, respectively. Treatment-related death confirmed by the independent data safety monitoring committee was observed in two patients (1.8%) in the irinotecan group. Causes of death included serious pneumonia in one patient and gastric perforation in the other.

Table 2. Adverse Events

	Weekly Paclitaxel (n = 108)				Irinotecan (n = 110)			
	All Grade		Grade 3 to 4		All Grade		Grade 3 to 4	
Adverse Event	No.	%	No.	%	No.	%	No.	%
Leukocytopenia	88	81.4	22	20.4	76	69.4	21	19.1
Neutropenia	85	78.7	31	28.7	77	70.0	43	39.1
Hemoglobin	69	63.9	23	21.3	84	76.4	33	30.0
Thrombocytopenia	6	5.6	1	0.9	15	13.6	2	1.8
Febrile neutropenia	3	2.8	3	2.8	10	9.1	10	9.1
Nausea	33	30.6	2	1.9	61	55.5	5	4.5
Vomiting	22	20.4	3	2.8	40	36.4	1	0.9
Anorexia	50	46.3	8	7.4	78	70.1	19	17.3
Diarrhea	21	19.4	1	0.9	49	44.5	5	4.5
Neuropathy (sensory)	62	57.4	8	7.4	2	1.8	0	0
Bilirubin	10	9.3	3	2.8	21	19.1	4	3.6
AST	32	29.6	4	3.7	42	38.2	9	8.2
ALT	24	22.2	3	2.8	41	37.3	3	2.7
Hyponatremia	21	19.4	4	3.7	35	31.8	17	15.5
Treatment-related death	0	0	0	0	2	1.8	2	1.8

DISCUSSION

To our knowledge, this was the first randomized phase III trial comparing paclitaxel and irinotecan in second-line chemotherapy for advanced gastric cancer. No statistically significant differences were observed between paclitaxel and irinotecan for the primary end point of OS or for other parameters evaluated in this study, including PFS and RR. Activity, feasibility, and tolerability of paclitaxel and irinotecan were comparable for second-line treatment of advanced gastric cancer.

When we planned this study, OS in patients who received second-line chemotherapy seemed to be longer than OS in patients who received BSC alone in previous trials.[12-16,19,20] Because > 70% of patients were receiving second-line chemotherapy as part of routine clinical practice at that time, conducting a trial of second-line chemotherapy compared with BSC alone was difficult in Japan. Since then, the survival benefit of second-line chemotherapy over BSC has been demonstrated in two randomized trials[22,23]: the AIO (Arbeitsgemeinschaft Internistische Onkologie) trial using irinotecan and Korean trial using irinotecan or docetaxel during the same time period as this WJOG 4007 study. On the basis of these results, second-line chemotherapy using irinotecan or docetaxel has been recognized as the standard of care for patients with gastric cancer. However, further comparison between irinotecan and taxane regimens would be valuable for strategic planning of treatment in patients with advanced gastric cancer.

In the Korean trial, choice of chemotherapy regimen—docetaxel or irinotecan—depended on investigator discretion. A subgroup analysis showed no significant difference in survival between regimens (median OS: docetaxel, 5.2 months *v* irinotecan, 6.6 months; $P = .116$).[23] In addition, Ji et al[24] conducted a retrospective analysis of 725 patients with gastric cancer treated with second-line chemotherapy; they found no relevant difference in OS between taxane and irinotecan treatment. In our exploratory subgroup analysis, no interaction was observed among several clinical factors; results favored neither paclitaxel nor irinotecan. Thus, either taxane or irinotecan can be recommended as a treatment option for second-line chemotherapy in patients with advanced gastric cancer.

Longer OS was achieved in this study than in previous phase III studies.[22,23] Many patients in good condition with small tumor burdens were enrolled onto our study. ECOG PS of 0 or 1 was recorded in almost all patients, and only one metastatic site was detected in > half of all patients. Additionally, excluding patients with severe peritoneal metastasis resulted in a lower proportion of patients (25.6%) with peritoneal metastasis, compared with those in the AIO (43%) and Korean (45%) trials.[22,23] These are well known as prognostic factors in advanced gastric cancer, and these patient-selection biases might have led to longer survival in our study.

In gastric cancer, peritoneal metastasis often develops along with disease progression, and irinotecan would be toxic for patients with

severe peritoneal metastasis. Indeed, the proportion of patients receiving subsequent irinotecan after second-line paclitaxel was only 24% in the previous report.[16] In this study, excluding patients with severe peritoneal metastasis seemed to result in a high proportion of patients (> 70%) receiving third-line chemotherapy, whereas 30% to 40% of patients did so in previous studies.[23,24] Although evidence is limited with regard to the efficacy of third-line chemotherapy in advanced gastric cancer, this therapy may have contributed to prolonged OS, and the unexpected higher proportion of those receiving third-line chemotherapy might have diluted a difference in OS between the paclitaxel and irinotecan groups.

Overall toxicity in both treatment arms was acceptable for second-line chemotherapy. In the paclitaxel group, common grade 3 or 4 toxicities (≥ 10%) included leukocytopenia, neutropenia, and anemia. Grade 3 sensory neuropathy, which was specific to paclitaxel, occurred at an incidence < 10% in this study. These toxicity profiles and severity levels are consistent with those in previous reports.[15,16] In the irinotecan group, leukocytopenia, neutropenia, anemia, anorexia, and hyponatremia were commonly observed. Frequency and severity of these toxicities were also consistent with those in previous reports.[22,23] Severe diarrhea, which is a well-known adverse reaction to irinotecan, generally occurs less frequently in Asian patients than in Western patients. In fact, grade 3 or 4 diarrhea was observed in 4.5% of patients in this trial, 8% of those in the Korean trial,[23] and 26% of those in the AIO trial.[22] Although ethnic diversity in metabolism of irinotecan has been suggested, the dosage of irinotecan is commonly higher in Western countries than in Asian countries. This may explain the different incidence of severe diarrhea between this and other studies.

Our study has several limitations. Participants were all Japanese; tumor biology may differ from that in Western patients.[25] In addition, a majority of patients received S-1 plus cisplatin as first-line chemotherapy, whereas S-1 is not popular in Western countries. However, a large, global phase III study (FLAGS [First-Line Therapy in Patients With Advanced Gastric Cancer Study] trial) demonstrated S-1 plus cisplatin to be similar in efficacy to fluorouracil plus cisplatin.[7] This difference in regimens used as first-line chemotherapy may have had little influence on interpretation of results of our study. Because patients with severe peritoneal metastasis were excluded from our study to avoid confounding effects of serious adverse events resulting from irinotecan, our results are not applicable to patients with severe peritoneal metastasis. Another trial is needed to determine the most appropriate treatment in such patients. As for statistical consideration, our hypothesis was 50% improvement in median OS in the irinotecan group over weekly paclitaxel group, and this resulted in a relatively small sample size. Therefore, if a small but true benefit existed in either group, this study may have been underpowered to detect it.

In conclusion, no difference in OS between paclitaxel and irinotecan groups was observed in this study. Both are considered reasonable second-line treatment options. The differences in toxicity profile and treatment schedule between both treatments will help in choosing either irinotecan or paclitaxel. Currently, several randomized trials investigating additional benefits of molecular targeting agents in second-line chemotherapy are planned or being conducted using weekly paclitaxel or irinotecan as a platform or reference regimen. The findings of our study are relevant to these future trials.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) and/or an author's immediate family member(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.
Employment or Leadership Position: None **Consultant or Advisory Role:** None **Stock Ownership:** None **Honoraria:** Tomohiro Nishina, Yakult Pharmaceutical Industry, Bristol-Myers Squibb; Satoshi Morita, Bristol-Myers Squibb, Yakult Pharmaceutical Industry, Daiichi Sankyo; Narikazu Boku, Daiichi Sankyo, Yakult Pharmaceutical Industry; Ichinosuke Hyodo, Yakult Pharmaceutical Industry **Research Funding:** Naotoshi Sugimoto, Yakult Pharmaceutical Industry; Taito Esaki, Yakult Pharmaceutical Industry; Ichinosuke Hyodo, Yakult Pharmaceutical Industry, Daiichi Sankyo **Expert Testimony:** None **Patents:** None **Other Remuneration:** None

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Acknowledgment

We thank Y. Hirai, N. Ozumi, K. Nonogaki, and S. Nakamura, MD, for data management and all members of the data safety monitoring committee and audit committee of the West Japan Oncology Group.

Surg Today (2013) 43:289–292
DOI 10.1007/s00595-012-0462-8

ORIGINAL ARTICLE

A rare point mutation in the Ras oncogene in hepatocellular carcinoma

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Received: 16 May 2011 / Accepted: 17 May 2011 / Published online: 26 December 2012

Abstract
Purpose The Ras gene is one of the oncogenes most frequently detected in human cancers, and codes for three proteins (K-, N-, and H-Ras). The aim of this study was to examine the mutations in codons 12, 13 and 61 of the three Ras genes in cases of human hepatocellular carcinoma (HCC).
Methods Paired samples of HCC and corresponding non-malignant liver tissue were collected from 61 patients who underwent hepatectomy. A dot-blot analysis was used to analyze the products of the polymerase chain reaction (PCR) amplification of codons 12, 13, and 61 of K-, N- and H-Ras for mutations.
Results Only one mutation (K-Ras codon 13; Gly to Asp) was detected among the 61 patients. Interestingly, this patient had a medical history of surgery for both gastric cancer and right lung cancer. No mutations were found in codons 12 and 61 of K-Ras or codons 12, 13 and 61 of the N-Ras and H-Ras genes in any of the HCCs or corresponding non-malignant tissues.
Conclusions These findings indicated that the activation of Ras proto-oncogenes by mutations in codons 12, 13, and 61 does not play a major role in hepatocellular carcinogenesis.

Keywords Ras · Mutation · Hepatocellular carcinoma · Sorafenib

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Abbreviations

Asp	Asparagine
Glu	Glutamate
Gly	Glycine
HCC	Hepatocellular carcinoma
Lys	Lysine
PCR	Polymerase chain reaction
TTP	Time to progression
Val	Valine

Introduction

Hepatocellular carcinoma (HCC) is a global health problem, accounting for more than 80 % of all primary liver cancers, and is one of the most common malignancies worldwide [1]. Most patients with HCC also present with concomitant cirrhosis, which is the major clinical risk factor for hepatic cancer, and results from alcoholism or infection with the hepatitis B or hepatitis C virus. Primary liver malignancies (95 % of which are HCC) are the third and fifth leading causes of cancer death among males and females, respectively, in Japan [2]. Both liver resection and liver transplantation are potentially curative treatments for HCC [3–5]. Although other treatment options, including percutaneous radiofrequency ablation or chemolipiodolization are also available, there is no standard systemic therapy for advanced cases.

Sorafenib (BAY 43-9006, Nexavar) is a novel oral kinase inhibitor that targets multiple tyrosine kinases in vivo and in vitro, and is widely used for HCC [6]. The main targets of sorafenib are the receptor tyrosine kinase pathways which are frequently deregulated in cancer, such as the Ras pathway. The Ras pathway represents a dominant signaling network promoting cell proliferation and

survival. The binding of different growth factors (e.g. epidermal growth factor: EGF) to their receptors (e.g. epidermal growth factor receptor: EGFR) induces the activation of Ras, which in turn activates c-raf, MEK and ERK. Phosphorylated ERK in the nucleus activates transcription factors that regulate the expression of genes involved in cell proliferation and survival.

A phase II trial involving 137 patients with advanced HCC showed that sorafenib induced partial responses in less than 5 % of patients, but the observed median survival of 9.2 months with a median time to progression of 5.5 months was classified as evidence of potential clinical benefit, since the expected median survival of these patients is 6 months [7]. Consequently, a large phase III clinical trial (SHARP) was conducted in 602 patients with advanced HCC. The results showed a 31 % decrease in the risk of death, with a median survival of 10.6 months in the sorafenib arm versus 7.9 months for placebo [8]. In addition, sorafenib showed a significant benefit in terms of the time to progression (TTP) as assessed by independent radiological review, with a median TTP of 5.5 months for the sorafenib and 2.8 months for the placebo arm.

Because Ras is one of the targets of sorafenib, it is important to determine whether mutations in the Ras gene result in the activation of the Ras/MAPK pathway in human HCCs. However, the relationship between Ras mutations and human HCC has not been fully evaluated. The present study was designed to investigate K-, N- and H-Ras (*KRAS, NRAS, HRAS*) somatic mutations in human HCC.

Materials and methods

Patients and tumor samples

Tumor tissue samples were obtained from 61 Japanese patients who underwent surgical resection for HCC during the period between December 1989 and April 1992 in the Department of Surgery and Science, Kyushu University Hospital, Fukuoka, Japan. Surgically resected tissue samples were frozen at −80 °C immediately after resection and were stored until use in this study. Written informed consent was obtained from all patients examined, and the current study was approved by the Kyushu University ethics committee.

DNA preparation and detection of Ras point mutations

High molecular weight DNA was isolated from frozen tumor samples, as described elsewhere [9]. Selective amplification of the Ras gene sequence was done using a PCR technique. The nucleotide sequences of the primers used are listed in Table 1. The PCR was performed at

Table 1 Ras gene primers used in this study

Gene/codon	Length (bp)	Sequence	
KRAS/12, 13	108	Forward	GACTGAATATAAACTTGTGG
		Reverse	CTATTGTTGGATCATATTCG
KRAS/61	128	Forward	TTCCTACAGGAAGCAAGTAG
		Reverse	CACAAAGAAAGCCCTCCCCA
HRAS/12, 13	63	Forward	GACGGAATATAAGCTGGTGG
		Reverse	TGGATGGTCAGCGCACTCTT
HRAS/61	73	Forward	AGACGTGCCTGTTGGACATC
		Reverse	CGCATGTACTGGTCCCGCAT
NRAS/12, 13	109	Forward	GACTGAGTACAAACTGGTGG
		Reverse	CTCTATGGTGGGATCATATT
NRAS/61	103	Forward	GGTGAAACCTGTTTGTTGGA
		Reverse	ATACACAGAGGAAGCCTTCG

bp base pairs

96 °C to denature the DNA (1 min), at 55 °C (*NRAS*), 57 °C (*KRAS*), 62 °C (*HRAS*) to anneal the primer (30 s), and at 72 °C to synthesize DNA (10 s to 1 min) using Taq DNA polymerase for 35–40 cycles in a DNA thermal cycler (Perkin-Elmer-Cetus). Amplified DNA samples were spotted onto nylon membranes (Hybond N+) for the hybridization analysis. All of the DNA isolated from the 61 tumor samples and the corresponding non-malignant liver tissues were screened for activated point mutations in codons 12, 13, and 61 of all three Ras genes using an oligonucleotide specific for the different sequences. The filters were prehybridized for 1 h at 55 °C in solution A (3.0 M tetramethylammonium chloride, 50 mM Tris-HCI, 2 HIMEDTA, 0.1 % SDS, 5× Denhardt's solution, 100 fg/ml denatured herring sperm DNA), and hybridized for 1 h at 55 °C in the same solution with 5 pmol ^{32}P-labeled probe. These filters were washed twice in 0.3 M NaCl, 0.02 M NaH2PO4, 2 mM EDTA and 0.1 % SDS at room temperature for 5 min, and in solution A without Denhardt's solution and herring sperm DNA, once for 5 min at room temperature and twice for 10 min at 60 °C. These filters were then exposed to Kodak XAR5 film. Human cancer cell lines carrying Ras genes mutations were used as positive controls. The colon cancer cell lines: SW620 (*KRAS* codon 12 GTT:Val), LSI80 (*KRAS* codon 12 GAT:Asp), and LOVO (*KRAS* codon 13 GAC:Asp) were obtained from the Japanese Cancer Research Resources Bank, and KMS4 (*KRASs* codon 12 TGT:Cys) was provided by Dr. Sugio (Institution?).

Results

The age of the 61 patients ranged from 43 to 79 years (average, 64.1 years), and 46 were males and 15 were

females. The positive rate of hepatitis surface B antigen was 12.9 %, and the positive rate of anti-hepatitis C virus antibody was 72.7 %. The mean tumor size was 4.47 cm.

One of the 61 HCCs (1.6 %) carried a point mutation, which was a G to A transition at codon 13 of the *KRAS* gene (Fig. 1). DNA extracted from the corresponding non-malignant liver tissue had the normal codon, suggesting that mutational activation of K-ras was involved in the malignant transformation in this case. This patient was positive for anti-hepatitis C virus antibodies, and was classified to have Child-Pugh A disease. The diameter of this patient's tumor was 12 cm, and the tumor was composed of well to moderately differentiated hepatocellular carcinoma. Interestingly, this patient had undergone surgery for gastric cancer 18 years before and lung cancer 12 years before the surgery for HCC.

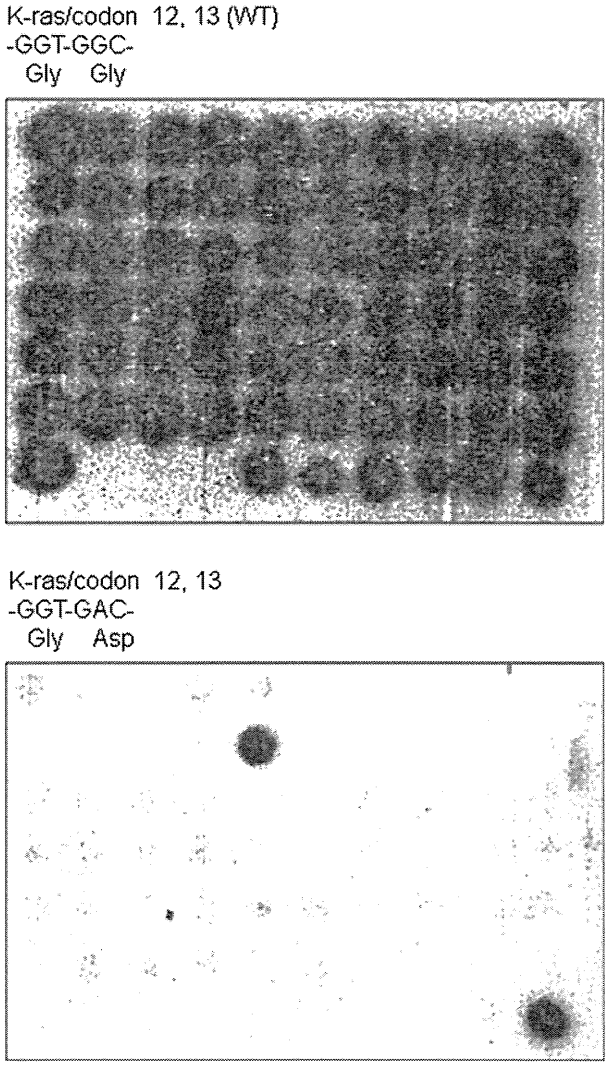

Fig. 1 Detection of a *KRAS* gene mutation in a patient with hepatocellular carcinoma. PCR-amplified DNA from 61 tumor samples was dotted onto nylon membranes and hybridized to a ^{32}P-labeled oligonucleotide probe. *WT* wild type *KRAS*

No mutational activation was found in codons 12 and 61 of *KRAS* or codons 12, 13 and 61 of the *NRAS* and *HRAS* genes in any of the HCCs or corresponding non-malignant tissue samples.

Discussion

This study examined 61 HCC tissues and their corresponding non-malignant liver tissues for a somatic mutation in codons 12, 13, and 61 of the *KRAS*, *HRAS*, or *NRAS* genes, which are known hot spots in various malignancies. However, the study showed the only one of the 61 HCCs (1.6 %) had a somatic mutation in codon 13 of the *KRAS* gene, indicating that Ras gene mutations do not appear to be related to the pathogenesis of most HCCs.

There have been several reports with small sample sizes regarding Ras gene mutations in HCC (Table 2). Most have reported that somatic mutations of the Ras gene in HCCs are uncommon, similar to the current study. Tsuda et al. [10] found only two tumors with Ras point mutations in surgically resected specimens from 30 HCC patients. In their patients, codon 12 of *KRAS* was altered from GGT, coding for Gly, to GTT, coding for Val in one case, and codon 61 of *NRAS* was altered from CAA, coding for Glu, to AAA, coding for Lys, in the other case. Tada et al. analyzed the mutations of the three Ras genes in 23 primary hepatic malignant tumors (12 hepatocellular carcinomas, nine cholangiocarcinomas, and two hepatoblastomas). Point mutations in *KRAS* codon 12 or *KRAS* codon 61 were found in 6 of the 9 cholangiocarcinomas. In contrast, there were no point mutations in any of 12 HCCs or two hepatoblastomas in codons 12, 13, or 61 of the Ras genes. The authors concluded that Ras gene mutations are not related to the pathogenesis of HCC, but play an important role in pathogenesis of cholangiocarcinoma.

Sorafenib is the first molecule with specific targets involved in the pathogenesis of HCC that has become available for routine clinical use. It is an orally applicable

Table 2 Reported Ras gene mutations in HCC patients

Author [references]	No. of patients	Ras gene mutation		
		KRAS	*NRAS*	*HRAS*
Tsuda et al. [10]	30	1 (codon 12)	1 (codon 61)	0
Tada et al. [14]	12	0	0	0
Ogata et al. [15]	19			2
Challen et al. [16]	19	1 (codon 61)	3 (codon 61)	0
Leon et al. [17]	12	1 (codon 61)	0	0
This study	61	1 (codon 13)	0	0

multi-kinase inhibitor that acts by blocking tumor cell proliferation and angiogenesis through the inhibition of serine/threonine kinases [11]. Sorafenib can increase survival by up to 3 months in patients with advanced HCC and acceptable liver function [8]. On the other hand, severe side effects have been reported with sorafenib, including hand-foot skin reactions or liver dysfunction [7, 8]. Therefore, it is important to identify prognostic markers and to establish the proper selection criteria for using sorafenib. Mutations of the Ras genes in cases of HCCs were systemically evaluated in this study because the Ras signaling pathway is the main target of sorafenib. The results indicated that mutational activation of Ras genes is uncommon in the pathogenesis of HCCs. Caraglia et al. [12] reported that the presence of phosphorylated ERK activity in peripheral blood mononuclear cells is valuable for predicting the response to sorafenib therapy in HCC patients. An in vitro study confirmed that phosphorylated ERK was a potential biomarker predicting the sensitivity of HCC to sorafenib [13]. Therefore, a mutation in the RAF/MEK/ERK pathway may be involved in the drug resistance to sorafenib, rather than a Ras mutation.

In summary, only one of 61 HCCs (1.6 %) in the present study carried a point mutation, which was a G to A transition in codon 13 of the *KRAS* gene. No mutational activation was found in codons 12 and 61 of *KRAS* or in codons 12, 13 and 61 of the *NRAS* or *HRAS* genes in any of the HCCs or corresponding non-malignant tissue samples. These findings suggested that Ras gene mutations are not related to the pathogenesis of most HCCs. The signaling pathways downstream of Ras should be examined to identify markers to predict a response to sorafenib.

Acknowledgments We thank Professor Brian Quinn for his review of this manuscript. No financial support was received for this work from any company. This study was supported in part by a grant from the Scientific Research Fund of the Ministry of Education of Japan.

Conflict of interest None of the authors has any conflict of interest.

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HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

Identification of Novel Serum Biomarkers of Hepatocellular Carcinoma Using Glycomic Analysis

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The altered *N*-glycosylation of glycoproteins has been suggested to play an important role in the behavior of malignant cells. Using glycomics technology, we attempted to determine the specific and detailed *N*-glycan profile for hepatocellular carcinoma (HCC) and investigate the prognostic capabilities. From 1999 to 2011, 369 patients underwent primary curative hepatectomy in our facility and were followed up for a median of 60.7 months. As normal controls, 26 living Japanese related liver transplantation donors were selected not infected by hepatitis B and C virus. Their mean age was 40.0 and 15 (57.7%) were male. We used a glycoblotting method to purify *N*-glycans from preoperative blood samples from this cohort (10 μL serum) which were then identified and quantified using mass spectrometry (MS). Correlations between the *N*-glycan levels and the clinicopathologic characteristics and outcomes for these patients were evaluated. Our analysis of the relative areas of all the sugar peaks identified by MS, totaling 67 *N*-glycans, revealed that a proportion had higher relative areas in the HCC cases compared with the normal controls. Fourteen of these molecules had an area under the curve of greater than 0.80. Analysis of the correlation between these 14 *N*-glycans and surgical outcomes by univariate and multivariate analysis identified G2890 (*m/z* value, 2890.052) as a significant recurrence factor and G3560 (*m/z* value, 3560.295) as a significant prognostic factor. G2890 and G3560 were found to be strongly correlated with tumor number, size, and vascular invasion. *Conclusion*: Quantitative glycoblotting based on whole serum *N*-glycan profiling is an effective approach to screening for new biomarkers. The G2890 and G3560 *N*-glycans determined by tumor glycomics appear to be promising biomarkers for malignant behavior in HCCs. (HEPATOLOGY 2013;57:2314-2325)

Hepatocellular carcinoma (HCC) is a common and fatal malignancy with a worldwide occurrence.[1] Liver resection has shown the highest level of control among the local treatments for HCC and is associated with a good survival rate.[2,3] However, the recurrence rates for HCC are still high even when a curative hepatectomy is performed.[4] Many factors associated with the prognosis and recurrence of HCC have now been reported. Vascular invasion of the portal vein and/or hepatic vein and tumor differentiation are important factors affecting survival and recurrence in HCC cases after a hepatectomy.[5,6] However, microvascular invasion and differentiation can only be detected by pathological examination just after a hepatectomy, and cannot be diagnosed preoperatively, and thus cannot be identified preoperatively either. Hence, the serum biomarkers alpha-fetoprotein (AFP) and protein induced by vitamin K absence-II (PIVKA-II) are used as prognostic markers[7,8] and also as surrogate markers for microvascular invasion and tumor differentiation.[9,10] AFP is associated with grade differentiation,[11] whereas PIVKA-II is related to vascular

Abbreviations: AFP, alpha-fetoprotein; AFP-L3, lens culinaris agglutinin-reactive fraction of alpha-fetoprotein; AUC, area under the curve; DFS, disease-free survival; HCC, hepatocellular carcinoma; ICGR15, indocyanin green retention rate at 15 minutes; PIVKA-II, protein induced by vitamin K absence or antagonism factor II; PS, patient survival; RF, risk factor; ROC, receiver operating characteristics.

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Received May 8, 2012; accepted December 19, 2012.

Supported by grants for "Development of Systems and Technology for Advanced Measurement and Analysis (SENTAN)" from the Japan Science and Technology Agency (JST).

invasion.[12,13] However, these tumor markers have limited sensitivity and are less predictive than microvascular invasion,[14,15] which is the most potent determinant of recurrence and survival in HCC patients undergoing a hepatectomy.[5] Therefore, new biomarkers that are more strongly associated with prognosis and recurrence in HCC than AFP or PIVKA-II are highly desirable.

Glycosylation is one of the most common posttranslational protein modifications. Alterations in the *N*-glycosylation profiles of glycoproteins have been suggested to play important roles in the proliferation, differentiation, invasion, and metastasis of malignant cells. Glycan species can be analyzed and characterized using mass spectrometry (MS) and the profiling of these molecules when they are secreted or shed from cancer cells is also performed. Hence, some glycoproteins have been suggested as biomarkers of human carcinomas such as ovarian cancer, breast cancer, and HCC.[16-19] Of note, changes to the N-linked glycan modification of glycoproteins occur during the tumorigenesis and progression of HCC lesions. However, the correlation between the *N*-glycan profile and tumor-associated characteristics such as the degree of malignancy and prognosis has not been previously evaluated in HCC. Recently, we developed a novel glycomics method that facilitates high-throughput and large-scale glycome analysis using an automated glycan purification system, SweetBlot. This approach enables us to profile serum *N*-glycans quantitatively. Using this quantitative *N*-glycomics procedure by way of glycoblotting technology, which is both highly accurate and can be conducted on a large scale, we have previously evaluated the potential of using *N*-glycans as markers of the prognosis and recurrence of HCC.[20]

In our current study we evaluated preoperative blood samples from an HCC patient cohort from which we purified serum *N*-glycans using our glycoblotting method.[21,22] We performed *N*-glycan profiling using MS to search for factors related to prognosis and recurrence by analysis of patient outcomes in 369 consecutive HCC cases that had undergone a primary curative hepatectomy at our medical facility. Through this screen we sought to correlate *N*-glycan levels on glycoproteins with the clinicopathologic characteristics and the outcomes of HCC.

Patients and Methods

Patients. Between April 1999 and March 2011, 369 consecutive adult patients underwent a hepatectomy procedure for HCC at our center and this sample population was examined in the current study. Patients with extrahepatic metastases had been excluded from this cohort because the outcomes of a hepatectomy in these cases are typically very poor. The mean age of the patients in the final study group was 62.7 ± 10.6 years (range, 33-90), 301/369 (81.6%) cases were male, 176 (47.7%) were hepatitis B virus surface antigen-positive, 119 (32.2%) were hepatitis C virus antibody-positive, and 120 (32.5%) were designated as F4 based on the New Inuyama Classification system.[23] The preoperative serum AFP and PIVKA-II levels were simultaneously measured in the patients using standard methods at least 2 weeks before the hepatectomy at the time of the imaging studies. Among the 369 patients in the cohort, 358 (97.0%) were categorized as Child-Pugh class A. According to the TNM stage revised by the Liver Study Group of Japan in 2010,[24] 26 (7.0%) patients were in stage I, 172 (46.6%) in stage II, 111 (30.1%) in stage III, and 60 (16.3%) in stage IVA. The patients were followed up for a median of 60.7 months (range, 9.8-155.1). As a normal control group, 26 living related liver transplantation donors were selected. They were evaluated for eligibility as donors by liver function tests, measurements of the tumor markers AFP and PIVKA-II, and also by x-ray photographs of chest and abdomen and dynamic computed tomography (CT). Their mean age was 40.0 with a range of 20-48. Of 26 controls, 15 (57.7%) were male and 11 (42.3%) were female. All controls were Japanese and not infected by hepatitis B and C virus. This study was approved by the Institutional Review Board of the Hokkaido University, School of Advanced Medicine. Informed consent was obtained from each patient in accordance with the Ethics Committees Guidelines for our institution.

Experimental Procedures: Serum* N-*Glycomics by Way of Glycoblotting. *N*-glycans from serum samples were purified by glycoblotting using BlotGlycoH. These are commercially available synthetic polymer beads with high-density hydrazide groups (Sumitomo Bakelite,

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View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep.26262
Potential conflict of interest: Nothing to report.

Tokyo, Japan). All procedures used the SweetBlot automated glycan purification system containing a 96-well plate platform (System Instruments, Hachioji, Japan).

Enzymatic Degradation of Serum* N-*Glycans. Each 10-μL serum sample aliquot was dissolved in 50 μL of a 106-mM solution of ammonium bicarbonate containing 12 mM 1,4-dithiothreitol and 0.06% 1-propanesulfonic acid, 2-hydroxyl-3-myristamido (Wako Pure Chemical Industries, Osaka, Japan). After incubation at 60°C for 30 minutes, 123 mM iodoacetamide (10 μL) was added to the mixtures followed by incubation in the dark at room temperature to enable reductive alkylation. After 60 minutes, the mixture was treated with 200 U of trypsin (Sigma-Aldrich, St. Louis, MO) at 37°C for 2 hours, followed by heat-inactivation of the enzyme at 90°C for 10 minutes. After cooling to room temperature, the *N*-glycans were released from the tryptic glycopeptides by incubation with 325 U of PNGase F (New England BioLabs, Ipswich, MA) at 37°C for 6 hours.

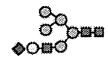
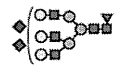
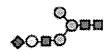
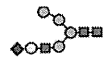
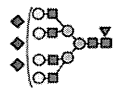
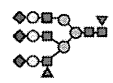
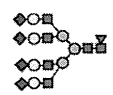
N-*Glycan Purification and Modification by Glycoblotting.* Glycoblotting of sample mixtures containing whole serum *N*-glycans was performed in accordance with previously described procedures. Commercially available BlotGlyco H beads (500 μL) (10 mg/ml suspension; Sumitomo Bakelite) were aliquoted into the wells of a MultiScreen Solvinert hydrophilic PTFE (polytetrafluoroethlene) 96-well filter plate (EMD Millipore, Billerica, MA). After removal of the water using a vacuum pump, 20 μL of PNGase F-digested samples were applied to the wells, followed by the addition of 180 μL of 2% acetic acid in acetonitrile. The filter plate was then incubated at 80°C for 45 minutes to capture the *N*-glycans onto the beads by way of a chemically stable and reversible hydrazone bond. The beads were then washed using 200 μL of 2 M guanidine-HCl in 10 mM ammonium bicarbonate, followed by washing with the same volume of water and of 1% triethyl amine in methanol. Each washing step was performed twice. The *N*-glycan linked beads were next incubated with 10% acetic anhydride in 1% triethyl amine in methanol for 30 minutes at room temperature so that unreacted hydrazide groups would become capped by acetylation. After capping, the reaction solution was removed under a vacuum and the beads were serially washed with 2 $\times$ 200 μL of 10 mM HCl, 1% triethyl amine in methanol, and dioxane. This is a pretreatment for sialic acid modification. On-bead methyl esterification of carboxyl groups in the sialic acids was carried out with 100 μL of 100 mM 3-methyl-1-*P*-tolyltriazene (Tokyo Chemical Industry, Tokyo, Japan) in dioxane at 60°C for 90 minutes to dryness. After methyl esterification of the more stable glycans, the beads were serially washed in 200 μL of dioxane, water, 1% triethyl amine in methanol, and water. The captured glycans were then subjected to a *trans*-iminization reaction with BOA (O-benzylhydroxylamine) (Tokyo Chemical Industry) reagent for 45 minutes at 80°C. After this reaction, 150 μL of water was added to each well, followed by the recovery of derivatized glycans under a vacuum.

Matrix-Assisted Laser Desorption Ionization, Time-of-Flight (MALDI-TOF) and TOF/TOF Analysis. The *N*-glycans purified by glycoblotting were directly diluted with α-cyano-4-hydroxycinnamic acid diethylamine salt (Sigma-Aldrich) as ionic liquid matrices and spotted onto the MALDI target plate. The analytes were then subjected to MALDI-TOF MS analysis using an Ultraflex time-of-flight mass spectrometer III (Brucker Daltonics, Billerica, MA) in reflector, positive ion mode and typically summing 1,000 shots. The *N*-glycan peaks in the MALDI-TOF MS spectra were selected using FlexAnalysis v. 3 (Brucker Daltonics). The intensity of the isotopic peak of each glycan was normalized using 40 μM of internal standard (disialyloctasaccharide, Tokyo Chemical Industry) for each status, and its concentration was calculated from a calibration curve using human serum standards. The glycan structures were estimated using the GlycoMod Tool (http://br.expasy.org/tools/glycomod/), so that our system could quantitatively measure 67 *N*-glycans.

Hepatectomy. Anatomical resection is defined as a resection in which lesion(s) are completely removed on the basis of Couinaud's classification (segmentectomy, sectionectomy, and hemihepatectomy or more) in patients with a tolerable functional reserve. Nonanatomical partial, but complete resection was achieved in all of our cases. R0 resections were performed while the resection surface was found to be histologically free of HCC. The indocyanin green retention rate at 15 minutes was measured in each case to evaluate the liver function reserve, regardless of the presence or absence of cirrhosis.

HCC Recurrence. For the first 2 years after the hepatectomy procedure, the HCC patients in our cohort were monitored every 3 months using liver function tests, measurements of the tumor markers AFP and protein induced by PIVKA-II, and also by ultrasonography and dynamic CT. At 2 years postsurgery, routine CT was performed only once in 4 months. If recurrence was suspected, both CT and magnetic resonance imaging (MRI) were performed and, if necessary, CT during angiography and bone scintigraphy were undertaken.

Table 1. List of the 14 Serum N-Glycans That Were Evaluated to be Specific for Hepatocellular Carcinoma Compared with Normal Controls by Receiver Operating Characteristic (ROC) Analysis

N-glycans	m/z		Specificity (%)	Sensitivity (%)	Cutoff Value	AUC
G2032	2032.724		100	86.45	1.115	0.968
G2890	2890.052		92.31	82.66	0.844	0.91
G1793	1793.672		92.31	75.61	1.963	0.9
G1708	1708.619		88.46	77.51	0.604	0.896
G1870	1870.672		88.46	75.88	2.886	0.873
G1955	1955.724		100	59.89	3.913	0.873
G3195	3195.163		92.31	71.27	6.109	0.864
G3560	3560.295		88.46	71.27	0.091	0.851
G2114	2114.778		88.46	75.88	2.208	0.839
G1809	1809.666		84.62	72.9	0.679	0.838
G3341	3341.221		84.62	69.92	0.086	0.821
G1590	1590.592		80.77	69.92	10.696	0.817
G1362	1362.481		65.38	87.26	1.381	0.813
G3865	3865.407		92.31	56.37	0.121	0.812

The area-under-the-curve (AUC) values of these 14 serum N-glycan were greater than 0.80. These glycan structures are represented with the symbol nomenclature explained in http://www.functionalglycomics.org/static/consortium/Nomenclature.shtml.

This enabled a precise diagnosis of the site, number, size, and invasiveness of any recurrent lesions.

Statistics. The specificity, the sensitivity, cutoff, and AUC (area under the curve) values of selected *N*-glycans are shown in Table 1. This ROC (receiver operating characteristics) analysis was carried out using R v. 2.12.1. The patient survival (PS) and disease-free survival rates (DFS) were determined using the Kaplan-Meier method and compared between groups by the log-rank test. Univariate analysis of variables was also performed, and selected variables using Akaike's Information Criterion (AIC)[25] were analyzed with the Cox proportional hazard model for multivariate analysis. Statistical analyses were performed using