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.

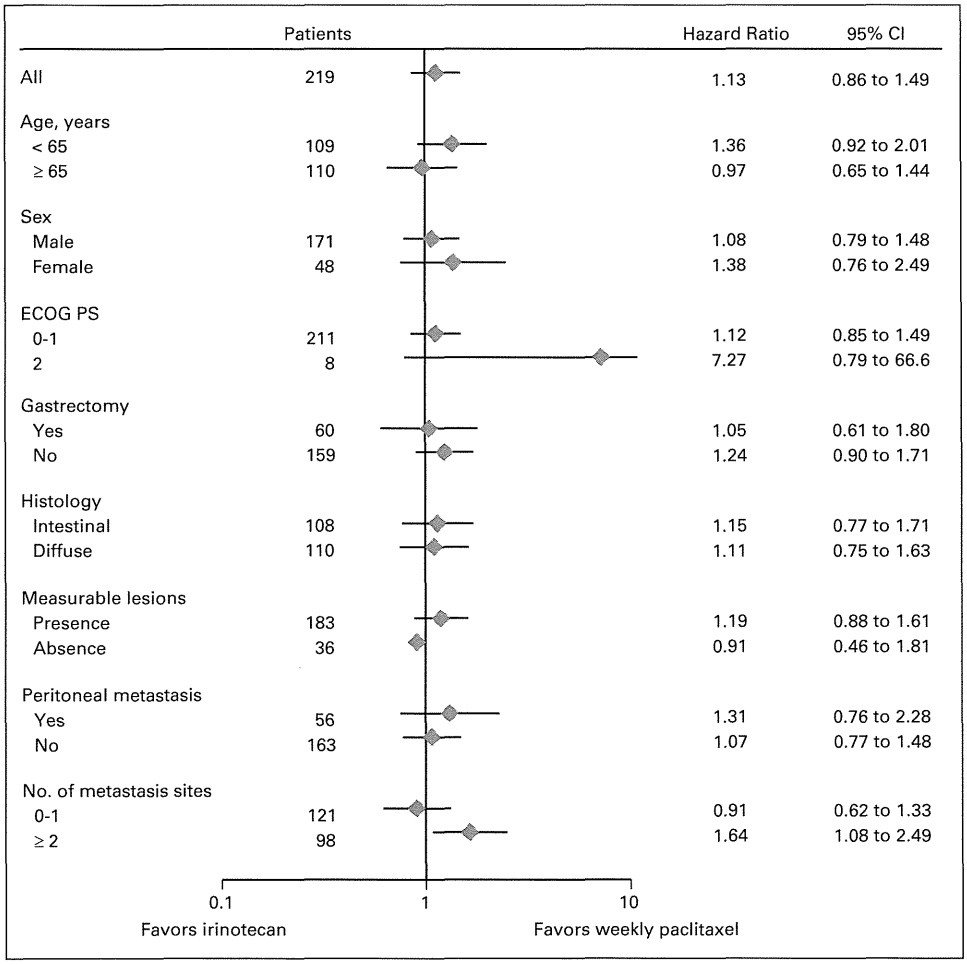


**Fig 3.** Forest plot of subgroup analyses. ECOG PS, Eastern Cooperative Oncology Group performance status.

### 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

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ORIGINAL ARTICLE

# A rare point mutation in the Ras oncogene in hepatocellular carcinoma

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**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 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 (*KRAS*s codon 12 TGT:Cys) was provided by Dr. Sugio (Institution?).

**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

## 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.

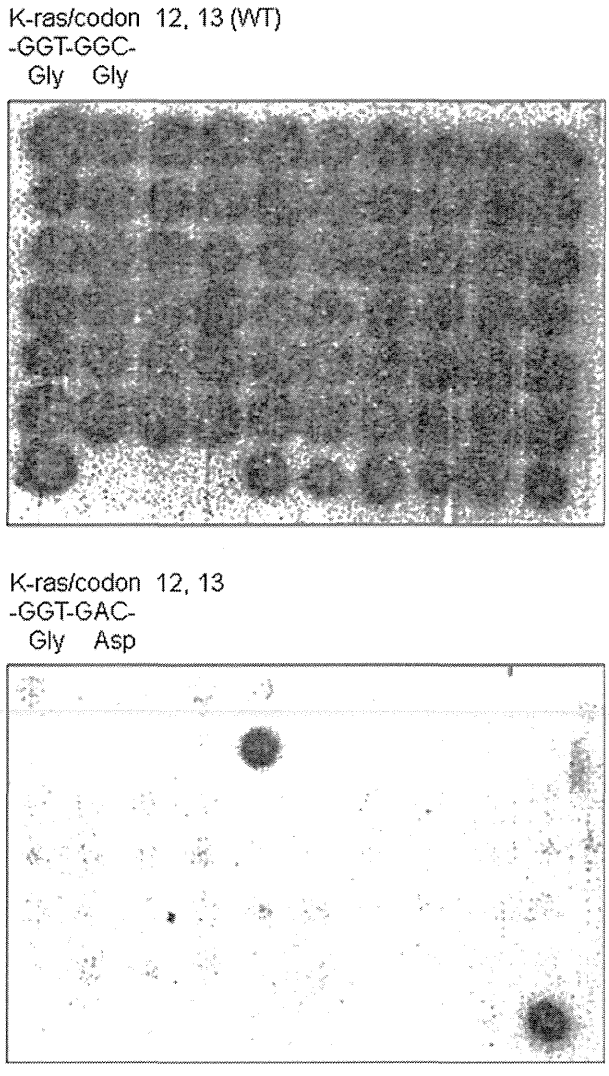


**Fig. 1** Detection of a *KRAS* gene mutation in a patient with hepatocellular carcinoma. PCR-amplified DNA from 61 tumor samples was dotted onto nylon membranes and hybridized to a $^{32}$P-labeled oligonucleotide probe. *WT* wild type *KRAS*

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.

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

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# 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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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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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.

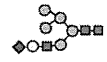
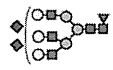
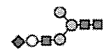
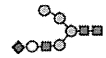
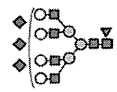
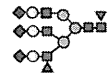
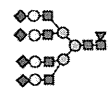
***N-Glycan Purification and Modification by Glycoblotting.*** Glycoblotting of sample mixtures containing whole serum *N*-glycans was performed in accordance with previously described procedures. Commercially available BlotGlyco H beads (500 μL) (10 mg/ml suspension; Sumitomo Bakelite) were aliquoted 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 × 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

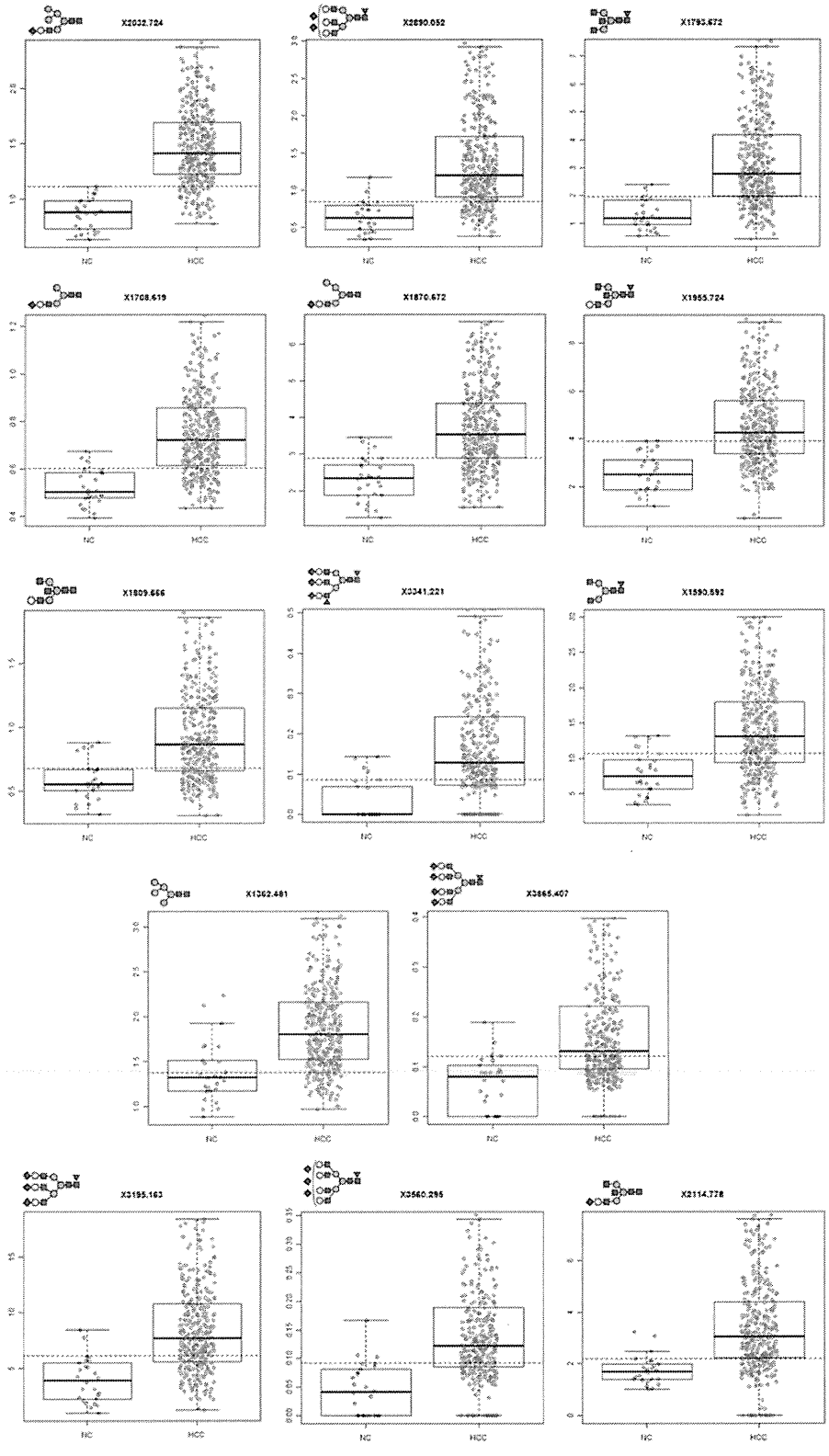



Fig. 1. Boxplots of the disease-free individuals (NC) and HCC patients for the selected 14 *N*-glycans. The dotted lines in the graphs represent the cutoff values determined in this analysis. These graphs were drawn using R v. 2.12.1.

**Table 2. Univariate Analysis of Predictive Values (the Selected 14 *N*-Glycans) of Patient Survival (PS) and Disease-Free Survival (DFS)**

| | | (n) | PS Hazard Ratio | PS *P*-value | DFS Hazard Ratio | DFS *P*-value |
|---|---|---|---|---|---|---|
| G2032 | Low | 206 | 1 | 0.9362 | 1 | 0.1054 |
| | High | 163 | 1.017 | | 1.243 | |
| G2890 | Low | 152 | 1 | <0.0001 | 1 | 0.0001 |
| | High | 217 | 3.044 | | 1.705 | |
| G1793 | Low | 112 | 1 | 0.6829 | 1 | 0.2897 |
| | High | 257 | 1.095 | | 1.168 | |
| G1708 | Low | 145 | 1 | 0.0016 | 1 | 0.0043 |
| | High | 224 | 2.017 | | 1.485 | |
| G1870 | Low | 151 | 1 | 0.5552 | 1 | 0.4008 |
| | High | 218 | 1.132 | | 1.122 | |
| G1955 | Low | 113 | 1 | 0.4213 | 1 | 0.795 |
| | High | 256 | 1.2 | | 1.038 | |
| G3195 | Low | 206 | 1 | <0.0001 | 1 | 0.0001 |
| | High | 163 | 3.238 | | 1.662 | |
| G3560 | Low | 246 | 1 | <0.0001 | 1 | <0.0001 |
| | High | 123 | 4.209 | | 1.74 | |
| G2114 | Low | 275 | 1 | 0.0056 | 1 | 0.1627 |
| | High | 94 | 1.776 | | 1.232 | |
| G1809 | Low | 238 | 1 | 0.0027 | 1 | 0.055 |
| | High | 131 | 1.824 | | 1.306 | |
| G3341 | Low | 188 | 1 | <0.0001 | 1 | 0.0005 |
| | High | 181 | 3.185 | | 1.592 | |
| G1590 | Low | 167 | 1 | 0.0956 | 1 | 0.9102 |
| | High | 202 | 1.413 | | 0.985 | |
| G1362 | Low | 261 | 1 | 0.0399 | 1 | 0.0004 |
| | High | 108 | 1.526 | | 1.634 | |
| G3865 | Low | 192 | 1 | <0.0001 | 1 | 0.0014 |
| | High | 177 | 3.145 | | 1.532 | |

standard tests ($\chi^2$, $t$ test) where appropriate using StatView 5.0 for Windows (SAS Institute, Cary, NC). Significance was defined as $P < 0.05$.

## Results

***Profiling of Human Serum Glycoforms and ROC Analysis in HCC Patients and Normal Controls.*** *N*-glycan profiles of blood samples from our HCC cohort were obtained by MALDI-TOF MS analysis using the high-throughput features of the instrument. We thereby identified 67 *N*-glycans from which we selected molecules that showed statistical differences by ROC analysis between HCC and disease-free individuals (normal controls, NC) comprising living related liver transplantation donors. Glycans with an AUC value greater than 0.80 were selected for analysis (Table 1) and boxplots for these selected molecules (14 in total) are shown in Fig. 1. Clear differences in the distribution of these factors are evident between the NC and HCC patients. The cutoff values were determined using the maximum values for specificity plus sensitivity. G2890 was elevated more than a cutoff value in 305 (82.7%) of HCC patients and G3560 in 261 (70.7%).

***Causes of Death.*** There were 115 deaths in total among our 369 HCC patient cohort (31.2%). The causes of death were as follows: HCC recurrence (n = 97; 84.3%), liver failure (n = 6; 5.2%), and other causes (n = 12; 10.4%).

***Univariate Analysis and Multivariate Analysis of Overall Patient and Disease-Free Survival.*** The overall PS rates at 1, 3, and 5 years in our HCC cohort were 88.8%, 76.4%, and 67.6%, respectively. The DFS values for this groups at 1, 3, and 5 years were 64.0%, 35.5%, and 27.4%, respectively. The 14 serum N-glycans that were highly specific for HCC were evaluated for 3-year recurrence-free survival by ROC analysis to determine the cutoff values about these N-glycans. The patients were divided to two groups by these cutoff values. The PS and DFS measurements associated with the selected 14 selected *N*-glycans were evaluated by univariate analysis. The *P* values for the PS rates associated with G2890, G1708, G3195, G3560, G2114, G1809, G3341, G1362, and G3865 were all less than 0.05. The DFS *P* values for G2890, G1708, G3195, G3560, G3341, G1362, and G3865 were also less than 0.05 (Table 2). When clinical and tumor-associated factors were evaluated by univariate analysis, albumin, Child-Pugh classification,

**Table 3. Univariate Analysis of Predictive Values (Clinical and Tumor Associated Factors) for Patient Survival (PS) and Disease-Free Survival (DFS)**

| | | (n) | PS Hazard Ratio | PS *P*-value | DFS Hazard Ratio | DFS *P*-value |
|---|---|---|---|---|---|---|
| Sex | Male | 301 | 1 | 0.7486 | 1 | 0.6535 |
| | Female | 68 | 0.913 | | 0.943 | |
| Age (years) | <=62 | 160 | 1 | 0.3272 | 1 | 0.6320 |
| | 62< | 209 | 1.211 | | 1.106 | |
| HBV | Positive | 176 | 1.259 | 0.1911 | 1.007 | 0.8093 |
| | Negative | 192 | 1 | | 1 | |
| HCV | Positive | 119 | 1.291 | 0.2433 | 1.008 | 0.8183 |
| | Negative | 250 | 1 | | 1 | |
| Albumin (mg/dL) | <=4.05 | 147 | 2.128 | <0.0001 | 1.626 | 0.0001 |
| | 4.05< | 222 | 1 | | 1 | |
| Total bilirubin (mg/dL) | <=0.82 | 235 | 1 | 0.5831 | 1 | 0.5241 |
| | 0.82< | 134 | 1.122 | | 1.128 | |
| ICGR15 (%) | <=16.7 | 223 | 1 | 0.1223 | 1 | 0.0106 |
| | 16.7< | 146 | 1.349 | | 1.375 | |
| Child-Pugh | A | 358 | 1 | <0.0001 | 1 | 0.0374 |
| | B | 11 | 4.292 | | 2.169 | |
| Anatomical resection | Anatomical | 282 | 1 | 0.8569 | 1 | 0.1435 |
| | Nonanatomical | 87 | 0.949 | | 1.225 | |
| AFP (ng/mL) | <=20 | 183 | 1 | <0.0001 | 1 | 0.0008 |
| | 20<<=1000 | 115 | 2.395 | | 1.449 | |
| | 1000< | 71 | 4.433 | | 1.870 | |
| AFP-L3 (%) | <=15 | 255 | 1 | <0.0001 | 1 | 0.0567 |
| | 15< | 113 | 2.366 | | 1.285 | |
| PIVKA-II (mAU/mL) | <=40 | 109 | 1 | <0.0001 | 1 | 0.0095 |
| | 40<<=1000 | 133 | 1.593 | | 1.240 | |
| | 1000< | 123 | 3.784 | | 1.635 | |
| Number | Single | 235 | 1 | <0.0001 | 1 | <0.0001 |
| | 2,3 | 89 | 3.731 | | 2.252 | |
| | 4<= | 45 | 7.299 | | 3.788 | |
| Size (cm) | <=3 | 116 | 1 | <0.0001 | 1 | 0.0086 |
| | 3<<=5 | 96 | 2.688 | | 1.260 | |
| | 5< | 157 | 4.049 | | 1.570 | |
| Differentiation | Well | 17 | 1 | 0.0003 | 1 | 0.0002 |
| | Moderately | 190 | 2.568 | | 2.990 | |
| | Poorly | 159 | 5.358 | | 4.361 | |
| Vp | Positive | 94 | 4.630 | <0.0001 | 2.156 | <0.0001 |
| | Negative | 275 | 1 | | 1 | |
| Vv | Positive | 35 | 5 | <0.0001 | 1.969 | 0.0004 |
| | Negative | 334 | 1 | | 1 | |
| Macroscopic vascular invasion | Positive | 48 | 6.135 | <0.0001 | 1.961 | <0.0001 |
| | Negative | 321 | 1 | | 1 | |
| Stage | 1 | 26 | 1 | <0.0001 | 1 | <0.0001 |
| | 2 | 172 | 2.844 | | 1.206 | |
| | 3 | 111 | 9.901 | | 2.404 | |
| | 4A | 60 | 15.625 | | 3.106 | |
| Noncancerous liver | Cirrhosis | 120 | 1.199 | 0.3105 | 1.293 | 0.0398 |
| | Noncirrhosis | 249 | 1 | | 1 | |

AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonism factor II; AFP-L3, lens culinaris agglutinin-reactive fraction of alpha-fetoprotein; vp, microscopic tumor thrombus in the portal vein; vv, microscopic tumor thrombus in the hepatic vein; HBV, hepatitis B virus s antigen; HCV, anti-hepatitis C virus antibody; ICGR15, indocyanin green retention rate at 15 minutes.

AFP, AFP-L3 (lens culinaris agglutinin-reactive fraction of alpha-fetoprotein), PIVKA-II, tumor number, tumor size, differentiation, microscopic portal vein invasion, microscopic hepatic vein invasion, macroscopic vascular invasion, and stage were found to be significantly associated with the PS rate. When the same analysis was undertaken for the DFS rate by univariate analysis, albumin, indocyanin green retention rate at 15 minutes, Child-Pugh classification, AFP, PIVKA-II, tumor number, tumor size, differentiation, microscopic portal vein invasion, microscopic hepatic vein invasion, macroscopic vascular invasion, stage, and noncancerous liver were found to be significantly associated with this measure (Table 3).

The variable selection from 19 clinical and tumor-associated factors in Table 3 and the 14 serum

**Table 4. Multivariate Analysis of Values That Is Predictive for Overall HCC Patient Survival**

| | | *P* | Hazard Ratio | 95% Confidence Interval | |
|---|---|---|---|---|---|
| ICGR15 (%) | 16.7< | 0.000209 | 2.435 | 1.5213 | 3.898 |
| Child-Pugh | B | 0.011136 | 3.007 | 1.2852 | 7.037 |
| AFP (ng/mL) | 20<<=1000 | 0.0003 | 2.558 | 1.5372 | 4.256 |
| | 1000< | 0.000217 | 2.782 | 1.6177 | 4.786 |
| Tumor number | 2,3 | 0.011844 | 1.937 | 1.1575 | 3.241 |
| | 4<= | <0.0001 | 2.989 | 1.7693 | 5.049 |
| Size (cm) | 3<<=5 | 0.278625 | 1.483 | 0.7269 | 3.026 |
| | 5< | 0.016071 | 2.237 | 1.1613 | 4.307 |
| Vp | Positive | <0.0001 | 2.982 | 1.8446 | 4.822 |
| C3560 | >0.158 | <0.0001 | 2.52 | 1.6191 | 3.923 |

ICGR15, indocyanin green retention rate at 15 minutes, AFP, alpha-fetoprotein; vp, microscopic tumor thrombus in the portal vein.

**Table 5. Multivariate Analysis of Values That Are Predictive of Disease-Free Survival in HCC Patients**

| | | *P* | Hazard Ratio | 95% Confidence Interval | |
|---|---|---|---|---|---|
| ICGR15 (%) | 16.7< | 0.00334 | 1.519 | 1.149 | 2.008 |
| AFP (ng/mL) | 20<<=1000 | 0.04904 | 1.366 | 1.001 | 1.864 |
| | 1000< | 0.01851 | 1.591 | 1.081 | 2.342 |
| Tumor number | 2,3 | 0.0072 | 1.551 | 1.126 | 2.135 |
| | 4<= | <0.0001 | 2.649 | 1.704 | 4.118 |
| Differentiation | Moderately | 0.01495 | 2.838 | 1.225 | 6.577 |
| | Poor | 0.00501 | 3.398 | 1.446 | 7.984 |
| vp | Positive | 0.01023 | 1.544 | 1.108 | 2.152 |
| C2890 | >1.12 | 0.01125 | 1.443 | 1.087 | 1.915 |

ICGR15, indocyanin green retention rate at 15 minutes, AFP, alpha-fetoprotein; vp, microscopic tumor thrombus in the portal vein.

N-glycans using the AIC was performed and the selected valuables were analyzed with PS and DFS by multivariate analysis. G3560 were found to be independent risk factors for PS (Table 4) and G2890 for DFS (Table 5).

The PS rates of HCC cases with low serum G3560 levels at 5 years were 80.5% and of high serum G3560 at 5 years were 40.4%. The DFS outcomes associated with low and high serum G2890 levels at 5 years were 21.3% and 35.1%, respectively (Fig. 2).

***Relationship Between Clinical and Tumor-Associated Factors in HCC and Specific Glycans.*** Among the low and high G2890 HCC groups, there were significant differences found in a number of clinical and tumor-associated factors including albumin, Child-Pugh classification, AFP, PIVKA-II, tumor number, tumor size, microscopic portal vein invasion, microscopic hepatic vein invasion, macroscopic vascular invasion, and stage (Table 6). In comparing the low and high G3560 HCC patients, significant differences were found in albumin, Child-Pugh Classification, operative procedures, AFP, AFP-L3, PIVKA-II, tumor number, tumor size, differentiation profiles, microscopic portal vein invasion, microscopic hepatic vein invasion, macroscopic vascular invasion, and stage (Table 6).

## Discussion

The *N*-glycan profiles of a large cohort of HCC patients were obtained in our current study by MALDI-TOF MS analysis and 67 of these molecules were thereby quantified. Of this group of factors, 14 *N*-glycans showed higher relative peaks in the HCC patients compared with normal controls and were

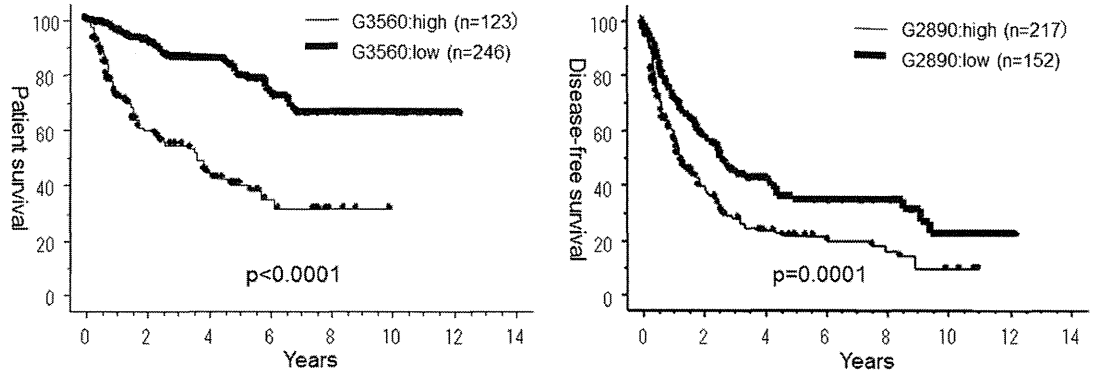



Fig. 2. The PS rates of HCC cases with low and high serum G3560 levels at 5 years were 80.5% and 40.4%, respectively. The DFS outcomes associated with low and high serum G2890 levels at 5 years were 21.3% and 35.1%, respectively.

**Table 6. Correlation Between the G2890 and G3560 *N*-Glycans and Clinical and Tumor Associated Factors in HCC Cases**

| | | G2890 | | | G3560 | | |
|---|---|---|---|---|---|---|---|
| | | High (n=217) | Low (n=152) | *P* | High (n=123) | Low (n=246) | *P* |
| Sex | Male | 184 | 117 | 0.0767 | 105 | 196 | 0.2286 |
| | Female | 33 | 35 | | 18 | 50 | |
| Age | ≦62 | 90 | 70 | 0.4433 | 49 | 111 | 0.393 |
| | >62 | 127 | 82 | | 74 | 135 | |
| HBV | Positive | 107 | 69 | 0.5254 | 59 | 117 | 0.9706 |
| | Negative | 110 | 83 | | 64 | 129 | |
| HCV | Positive | 63 | 56 | 0.1425 | 32 | 87 | 0.0904 |
| | Negative | 154 | 96 | | 91 | 159 | |
| Albumin (mg/dL) | ≦4.05 | 109 | 38 | <0.0001 | 73 | 74 | <0.0001 |
| | >4.05 | 108 | 114 | | 50 | 172 | |
| Total bilirubin (mg/dL) | ≦0.82 | 136 | 99 | 0.7088 | 82 | 153 | 0.4671 |
| | >0.82 | 81 | 53 | | 41 | 93 | |
| ICGR15 (%) | ≦16.7 | 125 | 98 | 0.2224 | 77 | 146 | 0.6246 |
| | >16.7 | 92 | 54 | | 46 | 100 | |
| Child-Pugh | A | 206 | 152 | 0.0034 | 115 | 243 | 0.008 |
| | B | 11 | 0 | | 8 | 3 | |
| Anatomical resection | Anatomical | 172 | 110 | 0.1583 | 106 | 176 | 0.0028 |
| | Nonanatomical | 45 | 42 | | 17 | 70 | |
| AFP (ng/mL) | ≦20 | 102 | 81 | 0.0461 | 52 | 131 | <0.0001 |
| | 20< & ≦1000 | 64 | 51 | | 30 | 85 | |
| | >1000 | 51 | 20 | | 41 | 30 | |
| AFP-L3 (%) | ≦15 | 143 | 112 | 0.1147 | 68 | 187 | <0.0001 |
| | >15 | 74 | 40 | | 55 | 59 | |
| PIVKA II (mAU/mL) | ≦40 | 52 | 58 | 0.0001 | 22 | 88 | <0.0001 |
| | 40< & ≦1000 | 74 | 60 | | 33 | 101 | |
| | >1000 | 91 | 34 | | 68 | 57 | |
| Number | Single | 122 | 113 | 0.0009 | 68 | 167 | <0.0001 |
| | 2, 3 | 60 | 29 | | 27 | 62 | |
| | ≧4 | 35 | 10 | | 28 | 17 | |
| Size (cm) | ≦3 | 48 | 68 | <0.0001 | 15 | 101 | <0.0001 |
| | 3< & ≦5 | 60 | 36 | | 21 | 75 | |
| | >5 | 109 | 48 | | 87 | 70 | |
| Differentiation | Well | 12 | 8 | 0.0981 | 6 | 14 | 0.0003 |
| | Moderately | 102 | 88 | | 46 | 144 | |
| | Poorly | 103 | 56 | | 71 | 88 | |
| vp | Positive | 67 | 27 | 0.0065 | 49 | 45 | <0.0001 |
| | Negative | 150 | 125 | | 74 | 201 | |
| vv | Positive | 29 | 6 | 0.0043 | 24 | 11 | <0.0001 |
| | Negative | 188 | 146 | | 99 | 235 | |
| Macroscopic vascular invasion | Positive | 43 | 5 | <0.0001 | 32 | 16 | <0.0001 |
| | Negative | 174 | 147 | | 91 | 230 | |
| Stage | 1 | 7 | 19 | <0.0001 | 3 | 23 | <0.0001 |
| | 2 | 88 | 84 | 45 | 127 | | |
| | 3 | 71 | 40 | 35 | 76 | | |
| | 4A | 51 | 9 | 40 | 20 | | |
| Noncancerous liver | Cirrhosis | 71 | 49 | 0.9876 | 35 | 85 | 0.2888 |
| | Noncirrhosis | 146 | 103 | | 88 | 161 | |

AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonism factor II; AFP-L3, lens culinaris agglutinin-reactive fraction of alpha-fetoprotein; vp, microscopic tumor thrombus in the portal vein; vv, microscopic tumor thrombus in the hepatic vein; HBV, hepatitis B virus s antigen; HCV, anti-hepatitis C virus antibody; ICGR15, indocyanin green retention rate at 15 minutes.

chosen for further analysis. These selected molecules were assessed for any correlation with surgical outcomes in the HCC cohort (i.e., prognosis and recurrence) by univariate and multivariate analysis. G3560 *N*-glycan was found to be a significant prognostic factor and G2890 *N*-glycan was found to be a significant recurrence factor for this disease. Moreover, G2890 and G3560 were found to strongly correlate with a number of well-known tumor-related prognostic and recurrent factors. These results show that quantitative glycoblotting based on whole serum *N*-glycan profiling is a potent screening approach for novel HCC biomarkers, and that the G3560 and G2890 *N*-glycans are promising biomarkers of the PS, DFS, and malignant behavior characteristics of HCC after hepatectomy.

Although glycans, once released from glycoproteins or glycopeptides, have been subjected to fluorescent labeling and purification for detection by high-performance liquid chromatography (HPLC) previously, this method is time-consuming and therefore not suited to clinical diagnosis. Our novel analytical method, which we refer to as glycoblotting, is far more rapid and accurate, as evidenced by the number of *N*-glycans detected in our current analysis. This chemoselective glycan enrichment technology known as glycoblotting was developed in our laboratory to purify oligosaccharides derived from glycoproteins in an effective and quantitative manner, thus enabling serum glycan profiling by way of a simpler method.[20] Our method is also applicable to the fully automated analysis of multiple samples simultaneously. It readily combines the isolation and labeling of oligosaccharides, which can then be subjected to conventional analytical methods including MS. We had already achieved high-speed quantitative and qualitative profiling of glycan expression patterns in biological materials using this technology. In our present study, we improved the method to allow quantitative analysis of high reproducibility and accuracy using a calibration curve of human serum standards. The analysis of the obtained 67 glycan profiles was performed using this new developed technology. The effectiveness of our method is evidenced by the identification of the G2890 and G3560 *N*-glycans as highly promising clinical markers of HCC associated with the PS, DFS, and tumor malignancy rates of these cancers.

It has been reported that AFP is the most significant tumor marker and independent predictor of prognosis for HCC,[26] even in patients who have received a hepatectomy.[27] Although high levels of AFP in cases of fully developed HCC, or in the serum of the host, are known to be associated with more aggressive behavior, and increased anaplasis,[28] AFP can also cause apoptosis in tumor cells.[29] Moreover, it has been suggested that AFP regulates the immune response and induces either stimulatory or inhibitory growth activity.[30] On the other hand, it is well known that AFP may increase in some patients with acute and chronic hepatitis without HCC,[31,32] and that the elevation of AFP correlates with inflammation of background disease and hepatocyte regeneration.[33] Hence, because the AFP profile does not always directly reflect the extent of tumor malignancy, the AFP levels do not influence patient survival and recurrence. On the other hand, AFP and many important tumor markers, such as carcinoembryonic antigen, carbohydrate antigen 125, and carbohydrate antigen 19-9, are glycoproteins, and this means that the glycan profiles in serum are altered by the onset of cancer. Indeed, the profiling of serum glycans has been performed previously as a screen for distinct potential glycan biomarkers of ovarian cancer and breast cancer.[18,19] Hence, we surmised that highly specific glycoprotein markers of HCC should be detected by monitoring the serum glycosylation profile in these patients. In glycan structure, both G2890 and G3560 are multiply branched (G2890 is tri-antennary and G3560 is tetra-antennary) glycans with a core fucose. In addition, both glycans have one nonsialylated branch, i.e., G2890 and G3560, are tri-antennary disialylated glycan, and tetra-antennary tri-sialylated glycan, respectively. The structure of G2890 and G3560 is quite different from the AFC-L3 (core fucosylated bi-antennary glycan) and CA19-9 (sialylated Lewis (a) antigen), which are well-known biomarkers related to HCC except for the core fucosylation.

There have been several previous studies of glycans in HCC. Kudo et al.[34] reported that *N*-glycan alterations are associated with drug resistance in HCC *in vitro*. In other reported clinical studies, only specific glycans have been assessed in relation to HCC. Vanhooren et al.[17] were the first to analyze the function of HCC-specific glycans, and reported that a triantennary glycan (NA-3Fb) correlated with the tumor stage and AFP levels in HCC patients. However, that study analyzed 44 patients with HCC but did not evaluate the relationship between the *N*-glycans and the clinical and pathological factors of this disease, the clinical course after hepatectomy, or prognosis and recurrence. In our current study, in contrast, we analyzed a far larger cohort than any other previous report, and evaluated a comprehensive panel of clinical and pathological parameters in relation to the *N*-glycan profile in HCC. Tang et al.[35] also described some HCC-specific glycans in their previous study that we did not find to be significant in our current analyses. This is likely due to the fact that the patient number in their study was smaller than ours, and the fact that the *N*-glycome profile in serum is gender- and age-dependent.[36] In this study, the mean age and the distribution of gender and infection of hepatitis B and C virus were the difference between NC and HCC patients. However, the selected 14 serum *N*-glycans were quantified by our MALDI-TOF MS analysis and compared with NC by ROC analysis. These were statistically different between HCC and NC with respect to the quantity. Because these 14 serum *N*-glycans of which the AUC values were greater than 0.80 were revealed to be specific for HCC, they had a high discriminating ability to differentiate HCC from NC. Further analyses are

required to determine whether G2890 and G3560 are elevated in patients with hepatitis B, hepatitis C, and/or cirrhosis without HCC.

The most important adverse prognostic factor for liver resection and transplantation in HCC has been found to be microscopic venous invasion.[5] However, microscopic portal invasion is not diagnosed preoperatively, and is revealed only by pathological examination. New biomarkers that are more strongly associated with prognosis and recurrence of HCC than AFP, AFP-L3, or PIVKA-II are therefore highly desirable. Our current data show that the *N*-glycans G2890 and G3560 correlate closely with well-known tumor-related prognostic and recurrent factors such as tumor number, size, microscopic portal vein invasion, microscopic hepatic vein invasion, differentiation, macroscopic vascular invasion, stage, AFP, AFP-L3, and PIVKA-II (Table 6). Moreover, when G2890 and G3560 were simultaneously included in multivariate analysis for PS and DFS with AFP, AFPL3 and PIVKA-II, *P*-values of G2890 and G3560 were lower than AFP, and AFPL3, and PIVKA-II were not selected as valuables by AIC. We demonstrate that these are novel independent prognostic factors for HCC that are related to the survival and recurrence of this disease and that show a lower *P*-value than other established tumor factors. Hence, we predict that G2890 and G3560 will prove to be markers that can preoperatively predict HCC tumor malignancy including microscopic portal vein invasion, and the PS and DFS rates more accurately and with more potency than the more well-known biomarkers.

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