Table IV. Characteristics and survival time according to HER2 status and trastuzumab therapy after brain metastases.

Characteristics	Positive HER2 with trastuzumab	Positive HER2 without trastuzumab	Negative HER2
Patient no.	12	15	35
Median age at first brain metastasis, years (range)	47 (35-67)	54 (39-64)	53 (35-79)
RPA class 1/2/3	0/10/2	4/9/2	10/21/4
ER-brain metastases +/-	0/12	4/11	6/29
PR-brain metastases +/-	1/11	2/13	4/31
Any 2nd brain metastases including LMM	8 (67%)	7 (47%)	21 (60%)
(within 6 months after surgery)	3 (25%)	5 (33%)	8 (23%)
(within 12 months after surgery)	6 (50%)	7 (47%)	17 (49%)
LMM	0 (0%)	4 (27%)	6 (17%)
(within 6 months after surgery)	0 (0%)	4 (27%)	4 (11%)
2nd brain metastatic-free survival time, years (95% CI)	0.6 (0.2-3.3)	0.5 (0.1-0.6)	0.7 (0.5-0.9)
Survival time after brain metastases, years (95% CI)	3.2 (0.4-3.9)	0.7 (0.3-1.1)	1.3 (0.9-1.8)

HER2, human epidermal growth factor receptor type 2; RPA, recursive partitioning analysis; ER, estrogen receptor; PR, progesterone receptor; LMM, leptomeningeal metastases; CI, confidence interval.

(P=0.0005) (Fig. 2A and B, Table IV). Patient characteristics, including age at brain metastasis and proportion of RPA classes, were not different in the HER2-positive group with or without trastuzumab (Table IV). The ER, PR and HER2 status of brain metastatic lesions exhibited no correlation with survival time after brain metastases. There was no difference in survival time after brain metastases between TNBC and HER2-positive patients.

Trastuzumab therapy and postoperative LMM. The incidence of LMM after surgery for brain metastases was 27% (4/15) in patients with HER2-positive central nervous system (CNS) samples who did not receive trastuzumab and 17% (6/35) in patients with HER2-negative CNS samples. All the patients with LMM exhibited neuronal death. Of five patients with recurrent brain metastases who did not receive trastuzumab, four patients (80%) developed LMM within 6 months after surgery for brain metastases. However, none of the 12 patients who were administered trastuzumab after surgery presented with LMM. Thus, trastuzumab therapy after brain metastases decreased the incidence of postoperative LMM (Chi-square test, P=0.053).

Discussion

Breast cancer is the second most common cause of brain metastases. Brain metastases occur in 14-20% of breast cancer patients and usually occur late in the progression of metastatic disease (16). The results of the present study have demonstrated that, among patients with HER2-positive brain metastases, those who received trastuzumab had longer survival times following surgery for brain metastases, compared to those without trastuzumab treatment.

The prolongation of the OS time in HER2-positive breast cancer patients with trastuzumab may be attributed to the decrease in LMM and neuronal death and the effects of trastuzumab on the control of systemic metastases (17-21). A positive HER2 status in primary breast cancer was considered a risk factor for the development of brain metastases (22). Although trastuzumab does not cross the blood-brain barrier (BBB) and has no direct activity on brain metastases, previous studies reported a survival benefit with trastuzumab in HER2-positive patients with brain metastases, who had a significantly longer survival compared to that of HER2-negative patients (17-21), which was consistent with our findings. Trastuzumab therapy prior to brain metastases did not correlate with the first brain metastatic-free survival time in our study, since trastuzumab therapy was not associated with an increased incidence of brain metastases (23,24). The patients with HER2-positive brain metastases who received trastuzumab exhibited longer OS after brain metastases compared to the patients with HER2-positive brain metastases without trastuzumab treatment in our study. The development of LMM was a rare manifestation encountered in 5-8.1% of patients with HER2-positive primary tumors (17,20). None of the patients who received trastuzumab therapy after surgery for brain metastases presented with LMM in our study. Of note, radiotherapy with doses of 20-30 Gy with a fraction size of 2 Gy was reported to increase the permeability of the BBB and may enhance the effect of chemotherapy (25). Thus, surgery followed by WBRT may disrupt the BBB and facilitate the delivery of trastuzumab to the brain in HER2-positive patients.

Hormone receptor and HER2 status are important predictive markers in breast cancer. ER negativity was associated with an increased risk of brain metastases (26-28) and HER2

amplification/overexpression was shown to be a prognostic and predictive factor for the development of brain metastases (29). Approximately two-thirds of early breast cancer patients were found to be ER-positive (30) and the HER2-positivity rate in early breast cancer was reported to be ~15% by a previous study (31). In our study, the ER and HER2-positivity rate in primary tumors was lower (11.7%) and higher (41.4%), respectively, compared to those reported by previous studies on primary breast tumors.

Several previous studies demonstrated that the discordance in biomarker expression between primary tumors and metastases and the alteration of hormone receptor and HER2 status is affected by adjuvant chemotherapy associated with hormone therapy and may affect the prognosis (1-5,32,33). The alterations were possibly attributed to a genetic drift or clonal selection during tumor progression (7) or to the presence of intratumoral heterogeneity in ER, PR and HER2 status (8,9). In previous studies that investigated primary breast cancer and distant metastases, the locoregional recurrence or lymph node metastasis, including brain metastasis, exhibited discordance rates of 0-37.5% (34-40). In our study, the immunohistochemical profiles for ER, PR and HER2 differed between the primary tumors and the brain metastases in 29.3% of the patients. Prior hormone therapy or chemotherapy exerted an effect on this discordance phenomenon (1-5,32,33). All the patients with ER or PR alterations (positive-negative and negative-positive) had received hormone therapy prior to the development of brain metastases. Negative-positive alterations were observed in 15.1% of ER-negative, 7.5% of PR-negative and 11.8% of HER2-negative primary tumors in our study. The HER2 status was highly maintained and the concordance rate between primary tumors and systemic metastases was shown to be 97% by a previous study (41). We demonstrated that 89% of HER2-positive patients treated with trastuzumab prior to the development of brain metastases maintained a positive status in brain metastases. The possibility of the discordance rate in HER2 expression between primary and metastatic tumors was less frequent compared to ER or PR (10,11,34-40,42); however, the possibility of intratumoral heterogeneity must be considered, with re-evaluation of the HER2 status in brain metastases for further systemic treatment after brain metastases. New HER2-targeted drugs, such as lapatinib, are able to cross the BBB and thereby control brain metastases and other systemic breast cancer metastases more effectively (43).

In conclusion, 11.8% of HER2-negative patients with primary breast cancers had positive HER2 status alterations in the brain metastases. Patients treated with trastuzumab after surgery for brain metastases and radiotherapy exhibited a better prognosis. Thus, the HER2 status in brain metastases requires re-evaluation and the administration of trastuzumab or lapatinib in HER2-positive patients should be considered even after brain metastases.

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ORIGINAL ARTICLE

RASSF1A methylation indicates a poor prognosis in hepatoblastoma patients

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Abstract
Purpose The RAS association domain family protein 1 (RASSF1A) is known to be frequently inactivated by promoter hypermethylation in cancers. This study investigated the association of *RASSF1A* methylation with clinical outcomes in hepatoblastoma patients and whether it is correlated with the histological phenotype of hepatoblastoma tumors.
Methods Seventy-four hepatoblastoma tumors were obtained from patients enrolled in the Japanese study group for pediatric liver tumor protocol-2. From nine formalin-fixed, paraffin-embedded specimens, we extracted DNA by dissection under a light microscope. We examined the methylation status of the *RASSF1A* promoter region by bisulfite pyrosequencing.
Results Twenty-five (33.8 %) hepatoblastoma tumors were classified as having methylated *RASSF1A*. The *RASSF1A* methylation was significantly associated with metastatic tumors and a poor prognosis. Despite the complete resection, five pretreatment extent of disease II tumors showed recurrence or distant metastasis postoperatively. Among these cases, four tumors were found to show *RASSF1A* methylation. When compared to histologically different types of cell, *RASSF1A* methylation values in samples of the normal liver, fetal type, and embryonal type, were significantly elevated in ascending order.
Conclusions We confirmed that *RASSF1A* methylation is a significant prognostic indicator in hepatoblastomas, and it may become a promising molecular marker to stratify patients into appropriate risk groups.

Keywords Hepatoblastoma · *RASSF1A* methylation · Prognostic marker

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Introduction

Hepatoblastoma is the most common malignant neoplasm of the liver in children. Despite the progress of therapy, the mortality rate remains at 35–50 % in high-risk patients, such as those with extrahepatic tumors, macroscopic invasion of large vessels, or distant or lymph node metastases [1]. Complete surgical resection or liver transplantation and mainstream treatment with cytotoxic drugs are essential for achieving a favorable long-term outcome. To

improve the mortality of hepatoblastoma patients in advanced stages, innovative treatment and potent prognostic markers for better therapy planning are needed.

Histologically, hepatoblastoma tumors are classified as wholly epithelial, or mixed epithelial and mesenchymal types. In the wholly epithelial type, there are two major subtypes, the fetal subtype and the mixed fetal and embryonal subtype [2]. Fetal and embryonal components often develop in combination, that is, heterogeneity is present. The RAS association domain family protein 1 (RASSF1A) is known to be frequently inactivated by promoter hypermethylation in many adult and childhood cancers [3]. We previously reported that *RASSF1A* methylation was correlated with a poor outcome by multivariate analysis, and suggested that *RASSF1A* may be a promising molecular–genetic marker predicting the treatment outcome in hepatoblastoma patients [4]. The association between the histological type and *RASSF1A* methylation is ambiguous despite the fact that the histologic features are associated with different prognoses; a pure fetal histology is favorable and small cell undifferentiated and macrotrabecular histologies are unfavorable [1]. Therefore, the current study was undertaken to determine the association with histological types by examining each type of hepatoblastoma cell dissected separately.

In this study, we investigated the methylation status of *RASSF1A* in hepatoblastoma tumors by bisulfite pyrosequencing, which is a rapid and accurate method to quantify DNA methylation. We analyzed the results with regard to patients' clinicopathological characteristics and prognosis, and evaluated its association with the histological phenotype on the basis of the epigenetic alteration of hepatoblastomas.

Methods

Patients and samples

Seventy-four hepatoblastoma patients with a median age of 18 months underwent tumor resection and partial hepatectomy between December 1999 and December 2008 at the institutions of the Japanese Study Group for Pediatric liver Tumors (JPLT). All patients were treated in the JPLT-2 study [5]. The extent of disease was determined at the time of initial biopsy or resection according to the classification of the pretreatment extent of disease (PRETEXT) staging system [6]. Metastatic tumors were found in 15 % of the patients (Table 1). The 5-year overall survival and event-free survival rates were 86.7 and 73.4 % for the 74 patients, respectively.

The DNA samples of the 74 hepatoblastoma tumors were supplied by JPLT, and they were extracted from fresh-frozen specimens. Furthermore, formalin-fixed, paraffin-embedded (FFPE) specimens were obtained from nine patients referred to our institution for surgical treatment between 1995 and 2011. We extracted DNA from different types of cell: fetal type, embryonal type, and normal liver, by dissection under a light microscope in order to avoid contamination with normal tissues and mesenchymal components. The ethics committee of our institution approved the study protocol, and signed

Table 1 Clinicopathological factors and *RASSF1A* methylation status in 74 patients with hepatoblastoma

Clinicopathological factors		No. of patients	*RASSF1A*		p value[1]
			Methylated	Unmethylated	
Sex	Male	45	14	31	0.360
	Female	29	11	18	
Age at diagnosis	<365 days	22	0	22	0.000064
	≥365 days	52	25	27	
PRETEXT	I	5	1	4	0.319
	II	27	7	20	
	III	29	10	19	
	IV	13	7	6	
Metastasis	No	63	15	48	0.000039
	Yes	11	10	1	
Histological type	Fetal	28	9	19	0.508
	Mixed fetal and embryonal	40	14	26	
	Unknown	6			
Outcome	Alive	63	15	48	0.000039
	Dead	11	10	1	

[1] Fisher's exact test

informed consent was obtained in all cases by local physicians of the participating institutions.

Evaluation of *RASSF1A* methylation level

We examined the methylation status of the *RASSF1A* promoter region by bisulfite pyrosequencing, which can calculate the level of methylation at each CpG site in samples after bisulfite treatment. Genomic DNA (500 ng) was modified with sodium bisulfite using an EpiTect bisulfite kit (Qiagen, Netherlands). Bisulfite pyrosequencing was carried out as described previously [7]. After PCR, the biotinylated PCR product was purified, made single-stranded, and used as a template in the pyrosequencing reaction. Briefly, the PCR products were bound to streptavidin sepharose beads HP (Amersham Biosciences, USA), after which beads containing the immobilized PCR product were purified, washed, and denatured using a 0.2 mol/L NaOH solution. After the addition of 0.3 μmol/L sequencing primer to the purified PCR product, pyrosequencing was carried out using a PSQ96MA system (Biotaga) and Pyro Q-CpG software (Biotage). The mean value of the methylation levels at two CpG sites in the *RASSF1A* promoter region was calculated. Primer sequences used in this study were as follows: forward, GAAGGAGGGAAGGAAGGGTAAG; reverse, GCCTCC CCCAAAATCCAA; sequencing primer, TTGTATTTAG GTTTTTATTG.

Statistical analysis

Correlations between the *RASSF1A* methylation status and clinicopathological factors were analyzed using the Fisher's exact test. Survival curves were constructed according to the methods of Kaplan and Meier, and comparisons of survival curves were performed with a log-rank test. One-way ANOVA followed by Student's *t* test with Bonferroni correction was used to compare methylation values of histologically different types of cell. A P value <0.05 was considered statistically significant.

Results

RASSF1A methylation status in 74 hepatoblastomas

The average of the *RASSF1A* methylation values in 74 hepatoblastoma tumors was 25.8 % (2.0–74.8 %). We performed the ROC analysis to determine the cutoff value of the *RASSF1A* methylation and adopted a cutoff value of 36.2 % in this study (Fig. 1). On the basis of this cutoff value, 25 (33.8 %) tumors were classified as having methylated *RASSF1A*, and the sensitivity and specificity for

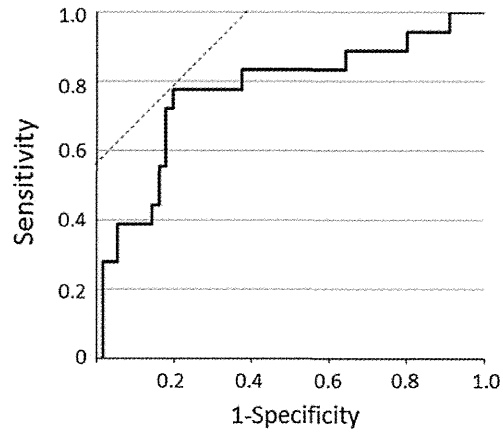

Fig. 1 ROC analysis to determine the cutoff value of the *RASSF1A* methylation

the patients having an event postoperatively were 77.8 and 80.4 %, respectively. There was only one patient who died in those with tumors with *RASSF1A* unmethylated (Fig. 2).

Associations between clinicopathological factors and *RASSF1A* methylation status

We evaluated the associations between the clinicopathological factors and *RASSF1A* methylation status in 74 patients. As Table 1 shows, there were no patients aged under 1 year who had a tumor with *RASSF1A* methylated; however, about half of the patients aged over 1 year were found to have a tumor with *RASSF1A* methylated. 10 of 25 patients (40 %) with a tumor with *RASSF1A* methylated suffered from metastatic tumors, although there was only one patient with metastasis in those with a tumor with *RASSF1A* unmethylated. This demonstrated that age at diagnosis and metastatic tumors were significantly associated with *RASSF1A* methylation. In Kaplan–Meier analyses, the patients with a tumor with methylated *RASSF1A* were significantly associated with a poor outcome: the 5-year overall survival and event-free survival rates were 63.6 and 35.5 %, respectively (Fig. 3).

The *RASSF1A* methylation was detected in 1 of 5 PRETEXT I tumors and 7 of 27 PRETEXT II tumors (Table 1). Despite complete resection, five PRETEXT II tumors showed recurrence or distant metastasis postoperatively, and three patients died. Among these cases, four tumors were found to have *RASSF1A* methylated.

Associations between histological types and *RASSF1A* methylation status

Next, we evaluated whether *RASSF1A* methylation is associated with the histopathological subtypes. Four of the

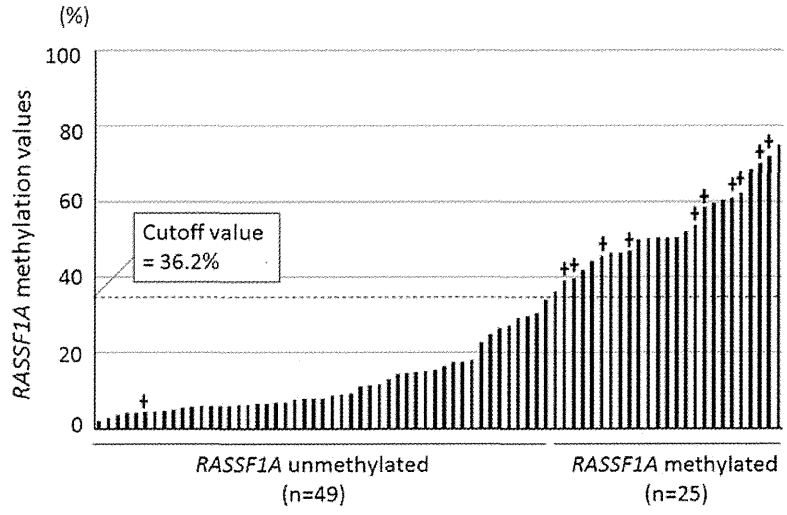

Fig. 2 *RASSF1A* methylation values in 74 patients with hepatoblastoma. *Plus* indicates the patient who died of the disease

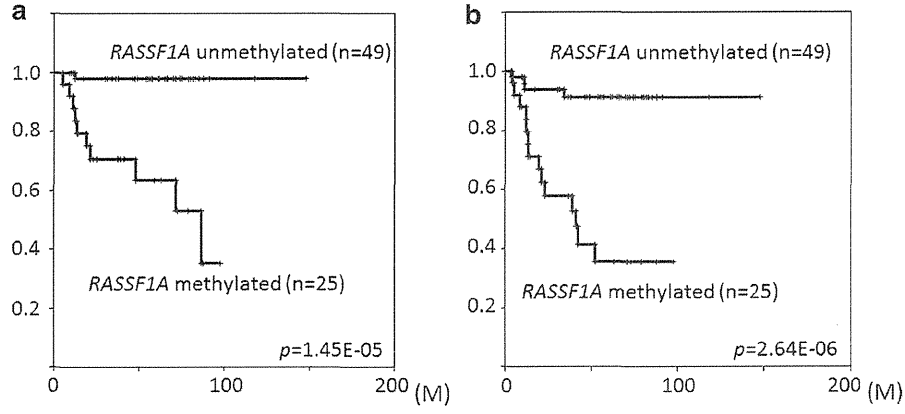

Fig. 3 **a** Overall survival curves and **b** event-free survival curves for hepatoblastoma patients classified by the methylation status of *RASSF1A*

nine tumors in FFPE specimens were classified pathologically into the mixed fetal and embryonal subtype, and DNA was extracted from the tumor cells of each subtype and the normal liver. The other five tumors were the pure fetal subtype, so DNA was extracted from fetal hepatoblastoma and normal liver cells.

The mean methylation values of *RASSF1A* in nine normal liver, nine fetal type, and four embryonal type samples were 8.6, 19.7, and 42.2 %, respectively (Fig. 4). This showed that fetal and embryonal types were significantly associated with *RASSF1A* methylation. Moreover, *RASSF1A* methylation values in samples of the normal liver, fetal type, and embryonal type were elevated in ascending order, when compared to each type of cell taken from the same patient.

Discussion

Complete surgical resection and chemotherapy including cisplatin remains the mainstay of hepatoblastoma treatment. In contrast to standard-risk patients, of who over 90 % achieve long-term survival, the treatment of patients with unrespectable and metastatic disease remains a challenge. Furthermore, there seems to exist a group of patients with high-risk tumors in PRETEXT II, which have a poorer prognosis despite the high-level resectability [5]. First, this study demonstrated that *RASSF1A* methylation was significantly associated with metastatic tumors and a poor prognosis, and that *RASSF1A* methylation may be useful to identify high-risk tumors in PRETEXT II. Secondly, *RASSF1A* methylation was also shown to be histologically correlated with different types of tumor. These findings suggest that *RASSF1A* may be a promising molecular marker to stratify the patients into appropriate risk groups in order to develop better therapeutic approaches.

The present factors predicting the outcome in hepatoblastoma patients include the age at diagnosis, histology, local growth pattern of the tumor, presence of metastasis, and the level of alpha–feto protein [1]. Chromosomal gains of 2q, 8q, and 20, high-level expression of *TERT* or *PLK1*, *CTNNB1* mutation, and *RASSF1A* methylation were shown

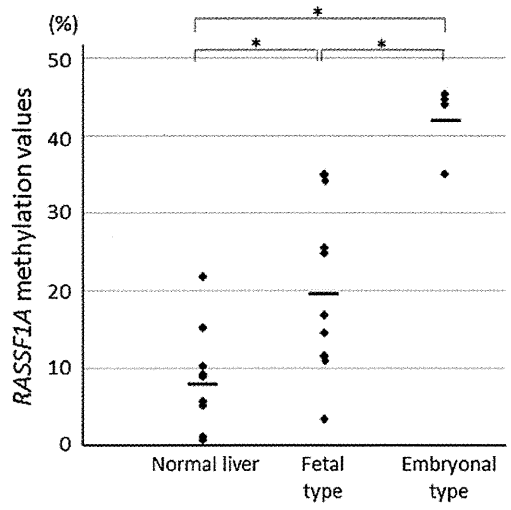

Fig. 4 *RASSF1A* methylation value for each sample is plotted by histological type. *Horizontal bars* indicate the mean value of each type. The *p* values were calculated by one-way ANOVA followed by Student's *t* test with Bonferroni correction ($*p < 0.017$)

to be molecular–genetic markers predicting a poor outcome [4, 8, 9]. We have been focusing on *RASSF1A* methylation in hepatoblastomas, since it has been proven to be an independent prognostic factor by multivariate analysis [4]. RASSF1A inhibits tumor formation by apoptosis, and regulates microtubule dynamics and mitotic arrest via multiple effectors. By dysregulation of the Ras-signaling pathway, *RASSF1A* methylation is correlated with poor differentiation and vascular invasion of cancer cells, and an unfavorable outcome [10]. In child cancers, *RASSF1A* methylation was shown to be associated with a poor outcome in neuroblastoma and Wilms tumor [11, 12]. In this study, we newly adopted bisulfite pyrosequencing as a tool for methylation analysis because it is a highly effective and practical method and offers higher throughput compared to quantitative methylation-specific PCR used in the previous study [4]. We believe that bisulfite pyrosequencing can be a reliable tool when used in a clinical setting.

Cairo et al. [13] identified a 16-gene signature discriminating tumors with a fairly well-differentiated histology and a favorable prognosis against advanced and poorly differentiated tumors with a dismal outcome. In this study, *RASSF1A* methylation was also shown to be correlated with different types of histological phenotype by examining FFPE samples dissected separately. As shown in Table 1, there was no apparent difference in *RASSF1A* methylation values between the fetal subtype and the mixed fetal and embryonal subtype, probably because contamination with different types of tumor cell, normal tissues, and mesenchymal components could not be avoided using fresh-frozen specimens. With these gene signatures based on different phenotypes, the molecular classification of hepatoblastoma tumors may become possible after thorough clinical testing. Although the number of cases in this study is too small to draw definite conclusions, we expect that these molecular markers can be used as prognostic markers predicting the treatment outcome when larger clinical trials are carried out.

In conclusion, *RASSF1A* methylation was significantly associated with metastatic tumors and a poor prognosis in hepatoblastoma patients, and it may be especially useful to identify high-risk tumors in PRETEXT II. The *RASSF1A* methylation was also shown to be correlated with different histological phenotypes. We hope that this work will contribute to establishing a useful molecular marker to predict the outcome of hepatoblastoma patients, stratify the patients efficiently, and develop better therapeutic strategies.

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Conflict of interest The authors who have taken part in this study declare that they do not have anything to disclose regarding funding or any conflict of interest with respect to this manuscript. The first and the corresponding authors are JSPS members, and this abstract was selected for presentation at the 50th Annual Meeting of the Japanese Society of Pediatric Surgeons.

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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.

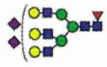
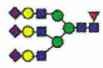
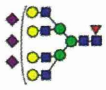
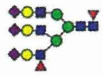
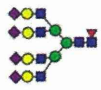
N-*Glycan Purification and Modification by Glycoblotting.* Glycoblotting of sample mixtures containing whole serum *N*-glycans was performed in accordance with previously described procedures. Commercially available BlotGlyco H beads (500 μL) (10 mg/ml suspension; Sumitomo Bakelite) were aliquoted into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

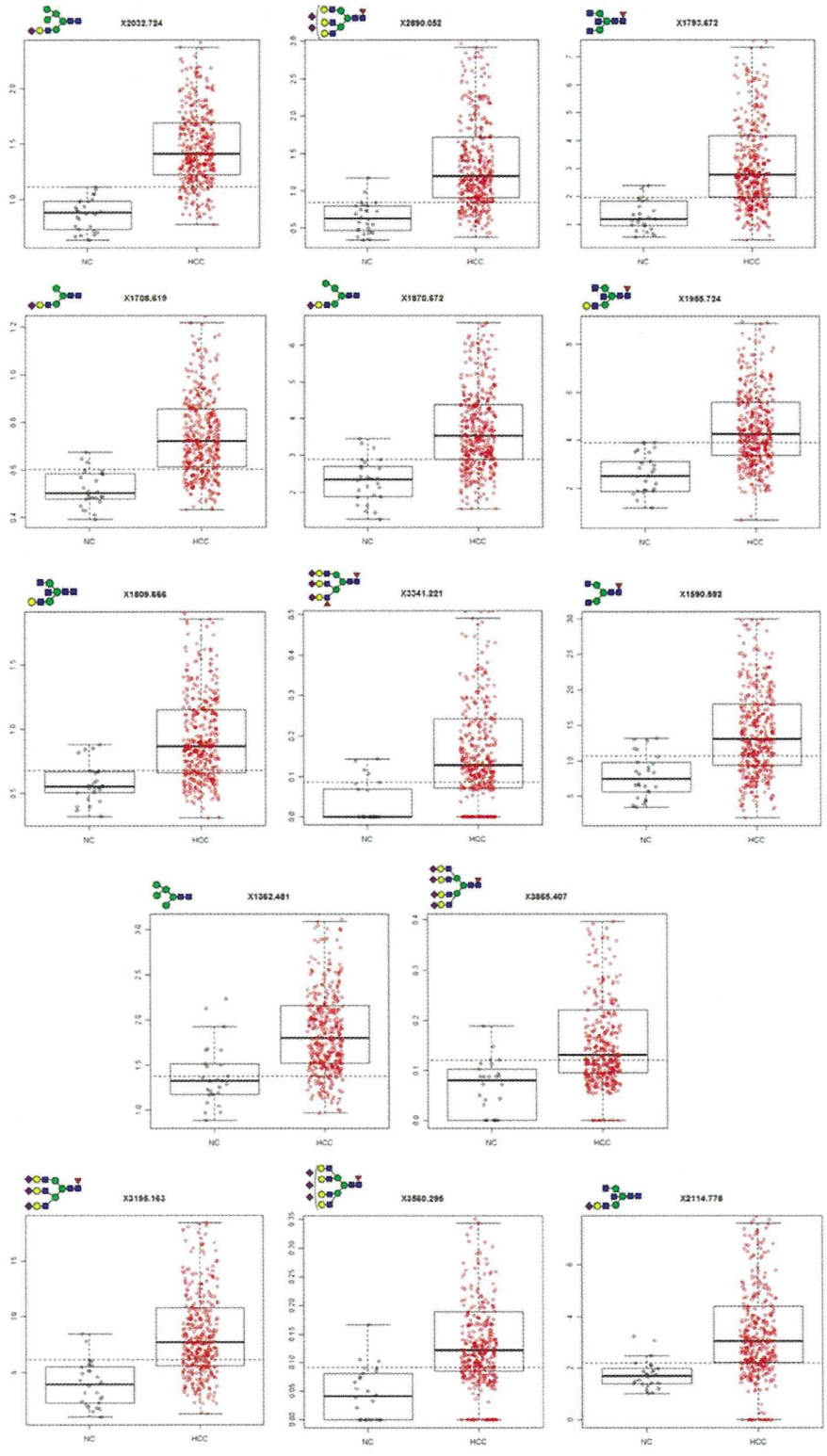


Fig. 1. Boxplots of the disease-free individuals (NC) and HCC patients for the selected 14 *N*-glycans. The dotted lines in the graphs represent the cutoff values determined in this analysis. These graphs were drawn using R v. 2.12.1.

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 (χ^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

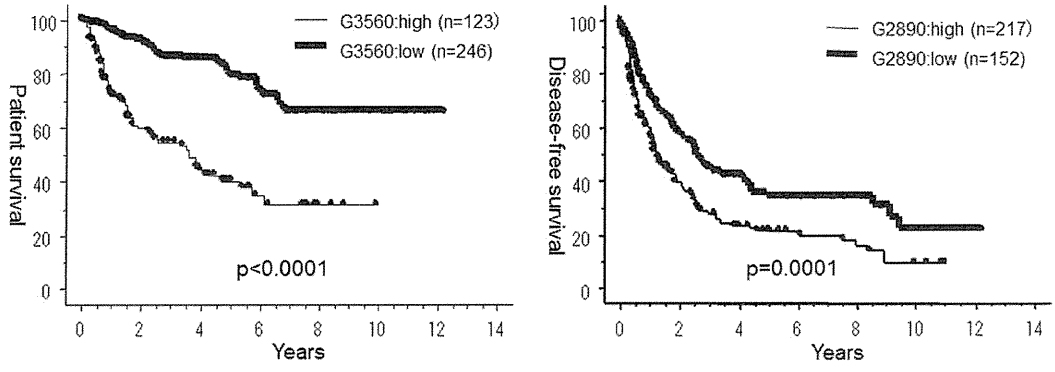


Fig. 2. The PS rates of HCC cases with low and high serum G3560 levels at 5 years were 80.5% and 40.4%, respectively. The DFS outcomes associated with low and high serum G2890 levels at 5 years were 21.3% and 35.1%, respectively.

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 di-sialylated 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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