biomarkers of the PS, DFS, and malignant behavior characteristics of HCC after a 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 via 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 mass spectrometry. 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 obtained 67 glycans profile 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 the view point of 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 non-sialylated 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 the well-known biomarker related to HCC except for the core fucosylation.

There have been several previous studies of glycans in HCC. Kudo et al reported that *N*-glycan alterations are associated with drug resistance in HCC in vitro (34). In other reported clinical studies, only specific glycans have been assessed in relation to HCC. Vanhooren et al 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 (17). However, this study analyzed 44 patients with HCC but did not evaluate 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 also described some HCC-specific glycans in their previous study (35) which 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 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-glycan 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 hepatocellular carcinoma.

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 which 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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Figure legends

Figure 1. Box plots of the disease-free individuals (NC) and HCC patients for the selected 14 *N*-glycans. The dotted lines in the graphs represent the cut-off values determined in this analysis. These graphs were drawn using R version 2.12.1.

Figure 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.

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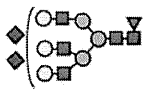
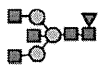
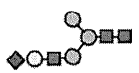
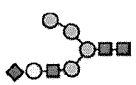
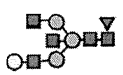
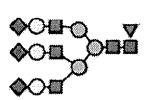
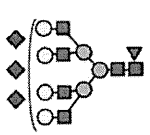
N-glycans	m/z		specificity (%)	sensitivity (%)	cut-off value	AUC
G2032	2032.724		100	86.45	1.115	0.968
G2890	2890.052		92.31	82.66	0.844	0.91
G1793	1793.672		92.31	75.61	1.963	0.9
G1708	1708.619		88.46	77.51	0.604	0.896
G1870	1870.672		88.46	75.88	2.886	0.873
G1955	1955.724		100	59.89	3.913	0.873
G3195	3195.163		92.31	71.27	6.109	0.864
G3560	3560.295		88.46	71.27	0.091	0.851

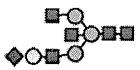
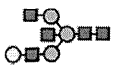
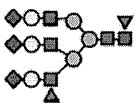
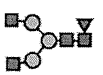
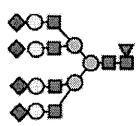
G2114	2114.778		88.46	75.88	2.208	0.839
G1809	1809.666		84.62	72.9	0.679	0.838
G3341	3341.221		84.62	69.92	0.086	0.821
G1590	1590.592		80.77	69.92	10.696	0.817
G1362	1362.481		65.38	87.26	1.381	0.813
G3865	3865.407		92.31	56.37	0.121	0.812

Table 1

List of the 14 serum N-glycans which were evaluated to be specific for hepatocellular carcinoma compared with normal controls by receiver operating characteristic (ROC) analysis. 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.

		(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

Table2

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
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
	Non anatomical	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	
differebntiation	well	17	1	0.0003	1	0.0002
	moderetely	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	
Non cancerous liver	Chirosis	120	1.199	0.3105	1.293	0.0398
	Non chirosis	249	1		1	

Table 3

Univariate analysis of predictive values (clinical and tumor associated factors) for patient survival (PS) and disease free survival (DFS). 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.

		P value	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

Table 4

Multivariate analysis of values that is predictive for overall HCC patient survival. ICGR15, indocyanin green retention rate at 15 minutes, AFP, alpha-fetoprotein; vp, microscopic tumor thrombus in the portal vein.

		P value	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
Differenciation	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

Table 5

Multivariate analysis of values that are predictive of disease free survival in HCC patients. ICGR15, indocyanin green retention rate at 15 minutes, AFP, alpha-fetoprotein; vp, microscopic tumor thrombus in the portal vein.

		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
	Non anatomical	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	

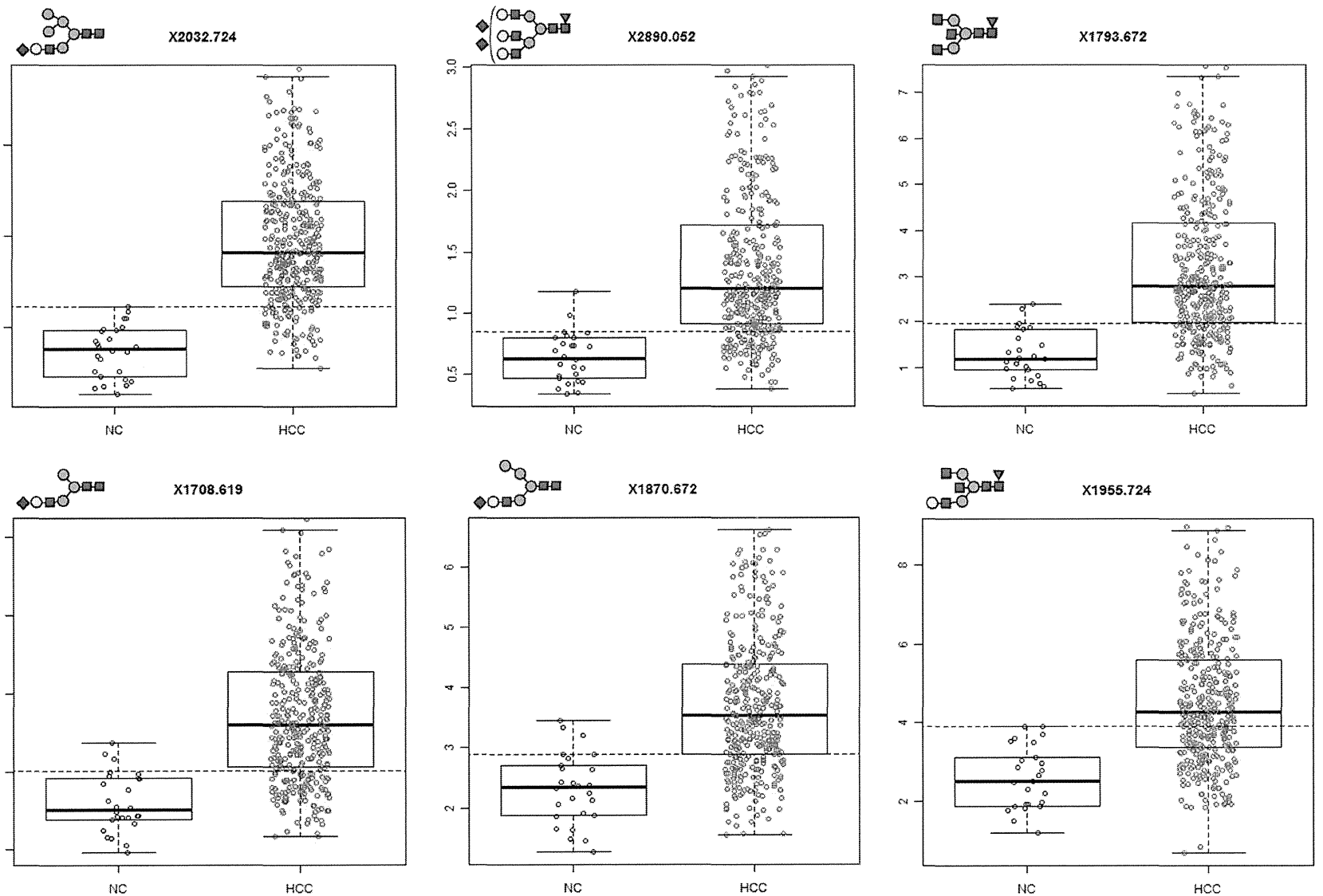
Number	Single	122	113		68	167	
	2, 3	60	29	0.0009	27	62	<0.0001
	≧4	35	10		28	17	
Size(cm)	≦3	48	68		15	101	
	3< & ≦5	60	36	<0.0001	21	75	<0.0001
	>5	109	48		87	70	
Differentiation	well	12	8		6	14	
	moderately	102	88	0.0981	46	144	0.0003
	poorly	103	56		71	88	
vp	positive	67	27	0.0065	49	45	<0.0001
	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	
Non cancerous liver	Cirrhosis	71	49	0.9876	35	85	0.2888
	Non cirrhosis	146	103		88	161	

Table 6

Correlation between the G2890 and G3560 *N*-glycans and clinical and tumor associated factors in HCC cases.

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.

Fig1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NC
HCC