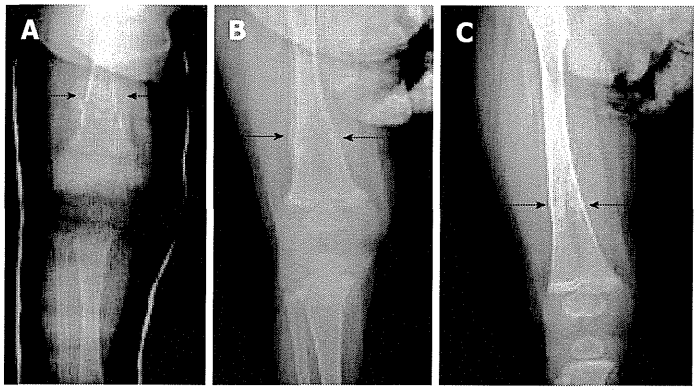

Figure 3 Plain skeletal radiographic features at the 8 d after the application of an immobilizing plaster bandage for the femur fracture in the case 1. Callus formation (arrows) was seen 8 d after the application of an immobilizing plaster bandage (A) in case 1. The plaster bandage was removed after 20 d (B) and the fracture of the right femur was cured 6 mo post-fracture (C).

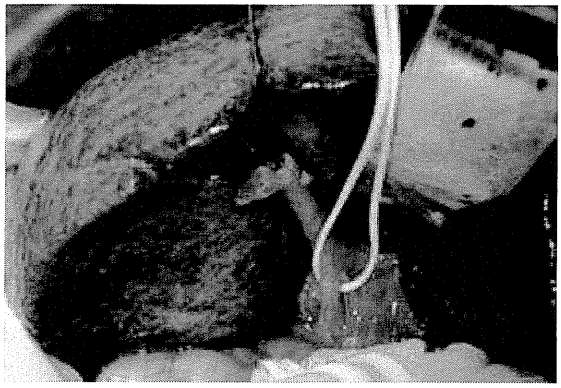

Figure 4 Intraoprative features. On laparotomy, the liver was brown and firm with a dull edge, suggesting cholestasis.

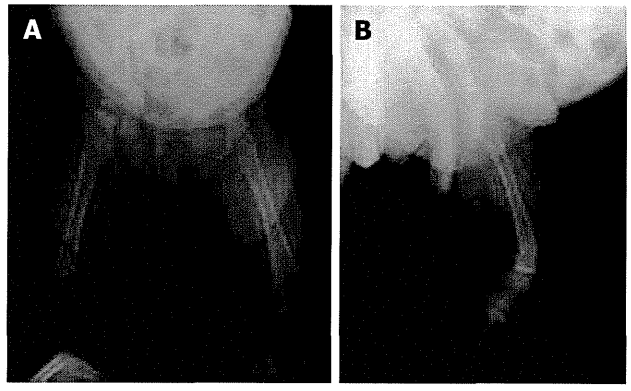

Figure 5 Plain skeletal radiographic features at the 28 d after hepaticojejunostomy in the case 2. Anteroposterior (A) and lateral (B) plain radiographs showing a displaced fracture (arrows) of the left distal femur.

jaundice at a medical check-up at 1 mo of age. The patient presented with acholic stools and increased jaundice at the age of 3 mo at a medical check-up, and was consequently admitted to our institution for further examinations. Laboratory studies upon admission revealed the following: AST 573 IU/L, ALT 377 IU/L, TB 6.6 mg/dL, DB 4.4 mg/dL, ALP 2248 IU/L, γ-GTP 666 IU/L, choline esterase 181 IU/L, and serum calcium 9.2 mg/dL. The findings on abdominal US and MRCP were just as same as those of the case 1. Therefore, BA was suspected, and the infant underwent an exploratory laparotomy at 113 d of age. The patient started oral vitamin D at 3 mo of age.

On laparotomy, the liver was brown and firm with a dull edge, suggesting cholestasis (Figure 4). Intraoperative cholangiography revealed a patent gallbladder and no patency of the extrahepatic bile duct. The infant was diagnosed as BA (II b1γ)[5] based on cholangiographic and macroscopic findings. The remnants were totally removed en block and a Roux-en-Y hepaticojejunostomy was performed with a Roux loop of 60 cm applied antecolically. Microscopic findings of the liver biopsy specimen were cirrhotic.

The patient could not move her left leg at 28 d post-laparotomy. A displaced fracture of the left distal femur was shown by plain skeletal radiograph (Figure 5A and B). Hepatic osteodystrophy was suspected based on the fact that there was no history of femur trauma and the patient suffered from chronic cholestasis. Child abuse by the family was not considered from the situation. Callus formation was seen 14 d after the application of an immobilizing plaster bandage. The plaster bandage was removed after 20 d and the fracture of the left femur was cured at 6 mo after post-fracture. Jaundice has been resolved and she is currently well at 11 mo of age.

DISCUSSION

BA is a rare disease with an incidence of approximately 1:10 000 live births in Japan and the Far East[6]. The most frequent symptom is prolonged jaundice. Several reports have shown that osteodystrophy was associated with severe chronic liver disease despite the administration of vitamin and mineral supplements[1]. Argao *et al*[7] suggested that the bone mineral content of patients with hepatic osteodystrophy did not improve despite successful normalization of the serum 25-OH vitamin D concentration by enhancing vitamin D absorption from the gastrointestinal tract. Chongsrisawat *et al*[8] reported that osteoporosis was recognized in up to 80% of a group of jaundiced BA patients in comparison with only 13.6% in a non-jaundiced group.

In BA, metabolic disturbance results from impairment of the passage of bile salts into the alimentary canal. As a consequence, the inadequate emulsification of fat results

in the incomplete absorption of vitamin D. Vitamin D is hydroxylated to 25-OH-D in the liver[2]. Additionally, over the course of the disease, liver cirrhosis develops and the hydroxylation of vitamin D is impaired. Vitamin D and hence calcium absorption are thus diminished. 25-OH-D is thought to be converted to more active forms, 125- or 2125-dehydro-OH-D. Rickets and osteoporosis were reported to be found in 23 of 39 patients (59%) with surgically unrepaired BA[1].

We herein report two infants: one infant with BA who initially presented with a bone fracture before Kasai hepatic portoenterostomy, and the other at 4 wk after Kasai hepatic portoenterostomy. There are a number of factors which may be important in the etiology of bone fractures in children, including trauma, metabolic bone disease, drugs, and immobilization[3]. However, the lack of significant trauma in the majority of cases (91%) is a notable feature in children with BA[3]. Hill *et al*[3] reported 12 (19%) children with fractures before and after transplantation out of 63 undergoing liver transplanatation. Eight of 12 children with fractures in BA had no identifiable trauma. The age at the time of fracture in BA ranged from 3 to 16 mo after birth, and the affected children suffered from osteopenia (generalized reduction in bone density). The fracture site was the ribs or long bones, and multiple fractures were seen in 2 children with BA (7 and 8 mo after birth). However, Hill *et al*[3] did not describe administering vitamin D supplements. BA patients with severe cholestasis have a risk of bone fracture despite the administration of essential vitamins and minerals such as our cases. In our cases, BA was diagnosed at 6 mo after birth in case 1 and at 3 mo after birth in case 2, with suspected severe cholestasis.

Conservative management such as immobilization using plaster bandages is generally effective for fractures in BA, and there were no complications related to fractures in our cases. In the literature, internal fixation was required in one case with oxalosis for a fractured neck of the femur[1]. The early diagnosis and treatment of BA before the occurrence of bone fracture is important. The measurement of reflected light from the surface of feces by near infrared reflectance spectroscopy was introduced by Akiyama *et al*[9] for the differential diagnosis of cholestatic diseases in infants. Another method, mass screening using color picture cards depicting normal and acholic stools, was carried out at 1 and 2 mo after birth in a Japanese prefecture[10]. Eight cases of BA were detected using this mass screening method during a 3-year period, with a specificity of 99.9% and a sensitivity of 80.0%. Such screening procedures could result in improved detection of BA in infants before bone disorders occur.

In summary, clinical awareness of BA should be maintained both in terms of careful handling to prevent possible bone fracturing and also in considering fractures as a possible diagnostic factor in children with reluctance to use a limb, even in the absence of previous trauma, before Kasai hepatic portoenterostomy. Radiological awareness is also important to avoid missing unsuspected fractures on radiographs.

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Identification of novel serum biomarkers of hepatocellular carcinoma using glycomic analysis

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Running Title: New biomarkers of hepatocellular carcinoma determined by glycomics

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Key words: hepatocellular carcinoma; glycomics; hepatectomy; biomarker.

Conflicts of interest

The authors declare no conflicts of interest.

Abstract

Background: The altered *N*-glycosylation of glycoproteins has been suggested to play an important role in the behavior of malignant cells. Using novel glycomics technology, we attempted to determine the specific and detailed *N*-glycan profile for hepatocellular carcinoma (HCC) and investigate the prognostic capabilities.

Method: 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, Japanese 26 living related liver transplantation donors were selected not infected by hepatitis B and C virus. Their mean age was 40.0. Fifteen (57.7%) were male. We used a glycoblotting method to purify *N*-glycans from preoperative blood samples from this cohort (10μl 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.

Results: 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 significant recurrent factor and G3560 (*m/z* value,

3560.295) as 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.

Abbreviations

HCC: hepatocellular carcinoma

PS: patient Survival

DFS: disease-free survival

RF: risk factor

ICGR15: indocyanin green retention rate at 15 minutes

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

AUC: area under the curve

ROC: receiver operating characteristics

Introduction

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, this 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 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 post-translational 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 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 *via* 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 have evaluated preoperative blood samples from a HCC patient cohort from which we purified serum *N*-glycans using our glycoblotting method (21, 22). We performed *N*-glycan profiling using mass spectrometry 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. We sought through this screen to correlate *N*-glycan levels on glycoproteins with the clinicopathologic characteristics and the outcomes of HCC.

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 two 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 of eligibility by for 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. 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 via 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 Co., Ltd., Tokyo, Japan). All procedures utilized the SweetBlot automated glycan purification system containing a 96 well plate platform (System Instruments Co. Ltd. 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 Ltd., Osaka, Japan). After incubation at 60°C for 30 min, 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 min, the mixture was treated with 200 U of trypsin (Sigma-Aldrich, St. Louis, MO) at 37 °C for 2 h, followed by heat-inactivation of the enzyme at 90°C for 10 min. 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 h.

***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 Co., Tokyo, Japan) were aliquoted into the wells of a MultiScreen Solvinert hydrophilic PTFE (polytetrafluoroethlene) 96-well filter plate (EMD Millipore Co., 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 min to capture the *N*-glycans onto the beads via 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 min at room temperature so that un-reacted 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 x 200 μl of 10 mM HCl, 1% triethyl amine in methanol and dioxane. This is a pre-treatment 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 Co., Tokyo, JAPAN) in dioxane at 60°C for 90 min 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 Co., Tokyo, JAPAN) reagent for 45 min 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.

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 1000 shots. The *N*-glycan peaks in the MALDI-TOF MS spectra were selected using FlexAnalysis ver. 3 (Brucker Daltonics, Billerica, MA). The intensity of the isotopic peak of each glycan was normalized using 40 μM of internal standard (disialyloctasaccharide, Tokyo Chemical Industry Co., Tokyo, JAPAN) 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 measure quativetatively 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. Non-anatomical partial, but complete resection was achieved in all of our cases. R0 resections were performed whilst 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 two years after the hepatectomy procedure, the HCC patients in our cohort were monitored every three months using liver function tests, measurements of the tumor markers AFP and protein induced by PIVKA-II, and also by ultrasonography and dynamic computed tomography. At two years post-surgery,

routine computed tomography was performed only once in 4 months. If recurrence was suspected, both computed tomography and magnetic resonance imaging were performed, and if necessary, computed tomography during angiography and bone scintigraphy were undertaken. This enabled a precise diagnosis of the site, number, size, and invasiveness of any recurrent lesions.

Statistics

The specificity, the sensitivity, cut-off 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 version 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 standard tests (X^2, *t*-test) where appropriate using StatView 5.0 for Windows (SAS Institute Inc., Cary, NC). Significance was defined by a *P* value of < 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 utilizing 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 box plots for these selected molecules (14 in total) are shown in Figure 1. Clear differences in the distribution of these factors are evident between the NC and HCC patients. The cut-off values were determined using the maximum values for specificity plus sensitivity. G2890 was elevated more than cut-off 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 which were highly specific for HCC were evaluated for 3-year recurrence free survival by ROC analysis to determine the cut-off values about these N-glycans. The patients were divided to 2 groups by these cut-off 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 (Table2). When clinical and tumor associated factors were evaluated by univariate analysis, albumin, Child-Pugh classification, AFP, AFP-L3, 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 non-cancerous liver were found to be significantly associated with this measure (Table 3) 5 .

The variable selection from 19 clinical and tumor associated factors in Table 3 and the 14 serum N-glycans using Akaike's Information Criterion (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 (Tables 4) and G2890 for DFS (Tables 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 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 significant prognostic factor and G2890 *N*-glycan was found to be 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