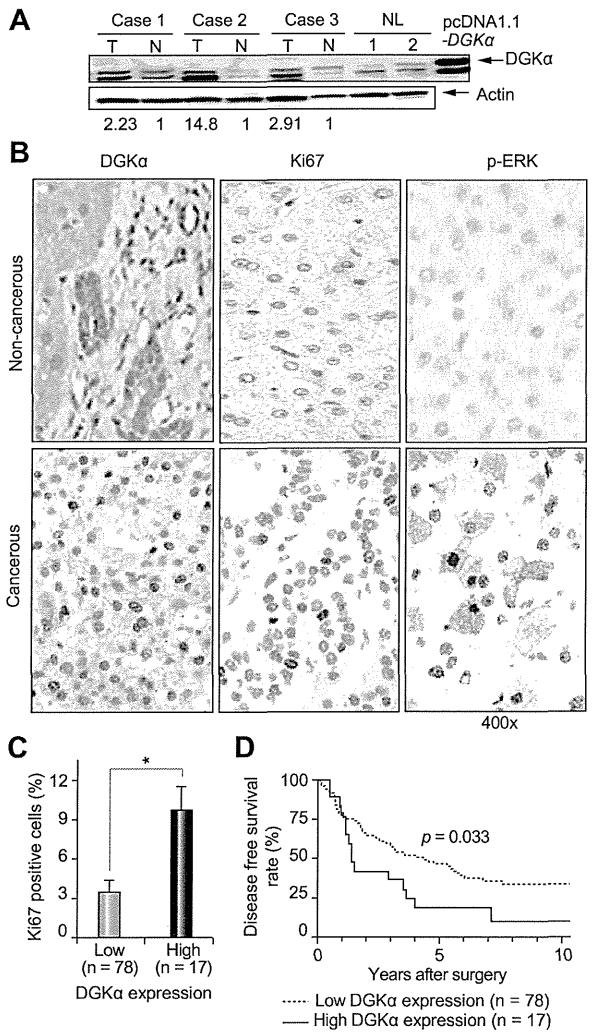


Fig. 4. DGKα expression in HCC samples. (A) DGKα expression in cancerous tissues (T), adjacent non-cancerous tissues (N) collected from surgical resection, and normal liver tissues (NL) was analyzed by Western blotting. DGKα expression levels were quantified by densitometry and normalized to β-actin. (B) DGKα, Ki67 and p-ERK expression in liver cancer samples was analyzed by immunohistochemistry. Normal bile duct cells indicated positive immunostaining for DGKα (400 × magnification). (C) Cell numbers positive for Ki67 are shown as mean ± S.D. Asterisks (*) indicate significant difference. (D) Disease-free survival curves after hepatectomy of the HCC patients comparing high and low DGKα expression.

A multivariate analysis of recurrence-free survival after hepatectomy was carried out using the Cox proportional hazards regression model. High DGKα expression was one of the independent risk factors for determining HCC recurrence after surgery ($p = 0.0184$; Table 2), as were intrahepatic metastasis ($p = 0.0004$), AFP >40 ng ($p = 0.0287$), and liver cirrhosis ($p = 0.0295$). The relative risk for HCC recurrence after hepatectomy in patients with high DGKα-expressing tumors was 2.32 times greater than that of patients with low DGKα-expressing tumors.

Table 2. Multivariate analysis of recurrence of HCC by Cox's proportional hazard model.

Factors	Odds ratio (95% CI)	*p* value
Intrahepatic metastasis		
Positive *vs.* negative	3.32 (1.75-6.10)	0.0004
DGKα		
High *vs.* low expression	2.32 (1.15-4.42)	0.0184
AFP >40 (ng/ml)	2.06 (1.07-3.89)	0.0287
Liver cirrhosis	1.92 (1.07-3.40)	0.0295

CI, confidence interval; DGKα, diacylglycerol kinase α; AFP, α-fetoprotein.

Discussion

Previous reports showed that DGKα enhanced interleukin 2-induced G1 to S transition and subsequent proliferation of T cells [18]. Another study reported that HGF induces DGKα activation in breast cancer cells, and is required for invasion [33]. Taken together, these findings imply that DGKα induces cell proliferation and invasion. Our findings have demonstrated the importance of DGKα as a potential tumor growth promoter in HCC. Furthermore, the inactive mutant, KD DGKα, failed to affect the extent of cell proliferation, thus, DG consumption or PA production might play an important role in the regulation of cell proliferation. It is known that the level of PA is also increased in cells transformed by oncogenes to promote proliferation [34,35]. As our study did not directly demonstrate DG consumption or PA production by DGKα in HCC, further studies are required to fully account for the mechanism as to how DGKα contributes to the progression of HCC. However, our results suggest that inhibition of DGKα or the catalytic activity could reduce HCC growth.

The therapeutic target for cancer should be the cancer-specific gene, whose upregulated or downregulated expression is limited to only cancerous cells, in order to enhance the effect of the therapy and to reduce side effects. In this study, DGKα expression was upregulated in HCC cell lines compared with normal hepatocytes, and was also increased in primary HCC tissue compared with adjacent non-tumor tissue *in vivo*. DGKα may be a novel target for HCC because of the specificity of HCC.

The MAPK signaling cascade is essential for the transduction of extracellular signals to the nucleus, regulating a wide variety of pathophysiological processes such as proliferation, differentiation, migration and carcinogenesis [36], and is the prominent pathway upregulated in HCC [37], making it an obvious target in the strategy for HCC treatment. This current study provides the first evidence that DGKα is a critical component in the MAPK pathway activated by HGF in HCC. Knockdown of DGKα impaired phosphorylation of ERK and of MEK, but not Ras activity. In addition, *DGKα* silencing activated Ras more strongly than the control, following 60 min of HGF stimulation. Ras was inactivated by ERK phosphorylation through negative feedback mechanisms [38]. These facts imply that the target component of DGKα might be Raf. We previously showed that DGKη activated the MAPK pathway induced by EGF, by augmenting the activity and heterodimerization of Raf in HeLa [39]. DGKα may contribute to activation of the MAPK pathway instead of Ras activation in HCC progression.

In conclusion, DGKα expressed in human HCC appears to have an important role in cell proliferation and invasion in HCC via

activation of the MAPK cascade, and may determine the degree of malignancy of HCC. Inhibition of DGKα might contribute to suppression of HCC growth, thus DGKα could be a novel target for HCC therapeutics as well as a prognostic marker.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2012.02.026.

References

[1] Kiyosawa K, Umemura T, Ichijo T, Matsumoto A, Yoshizawa K, Gad A, et al. Hepatocellular carcinoma: recent trends in Japan. Gastroenterology 2004;127:S17–S26.

[2] Parkin DM. Global cancer statistics in the year 2000. Lancet Oncol 2001;2:533–543.

[3] Taketomi A, Sanefuji K, Soejima Y, Yoshizumi T, Uhciyama H, Ikegami T, et al. Impact of des-gamma-carboxy prothrombin and tumor size on the recurrence of hepatocellular carcinoma after living donor liver transplantation. Transplantation 2009;87:531–537.

[4] Taketomi A, Kitagawa D, Itoh S, Harimoto N, Yamashita Y, Gion T, et al. Trends in morbidity and mortality after hepatic resection for hepatocellular carcinoma: an institute's experience with 625 patients. J Am Coll Surg 2007;204:580–587.

[5] Shirabe K, Kanematsu T, Matsumata T, Adachi E, Akazawa K, Sugimachi K. Factors linked to early recurrence of small hepatocellular carcinoma after *hepatectomy: univariate and multivariate analyses. Hepatology* 1991;14:802–805.

[6] Taketomi A, Fukuhara T, Morita K, Kayashima H, Ninomiya M, Yamashita Y, et al. Improved results of a surgical resection for the recurrence of hepatocellular carcinoma after living donor liver transplantation. Ann Surg Oncol 2010;17:2283–2289.

[7] Lai JP, Sandhu DS, Moser CD, Cazanave SC, Oseini AM, Shire AM, et al. Additive effect of apicidin and doxorubicin in sulfatase 1 expressing hepatocellular carcinoma in vitro and in vivo. J Hepatol 2009;50:1112–1121.

[8] Schmitz KJ, Wohlschlaeger J, Lang H, Sotiropoulos GC, Malago M, Steveling K, et al. Activation of the ERK and AKT signalling pathway predicts poor prognosis in hepatocellular carcinoma and ERK activation in cancer tissue is associated with hepatitis C virus infection. J Hepatol 2008;48:83–90.

[9] Grant S. Cotargeting survival signaling pathways in cancer. J Clin Invest 2008;118:3003–3006.

[10] Sakane F, Imai S-i, Kai M, Yasuda S, Kanoh H. Diacylglycerol kinases: why so many of them? Biochim Biophys Acta 2007;1771:793–806.

[11] Topham MK. Signaling roles of diacylglycerol kinases. J Cell Biochem 2006;97:474–484.

[12] Goto K, Hozumi Y, Kondo H. Diacylglycerol, phosphatidic acid, and the converting enzyme, diacylglycerol kinase, in the nucleus. Biochim Biophys Acta 2006;1761:535–541.

[13] van Blitterswijk WJ, Houssa B. Properties and functions of diacylglycerol kinases. Cell Signal 2000;12:595–605.

[14] Merida I, Avila-Flores A, Merino E. Diacylglycerol kinases: at the hub of cell signalling. Biochem J 2008;409:1–18.

[15] Sakane F, Yamada K, Kanoh H, Yokoyama C, Tanabe T. Porcine diacylglycerol kinase sequence has zinc finger and E–F hand motifs. Nature 1990;344:345–348.

[16] Jiang Y, Qian W, Hawes JW, Walsh JP. A domain with homology to neuronal calcium sensors is required for calcium-dependent activation of diacylglycerol kinase alpha. J Biol Chem 2000;275:34092–34099.

[17] Sakane F, Yamada K, Imai S, Kanoh H. Porcine 80-kDa diacylglycerol kinase is a calcium-binding and calcium/phospholipid-dependent enzyme and undergoes calcium-dependent translocation. J Biol Chem 1991;266:7096–7100.

[18] Flores I, Casaseca T, Martinez AC, Kanoh H, Merida I. Phosphatidic acid generation through interleukin 2 (IL-2)-induced alpha-diacylglycerol kinase activation is an essential step in IL-2-mediated lymphocyte proliferation. J Biol Chem 1996;271:10334–10340.

[19] Yamada K, Sakane F, Kanoh H. Immunoquantitation of 80 kDa diacylglycerol kinase in pig and human lymphocytes and several other cells. FEBS Lett 1989;244:402–406.

[20] Olenchock BA, Guo R, Carpenter JH, Jordan M, Topham MK, Koretzky GA, et al. Disruption of diacylglycerol metabolism impairs the induction of T cell anergy. Nat Immunol 2006;7:1174–1181.

[21] Zha Y, Marks R, Ho AW, Peterson AC, Janardhan S, Brown I, et al. T cell anergy is reversed by active Ras and is regulated by diacylglycerol kinase-alpha. Nat Immunol 2006;7:1166–1173.

[22] Kanoh H, Iwata T, Ono T, Suzuki T. Immunological characterization of sn-1,2-diacylglycerol and sn-2-monoacylglycerol kinase from pig brain. J Biol Chem 1986;261:5597–5602.

[23] Liver Cancer Study Group of Japan. The General Rules for the Clinical and Pathological Study of Primary Liver Cancer. 2nd English ed. Kanehara: Tokyo; 2003.

[24] Yamada K, Sakane F, Imai S, Tsushima S, Murakami T, Kanoh H. Regulatory role of diacylglycerol kinase gamma in macrophage differentiation of leukemia cells. Biochem Biophys Res Commun 2003;305:101–107.

[25] Itoh S, Taketomi A, Tanaka S, Harimoto N, Yamashita Y, Aishima S, et al. Role of growth factor receptor bound protein 7 in hepatocellular carcinoma. Mol Cancer Res 2007;5:667–673.

[26] Anegawa G, Kawanaka H, Yoshida D, Konishi K, Yamaguchi S, Kinjo N, et al. *Defective endothelial nitric oxide synthase signaling is mediated by rho-kinase activation in rats with secondary biliary cirrhosis. Hepatology* 2008;47:966–977.

[27] Sharma D, Wang J, Fu PP, Sharma S, Nagalingam A, Mells J, et al. Adiponectin antagonizes the oncogenic actions of leptin in hepatocellular carcinogenesis. Hepatology 2010;52:1713–1722.

[28] Nakabayashi H, Taketa K, Yamane T, Miyazaki M, Miyano K, Sato J. Phenotypical stability of a human hepatoma cell line, HuH-7, in long-term culture with chemically defined medium. Gann 1984;75:151–158.

[29] MacNab GM, Alexander JJ, Lecatsas G, Bey EM, Urbanowicz JM. Hepatitis B surface antigen produced by a human hepatoma cell line. Br J Cancer 1976;34:509–515.

[30] Baldanzi G, Mitola S, Cutrupi S, Filigheddu N, van Blitterswijk WJ, Sinigaglia F, et al. Activation of diacylglycerol kinase alpha is required for VEGF-induced angiogenic signaling in vitro. Oncogene 2004;23:4828–4838.

[31] Calvisi DF, Ladu S, Gorden A, Farina M, Conner EA, Lee JS, et al. Ubiquitous activation of Ras and Jak/Stat pathways in human HCC. Gastroenterology 2006;130:1117–1128.

[32] Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol 2000;182:311–322.

[33] Filigheddu N, Cutrupi S, Porporato PE, Riboni F, Baldanzi G, Chianale F, et al. Diacylglycerol kinase is required for HGF-induced invasiveness and anchorage-independent growth of MDA-MB-231 breast cancer cells. Anticancer Res 2007;27:1489–1492.

[34] Hurst-Kennedy J, Boyan BD, Schwartz Z. Lysophosphatidic acid signaling promotes proliferation, differentiation, and cell survival in rat growth plate chondrocytes. Biochim Biophys Acta 2009;1793:836–846.

[35] Sugimoto Y, Whitman M, Cantley LC, Erikson RL. Evidence that the *Rous sarcoma* virus transforming gene product phosphorylates phosphatidylinositol and diacylglycerol. Proc Natl Acad Sci U S A 1984;81:2117–2121.

[36] Marshall CJ. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 1995;80:179–185.

[37] Saxena NK, Sharma D, Ding X, Lin S, Marra F, Merlin D, et al. Concomitant activation of the JAK/STAT, PI3K/AKT, and ERK signaling is involved in leptin-mediated promotion of invasion and migration of hepatocellular carcinoma cells. Cancer Res 2007;67:2497–2507.

[38] Cirit M, Wang CC, Haugh JM. Systematic quantification of negative feedback mechanisms in the extracellular signal-regulated kinase (ERK) signaling network. J Biol Chem 2010;285:36736–36744.

[39] Yasuda S, Kai M, Imai S, Takeishi K, Taketomi A, Toyota M, et al. Diacylglycerol kinase eta augments C-Raf activity and B-Raf/C-Raf heterodimerization. J Biol Chem 2009;284:29559–29570.

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

Identification of Novel Serum Biomarkers of Hepatocellular Carcinoma Using Glycomic Analysis

Toshiya Kamiyama,[1] Hideki Yokoo,[1] Jun-Ichi Furukawa,[2] Masaki Kurogochi,[2] Tomoaki Togashi,[2] Nobuaki Miura,[2] Kazuaki Nakanishi,[1] Hirofumi Kamachi,[3] Tatsuhiko Kakisaka,[1] Yosuke Tsuruga,[1] Masato Fujiyoshi,[1] Akinobu Taketomi,[1] Shin-Ichiro Nishimura,[2] and Satoru Todo[3]

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.

From the [1]Department of Gastroenterological Surgery I, Hokkaido University Graduate School of Medicine, Hokkaido, Japan; [2]Graduate School of Life Science and Frontier Research Center for Post-Genome Science and Technology, Hokkaido University, Hokkaido, Japan; [3]Department of Transplantation Surgery, Hokkaido University Graduate School of Medicine, Hokkaido, Japan

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

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

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

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

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

Patients and Methods

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

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

Address reprint requests to: Toshiya Kamiyama, M.D., Department of Gastroenterological Surgery I, Hokkaido University Graduate School of Medicine, North 15, West 7, Kita-ku, Sapporo 060-8638 Japan. E-mail: t-kamiya@med.hokudai.ac.jp; fax: +81-11-717-7515.

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

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

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

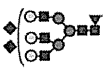
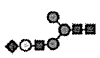
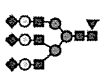
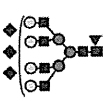
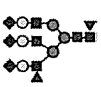
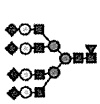
N-*Glycan Purification and Modification by Glycoblotting.* Glycoblotting of sample mixtures containing whole serum *N*-glycans was performed in accordance with previously described procedures. Commercially available BlotGlyco H beads (500 μL) (10 mg/ml suspension; Sumitomo Bakelite) were aliquoted into the wells of a MultiScreen Solvinert hydrophilic PTFE (polytetrafluoroethlene) 96-well filter plate (EMD Millipore, Billerica, MA). After removal of the water using a vacuum pump, 20 μL of PNGase F-digested samples were applied to the wells, followed by the addition of 180 μL of 2% acetic acid in acetonitrile. The filter plate was then incubated at 80°C for 45 minutes to capture the *N*-glycans onto the beads by way of a chemically stable and reversible hydrazone bond. The beads were then washed using 200 μL of 2 M guanidine-HCl in 10 mM ammonium bicarbonate, followed by washing with the same volume of water and of 1% triethyl amine in methanol. Each washing step was performed twice. The *N*-glycan linked beads were next incubated with 10% acetic anhydride in 1% triethyl amine in methanol for 30 minutes at room temperature so that unreacted hydrazide groups would become capped by acetylation. After capping, the reaction solution was removed under a vacuum and the beads were serially washed with 2 × 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

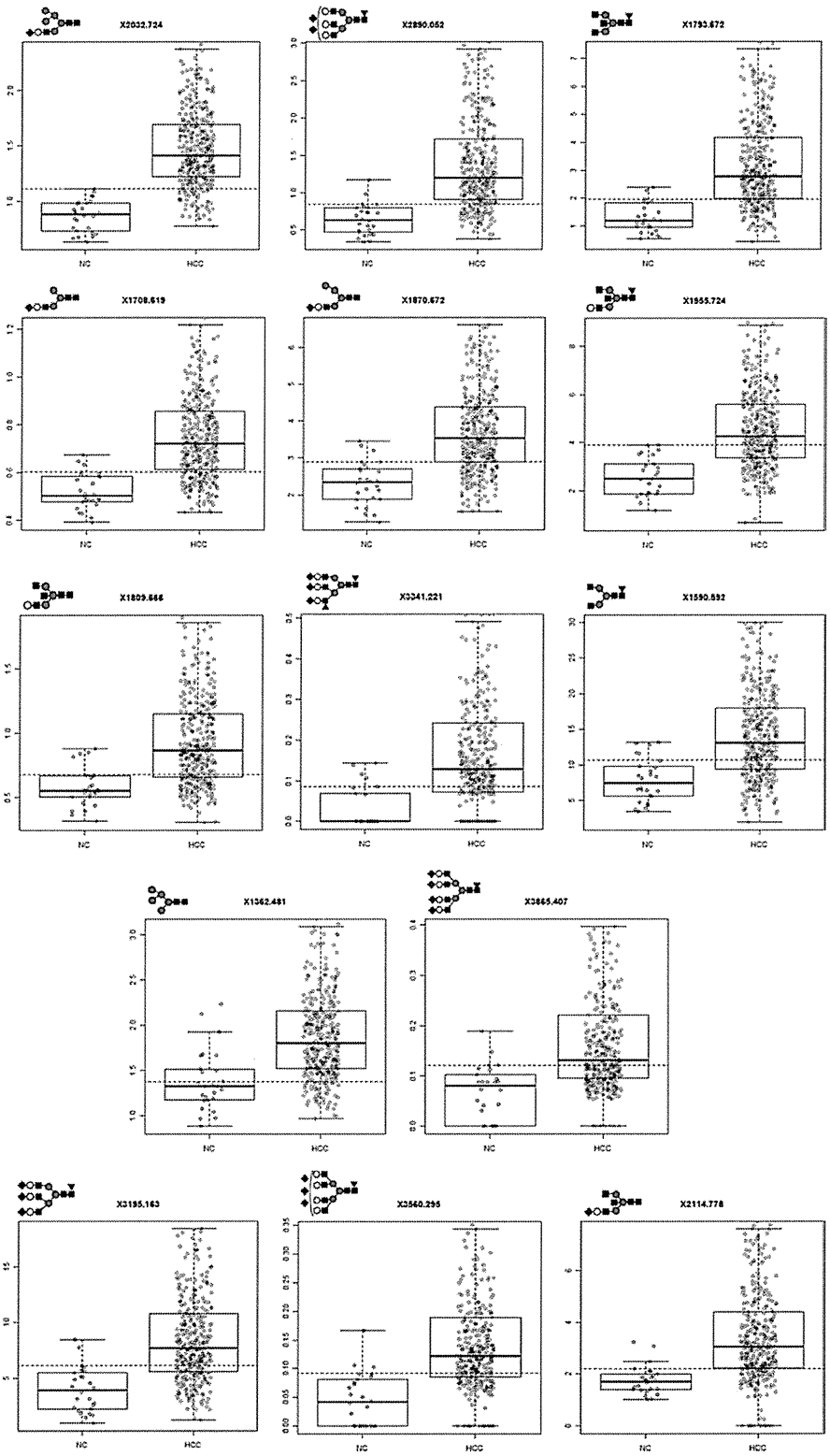


Fig. 1. Boxplots of the disease-free individuals (NC) and HCC patients for the selected 14 *N*-glycans. The dotted lines in the graphs represent the cutoff values determined in this analysis. These graphs were drawn using R v. 2.12.1.

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

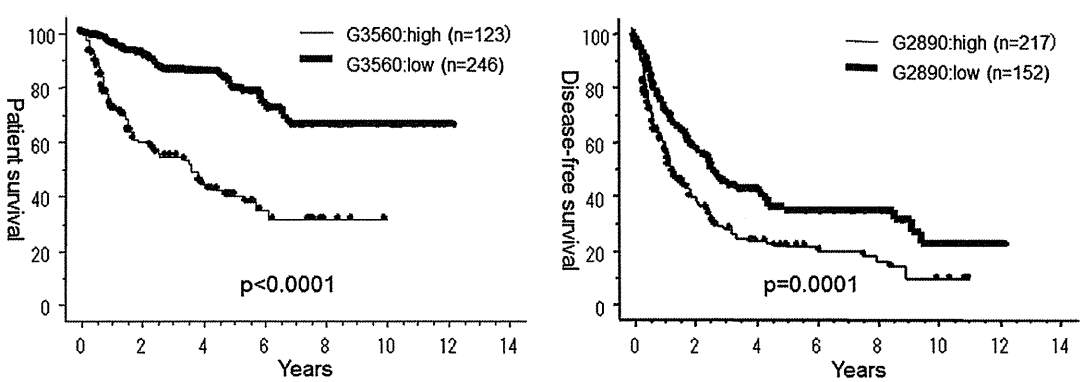


Fig. 2. The PS rates of HCC cases with low and high serum G3560 levels at 5 years were 80.5% and 40.4%, respectively. The DFS outcomes associated with low and high serum G2890 levels at 5 years were 21.3% and 35.1%, respectively.

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.

Acknowledgment: We thank the staff of the Gastroenterological Surgery I, Graduate School of Medicine, and Faculty of Advanced Life Science, Frontier Research Center for Post-Genome Science and Technology, Hokkaido University, and System Instruments Co. Ltd., Science & Technology Systems Inc., Bruker Daltonics K. K., for their kind cooperation during this study.

References

1. Farazi PA, DePinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment. Nat Rev Cancer 2006;6:674-687.
2. Arii S, Yamaoka Y, Futagawa S, Inoue K, Kobayashi K, Kojiro M, et al. Results of surgical and nonsurgical treatment for small-sized hepatocellular carcinomas: a retrospective and nationwide survey in Japan. The Liver Cancer Study Group of Japan. HEPATOLOGY 2000;32:1224-1229.
3. Hasegawa K, Kokudo N, Imamura H, Matsuyama Y, Aoki T, Minagawa M, et al. Prognostic impact of anatomic resection for hepatocellular carcinoma. Ann Surg 2005;242:252-259.
4. Kamiyama T, Nakanishi K, Yokoo H, Kamachi H, Tahara M, Suzuki T, et al. Recurrence patterns after hepatectomy of hepatocellular carcinoma: implication of Milan criteria utilization. Ann Surg Oncol 2009;16:1560-1571.
5. Ikai I, Arii S, Kojiro M, Ichida T, Makuuchi M, Matsuyama Y, et al. Reevaluation of prognostic factors for survival after liver resection in patients with hepatocellular carcinoma in a Japanese nationwide survey. Cancer 2004;101:796-802.
6. Shah SA, Cleary SP, Wei AC, Yang I, Taylor BR, Hemming AW, et al. Recurrence after liver resection for hepatocellular carcinoma: risk factors, treatment, and outcomes. Surgery 2007;141:330-339.
7. Imamura H, Matsuyama Y, Miyagawa Y, Ishida K, Shimada R, Miyagawa S, et al. Prognostic significance of anatomical resection and des-gamma-carboxy prothrombin in patients with hepatocellular carcinoma. Br J Surg 1999;86:1032-1038.
8. Shimada M, Takenaka K, Fujiwara Y, Gion T, Kajiyama K, Maeda T, et al. Des-gamma-carboxy prothrombin and alpha-fetoprotein positive status as a new prognostic indicator after hepatic resection for hepatocellular carcinoma. Cancer 1996;78:2094-2100.
9. Shirabe K, Itoh S, Yoshizumi T, Soejima Y, Taketomi A, Aishima S, et al. The predictors of microvascular invasion in candidates for liver transplantation with hepatocellular carcinoma-with special reference to the serum levels of des-gamma-carboxy prothrombin. J Surg Oncol 2007;95:235-240.
10. Esnaola NF, Lauwers GY, Mirza NQ, Nagorney DM, Doherty D, Ikai I, et al. Predictors of microvascular invasion in patients with hepatocellular carcinoma who are candidates for orthotopic liver transplantation. J Gastrointest Surg 2002;6:224-232; discussion 232.
11. Tamura S, Kato T, Berho M, Misiakos EP, O'Brien C, Reddy KR, et al. Impact of histological grade of hepatocellular carcinoma on the outcome of liver transplantation. Arch Surg 2001;136:25-30; discussion 31.
12. Toyoda H, Kumada T, Kiriyama S, Sone Y, Tanikawa M, Hisanaga Y, et al. Prognostic significance of simultaneous measurement of three tumor markers in patients with hepatocellular carcinoma. Clin Gastroenterol Hepatol 2006;4:111-117.
13. Inoue S, Nakao A, Harada A, Nonami T, Takagi H. Clinical significance of abnormal prothrombin (DCP) in relation to postoperative survival and prognosis in patients with hepatocellular carcinoma. Am J Gastroenterol 1994;89:2222-2226.
14. Imamura H, Matsuyama Y, Tanaka E, Ohkubo T, Hasegawa K, Miyagawa S, et al. Risk factors contributing to early and late phase intrahepatic recurrence of hepatocellular carcinoma after hepatectomy. J Hepatol 2003;38:200-207.
15. Sumie S, Kuromatsu R, Okuda K, Ando E, Takata A, Fukushima N, et al. Microvascular invasion in patients with hepatocellular carcinoma and its predictable clinicopathological factors. Ann Surg Oncol 2008;15:1375-1382.
16. Kang P, Madera M Jr WRA, Goldman R, Mechref Y, Novotny MV. Glycomic alterations in the highly-abundant and lesser-abundant blood serum protein fractions for patients diagnosed with hepatocellular carcinoma. Int J Mass Spectrom 2011;305:185-198.
17. Vanhooren V, Liu XE, Franceschi C, Gao CF, Libert C, Contreras R, et al. N-glycan profiles as tools in diagnosis of hepatocellular carcinoma and prediction of healthy human ageing. Mech Ageing Dev 2009;130:92-97.
18. Kirmiz C, Li B, An HJ, Clowers BH, Chew HK, Lam KS, et al. A serum glycomics approach to breast cancer biomarkers. Mol Cell Proteomics 2007;6:43-55.
19. An HJ, Miyamoto S, Lancaster KS, Kirmiz C, Li B, Lam KS, et al. Profiling of glycans in serum for the discovery of potential biomarkers for ovarian cancer. J Proteome Res 2006;5:1626-1635.
20. Miura Y, Hato M, Shinohara Y, Kuramoto H, Furukawa J, Kurogochi M, et al. BlotGlycoABCTM, an integrated glycoblotting technique for rapid and large scale clinical glycomics. Mol Cell Proteomics 2008;7:370-377.
21. Nishimura S, Niikura K, Kurogochi M, Matsushita T, Fumoto M, Hinou H, et al. High-throughput protein glycomics: combined use of

chemoselective glycoblotting and MALDI-TOF/TOF mass spectrometry. Angew Chem Int Ed Engl 2004;44:91-96.
22. Furukawa J, Shinohara Y, Kuramoto H, Miura Y, Shimaoka H, Kurogochi M, et al. Comprehensive approach to structural and functional glycomics based on chemoselective glycoblotting and sequential tag conversion. Anal Chem 2008;80:1094-1101.
23. Ichida F, Tsuji T, Omata M, Ichida T, Inoue K, Kamimura T, et al. New Inuyama Classification; new criteria for histlogical assessment of chronic hepatitis. Int Hepatol Commun 1996;6:112-119.
24. The Liver Study Group of Japan. The general rules for the clinical and pathological study of primary liver cancer. 3rd English ed. Tokyo, Japan: Kanehara & Co.
25. Akaike H. A new look at the statistical model identification. IEEE Trans Autom Control 1974;19:716-723.
26. Nomura F, Ohnishi K, Tanabe Y. Clinical features and prognosis of hepatocellular carcinoma with reference to serum alpha-fetoprotein levels. Analysis of 606 patients. Cancer 1989;64:1700-1707.
27. Hanazaki K, Kajikawa S, Koide N, Adachi W, Amano J. Prognostic factors after hepatic resection for hepatocellular carcinoma with hepatitis C viral infection: univariate and multivariate analysis. Am J Gastroenterol 2001;96:1243-1250.
28. Matsumoto Y, Suzuki T, Asada I, Ozawa K, Tobe T, Honjo I. Clinical classification of hepatoma in Japan according to serial changes in serum alpha-fetoprotein levels. Cancer 1982;49:354-360.
29. Yang X, Zhang Y, Zhang L, Mao J. Silencing alpha-fetoprotein expression induces growth arrest and apoptosis in human hepatocellular cancer cell. Cancer Lett 2008;271:281-293.
30. Mizejewski GJ. Alpha-fetoprotein structure and function: relevance to isoforms, epitopes, and conformational variants. Exp Biol Med (Maywood) 2001;226:377-408.
31. Smith JB. Occurrence of alpha-fetoprotein in acute viral hepatitis. Int J Cancer 1971;8:421-424.
32. Silver HK, Gold P, Shuster J, Javitt NB, Freedman SO, Finlayson ND. Alpha(1)-fetoprotein in chronic liver disease. N Engl J Med 1974;291:506-508.
33. Fujiyama S, Tanaka M, Maeda S, Ashihara H, Hirata R, Tomita K. Tumor markers in early diagnosis, follow-up and management of patients with hepatocellular carcinoma. Oncology 2002;62(Suppl 1):57-63.
34. Kudo T, Nakagawa H, Takahashi M, Hamaguchi J, Kamiyama N, Yokoo H, et al. N-glycan alterations are associated with drug resistance in human hepatocellular carcinoma. Mol Cancer 2007;6:32.
35. Tang Z, Varghese RS, Bekesova S, Loffredo CA, Hamid MA, Kyselova Z, et al. Identification of N-glycan serum markers associated with hepatocellular carcinoma from mass spectrometry data. J Proteome Res 2010;9:104-112.
36. Ding N, Nie H, Sun X, Sun W, Qu Y, Liu X, et al. Human serum N-glycan profiles are age and sex dependent. Age Ageing 2011;40:568-575.

J Gastroenterol (2013) 48:777–778
DOI 10.1007/s00535-013-0800-7

LETTER TO THE EDITOR

Serum HBV RNA as a possible marker of HBV replication in the liver during nucleot(s)ide analogue therapy

Masayuki Kurosaki · Kaoru Tsuchiya · Hiroyuki Nakanishi · Jun Itakura · Namiki Izumi

Received: 5 March 2013 / Accepted: 5 March 2013 / Published online: 30 March 2013

We read with interest the article by Tsuge et al. [1] published in the recent issue of the Journal of Gastroenterology. Treatment with nucleot(s)ide analogue (NUC) strongly suppresses the replication of hepatitis B virus (HBV) leading to a high rate of serum HBV DNA negativity. However, the incidence of relapse after the cessation of NUCs is high. Criterion for safe discontinuation of NUC therapy after long term therapy is not established to date. In HBe antigen positive patients, seroconversion, HBV DNA negativity and consolidation therapy of >6 months may be a consensus criteria but 30–50 % of patients fulfilling this criteria experience a relapse. In HBe antigen negative patients, NUC therapy is generally recommended until HBs antigen becomes undetected. Tsuge et al. [1] measured serum HBV RNA plus DNA by real time PCR and showed that the serum HBV DNA + RNA titer following 3 months of NUC treatment was a significant predictor of early (within 24 weeks) HBV DNA rebound after discontinuation of NUC. The serum HBV DNA + RNA titer was also associated with ALT rebound in HBe antigen positive patients. The results of the study by Tsuge et al. indicate that serum HBV DNA + RNA titer may serve as predictor of relapse after discontinuation of NUC.

The high rate of relapse after discontinuation of NUC is due to the persistence of HBV replication in the liver even during the NUC therapy. The replicative intermediate form of HBV, covalently closed circular DNA (cccDNA), may not be eliminated by NUC therapy and serves as a template for viral pre-genomic messenger RNA [2]. This concept was proved by a study showing that quantification of intrahepatic HBV cccDNA had a high accuracy of predicting sustained virological response after NUC discontinuation [3]. Still, we need a non-invasive and clinically usable marker for the assessment of HBV replication in the liver during NUC therapy. The measurement of HBV core related antigen may be an alternative [4]. The rationale of measuring HBV RNA in serum was that immature HBV particles including HBV RNA are released from hepatocytes during NUC treatment under the circumstances that pre-genomic HBV RNA are transcribed from HBV cccDNA, packaged into HBV core particles, but not reverse transcribed into plus-strand HBV DNA due to strong interference by NUC, and the excessive amounts of these immature particles are accumulated in hepatocytes [5, 6]. Tsuge et al. showed that serum HBV DNA + RNA titer following 3 months of NUC treatment was significantly lower in patients with no rebound of HBV DNA. By using a cut-off value of 4.8 log copies/mL, the cumulative incidence of HBV DNA rebound was significantly lower in patients with serum HBV DNA + RNA titer < 4.8 log at 3 months of NUC treatment. The same groups previously showed that HBV RNA levels at 3 months of lamivudine treatment were predictor of early emergence of resistant mutations [7]. Taken together, serum HBV DNA + RNA titer may be linked to the level of HBV replication in the liver during NUC therapy. Monitoring of serum HBV DNA + RNA response may be utilized in various decision makings in treatment of HBV patients with NUC therapy.

Based on these important findings, several questions may remain for future elucidation. Commercially available transcription-mediated amplification and hybridization assay

An answer to this letter to the editor is available at doi:10.1007/s00535-013-0801-6.

M. Kurosaki (✉) · K. Tsuchiya · H. Nakanishi · J. Itakura · N. Izumi
Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, 1-26-1 Kyonan-cho, Musashino-shi, Tokyo 180-8610, Japan
e-mail: kurosaki@musashino.jrc.or.jp

(TMA) detects both HBV DNA and RNA. We do recognize that detection sensitivity of this assay is not sensitive but could this assay be used in alternative to real time PCR? In the present study, duration of therapy was 36 weeks in average. The question is whether serum HBV DNA + RNA decrease further by a longer duration of therapy and whether monitoring of serum HBV DNA + RNA (at the end of treatment) serve as a predictor of safe discontinuation after long term NUC therapy. Various protocols of sequential interferon therapy starting with NUC are reported in an attempt to enhance the antiviral activity or to achieve drug-free status [8]. However, their outcome varies considerably and negative HBe antigen at the start of interferon is the only predictor of response [9]. Since 26 out of 36 patients in the study by Tsuge et al. received sequential interferon therapy, serum HBV DNA + RNA titer may be an alternative predictor of favorable response to sequential interferon therapy. Further investigation may be necessary to solve these issues but readers of the journal may be interested if comments can be made by the authors.

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

References

1. Tsuge M, Murakami E, Imamura M, Abe H, Miki D, Hiraga N, et al. Serum HBV RNA and HBeAg are useful markers for the safe discontinuation of nucleotide analogue treatments in chronic hepatitis B patients. J Gastroenterol. 2013. Epub 2013/02/12.
2. Moraleda G, Saputelli J, Aldrich CE, Averett D, Condreay L, Mason WS. Lack of effect of antiviral therapy in nondividing hepatocyte cultures on the closed circular DNA of woodchuck hepatitis virus. J Virol. 1997;71(12):9392–9. Epub 1997/11/26.
3. Sung JJ, Wong ML, Bowden S, Liew CT, Hui AY, Wong VW, et al. Intrahepatic hepatitis B virus covalently closed circular DNA can be a predictor of sustained response to therapy. Gastroenterology. 2005;128(7):1890–7. Epub 2005/06/09.
4. Kimura T, Rokuhara A, Sakamoto Y, Yagi S, Tanaka E, Kiyosawa K, et al. Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. J Clin Microbiol. 2002;40(2):439–45. Epub 2002/02/05.
5. Su Q, Wang SF, Chang TE, Breitkreutz R, Hennig H, Takegoshi K, et al. Circulating hepatitis B virus nucleic acids in chronic infection: representation of differently polyadenylated viral transcripts during progression to nonreplicative stages. Clin Cancer Res. 2001;7(7):2005–15. Epub 2001/07/13.
6. Zhang W, Hacker HJ, Tokus M, Bock T, Schroder CH. Patterns of circulating hepatitis B virus serum nucleic acids during lamivudine therapy. J Med Virol. 2003;71(1):24–30. Epub 2003/07/15.
7. Hatakeyama T, Noguchi C, Hiraga N, Mori N, Tsuge M, Imamura M, et al. Serum HBV RNA is a predictor of early emergence of the YMDD mutant in patients treated with lamivudine. Hepatology. 2007;45(5):1179–86. Epub 2007/04/28.
8. Yokosuka O, Kurosaki M, Imazeki F, Arase Y, Tanaka Y, Chayama K, et al. Management of hepatitis B: consensus of the Japan Society of Hepatology 2009. Hepatol Res. 2011;41(1):1–21. Epub 2010/11/13.
9. Enomoto M, Nishiguchi S, Tamori A, Kobayashi S, Sakaguchi H, Shiomi S, et al. Entecavir and interferon-alpha sequential therapy in Japanese patients with hepatitis B e antigen-positive chronic hepatitis B. J Gastroenterol. 2012. (Epub 2012/08/02).

Hepatology Research

Hepatology Research 2013 doi: 10.1111/hepr.12254

Original Article

Hepatic oxidative stress in ovariectomized transgenic mice expressing the hepatitis C virus polyprotein is augmented through suppression of adenosine monophosphate-activated protein kinase/proliferator-activated receptor gamma co-activator 1 alpha signaling

Yasuyuki Tomiyama, Sohji Nishina, Yuichi Hara, Tomoya Kawase and Keisuke Hino

Department of Hepatology and Pancreatology, Kawasaki Medical School, Kurashiki, Japan

Aim: Oxidative stress plays an important role in hepatocarcinogenesis of hepatitis C virus (HCV)-related chronic liver diseases. Despite the evidence of an increased proportion of females among elderly patients with HCV-related hepatocellular carcinoma (HCC), it remains unknown whether HCV augments hepatic oxidative stress in postmenopausal women. The aim of this study was to determine whether oxidative stress was augmented in ovariectomized (OVX) transgenic mice expressing the HCV polyprotein and to investigate its underlying mechanisms.

Methods: OVX and sham-operated female transgenic mice expressing the HCV polyprotein and non-transgenic littermates were assessed for the production of reactive oxygen species (ROS), expression of inflammatory cytokines and antioxidant potential in the liver.

Results: Compared with OVX non-transgenic mice, OVX transgenic mice showed marked hepatic steatosis and ROS production without increased induction of inflammatory cytokines, but there was no increase in ROS-detoxifying enzymes such as superoxide dismutase 2 and glutathione peroxidase 1. In accordance with these results, OVX transgenic mice showed less activation of peroxisome proliferator-activated receptor-γ co-activator-1α (PGC-1α), which is required for the induction of ROS-detoxifying enzymes, and no activation of adenosine monophosphate-activated protein kinase-α (AMPKα), which regulates the activity of PGC-1α.

Conclusion: Our study demonstrated that hepatic oxidative stress was augmented in OVX transgenic mice expressing the HCV polyprotein by attenuation of antioxidant potential through inhibition of AMPK/PGC-1α signaling. These results may account in part for the mechanisms by which HCV-infected women are at high risk for HCC development when some period has passed after menopause.

Key words: antioxidant potential, glutathione peroxidase, reactive oxygen species, superoxide dismutase

INTRODUCTION

PERSISTENT HEPATITIS C virus (HCV) infection is a major risk factor for the development of hepatocellular carcinoma (HCC) in Japan. Approximately 70% of Japanese HCC patients are currently diagnosed with HCV-associated cirrhosis or chronic hepatitis C.[1] Nevertheless, the mechanisms underlying HCV-associated hepatocarcinogenesis are incompletely understood. Notably, there is sex disparity in HCC development, that is, male sex has been demonstrated to be an independent risk factor associated with HCC development.[2–4] It is proposed that estrogen-mediated inhibition of interleukin (IL)-6 production by Kupffer cells reduces the HCC risk in females.[5] In addition, the proportion of females among elderly patients with HCV-related HCC has recently increased in Japan.[6] These results suggest that menopause may be a risk factor associated with HCC development in female patients with HCV infection.

Numerous studies have shown that oxidative stress is present in chronic hepatitis C to a greater degree than in other inflammatory disease,[7,8] and is related to

Correspondence: Professor Keisuke Hino, Department of Hepatology and Pancreatology, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192, Japan. Email: khino@med.kawasaki-m.ac.jp

Received 9 September 2013; revision 24 September 2013; accepted 30 September 2013.

hepatocarcinogenesis in HCV-associated chronic liver diseases.[9,10] We have previously demonstrated that transgenic mice expressing the HCV polyprotein develop liver tumors including HCC, in connection with oxidative stress induced by HCV and iron overload.[11] Interestingly, such hepatocarcinogenesis was observed only in male transgenic mice, suggesting that females are resistant to oxidative stress in these transgenic mice. On the other hand, it is reported that ovariectomy increases nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity[12] and decreases mitochondrial-reduced glutathione levels in rats.[13] However, it remains unknown how HCV affects ovariectomy-induced oxidative stress. Investigation of this issue may provide a clue for understanding why the incidence of HCC increases in elderly postmenopausal women with HCV infection. The aim of this study was to determine whether HCV proteins amplify oxidative stress induced by ovariectomy and to investigate the mechanisms underlying this.

METHODS

Animals

CONTAINING THE FULL-LENGTH polyprotein-coding region under the control of the murine albumin promoter/enhancer, the transgene pAlbSVPA-HCV has been described in detail.[14,15] Of the four transgenic lineages with evidence of RNA transcription of the full-length HCV-N open reading frame (FL-N), the FL-N/35 lineage proved capable of breeding in large numbers. There is no inflammation in the transgenic liver.[15]

Experimental design

Female FL-N/35 transgenic mice and their normal female C57BL/6 littermates were anesthetized for surgery and underwent either a bilateral ovariectomy or sham operation at the age of 4–6 weeks. We studied ovariectomized (OVX) transgenic mice ($n = 5$), sham-operated transgenic mice ($n = 5$), OVX non-transgenic mice ($n = 5$) and sham-operated non-transgenic mice ($n = 5$). These mice were fed a normal rodent diet, bred, maintained, and killed by i.p. injection of 10% pentobarbital sodium preceded by 20-h fasting at the age of 24 weeks. All experimental protocols and animal maintenance procedures used in this study were approved by the Ethics Review Committee for Animal Experimentation of Kawasaki Medical School.

Histological procedures

A portion of liver tissue was immediately snap-frozen in liquid nitrogen for determination of the hepatic triglyceride concentration. The remaining liver tissue was fixed in 4% paraformaldehyde in phosphate-buffered saline and embedded in paraffin for histological analyses. Liver sections were stained with hematoxylin–eosin.

Serum leptin concentration

The serum leptin level was measured using a Rat Leptin Elisa kit (Morinaga Institute of Biological Science, Yokohama, Japan) according to the manufacturer's instructions.

Hepatic triglyceride content

Lipids were extracted from the homogenized liver tissue by the method of Bligh and Dyer.[16] The triglyceride level was measured with a TGE-test Wako kit (Wako Pure Chemicals, Tokyo, Japan), according to the manufacturer's instructions. Protein concentrations in liver were determined by the method of Lowry *et al.*,[17] using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

In situ detection of reactive oxygen species (ROS)

In situ ROS production in the liver was assessed by staining with dihydroethidium, as described previously.[18] In the presence of ROS, dihydroethidium (Invitrogen, Carlsbad, CA, USA) is oxidized to ethidium bromide and stains nuclei bright red by intercalating with the DNA.[19] Fluorescence intensity was quantified using National Institutes of Health image analysis software for 3 randomly selected areas of digital images for each mouse.

Hepatic iron content

Hepatic iron content was measured by atomic absorption spectrometry, as described previously,[11] and expressed as micrograms Fe per gram of tissue (wet weight).

Derivatives of reactive oxygen metabolites (dROM) and biological antioxidant potential (BAP)

The levels of dROM and BAP were measured using a Free Radical Elective Evaluator (Wismerll, Tokyo, Japan), as described previously.[20] Measurement of dROM is based on the ability of the transition metal ions to catalyze the formation of alkoxy and peroxy radicals from hydroper-

oxides present in serum. The results are expressed in conventional units as Carrtelli units (U.CARR), where 1 U.CARR corresponds to 0.8 mg/L H_2O_2. Measurement of BAP is based on the ability of antioxidants to reduce ferric (F^{3+}) ions to ferrous (Fe^{2+}) ions.

RNA isolation and real-time reverse transcription polymerase chain reaction (RT–PCR)

Total RNA was isolated using an RNeasy mini kit (QIAGEN, Hilden, Germany) and reverse-transcribed into cDNA by using a Superscript III reverse transcription kit (Invitrogen). The PCR reactions were run in the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster, CA, USA). The levels of mRNA were determined using cataloged primers (Applied Biosystems) for mice (tumor necrosis factor [TNF]-α, Mm00443258_m1; IL-1β, Mm00434228_m1; IL-6, Mm00446190_m1; HAMP [gene encoding hepcidin], Mm00519025_mL; superoxide dismutase 2 [SOD2], Mm01313000_m1; glutathione peroxidase 1 [GPx1], Mm00656767_g1; and sirtuin 3 [SIRT3], Mm00452131_m1). Expression of these genes was normalized to expression of glyceraldehyde 3-phosphate dehydrogenase mRNA (GAPDH, Mm99999915_g1).

Isolation of mitochondria and nuclear fraction

Mitochondrial extraction from liver tissue was performed using a Qproteome Mitochondrial Isolation kit (QIAGEN) according to the manufacturer's instructions. The nuclear fraction from liver tissue was prepared using a Nuclear Extraction kit (Panomics, Fremont, CA, USA) according to the manufacturer's instructions.

Immunoblotting

Liver lysates and the mitochondrial and nuclear fractions from liver were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bradford, MA, USA), blocked overnight at 4°C with 5% skim milk and 0.1% Tween-20 in Tris-buffered saline, and subsequently incubated for 1 h at room temperature with goat antihuman SOD2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit antihuman GPx1 antibody (Abcam, Cambridge, MA, USA), rabbit antihuman SIRT3 antibody (Abcam), rabbit antihuman peroxisome proliferator-activated receptor-γ co-activator-1α (PGC-1α) antibody (Abcam), rabbit antihuman adenosine monophosphate-activated protein kinase-α (AMPKα) antibody (Cell Signaling Technology, Boston, MA, USA), rabbit antihuman phospho-AMPKα (Thr172) antibody (Cell Signaling Technology), rabbit antihuman mitochondrial heat shock protein 70 antibody (HSP70; Thermo Scientific, Rockford, IL, USA), rabbit antihuman β-actin antibody (Cell Signaling Technology) or rabbit antimouse lamin B1 antibody (Abcam). The membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated donkey antigoat immunoglobulin (Ig)G (Santa Cruz Biotechnology) or HRP-conjugated donkey antirabbit IgG (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

Statistical analysis

Quantitative values are expressed as mean ± standard deviation. Two groups among multiple groups were compared by the rank-based Kruskal–Wallis ANOVA test followed by Scheffé's test. The statistical significance of correlation was determined by the use of simple regression analysis. $P < 0.05$ was considered to be significant.

RESULTS

Ovariectomy enhanced hepatic steatosis in FL-N/35 transgenic mice

AS CONFIRMATION OF successful ovariectomy-induced suppression of endogenous estrogen production, the uterine weight of OVX mice was significantly decreased compared with that of sham-operated mice (Table 1). Dietary intake, bodyweight, liver weight and serum leptin levels were significantly greater in OVX mice than in sham-operated mice regardless of whether they were transgenic or non-transgenic (Table 1). Interestingly, the serum alanine aminotransferase (ALT) level was significantly higher in OVX transgenic mice than in mice in the other three groups, but the levels were comparable in OVX non-transgenic and sham-operated non-transgenic mice (Table 1). To determine why OVX transgenic mice have a higher ALT level, we investigated the liver histology of the mice in the four groups (OVX transgenic, sham-operated transgenic, OVX non-transgenic and sham-operated non-transgenic mice). In contrast to the mild to moderate degree of hepatic steatosis noted in OVX non-transgenic mice and sham-operated transgenic mice, OVX transgenic mice developed severe hepatic steatosis (Fig. 1a) without infiltration of inflammatory mononuclear cells. Hepatic triglyceride content was measured to quantify the degree of steatosis. The triglyceride content was significantly greater in OVX transgenic mice than in mice in the other three groups (Fig. 1b), which was consistent with the

Table 1 Body, liver and uterus weight and serum biochemical parameters

Body, liver, and uterus weight and serum biochemical parameters	Non-transgenic		Transgenic	
	Sham-operated	OVX	Sham-operated	OVX
Bodyweight (g)	21.5 ± 1.2	30.7 ± 4.9*	27.7 ± 4.6	34.2 ± 3.8**
Liver weight (g)	0.86 ± 0.075	1.09 ± 0.236*	0.90 ± 0.102	1.18 ± 0.156**
Ratio of liver to bodyweight	0.038 ± 0.037	0.035 ± 0.003	0.031 ± 0.002	0.034 ± 0.006
Uterus weight (g)	0.08 ± 0.01	0.01 ± 0.02*	0.09 ± 0.01	0.01 ± 0.01**
Total dietary intake (g)	337 ± 24	429 ± 13*	368 ± 28	490 ± 31**
Serum glucose (mg/dL)	222.9 ± 110.0	275.1 ± 121.4	284.0 ± 84.1	259.7 ± 108.9
Serum ALT (IU/L)	15.5 ± 6.5	30.6 ± 38.1	21.8 ± 11.4	281.2 ± 165.1***
Serum triglyceride (mg/dL)	99.9 ± 9.7	78.9 ± 10.8	98.3 ± 11.4	89.7 ± 13.3
Serum leptin (ng/mL)	0.45 ± 0.14	1.31 ± 0.31*	0.65 ± 0.22	1.60 ± 0.28**

Data are mean ± standard deviation.
*$P < 0.05$ compared with sham-operated non-transgenic mice. **$P < 0.05$ compared with sham-operated transgenic mice. ***$P < 0.01$ compared with mice in the other three groups.
ALT, alanine aminotransferase; OVX, ovariectomized.

results for hepatic steatosis. Thus, the increase in the serum ALT level in the OVX transgenic mice was thought to reflect the hepatic steatosis.

Ovariectomy increased ROS and IL-6 production in the liver

Only OVX transgenic mice showed marked hepatic steatosis, regardless of the comparable diet intake and the ratio of liver to bodyweight of OVX non-transgenic mice (Table 1). We have previously demonstrated that iron-overloaded male FL-N/35 transgenic mice expressing the HCV polyprotein develop severe hepatic steatosis through increased ROS production.[11] Therefore, we examined whether ROS production was relevant to the marked hepatic steatosis observed in the OVX transgenic mice. Ovariectomy significantly increased ROS (super-

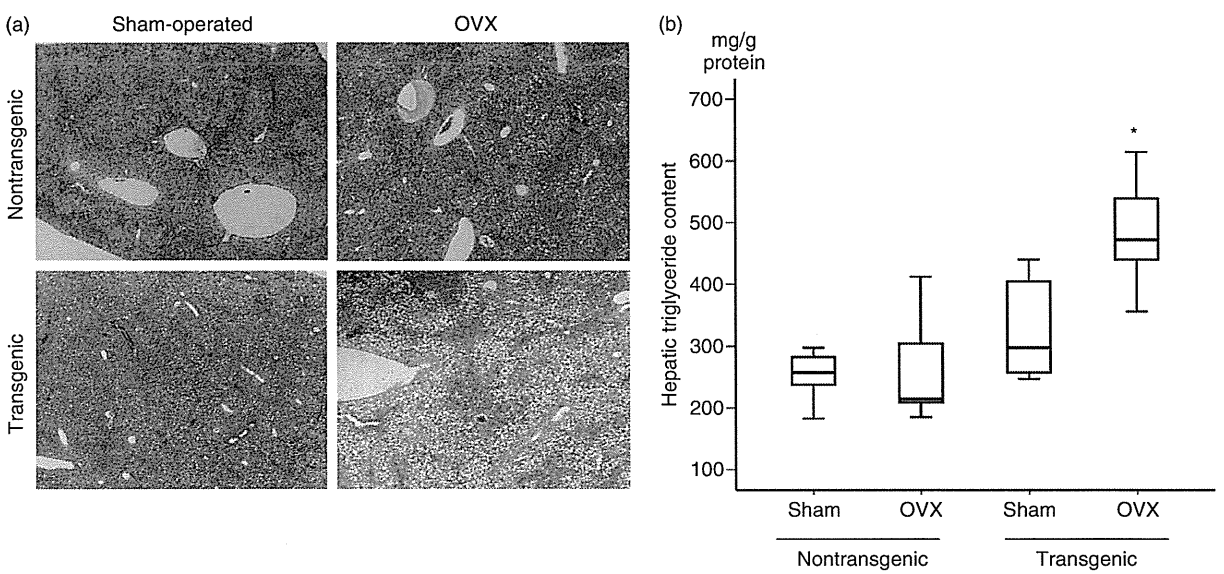


Figure 1 Hepatic steatosis and triglyceride content in sham-operated and ovariectomized (OVX) FL-N/35 transgenic and non-transgenic mice. (a) Hepatic steatosis in mice in each group (H&E, original magnification × 100). (b) Hepatic triglyceride content in mice in each group ($n = 5$). The results are shown as box plot profiles. The bottom and top edges of the boxes are the 25th and 75th percentiles, respectively. Median values are shown by the line within each box. *: $P < 0.05$ versus mice in the other three groups.