Use of targeted glycoproteomics to identify serum glycoproteins that correlate with liver cancer in woodchucks and humans

Timothy M. Block*†, Mary Ann Comunale*, Melissa Lowman*, Laura F. Steel*, Patrick R. Romano*, Claus Fimmel‡, Bud C. Tennant§, W. Thomas London¶, Alison A. Evans¶, Baruch S. Blumberg¶‖, Raymond A. Dwek‖, Tajinder S. Mattu*, and Anand S. Mehta*†

*Drexel Institute for Biotechnology and Virology Research, Drexel University, Doylestown, PA 18901; ‡Division of Gastroenterology, Saint Louis University School of Medicine, John Cochran Veterans Affairs Medical Center, St. Louis, MO 63106; §Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853; ¶Fox Chase Cancer Center, Philadelphia, PA 19111; and ‖Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, United Kingdom

Contributed by Baruch S. Blumberg, December 7, 2004

Chronic infection with hepatitis B virus (HBV) is associated with the majority of hepatocellular carcinoma (HCC). The diagnosis of HCC is usually made in the late stages of the disease, when treatment options are limited and prognosis is poor. We therefore have developed a method of glycoproteomic analysis in an attempt to discover serum markers that can assist in the early detection of HBV-induced liver cancer. Briefly, a comparative method for analysis of oligosaccharides released from serum glycoproteins and for recovery and identification of proteins with aberrant glycosylation, as a function of cancer diagnosis, is described. The model we have used is the woodchuck (*Marmota monax*), which shares similarities in the glycosylation pattern associated with liver proteins in human HCC. In this report, we show that woodchucks diagnosed with HCC have dramatically higher levels of serum-associated core α-1,6-linked fucose, as compared with woodchucks without a diagnosis of HCC. The coupling of this methodology with 2D gel proteomics has permitted the identification of several glycoproteins with altered glycosylation as a function of cancer. One such glycoprotein, Golgi Protein 73 (GP73), was found to be elevated and hyperfucosylated in animals with HCC. Further, the study showed GP73 to be elevated in the serum of people with a diagnosis of HCC, providing a validation of our approach. The potential of this technology for biomarker discovery and the implications of increased levels of GP73 in liver cancer are discussed.

glycomics | hepatitis B virus | hepatocellular carcinoma | proteomics

Despite being fifth in cancer incidence worldwide, hepatocellular carcinoma (HCC) is the third leading cause of cancer death (1, 2). The high mortality associated with HCC is partly due to unresponsiveness to treatment, with a 5-year survival rate after diagnosis of <5% (1). As therapeutic options increase, early detection of HCC is important to improving the prognosis (3). Because the major cause of HCC is chronic infection with either hepatitis B virus (HBV) or hepatitis C virus (HCV), high-risk populations can be monitored for biomarkers of disease, as these markers become available. Moreover, the long latency between infection and disease onset in this high-risk population provides an opportunity for early detection well before the onset of advanced disease.

Currently, disease status is most often monitored by physical examination of the patient, ultrasound imaging of the liver, and analysis of serum for a panel of markers (2). Because there is a correlation between elevated levels of α-fetoprotein (AFP) and the occurrence of HCC, determination of AFP levels is often included as a serum marker of disease (4). However, AFP as a sole indicator of HCC is of limited value, often being elevated in the absence of serious disease and not elevated in as many as 50% of liver cancers (5). Its value in the detection of HCV-associated HCC is even less clear. Nevertheless, even the limited correlation between AFP and HCC underscores the value of serum as a source of biomarkers of liver disease.

To identify serum biomarkers of HCC, we have developed a targeted glycoproteomic approach. That is, we have used a semiquantitative method to identify alterations in the glycosylation profile of serum derived from those with HCC compared with healthy subjects and to identify the glycoproteins with the altered glycosylation by 2D electrophoresis (2DE)-based proteomics.

The literature reports many biomolecular changes that occur during the development of HCC, including glycosylation (6–11). The most notable change in glycosylation is an increase in the level of fucosylation of AFP (12–16). Although the molecular mechanism of increased fucosylation in HCC is not clear (17, 18), it is known that the increase is not restricted to AFP (19, 20). However, a comprehensive comparative analysis of all of the fucosylated glycoproteins in HCC patients has not been performed. This type of study has been limited because of the absence of a suitable technology to allow the examination of large pools of unknown proteins. With the advent of sensitive glycan analysis and proteomic technologies, the ability to comprehensively identify all of the fucosylated proteins in patients with HCC and to identify those proteins for the development of diagnostic markers is now a possibility.

Woodchucks (*Marmota monax*) infected with woodchuck hepatitis virus (WHV), a hepadnavirus related to the human HBV, develop HCC (reviewed in ref. 2). Here, we report the results of a study with woodchucks chronically infected with WHV with and without a diagnosis of HCC. The sera of animals diagnosed with cancer were found to posses dramatically higher levels of core α-1,6-linked fucose. In addition, this methodology has allowed for the identification of several polypeptides that appear to possess a glycosylation profile that altered as a function of cancer. One such glycoprotein, Golgi Protein (GP73), was found to be elevated and hyperfucosylated in animals with HCC. Further analysis of human serum from different individuals with varying disease states confirmed the specific association of GP73 with the development of HCC. GP73 is a resident Golgi membrane protein that has been reported to be up-regulated, on the basis of immunostaining, in the hepatocytes (but not circulation) of those with liver disease, including hepatitis virus infection

Abbreviations: AFP, α-fetoprotein; CRC, colorectal cancer; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; WHV, woodchuck hepatitis virus; 2DE, 2D electrophoresis.

†To whom correspondence may be addressed at: Drexel Institute of Biotechnology and Virology Research, 700 East Butler Avenue, Doylestown, PA 18901. E-mail: tim.block@drexel.edu or anand.mehta@drexel.edu.

(21). Although its function has yet to be elucidated, its expression profile suggests that GP73 may serve as a biomarker of liver cancer. The potential of coupling glycan profiling to 2DE in the discovery of disease biomarkers in general and of GP73 as a correlate of liver cancer in particular is discussed.

Materials and Methods

Human Subjects. Archived human serum samples from two sources were used for the analysis of GP73. HBV and HCC serum samples were provided by W.T.L. Sera from patients diagnosed with colorectal cancer (CRC) were obtained from Ying-Hsiu Su (Drexel University), through collaboration with the Early Detection Research Network of the National Cancer Institute and Dean Brenner (University of Michigan, Ann Arbor, MI). HBV infection status was based on HBV DNA detection, hepatitis B surface antigen (HBsAg), and HBV "*e*" antigen (HBeAg). Detailed descriptions of the HBV clinical categorization are in ref. 22. Diagnosis of HCC was confirmed by ultrasound as described in ref. 22. Diagnosis of CRC was made by biopsy (23). HBV-, HCV-, and HIV-negative human serum was purchased from Sigma.

Woodchucks and Woodchuck Serum. Experimental laboratory-bred woodchucks were maintained in the College of Veterinary Medicine facilities of Cornell University (Ithaca, NY). Chronic infection with WHV resulted from neonatal infection with WHV strain 7P1 and was confirmed by WHV surface antigen (WHsAg) assay for envelope protein in the serum and by dot blot of serum for WHV-specific DNA (24). HCC was determined by ultrasound and confirmed by biopsy or autopsy. Serum γ-glutamyl transferase levels were performed as described in ref. 25.

Glycan and glycoproteomic analysis was performed on serum from 2- to 3-year-old male and female woodchucks that were either uninfected or chronically infected with WHV. Both the normal and HCC group contained three animals that were 2 years old and two animals that were 2 years old. Infected woodchucks without HCC were all 1 year old (26). Woodchuck serum samples were provided, coded, by B.C.T. and only decoded after the completion of analysis.

Glycan Analysis. N-linked glycans were removed from total serum with peptide *N*-glycosidase F and the released glycans fluorescently labeled at their reducing end with 2-aminobenzamide using the Signal Labeling Kit (LudgerClean H, Ludger, Oxford) as described in refs. 21 and 22. Glycans were analyzed subsequently by normal-phase HPLC. Glycan structures were identified by comparison with known standards and by sequential exoglycosidase digestion (21). HPLC analysis was performed by using the Waters Alliance HPLC System and quantified by using the Millennium Chromatography Manager (Waters).

Lectin Extraction and Analysis. Immunoglobulins (Igs) were removed from the samples (media and serum) by using a Protein A/G column (Pierce) before the lectin extraction. Samples were resuspended in 20 mM Tris-buffered saline (pH 7.0), 1 mM calcium chloride, 1 mM magnesium chloride, and 1 mM manganese chloride and incubated for 16 h at 4°C with an array of fucose recognizing lectins from *Lens culinaris, Pisum sativum*, and *Vicia faba*. The lectin column was washed thoroughly with lectin-binding solution before the bound fraction was eluted by using the appropriate inhibitory monosaccharides (200 mM methyl-α-D-glucopyranoside/200 mM α-methyl-D-mannopyranoside). The bound and unbound fractions were buffer-exchanged into TBS by using Milllipore YM-3 Centricon devices and subjected to glycan analysis or 2DE as described. Protein levels were monitored throughout all extractions.

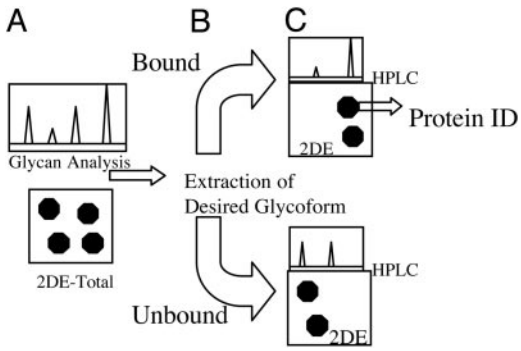

Fig. 1. A system for the identification of altered glycoproteins in cancer. (*A*) Glycan sequencing of an aliquot of total serum is performed to determine which glycoform(s) to target. 2DE is performed on a separate aliquot of total protein. (*B*) Affinity-mediated extraction using lectin chromatography of sample and fractionation of sample into a bound (fucosylated) and unbound (nonfucosylated) sample and analysis with 2DE. (*C*) Extraction efficiency can be determined by HPLC-based analysis of unbound fraction. Features of interest can be identified by mass spectroscopy. See *Materials and Methods* for details.

2DE and Gel Imaging and Analysis. 2DE and analysis were performed as described in ref. 27. Gels were digitally imaged by using a 16-bit cooled charge-coupled device camera (FluorChem 8000, Alpha Innotech, San Leandro, CA) and analyzed by using the NONLINEAR DYNAMICS PROGENESIS WORKSTATION gel imaging software package (Nonlinear USA, Durham, NC). Polypeptide features were normalized by using the integrated intensity of each feature and expressing it as a percentage of the sum of integrated intensities of the entire gel. Spot identification was performed with mass spectroscopy as described in ref. 22.

Immunoblot Analysis for GP73. Equal volumes of patient sera (0.5 μl per lane) were resolved by SDS/PAGE on 4–20% polyacrylamide gradient gels, and GP73 was detected as described in ref. 21. Densitometric analysis of the immunoblots was performed to quantify the amounts of GP73 protein in patient sera relative to the signal present in the Sigma control standard. The signal for the Sigma control was set to a value of 1.0. GP73 specific signals from the 73-kDa species were quantified from x-ray films by using an Alpha Innotech FluorChem charge-coupled device camera with ALPHAEASE spot densitometry software (Alpha Innotech) and expressed as integrated intensity units relative to the GP73 signal detected in Sigma control serum standard. To assess the reproducibility of the assay, all samples were tested in triplicate, and GP73 concentration was calculated as the mean of triplicate determinations for each serum sample. There was $<10\%$ variation in sample-to-sample analysis.

Results

System for the Identification of Altered Glycoproteins in Cancer. To identify glycan modifications of interest in clinical samples, and the N-linked glycosylated polypeptides that contain these modifications, a system of glycan profiling followed by polypeptide identification was developed. This system is outlined in Fig. 1. The methods can be applied to any samples but are used in this study for analysis of serum samples from those with and without a diagnosis of HCC. Briefly, polypeptides from a sample are subjected to chemical or enzymatic treatment to release N-glycans (as in *Materials and Methods*). The N-glycan pools, representing contributions from all glycoproteins present in the sample, are resolved by neutral-phase HPLC. This procedure provides a guide to N-glycan structures of interest, shown as peaks in Fig. 1*A*, because each peak corresponds to a specific glycan structure. If a peak appears to be specific to sample sets,

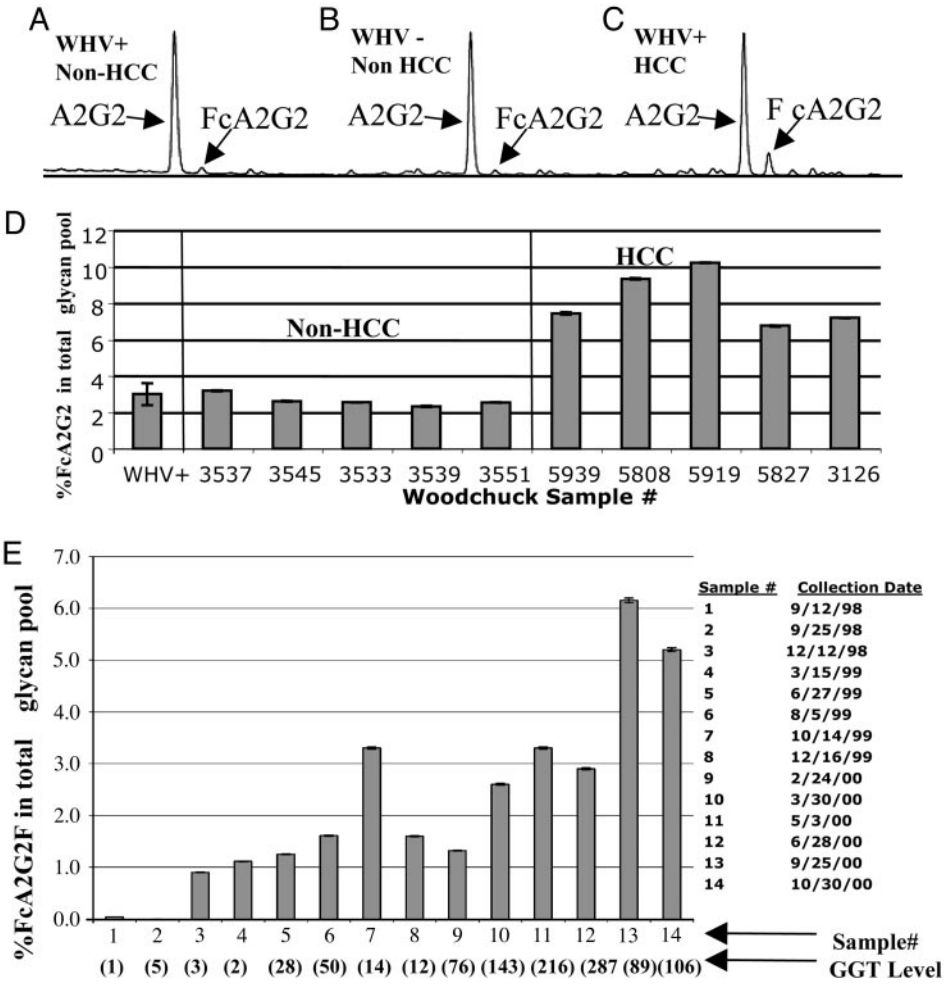

Fig. 2. The level of FcA2G2 glycan in normal, WHV-infected woodchucks and WHV-infected woodchucks that have developed HCC. (*A*) Glycan profile from a representative infected (WHV+) non-HCC woodchuck. (*B*) Glycan profile from a representative normal (WHV−) non-HCC woodchuck. (*C*) Glycan analysis of a representative HCC+ (WHV+) woodchuck. For *A–C*, the major glycan species are indicated. (*D*) Average amounts (as percentage of total detected glycan) of the FcA2G2 structure in 31 woodchucks that are virally infected (labeled as WHV+) or from 10 individual woodchucks either with or without cancer (labeled by their four-digit identifier on the *x*-axis). The *y*-axis is the percent of FcA2G2 structure in each woodchuck as a function of total released glycan. (*E*) Glycan analysis of chronically infected woodchuck 97-13 over a period of 2 years. The levels of the FcA2G2 structure as a function of the total glycan pool minus the average of the normal woodchucks is indicated on the *y*-axis, and the sample identifier is provided on the *x*-axis. The time of collection is provided for each sample. The levels of γ-glutamyl transferase (GGT) in units per liter are given below the sample number.

such as serum samples from those with liver cancer, affinity chromatography to "extract" glycoproteins containing the glycan structures of interest is implemented, as indicated in Fig. 1*B*. Lectins are the usual affinity reagent used for the extraction of specific glycoproteins. An aliquot of the sample that bound the affinity ligand (called "bound") is subjected to chemical or enzymatic methods to release N-glycans, followed by neutral-phase chromatography, to confirm that the affinity chromatography successfully recovered the glycan structures of interest (Fig. 1*C*). The balance of the sample from the affinity purification of the glycoproteins is resolved by 2DE (Fig. 1*C*), and polypeptides of interest are recovered and identified by immunological or biochemical methods. The "unbound" sample is the component of the sample that did not bind to the affinity reagent and is resolved in parallel with the bound to permit determination of the efficiency of recovery by the affinity reagent.

Glycan Analysis of Serum Glycoproteins from Woodchucks With and Without HCC. The approach described above was applied to disease-related samples by using the woodchuck model of HBV-induced HCC. WHV is a HBV-like virus that infects and causes chronic hepatitis infection in woodchucks (24, 25). Chronic infection of neonatal woodchucks with WHV results in HCC in almost all animals, after a period of years (25). Thus, the woodchuck has been a very attractive model to study hepadnavirus disease. We reasoned that WHV-induced HCC in woodchucks would be a good place to gain a proof of concept of our system for discovery of HCC biomarkers.

Fig. 2 shows the total serum glycoprotein glycan analysis from a representative infected woodchuck without evidence of disease (Fig. 2*A*), an uninfected woodchuck without evidence of disease (Fig. 2*B*), and a representative infected woodchuck that had developed HCC (Fig. 2*C*). As Fig. 2 shows, the serum from animals without evidence of disease (Fig. 2 *A* and *B*) possessed very little of the core fucosylated α-1,6-linked biantennary glycan structure (FcA2G2). This result was true for each of the 31 infected and 5 noninfected woodchucks without disease tested, as summarized in Fig. 2*D*, where quantification of the FcA2G2 peak for each sample is shown. These data are consistent with previous studies of the glycan profile of the serum of woodchucks without HCC (26, 28) and were independent of the age of the animal (data not shown and Fig. 2*F*). In contrast, the

animal shown in Fig. 2*C* that developed HCC had a much greater level of the FcA2G2 structure. Again, this result was true for all five of the animals with HCC that were tested, as shown in Fig. 2*D* (HCC), where quantification of the FcA2G2 peak is presented for each sample. Whereas animals without cancer had an average of 2.64% (± 0.33%) of the FcA2G2 structure, animals with HCC had an average of 8.18% (± 1.51%) of the FcA2G2 structure. This difference was statistically different with $P = 0.0008$. Because these woodchucks did not develop cirrhosis (data not shown), the increase in fucosylation appears to be related directly to the development of HCC. This finding is consistent with prior work highlighting the fucosylation of AFP in the woodchuck model of cancer (29).

Because the woodchucks had been under observation since infection, it was possible to study the amount of FcA2G2 in the circulation as a function of time after infection and up to development of HCC. Fig. 2*E* shows the amount of FcA2G2 derived from the serum of a single woodchuck (no. 97–13) that developed HCC over a period of 2 years. This animal was born on June 27, 1997, and was experimentally infected with WHV shortly after birth. Glycan analysis over time showed a steady rise in the level of the FcA2G2 structure with levels peaking on September 25, 2000. The animal was killed shortly after the last time point, October 30, 2000, because of the tumor burden. The levels of γ-glutamyl transferase are indicated in Fig. 2, and values >10 units/liter are indicative of the development of cancer (24). As Fig. 2*E* shows, the most dramatic rise in the level of the FcA2G2 structure came after the rise in γ-glutamyl transferase. It is also noted that examination of serial samples from noninfected animals showed no change in fucosylation as a consequence of time (data not shown).

Identification of Fucosylated Glycoproteins in Woodchucks with Cancer. Because it was observed that the sera of HCC woodchucks contained glycoproteins with an increased amount of fucosylation, it was of interest to determine the identity of specific glycoproteins containing fucosylation. Glycoproteins containing fucose were extracted from Ig-depleted serum samples with the lectins *L. culinaris*, *P. sativum*, and *V. faba,* which recognize core fucosylated glycoproteins (22). In contrast to human Igs, woodchuck Igs are not fucosylated. Nevertheless, all serum was depleted of Igs to establish a protocol that could be used for analysis of non-Ig associated fucosylated glycan from either animal or human serum. Fig. 3*A* shows the serum 2DE profile from a representative woodchuck with HCC (no. 5919 from Fig. 2) after removal of the Igs by using a protein A/G column. Fig. 3*B* shows the 2DE profile from both the unbound and bound fractions from same woodchuck after lectin extraction. As Fig. 3 shows, there are many glycoproteins that are fucosylated in the serum of woodchucks that have cancer. The efficiency of extraction was measured by glycosylation analysis of the bound and unbound fraction (Fig. 3*C*) and was determined to be >95%.

A comparative analysis of the fucosylated proteome from a representative non-HCC woodchuck (no. 3533 in Fig. 2) and a HCC-positive woodchuck (no. 5919 in Fig. 2) is shown in Fig. 4. As Fig. 4 *A* and *B* shows, consistent with the glycan analysis in Fig. 2, a larger number of proteins appear to be fucosylated in the HCC sample as compared with the non-HCC sample. Although features of interest can be seen throughout the gel, specific areas of interest are shown in the circled areas.

Identification of woodchuck proteins is difficult because of the limited information about the woodchuck proteome and genome. However we have identified several proteins of interest by *de novo* sequencing and immunoblot. For example, we have determined by immunoblot, using rat-specific antibodies, that α-1-acid glycoprotein, AFP, α-1-antitrypsin, and α-1-antichymotrypsin are hyperfucosylated in woodchucks that have cancer (circled areas). Hyperfucosylation (in cancer vs. noncancer samples) of these polypeptides was somewhat expected, based on reports in the literature that used human samples (20).

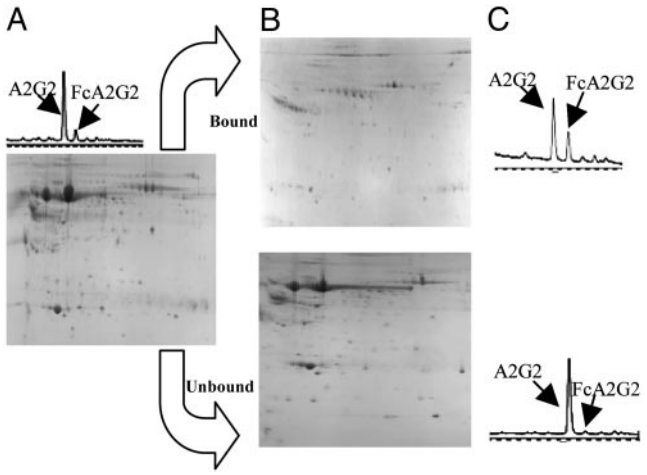

Fig. 3. Lectin extraction and glycan analysis of fractionated glycoproteins from the serum of a representative woodchuck with cancer. (*A*) Glycan analysis and 2DE of the Ig-depleted serum proteins. (*B*) Lectin fractionation of the Ig-depleted serum showing the bound (fucosylated) and unbound (nonfucosylated) glycoproteins resolved by means of 2DE. (*C*) The glycan profiles from each fraction are shown on the right of the 2DE and highlight the extraction efficiency (>95%).

However, one of the fucosylated glycoproteins observed to be up-regulated in HCC-positive woodchucks was not expected. It had amino acid sequence homology to a human glycoprotein, GP73, along with a similar molecular weight and pI (21). Because GP73 is a resident Golgi protein that had been reported previously to be associated with HBV- and HCV-infected hepatocytes (30), our interest was heightened. Immunoblot analysis with hyperimmune serum specific for human GP73 on the fucosylate fraction confirmed the initial finding, and the results are shown in Fig. 4 *C* and *D*. As Fig. 4 *C* and *D* shows, the amount of reactive

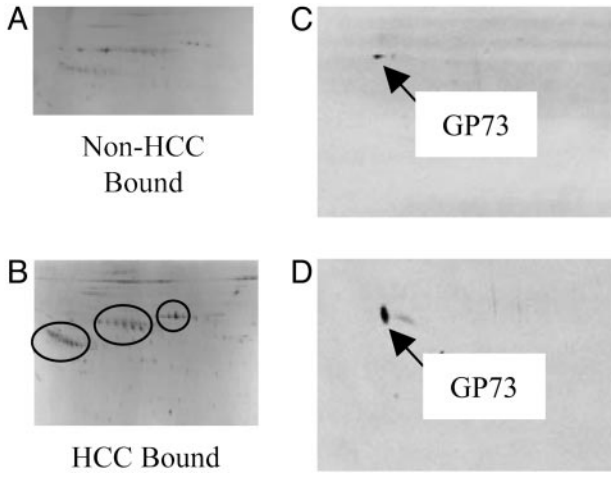

Fig. 4. Comparison of the fucosylated fraction from representative non-HCC and HCC woodchuck serum resolved by 2DE and the levels of GP73 in the fucosylated serum proteome from those woodchucks. (*A*) The fucosylated glycoproteins isolated from a representative normal, non-HCC woodchuck. (*B*) The bound, fucosylated proteins from a representative HCC woodchuck. The circles indicate areas that are substantially different. (*C* and *D*) GP73 was detected by using a polyclonal antibody to human GP73 in both normal and HCC woodchucks (2DE blots) as described in *Materials and Methods*. The position of GP73 is indicated. For each gel, an equal volume was loaded from both the non-HCC- and HCC-bound fractions.

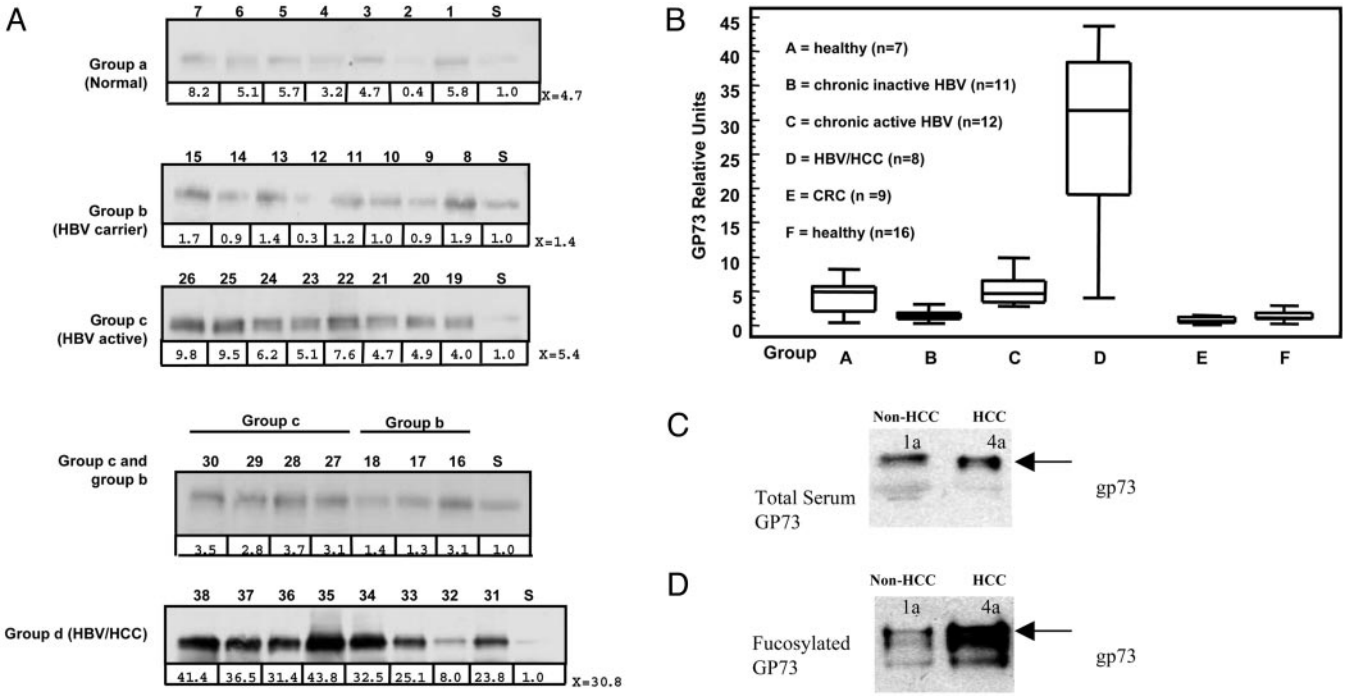

Fig. 5. Immunoblot analysis of GP73 serum levels in human subjects with and without viral hepatitis, HCC, and CRC. (*A*) Patient serum levels of GP73 are indicated at the bottom of each lane as relative values, compared with the level of GP73 detected in the normalization lane S included on the immunoblot (commercially purchased HBV-, HCV-, and HIV-negative serum). The average value of GP73 signal intensity (*x*) for each group vs. the normalization ''S'' control is also indicated to the right of each immunoblot. Immunoblot analyses of sera from control subjects with no evidence of HBV infection (a), from patients with inactive (b) or active (c) HBV infection or with HBV-associated HCC (d). These are the same serum samples and clinical categories as in ref. 12. (*B*) Box plot of the data shown in *A* with additional data from patients with CRC. The box indicates the interquartile range (25th to 75th percentile) with the middle line indicating the median and the vertical line extending from the minimum to maximum values. (*C*) Total serum analysis (immunoblot with GP73 antibody) of a noncancer HBV-infected patient (1a) and a HCC-positive HBV-infected patient (4a) with similar GP73 levels. For each sample, 20 μg of total protein was loaded. (*D*) Immunoblot of GP73 in the lectin extracted (fucosylated) fraction of serum of noncancer HBV-infected (1a) and HCC-positive HBV-infected (4a) patients. The entire fucosylated fraction from 200 μg of total protein was loaded for each lane.

GP73 species is increased dramatically in animals that have HCC.

GP73 Is Elevated in the Sera of People with HCC. Because GP73 appeared to be both up-regulated and hyperfucosylated in the serum of animals with cancer, it warranted further examination in the human disease. Therefore, serum from age- and sex-matched (all male) people with either no evidence of liver disease (HBV-negative) or evidence of chronic HBV infection, with and without a diagnosis of HCC, was resolved by 1D gel electrophoresis, and the amount of GP73 present was determined by Western blot, as described in *Materials and Methods*.

As Fig. 5 shows, only low levels of GP73 were detected in the total serum of healthy subjects (Group a), as well as HBV-infected individuals without (Group b) and with (Group c) active hepatitis. However, consistent with the results obtained in the woodchuck model, the majority of patients with HBV-induced HCC had elevated levels of GP73 (Group d) compared with the healthy subjects (Group a).

Fig. 5*B* shows quantification of the data presented in Fig. 5*A*, with additional data derived from an analysis of the sera from patients with CRC. As these data show, whereas patients with CRC have GP73 levels close to healthy subjects, patients with HBV-induced HCC have a >30-fold increase in the level of GP73. Statistical analysis shows a significant difference between the HCC group and all other groups, with $P < 0.001$.

Although compared with ''healthy'' subjects and those with CRC, the majority of HCC patients do generally have the greatest amounts of GP73, there was one HCC-derived sample (no. 32, Fig. 5*A*) where the amount of GP73 was not significantly different from the non-HCC-derived samples. Because GP73 from woodchucks with HCC appeared to be hyperfucosylated, it was reasoned that GP73 in the serum of patients with HCC but nearly ''normal'' GP73 levels might be hyperfucosylated relative to individuals without HCC. This hypothesis was confirmed by the examination of serum from a HCC-positive patient with nearly normal levels of GP73, either by total serum analysis for GP73 (Fig. 5*C*) or by analysis of only the fucosylated fraction (Fig. 5*D*). As this figure shows, compared with analysis of ''total GP73'' levels, restricting examination to only the fucosylated fraction may provide an even better resource for the correlation between GP73 and HCC and further increase the specificity of this marker. It is noted that elevated fucosylation has been seen in all ($n = 10$) HCC-derived GP73 samples examined to date (A.S.M., M.A.C., and P.R.R., unpublished data). Although this is a small number, the results are consistent and highlight the potential of this methodology and marker.

Discussion

Changes in N-linked glycosylation have long been associated with the development of disease (12, 18, 20, 31, 32). However, in many situations, truly quantitative methods for analysis have not been used to assess these changes. In this study, we have used a rapid, highly sensitive, and quantitative method of glycan sequencing to identify specific changes that occur with the development of liver cancer. Once specific changes in glycosylation were identified, the specific glycoproteins were identified by using semiquantitative 2DE and immunoblotting. Indeed, as Fig.

2 shows, in a blinded study, glycan analysis can be used to identify animals that have developed cancer based on the level of core fucosylation of total serum glycoproteins. The mechanisms responsible for the increase in fucosylation seen with the development of HCC are unknown, but they may involve an increase in both enzymes (i.e., fucosyltransferase) and substrates (i.e., GDP-L-fucose) and alterations in the substructure of the Golgi apparatus (33, 34). It is also possible that the half-life of the serum associated fucosylated glycoproteins is longer and, hence, this finding could be reflected in our glycan analysis. However, several reports have indicated that core fucosylation has no impact on serum clearance rates (35, 36).

It is noted that in addition to the data presented here, glycan sequencing has been used previously to monitor drug efficacy (26, 28) and to identify specific enzyme defects in patients with congenital disorders of glycosylation (37). Thus, if truly quantitative methods of glycan identification can be automated, it may have significant clinical value. A recent report has indicated that fucosylation of serum Igs also may change with disease (38).

The targeted glycoproteomic methodology presented here enabled the discovery of the woodchuck homologue of GP73 in the circulation of woodchucks with HCC. The elevation of GP73 was not restricted to animals with HCC, because it was clearly increased in abundance in seven of eight people chronically infected with HBV and a diagnosis of HCC. Elevation of GP73 thus was not a function of species, because it was elevated in both people and animals with a diagnosis of HCC. Chronic infection with HBV was not, in itself, sufficient to be associated with elevated GP73, because GP73 was not elevated (compared with "uninfected," age- and sex-matched control samples) in people with HBV infection in the absence of HCC. This finding is somewhat surprising because elevation of GP73 has been detected in liver tissue from HCV- and HBV-infected individuals, in the absence of a diagnosis of HCC (21). The secretion of a Golgi resident glycoprotein such as GP73 and the alteration in glycosylation (that occurs in the Golgi) may indicate a larger dysfunction of the Golgi apparatus during HCC.

The correlation between serum GP73 levels and a diagnosis of HCC in people has recently been confirmed in a blinded study with >240 samples through collaboration with the Early Detection Research Network of the National Cancer Institute (41). Total GP73 levels, as determined by Western blots, were shown to have a positive predicative value equal to or greater than the currently used marker, AFP. This finding further validates the methods described in the present study as useful in the identification of possible biomarkers of disease.

In addition to identifying the correlation between serum GP73 and liver cancer, this technique also has identified the hyperfucosylation of AFP, α-1-acid glycoprotein, α-1-antitrypsin, and transferrin as a function of liver cancer. These glycoproteins previously have been reported to be hyperfucosylated in human liver cancer (20, 39, 40), and in many ways the detection of these glycoproteins by our system is a validation of our approach.

In conclusion, the results reported here demonstrate the discovery value of a targeted glycoproteomic methodology. Further studies, with larger cohorts, are needed to determine the true usefulness of an assay for fucosylated and unfucosylated GP73 in cancer detection.

We thank Ms. Melissa Moxley for help with manuscript preparation and Dr. Olga V. Nikolaeva for insight and work related to this study. This work was supported by the National Cancer Institute Innovative Molecular Analysis Technologies Program and Early Research Detection Network, the Great Lakes New England Cancer Consortium, an appropriation from The Commonwealth of Pennsylvania and the Hepatitis B Foundation. A.S.M. is the Bruce Witte Research Scholar of the Hepatitis B Foundation.

1. El-Serag, H., Mason, A. & Key, C. (2001) *Hepatology* **33,** 62–65.
2. Block, T., Mehta, A., Fimmel, C. J. & Jordan, R. (2003) *Oncogene* **22,** 5093–5107.
3. Hoofnagle, J. H. & di Bisceglie, A. M. (1997) *N. Engl. J. Med.* **336,** 347–356.
4. Buamah, P. K., Gibb, I., Bates, G. & Ward, A. M. (1984) *Clin. Chim. Acta* **139,** 313–316.
5. Nguyen, M. H., Garcia, R. T., Simpson, P. W., Wright, T. L. & Keeffe, E. B. (2002) *Hepatology* **36,** 410–417.
6. Pirisi, M., Fabris, C., Luisi, S., Santuz, M., Toniutto, P., Vitulli, D., Federico, E., Del Forno, M., Mattiuzzo, M., Branca, B. & Petraglia, F. (2000) *Cancer Detect. Prev.* **24,** 150–155.
7. Newsome, P. N., Beldon, I., Moussa, Y., Delahooke, T. E., Poulopoulos, G, Hayes, P. C. & Plevris, J. N. (2000) *Aliment. Pharmacol. Ther.* **14,** 1295–1301.
8. Baumann, N. A., Vidugiriene, J., Machamer, C. E. & Menon, A. K. (2000) *J. Biol. Chem.* **275,** 7378–7389.
9. Inoue, G., Horiike, N. & Onji, M. (2001) *Int. J. Mol. Med.* **7,** 67–71.
10. Zondervan, P. E., Wink, J., Alers, J. C., IJzermans J. N., Schalm, S. W., de Man, R. A. & van Dekken, H. (2000) *J. Pathol.* **192,** 207–215.
11. Kawai, H. F., Kaneko, S., Honda, M., Shirota, Y. & Kobayashi, K. (2001) *Hepatology* **33,** 676–691.
12. Breborowicz, J., Mackiewicz, A. & Breborowicz, D. (1981) *Scand. J. Immunol.* **14,** 15–20.
13. Miyazaki, J., Endo, Y. & Oda, T. (1981) *Acta Hepatol. Jpn.* **22,** 1559–1568.
14. Taketa, K., Ichikawa, E., Taga, H. & Hirai, H. (1985) *Electrophoresis* **6,** 492–497.
15. Taketa, K., Sekiya, C., Namiki, M., Akamatsu, K., Ohta, Y., Endo, Y. & Kosaka, K. (1990) *Gastroenterology* **99,** 508–518.
16. Shiraki, K., Takase, K., Tameda, Y., Hamada, M., Kosaka, Y. & Nakano, T. (1995) *Hepatology* **22,** 802–807.
17. Hutchinson, W. L., Du, M. Q., Johnson, P. J. & Williams, R. (1991) *Hepatology* **13,** 683–688.
18. Miyoshi, E., Noda, K., Yamaguchi, Y., Inoue, S., Ikeda, Y., Wang, W., Ko, J. H., Uozumi, N., Li, W. & Taniguchi, N. (1999) *Biochim. Biophys. Acta* **1473,** 9–20.
19. Yamashita, K., Koide, N., Endo, T., Iwaki, Y. & Kobata, A. (1989) *J. Biol. Chem.* **264,** 2415–2423.
20. Naitoh, A., Aoyagi, Y. & Asakura, H. (1999) *J. Gastroenterol. Hepatol.* **14,** 436–445.
21. Kladney, R. D., Bulla, G. A., Guo, L., Mason, A. L., Tollefson, A. E., Simon, D. J., Koutoubi, Z. & Fimmel, C. J. (2000) *Gene* **249,** 53–65.
22. Comunale, M. A., Mattu, T. S., Lowman, M. A., Evans, A. A., London, W. T., Semmes, O. J., Ward, M., Drake, R., Romano, P. R., Steel, L. F., *et al.* (2004) *Proteomics* **4,** 826–838.
23. Su, Y. H., Wang, M., Brenner, D. E., Ng, A., Melkonyan, H., Umansky, S., Syngal, S. & Block, T. M. (2004) *J. Mol. Diagn.* **6,** 101–107.
24. Tennant, B. C. (2001) *Clin. Liver Dis.* **5,** 43–68.
25. Gerin, J. L., Cote, P. J., Korba, B. E. & Tennant, B. C. (1989) *Cancer Detect. Prev.* **14,** 227–229.
26. Block, T. M., Lu, X., Mehta, A., Blumberg, B. S., Tennant, B., Ebling, M., Korba, B., Lansky, D., Jacob, G. S. & Dwek, R. A. (1998) *Nat. Med.* **4,** 610–614.
27. Rudd, P. M., Mattu, T. S., Zitzmann, N., Mehta, A., Colominas, C., Hart, E., Opdenakker, G. & Dwek, R. A. (1999) *Biotechnol. Genet. Eng. Rev.* **16,** 1–21.
28. Mehta, A., Zitzmann, N., Rudd, P. M., Block, T. M. & Dwek, R. A. (1998) *FEBS Lett.* **430,** 17–22.
29. Kelleher, P. C., Walters, C. J., Myhre, B. D., Tennant, B. C., Gerin, J. L. & Cote, P. J. (1992) *Cancer Lett.* **63,** 93–99.
30. Kladney, R. D. Cui, X., Bulla, G. A., Brunt, E. M. & Fimmel, C. J. (2002) *Hepatology* **35,** 1431–1440.
31. Aoyagi, Y. (1995) *Glycoconj. J.* **12,** 194–199.
32. Steel, L. F., Mattu, T. S., Mehta, A., Hebestreit, H., Dwek, R., Evans, A. A., London, W. T. & Block, T. (2000) *Dis. Markers* **17,** 179–183.
33. Noda, K., Miyoshi, E., Gu, J., Gao, C. X., Nakahara, S., Kitada, T., Honke, K., Suzuki, K., Yoshihara, H., Yoshikawa, K., *et al.* (2003) *Cancer Res.* **63,** 6282–6289.
34. Kellokumpu, S., Sormunen, R. & Kellokumpu, I. (2002) *FEBS Lett.* **516,** 217–224.
35. Hoffmann, T., Penel, C. & Ronin, C. (1993) *J. Endocrinol. Invest.* **16,** 807–816.
36. Camani, C., Gavin, O., Bertossa, C., Samatani, E. & Kruithof, E. K. (1998) *Eur. J. Biochem.* **251,** 804–811.
37. Butler, M., Quelhas, D., Critchley, A. J., Carchon, H., Hebestreit, H. F., Hibbert, R. G., Vilarinho, L., Teles, E., Matthijs, G., Schollen, E., *et al.* (2003) *Glycobiology* **13,** 601–622.
38. Callewaert, N., Van Vlierberghe, H., Van Hecke, A., Laroy, W., Delanghe, J. & Contereras, R. (2004) *Nat. Med.* **10,** 429–434.
39. Saitoh, A., Aoyagi, Y. & Asakura, H. (1993) *Arch. Biochem. Biophys.* **303,** 281–287.
40. Aoyagi, Y., Isokawa, O., Suda, T., Watanabe, M., Suzuki, Y. & Asakura, H. (1998) *Cancer* **83,** 2076–2082.
41. Marrero, J. A. & Lok, A. S. (2004) *Gastroenterology* **127,** Suppl. 1, S113–S119.