# Novel Fucosylated Biomarkers for the Early Detection of Hepatocellular Carcinoma

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## Abstract

**Changes in glycosylation, most notably fucosylation, have been associated with the development of hepatocellular carcinoma (HCC). In this report, the levels of fucosylated kininogen (Fc-Kin) and fucosylated α-1-antitrypsin were analyzed individually and in combination with the currently used marker, α-fetoprotein, and a previously identified biomarker, Golgi protein 73 (GP73), for the ability to distinguish between a diagnosis of cirrhosis and HCC. This analysis was done on serum from 113 patients with cirrhosis and 164 serum samples from patients with cirrhosis plus HCC. The levels of Fc-Kin and fucosylated α-1-antitrypsin were significantly higher in patients with HCC compared with those with cirrhosis ($P < 0.0001$). Greatest performance was achieved through the combination of Fc-Kin, α-fetoprotein, and GP73, giving an optimal sensitivity of 95%, a specificity of 70%, and an area under the receiver operating characteristic of 0.94. In conclusion, the altered glycosylation of serum glycoproteins can act as potential biomarkers of primary HCC when used independently or in combination with other markers of HCC.** (Cancer Epidemiol Biomarkers Prev 2009;18(6):1914–21)

## Introduction

Infection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV) is the major etiology of hepatocellular cancer (HCC; refs. 1-4). Both HBV and HCV cause acute and chronic liver infections and most chronically infected individuals remain asymptomatic for many years (5). Ten percent to 40% of all chronic HBV carriers eventually develop liver cancer, and it is estimated that over 1 million people worldwide die because of HBV/HCV-associated liver cancer (2, 6, 7). Indeed, HBV and HCV infections are associated with >80% of all HCC cases worldwide and can be as high as 96% in regions where HBV is endemic (3).

The progression of liver disease into liver cancer is primarily monitored by serum levels of the oncofetal glycoprotein, α-fetoprotein (AFP), or the core fucosylated glycoform of AFP (AFP-L3). However, AFP can be produced under many circumstances, including other liver diseases (8-10), and is not present in all those with HCC. Therefore, the use of AFP as a primary screen for HCC has been questioned (11) and more sensitive serum biomarkers for HCC are desired.

The glycosylation of proteins is cell specific and the N-linked glycan a protein carries reflects modifications that occurred in the cell from which it came (12). Sugar (glycan) structures on the same protein secreted from malignant or diseased tissue and normal cells may, and often do, differ (13). We and others have observed changes in N-linked glycosylation with the development of cirrhosis and HCC (14-18). Specifically, the amount of fucosylated N-linked glycan derived from total protein preparations isolated from the serum of individuals chronically infected with HCV and from those with a diagnosis of HCC was consistently greater than healthy subjects or those with HCV and "inactive" disease (18).

Using fucose-specific lectins to identify the proteins that become fucosylated with liver disease, we have identified >18 glycoproteins that contained increased fucosylation with HCC and/or cirrhosis (18). We have previously described the analysis of fucosylated Golgi protein 73 (GP73) and fucosylated hemopexin by immunoblot of the fucosylated proteome (19).

In the present study, the aim was to determine the correlation of two identified proteins, fucosylated kininogen (Fc-Kin) and fucosylated α-1-antitrypsin (Fc-AAT; ref. 14), with the development of HCC in two independent patient cohorts consisting of 113 patients with cirrhosis, 108 patients with stage I or II HCC, and 56 patients with stage III or IV HCC. The performance of these markers and their potential use in the management of liver cancer are discussed.

## Materials and Methods

**Patients.** Serum samples were obtained from Saint Louis University School of Medicine or the University of Michigan. For samples obtained from the University of Michigan, the University of Michigan's Institutional Review Board approved the study protocol and written informed consent was obtained from each subject. Demographic and clinical information were obtained

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and a blood sample was collected from each subject. Consecutive patients with HCC and patients with cirrhosis that were age, gender, and race/ethnicity matched to the HCC patients were enrolled from the Liver Clinic during this period. The diagnosis of HCC was made by histopathology, including all T1 lesions, and, if histopathology was not available, by two imaging modalities [ultrasound (US), magnetic resonance imaging (MRI), or computed tomography (CT)] showing a vascular enhancing mass of >2 cm. Diagnosis of cirrhosis was based on liver histology or clinical, laboratory, and imaging evidence of hepatic decompensation or portal hypertension. Each of the patients with cirrhosis had a normal US and, if serum AFP was elevated, a MRI of the liver within 3 mo before enrollment and another one 6 mo after enrollment that showed no liver mass. The cirrhotic controls have been followed for a median of 12 mo (range, 7-18 mo) after enrollment and no one has developed HCC. Tumor staging was determined using the United Network of Organ Sharing–modified tumor-node-metastasis staging system for HCC. Early HCC was defined as T1 (single lesion <2 cm in diameter) and T2 (single lesion between 2 and 5 cm in diameter or <3 lesions each <3 cm in diameter), which met criteria for liver transplantation in the United States. A 20-mL blood sample was drawn from each subject, spun, and aliquoted, and the serum was stored at −80°C until testing. Blood samples were drawn before initiation of HCC treatment. AFP was tested using commercially available immunoassays using enhanced chemiluminescence at the University of Michigan Hospital Clinical Diagnostic Laboratory. The upper limit of normal was 8 ng/mL.

For samples obtained from Saint Louis University School of Medicine, the Saint Louis University Institutional Review Board approved the study protocol and written informed consent was obtained from each subject. Demographic and clinical information were obtained and a blood sample was collected from each subject in a serum separator tube and spun within 2 h, and serum was stored at −80°C until testing. For the HCC group, consecutive patients were enrolled from the Saint Louis University Liver Cancer Clinic using criteria for HCC diagnosis established for the HALT-C trial. Subjects had either HCC on biopsy, a new hepatic defect showing vascular enhancement on one imaging modality (US, MRI, or CT) with AFP >1,000 ng/mL, or presumed HCC. Subjects were presumed to have HCC if they had a discrete hepatic defect on US with AFP <1,000 ng/mL and either two other scans (MRI, CT, or angiography) indicating malignancy with at least one of the following characteristics: hypervascularity, arterial to portal vein shunts, portal vein thrombosis near the defect, tumor in the portal vein, or one other scan (MRI or CT) showing features characteristic of HCC and either an increase in size over time after initial discovery (at least doubling if <1 cm) or an increase in AFP to >200 ng/mL. Tumor staging was determined using the United Network of Organ Sharing–modified tumor-node-metastasis staging system for HCC. For the cirrhosis group, patients with hepatitis C–proven and biopsy-proven cirrhosis were enrolled. All cirrhotic controls were screened for HCC using US, CT, or MRI before enrollment.

**Lectin Fluorophore-Linked Immunosorbent Assay.** In our previous work, we analyzed the levels of fucosylated GP73 and fucosylated hemopexin in patients with HCC via immunoblotting of lectin-enriched fractions (19). This method involved the depletion of immunoglobulin from serum samples followed by lectin extraction of all fucosylated proteins. Subsequently, proteins were resolved through polyacrylamide gels and proteins of interest were detected via immunoblotting. As this technique was not suitable for the analysis of larger sample numbers, a lectin fluorophore-linked immunosorbent assay (FLISA) was developed.

A diagram of a typical lectin FLISA is shown in Fig. 1A. Briefly, to remove the fucosylation of the capture antibody (mouse anti-human AAT or rabbit anti-human LMW kininogen; AbD Serotec), the antibody was incubated with 10 mmol/L sodium periodate for 1 h at 4°C. An equal volume of ethylene glycol was added and the oxidized antibody was brought to a concentration of 10 μg/mL with sodium carbonate buffer (pH 9.5). Antibody (5 μg/well) was added to the plate and, following incubation, washed with 0.1% Tween 20/PBS 7.4 and blocked overnight with 3% bovine serum albumin/PBS. For analysis, 5 μL serum was diluted in 95 μL of heterophilic blocking tubes (Scantibodies Laboratory, Inc.) and incubated at room temperature for 1 h. Subsequently, samples were added to the plates for 2 h and washed five times in lectin incubation buffer [10 mmol/L Tris (pH 8.0), 0.15 mol/L NaCl, 0.1% Tween 20] before fucosylated protein was detected with a biotin-conjugated *Aleuria aurantia* lectin (Vector Laboratories). Bound lectin was detected using IRDye 800–conjugated streptavidin and signal intensity was measured using the Odyssey IR Imaging System (LI-COR Biotechnology). In all cases, signal intensity was compared with signals detected with commercially purchased human serum (Sigma Chemical). It is noted that the lectin FLISA detects the amount of fucosylation present on an equal amount of captured molecules from each patient sample and is done in a manner that is independent of the total amount of protein in any given patient.

**Immunoblotting for GP73.** Equal volumes of patient sera were resolved by SDS-PAGE on 10% polyacrylamide gels and the proteins were transferred to a polyvinylidene difluoride membrane by immunoblotting. The membranes were blocked by incubating with a blocking buffer of 1× TBS [50 mmol/L Tris-HCl (pH 7.6), 150 mmol/L sodium chloride], 5% nonfat dried milk, and 0.1% Tween 20 for 1 h at room temperature. The blots were incubated overnight with polyclonal anti-GP73 antibody (1:2,000) and incubated with rocking at room temperature for 2 h. Blots were subsequently washed thrice for 10 min in 0.1% Tween-PBS and GP73 was visualized using an IRDye 700–conjugated mouse anti-rabbit secondary antibody (1:10,000). Signal intensity was measured using the Odyssey IR Imaging System. In all cases, sample intensity was compared with commercially purchased human serum (Sigma Chemical).

**Statistical Analysis.** Descriptive statistics for stage patients were compared by scatter plots that included the outliers. All values were reported as mean values ± SD unless otherwise stated. As the data did not follow typical Gaussian distributions, a nonparametrical test (two-tailed, 95% confidence, Mann-Whitney test) was used to determine statistical difference between groups. To evaluate the performance of combining multiple markers,

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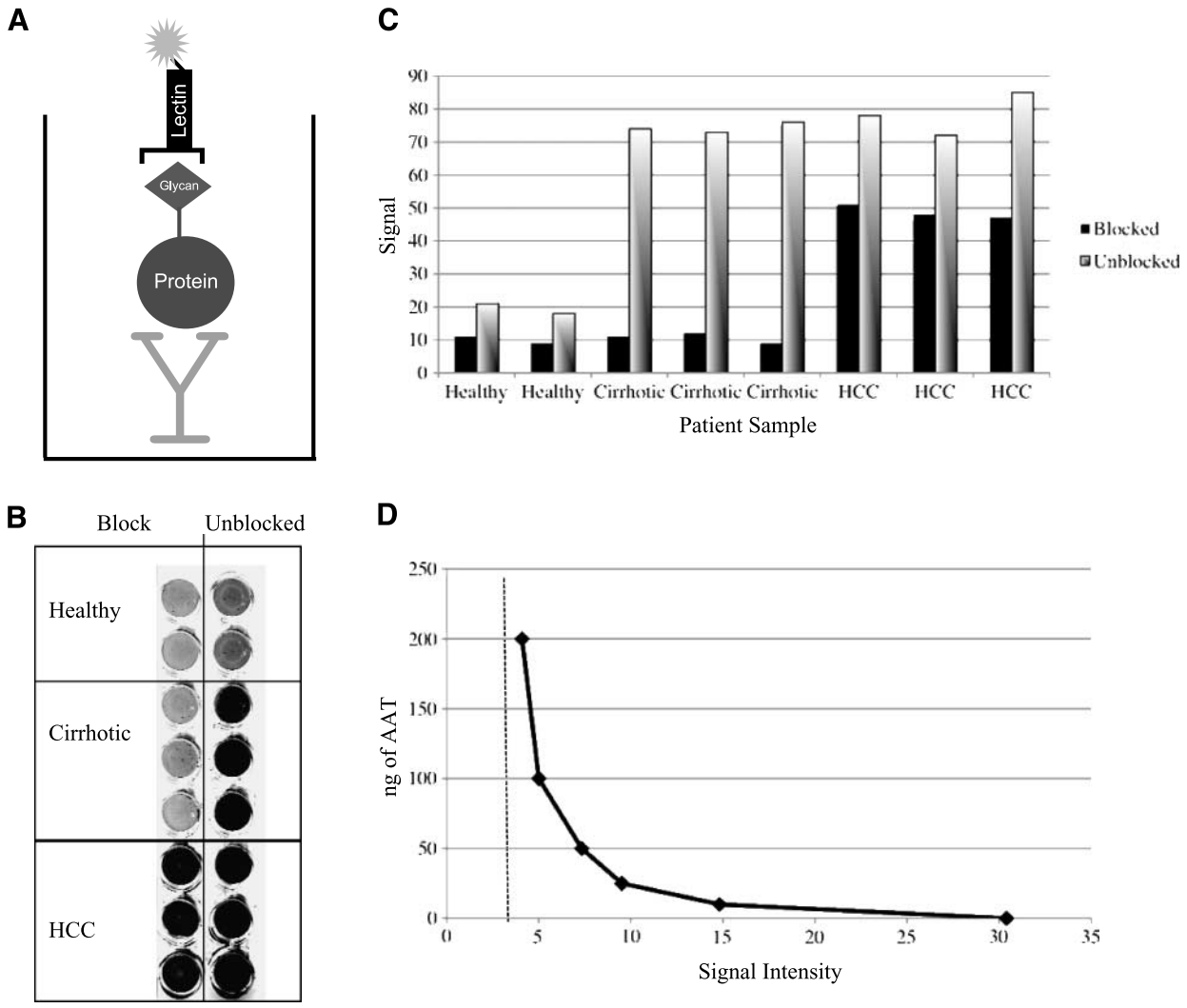


**Figure 1.** Lectin FLISA for the measurement of fucosylated glycoproteins. **A.** General lectin FLISA methodology. Periodate-oxidized mouse anti-human AAT was used as the capture antibody and the level of fucosylated protein was determined by a biotin-conjugated lectin (*Aleuria aurantia*) and detected using IRDye 800–conjugated streptavidin. Signal intensity was measured using the Odyssey IR Imaging System. In all cases, sample intensity was compared with commercially purchased human serum (Sigma Chemical). **B.** Example of lectin ELISA for the detection of Fc-AAT. *Left column,* results of lectin FLISA following blocking of heterophilic antibodies; *right column,* results obtained without blocking. **C.** Quantification of results obtained from the results in **B**. **D.** Titration of non–Fc-AAT into the lectin FLISA reaction can reduce the signal from a cancer patient. Up to 200 ng of non–Fc-AAT were titrated into a standard lectin ELISA using 5 μL of patient serum. *Dashed line,* background of the assay.

values of multiple markers were input into a multiple logistic regression model. In each case, the output (predicted value) was between 0 and 1, with 0 being cirrhosis and 1 being cancer. A $P$ value of 0.5 was used as a fixed cutoff and patients were classified as being HCC positive when $P \geq 0.5$, otherwise they were classified as cirrhotic ($P < 0.5$). To determine the optimal cutoff value for each marker, the receiver operating characteristic (ROC) curves were constructed using all possible cutoffs for each assay. The area under the ROC (AUROC) curves were constructed and compared as described previously. A two-tailed $P$ value of 0.05 was used to determine statistical significance. All analyses were done using GraphPad Prism.

## Results

**Development of a Lectin FLISA.** To allow for the high-throughput analysis of Fc-AAT and Fc-Kin, a plate-based assay was developed (Fig. 1A). The key to the development of this assay is the blocking of heterophilic antibodies. That is, we have previously shown that there is a large increase in the amount of heterophilic antibodies in people with liver fibrosis and cirrhosis and that, in fibrosis, these antibodies become highly reactive with fucose binding lectins (20, 21). Thus, as most liver cancers develop in the background of fibrosis and cirrhosis, assays that do not account for these heterophilic antibodies may give false results.



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**Table 1. Sample population characteristics**

| Obtained from | Saint Louis University* | | | University of Michigan* | | |
|---|---|---|---|---|---|---|
| Disease diagnosis | HCC† | Cirrhosis† | *P* | HCC† | Cirrhosis† | *P* |
| Number | 65 | 32 | N/A | 99 | 81 | |
| Etiology% (HBV/HCV/crypto/alcohol/other)‡ | 14/52/6/20/8 | 0/100/0/0/0 | <0.0001 | 11/51/24/10/4 | 5/48/20/18/9 | 0.1405 |
| Age (mean ± SD) | 58.04 ± 11 | 50 ± 8 | <0.0001 | 58.6 ± 12 | 58 ± 3 | 0.3165 |
| Ethnicity (non-Hispanic White/African-American/Hispanic/Asian) | N/A | N/A | N/A | 86/8/4/2 | 81/11/6/2 | 0.7956 |
| Gender (M:F)% | 71:29 | 84:16 | 0.4079 | 75/25 | 60/40 | 0.053 |
| MELD score (mean ± SD) | 11.8 ± 5 | NA | N/A | 10.3 ± 4 | 9 ± 2 | 0.0026 |
| Child class (A/B/C) or NA%§ | 52:29:9:10 | 88:8:4:0 | <0.0001 | 48/42/10/0 | 40/54/6/0 | 0.1992 |
| Tumor stage (I/II/III/IV)%‖ | 26:48:12:14 | NA | N/A | 21/40/26/13 | NA | N/A |

Abbreviations: MELD, model for end-stage liver disease; N/A, not available.
*Samples were provided coded from Saint Louis University Medical School or from the University of Michigan. See text for more details.
†Disease diagnosis was determined by MRI or by liver biopsy.
‡For etiology: crypto, cryptogenic liver disease; alcohol, alcohol-induced liver disease; other, liver disease of unknown origin.
§The percentage of patients with each Child-Pugh score is given as a percentage in each group.
‖Tumor staging was determined using the United Network of Organ Sharing–modified tumor-node-metastasis staging system for HCC. The percentage of patients within each stage is given. Patient's characteristics were analyzed through the use of $\chi^2$ test, Fisher's exact test, or Welch's approximate *t* test as appropriate. All test were two sided, and $P < 0.05$ was considered significant.

Figure 1B shows typical results obtained for Fc-AAT from two healthy controls, three serum samples from cirrhotic patients, or three serum samples from patients with cirrhosis plus HCC. On the right side of this panel, samples were depleted of heterophilic IgG molecules (blocked) or mock treated (unblocked). As this figure shows, when samples are left unblocked (on the right), strong binding is observed in all cirrhotic and HCC samples. Proof that this is IgG comes from the use of a secondary anti-human IgG antibody, which shows strong binding in all samples (data not shown). In contrast, when immunoglobulins have been removed using a protein A/G resin, strong signal is only observed from the sera of those patients with HCC. Figure 1C shows the quantification of results obtained from Fig. 1B. As Fig. 1C shows, unless heterophilic antibodies are effectively blocked, the change in glycosylation of fucosylated proteins cannot be accurately measured. Evidence that signal is obtained from captured AAT comes from competition experiments using purchased AAT that lacks fucose to compete out binding of AAT from HCC sera. As Fig. 1D shows, titration of non–Fc-AAT into human sera can effectively block the signal obtained, providing evidence that these signals are coming from captured AAT. Similar experiments were done for the other fucosylated proteins used in this article (data not shown). This assay was used for all subsequent analysis.

**Correlation of Fc-AAT and Fc-Kin with HCC.** The correlation of Fc-AAT and Fc-Kin was examined in a two-cohort coded study comprising 113 patients (see Tables 1 and 2) with biopsy-confirmed cirrhosis, 108 patients with cirrhosis plus stage I or II HCC, and 56 patients with cirrhosis plus stage III or IV HCC. The method used was the lectin FLISA as described in Fig. 1 and the results are presented in Fig. 2. For both Fc-AAT and Fc-Kin, relative levels were compared with commercially purchased serum. As Fig. 2 shows, there was a substantial increase in the level of both Fc-AAT (Fig. 2A) and Fc-Kin (Fig. 2B) in patients with HCC compared with those without HCC. Using commercially purchased serum (from HBV- and HCV-negative donors), the level of Fc-AAT was 1.8-fold elevated in serum from patients with cirrhosis, 2.9-fold elevated in patients with stage I or II HCC, and 3.6-fold in patients with stage III or IV HCC (Fig. 2A). Statistical significance was observed between the cirrhosis group and all HCC groups ($P < 0.0001$) but not between the HCC groups ($P = 0.05$).

A similar pattern of alterations was observed for Fc-Kin, which had a mean increase of 1.1-fold in patients with cirrhosis, 2.3-fold in patients with stage I or II HCC, and 2.9-fold in patients with stage III or IV HCC (Fig. 2B). As with Fc-AAT, statistical significance was observed between the cirrhosis group and all HCC groups ($P < 0.0001$) but not between the individual HCC groups ($P = 0.32$).

**Table 2. Sensitivities and specificities of individual markers at the detection of stage I or II cancer**

| | Fc-AAT* | Fc-Kin* | GP73† | AFP‡ |
|---|---|---|---|---|
| AUROC | 0.74 | 0.79 | **0.89** | 0.83 |
| SE | 0.04 | 0.03 | 0.02 | 0.03 |
| 95% CI | 0.67-0.81 | 0.73-0.85 | **0.85-0.93** | 0.77-0.88 |
| *P* | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| % Specificity at 50% sensitivity | 81 | 84 | 97§ | 98§ |
| % Specificity at 75% sensitivity | 64 | 67 | **86**§ | 74 |
| % Specificity at 90% sensitivity | 41 | 47 | **68**§ | 36 |
| % Specificity at 95% sensitivity | 28 | 42§ | **43**§ | 28 |
| % Specificity at 100% sensitivity | 4 | 23§ | **25**§ | 4 |

*Analysis of Fc-AAT or Fc-Kin via lectin FLISA as described in Fig. 1.
†GP73 was analyzed by immunoblot.
‡AFP was measured using a commercially available AFP ELISA kit. The best values for each category are given in bold.
§Statistically different than the other values in the given group ($P < 0.05$).



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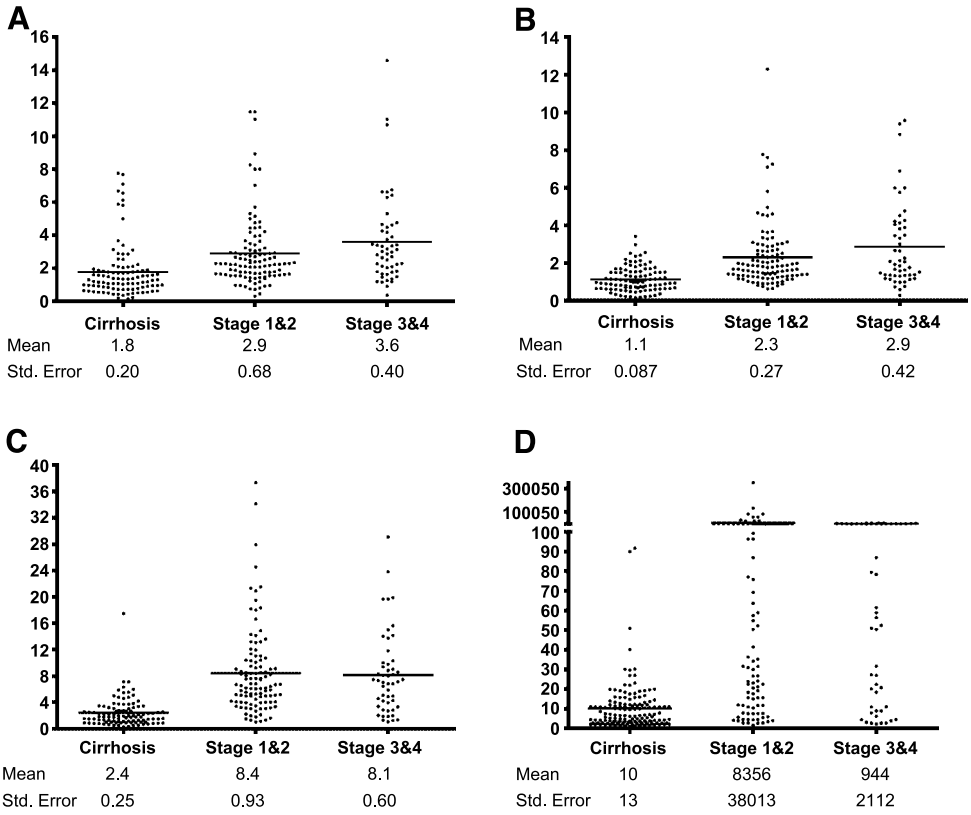


**Figure 2.** Scatter plots of the relative levels of Fc-AAT (**A**), Fc-Kin (**B**), total GP73 (**C**), or AFP (**D**) in patients with either cirrhosis, stage I or II HCC, or stage III and IV HCC. In the case of GP73, Fc-AAT or Fc-Kin values are presented as fold over commercially purchased sera. Values for AFP are given as ng/mL. In all panels, the line indicates the mean. Analysis of Fc-AAT and Fc-Kin was done via the lectin FLISA described in Fig. 1A. Total GP73 was analyzed via immunoblotting. AFP was measured using a commercially available AFP ELISA kit.

GP73, a Golgi protein we have previously reported to be in the circulation of people with HCC (17, 20), was also examined. In the present study, relative to the amount detected in commercial serum, GP73 had a mean increase of 2.4-fold in patients with cirrhosis, 8.4-fold in patients with stage I or II HCC, and 8.1-fold in patients with stage III or IV HCC (Fig. 2C). Again, whereas no statistical significance was observed between the two HCC groups ($P = 0.79$), statistical significance was observed between the cirrhosis group and the HCC groups ($P < 0.0001$).

For comparison, the levels of the currently used marker for HCC, AFP, were also measured in these samples. In these samples, AFP had a mean level of 10 ng/mL in patients with cirrhosis, 8,356 ng/mL in patients with stage I or II HCC, and 944 ng/mL in patients with stage III or IV HCC (Fig. 2D).

Changes in glycosylation and, more importantly, fucosylation are known to occur with the development of cancer. The possibility that the increase in fucosylation would be observed in patients with cancer was tested through the examination of serum samples from patients with prostate cancer ($n = 20$), ovarian cancer ($n = 22$), lung cancer ($n = 10$), cervical cancer ($n = 16$), and colorectal cancer ($n = 10$). These patients had similar values of Fc-AAT, Fc-Kin, and total GP73 to those obtained from serum of control subjects, suggesting specificity of this marker for liver disease (data not shown).

**Statistical Analysis of Fc-AAT and Fc-Kin.** ROC curves were plotted to determine overall performance and to identify the sensitivity and specificity for each marker in differentiating HCC from cirrhosis. As a major

**Table 3. Sensitivities and specificities of individual markers at the detection of all HCC**

| | Fc-AAT* | Fc-Kin* | GP73† | AFP‡ |
|---|---|---|---|---|
| AUROC | 0.75 | 0.79 | **0.88** | 0.82 |
| SE | 0.03 | 0.03 | 0.02 | 0.03 |
| 95% CI | 0.69-0.81 | 0.74-0.85 | **0.84-0.92** | 0.77-0.87 |
| *P* | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| % Specificity at 50% sensitivity | 81 | 85 | 97§ | **98**§ |
| % Specificity at 75% sensitivity | 65 | 67 | **86**§ | 64 |
| % Specificity at 90% sensitivity | 47 | 47 | **54** | 36 |
| % Specificity at 95% sensitivity | 28 | 35 | **35** | 21 |
| % Specificity at 100% sensitivity | 4 | 8 | **25**§ | 4 |

*Analysis of Fc-AAT or Fc-Kin via lectin FLISA as described in Fig. 1.
†GP73 was analyzed by immunoblot.
‡AFP was measured using a commercially available AFP ELISA kit. The best values for each category are given in bold.
§Statistically different than the other values in the given group ($P < 0.05$).



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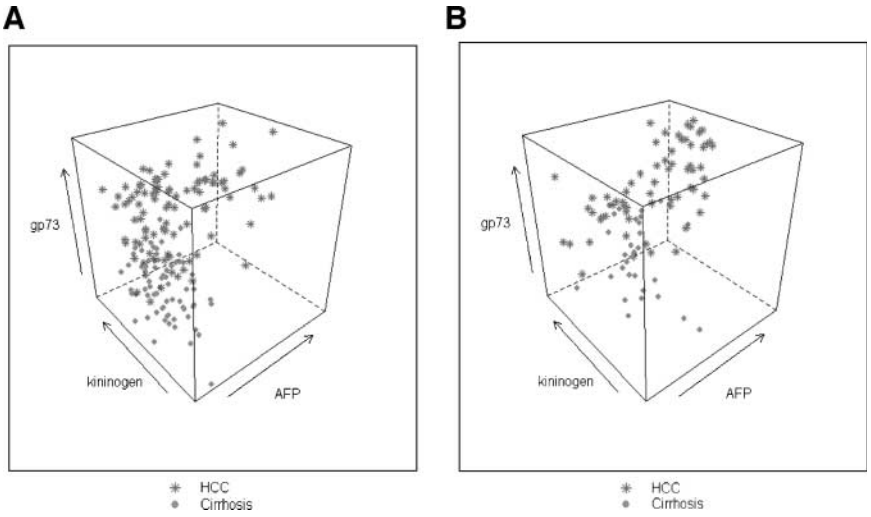


**Figure 3.** Perspective plot of the relative levels of Fc-Kin, total GP73, and total AFP in the Saint Louis (**A**) or University of Michigan (**B**) patients. For both **A** and **B**, the asterisks represent individual HCC patients and closed circles represent the cirrhotic patients. For the plot, log-transformed values for each marker were used.

goal of our biomarker discovery work is the development of a more sensitive marker of early cancer, the specificity of each marker was determined at fixed points of sensitivity (see Table 2). As detailed in Table 2, in differentiating cirrhosis from stage I or II HCC, the AUROC curve for Fc-Kin was 0.79 with a specificity of 42% at a fixed sensitivity of 95%. Comparable results were obtained when comparing cirrhosis with all HCC stages (see Table 3). Similarly, Fc-AAT had an AUROC of 0.74 with a specificity of 28% at a fixed sensitivity of 95%. Like Fc-Kin, results were similar when comparing cirrhosis with all HCC stages.

The marker GP73 had the best individual performance characteristics. As detailed in Table 2, GP73 had an AUROC of 0.89 with a specificity of 43% at a fixed sensitivity of 95%, in differentiating cirrhosis from stage I or II HCC. The addition of stage III or IV HCC patients did not alter the performance of GP73 (see Table 3). For comparison, AFP had a similar performance as GP73 with specificity of 28%, at a fixed sensitivity of 95%, and an AUROC of 0.83.

**Combinatorial Analysis of Fc-AAT, Fc-Kin, and Total GP73 in the Detection of HCC.** Figure 3 shows a perspective plot of the relative levels of GP73, Fc-Kin, and AFP in the two separate patient cohorts. As this figure shows, most HCC patients (represented by *asterisks*) were found in the upper back corner of the plot, indicating that they were positive for GP73, Fc-Kin, and AFP. This was true for the HCC patients in both the Saint Louis cohort (Fig. 3A) and the University of Michigan cohort (Fig. 3B). In contrast, the cirrhotic samples (represented by *circles*) cluster in the bottom left-hand corner of the plot, indicative of low levels of the individual markers.

The performance of these markers when used in combination was also tested. This was done using a combination of any two to four markers using logistic regression analysis (see Tables 4 and 5). The combination of GP73, Fc-Kin, and AFP gave the best overall results with an AUROC of 0.94 with a specificity of 70% at a fixed sensitivity of 95%. This was much greater than any marker alone, as shown in Tables 2 or 3 ($P < 0.05$). For all markers used in combination, performance was similar in both early tumors (stage I or II) and with the analysis of all cases of HCC.

## Discussion

We have previously identified several glycoproteins that contained altered fucosylation with the development of HCC (17, 18). In an attempt to determine how useful these

**Table 4. Sensitivities and specificities of combined markers for the detection of stage I or II HCC**

| | Fc-AAT and Fc-Kin* | Fc-Kin and GP73† | Fc-AAT and GP73 | Fc-AAT and AFP‡ | Fc-Kin and AFP | GP73 and AFP | GP73, AFP, and Fc-Kin§ |
|---|---|---|---|---|---|---|---|
| AUROC | 0.81 | 0.92 | 0.89 | 0.86 | 0.89 | 0.92 | **0.94** |
| SE | 0.03 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 |
| 95% CI | 0.75-0.87 | 0.89-0.96 | 0.85-0.94 | 0.81-0.91 | 0.85-0.93 | 0.88-0.96 | **0.91-0.97** |
| *P* | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| % Specificity at 50% sensitivity | 85 | 98 | 97 | 98 | 99 | 99 | **99** |
| % Specificity at 75% sensitivity | 66 | **95**‖ | 84 | 79 | 85 | 90 | **95**‖ |
| % Specificity at 90% sensitivity | 55 | 65 | 68 | 46 | 57 | 75‖ | **77**‖ |
| % Specificity at 95% sensitivity | 44 | 56 | 52 | 37 | 43 | 62 | **70**‖ |
| % Specificity at 100% sensitivity | 31 | 26 | 27 | 20 | 21 | 14 | **36**‖ |

*Analysis of Fc-AAT or Fc-Kin via lectin FLISA as described in Fig. 1.
†GP73 was analyzed by immunoblot.
‡AFP was measured using a commercially available AFP ELISA kit. The best values for each category are given in bold.
§Statistical difference for all values compared with GP73 or AFP alone ($P < 0.05$).
‖Statistically different than the other values in the given group ($P < 0.5$).



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**Table 5. Sensitivities and specificities of combined markers for the detection of all HCC**

| | Fc-AAT and Fc-Kin* | Fc-Kin and GP73† | Fc-AAT and GP73 | Fc-AAT and AFP‡ | Fc-Kin and AFP | GP73 and AFP | GP73, AFP, and Fc-Kin |
|---|---|---|---|---|---|---|---|
| AUROC | 0.81 | 0.91 | 0.89 | 0.86 | 0.89 | 0.91 | 0.93 |
| SE | 0.03 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.01 |
| 95% CI | 0.76-0.86 | 0.88-0.94 | 0.85-0.93 | 0.82-0.91 | 0.85-0.93 | 0.88-0.94 | 0.90-0.96 |
| *P* | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| % Specificity at 50% sensitivity | 86 | 99 | 96 | 97 | **100**§ | 99 | 99 |
| % Specificity at 75% sensitivity | 70 | 93 | 82 | 80 | 86 | 87 | 92 |
| % Specificity at 90% sensitivity | 53 | 64 | 66 | 47 | 52 | 68 | **74**§ |
| % Specificity at 95% sensitivity | 41 | 48 | 51 | 40 | 38 | 55 | **60**§ |
| % Specificity at 100% sensitivity | 17 | 26 | 25 | 20 | 21 | 13 | 27 |

*Analysis of Fc-AAT or Fc-Kin via lectin FLISA as described in Fig. 1.
†GP73 was analyzed by immunoblot.
‡AFP was measured using a commercially available AFP ELISA kit. The best values for each category are given in bold.
§Statistically different than the other values in the given group ($P < 0.05$).

proteins would be as biomarkers of HCC, we developed a simple FLISA-styled method to measure the level of altered glycosylation of two proteins: Fc-Kin and Fc-AAT. Although simple in design, this assay (shown in Fig. 1) was initially hampered by the interference of heterophilic antibodies (20).

Because the antibodies used to capture the proteins of interest are made in animals, they contain epitopes or saccharide structures that are considered foreign and can be reactive to human antibodies. Hence, they can bind to the antibody used to capture the antigen of interest, which leads to signal backgrounds/interference. This is particularly applicable in our situation, as we have recently determined that heterophilic antibodies reactive toward oligosaccharide Galα-1-3Galβ1-(3) 4GlcNAc-R (referred to as the α-gal epitope) are increased and become reactive to fucose binding lectins with the development of fibrosis and cirrhosis (20). As almost all patients develop HCC in the background of fibrosis/cirrhosis, these α-gal antibodies must be blocked (neutralized) before analysis of a specific protein of interest could be determined.

Using the lectin FLISA method, we were able to examine the level of Fc-AAT and Fc-Kin in 277 patients with cirrhosis or cirrhosis plus HCC. Patients with cirrhosis plus HCC generally had higher levels of both Fc-AAT and Fc-Kin than patients with cirrhosis alone. Unfortunately, the sensitivity, specificity, and accuracy of these markers were not superior to that obtained with another identified marker, GP73, or with the currently used marker AFP (see Table 2). However, when used in combination, these markers lead to an enhanced detection of HCC ($P < 0.001$). Specifically, as Tables 4 and 5 show, when used in combination, total GP73, AFP, and Fc-Kin had an AUROC of 0.94 in discriminating cirrhosis from stage I or II HCC.

Core fucosylation of N-linked glycoproteins occurs in the Golgi apparatus (22). The exact mechanisms for increased fucosylation in HCC are unknown but are thought to involve increases in both the levels of the enzymes and substrate involved in core fucosylation (16). As both GP73 and fucosylation are associated with the Golgi apparatus, it is possible that these markers reflect some alteration in the Golgi apparatus. Indeed, recent reports have suggested that fucosylation of proteins in the liver plays a role in cell sorting (23). Thus, it is conceivable that the appearance of GP73 and fucosylated proteins in the serum may reflect a common defect in the Golgi apparatus. This is currently under investigation. It is also noted that many HCC-positive patients were also negative for all three markers. Proteomic analysis is under way to attempt to identify novel markers that exist in these patients that could be used in combination to increase the sensitivity of this assay.

In summary, we have developed a lectin FLISA–based method for the analysis of fucosylated glycoforms of two secreted liver glycoproteins. These markers when used in combination with GP73 had an overall performance that was better than AFP alone. It is postulated that these markers could be used to supplement AFP as a general screen in those patients at high risk for HCC development either alone or in combination with US, as is the current practice. These markers may also be useful in the monitoring of HCC patients following treatment as well as identifying those patients with reoccurrence.

These data need to be confirmed in larger cohorts of patients to determine if these markers are true indicators of early HCC and to compare its accuracy with AFP in patients of diverse gender, ethnicity, and etiologies of liver disease and to determine its role in HCC surveillance. Future studies should also test the benefit of combinatorial analysis with other potential markers of HCC, such as des-γ-carboxy prothrombin.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest exist.

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