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Research paper

Biomarker analysis of fucosylated kininogen through depletion of lectin reactive heterophilic antibodies in hepatocellular carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) accounts for > 700,000 deaths worldwide, largely related to poor rates of diagnosis. Our previous work identified glycoproteins with increased levels of fucosylation in HCC. Plate-based assays to measure this change were compromised by increased levels of heterophilic antibodies with glycan lacking terminal galactose residues, which allowed for increased binding to the lectins used in these assays. To address this issue, we developed a multi-step protein A/G incubation and filtration method to remove the contaminating signal. However, this method was time consuming and expensive so alternative methods were desired. Herein, we describe a simple method relying on PEG precipitation that allows for the removal of IgG and IgM but retention of glycoproteins of interest. This method was tested on three sample sets, two internal and one external. PEG depletion of heterophilic IgG and IgM reduced in the coefficient of variation as observed with the protein A/G filtration method from 26.82% to 7.50% and allowed for the measurement of fucosylated protein. This method allowed for the measurement of fucosylated kininogen, which could serve as a biomarker of HCC. In conclusion, a new and simple method for the depletion of heterophilic IgG and IgM was developed and allowed for the analysis of fucosylated kininogen in patients with liver disease.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and the incidence in the United States (USA) is increasing (Block et al., 2003; Singal and El-Serag, 2015; Atiq et al., 2017). The progression of liver disease into liver cancer can be monitored with serum levels of alpha-fetoprotein (AFP). However, AFP's limited sensitivity and specificity has reduced its reliability as a primary screening tool for HCC (Sherman, 2005), and more sensitive biomarkers for HCC are desired.

Using fucose-specific lectins, we previously identified > 50 glycoproteins that contained increased fucosylation in HCC (Comunale et al., 2006) as compared to those with liver disease but without HCC, and have used these in plate-based assays to detect HCC in the background of cirrhosis (Wang et al., 2009a; Comunale et al., 2009a; Comunale et al., 2010). However, the plate-based assays were hampered by the presence of heterophilic antibodies (human antibodies that can interact with assay antibodies) that themselves had altered glycan (Mehta et al., 2008). This alteration in glycosylation occurred with liver fibrosis and cirrhosis, the background from which most HCC develops (Atiq et al., 2017; Singal et al., 2014). To deal with this issue, we have utilized several methods to deplete heterophilic immunoglobulins from

Abbreviations: HCC, Hepatocellular carcinoma; PEG, Poly-ethylene glycol; AUROC, Area under receiver operator curve; AUC, Area under curve; AAL, Aleuria aurantia lectin; AFP, Alpha-fetoprotein

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serum. Unfortunately, all of these methods required expensive protein A/G mixtures and several time consuming filtration steps limiting their translation and clinical use (Mehta et al., 2008; Balagopal et al., 2008). In the current study, we have developed a simple and rapid method for the removal of these contaminants before plate-based lectin analysis. This method was used in three independent sample sets to validate the method and the performance of fucosylated kininogen as a biomarker of HCC. Fucosylated kininogen is of interest as we have shown in prior studies that this marker can combine with AFP and other clinical factors to achieve AUC of > 0.90 in the differentiation of HCC from cirrhosis (Wang et al., 2017a). The potential use of this method as a diagnostic tool for the detection of liver cancer is discussed.

2. Material and methods

2.1. Patient samples and ethics statements

Three sets of serum samples were utilized for this study. The first was from the University of Texas Southwestern (UTSW) Medical Center. Serum samples were obtained via a study protocol approved by the UTSW Institutional Review Board. In all cases, written informed consent was obtained from each subject. Diagnosis of cirrhosis was based on liver histology or clinical, laboratory and imaging evidence of hepatic decompensation or portal hypertension (15). Each non-HCC patient had a normal ultrasound; if serum AFP was elevated, a CT or MRI showed no liver mass. For HCC patients, the diagnosis of HCC was made by histopathology or imaging (magnetic resonance imaging [MRI] or computed tomography [CT]) showing a vascular enhancing mass with delayed washout (5). Tumor staging was determined using the Milan Criteria (Mazzaferro et al., 1996). Demographic and clinical information were obtained, and a blood sample was collected from each subject. A 20-ml blood sample was drawn from each subject, spun, aliquoted, and serum stored at −80°C until testing. Blood samples for HCC patients were drawn prior to initiation of HCC treatment. Patient details are provided in Supplementary Table S1.

The second set was obtained from the University Michigan under a study protocol approved by the University Michigan Review Board and written informed consent was obtained from each subject. Patients details regarding samples these are found in our previous publication (Comunale et al., 2013a).

The third patient set was from the Fudan University Shanghai Cancer Center. As before these samples sets were collected under study protocols approved by the Institutional Review Board and written informed consent was obtained from each subject. Detailed information regarding patients from the Fudan University Shanghai Cancer Center is found in Table 1.

Table 1
Patient characteristics for validation study.

Disease Diagnosis	HCC[1]	Cirrhosis[2]	*p* Value[3]
Number	75	75	
Etiology% (HBV/HCV/other) [4]	100/0/10	100/0/0	
Age (mean, SD) [5]	54.1 (11.7)	47.8 (11.9)	0.0005
AFP ng/mL (mean, SD) [6]	842.7 (1291)	5.2 (9.2)	< 0.0001
AST IU/mL (mean, SD) [7]	50.9 (65.4)	30.5 (21.6)	0.0003
ALK IU/mL (mean, SD) [8]	126.2(113.7)	69.2 (21.1)	< 0.0001
Gender(M:F)% [9]	80/20	48/52	< 0.0001
Tumor Stage (1/2/3/4/unk)%[10]	3/24/25/8/40	N/A	

1&2) Disease diagnosis was determined by MRI (HCC) or by liver biopsy (in the case of cirrhosis). All HCC patients had cancer in the background of cirrhosis. 3) P value comparing two groups. Patient's characteristics were analyzed through the use of Chi-Square 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. 4) For Etiology: HBV, hepatitis B virus; HCV, hepatitis C virus; other, liver disease consisting of cryptogenic liver disease or alcohol induced liver disease. 5) Mean age of groups. 6) Mean level of AFP. 7) Mean level of aspartate aminotransferase (AST) with the standard deviation indicated (in U/L). 8) Mean level of alkaline phosphatase with the range indicated (in U/L). 9) Gender as a % male or female. 10) Tumor staging information. Unknown indicates patients with unknown tumor stage.

2.2. Lectin fluorescence-linked immunosorbent assay (FLISA)

The basic design of the commonly used lectin FLISA is described elsewhere (Wang et al., 2009a). Liver fibrosis (and cirrhosis) is associated with increased levels of heterophilic immunoglobulins and significantly, an alteration in the glycosylation of these molecules (Wang et al., 2016a;Comunale et al., 2013b;Wang et al., 2009b). This change in glycosylation increases their reactivity to fucose binding lectins. Two methods to deal with these lectin reactive heterophilic antibodies were used. For the protein A/G method (Wang et al., 2016a; Comunale et al., 2013b; Wang et al., 2009b), a Pall Omega Nanosep 100 K spin filter (Pall Corporation, #OD100C35) is washed twice with 300 µl of X PBS to prepare the filter. Subsequently, 150 µL of Pierce Protein A/G Plus™ (Pierce Chemicals, # 20423/4) is added to the filter, spun at 14,000 g for 5 min and the Protein A/G beads re-washed twice in 300 µl 1× PBS. In a separate tube, 12.5 µL of serum is diluted to a final volume of 125 µL in 1× PBS and transfered to the washed Pierce Protein A/G Plus™ in the centrifugal filter. The filter lid is wrapped with parafilm to ensure no leaks and vortexed. Tubes are placed on a shaker for 2 h at 37 °C. Samples are spun at 10,000 g for 5 min and flow through is collected and used for subsequent analysis. In the final PEG method, 20 µL of serum is incubated with 20 µl of 40% polyethylene glycol (PEG)-8000 for 30 min at room temperature with shaking and the samples centrifuged at 14,000 *g* for 30 min at 4 °C. The supernatant, free of all immunoglobulins, contains fucosylated kininogen (as well as many other fucosylated proteins of interest such as fucosylated A1AT and fetuin-A) and is used for subsequent analysis.

Two assays for the measurement of fucosylated kininogen were performed. Our plate based assays that utilized detection of bound biotinylated lectin using IRDye-labelled streptavidin and visualized using the LI-COR Odyssey imaging system (Comunale et al., 2009a;Comunale et al., 2010;Wang et al., 2017a;Comunale et al., 2013a;Wang et al., 2016a;Comunale et al., 2011;Lamontagne et al., 2013) and a second method using more conventional horse radish peroxidase-labelled streptavidin where development occurs with 3,3′,5,5′-tetramethylbenzidine (TMB) chromogenic substrate. In this case, fucosylated protein standards (66%, 55%, 44%, 33%, 22% 11% 7% and 0%) are used to generate the calibration curve and assign % fucosylation values for each sample. In all cases, fucosylation is detected using a recombinant Aleuria aurantia lectin (AAL) that is modified at site 3 (N224Q). Information on this lectin is found elsewhere (Norton et al., 2016;Houser et al., 2017;Romano et al., 2011).

2.3. Immunoblotting

Specific proteins or human serum (Comunale et al., 2009b) depleted as above were resolved via SDS-PAGE and either stained with colloidal Coomassie brilliant blue (Colloidal Blue Staining Kit, Thermo Fisher) or transferred PVDF membranes for immunoblot analysis. IgM, IgG, or kininogen was detected using polyclonal antibodies (Abcam, Cambridge, MA and AbBiotec, San Diego, CA, GenScript, Piscataway, NJ). Bound antibody was visualized using IRDye® 800-conjugated anti-mouse-800, IRDye®-conjugated anti-mouse-antibody, IRDye® 800-conjugated anti-goat antibody.

2.4. Statistical analysis

Descriptive statistics for patient groups were compared by scatter plots that included the outliers. All values were reported as mean values ± SD unless otherwise stated. For Lectin-FLISA data, as the signal intensity varied following sample processing, signal intensity was