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Identification of IgM as a contaminant in lectin-FLISA assays for HCC detection

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ABSTRACT

Liver disease, in the form of hepatocellular carcinoma (HCC) accounts for > 700,000 deaths worldwide. A major reason for this is late diagnosis of HCC. The currently used biomarker, serum alpha-fetoprotein (AFP) is elevated in 40–60% of those with HCC and other markers that can either compliment or replace AFP are desired. Our previous work has identified a number of proteins that contain altered glycans in HCC. Specifically, these altered glycans were increased levels of core and outer arm fucosylation. To determine the clinical usefulness of those identified glycoproteins, a plate based assay was developed that allowed for the detection of fucosylated glycoforms. While this method was applicable to a number of independent patient sets, it was unable to specifically detect fucosylated glycoforms in many patient samples. That is, some material was present in serum that led to non-specific signal in the lectin-fluorescence -linked immunosorbent assay (lectin-FLISA). To address this issue, a systematic process was undertaken to identify the material. This material was found to be increased levels of lectin reactive IgM. Removal of both IgG and IgM using a multi-step protein A/G incubation and filtration step removed the contaminating signal and allowed for the analysis of specific protein glycoforms. This assay was subsequently used on two sample sets, one that was shown previously to be unable to be tested via a lectin FLISA and in a larger independent sample set. The clinical usefulness of this assay in the early detection of HCC is discussed.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and the incidence in the United States (USA) is increasing [1,2]. The progression of liver disease into liver cancer has been monitored with serum levels of alpha-fetoprotein (AFP). However, AFP's limited sensitivity and specificity has resulted in the questioning of AFP as a primary screen for HCC [3] and more sensitive biomarkers for HCC are desired.

Using fucose-specific lectins we have previously identified more than 50 glycoproteins that contained increased fucosylation with HCC [4] and have used these in plate-based assays to diagnosis HCC [5–7]. While this method was applicable to a number of independent patient sets, it was unable to specifically detect fucosylated glycoforms in certain patient samples. That is, some material was present in serum that led to non-specific signals in the lectin-FLISA [8,9]. In the current study, we have identified the contaminating lectin reactive factors present in the serum. This lectin reactive factor was shown to be IgM and when this was removed from the serum prior to lectin-FLISA, specific glycoprotein associated lectin reactive signal could be detected. This method was used in two independent sample sets to validate the method and also to validate the performance of the fucosylated glycoforms as biomarkers of HCC. The potential use of this method as a diagnostic tool for the detection of liver cancer is discussed.

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2. Material and methods

2.1. Patient samples

Serum samples were obtained from the University of Michigan and the University of California San Diego under a study protocol approved by the respective Institutional Review Board and written informed consent was obtained from each subject. Patients details regarding samples from the University of Michigan are found in our previous publication [10]. Detailed information regarding patients from the University of California at San Diego are found in Supplementary Table 2.

2.2. Lectin FLISA

The traditional lectin FLISA is described elsewhere [5]. The modified assay involves incubation of 1–5 μl of human serum (diluted into 45–49 μl PBS) with 20 μl of Pierce™ Protein A/G Plus (Thermo Fisher, Waltham, MA) for 1 h prior to filtration in a 100 KD Amicon Ultran 0.5 mL spin filter (EMD Millipore, Billerica, MA). Flow through was applied directly to plates and the lectin FLISA performed as before [5]. For the anti-alpha 1 anti-trypsin (A1AT) Lectin-FLISA, a polyclonal anti-A1AT (Sigma-Aldrich, St. Louis, MO) was used.

2.3. Immunoblotting

Pooled, HCC serum [11] was depleted of IgG using protein A/G coated agarose beads as described elsewhere [10] and serum incubated on 96-well plates coated with A1AT antibody for 2 h at room temperature. Captured proteins were resolved via SDS-PAGE and either stained with colloidal Coomassie brilliant blue (Colloidal Blue Staining Kit, Thermo Fisher) or proteins transferred using western-blotting method to PVDF membranes for immunoblot analysis. Fucosylation was detected using biotin-conjugated *Aleuria aurantia* lectin (AAL). A1AT was detected using a polyclonal anti-A1AT (Sigma-Aldrich). IgM or IgG was detected using polyclonal antibodies (Abcam, Cambridge, MA). Bound AAL or antibody was visualized using IRDye®800-conjugated streptavidin or IRDye®700-conjugated anti-rabbit antibody.

2.4. Proteomic identification of contaminating factors

For proteomic analysis well-associated proteins were digested with Trypsin Gold (Promega, Madison, WI). Samples were analysed by the Biological Mass Spectrometry Facility at Rutgers, the State University of New Jersey using a Velos LTQ Orbitrap tandem mass spectrometer coupled to a Dionex UltiMate 3000 Rapid Separation LC System (Thermo Scientific) using methods similar to previous reports [11]. The LC-MS/MS data was searched against the most up-to-date complete protein database (ensembl.org) using a local version of the Global Proteome Machine (GPM cyclone, Beavis Informatics Ltd, Winnipeg, Canada) with carbamidoethyl on cysteine as fixed modification and oxidation of methionine and tryptophan as variable modifications using a 10 ppm precursor ion tolerance and a 0.4 Da fragment ion tolerance.

2.5. 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. As 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.

3. Results

3.1. Identification of IgG and IgM as a contaminant in lectin-FLISA assays for HCC detection

We have previously identified proteins that become hyper-fucosylated in HCC and developed a plate-based assay to assess the level of fucosylation in samples [5,12]. This method worked well in many sample set but in others, was unable to detect a lectin reactive signal that could be attributed specifically to the protein that was captured [10]. An example of such a result is shown in Fig. 1A. Here a lectin-FLISA was performed for fucosylated alpha-1-anti-trypsin (A1AT). As Fig. 1A show, when such an assay is done using HCC serum, a lectin reactive signal is observed (Fig. 1A; wells A-D). However, when attempts were made to compete out this signal using non fucosylated native A1AT (nA1AT), we were unable

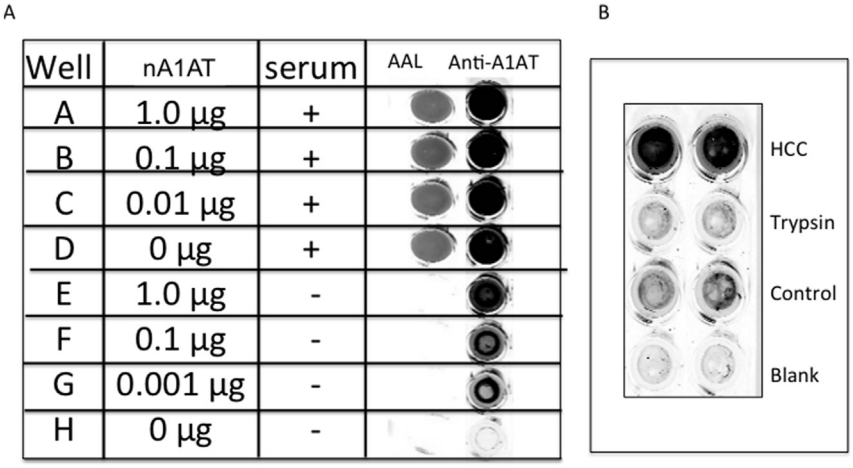

Fig. 1. Serum contains a protein based nonspecific lectin reactive signal that confounds a plate-based lectin-FLISA. A) Lectin-FLISA using serum from a HCC patient that was shown to be positive by Lectin-FLISA assay (well D). Attempts were made to block the signal using purchased native A1AT (nA1AT) that was not AAL reactive (wells E-H). While A1AT from both serum and exogenously sources can be detected by using an anti-A1AT antibody, an AAL signal is obtained when serum is used and cannot be competed with non-lectin reactive A1AT. B) Treatment of sample with trypsin leads to the complete loss of signal. In panel B, the HCC sample is the same as panel A, trypsin is the sample following digestion with trypsin, control is normal human sera purchased from Sigma Chemicals, blank is no sera added.