Blood serum alpha fetoprotein enhancer binding protein, a tumor suppressor, decreases in chronic HBV hepatitis patients as hepatocellular cancer appears

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Abstract. Chronic hepatitis increases the risk of hepatocellular carcinoma (HCC). To test whether circulating proteins reflect hepatic carcinogenesis, sera from patients and controls were albumin depleted, enriched for glycoproteins, digested with trypsin, and subjected to reverse phase chromatography and tandem mass spectrometry. Alpha-fetoprotein enhancer binding protein (AFPebp), a tumor suppressor, was repeatedly identified in sera from chronic HBV hepatitis patients. We independently identified and quantified AFPebp with a deuterated, phenylisocyanate-labeled synthetic peptide standard. Elevated AFPebp levels in sera from chronic HBV hepatitis patients decreased as cancer developed. These data suggest that rising AFPebp levels in chronic HBV hepatitis may be protective, while falling levels may contribute to HCC development.

Keywords: Alpha-fetoprotein, alpha-fetoprotein enhancer binding protein, tumor suppressor, hepatitis B virus, hepatitis C virus, hepatocellular carcinoma, liquid chromatography, tandem mass spectrometry, phenylisocyanate, deuterium

1. Introduction

HCC ranks fifth in worldwide cancer incidence with approximately 250,000 to 500,000 new cases each year [1]. The underlying causes for HCC are not known, though it usually follows many years of chronic viral hepatitis (CVH). The rate of development of HCC in CVH and cirrhosis is approximately 1% and 2.5% per year, respectively [1]. Clinical assessments used to assess the onset of HCC are liver ultrasound, biopsy, and serum measures for liver function and alpha-fetoprotein (AFP) [2]. Ultrasound only allows for the detection of tumors after onset of HCC. Liver function tests are non-specific for the diagnosis of HCC.

AFP, the current standard, has been shown to increase in some HCC cases (30–60%) [3–11], glycosylation changes alter in HCC [12–14], and it may be useful as a prognostic marker during treatment. A more specific and sensitive test to detect the onset of HCC is needed [3].

Research has focused on finding protein biomarkers for HCC in the sera of at-risk patients using LC-MS, MALDI-MS, SELDI-MS, or 2D gel technologies [9, 10,15–23]. SELDI-TOF has been used to identify complement C3a as a potential marker for HCV+ induced HCC [19]. MALDI-TOF has been used to identify small peptides that appear related to HCC [24]. Changes in DNA methylation and changes in protein modification have been probed as diagnostic markers [25,26]. In a study of sera from patients with hepatitis and hepatitis-induced HCC, hyper-fucosylation of glycoproteins has been related to the development of HCC [13]. HNF4alpha, p53, and Ki-67 have also been

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Table 1
Diagnostic criteria for patient class

	Antibody	HBV-DNA	HCV-RNA	ALT	Histology
Normal ($n = 16$)	No AB	−	−	normal	normal
HBV Carrier ($n = 11$)	HVsAg	+	−	normal	normal
HCV Carrier ($n = 6$)	Anti-HCV	−	+	normal	normal
Chronic HBV Hepatitis ($n = 42$)	HBsAg	+	−	elevated	inflammation/cirrhosis
Autoimmune Hepatitis ($n = 17$)	ANA	−	−	elevated	inflammation/cirrhosis
HBV/HCC ($n = 37$)	HBsAg	+	−	elevated	HCC & cirrhosis*

ALT-alanine aminotransferase.
ANA-anti-nuclear antibodies.
*liver inflammation is observed in only some HCC patients.

suggested as possible markers of HCC [27,28]. It has yet to be determined whether any of these findings will measure HCC risk or at what stage of the disease they will be relevant for diagnosing cancer [29].

Using shotgun protein sequencing and data analysis, we have identified proteins that change between the time of chronic HBV hepatitis and the onset of HCC. One biomarker candidate identified with this method, alpha-fetoprotein enhancer binding protein (AFPebp), was independently verified using quantitative mass spectrometry. Our data demonstrate that AFPebp levels rise with chronic HBV hepatitis and drop as HCC develops. Thus AFPebp changes reveal pathophysiology between chronic HBV hepatitis and its transition to HCC.

2. Materials and methods

2.1. Study population

Sera from 129 participants (5–88 years of age) included both archival and prospective samples after giving informed consent based on an ethically approved protocol (human subjects assurance # FWA 00002338) with diagnostic criteria in Table 1. Sixteen age-, gender- and race-matched controls had no known liver disease, and were negative for hepatitis B surface antigen (HBsAg) and HCV antibodies. Eleven carrier HBV patients tested positive for HBsAg without elevated alanine aminotransferase (ALT) or aspartate aminotransferase (AST) levels. Six carrier HCV patients had HCV antibodies without elevated ALT/AST levels. Seventeen autoimmune hepatitis patients with antinuclear antibodies and elevated ALT/AST levels had no detectable HBsAg or HCV antibody levels. Forty-two chronic HBV infected patients were positive for HBsAg with elevated ALT/AST levels. Thirty-seven patients had either histological and/or diagnostic imaging

confirmation of HCC. Histology was available on all of the subset of ten paired, chronic HBV+ hepatitis patients that progressed to HCC and that were analyzed by quantitative assay for AFPebp: all of these patients had cirrhosis. Patients were excluded if there was evidence of confounding systemic disease, alcoholism, toxic drug exposure, other cancers, abnormal storage disease, HIV, or cytomegalovirus. Sera were obtained by centrifugation of freshly clotted blood and 0.5 mL aliquots were stored at −80°C until use.

2.2. Protein assay

Concentrations of protein in serum were determined using a microplate-based Quant-iT protein assay kit (Invitrogen, Carlsbad, CA) using pre-diluted kit BSA, 0–500 μg/mL, as a standard. Briefly, single aliquots of serum or protein standard in triplicates were added to a 96 well microtiter plate. Quant-iT protein reagent was diluted (200X) in Quant-iT protein buffer and 200 μL was added to each well. After 45 to 60 min, the fluorescence (excitation/emission at 470/570 nm) was measured using a microplate reader (Gemini XPS, Molecular Devices, Sunnyvale, CA) and protein concentrations in each sample were determined using Softmax software from Molecular Devices.

2.3. Serum fractionation

Our logic is to remove albumin and enrich for glycoproteins so that a search can be made by shotgun sequencing for lower abundance proteins that may reflect the transition from chronic HBV hepatitis to that of HCC. Thus we chose to enrich less abundant proteins and those that are glycosylated, since glycosylation is a major liver activity and has been associated with one of the existing biomarkers, AFP [8,30,31].

2.4. Albumin depletion

Albumin and albumin-like proteins were removed using a HiTrap Blue G column (GE Health Care). Briefly, samples were thawed at room temperature, vortexed and centrifuged to remove any particulate matter. Injections of 0.1 mL whole sera were made onto an Agilent 1050 LC system equipped with UV detection (280 nm) for eluting proteins. Serum was passed through GE Health Care HiTrap Blue G (5 mg) columns at a flow rate of 0.3 mL/min at ambient temperature in 0.01 mM sodium phosphate buffer at pH 7.4. Flow through fraction was observed between 2–5 min, and collected as Fraction 1 (F1). Elution of column bound proteins was completed by switching to 0.3 mL/min 0.01 mM sodium phosphate, pH 7.4, 0.5 M NaCl at 7 min post injection. Fraction 2 (F2) eluted between 15–17 min and was collected. The column was washed by returning the eluent conditions to 0.3 mL/min of 0.01 mM sodium phosphate buffer at pH 7.4 at 20 min post injection and maintained for an additional 10 min before another injection was made.

2.5. Glycoprotein fractionation

The flow through fraction (F1), which contains mostly non-albumin proteins, was fractionated further based on the glycosylation state of the proteins within the sample. Conconavalin A (ConA) bound Sepharose beads (GE Health Care) were used. F1 was placed onto the Con A Sepharose beads, preconditioned with binding buffer, in a ratio of 0.1 mL bead suspension to 0.4 mL of eluted fraction and 0.5 mL of 2X binding buffer (20 mM Tris-Cl (pH 7.4), 500 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$). Batch purification was completed by mixing the beads with the sample via gentle inversion for 1 hr at ambient temperature. After spinning the Con A Sepharose beads into a pellet, the supernatant, fraction 1A (F1A) (a mixture of glycosylated proteins that did not bind to Con A, and non-glycosylated proteins), was saved. The beads were then washed in 500 μL of 1X binding buffer to remove any non-specifically bound protein by inversion for 30 min at ambient temperature. The beads were pelleted and the supernatant was discarded. Glycosylated proteins were removed by adding 0.5 mL of 1X elution buffer (binding buffer + 500 mM D-glucose) and gently mixing for 1 hr at ambient temperature. The beads were pelleted and the supernatant was saved as fraction F1B.

2.6. Preparation of fractionated samples for LC/MS/MS analysis

Albumin-depleted fractions for both Con A fractions (F1A & F1B) were concentrated using vacuum centrifugation and resuspended to a final concentration of 1 mg/mL in 100 mM ammonium bicarbonate, pH 8.0. Samples were applied to 10 kDa MWCO Amicon Spin Filters (Millipore) and reduced, alkylated, and digested with trypsin as described [30].

2.7. LC/MS/MS of proteins

LC/MS/MS experiments of sera from 97 different study participants were performed using the Surveyor LC pump and autosampler connected in line with the ThermoFisher LCQ (San Jose, CA). For reproducibility, we did not do multiple shotgun sequence analyses of the same samples, but looked for replicates of each protein identified within each clinical group. Sera from 16 normal patients, 44 chronic HBV hepatitis patients, and 37 HBV+ patients with HCC were analyzed by LC/MS/MS. Samples were injected onto a peptide enrichment column (C18) in the full loop mode. The eluent for the first 22 minutes of the gradient was diverted to waste to remove any salts in the sample. Flow to the column was established at 22 min onto a PicoFrit Biobasic 5 μm C18 capillary column, (10 cm by 75 μm ID) (New Objective, Woburn, MA). The sample was then eluted using a gradient of 0.1% formic acid in water (J.T. Baker, solvent A) and 0.1% formic acid in acetonitrile (J.T. Baker, solvent B). The gradient was as follows: 0–22 min, isocratic 100% A; 22–50 min, linear change to 60% B; 50–100 min, linear change to 80% B; 100–125 min, isocratic 80% B; 125–135 min, linear change to 100% A; 135–150 min, isocratic 100% A. The eluent from the capillary column was introduced into the LCQ source using nanospray ionization (capillary voltage at 2.2 V). Gas phase fractionation was completed to survey the peptide masses from *m/z 350–750* and *m/z 750–2000*. MS/MS was acquired in a data-dependent manner consisting of a single full MS followed by three successive MS/MS experiments. Data-dependent scanning was completed with an allowed repeat count of 5 over 90 seconds with dynamic exclusion for 25 different ions set at 180 seconds duration.

2.8. Database searching

MS/MS data are analyzed using ThermoElectron's BioWorks 3.0 software using a human database of proteins (Swiss Prot database, release 7459). Protein identification was dependent upon Xcorr score fit. Protein matches are identified using two sets of criteria. First, strict Washburn criteria [32] (as distinct from probability-based criteria [33]) were used to identify proteins, based on the charge of the precursor peptide ion and the Xcorr assigned by Bioworks software. These criteria dictate that for ion trap data, the required Xcorr value for identification increases with increasing charge ($z = +3$, Xcorr > 3.5; $z = +2$, Xcorr > 2.2; and $z = +1$, Xcorr > 1.9). These criteria allow for increased confidence in correct peptide sequence assignment within a single sample run.

Depending on the criteria we choose to match MS/MS spectra to peptides in the human database, the number of identified proteins changes. Decreasing the threshold for accepting a peptide match increases the number of likely false-positive matches. Since the ultimate aims of this project are to use identified peptides/proteins in a quantitative sense, we relaxed the criteria and allowed more protein identifications, based only on Xcorr score of > 1.2 for all charge states. Our justification for this is two-fold. First, we anticipate the probability for the presence of a protein in serum is increased if it is repeatedly observed in our Sequest searches of multiple serum samples ($\geqslant 50\%$ of samples per group). Second, we are able to pick out peptides that are more difficult to detect. These difficulties may be because the tryptic peptides for a given protein may be derived from low abundance molecules in the serum or because a peptide may give sub-optimal MS/MS spectra due to its chemical/physical properties. While we are aware that loosening our exclusion criteria will give greater uncertainty in our protein identifications, we feel it is necessary to do this to increase our overall yield of serum changes that may occur in the transition from hepatitis infection to HCC. Because we are taking liberties with our identification strategy, it is especially important to verify our identifications by using other independent methods that validate our protein matches (the stable isotope-based quantitative mass spectrometry detailed below).

2.9. Analysis and statistics

Samples and molecular data were encoded without personal identifiers. Data from BioWorks searches were exported as text files. Proteins were accepted as related to a disease state if a peptide corresponding to an identified protein was found in at least half of all peptide searches for samples in a participant's subgroup and were present in less than 50% of all other subgroups. Protein lists were then compared amongst subgroups to assign differences. Derived data was kept in Excel spreadsheets, where initial sorting and comparisons were done, with extensive use of Excel's Pivot Table function. Statistics were then analyzed using Prism software, starting with basic descriptive statistics to review the data content for each measure.

2.10. Phenylisocyanate (PIC) labeling of peptide digests for quantitative analysis

The paired sera samples from 10 chronic HBV hepatitis patients who progressed to HCC, along with sera from all other comparative groups (11 controls, 11 carriers for HBV, 6 carriers with HCV, and 17 patients with autommimune hepatitis) were randomly selected for measurement of AFPebp. PIC labeling of peptides used for quantitative analysis was carried out as described by Mason et al. [30]. AFPebp peptide (ISFPGSSESPLSSKR), a unique sequence from AFPebp, was purchased from New England Peptide (Gardner, MA) and diluted in water for labeling. PIC-d_0 and PIC-d_5 were purchased from Sigma-Aldrich, and a standard curve of AFPebp- PIC-d_5 was made for quantitative purposes. 1.5 ng of pure PIC-d_5 labeled peptide was then added as an internal standard post digestion to a mixture of PIC-d_0 labeled tryptic peptides from a digest of whole serum. The peptides were then analyzed by LC/MS/MS using the same gradient described above, with the mass spectrometer set to probe masses from 500–900 amu, allowing one data-dependent scan per full scan. The ratio of the PIC-d_5 peak area with the PIC-d_0 area was then used to calculate the amount of the peptide in the sample. Amounts of AFPebp were calculated and p values for differences in patient groups were determined using Prism software (GraphPad Software Inc., San Diego, CA). The standard deviation for AFPebp quantification in this method was $< 20\%$.

3. Results

3.1. Fractionation of sera

Albumin depletion was generally 85–90% for one injection and yielded approximately 3–5 mg of protein from 0.1 mL of serum in the flow through fraction (F1).

Table 2
Unique proteins identified in chronic HBV hepatitis and HCC samples

	ACCESSION #	SWISSPROT #	PROTEIN NAME
chronic HBV hepatitis ONLY	Q02880	TOP2B_HUMAN	DNA topoisomerase 2-beta
	Q15911	ATBF1_HUMAN	Alpha-fetoprotein enhancer-binding protein
	P52757	CHIO_HUMAN	Beta-chimaerin
	Q14767	LTBP2_HUMAN	Latent-transforming growth factor beta-binding protein 2
	P43353	AL3B1_HUMAN	Aldehyde dehydrogenase
	Q9UM73	ALK_HUMAN	ALK tyrosine kinase receptor
	O00512	BCL9_HUMAN	B-cell lymphoma 9 protein
	O14514	BAI1_HUMAN	Brain-specific angiogenesis inhibitor 1
	O60241	BAI2_HUMAN	Brain-specific angiogenesis inhibitor 2
	O75363	BCAS1_HUMAN	Breast carcinoma amplified sequence 1
	Q99075	HBEGF_HUMAN	Proheparin-binding EGF-like growth factor
	O15111	IKKA_HUMAN	Inhibitor of nuclear factor kappa-B kinase subunit alpha
	O60494	CUBN_HUMAN	Cubilin [Precursor]
	Q8N282	Q8N282_HUMAN	Similar to development- & differentiation-enhancing factor 2
	P29320	EPHA3_HUMAN	Ephrin type-A receptor 3
	Q53GG0	Q53GG0_HUMAN	Epithelial protein lost in neoplasma beta variant
	P16591	FER_HUMAN	Proto-oncogene tyrosine-protein kinase FER
	P26927	HGFL_HUMAN	Hepatocyte growth factor-like protein
	Q01101	INSM1_HUMAN	Insulinoma-associated protein 1
	P08581	MET_HUMAN	Hepatocyte growth factor receptor
	Q13330	MTA1_HUMAN	Metastasis-associated protein MTA1
	P20742	PZP_HUMAN	Pregnancy zone protein
	P07949	RET_HUMAN	Proto-oncogene tyrosine-protein kinase receptor ret
	Q9P2Y5	UVRAG_HUMAN	UV radiation resistance-associated gene protein
	Q03167	TGBR3_HUMAN	TGF-beta receptor type III
	Q49AT7	Q49AT7_HUMAN	TGFBI protein
	O75410	TACC1_HUMAN	Transforming acidic coiled-coil-containing protein 1
HBV/HCC	Q9UNA1	RHG26_HUMAN	Rho GTPase-activating protein 26
	Q92900	RENT1_HUMAN	Regulator of nonsense transcripts 1
	Q9H2X0	CHRD_HUMAN	Chordin [Precursor]
	Q16630	CPSF6_HUMAN	Cleavage and polyadenylation specificity factor 6
	Q9Y2E4	DIP2C_HUMAN	Disco-interacting protein 2 homolog C
	Q9Y467	SALL2_HUMAN	Sal-like protein 2
	P18583	SON_HUMAN	SON protein
	Q92797	SYMPK_HUMAN	Symplekin

	Normal	Chronic HBV hepatitis	HBV/HCC
Number of patients	16	44	37
Total proteins identified (Washburn criteria)	85	70	95
Total proteins identified (relaxed criteria)	315	410(370 unique)	355(61 unique)

Repeat injection of F1 did not yield further albumin depletion. Recovery of glycoproteins from the batch purification method was generally $> 70\%$. It resulted in a fraction combined of non-glycosylated proteins with glycoproteins that do not bind Con A (F1A), and a Con A-bound glycoprotein fraction (F1B). Yields were generally higher in F1A (~ 2.5 mg total protein per 100 μL serum prep) as compared to yields for F1B ($\sim$1.5 mg total protein per 100 μL serum prep).

3.2. Identification of proteins in serum

Our key findings from LC/MS/MS of combined F1A and F1B proteins are detailed in Table 2, an abbreviated table of protein identifications relevant to our work, based on proteins identified in 16 control samples, 44 chronic HBV hepatitis without cancer, and 37 chronic HBV hepatitis with HCC. For a peptide match from our Bioworks database to be considered as an identified protein, a given protein must be identified by at least
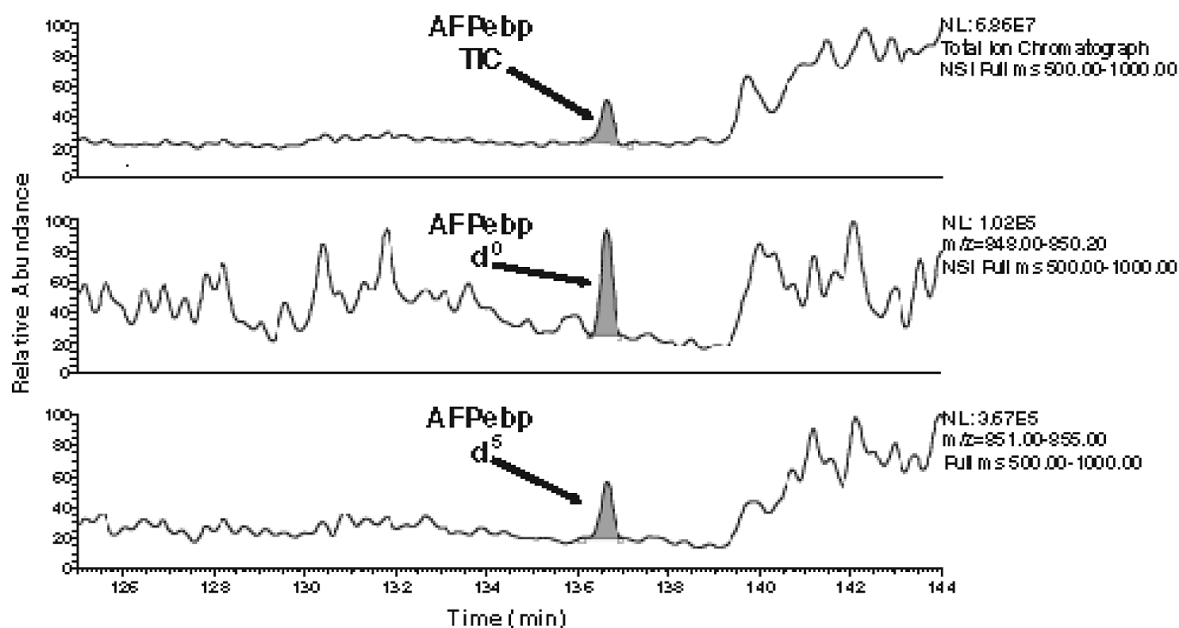
Fig. 1. Total ion chromatograph (TIC) of AFPebp in serum. Serum was spiked with d5-PIC labeled AFPebp peptide, digested with trypsin and subjected to LC/MS/MS. TIC was obtained and the area under the curve highlighted for the d^0 (endogenous) and d^5 (spiked) isotopes of AFPebp at M^{2+} 849 (B) and 852 (C) amu, respectively. This chromatograph is representative of the 64/65 of our experiments detecting AFPebp.

one peptide in $\geqslant$ 50% of all samples run per group and give an Xcorr score of $>$ 1.2. By loosening our criteria, the number of identified proteins increases approximately 4–5 fold. For the identifications under relaxed criteria, a larger number of proteins are observed for the two disease states than in controls. Since the higher abundance proteins are the same between groups, we think the greater number of lower abundance proteins in HBV states is most likely due to disease activity rather than the smaller number of control samples assayed. From these data, proteins of interest have been listed that are unique to a given patient subgroup and warrant further investigation (Table 2).

3.3. Quantitative analysis of alpha-fetoprotein enhancer binding protein (AFPebp) peptide by LC/MS/MS

Table 2 proteins are identified in serum from patients with the chronic HBV hepatitis state but not in control samples, are enriched in either the pre-cancer or HCC state (i.e., they disappear or appear as HCC develops), and are known to be involved in regulation of DNA repair, growth, or embryonic activities. These are thus candidate biomarkers for which there are some pathophysiological interpretations relevant to the transition from chronic HBV hepatitis to HCC. While these pro-

teins are all identified repeatedly in $\geqslant$ 50% of serum samples for either disease state, all of these functionally interesting proteins have low Xcorr values between 1.2 and 1.8. Therefore, we would not consider the assignments to be correct without further study.

To test whether any of the biomarker candidates are correctly identified in these serum samples, we selected AFPebp to be further evaluated. Since AFP is the standard biomarker for HCC, its regulatory transcription factor (AFPebp) [34] might be relevant pathophysiologically. Very little previous work has been completed on AFPebp and it's role in HCC, with reports detailing its role in the regulation of AFP expression in hepatoma [35], and other cancers [36–40].

Protein digests of whole serum were labeled using phenylisocyanate (PIC-d_0), spiked with PIC-d_5 standard, and analyzed using LC/MS/MS as described in "Methods." Any AFPebp that was identified was quantified, based on the spiked PIC-d_5 label. Sera from controls, and patients with HBV (carrier and chronic hepatitis states), autoimmune hepatitis, HCV carrier or HBV/HCC were tested to evaluate their levels of AFPebp. Figure 1 shows the selective identification in serum of the two PIC labeled peptides (d_0 & d_5) for AFPebp. The total ion chromatograph looks barren as most peptides are +3 and $>$ 500 amu.

Using the d_0/d_5 peptide labeling strategy, we obtained comparative data from serum samples. Figure 2

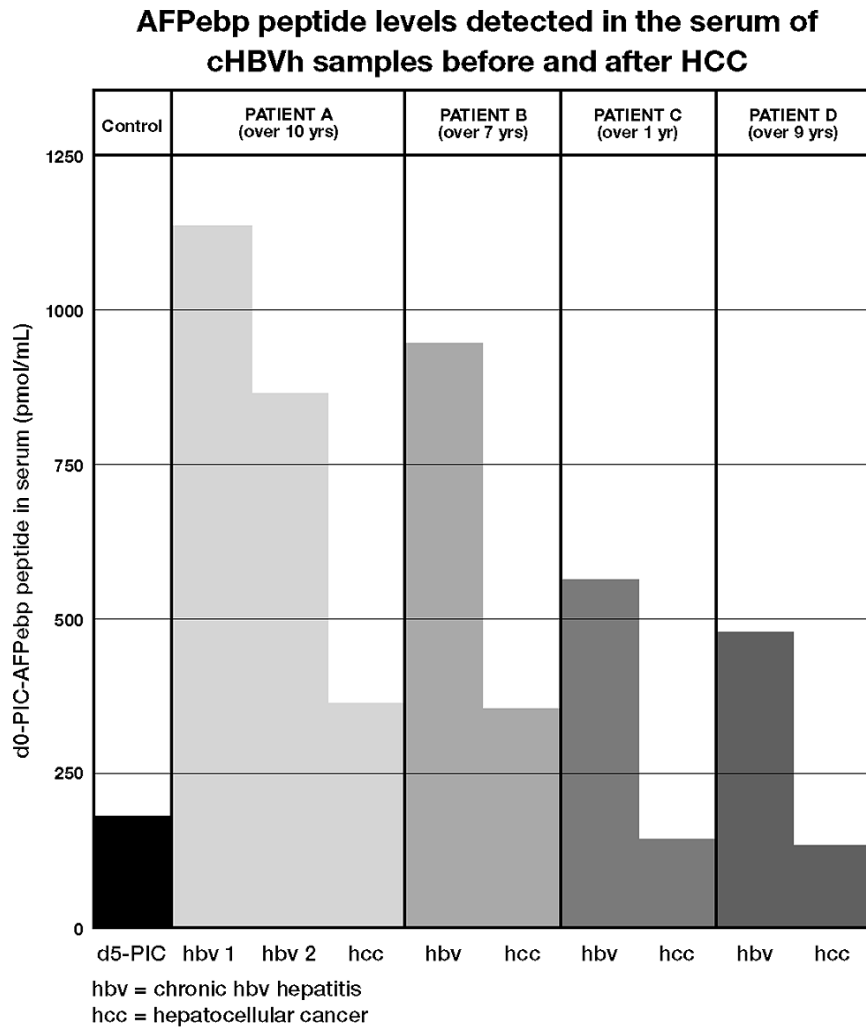## AFPebp peptide levels detected in the serum of cHBVh samples before and after HCC

Fig. 2. Serum AFPebp levels in chronic HBV hepatitis patients. AFPebp levels were measured in serum samples before and after HCC was detected over short (1 year) to long duration (10 years). Amounts of AFPebp (pmol / mL) were calculated as described in materials and methods.

shows levels of AFPebp peptide determined from four representative historical samples from chronic HBV hepatitis patients before and after they developed HCC. We observe the levels of AFPebp peptide derived from serum are greatest in samples during the pre-cancer stage, and they decrease when serum is taken when the patient has progressed to HCC regardless of the time between the serum sampling (1–10 yrs). The AFPebp levels decreased in all 10 of the paired cases ($p < 0.005$) (Fig. 3). The levels of AFPebp detected in the paired samples in the chronic HBV hepatitis state was variable, which may explain the differences observed in the overall decrease in AFPebp levels during HCC.

AFP levels in our 10 paired samples did not change as consistently as the AFPebp levels (data not shown).

In six of the pairs, the AFP levels rose with HCC development, but in two cases there was no rise, in one case the levels fell with HCC, and in one case, the AFP levels were already high with chronic HBV hepatitis and did not change with HCC.

Levels of AFPebp found in the sera from different participant classes were compared and subgroup differences ($p < 0.0001$) were determined by ANOVA. Figure 4 shows the distribution and mean levels of AFPebp in the different groups. Notice that normal controls and HBV and HCV carriers had low levels of AFPebp. AFPebp levels in chronic HBV hepatitis were the highest. HBV/HCC patients had higher levels than those for normal controls and HBV and HCV carriers, but lower levels than those observed for chronic HBV hep-
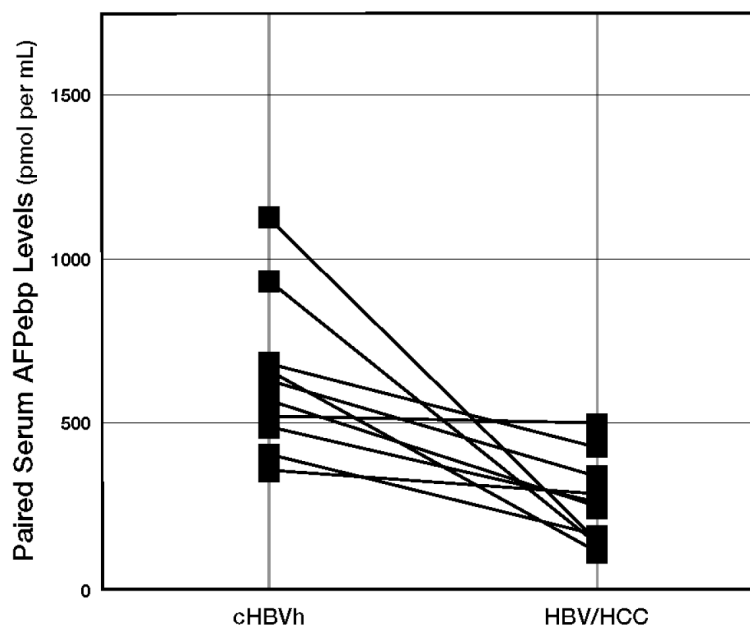
Fig. 3. AFPebp serum levels in 10 paired samples from patients transitioning from chronic HBV hepatitis (cHBVh) to HCC. Amounts of AFPebp (pmol / mL) were calculated as described in materials and methods. AFPebp levels consistently dropped in this transition to HCC ($p < 0.005$).

atitis. Levels of AFPebp in patients with autoimmune hepatitis were similar to those in HBV/HCC.

4. Discussion and conclusions

HCC is the major type of liver cancer, yet there is no reliable diagnostic or prognostic test. Genetic screening is predicted to reveal at risk individuals, but genes for HCC are not fully characterized [41]. In addition to genetic screening, expression of proteins in serum [29], including epigenetic markers [42], may indicate the development or the extent of cancer development.

To identify candidates for protein cancer biomarkers, recent advances in mass spectrometry have made it possible to detect thousands of proteins in serum [43], but there are seemingly endless protocol variations. Different sample preparations, such as depletion of abundant proteins, can focus the search on selected proteins, and different protease digestion protocols have been employed to improve peptide coverage. The different identification algorithms employed yield varied results, as do replicated runs of the same sample. There is limitation in the dynamic range, sensitivity, and capability of different instruments, with the result that there is no single, best approach. There are three sequences in the initial discovery of a protein marker: identification, verification of its presence, and determination of the

sensitivity, specificity, and predictive value. Our study addresses only the first two requirements, and further studies are needed to assess the sensitivity, specificity, and predictive value for AFPebp and HCC.

An additional method we used to increase the sensitivity to identify low abundance proteins from those identified with the more rigorous Washburn criteria [32] was to relax the Sequest criteria. Only when we relaxed the Sequest criteria did we identify proteins whose known functions were of potential interest for roles in the development of HCC (Table 2). Since we relaxed the typically used high confidence criteria for assignments, our final protein identifications may be spurious. Hence the critical need to confirm and quantify their presence, for which an alternative, reproducible method is required. A time-honored method to quantify peptides has been some form of immunoassay but, as yet, there is no available AFPebp-specific antibody. Stable isotope labeling offers a strategy both to validate as well as quantify peptides from shotgun sequencing studies. Using this approach, we confirm that a peptide that is specific to one of the low abundance proteins in Table 2, AFPebp, is present in serum (Fig. 1). Indeed, AFPebp was measured in whole serum from all but one of the 65 samples assayed, a much higher sensitivity than from the shotgun sequencing when only 50–95% of samples in chronic HBV hepatitis samples and < 50% of the other samples had any AFPebp peptide
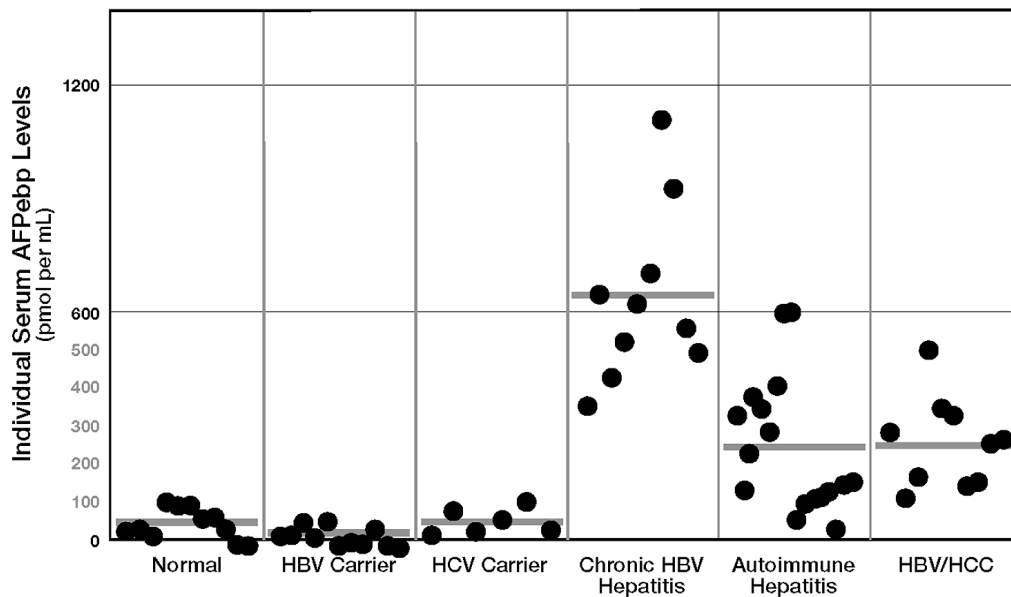
Fig. 4. Sixty-five AFPebp serum levels in controls and different disease subgroups. Serum AFPebp levels (pmol/mL) were measured in healthy controls and 5 different disease groups. The bar represents the mean for all samples included in the study. The changes were determined to be significant by ANOVA ($p < 0.0001$).

identified. This is an important verification, since its Xcorr value from shotgun sequencing was always well below the normal cutoff (2.2 for M^{2+}) in all samples tested (data not shown).

AFPebp is not glycosylated and, consequently, was present in both Con A bound and unbound fractions. Though our glycoprotein fractionation was not necessarily relevant for its enrichment, this does not invalidate the strategy for other proteins that will only be determined in further experiments. We have only verified the presence of AFPebp, but we propose that the other proteins in Table 2 merit experiments to evaluate them.

Our quantitative data identify that AFPebp is present in low abundance in normal blood sera; the levels increase during chronic HBV hepatitis, and then decrease as HCC develops. For an initial exploration of the specificity of AFPebp, we measured levels in controls, patients with carrier hepatitis B or C status, chronic autoimmune hepatitis, chronic HBV hepatitis, and HCC associated with HBV (Fig. 4). The prevalence of AFPebp in the sera from controls is low and equivalent to viral carriers (HBV or HCV). Patients with autoimmune hepatitis have levels similar to patients with HBV/HCC, but lower than the levels observed in the chronic HBV hepatitis state. Further studies of more patients in these categories will allow correlation testing of AFPebp, including tumor size, cirrhosis,/noncirrhosis, viral loads, and treatments. These variables

along with examination of patients with other liver diseases such as chronic HCV hepatitis or non-viral cirrhosis, will be necessary before the clinical specificity and predictive value of AFPebp can be determined. Additional technical studies would also be informative, including the use of different AFPebp peptide sequences for stable isotope-labeling studies.

AFP levels did not change as consistently as did AFPebp levels when the 10 patients with chronic HBV hepatitis transitioned to HCC. This inconsistency with AFP is well known as referenced in the introduction. Only further studies will clarify if AFPebp measurement has potential as a clinically useful marker of this transition.

Our data provides evidence that AFPebp is present in serum, increases in chronic HBV hepatitis, and reduces as HCC develops. Since AFPebp is a tumor suppressor [38], our data is consistent with its pathophysiological role to protect against the formation of HCC in patients with chronic HBV hepatitis. We also propose that decreasing AFPebp levels in chronic HBV hepatitis patients predisposes to HCC. We do not know the mechanism for this initial AFPebp upregulation, followed by its relative downregulation, but its role may be protective and its reduction could predispose to cancer. Thus, the AFPebp changes we have identified may represent an important regulatory mechanism in the pathophysiology of HCC that merits further study

Acknowledgments

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