

Preneoplastic Markers of Hepatitis B Virus-Associated Hepatocellular Carcinoma

Hie-Won L. Hann,¹ Jungmin Lee,^{1,3} Anne Bussard,² Catherine Liu,² Yongri R. Jin,³ Koel Guha,² Marcia M. Clayton,³ Kristin Ardlie,⁵ Michael J. Pellini,⁵ and Mark A. Feitelson^{3,4}

¹Department of Medicine, Division of Gastroenterology and Hepatology, Jefferson Medical College, Thomas Jefferson University Hospital, Philadelphia, Pennsylvania; ²Jefferson Medical College, Philadelphia, Pennsylvania; ³Department of Pathology, Anatomy, and Cell Biology, and ⁴Department of Microbiology and Immunology, Thomas Jefferson University, Philadelphia, Pennsylvania; and ⁵Genomics Collaborative, Cambridge, Massachusetts

ABSTRACT

Hepatitis B virus (HBV) carriers are at high risk for the development of hepatocellular carcinoma (HCC), but there are no reliable markers that will identify such high-risk carriers. The objective of this work is to identify serologic markers that may indicate the early presence of HCC. Since HBV-encoded X antigen (HBxAg) likely contributes to HCC by up- or down-regulation of host gene expression, X positive and negative HepG2 cells were made and subjected to cDNA subtraction. When specific ELISAs were constructed measuring differentially expressed antigens and corresponding antibodies, antibodies to several differentially expressed genes were detected. In cross-sectional and longitudinal studies, antibodies were predominantly present in patients with HBV-associated cirrhosis and HCC, but not in most carriers with hepatic inflammation alone or without active liver disease. Antibodies were also present in patients with hepatitis C virus (HCV)-related HCC, but rarely detected in sera from uninfected individuals, those with tumors other than HCC, or those with drug-induced hepatitis. Statistical analysis showed that HCC patients with four or more antibodies detectable before the appearance of HCC had decreased survival, suggesting that these markers may reflect stepwise hepatocarcinogenesis. Hence, these antibodies may serve as preneoplastic markers for HCC in HBV carriers with chronic liver disease, and may be identified by a simple blood test.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, with ~250,000 new cases diagnosed each year (1). The Hepatitis B virus (HBV) carrier state and chronic liver disease are the most important risk factors for HCC (2, 3). Given the roughly 350 million carriers of HBV globally, and that it takes 30 to 50 years to develop HCC (4), ample opportunity exists for early detection, chemoprevention, or treatment of patients with small tumors. Although easily treatable, patients with early HCC are often asymptomatic, and, therefore, most diagnoses are made in patients with late-stage, advanced HCC. Hence, identifying carriers at high risk for tumor development, or with early HCC, would be very important in improving survival.

The relative risk of HBV carriers with chronic liver disease developing HCC is in excess of 100 (2, 3), which is one of the strongest associations between an environmental carcinogen and a tumor type. The finding that the HBV-like woodchuck hepatitis virus causes HCC in nearly 100% of chronically infected woodchucks (5) suggests that woodchuck hepatitis virus is a complete carcinogen and that it makes a genetic contribution to HCC. This contribution involves expression of the X antigen, which promotes the development and persistence of

the carrier state (6), is strongly expressed in the liver of animals and patients with chronic liver disease (7–9), and is capable of hepatocellular transformation *in vitro* (10, 11) and *in vivo* (12, 13).

Hepatitis B x antigen (HBxAg) is a *trans*-activating protein (14, 15) that may *trans*-activate virus gene expression and replication (16, 17) but also may alter patterns of cellular gene expression that contribute to tumor development (18, 19). In support of this hypothesis, HBV DNA that encodes HBxAg (20) is found integrated into many regions of the host DNA in the livers of HBV carriers, resulting in the intracellular accumulation of HBxAg (9, 21). HBxAg also stimulates several signal transduction pathways and binds to selected transcription factors (15, 22), which may result in up-regulated expression of several cellular genes (23–27). One of these, α -fetoprotein, has been used as a tumor marker for HCC (28), although its low specificity has made its continued use controversial (29). The HBV-encoded “e” antigen (HBeAg) is also associated with HCC development (30), although most carriers with HCC are anti-HBe positive years before tumor diagnosis. Other reports, with microarray analysis, show altered expression of host gene products in tumor compared with nontumor (31–34), but it is not clear whether these changes reflect early or late events in hepatocarcinogenesis, or whether any will be useful as diagnostic or prognostic markers of HCC. Recently, antibodies in HCC sera, binding to a subset of proteins in a cDNA expression library, suggest that changes in gene expression in tumor compared with normal liver could trigger antibody responses that might identify tumor-bearing patients (35).

Since HBxAg expression precedes tumor development (7–9), at least some HBxAg-mediated alterations in gene expression occur before the appearance of HCC. To identify these changes, HBxAg was stably transfected into the human hepatoblastoma cell line HepG2 (36), and the expression profiles of RNAs from HBxAg positive ([+]) and negative ([–]) cells were compared by PCR select cDNA subtraction (37). The results revealed proteins that were up-regulated in HBxAg [+] compared with [–] HepG2 cells, and in the peritumor liver from tumor-bearing patients (37–41). Hence, experiments were designed to test whether HBxAg up-regulated proteins in the liver triggered corresponding antibodies in patients with chronic liver disease who later developed HCC.

MATERIALS AND METHODS

Populations and Sera. All of the test serum samples used in this study were obtained from Korean immigrants residing on the East Coast of the United States. For cross-sectional studies, single serum samples were obtained from members of the Korean Immigrant Churches who were screened for HBsAg and anti-HBs, and for alanine aminotransferase (ALT) as part of a hepatitis B campaign for high-risk populations. This effort identified asymptomatic HBV carriers who would benefit from subsequent medical intervention, thereby reducing the incidence of chronic liver disease and HCC. The latter group consisted, in part, of 190 males and 126 females ranging in age from 30 to 60 years old. This group tested negative for HBsAg and anti-HBs, had normal ALT, and were considered uninfected (group 1). Among the HBV carriers identified in these screenings, 109 were selected: 21 with normal ALT (<40 U/L; group 2), 40 with elevated ALT (>40; group 3), 28 with cirrhosis

Received 3/30/04; revised 7/30/04; accepted 8/11/04.

Grant support: Supported by NIH grants CA48656 and CA66971 (M. Feitelson) and 5R25 CA48010 (summer research experience for medical students, A. Bussard, C. Liu, and K. Guha).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Mark Feitelson, Room 222 Alumni Hall, Department of Pathology, Anatomy, and Cell Biology, Thomas Jefferson University, 1020 Locust Street, Philadelphia, PA 19107-6799 phone: 215-503-1036; fax: 215-503-9982; E-mail: Mark.Feitelson@jefferson.edu.

©2004 American Association for Cancer Research.

Table 1 Antibody (Ab) frequencies in cross-sectional studies

Population	No. of patients	No. (%) of Ab markers			
		0	1	2	≥3
Tumor type					
HCC (HBV) (group 5)	20	2 (10)	0 (0)	2 (10)	16 (80)
HCC (HCV)	13	2 (15)	4 (31)	3 (23)	4 (31)
Breast	60	50 (83)	6 (10)	3 (5)	1 (2)
Colon	50	38 (76)	9 (18)	2 (4)	1 (2)
Head/neck	41	36 (88)	5 (12)	0	0
Prostate	50	41 (82)	7 (14)	2 (4)	0
Korean					
Uninfected (group 1)	316	216 (68)	70 (22)	27 (8.5)	3 (0.95)
HBV carriers (group 2)	21	14 (67)	4 (19)	2 (9.5)	1 (4.8)
HBV, hepatitis (group 3)	40	30 (75)	4 (10)	3 (7.5)	3 (7.5)
HBV, cirrhosis (group 4)	28	8 (29)	5 (18)	6 (21)	9 (32)
Drug-induced hepatitis	51	37 (72)	11 (22)	3 (6)	0
Icelandic blood donors	156	155 (99)	1 (0.65)	0	0

(group 4), and 20 with HCC (group 5; all diagnosed by physical exam, blood chemistry, and magnetic resonance imaging; Table 1).

Control samples collected for cross-sectional studies included 13 from as many HCC patients who were negative for HBsAg, anti-HBs, and HBV DNA but positive for hepatitis C virus (HCV) antibodies and RNA. These patients had similar age, race, and gender distribution compared with HBV tumor-bearing patients. Additional controls were obtained from patients with breast, colon, head/neck, or prostate cancer, and were provided by Genomics Collaborative, Inc. (Table 1). Genomics Collaborative, Inc. also provided samples from patients with drug-induced hepatitis. Additional samples were collected from blood donors in Iceland (kindly provided by Dr. Olafur Jensson, Icelandic Red Cross, Reykjavik, Iceland), in which HBV infection is very low in the general population (Table 1).

For longitudinal studies, serial serum samples were collected during routine office visits from 25 HBsAg [+] patients with HCC and another 24 HBsAg [+] cirrhotic patients with regenerative and/or dysplastic nodules but no HCC. HCC patients were treated with cryosurgery, transarterial chemoembolization, percutaneous EtOH injection, radiofrequency tumor ablation, and/or combination chemotherapy. Additional characteristics of these populations are presented in Table 2.

In all of the populations, each serum sample was stored at -80°C until it was decided to conduct the present studies. All of the samples were then tested blindly with no identifying information that could be traced back to the participating donors. These studies were reviewed and approved by the Insti-

tutional Review Board (IRB) at Thomas Jefferson University and by multiple, local or central IRBs working with Genomics Collaborative, Inc..

Establishment of HBxAg [+] and [-] HepG2 Cells. HBxAg [+] and [-] HepG2 cell lines were constructed exactly as described previously (37). Whole cell RNA from these cells was isolated and was subjected to PCR select cDNA subtraction. Characterization of several differentially expressed genes have shown that they enhance hepatocellular growth in culture and in soft agar, promote resistance to apoptosis, and/or stimulate tumor development (37–41).

ELISA for Detection of Antibodies to Differentially Expressed Proteins.

To construct an ELISA to detect each antibody, sequences spanning hydrophilic domains were selected for solid-phase peptide synthesis. Solid-phase peptide synthesis was conducted at Thomas Jefferson University and resulted in the peptide sequences listed in Table 3. Peptides were individually coupled to keyhole limpet hemocyanin (KLH, Sigma Chemical Co., St. Louis, MO), and antibodies were raised in rabbits, as described previously (42). These sera had been previously characterized (37–41) and were used to verify ELISA specificity by radioimmunoprecipitation (see below).

Each ELISA was constructed and performed the same way with the exception of the peptides used for coating the wells. To detect antibodies to up-regulated gene 4 (anti-URG4), for example, a mixture containing 1 μg of each URG4 synthetic peptide (Table 3) in 50 μL of phosphate buffered saline (PBS), was used to coat each well (Immunolon 4, Thermo Lab Systems, Franklin, MA) in a 96-well plate. After overnight incubation at 4°C , the wells were washed six times with PBS and a Nunc ImmunoWash 120 and then were blocked overnight with PBS containing 10% fetal calf serum (FCS). After washing, a test serum (50 μL /well at a 1:10 dilution in PBS/FCS) was added to each well, and the plates were incubated overnight at 4°C . After washing six times with PBS, affinity-purified horseradish peroxidase-conjugated anti-human immunoglobulin (50 μL /well at a 1:100 dilution in PBS/FCS; Cooper Biomedical, Malvern, PA) was added to each well. Plates were incubated for 1 hour at 37°C and were washed six times with PBS; binding was determined by the addition of *o*-phenylenediamine (OPD, Abbott Labs, North Chicago, IL) with an automated ELISA plate reader at 450 nm.

Controls included performing the assays in wells coated with irrelevant peptides (Table 3) or PBS/FCS only, or by preincubating antibody [+] human sera with an excess of synthetic peptides (25 μg of each peptide) for 1 hour at 37°C before the assay. A positive value for a serum tested in wells coated with relevant peptides was two SDs above the mean of the negative control wells. The latter was the average absorbance (*A*) value of six human serum samples from individuals with no serologic evidence of HBV infection or biochemical evidence of liver disease. A true positive serum tested on PBS/FCS coated wells also yielded *A* values which were less than two SDs above the average

Table 2 Characteristics of HBV-infected populations used for longitudinal studies

HCC	No. of cases	Mean age, y	Gender M/F	Average no. of sera	Average duration of collection, mo	HBeAg	anti-HBe	HBeAg → anti-HBe seroconversion
+	25	53.6 (31–67)	21/4	8.8 (4–23)	43.5 (5–155)	9	12	4
–	24	50.5 (34–66)	22/2	11.3 (3–28)	58.6 (7–157)	10	9	5

NOTE. The numbers in parentheses represent the range of values for each variable measured.

Table 3 Peptides used for construction of specific ELISAs

Protein	Sequence (NH ₂ → COOH terminus)	Amino acids	Ref.
URG4	CEECQKAKDRMERITRRIKIDSDAY SVPGPRPRDKRQLLDPPGC	457–480 817–834	52
URG7	GVWNQTEPEPAATSC HHHGRGYLRMSPLFK	12–25 56–70	51
URG11	PCPELACPREEWRLGP DPSRSPHSTSSFPGRSSATSCDSR HPPDGSFSTFHDGPPLEDPC	254–269 568–591 611–630	53
S15a	KSINNAEKRGKRQC DHEEARRKHTGGKC	12–23 112–124	
Sui1	DDYDKKKLVKAFKKKFC EHPEYGEVIQLQGDRKKNIC	52–69 75–94	50
control peptide 1	YADSDKIKRTIREMRDKAKQCEEC	inverse of URG4 457–480	
control peptide 2	RSDCSTASSGRPFSSSTSHPSRSPD	inverse of URG11 568–591	

A value of the negative serum controls. All of the test sera were evaluated blindly and in duplicate. To monitor sensitivity, rabbit peptide antisera in each test were serially diluted and tested to insure that the A values and titer were reproducible in each test plate.

Radioimmunoprecipitation. Full-length cDNAs for URG4 (39), URG7 (38), URG11 (40), S15a (41), and Sui1 (37) were each subcloned into pcDNA3. Each recombinant was linearized, *in vitro* transcribed, and then translated in rabbit reticulocyte lysates (Promega, Madison, WI) with [³⁵S]methionine and [³⁵S]cysteine (Amersham, Piscataway, NJ). Incorporation was verified by SDS/PAGE followed by autoradiography. For radioimmunoprecipitation, 5 × 10⁴ counts per minute of each protein was incubated for 1 hour at 37°C with 5 μL of human serum samples that were [+] or [-] for the corresponding antibody by ELISA. Samples were then incubated with 2 μL of Sepharose 4B-protein G beads (Pharmacia, Piscataway, NJ) for 15 min on ice, were washed three times in reticulocyte lysate antibody buffer [100 mmol/L NaCl, 0.1 mol/L Tris-HCl (pH 8.0), 1% NP40], and then were analyzed by SDS/PAGE (on 12% gels) and autoradiography.

Detection of HBsAg, anti-HBs, and Alanine Aminotransferase. HBsAg and anti-HBs were measured with the Auszyme and Ausab kits, respectively (Abbott Laboratories, North Chicago, IL). ALT was measured with a commercially available assay (Sigma).

Quantitative Determination of HBV DNA and HCV RNA. Quantitation of HCV RNA in 100 μL of serum was carried out with the Cobas Amplicor Hepatitis C Monitor kit (Roche) following the manufacturer's enclosed instructions.

Quantitation of HBV DNA in serum samples was conducted by real-time PCR. Briefly, 100 μL of serum was digested with 1 μL of RNase-free DNaseI (10–50 units/μL; Roche, Indianapolis, IN) at 37°C for 30 minutes. DNA extraction was performed with QIAamp DNA Blood minikit (Qiagen, Valencia, CA). Nucleic acids were recovered in 200 μL. Ten microliters of the latter were used for amplification in 25 μL of Ready-To-Go PCR Beads (Pharmacia Biotech, St. Louis, MO) containing primers HBSF1 [5'-CACATCAGGATTCCTAGGACC-3', nucleotides (nts.) 168–188], HBSR1 (5'-GGTGAGTGATTGGAGGTTG-3', nts. 341–323), and the TaqMan probe: HBSPI (5'-FAM-CAGAGTCTAGACTCGTGGTGGACTTCT-AMRA-3', nts. 247–270), all at 200 nmol/L and 3.5 mmol/L manganese acetate. PCR consisted of an initial step of 2 minutes at 95°C, followed by 40 cycles (15 seconds at 95°C and 30 seconds at 60°C) with a Smart Cycler (Cepheid, Sunnyvale, CA). The final titer was normalized to copies per milliliter of serum.

Statistical Analysis. The Student's *t* test was used to compare the mean difference in survival time for patients with different numbers of antibodies and the mean difference in the number of antibodies in patients who developed HCC compared with patients who did not develop tumor. The relationship between different antibodies was examined with the χ^2 test.

RESULTS

Selection of Antibodies and Specificity of ELISAs. ELISAs were constructed based on the results of PCR select cDNA subtraction of HepG2X compared with HepG2CAT cells, and verification that proteins up-regulated in HepG2X cells were also up-regulated in infected liver (37–41). To establish the specificity of each ELISA, human serum samples were assayed in wells coated with or without the synthetic peptides of interest. Antibody [+] sera were independently checked for specificity by preincubating selected serum samples with relevant or irrelevant synthetic peptides before assay. The results show that when antibody [+] serum samples were preincubated with different amounts of homologous synthetic peptides, but not with irrelevant synthetic peptides, the binding was blocked (Fig. 1). Although the data for anti-URG4 (Fig. 1A) and anti-URG11 (Fig. 1B) are presented, analogous data were obtained in the ELISAs for anti-URG7, anti-S15a, and anti-Sui1 (data not shown). These findings demonstrate the specificity of the ELISAs used herein.

Specificity was also demonstrated by radioimmunoprecipitation of *in vitro* translated URG4, URG7, URG11, S15a, and sui1 polypeptides. The results show that serum samples [+] for a single antibody by ELISA were also [+] for antibody by immunoprecipitation,

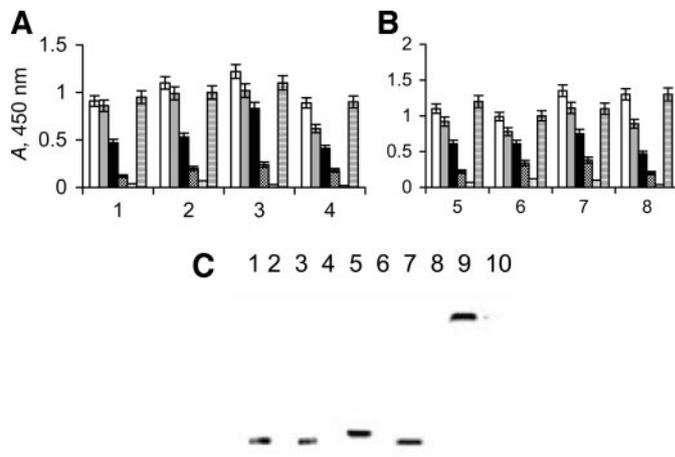


Fig. 1. Specificity of ELISAs. A serum sample from each of four patients was preincubated with 0 (white bars), 0.5 (gray bars), 5 (black bars), 10 (dark gray bars), or 25 μg (vertical strips) of a mixture containing homologous synthetic peptides before assay. Alternatively, each serum sample was preincubated with 25 μg of irrelevant synthetic peptides (horizontal stripes) before assay. A, anti-URG4 assay. B, anti-URG11 assay. C, immunoprecipitation of ³⁵S-labeled *in vitro* translated URG7 (*M_r* 10,700; Lanes 1 and 2), URG4 (*M_r* 10,400; Lanes 3 and 4), S15a (*M_r* 14,300; Lanes 5 and 6), Sui1 (*M_r* 13,300; Lanes 7 and 8), or URG11 (*M_r* 68,000; Lanes 9 and 10) with human serum samples that were positive for the corresponding antibody by ELISA (Lanes 1, 3, 5, 7, and 9) or with human serum samples that were negative for all of these antibodies by ELISA (Lanes 2, 4, 6, 8, and 10). Immunoprecipitates were analyzed by SDS/PAGE, the gel dried down, and the image revealed by autoradiography. (A, absorbance.)

whereas serum samples [-] for all antibodies were unable to immunoprecipitate any of the radiolabeled antigens (Fig. 1C). In addition, Sepharose 4B-protein G beads did not bind to any of the *in vitro* radiolabeled proteins in the absence of human serum, which suggested there was no nonspecific binding to the beads (data not shown).

Assay sensitivity was determined by measuring the binding of a single dilution of rabbit antibody to wells coated with known serial dilutions of corresponding synthetic peptides. Because one molecule of synthetic peptide probably binds to one antibody molecule, anti-URG4 could reproducibly be detected at a sensitivity of 2.6 × 10⁻⁸ mol/L (or 1.3 pmol). The sensitivities for the other antibodies were 3.5 × 10⁻⁸ mol/L (or 2.1 pmol) for anti-URG7, 1.56 × 10⁻⁷ mol/L (or 7.8 pmol) for anti-URG11, 8.8 × 10⁻⁸ mol/L (or 4.4 pmol) for anti-S15a, and 6.2 × 10⁻⁸ mol/L (or 3.1 pmol) for anti-Sui1.

Cross-sectional Studies. To test whether HBV patients with cirrhosis and/or HCC had antibodies against these differentially expressed proteins, serum samples from Korean populations (groups 1–5, Table 1) were tested. Individual antibodies were found in a small percentage of uninfected individuals, in HBV carriers, and among patients with chronic hepatitis (Table 1, groups 1–3). In contrast, many of the patients with cirrhosis, and an even larger percentage of patients with HCC, were antibody [+] in each of the assays (Table 1, groups 4 and 5). Hence, antibodies were detectable primarily in HBV patients at highest risk for tumor development (*i.e.*, those with cirrhosis) and in HCC patients.

The number of antibodies in individual serum samples also differed in these groups. Three or more antibodies were found in 16 (80%) of 20 antibody [+] HCC patients, in 9 (32%) of 28 antibody [+] patients with cirrhosis, and in only 3 (0.95%) of 316 uninfected individuals (Table 1). Five to ten percent of HBV patients with normal or elevated ALT had 3 antibodies (Table 1). These results suggest that the number of antibodies in a sample were related to an increased risk for HCC and were not associated with chronic HBV infection, *per se*. In addition, among patients with HCC or who were likely to develop HCC, there was a 30-or-more-fold increase of having three or more antibodies, relative to the uninfected population.

Table 4 Selected characteristics of antibodies (Abs) in HCC positive and negative patients.

HCC	Ab [+] patients, n (%)	Average no. of Abs/patient	α -Fetoprotein		Frequency of Abs (anti-)				
			Before dx n (%)	After dx n (%)	URG4 n (%)	URG7 n (%)	URG11 n (%)	S15a n (%)	Sui1 n (%)
+	23 (92)	3.6	8 (32)	5 (20)	15 (60)	17 (68)	19 (76)	19 (76)	13 (52)
-	18 (75)	2	6 (25)		10 (42)	12 (50)	4 (17)	9 (38)	13 (54)

Abbreviation: dx, diagnosis.

To determine whether these antibodies correlated with HBV DNA levels in serum, all of the samples from HBsAg [+] patients were assayed for HBV DNA by real-time PCR. More than 95% of patients in each group were HBV DNA [+], with titers ranging from 10^3 to 10^8 virus genome equivalents/mL of serum. However, there was no correlation between HBV DNA titer and the presence or number of antibodies (data not shown).

To test whether these antibodies were in patients with HCV-associated HCC, cross-sectional studies were conducted with serum samples from 13 HCC patients who were infected with HCV but not HBV. Eleven of 13 tumor-bearing patients (85%) had an average of two antibodies (range, 1–4 antibodies), suggesting that patients with HCV-associated HCC also had a high frequency of antibodies (Table 1). In contrast, when parallel studies were performed with samples obtained from individuals with breast, colon, head/neck, or prostate cancer, >75% of these patients had no detectable antibodies (Table 1). Hence, these antibodies appear to be associated with HCC and not with other tumor types. To determine whether these antibodies might be triggered in response to general liver cell injury, samples from patients with drug-induced hepatitis were evaluated. The results show that the majority (72%) lacked the antibodies (Table 1), suggesting that the antibodies were not commonly associated with liver cell damage, *per se*. Finally, when the antibodies were tested in samples from blood donors in Iceland, only one individual had antibody (anti-Sui1; Table 1). This is lower than that observed in the uninfected Korean population but may be related to the fact that Koreans come from a part of the world that is endemic for HBV, whereas residents from Iceland do not.

Longitudinal Studies. To test whether these antibodies were detectable in HBV patients with cirrhosis before the diagnosis of HCC and/or in newly diagnosed HCC patients, previously stored serial serum samples from each of 25 HBV patients who later developed HCC and another 24 cirrhotic patients who had regenerative or

dysplastic nodules, but no HCC, were tested blindly for these Abs. These populations had similar characteristics (Table 2), none of which were statistically different in the two groups ($P > 0.1$).

Among HBV patients who developed HCC, an average of 3.6 antibody specificities appeared before, or at the time of, tumor diagnosis in 23 (92%) of 25 patients (Table 4). There were no patients in whom these antibodies were first detected after the diagnosis of HCC, suggesting that they appeared just before and/or at early stages of tumor development. In contrast, when α -fetoprotein was measured in the same samples, elevated levels (>20 ng/ml) were detected before, or at the time of, tumor diagnosis in only eight (32%) of the HCC patients (Table 4). In addition, the percentage of patients in this group that were positive for a single antibody was much higher (52–76%) than the percentage (32%) with elevated α -fetoprotein before tumor diagnosis, suggesting that, individually, the new antibodies detected many more patients who later developed HCC than did α -fetoprotein. In contrast, among cirrhotic patients with no HCC, 18 (75%) had an average of only two different antibody specificities detectable (Table 4). This was significantly lower than the number of antibodies found in patients who developed HCC ($P < 0.001$). In addition, with the exception of anti-Sui1, the fraction of non-tumor-bearing patients with other antibody specificities was less than in those who later developed HCC. Hence, there were fewer antibodies in fewer patients who did not develop tumor compared with those who did.

An indicator that these antibodies may have prognostic value in HCC development is the time in which they appeared before tumor diagnosis. In the 18 HBV-infected patients whose samples were available, one or more antibodies appeared 31.3 months, on average, before diagnosis (range, 2–118 months; Table 5). Antibodies were detected in seven other patients at the time of HCC diagnosis (patients 8, 11, 13, 14, 18, 20, and 24 in Table 5), but unfortunately, in these patients, no serum samples were available before diagnosis. Hence,

Table 5 Presence, frequency, and distribution of antibodies (Abs) in HBV patients who developed HCC

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Gender	M	M	M	M	M	M	M	M	M	F	M	M	M	M	F	F	M	M	M	M	M	F	M	M	M
Age	57	53	56	52	50	61	53	57	59	51	49	58	65	58	48	64	54	35	31	48	43	67	61	52	58
Anti-URG4	+	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	-	+	-
Anti-URG7	+	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	-
Anti-URG11	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+
Anti-S15a	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	+	-	+	-
Anti-Sui1	±	-	-	-	±	±	±	-	-	-	±	±	-	-	±	-	±	±	±	±	-	-	±	-	±
No. of Ab*	4	0	2	4	4	3	3	4	4	4	5	5	4	4	5	3	5	3	3	0	3	2	4	4	2
First Ab(s)†	7	-	11	11	11	12‡	11	(4)§	4	4	(4)§	4	(4)§	(4)§	4	7	4	(7)§	7	(D2)§	-	11	11	(4)§	11
					D2			(11)	7	11	(11)	7	(7)	(11)	11	11	7	(D2)				12		(7)	(11)
								(12)	11			11		(12)	12		11							(11)	(12)
First time detected (mo)¶	2	-	10	10	14	3	3	0	23	50	0	9	0	0	16	118	24	0	15	0	-	101	85	0	18

NOTE. Case numbers 1–25.

* Number of Ab specificities in each patient.

† Lists the Ab specificity/specificities to first appear in the serum of each patient.

‡ 12 refers to anti-S15a.

§ For these patients, no serum samples were available prior to HCC diagnosis; therefore, although the Ab specificities in parentheses were detected at the time of diagnosis, it is not known how far in advance of diagnosis these Abs appeared in each patient.

|| D2 refers to anti-Sui1.

¶ First time detected (mo) refers to the time (in months) prior to HCC diagnosis that the first Ab or Abs were initially detected. For example, patient 10 developed four Ab specificities prior to tumor diagnosis. Anti-URG4 and anti-URG11 were the first detectable Abs that appeared prior to HCC diagnosis.

Table 6 Characteristics of antibodies (Abs) in HBV patients who had regenerative/dysplastic nodules

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Gender	M	M	M	M	M	M	M	M	M	M	F	M	M	M	M	M	M	M	M	M	M	M	F	M
Age	45	46	46	65	48	43	38	55	47	43	50	48	53	46	57	44	51	46	48	53	62	36	46	60
Anti-URG4	+	-	-	-	+	-	-	+	-	+	+	-	-	-	-	-	+	-	+	-	-	+	+	+
Anti-URG7	-	+	-	-	+	-	+	+	+	-	+	+	-	+	+	-	+	-	-	-	+	+	+	-
Anti-URG11	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Anti-S15a	-	-	-	-	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	-	+	-
Anti-Sui1	-	±	-	-	±	±	±	±	±	±	±	±	-	-	-	-	-	-	-	-	±	±	±	±
No. of Ab*	1	2	0	0	5	1	3	5	4	2	4	3	0	0	1	0	2	0	1	1	3	3	5	2
First Ab(s)† (type)	4	D2‡	-	-	D2	D2	D2	D2	7	4	D2	7	-	-	7	-	4	-	4	12	7	7	D2	D2
					11		7	7	12		12													
					12		12																	
Mo. of collection	27	127	80	39	157	8	12	34	27	29	161	54	35	34	22	7	70	57	13	46	100	57	56	152

NOTE. Case numbers 1 to 24.

* Number of Abs detected during the period of observation.

† Specificity of Abs initially detected in the sera of patients. When multiple Ab specificities are listed, they were detected in the initial serum obtained from these patients.

‡ D2 = Sui1.

one or more antibodies may precede the diagnosis of HCC by up to 3 years.

When the antibody specificities were examined in patients who later developed HBV-associated HCC, anti-URG11 and anti-S15a were the most frequent, with each present in 19 of the 23 antibody [+] HCC patients (Table 5). They coexisted in 16 of these patients ($P < 0.025$; Table 5). The other antibody specificities, although less frequent, were also present in more than one half of the HCC patients (Tables 4 and 5). In contrast, among HBV patients without HCC, anti-Sui1 and anti-URG7 were the most prevalent antibodies in 13 (54%) and in 12 (50%) patients, respectively. These antibody specificities coexisted in 10 of these patients ($P < 0.02$). Anti-URG11 and anti-S15a, the most prevalent antibodies in patients who developed cancer, were rarely found among patients without HCC (Table 6). Hence, the antibody specificities appear to differ between patients who develop HCC and those who do not.

When the data were analyzed to identify the earliest appearing antibody, anti-URG11 appeared first in 17 (74%) of the 23 antibody [+] HBV associated HCC patients (Table 5). In comparison, anti-URG4 appeared first in nine patients (39%), anti-URG7 in eight (35%), anti-S15a in six (26%), and anti-Sui1 in four (17%; Table 5). In contrast, anti-Sui1 and anti-URG7 were the most prevalent antibodies that appeared earliest among patients with regenerative or dysplastic nodules. Thirteen (72%) of the 18 antibody [+] patients with nontumor nodules developed either anti-Sui1 and/or anti-URG7, whereas the other antibodies appeared in only a few patients (Table 6). Hence, the antibody specificities that initially appeared in patients were different, depending on whether or not they developed HCC. Anti-URG11 stood out as an early antibody in patients who later developed cancer. Because carcinogenesis is multistep, it was of interest to see whether the number of antibodies that preceded HBV-associated HCC was related to patient survival after diagnosis. When this was determined, patients with 0 to 1 marker before diagnosis survived an average of 36 to 38 months. Patients with 2 to 3 markers survived 22 to 24 months, whereas patients with ≥ 4 antibodies before the diagnosis of HCC had a significantly shorter survival time (5.5 months; $P < 0.001$), suggesting that the number of antibodies appearing before diagnosis may have an impact on survival.

The patterns of antibodies in several HBV-infected patients are presented in Fig. 2. In one patient (Fig. 2A), anti-URG4 and anti-URG11 appeared about 45 months before tumor diagnosis, and anti-URG7 appeared about 34 months before diagnosis, which suggested that these were early markers of HCC. In Fig. 2B, four antibodies were detected 22 months before tumor diagnosis (and probably sooner if additional sera were available). Despite treatment, the tumor continued to grow, and antibodies were persistently present. Tumor growth

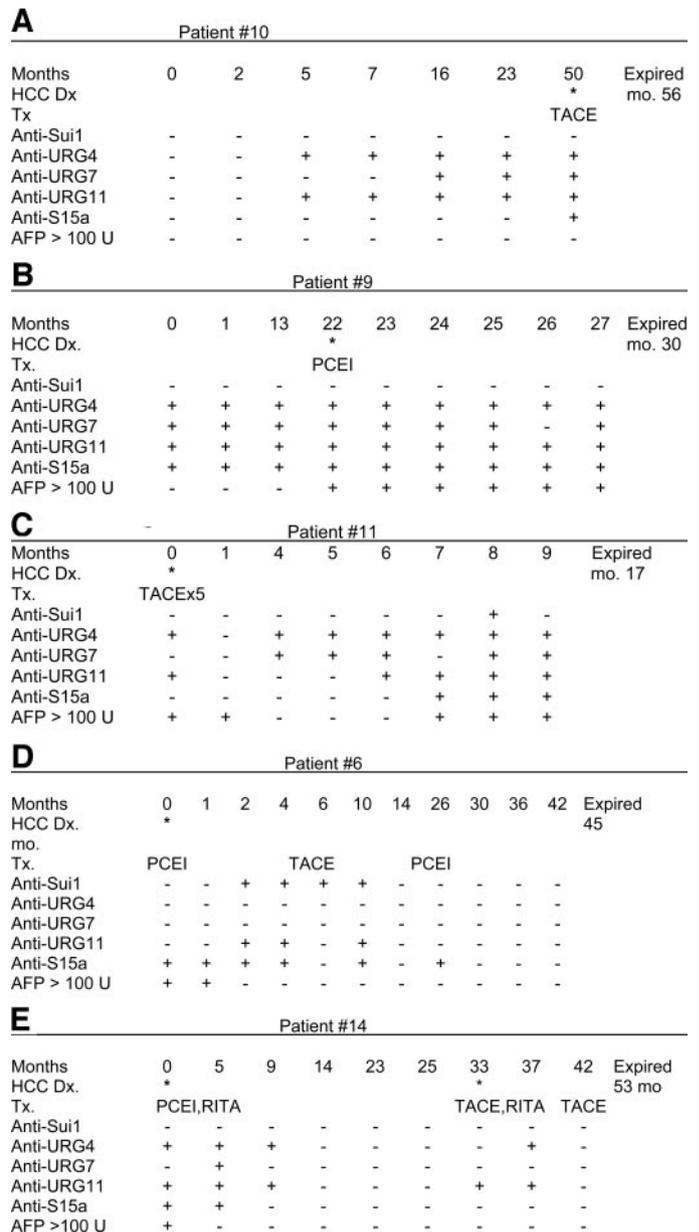


Fig. 2. Antibody profiles of individual HBV patients who developed HCC during the period of observation. *, the point in time when the diagnosis of HCC was made. Please see the Results section for additional information on each patient. (Dx, diagnosis; Tx, treatment; TACE, transarterial chemoembolization; AFP, α -fetoprotein; PCEI, percutaneous ethanol injection; RITA, radiofrequency tumor ablation.)

and persistent antibodies were also observed in seven other patients. In Fig. 2C, the patient was treated aggressively on diagnosis, and the existing antibodies became transiently undetectable, but the tumor reappeared, and multiple antibodies reappeared. Similar patterns were observed in four other patients. In a unique case (Fig. 2D) in which aggressive treatment after diagnosis resulted in the disappearance of tumor, antibodies also became undetectable. In another patient who was treated for HCC, and then relapsed (Fig. 2E), antibodies became undetectable when the magnetic resonance imaging showed no tumor (months 14–25) but became positive again when the tumor reappeared (month 33). Three other patients had a similar profile. Hence, the presence, frequency, and distribution of antibodies may reflect continued elevated risk for tumor relapse, at least in some patients.

DISCUSSION

The strong epidemiologic relationship between chronic HBV infection and HCC (2, 3) and the important contribution of HBxAg to HCC (10, 12, 13, 15, 22) suggest that the molecular pathways altered by HBxAg are important for tumor development. Previously, HBxAg has been shown to alter the expression of cellular genes that promote hepatocellular growth, survival, and tumorigenesis (37–41). Given that they are overexpressed in the cirrhotic liver and in peritumor tissue, and that liver cell damage and destruction are important features of progressive chronic liver disease, it is likely that these proteins are released into the bloodstream. It is proposed that these proteins trigger corresponding antibody responses that may accompany the onset of critical transforming events. In this way, the antibodies to these HBxAg “target” genes mirror changes that contribute to tumor development. This may partially explain the very strong association of these antibodies in HCC-bearing patients and in those at high risk for tumor development. Preliminary evidence to support this hypothesis has already been presented in abstract form (43, 44).

The finding of multiple antibodies in most patients developing HBV-associated HCC (Tables 4 and 5), relative to patients without HCC (Tables 4 and 6), and in very few uninfected individuals (Table 1), is consistent with the association of these antibodies with multistep carcinogenesis. In addition, the finding that HCC patients with four or more antibodies before tumor development have a significantly shorter survival, is consistent with the idea that the up-regulated expression of multiple pathways increases the risk for the appearance of single and/or multinodular HCC. If so, then the panel of antibodies characterized herein may be molecular-based risk factors and, therefore, preneoplastic markers for HCC.

The appearance of anti-URG11 as the first and most commonly detected antibody in patients who develop HBV-associated HCC (Tables 4 and 5), and that anti-Sui1 and anti-URG7 are the earliest and most common antibodies in patients with dysplastic and/or regenerative nodules (Tables 4 and 6), suggest that the antibody specificities that appear in patients with chronic liver disease may have prognostic value in predicting the outcome of chronic infection. URG11, for example, is a novel protein up-regulated by HBxAg that promotes cell growth in culture and colony formation in soft agar, and that accelerates tumor development in nude mice (40). Therefore, the appearance of anti-URG11 before tumor development suggests that up-regulation of this “oncoprotein” is turned on in the early stages of tumorigenesis. Similar arguments can be made for S15a, a ribosomal protein associated with enhanced cell growth (41), and the unique protein URG4, which also acts like an oncoprotein (39). URG7, on the other hand, inhibits Fas-mediated apoptosis (38). Because Fas is central to the destruction of virus-infected hepatocytes (45, 46), resistance to apoptosis is likely to be a major mechanism whereby HBxAg [+] hepatocytes may survive in the presence of recurring

cellular immune responses. Together, these considerations suggest that up-regulation of positive growth-regulatory proteins, and increased resistance to apoptosis, would be expected to promote tumor survival and growth. In this context, it is proposed that the appearance of multiple antibody specificities corresponding to these up-regulated proteins, contribute to the shortened survival of these patients after HCC diagnosis, although this observation needs to be verified in additional patients.

In contrast to patients who developed HBV-associated HCC, patients with dysplastic or regenerative nodules most commonly had anti-Sui1 and anti-URG7 (Tables 4 and 6). Sui1 is a translation initiation factor that helps to maintain the integrity of translation initiation by working with elongation initiation factor 2 (eIF-2) to enable initiator tRNA(Met) to establish ribosomal recognition of an AUG codon (47). Sui1 has also recently been shown to be a tumor suppressor in hepatocarcinogenesis (37). Combined with URG7, it is proposed that the altered expression of Sui1 in infected liver, negatively regulates hepatocellular growth, which may be one of the reasons why patients with predominantly anti-Sui1 and anti-URG7 in the absence of anti-URG11, anti-URG4, and anti-S15a, do not readily develop cancer, even after several years of monitoring (Table 6). In this context, it will be important to see whether these different antibody specificities correlate with outcome in larger populations of Korean HBV patients and in other populations at high risk for the development of chronic liver disease and HCC. The finding of these antibodies in the sera of most HCV patients with HCC suggests that there may be several common steps whereby HBV and HCV trigger HCC. Although any common denominators require further work, HBV and HCV constitutively activate some of the same signal transduction pathways that promote cell survival and growth. These include mitogen-activated protein kinase (MAPK; refs. 48, 49), insulin-like growth factor II (IGFII; refs. 50, 51), nuclear factor κ B (NF- κ B; refs. 52–54), activating protein 1 (AP-1; refs. 52–54), signal transducer and activator of transcription (STAT3; refs. 54, 55), and phosphatidylinositol 3-kinase (PI3K; refs. 56, 57). If so, these signaling pathways may target some of the same cellular genes and, in the context of chronic liver disease, may result in the development of overlapping antibody specificities. Meanwhile, the approaches used in this work, involving the elucidation of HBxAg-mediated alterations in hepatocellular gene expression, have resulted in the development of antibodies that may be useful in the identification of HBV patients with chronic liver disease who are most likely to develop HCC.

REFERENCES

1. Feitelson MA. HBV and cancer. In: Notkins AL, Oldstone MBA, editors. Concepts in viral pathogenesis. New York: Springer-Verlag; 1986. p. 269–75.
2. Beasley RP, Hwang LY. Epidemiology of hepatocellular carcinoma. In: Vyas GN, Dienstag JL, Hoofnagle JH, editors. Viral hepatitis and liver disease. New York: Grune and Stratton; 1994. p. 209–24.
3. Beasley RP, Hwang LY, Lin CC, Chien CS. Hepatocellular carcinoma and HBV. A prospective study of 22,707 men in Taiwan. *Lancet* 1981;2:1129–33.
4. Tiollais P, Pourcel C, Dejean A. The hepatitis B virus. *Nature (Lond)* 1985;317:489–95.
5. Popper H, Roth L, Purcell RH, Tennant BC, Gerin JL. Hepatocarcinogenicity of the woodchuck hepatitis virus. *Proc Natl Acad Sci USA* 1987;84:866–70.
6. Chen HS, Kaneko S, Girones R, et al. The WHV X gene is important for establishment of virus infection in woodchucks. *J Virol* 1993;67:1218–26.
7. Jin YM, Yun C, Park C, Wang HJ, Cho H. Expression of hepatitis B virus X protein is closely correlated with the high periportal inflammatory activity of liver diseases. *J Viral Hepat* 2001;8:322–30.
8. Feitelson MA, Lega L, Duan LX, Clayton MM. Characteristics of woodchuck hepatitis X-antigen in the livers and sera from infected animals. *J Hepatol* 1993;17(Suppl 3):S24–34.
9. Wang W, London WT, Lega L, Feitelson MA. HBxAg in the liver from carrier patients with chronic hepatitis and cirrhosis. *Hepatology* 1991;14:29–37.
10. Luber B, Arnold N, Sturzl M, et al. Hepatoma-derived integrated HBV DNA causes multi-stage transformation *in vitro*. *Oncogene* 1996;12:1597–608.
11. Seifer M, Hohne M, Schaefer S, Gerlich WH. *In vitro* tumorigenicity of hepatitis B virus DNA and HBx protein. *J Hepatol* 1991;13(Suppl 4):S61–5.

12. Kim CM, Koike K, Saito I, Miyamura T, Jay G. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. *Nature (Lond)* 1991;351:317–20.
13. Koike K, Moriya K, Iino S, et al. High-level expression of hepatitis B virus HBx gene and hepatocarcinogenesis in transgenic mice. *Hepatology* 1994;19:810–9.
14. Rossner MT. Hepatitis B virus X-gene product: a promiscuous transcriptional activator. *J Med Virol* 1992;36:101–17.
15. Henkler F, Koshy R. Hepatitis B virus transcriptional activators: mechanisms and possible role in oncogenesis. *J Viral Hepat* 1996;3:109–21.
16. Bouchard MJ, Wang LH, Schneider RJ. Calcium signaling by HBx protein in hepatitis B virus DNA replication. *Science (Wash DC)* 2001;294:2376–8.
17. Nakatake H, Chisaka O, Yamamoto S, Matsubara K, Koshy R. Effect of X protein on transactivation of HBV promoters and on viral replication. *Virology* 1993;195:305–14.
18. Caselmann WH. Transactivation of cellular gene expression by hepatitis B viral proteins: a possible mechanism of hepatocarcinogenesis. *J Hepatol* 1995;22(Suppl 1):34–7.
19. Koshy R, Hofschneider PH. Transactivation by hepatitis B virus may contribute to hepatocarcinogenesis. *Curr Top in Microbiol Immunol* 1989;144:265–81.
20. Wollersheim M, Debelka U, Hofschneider PH. A transactivating function encoded in the hepatitis B virus X gene is conserved in the integrated state. *Oncogene* 1988;3:545–52.
21. Diamantis ID, McGandy CE, Chen TJ, Liaw YF, Gudat F, Bianchi L. Hepatitis B X gene expression in hepatocellular carcinoma. *J Hepatol* 1992;15:400–3.
22. Feitelson MA, Duan LX. Hepatitis B virus X antigen in the pathogenesis of chronic infections and the development of hepatocellular carcinoma. *Am J Pathol* 1997;150:1141–57.
23. Zhou MX, Watabe M, Watabe K. The X-gene of human hepatitis B virus transactivates the c-jun and α -fetoprotein genes. *Arch Virol* 1994;134:369–78.
24. Su Q, Liu JF, Zhang SX, Li DF, Yang JJ. Expression of insulin-like growth factor II in hepatitis B, cirrhosis and hepatocellular carcinoma: its relationship with hepatitis B virus antigen expression. *Hepatology* 1994;20:788–99.
25. Kim SO, Park JG, Lee YI. Increased expression of the insulin-like growth factor I (IGF-I) receptor gene in hepatocellular carcinoma cell lines: Implications of IGF-I receptor gene activation by hepatitis B virus X gene product. *Cancer Res* 1996;56:3831–6.
26. Yoo YDI, Ueda H, Park K, et al. Regulation of transforming growth factor- β 1 expression by the hepatitis B virus (HBV) X transactivator. *J Clin Investig* 1996;97:388–95.
27. Han J, Yoo HY, Choi BH, Rho HM. Selective transcriptional regulations in the human liver cell by hepatitis B viral X protein. *Biochem Biophys Res Commun* 2000;272:525–30.
28. Oka H, Tamori A, Kuroki T, Kobayashi K, Yamamoto S. Prospective study of alpha-fetoprotein in cirrhotic patients monitored for development of hepatocellular carcinoma. *Hepatology* 1994;19:61–6.
29. Sherman M. Alpha-fetoprotein: an obituary. *J Hepatol* 2001;34:603–5.
30. Yang HI, Lu SN, Liaw YF, et al. Hepatitis B e antigen and the risk of hepatocellular carcinoma. *N Engl J Med* 2002;347:168–74.
31. Tackels-Horne D, Goodman MD, Williams AJ, et al. Identification of differentially expressed genes in hepatocellular carcinoma and metastatic liver tumors by oligonucleotide expression profiling. *Cancer* 2001;92:395–405.
32. Shirota Y, Kaneko S, Honda M, Kawai HF, Kobayashi K. Identification of differentially expressed genes in hepatocellular carcinoma with cDNA microarrays. *Hepatology* 2001;33:832–40.
33. Chen X, Cheung ST, So S, et al. Gene expression patterns in human liver cancers. *Mol Biol Cell* 2002;13:1929–39.
34. Xu XR, Huang J, Xu ZG, et al. Insight into hepatocellular carcinogenesis at transcriptome level by comparing gene expression profiles of hepatocellular carcinoma with those of corresponding noncancerous liver. *Proc Natl Acad Sci USA* 2001;98:15089–94.
35. Uemura M, Nouse K, Kobayashi Y, et al. Identification of the antigens predominantly reacted with serum from patients with hepatocellular carcinoma. *Cancer (Phila)* 2003;97:2474–9.
36. Knowles BB, Howe CC, Aden DP. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science (Wash DC)* 1980;209:497–9.
37. Lian Z, Pan J, Liu J, et al. The translation initiation factor, SUI1, may be a target of hepatitis B x antigen in hepatocarcinogenesis. *Oncogene* 1999;18:1677–87.
38. Lian Z, Liu J, Pan J, et al. A cellular gene upregulated by hepatitis B virus encoded X antigen promotes hepatocellular growth and survival. *Hepatology* 2001;34:146–57.
39. Tufan NLS, Lian Z, Liu J, et al. Hepatitis B x antigen stimulates expression of a novel cellular gene, URG4, that promotes hepatocellular growth and survival. *Neoplasia* 2002;4:355–68.
40. Lian Z, Liu J, Li L, et al. Up-regulated expression of a unique gene by hepatitis B x antigen promotes hepatocellular growth and tumorigenesis. *Neoplasia* 2003;5:229–44.
41. Lian Z, Liu J, Li L, et al. Human S15a is an effector which is up-regulated by HBV X protein. *Mol Carcinogen* 2004;40:34–46.
42. Feitelson MA, Millman I, Duncan GD, Blumberg BS. Presence of antibodies to the polymerase gene product(s) of hepatitis B and woodchuck hepatitis virus in natural and experimental infections. *J Med Virol* 1988;24:121–36.
43. Jin YR, Hann HWL, Clayton MM, Bussard A, Liu C, Feitelson MA. Preneoplastic markers of hepatocarcinogenesis in hepatitis B virus carriers [abstract]. *J Hepatol* 2002;36:184.
44. Hann HWL, Feitelson MA, Jin YR, Clayton MM, Bussard A, Liu C. Early markers of hepatocellular carcinoma in hepatitis B virus carriers[abstract]. *Hepatology* 2002;36:243A.
45. Diao J, Khine AA, Sarangi F, et al. X protein of hepatitis B virus inhibits Fas-mediated apoptosis and is associated with up-regulation of the SAPK/JNK pathway. *J Biol Chem* 2001;276:8328–40.
46. Hayashi N, Mita E. Involvement of Fas system-mediated apoptosis in pathogenesis of viral hepatitis. *J Viral Hepat* 1999;6:357–65.
47. Yoon HJ, Donahue TF. The sui1 suppressor locus in *Saccharomyces cerevisiae* encodes a translation factor that functions during tRNA_i^{Met} recognition of the start codon. *Mol Cell Biol* 1992;12:248–60.
48. Hayashi J, Aoki H, Kajino K, Moriyama M, Arakawa Y, Hino O. Hepatitis C virus core protein activates the MAPK/ERK cascade synergistically with tumor promoter TPA, but not with epidermal growth factor or transforming growth factor α . *Hepatology* 2000;32:958–61.
49. Benn J, Schneider RJ. HBV X protein activates ras-GTP complex formation and establishes a ras, raf, MAP kinase signaling cascade. *Proc Natl Acad Sci USA* 1994;91:10350–4.
50. D'Arville CN, Nouri-Aria KT, Johnson P, Williams R. Regulation of insulin-like growth factor II gene expression by hepatitis B virus in hepatocellular carcinoma. *Hepatology* 1991;13:310–5.
51. Lee S, Park U, Lee YI. Hepatitis C virus core protein transactivates insulin-like growth factor II gene transcription through acting concurrently on Egr1 and Sp1 sites. *Virology* 2001;283:167–77.
52. Kekule AS, Lauer U, Weiss L, Lubber B, Hofschneider PH. HBV transactivator HBx uses a tumour promoter signalling pathway. *Nature (Lond)* 1993;361:742–5.
53. Kato N, Yoshida H, Kiolo ONS, et al. Activation of intracellular signaling by hepatitis B and C viruses: C-viral core is the most potent signal inducer. *Hepatology* 2000;32:405–12.
54. Gong G, Waris G, Tanveer R, Siddiqui A. Human hepatitis C virus NS5A protein alters intracellular calcium levels, induces oxidative stress, and activates STAT-3 and NF- κ B. *Proc Natl Acad Sci USA* 2001;98:9599–604.
55. Lee YH, Yun Y. HBx protein of hepatitis B virus activates Jak1/STAT signaling. *J Biol Chem* 1998;273:25510–5.
56. He Y, Nakao H, Tan SL, et al. Subversion of cell signaling pathways by hepatitis C virus nonstructural 5A protein via interaction with Grb2 and p85 phosphatidylinositol 3-kinase. *J Virol* 2002;76:9207–17.
57. Shih WL, Kuo ML, Chuang SE, Cheng AL, Doong SL. Hepatitis B virus X protein inhibits transforming growth factor-beta-induced apoptosis through the activation of phosphatidylinositol 3-kinase pathway. *J Biol Chem* 2000;275:25858–64.

Preneoplastic Markers of Hepatitis B Virus-Associated Hepatocellular Carcinoma

Hie-Won L. Hann, Jungmin Lee, Anne Bussard, et al.

Cancer Res 2004;64:7329-7335.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/64/20/7329>

Cited articles This article cites 53 articles, 14 of which you can access for free at:
<http://cancerres.aacrjournals.org/content/64/20/7329.full#ref-list-1>

Citing articles This article has been cited by 1 HighWire-hosted articles. Access the articles at:
<http://cancerres.aacrjournals.org/content/64/20/7329.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.