Gene Expression Analysis in HBV Transgenic Mouse Liver: A Model to Study Early Events Related to Hepatocarcinogenesis

Michele Barone,[1] Daniela Spano,[2] Maria D'Apolito,[3] Marta Centra,[3] Carla Lasalandra,[3] Mario Capasso,[2] Alfredo Di Leo,[1] Stefano Volinia,[4] Diego Arcelli,[4] Natalia Rosso,[5] Antonio Francavilla,[1] Claudio Tiribelli,[5] Achille Iolascon[2,6]

[1]Sezione di Gastroenterologia, Dipartimento di Emergenza e di Trapianto d'Organo (D.E.T.O.), Università di Bari, Bari, Italia; [2]CEINGE Biotecnologie Avanzate s.c.ra.l., Napoli, Italia; [3]Laboratorio di Medicina Molecolare, Dipartimento di Scienze Mediche e del Lavoro, Università di Foggia, Foggia, Italia; [4]Dipartimento di Morfologia ed Embriologia, Università di Ferrara, Ferrara, Italia; [5]Centro Studi Fegato, AREA Science Park, Campus Basovizza Bldg Q, Università di Trieste, Trieste, Italia; [6]Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli, Napoli, Italia

Hepatitis B virus (HBV) is one of the major etiological factors responsible for the development of hepatocellular carcinoma (HCC). We used a transgenic mouse, containing HBV sequences, as a model system to unravel the molecular mechanisms of hepatocarcinogenesis induced by HBV. We chose this animal model because it consistently develops liver cancer after intermediate steps that mimic the natural history of HBV infection in humans. In this study, we focus our attention on the early events leading to liver cancer. We compared the gene expression profile of 3-month-old transgenic mice with that of 3-month-old wild-type (wt) animals. In the transgenic mouse, microarray data analysis showed a total of 45 significantly differentially expressed genes, 25 highly expressed (fold change ≥ 2; $P = 0.0025$), and 20 downregulated (fold change ≤ 0.5; $P = 0.0025$). These genes belong to several different functional categories such as the regulation of immunological response, transcription, intracellular calcium ion mobilization, regulation of cell cycle and proliferation, NF-κb signal transduction cascades, and apoptosis. In particular, the upregulation of the antiapoptotic gene *Nuprl* and the downregulation of the proapoptotic gene *Bnip3* were found. This observation was supported by an in vitro apoptosis assay that showed downregulation of apoptosis in hepatocytes of HBV transgenic mouse compared with wt mice treated with staurosporine. In conclusion, our experimental approach allowed identification of new genes modulated by HBV and showed that the apoptotic process was deregulated in transgenic mouse hepatocytes. These data shed light on one possible mechanism by which HBV induces hepatocarcinogenesis.

Online address: http://molmed.org
doi: 10.2119/2006-00015.Barone

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent primary solid tumor of the liver. It is a heterogeneous disease in terms of etiology and underlying associations as well as biologic and clinical behavior. HCC develops commonly, but not exclusively, in a setting of liver cell injury, which leads to inflammation, hepatocyte regeneration, liver matrix remodeling, fibrosis, and ultimately, cirrhosis. The vast majority of HCC worldwide (80%) is attributed to hepatitis B virus (HBV) or HCV infection, but other risk factors include dietary exposure to aflatoxin B_1, alcohol abuse, hemochromatosis, fatty liver disease, androgenic steroid use, and α-1 antitrypsin deficiency. The mechanisms by which these varied etiologies lead to cirrhosis and HCC are not well understood.

Numerous evidences indicate that HBV is one of the major etiological factors responsible for the development of HCC (1). The HBV genome is a circular, partially double-stranded DNA molecule containing 4 overlapping open-reading frames (ORFs), encoding PreS/S, PreC/C, P, and X proteins (HBx). HBV DNA integrates into the host genome, and this integration is believed, in part, to be carcinogenetic. In fact, the random integration of viral sequences could determine the fortuitous activation of an adjacent cellular oncogene (2). In particular, the integration of HBV genomic DNA encoding HBx is the most frequent viral marker found in HCC (3).

Address correspondence and reprint requests to *Prof Achille Iolascon, Chair of Medical Genetics, University Federico II – Naples, CEINGE Biotecnologie Avanzate s.c.ra.l., Via Comunale Margherita 482, 80145 Napoli, Italy. Phone: +39-0813722897; Fax: +39-0813722804; e-mail: iolascon@dbbm.unina.it.*
Submitted March 7, 2006; accepted for publication May 6, 2006.

Several evidences indicate that HBx has oncogenic function. This protein is essential for viral replication in vivo (4,5) and can be oncogenic in strains of transgenic mice (2). Furthermore, HBx induces cellular gene expression deregulation through its transactivational activity (6) by interacting with components of the basal transcription machinery and several transcription factors (7-10). HBx also activates several signal transduction pathways including NF-κB, mitogen-activated protein kinase, protein kinase C, and JAK/STAT pathways (11-16), which take part in many aspects of cell regulation. HBx has been shown to promote tumor cell invasion (17), and several studies showed that HBx plays a role in regulating apoptosis (18-22). Collectively, these studies indicate that HBx plays a prominent role in hepatocarcinogenesis induced by HBV infection. It should be remembered, however, that chronic liver injury initiates a cascade of events characterized by elevated cell proliferative activity and compromised cellular detoxification and repair function. These events may lead to accumulation of DNA mutations, probable in replicating cells, which in the long run would favor the selection of cells with a malignant phenotype (23). This cascade of events is consistent with the close correlation between chronic inflammation and carcinogenesis (24) and with the observation of a greater risk of HCC in HBV-infected patients with cirrhosis than in those without cirrhosis.

At present, it still remains uncertain which of the oncogenic functions of HBV are really involved in HCC development and what is the sequence of genomic events responsible for hepatocyte transformation. The HBV transgenic mouse, officially designed as Tg (Alb-1 HBV) Bri44, contains the HBV genomic sequences for pre-S, S, and X proteins. It represents an ideal model for the study of the steps (gene and protein expression alterations) that lead to HCC (25-27). In fact, this transgenic mouse develops progressive hepatocyte damage (25-29) which determines, in the first month of life, degenerative alterations (26), followed by an active inflammatory damage that is characterized by elevated damage-related compensatory proliferative response (27, 29). This is a precancerous condition which is consistently followed, after the eighth to ninth month after birth, by the appearance of dysplastic hepatic lesions (27,28), which become clearly neoplastic after the 12th month (27). Successively, neoplastic lesions progressively grow to macroscopic nodules that can be observed in all animals within the 16th to 18th month of life. Therefore, this transgenic mouse mimics many of the pathological events that occur before the development of HCC in chronic HBV infection in humans, representing a useful model system to study the processes of initiation, development, and progression of HCC upon HBV infection.

In the effort to elucidate the early molecular mechanisms of hepatocarcinogenesis induced by HBV, we investigated the gene expression profiling of HBV transgenic mice compared with that of wt mice. We focused our attention on the gene expression alterations occurring in 3-month-old HBV transgenic mice in the hypothesis that this age represents the start of the multistep process of hepatocarcinogenesis. Our data show the downregulation of apoptotic process in HBV transgenic mouse hepatocytes, indicating deregulation as a possible mechanism by which HBV induces hepatocarcinogenesis.

MATERIALS AND METHODS

Animals

Twelve-week-old male C57BL/6J (wt) mice were purchased from Charles River (Calco, Italy). In addition, 12-week-old male, transgenic mice designed as Tg (Alb-1HBV) Bri 44 were obtained from Jackson Laboratories (Bar Harbor, ME, USA). All mice were kept in temperature-, air-, and light-controlled (light on from 7 a.m. to 7 p.m.) conditions and received food and water ad libitum for at least 1 week before being used for our experiments. All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals.

RNA Extraction and Quantification

Mice were killed by CO_2 asphyxiation. The whole liver was isolated, snap frozen, and stored in liquid nitrogen. Total RNA from liver tissues was prepared using TRIzol Reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. After the ethanol precipitation step in the TRIzol extraction procedure, a cleanup was performed using RNeasy columns (Qiagen, Valencia, CA, USA). Total RNA concentration and purity were assessed by spectrophotometric analysis. The A_{260}/A_{280} ratio was between 1.9 and 2.1 for each sample. The integrity of total RNA was assessed on standard 1% agarose/formaldehyde gel.

Microarray

The microarray hybridization experiments were performed in triplicate. The cRNA "targets" were generated as described by the Affymetrix Expression Analysis Gene Chip Technical Manual, protocol P/N 900218rev.2, P/N70021rev.3. Briefly, double-stranded cDNA was synthesized using the SuperScript Choice System (Invitrogen) and a primer containing poly(dT) downstream to T7 RNA polymerase promoter sequence (MWG). In vitro transcription using double-stranded cDNA as a template in the presence of biotinylated UTP and CTP was carried out using BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostic, Farmingdale, NY, USA). Biotinylated cRNA was purified, fragmented, and hybridized to the Affymetrix MG-U74av2 chips (Affymetrix, Santa Clara, CA, USA), containing 12625 probe sets recognizing murine genes of mostly known function. The cRNA was detected with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR, USA), and analysis was completed by using a Hewlett-Packard Gene Array Scanner (Affymetrix). The

20x eukaryotic hybridization control kit was included, as recommended by the manufacturer (Affymetrix Data Analysis Fundamentals manual). The signal for each probe set was quantified using Micro Array Suite 5.0 software (Affymetrix). The recommended internal controls and quality checks were performed: briefly, average background and noise were verified to be comparable between different experiments; the percentages of probe sets scored as present were similar in the replicates; the ratios of 3′ and 5′ probe sets for GAPDH and actin were less than 3; finally the scaling/normalization factors were comparable between different arrays. Default parameters were used for the statistical algorithm and for probe set scaling using Microarray Suite 5.0 (with a target intensity of 100).

Analysis of Data

Potential clusters corresponding to subgroups were visualized with a 2-step approach. In the first step, the data were scaled from each array to a target intensity value of 100 (Microarray Suite 5.0) to enable interarray comparisons. Data were filtered so that the absolute value of the fold change was 2 or more. Additionally, genes that were scored as absent in experimental and baseline files were removed. The statistical significance in the comparison analyses was calculated using Wilcoxon signed rank test as described in the Affymetrix Data Analysis Fundamentals manual. The default cut off *P* value of 0.0025 was used. The sorting for change (2 fold) was chosen to remove false positives and to detect changes that were biologically more relevant. In the second step, all data were permutated 100 cycles using the multiclass response parameter of the Significance Analysis of microarrays algorithm (SAM) (Tusher et al., 2001),Q2 a statistical method for identifying significant changes in gene expression that takes into account multiple testing. For a user-defined threshold, based on the number of expected false-positive regulated genes, a list with the significantly regulated genes is generated.

The total set of 12,600 genes was reduced to the significant differentially expressed genes. The reduced set was prepared for biological interpretation.

Annotations about individual genes were obtained in the Net Affx Analysis Center (www.affymetrix.com) and the NCBI site (ncbi.nlm.nih.gov: OMIM and Pub Med).

RT-PCR

The results obtained by microarray analysis were validated by semiquantitative reverse transcription (RT) followed by polymerase chain reaction (PCR). The RNAs used for these analyses were not the same as those used for gene chip analysis, but derived from independent extraction. Total RNA (1 μg), previously treated with RNase-free DNase I (Roche Diagnostics S.p.a., Milano, Italy), was used for semiquantitative RT-PCR. Reverse transcription of mRNAs was carried out with the Superscript III reverse transcriptase kit (Invitrogen) using oligo(dT) as primer. Single-stranded cDNAs in 1 μL of a 20-μL reaction mixture were amplified by PCR using the following conditions: 25 pmol of each primer, 25 nmol of each deoxyribonucleoside triphosphate, 3 U Taq Gold DNA polymerase (Applied Biosystems, Branchberg, NJ, USA), 5 μL 10× PCR buffer in a final volume of 50 μL. Oligonucleotides were designed using the PRIMER3 program (http://www.genome.wi.mit.edu). The nucleotide sequences of primers and corresponding annealing temperatures used are available on request. β-Actin mRNA was amplified as an internal control for the reverse transcription reaction. Further controls were performed with RNA preparations amplified in absence of reverse transcriptase to verify the absence of DNA contamination.

Western Blot

Liver tissues were resuspended in the lysis buffer containing 10 mM Tris-HCl buffer (pH 7.5) and protease inhibitors (PIs) (2 μg/mL leupeptin, 2 μg/mL aprotinin, 1 μg/mL pepstatin, 100 μg/mL phenylmethylsulfonyl fluoride [Roche Diagnostics S.p.a., Milano, Italy], and 2 mM EDTA]. Liver tissues were homogenized using the Dunce homogenizer, and homogenates were centrifuged at 14000*g* for 10 min. The supernatants were collected and stored frozen at –80 °C. All procedures were carried out at 4 °C. Protein concentration was determined by the Bradford assay (Bio-Rad). Total proteins (50 μg) were diluted in Laemmli sample buffer, resolved by 12% SDS-PAGE, and transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). The membranes were incubated with polyclonal rabbit antibody against HMGB2 (PharMingen, Becton Dickinson Biosciences, San Diego, CA, USA) at 1:750 dilution and with antitubulin antibody (Chemicon International, Temecula, CA, USA) at 1:1000 dilution. Immunodetection was realized using enhanced chemiluminescence reagents (Cell Signaling Technology, New England Biolabs, Ipswich, MA, USA).

Hepatocyte Isolation

All liver perfusions were carried out at noon.Q3 Hepatocytes were isolated as previously described (30). Viability was determined by trypan blue exclusion assay, and only preparations having a viability higher than 80% were used. The purity of the preparations was more than 98% as demonstrated by the staining for glucose-6-phosphatase performed as previously described (30). Hepatocytes, resuspended in minimum essential medium (Eagle) with Earle's salts and supplemented with glucose (1.8 g/L), pyruvate (0.11 g/L), aspartate (0.022 g/L), serine (0.021 g/L), proline (0.030 g/L), glutamine (0.584 g/L), HEPES (4.75 g/L), hydrocortisone (50 nM), insulin (10^{-7} M) and 10% FCS, were plated at a density of 1.2×10^6 cells/4.5 mL medium per 60-mm Falcon dish, and maintained at 37 °C and 5% humidified atmosphere. After 4 h (attachment period), medium was replaced

with FCS-free medium (incubation medium), and hepatocytes were maintained for additional 12 or 16 h in the presence or in absence of 10 μM staurosporine, an inducer of apoptosis.

Thiazolyl Blue (MTT) Assay

After the exposure to the proapoptotic agent staurosporine, the number of untreated and treated cells was determined by MTT assay. The MTT assay was performed in 3 dishes for each experimental time point (12 and 16 h, in the presence or absence of staurosporine) as described by the manufacturer (Sigma, Milano, Italy; product no. M 5655).

DNA Ladder Assay

For the evaluation of DNA fragmentation, a modification of the method described by Cifone et al. was used (31). At the end of the incubation period, the medium was removed (dead cells in suspension were not collected because their number was very low) and adherent hepatocytes were collected by scraping and resuspended in 1× PBS. After centrifugation (5 min at 450*g*), the cells were resuspended in 300 μL lysis buffer (10 mM Tris-HCl pH 8.0, 25 mM EDTA, 100 mM NaCl, 0.5% SDS, 0.1 mg/mL proteinase K) and incubated overnight at 37 °C. Genomic DNA was extracted by phenol/chloroform (1:1, vol/vol), precipitated with ethanol, resuspended in TE 1× (Tris-HCl 10 mM, pH 8.0, 1 mM EDTA) and treated with RNase (final concentration 1 μg/mL) at 37 °C for 1 h. Finally, genomic DNA was analyzed on a 1.8% agarose gel using ethidium bromide staining for the detection.

RESULTS

Gene Expression Profile Analysis

To unravel the early molecular events of hepatocarcinogenesis induced by HBV, we performed a gene-profiling approach on the liver total RNAs of 3-month-old wt and HBV transgenic mice. Genes that were statistically differently expressed were grouped in categories according to subcellular localization, biological process, or molecular function as revealed by the gene ontology (GO) biological process description. The genes either increased or decreased in transgenic mouse are classified in Tables 1 and 2 based on the GO biological term.

Upregulated Genes

Table 1 shows the list of 25 liver cell transcripts that were upregulated (fold change ≥ 2, $P = 0.0025$) in transgenic mice. The most common GO functional category was immunological function. We also observed an overexpression of genes involved in NF-κb signal transduction (*Vcam1* and *Cxcr4*), regulation of transcription (*Hmgb2*, *Nfatc1*, *Nupr1*, and *Atf3*), and regulation of cell cycle and proliferation (*Cdkn2d* and *Slfn2*). In addition, we observed upregulation of some genes involved in intracellular calcium ion mobilization (*Guca1*, *Nucb2*, *Ptp4a3*, *Lcp2*) and actin cytoskeleton organization and biogenesis (*Tmsb10*, *Marcks*).

Downregulated Genes

A total of 21 genes were found to be downregulated (fold change ≤ 0.5, $P = 0.0025$) in transgenic mice (Table 2). The GO functional categories represented were 1) cholesterol metabolism, 2) amino acid metabolism, 3) blood coagulation, 4) extra matrix structure component, 5) cell adhesion, and 6) water transport. We also found downregulated genes encoding for the receptors involved in the control of proliferation (*Ghr*, *Erbb3*) and for the *Bnip3* proapoptotic gene.

Validation of Microarray Data by Semiquantitative RT-PCR

We checked the microarray data, analyzing the expression level of the statistically different genes in 3-month-old HBV transgenic and wt mouse livers. Semiquantitative RT-PCR experiments were carried out on several randomly selected differentially expressed genes (data not shown), which were shown to be modulated in HBV transgenic mouse liver as demonstrated in microarray analysis. In particular, Figure 1 shows the semiquantitative RT-PCR analysis performed on the *Nupr1*, *Cdkn2d*, and *Car2* genes. All 3 genes were clearly overexpressed in the transgenic versus the wt mice.

Western Blot Analysis

We performed a Western blot analysis to correlate the increase in *Hmgb2* gene expression to its protein expression. As shown in Figure 2, the analysis of liver total extracts, obtained from transgenic and wt mice, demonstrated that Hmgb2 protein was expressed at very low level in controls, whereas its expression was significantly increased in the transgenic mice (Figure 2).

Evaluation of Apoptosis in Cultured Hepatocytes

The DNA-laddering assay was performed on hepatocytes isolated from 3-month-old transgenic and wt mice and incubated for 12 or 16 h in presence of staurosporine (Figure 3). DNA fragmentation was observable in hepatocytes isolated from wt mice 12 h after incubation with staurosporine; this phenomenon was even more evident after 16 h of incubation. On the other hand, in hepatocytes isolated from transgenic mice, the phenomenon of DNA-laddering was less evident both 12 and 16 h after incubation with staurosporine (Figure 3). To exclude that this difference between transgenic and wt hepatocyte DNA-laddering could be related to a different cellular mass (number of functional active hepatocytes), this parameter was evaluated by MTT assay. Similar results were obtained in hepatocytes isolated from transgenic and wt mice (data not shown).

DISCUSSION

HCC is a heterogeneous disease in terms of etiology and biologic and clinical behavior. Very little is known about how many genes concur at the molecular level for tumor development, progression, and aggressiveness.

As in other solid tumors, development, progression, and metastasis of HCC are multistep processes and are believed to be caused by the accumulation of genetic alterations, including chromo-

Table 1. Upregulated genes in 3-month-old HBV transgenic mice.

Gene Function and GenBank Acc. No.	Gene Description	Gene Symbol	Fold Change
Immunological function			
M13018	Cysteine-rich protein 1 (intestinal)	Crip1	33.58
Z80112	Chemokine orphan receptor 1	Cmokor1	4.91
X52643	Histocompatibility 2, class II antigen A, alpha	H2-Aa	17.80
M21932	Histocompatibility 2, class II antigen A, beta 1	H2-Ab1	8.01
U16985	Lymphotoxin β	Ltb	8.76
X00496	Ia-associated invariant chain	Ii	7.38
J04170	CD72 antigen	Cd72	4.98
Ubiquitination			
AI788534	Ring finger protein 8	Rnf8	11.65
NF-κB signal transduction cascades			
M84487	Vascular cell adhesion molecule 1	Vcam1	5.06
AF000236	Chemokine (C-X-C motif) receptor 4	Cxcr4	5.04
One-carbon compound metabolism			
M25944	Carbonic anhydrase 2	Car2	6.51
Regulation of transcription			
X67668	High mobility group box 2	Hmgb2	5.44
AF087434	Nuclear factor of activated T-cells, cytoplasmic 1	Nfatc1	18.56
AI852641	Nuclear protein 1	Nupr1	72.44
AK217100	Activating transcription factor 3	Atf3	3.8
Intracellular calcium ion mobilization			
L36860	Guanylate cyclase activator 1a (retina)	Guca1	15.14
AJ222586	Nucleobindin 2	Nucb2	9.69
AF035645	Protein tyrosine phosphatase 4a3	Ptp4a3	4.99
U20159	Lymphocyte cytosolic protein 2	Lcp2	4.43
Regulation of cell cycle and proliferation			
U19597	Cyclin-dependent kinase inhibitor 2D (p19)	Cdkn2d	3.71
AF099973	Schlafen 2	Slfn2	9.76
Actin cytoskeleton organization and biogenesis			
AI852553	Thymosin, beta 10	Tmsb10	4.27
M600474	Myristoylated alanine rich protein kinase C substrate	Marcks	3.66
Protein modification			
X61232	Carboxypeptidase E	Cpe	4.63
Intracellular signalling cascade			
AA189555	RIKEN cDNA F630107H02 gene	F630107H02Rik	8.13

somal aberrations, oncogene activation, and inactivation of tumor suppressor genes (32-36). Recent studies have identified numerous epigenetic changes, such as promoter CpG island methylation, that are responsible for inactivation of tumor suppressor genes (37-40). Gene expression profiles of HCCs with different etiology show different molecular signatures (41,42), suggesting that heterogeneous hepatocarcinogenetic pathways, involved in cell proliferation, cell cycle, apoptosis, and angiogenesis, exist and are deregulated in hepatocarcinogenesis.

Despite efforts to unravel the molecular mechanisms of hepatocarcinogenesis, the biologic heterogeneity and multiple etiologies of HCC result in an incomplete understanding of the key molecular changes leading to HCC development. In particular, gene expression profile analysis, using DNA microarray technique, has paved a way to better understand molecular mechanisms in the development of HCC (41,42). Among the enormous number of deregulated genes, however, we still need to identify the "critical" genes that play a pivotal role in initiation and/or promotion of the development of HCC and distinguish them from "bystander" genes as a result of epigenetic interaction.

Current knowledge about the molecular pathogenesis of HCC is a result of investigations of fully developed HCC, and much less is known about the genetic basis of preneoplastic lesions, namely macroregenerative nodules and/or dysplastic nodules. This is the most critical issue to be addressed to get a clearer picture of the early molecular events for the HCC progression and to identify gene markers for developing effective therapeutic options. To address this issue, we used the HBV transgenic mouse as a model to study the processes of initiation and progression of HCC related to the HBV infection. Although lab-

Table 2. Downregulated genes in 3-month-old HBV transgenic mice.

Gene Function and GenBank Acc. No.	Gene Description	Gene Symbol	Fold Change
Metabolism of cholesteryl			
AW260404	PDZ domain containing 1	Pdzk1	-1.92
Extra matrix structure component			
U24703	Reelin	Reln	-2.26
D50586	Tissue factor pathway inhibitor 2	Tfpi2	-1.80
Enzymes			
AF026073	*N*-sulftransferase	Sultn	-1.72
X17069	FK506 binding protein 4	Fkbp4	-2.15
Blood coagulation			
M23109	Coagulation factor IX	F9	-2.01
U60473	CD59a antigen	Cd59a	-169
Cell adhesion			
M77196	CEA-related cell adhesion molecule 1	Ceacam1	-2.03
Receptors			
U15012	Growth hormone receptor	Ghr	-2.25
X99347	Lipopolysaccharide binding protein	Lbp	-2.13
AF05175	v-erb-b2 erythroblastic leukaemia viral oncogene homolog 3 (avian)	Erbb3	-2.1Q4
Electron transport			
U90535	Flavin containing monooxygenase 5	Fmo5	-2.12
AB016248	Sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) Homolog (*S. cerevisiae*)	Sc5d	-2.23
AF031170	Hydroxysteroid dehydrogenase-6, delta<5>3-beta	Hsd3b6	-2.15
Water transport			
AA967194	Aquaporin 9	Aqp9	-2.22
Oxidoreduttase activity			
A1787269	RIKEN cDNA 1200014D15 gene	1200014D15Rik	-2.17
Apoptosis			
AF041054	BCL2/adenovirus E1B 19kDa-interacting protein 1	Bnip3 (NIP3)	-1.69
Amino acid metabolism			
U28016	Phosphotriesterase related protein	Pter	-3.46
Unknown			
AA183875	ESTs		-1.80
AW125273	Plastin 3 (T-isoform)	Pls3	-2.00

oratory mice have represented a powerful experimental system for understanding the intricacies of human cancer pathogenesis, there are important differences between mice and humans that influence the way in which cancer develops (43-45). Among the main differences is the number of genetic events involved in cancer development. Fewer genetic, epigenetic, or gene-expression altering events are required to induce a malignant transformation in murine cells compared with human cells (43,44), and studies have shown that several important signaling pathways seem to function differently in human and rodent models of transformation. In spite of these differences, our animal model appears to be a useful tool to study human HCC development because it mimics many of the pathological events that occur before the development of HCC in chronic HBV infection in humans.

Using cDNA microarray technology, we have identified a number of genes differently expressed in normal and pathological specimens during the murine hepatocarcinogenesis process. This allowed us to obtain, for the first time, an almost complete picture of the early genetic alterations that are directly or indirectly involved in the development of HCC. The data obtained from gene expression profiling will allow us to acquire insights on molecular mechanisms of hepatocarcinogenesis and to identify specific genes (or gene products) that can be used for early molecular diagnosis, risk analysis, prognosis prediction, and development of new therapies. Therefore, the transgenic mouse represents a useful model system not only for basic research, but also for clinical research applied to therapeutic drug discovery.

In this article, we focused our attention on the early molecular events that occurred in HBV transgenic mice and probably represent the earliest ones that start the multistep process of hepatocarcinogenesis. By analyzing gene expres-

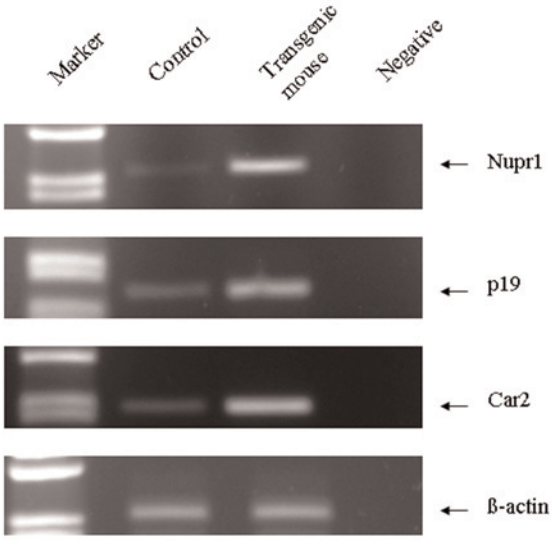

Figure 1. Semiquantitative RT-PCR analysis was performed on total RNAs prepared from livers of 3-month-old wt (control) and HBV transgenic mice. *Nupr1* (nuclear protein 1), *Cdkn2d* (p19), and *Car2* (carbonic anhydrase) expression levels were analyzed. β-Actin mRNA amplification was performed as an internal control. The reaction without cDNAs was the negative control. The results were concordant to microarray data.

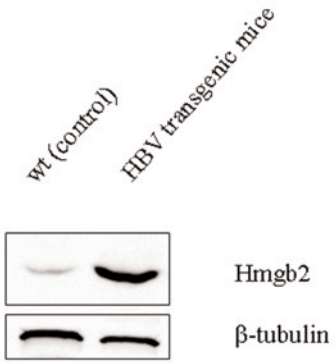

Figure 2. Hmgb2 protein expression in wt and HBV transgenic mice livers. Total protein extracts (50 μg of total protein/lane) were prepared from livers isolated from wt (control) and HBV transgenic mice at 3 months of age. All samples were analyzed by SDS-PAGE and immunoblotting with anti-Hmgb2 and anti–β-tubulin antibodies.

sion profile, we identified 25 upregulated and 20 downregulated genes in the HBV transgenic mouse, suggesting a direct effect of HBV in the modulation of these genes

We confirmed previously reported data on *Atf3* overexpression due to HBV (46). GO analysis showed that several differently expressed genes are involved in several cellular functions. In particular, a large number of upregulated genes were involved in the modulation of immunological functions, suggesting that the accumulation in the hepatocytes of viral proteins yielded cellular damage followed by an immune response (23,27,29). In addition, several upregulated transcripts have already been correlated to cellular dysplasia and tumor malignancy in different types of cancer (47-58). The microarray data showed the upregulation of *NuprI*, a transcription factor whose overexpression is inversely correlated with apoptosis in pancreatic cancer (59), and the downregulation of the proapoptotic gene *Bnip3* (60,61). This finding supports the conclusion that the deregulation of apoptosis, facilitating the escape of "abnormal" cells from death,

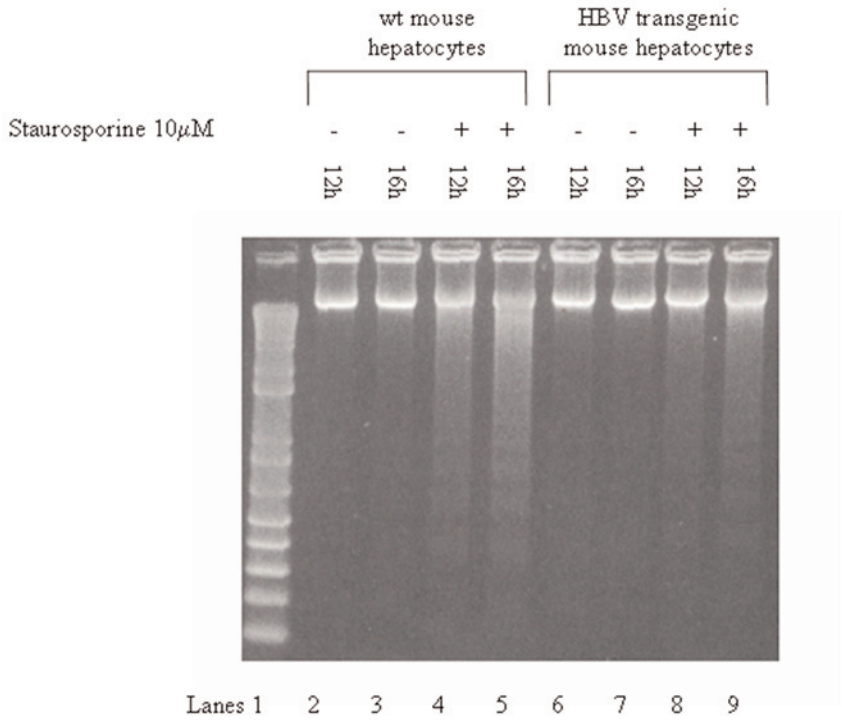

Figure 3. Apoptotic assay. The DNA-laddering assay was performed in primary cultured hepatocytes isolated from 3-month-old wt (lanes 2-5) and HBV transgenic (lanes 6-9) mice in the presence or absence of 10 μM staurosporine.

could be a mechanism by which HBV promotes HCC development. To confirm the influence of HBV on apoptosis, we analyzed DNA fragmentation, a hallmark of apoptosis, upon staurosporine treatment in hepatocytes isolated from 3-month-old transgenic and wt mice using an in vitro apoptotic assay. Our results showed a significant downregulation of apoptosis in hepatocytes isolated from HBV transgenic mice compared with controls, in spite of comparable cell viability. Therefore, the downregulation of the apoptotic process may represent a possible mechanism by which HBV induces malignant transformation. The role of HBx in regulating apoptosis is controversial because previous reports, indicating an abrogation of p53-induced apoptosis (18), were probably refuted by later data indicating a positive regulation of the apoptotic process (19-22).

In conclusion, gene expression profile is a useful tool to acquire new clues and generate new fields of research. Our experimental setup allowed us to acquire new insights on early molecular events of hepatocarcinogenesis induced by HBV, and in particular on possible mechanisms by which HBV induces HCC development. Further investigations are in progress in transgenic mice studied at different ages to confirm and expand these findings and clarify their role in more advanced stages of the disease.

ACKNOWLEDGMENTS

We thank Francioso Doriana for technical assistance. This work was supported in part by MIUR funds (grant 2003069954), FIST project (C.T.), and AIRC regional funds 2005.

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Queries

Q1. Throughout, it was sometimes difficult to determine the level of headings. Please check carefully.

Q2. Please supply the complete reference for "Tusher et al., 2001."

Q3. Please verify "noon"—or do you mean midnight?

Q4. Is this data line presented as you meant?