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Gene Expression Profile Related to the Progression of Preneoplastic Nodules toward Hepatocellular Carcinoma in Rats[1*]

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Abstract

In this study, we investigated the time course gene expression profile of preneoplastic nodules and hepatocellular carcinomas (HCC) to define the genes implicated in cancer progression in a resistant hepatocyte model. Tissues that included early nodules (1 month, ENT-1), persistent nodules (5 months, ENT-5), dissected HCC (12 months), and normal livers (NL) from adult rats were analyzed by cDNA arrays including 1185 rat genes. Differential genes were derived in each type of sample (*n* = 3) by statistical analysis. The relationship between samples was described in a Venn diagram for 290 genes. From these, 72 genes were shared between tissues with nodules and HCC. In addition, 35 genes with statistical significance only in HCC and with extreme ratios were identified. Differential expression of 11 genes was confirmed by comparative reverse transcription–polymerase chain reaction, whereas that of 2 genes was confirmed by immunohistochemistry. Members involved in cytochrome *P*450 and second-phase metabolism were downregulated, whereas genes involved in glutathione metabolism were upregulated, implicating a possible role of glutathione and oxidative regulation. We provide a gene expression profile related to the progression of nodules into HCC, which contributes to the understanding of liver cancer development and offers the prospect for chemoprevention strategies or early treatment of HCC.
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Introduction

Hepatocellular carcinoma (HCC) is a cancer that is prevalent worldwide. Detection of serum α-fetoprotein and liver imaging techniques are the conventional methods used for the identification of this malignancy [1–3]. However, these techniques are not reliable for early diagnosis [4]. Early detection of HCC is an important issue because effective treatment of small tumors is possible by surgical resection [5]. The early stages of human and animal hepatocarcinogenesis are characterized by the presence of preneoplastic lesions, namely, nodules that could be useful for the identification of early tumor markers, for the study of liver cancer progression, and for chemopreventive strategies [6,7]. However, human precancerous lesions such as dysplasic nodules are rather difficult to obtain because of their small size, incidental detection, and coexistence with other liver pathologies [8]. Thus, the rat model for hepatocarcinogenesis has been proven important as a tool for the analysis of nodules and liver cancer progression. Chemically induced nodules in the rat liver share several morphologic, biochemical, and molecular characteristics with human dysplasic nodules [9]. In the resistant hepatocyte (RH) model described by Solt and Farber [10] and Farber and Sarma [11], a necrogenic dose of diethylnitrosamine (DEN) induces resistant hepatocytes (RHs) during initiation. These cells can be stimulated to develop rapidly into altered hepatocyte foci and nodules by a selection procedure in which the carcinogen 2-acetylaminofluorene (2-AAF) is administered in combination with partial hepatectomy (PH) [12,13]. With this regime, which induces rapid growth of resistant altered hepatocytes, visible nodules are formed synchronously—some of them displaying sufficient genomic damage and progress to HCC without any additional treatment with the carcinogen.

As with other cancers, HCC is caused by the accumulation of genetic alterations resulting in a distorted expression of

Abbreviations: HCC, hepatocellular carcinoma; DEN, diethylnitrosamine; 2-AAF, 2-acetylaminofluorene; GSTP, glutathione *S*-transferase, placental form; GGT, γ-glutamyl transpeptidase

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thousands of genes. Hence, gene expression analysis with DNA microarray methodologies has been successfully used to study cancer development [14,15]. In this study, using a modified RH model, we investigated the time course of expression changes during the progression of nodules toward cancer using Atlas array membranes bearing 1185 well-typified rat genes. By comparing the expression data between tissues with nodules and HCC, we were able to provide a list of selectively expressed genes at different stages and those genes shared in both situations. We showed that some of the differentially regulated genes are involved in glutathione metabolism and redox control, agreeing with the proposition that altered cellular homeostasis may be a predisposing factor for disease progression in rat hepatocarcinogenesis.

Materials and Methods

Animals and Treatments

F344 rats weighing 180–200 g (UPEAL-Cinvestav, Mexico, DF, Mexico) were subjected to a 10-day carcinogen treatment. All experiments followed Institutional Animal Care and Use Committee Guidelines. Rats were initiated with an intraperitoneal dose of DEN (200 mg/kg; Sigma–Aldrich, Toluca, Mexico) and subjected to a modified selection regime 1 week later [6,16]. 2-AFF was administered by gavage at a dose of 25 mg/kg during three consecutive days, beginning on day 7 after initiation. On day 10, rats were subjected to PH. Animals were sacrificed by exsanguination under ether anesthesia at periods from 1 month after initiation up to 24 months. Livers were excised, washed in physiological saline solution, frozen in 2-methyl butane with liquid nitrogen or immersed in RNA*later* (Sigma, St. Louis, MO), and stored at −80°C.

Histologic Analyses

Representative 20-μm thick sections from liver slices were stained for γ-glutamyl transpeptidase (GGT) activity, in accordance with Rutenburg et al. [17]. In addition, 5-μm sections were processed for routine histologic examination using hematoxylin and eosin (H&E) staining. For immunostaining, formalin-fixed paraffin specimens were blocked for 1 hour in 0.1% H_2O_2 in phosphate-buffered saline, pH 7.4. Subsequently, they were incubated with commercial monoclonal antibodies specific to glutathione *S*-transferase, placental form (GSTP; DakoCytomation, Glostrup, Denmark), or anti–cyclin D1 (Cell-Marque, Hotspring, AR) diluted 1:50 and 1:20, respectively, in blocking buffer overnight. After washing with phosphate-buffered saline, the primary antibody was detected using an avidin–biotin complex immunoperoxidase technique (Zymed Laboratories, Inc., Carlsbad, CA). No staining was observed when the primary antibody was substituted with mouse isotype control.

Liver Section, Enrichment of Nodular Tissue, and Tumor Dissection

Hepatocellular tumors with diameters larger than 5 mm were easily dissected, and their corresponding nontumorous (Nt) liver tissues were obtained. Small nodular lesions 0.5 to 3 mm in diameter were distinguished by their sharp grayish white color demarcation from the surrounding reddish brown liver. Then a stainless steel cork borer (internal diameter, 1 mm) was introduced into frozen tissues. In this way, between 15 and 25 nodules per liver were collected, pooled, and stored in RNA*later* at −80°C. These samples were designated as enriched nodular tissues (ENTs). The increased presence of the hepatocarcinogenesis markers GSTP and GGT in ENT was verified by comparative reverse transcription–polymerase chain reaction (RT-PCR).

RNA Isolation, cDNA Synthesis, Labeling, and Purification

The total RNA of normal livers (NL), ENT of 1 month (ENT-1), ENT of 5 months (ENT-5), and individual HCC samples were obtained by tissue homogenization and extraction with Trizol (Life Technologies, Inc.). After DNase treatment, RNA was purified by phenol chloroform extraction. RNA quality and concentration were determined by capillary electrophoresis (RNA Nano LabChip; Agilent Technologies, Massy, France). For cDNA synthesis, 20 μg of RNA was reverse-transcribed for 1.5 hours at 42°C with 200 U of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA); 6 μl of 5× first strand buffer (Invitrogen); 1 μg of oligo(dT_{15}); 50 μCi of [α-^{33}P]dCTP (2500 Ci/mmol; Amersham Biosciences, Pittsburgh, PA); 5 μM dCTP; 0.8 mM each of dATP, dGTP, and dTTP; and 10 mM dithiothreitol, in a final volume of 30 μl. After 1 hour, 200 U of Superscript II reverse transcriptase was added to the reaction mixture. The resulting ^{33}P-labeled cDNA was purified with MicroSpin S-200 HR columns (Amersham Biosciences), according to the manufacturer's instructions.

cDNA Microarray Membrane and Hybridization

Atlas rat toxicology cDNA expression 1.2 arrays (7860-1) were purchased from Clontech Laboratories, Inc. (Palto Alto, CA) The membranes contained 1176 known rat genes and 9 housekeeping control cDNA. The arrays were prehybridized with 15 ml of Church buffer [18] and 0.5 mg of denatured salmon testes DNA at 65°C for 2 hours, with continuous agitation. Then, ^{33}P-labeled cDNA was added to the hybridization buffer at 65°C overnight. The membranes were washed at 65°C with 40 mM sodium phosphate buffer (pH 7.2) and 0.1% sodium dodecyl sulfate.

Imaging and Analysis

The membranes were then exposed to low-energy phosphorimage screens for 2 days. Images were acquired with a PhosphorImager 445 SI (Molecular Dynamics/Amersham Biosciences, Pittsburgh, PA). The spots were detected with XDotsReader (v. 1.8), and data were analyzed with the web service BioPlot (http://biopuce.insa-toulouse.fr/; Genopole-Toulouse, Toulouse, France). The intensity of each spot was corrected by subtracting the background; for normalization, the intensity of each spot was divided by the mean intensity of the nine housekeeping genes. From the RNA of rat livers (NL = 3; ENT-1 = 3; ENT-5 = 3; HCC = 3), 12 independent sets of data were obtained. Ratio was obtained from the

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