Identification of Novel Tumor Markers in Hepatitis C Virus-associated Hepatocellular Carcinoma

Maria W. Smith, Xiaohua N. Yue, Gary K. Geiss, Natalya Y. Sadovnikova, Victoria S. Carter, Loreto Boix, Catherine A. Lazaro, Gary B. Rosenberg, Roger E. Bumgarner, Nelson Fausto, Jordi Bruix, and Michael G. Katze

ABSTRACT

Hepatocellular carcinoma (HCC) is a common primary cancer associated with chronic hepatitis C virus (HCV). To gain insight into the molecular mechanisms of hepatocarcinogenesis, and to identify potential HCC markers, we performed cDNA microarray analysis on surgical liver samples from 20 HCV-infected patients. RNA from individual tumors was compared with RNA isolated from adjacent nontumor tissue that was cirrhotic in all of the cases. Gene expression changes related to cirrhosis were filtered out using experiments in which pooled RNA from HCV-infected cirrhotic liver without tumors was compared with pooled RNA from normal liver. Expression of ~13,600 genes was analyzed using the advanced analysis tools of the Rosetta Resolver System. This analysis revealed a set of 50 potential HCC marker genes, which were up-regulated in the majority of the tumors analyzed, much more widely than common clinical markers such as cell proliferation-related genes. This HCC marker set contained several cancer–related genes, including serine/threonine kinase 15 (STK15), which has been implicated in chromosome segregation abnormalities but which has not been linked previously with liver cancer. In addition, a set of genes encoding secreted or plasma proteins was identified, including plasma glutamate carboxypeptidase (PGCP) and two secreted phospholipases A2 (PLA2G13 and PLA2G7). These genes may provide potential HCC serological markers because of their strong up-regulation in more than half of the tumors analyzed. Thus, high throughput methods coupled with high-order statistical analyses may result in the development of new diagnostic tools for liver malignancies.

INTRODUCTION

Primary HCC is emerging as an important global health care problem largely because of the worldwide epidemic of hepatitis B and C viruses (1, 2). HCV causes a chronic infection in 70–80% of infected individuals, 10–20% of which develop liver fibrosis and cirrhosis within 2 decades (3). Once cirrhosis is pronounced, HCC develops at a rate between 1.5 and 8% per year (4–6). The process of HCV-induced carcinogenesis is still poorly understood because of the lack of a universally applicable small animal model and HCV-susceptible cell lines. Chronic inflammation, immune-mediated hepatocellular destruction, and liver regeneration underlie cirrhosis, and are thought to play central roles in primary carcinogenesis (7). Experimental evidence suggests that neoplastic cells originate from rapidly dividing hepatocytes or from liver stem cells that accumulate genetic alterations and chromosomal abnormalities (8).

Early identification of HCC, using imaging techniques and surveil-

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3 To whom requests for reprints should be addressed, at Department of Microbiology, University of Washington, Seattle, WA 98195-8070. Phone: (206) 732-6039; Fax: (206) 732-6055; E-mail: smitmv@u.washington.edu.

4 The abbreviations used are: HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIF, primary human fetal hepatocyte; RT-PCR, reverse transcription-PCR; PGCP, plasma glutamate carboxypeptidase; PAF, platelet activating factor; PLA2G7, phospholipases A2 group 7; PLA2G13, phospholipases A2 group 13.

MATERIALS AND METHODS

Patients and Tissue Samples. Surgical material was collected with prior informed consent in accordance with the guidelines of the Hospital Clinic at the University of Barcelona, Barcelona, Spain, and used according to protocols approved by the University of Washington Human Subjects Review Committee. Tumor and nontumor samples were obtained from 20 HCV-infected Caucasian patients with compensated liver cirrhosis (Child-Pugh grade A) who were treated according to published strategies (21). Patient clinical characteristics and tumor pathology are described in Table 1. A pool of RNA from early stage tumors (EaT sample) was prepared by mixing aliquots of RNA isolated from tumors 4, 15, 16, 19, and 23. A similar pool of RNA from tumors with one or two advanced-stage characteristics (AdT sample) was prepared using RNA isolated from tumors 1, 3, 14, and 24. RNA from 4 samples of nondis-
simplified liver (from noninfected individuals) was pooled to serve as a “normal liver” sample (N). Two independent pools of RNA from HCV-infected liver with signs of advanced cirrhosis (Child-Pugh grade B or C), but without tumors, were prepared from 8 samples isolated at the time of transplantation (samples CR1 and CR2).

HFHs were obtained according to protocols approved by the University of Washington Human Subjects Review Committee. Briefly, liver fragments from fetuses at the first trimester of gestation were minced, digested with collagenase A (3 mg/ml; Roche Molecular Biochemicals, Indianapolis, IN), and made into single cell suspensions. Cells in attachment medium, containing 5% FBS, were plated at a density of 0.5 × 10⁶ cells/60-mm dish. After 4 h, the attachment medium was replaced by serum-free medium containing epidermal growth factor (20 ng/ml). The human hepatoma cell line HuH7, kindly provided by Dr. Ralf Bartenschlager, University of Heidelberg, Heidelberg, Germany, was cultivated at 37°C in DMEM supplemented with 10% FBS, 2 mM l-glutamine, penicillin (100 units/ml), and streptomycin (100 µg/ml). Cell cultures were collected when ~80% confluent.

**Total RNA Isolation and mRNA Amplification.** Total RNA was isolated as described previously (22) and amplified using the AmpliScribe Transcription kit (Epicentre Technologies, Madison, WI) as described by the manufacturer. The quality of amplified RNA was checked by capillary electrophoresis using an Agilent Technologies (Palo Alto, CA) 2100 Bioanalyzer. cDNA Microarrays and Data Analysis. Microarray format, probe labeling, hybridization, slide treatment, and scanning were described previously (23). A single experiment comparing two samples consisted of two identical microarray sets (containing 13,597 unique IMAGE cDNA clones) in which the dye labels had been reversed, providing a total of at least four measurements per gene per experiment. Images were quantified as described previously (23), producing SDs and mean ratios between the expression levels of each gene in the analyzed sample pair. All of the data were entered into our custom-designed gene expression database, Expression Array Manager, and subsequently uploaded into the Rosetta Resolver System (Rosetta Biosoftware, Kirkland, WA). In accordance with proposed Minimum Information About a Microarray Experiment standards (24), raw data, including sample information, intensity measurements, error analysis, microarray content, and slide hybridization conditions will be made available at the Expression Array Manager web site.²⁶

**RT-PCR Validation of Microarray Data.** Total RNA was treated with DNase using the Ambion (Austin, TX) DNA-free kit and then used for first-strand cDNA synthesis using Retroscript (Ambion) as described by the manufacturer. Gene-specific primers and RT-PCR conditions used are shown in Supplementary Figure 1. For semiquantitative PCR, we used the Ambion Competimer technology as described by the manufacturer. All of the cDNA samples were used in parallel for multiplex PCR with gene-specific and endogenous-control (18S rRNA) primer pairs (Ambion QuantumRNA 18S Internal Standards). PCR products were quantified by capillary electrophoresis using the Agilent Technologies 2100 Bioanalyzer. The ratios of gene expression in tumor versus nontumor samples were calculated from the PCR data normalized against the endogenous control.

**RNA Blot Hybridization.** A 562-bp fragment of PLA2G13 and a 1004-bp fragment of PGCP were PCR-amplified, purified using a 1% agarose gel, and labeled with [³²P]CTP using Ready-To-Go DNA Labeling Beads (Amersham Pharmacia Biotech, Piscataway, NJ). The labeled probe was hybridized to a Multiplex Tissue Northern Blot and to a Matched Tumor/Normal Expression Array, both purchased from Clontech (Palo Alto, CA) and processed as recommended by the manufacturer. Signals were visualized using a Storm 840 Phosphor Imager (Molecular Dynamics, Sunnyvale, CA).

**RESULTS AND DISCUSSION**

**Microarray Analysis of Gene Expression Changes in HCC Tumors.** Of the tumors examined in this study, 8 were described as early, solitary, well-to-moderately differentiated HCC without additional nodules or vascular invasion (Table 1). The remaining tumors had one or more clinical or pathological characteristics associated with more advanced tumor types. In each microarray experiment, RNA from an individual HCC tumor was compared with RNA isolated from adjacent nontumor tissue that was cirrhotic in all of the cases. Thus, the observed gene expression changes reflected two pathological processes: carcinogenesis and cirrhosis. To distinguish between these processes, we included four additional experiments in our analyses. RNA pooled from samples of early-stage or advanced-stage tumors, or from liver with HCV-related cirrhosis but without tumors, was compared with RNA pooled from normal liver samples. To elucidate the gene expression changes between cancerous and normal liver cells in the absence of HCV infection, we also compared gene expression in HuH7 cells with that of cultured HFHs.

Expression microarrays containing ~13,600 individual genes were used to measure the ratios between the expression levels of each gene in an analyzed sample pair. Genes were selected as differentially expressed if the change in their ratio was at least 2-fold, with p < 0.05. For 10 differentially expressed genes, we validated the microarray data using semiquantitative RT-PCR with the endogenous control, 18S rRNA (Supplementary Figure 2). We found that the RT-PCR data were similar to that obtained by microarray, but that RT-PCR tended to underestimate the changes in gene expression level.

The number of genes differentially expressed in individual HCC tumors relative to the adjacent nontumor tissue showed significant tumor-to-tumor variation, ranging from about 17–48% of all of the...
genes present on the array. In total, 8139 genes (60% of all genes present on the arrays) were differentially regulated in at least 1 of 20 tumors.

**Identification of an HCC Marker Gene Set.** To search for potential HCC markers, we created a series of gene sets based on common patterns of differential expression in at least several tumors. Interestingly, none of genes were differentially regulated in all of the tumors, and only 10 genes were differentially expressed in 18 of 20 tumors analyzed. The most consistent changes in gene expression, observed in over half of all of the tumors examined, were associated with liver cirrhosis rather than with carcinogenesis (Supplementary Figure 3). However, a set of 674 genes, which were differentially expressed in at least 8 tumors, contained 273 genes that were up-regulated in HCC. These genes were selected for hierarchical two-dimensional clustering of all of the microarray experiments and for additional examination using the following criteria. First, the potential markers had to be up-regulated in HCC tumors. Second, the markers had to be cancer-specific, thus, they could not be differentially expressed in comparisons of HCV-infected cirrhotic liver with normal liver. This second criterion filtered out genes that were significantly down-regulated in cirrhotic liver but which stayed unchanged in tumor cells, producing a false pattern of tumor up-regulation and also genes that were highly up-regulated in relation to cirrhosis. Third, the markers had to be up-regulated in the pooled tumor samples compared with normal liver. This criterion helped to filter out the gene expression changes related to cirrhosis and also reduced the impact of individual variation, thus giving more weight to the observed gene expression patterns. Fourth, the markers could not be strongly down-regulated in HuH7 cells in comparison with fetal hepatocytes, thus supporting the cancer-related nature of the observed gene expression changes.

The resultant set of potential HCC markers contained 50 genes. As shown in Fig. 1, two-dimensional hierarchical clustering based on this set grouped 15 of 20 tumors into a large, tight cluster (shown in red) that showed up-regulation of the identified genes in HCC. The remaining 5 tumors fell into a separate cluster that showed up-regulation of only a few marker genes from the set. The early-stage tumors were split between the two clusters, showing that the gene set could not unequivocally distinguish between early and more advanced stages of tumor development. Clearly, the gene expression changes that contribute to the development of advanced tumor characteristics start to accumulate before any observable clinical features, thus making some of the early-stage tumors appear as more advanced in terms of gene expression. In general, this makes the HCC marker set potentially useful for predicting the biological evolution of liver tumor cells.

Several genes in the HCC marker set are related to the p53 tumor suppressor system, the RAS oncogene family, and tumor necrosis factor signaling (shown in red in Fig. 1). These genes have been implicated previously in hepatocarcinogenesis (8, 13, 15, 19, 25, 26). In addition, the HCC marker set also contains a group of genes that are known to be tumor-related but which have not been reported previously to be associated with liver carcinogenesis (also shown in red in Fig. 1). One such gene encodes serine/threonine kinase 15, STK15, a centrosome-associated kinase that is implicated in chromosome segregation abnormalities and aneuploidy in many cancer cell types (27).

In our experiments, STK15 was up-regulated in 17 of 20 HCC tumors (85%) and in HuH7 cells (Fig. 1). To our knowledge, this is the first evidence linking STK15 to hepatocarcinogenesis. This kinase may...
Fig. 2. A, two-dimensional hierarchical clustering results for genes of the cell proliferation-related set. These genes were differentially regulated in at least eight of all experiments (P ≤ 0.05 and fold change ≥ 2). Individual tumor versus nontumor liver experiments showing up-regulation of the majority of cell proliferation-related genes are depicted in red, and the corresponding data are highlighted with white borders. The gene cluster that is up-regulated in the majority of HCC tumors in comparison with the nontumor liver tissue is shown in red. B, two-dimensional hierarchical clustering results for the identified set of potential serological marker genes that encode putative plasma and secreted proteins. Individual tumor versus nontumor liver experiments showing up-regulation of the identified gene set are shown in red. The names of genes that were up-regulated in the majority of analyzed tumors, and which may be used as targets of additional biological and serological analysis, are shown in red. See legend of Fig. 1 for details on clustering, the applied color scheme, experiment representation, and gene names.

participate in neoplastic transformation of liver cells that is known to be accompanied by chromosomal instability.

Several members of the HCC marker gene set encode proteins involved in peroxisome proliferation and functioning (PXMP3 and PMVK), exocytosis (ANXA7), detoxification, and oxidative stress (ALDH1, GSTA4, and GSTM4; Fig. 1, black arrowheads). Previous microarray analyses of HCC tumors have also demonstrated up-regulation of oxidative stress-related genes (17). Studies of mouse models of hepatocarcinogenesis suggest that chronic stimulation of cell proliferation in the liver creates oxidative stress leading to massive DNA damage and acceleration of hepatocarcinogenesis (28). There is also some evidence that the HCV nonstructural protein 5A may induce oxidative stress (29). Our data on the widespread up-regulation of oxidative stress-related genes are consistent with an important role for this process in HCC development.

Expression of Clinical HCC Markers Related to Cell Proliferation. Many of the traditional clinical markers of carcinogenesis are proteins related to cell-cycle control and cell proliferation. A keyword search identified 59 cell-cycle-related genes present on our arrays. This set was filtered to include only those genes that were differentially regulated in at least eight experiments, yielding a total of 12 genes (Fig. 2A). Two genes, SPHAR and CCND1 (cyclin D1), were strongly up-regulated in cirrhotic liver, appearing to be down-regulated in the individual tumor versus nontumor liver experiments. The remaining 10 genes (shown in red in Fig. 2A) showed the opposite patterns of strong up-regulation in most tumors in comparison with cirrhotic and normal liver tissue. These genes were used to create a cell proliferation-related marker set, the up-regulation of which could be interpreted as indicative of a high cell proliferation rate in tumors.

We next compared expression of the identified cell proliferation-related marker gene set with tumor clinical characteristics (Table 1; Fig. 2A). High up-regulation of this set correlated with the characteristics of the more advanced tumor stage, such as multiple or satellite tumors, and the lack of tumor capsule accompanied by poor differentiation degree (these tumors are shown in red in Fig. 3, and the data are highlighted with white borders). Although the cell proliferation-related marker set was up-regulated in fewer tumors than the HCC marker gene set, the two gene sets might be combined for usage in microarray diagnostics and prognostics of HCC.

Identification of Potential Serological Markers. To identify genes encoding potential serological markers, we selected a set of 2302 genes that were differentially regulated in at least 4 of 20 tumors and subjected it to a keyword search to find genes encoding putative secreted, plasma, or blood proteins. Genes that were down-regulated in tumors were excluded from this list, and the remaining 11 genes were used for two-dimensional clustering of all of the experiments shown in Fig. 2B. Two of these genes, PGCP and PLA2G13 (shown in red in Fig. 2B), showed a pronounced up-regulation in about 60–70% of all of the tumors, and in the pooled tumor samples compared with normal liver. They were also up-regulated in the pooled cirrhotic versus normal liver but to a lesser degree than in tumors.

PGCP is a M₆, 56,000 glutamate carboxypeptidase that is produced...
in placenta and kidney, the majority of which is thought to be secreted into the bloodstream (30). The exact physiological substrates of PGCP are unknown, although this enzyme may play an important role in the hydrolysis of circulating peptides (30). Its closest homologue encodes an important brain glutamate carboxypeptidase II (NAALADase) identical to the prostate-specific membrane antigen (30), which serves as a marker for prostatic cancer metastasis (31). In contrast, PGCP has not been linked to any type of cancer, and it provides an attractive target for serological analysis in HCV-infected HCC patients. However, the most interesting of the identified potential serological markers was PLA2G13. This gene was up-regulated in 16 of 20 HCC tumors, and the least up-regulated in the cirrhotic liver relative to normal liver (Fig. 2B). In addition, the identified gene set contained a gene related to PLA2G13, PLA2G7, that was also up-regulated in tumors, although the magnitude of expression changes was much less than in the case of PLA2G13.

**PLA2G13 Is Expressed in the Liver and Up-regulated in HCC Tumors.** PLA2G13 and PLA2G7 belong to a large family of secreted phospholipases A2 (sPLA2). Generally, sPLA2 enzymes participate in inflammation, host defense against bacteria, tumor suppression, exocytosis, blood coagulation, and atherosclerosis (32, 33). Most sPLA2 are involved in lipid mediator production by releasing arachidonic acid from membranes, which then may serve as a precursor of prostaglandins or leukotrienes (33). In addition, sPLA2 may play a role as a scavenger of the oxidatively fragmented phospholipids in the blood, providing a protective mechanism against the damaging effects caused by oxidative stress (34).

The secreted PLA2G7 has a well-defined function as the plasma PAF acetylhydrolase. PAF is a potent phospholipid mediator functioning both in normal physiological events and in pathological responses, such as inflammation and allergy (35). PLA2G7 is thought to serve as a signal terminator for PAF function, providing an anti-inflammatory safety net (35).

The biological role and physiological substrates of PLA2G13 are not known, and although the PLA2G13 gene was originally cloned from a fetal liver and spleen library, its tissue distribution has not been determined. Therefore, we analyzed the tissue distribution of PLA2G13 using RNA blots. As shown in Fig. 3, the only detectable transcript species, a band of ~1.4 kb, was highly expressed in human adult liver and at a much lower level in kidney. This expression pattern differed dramatically from the much wider tissue distribution of another potential marker gene, PGCP (Fig. 3). These data were additionally supported using RNA dot blots containing matching pairs of tumor and nontumor samples from various human organs (Supplementary Figure 4). PLA2G13 mRNA expression was observed in 11 of 15 kidney samples, and the expression levels were decreased in renal carcinomas. Other organs had very low expression levels, except for slightly elevated expression in a single sample of normal colon and small intestine tissue. Among different tumors and cancer cell lines, the gene was significantly up-regulated only in 1 of 8 samples of stomach adenocarcinoma. Unfortunately, the RNA dot blots used in our analysis did not contain any liver tissue or liver-derived cell lines. PLA2G13 protein is homologous (46% identity, 70% similarity) to another member of the sPLA2 family, PLA2G12. However, the tissue distribution of PLA2G13 differed dramatically from that reported for PLA2G12 (32). Comparison of RT-PCR data for these two homologous genes showed very different, and sometimes opposite, transcript expression patterns (Supplementary Figure 5). As expected, the RT-PCR data for PLA2G13 were in good agreement with the microarray data, supporting our conclusion about liver-specific expression of this gene that is additionally induced in HCC tumors.

The role of PLA2G13 in the process of HCC carcinogenesis remains unclear. Assuming that a set of PLA2G13 coregulated genes might provide some clues to its role, we used Resolver similarity searches and identified 32 genes expressed in correlation with PLA2G13 (Fig. 4). This set contained several genes related to protein secretion, including TRA1, TLOC1, and ANXA7 (Fig. 4, black arrowheads), up-regulation of which may indicate the increased activity of protein trafficking in cancer cells. In addition, the set contained a cell surface-associated fibroglycan syndecan 2 (Fig. 4, red arrowhead), which may play the role of PLA2G13-specific receptor. Many sPLA2s are known to associate with specific membrane receptors, e.g., the cell surface proteoglycan glypican in fibroblastic cells (33). Interestingly, the PLA2G13-correlated set also contained 7 genes encoding peroxisomal components, and enzymes related to detoxification and the oxidative stress response (shown in red in Fig. 4), thus
indicating a possible involvement of PL2AG13 in these processes. Our data suggest that PL2AG13 provides a potential candidate for a serological marker of HCC tumors and at the same time a very attractive target for additional biological investigation of its role in hepatocarcinogenesis. Taken together, the identified HCC marker gene sets may ultimately be useful for diagnostics and prognostics of liver disease progression in chronically infected HCV patients, and also may provide some insight into the molecular mechanisms of liver carcinogenesis.

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