Molecular Profiling of Human Hepatocellular Carcinoma Defines Mutually Exclusive Interferon Regulation and Insulin-Like Growth Factor II Overexpression

Kai Breuhahn, 1 Sebastian Vreden, 1 Rami Haddad, 4 Susanne Beckebaum, 7 Dirk Stippel, 3 Peer Flemming, 6 Tanja Nussbaum, 1,2 Wolfgang H. Caselmann, 2 Brian B. Haab, 4 and Peter Schirmacher 1,2

1 Institute of Pathology, 2 Center for Molecular Medicine, and 3 Department of Visceral and Vascular Surgery, University of Cologne, Cologne, Germany; 4 Van Andel Research Institute, Grand Rapids, Michigan; 5 Department of Medicine I, University of Bonn, Bonn, Germany; 6 Institute of Pathology, Medical School of Hannover, Hannover, Germany; and 7 Department of Gastroenterology and Hepatology, University of Essen, Essen, Germany

ABSTRACT

Molecular subtyping of human hepatocellular carcinoma (HCC) with potential mechanistic and therapeutic impact has not been achieved thus far. We have analyzed the mRNA expression patterns of 43 different human HCC samples and 3 HCC cell lines in comparison with normal adult liver using high-density cDNA microarrays. Two main groups of HCC, designated group A (65%) and group B (35%), were distinguished based on clustering of the most highly varying genes. Group A HCCs were characterized by induction of a number of interferon (IFN)-regulated genes, whereas group B was characterized mainly by down-regulation of several apoptosis-relevant and IFN-regulated genes. The number of apoptotic tumor cells and tumor-infiltrating lymphocytes was significantly higher in tumors of group A as compared with those of group B. Based on the expression pattern, group B was further subdivided into two subgroups, designated subgroup B1 (6 of 43 tumors, 14%) and subgroup B2 (9 of 43 tumors, 21%). A prominent characteristic of subgroup B1 was high overexpression of insulin-like growth factor (IGF)-II. All tested HCC cell lines expressed equally high concentrations of IGF-II transcripts and co-segregated with group B1 in clustering. IGF-II overexpression and induction of IFN-related genes were mutually exclusive, even when analysis was extended to other cancer expression profile studies. Moreover, IFN-γ treatment substantially reduced IGF-II expression in HCC cells. In conclusion, cDNA microarray analyses provided subtyping of HCCs that is related to intratumor inflammation and tumor cell apoptosis. This profiling may be of mechanistic and therapeutic impact because IGF-II overexpression has been linked to reduced apoptosis and increased proliferation and may be accessible to therapeutic intervention.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent malignant tumors worldwide, with increasing incidence and a well-defined etiology [mainly chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection and alcohol abuse]. Only a minority of patients are amenable to surgery (partial hepatectomy or transplantation), which represents the only treatment with curative potential thus far. Alternative therapeutic prospects for HCC are restricted because local therapy, such as ethanol injections or thermoablative therapies, may only be effective in localized small HCCs, which are present only in a minority of patients (1). Because of the aggressive growth of the tumors and the lack of effective treatment options, the 5-year survival rate of HCC patients is extremely low (reviewed in ref. 2). Different macroscopic and histological growth patterns of HCC have been classified, but these classifications are not of mechanistic, therapeutic, or prognostic impact. Thus, conventional grading and staging are currently the only tumor parameters of significant prognostic impact. Therefore, a molecular classification of HCCs may lead to improved mechanistic understanding and the identification of novel markers.

A number of frequent chromosomal alterations affecting tumor-relevant genes [e.g., RB-1, E-cadherin, and COX-2 (3–5)] and point mutations in genes responsible for apoptosis [e.g., p53 (6, 7), or cell cycle regulation [e.g., cyclin-dependent kinase inhibitors, such as p16INK4a (8)] may alter gene expression in HCC cells constitutively. Furthermore, overexpression of pleiotropic growth factors, such as insulin-like growth factor (IGF)-II (9), transforming growth factor α (10), and vascular endothelial growth factor (11), may promote proliferation, antiapoptosis, neoangiogenesis, and invasive behavior.

DNA microarray studies of malignant lymphoma (12), breast cancer (13, 14), and kidney cancer (15) have demonstrated the existence of distinct subtypes of tumors defined by gene expression profiles. Expression data of HCC have been gathered in previous studies (16–26), and a variety of genes involved in the regulation of detoxification, mitosis, apoptosis, differentiation, metastasis, immune surveillance, and cytoskeletal architecture have been identified. However, a subtyping of HCC with potential therapeutic impact has not been achieved. Nevertheless, there is some support for the hypothesis that the current morphological classification of HCC comprises tumors of fundamentally different pathogenesis that may be unraveled by transcriptional profiling.

In the present work, we have used high-density cDNA microarray technology to obtain genome-wide expression profiles of human HCCs in comparison with normal liver and provide a molecular subtyping based mainly on the regulation of interferon (IFN)-dependent genes and down-regulation of apoptosis-relevant genes. There is evidence for a subcluster related to IGF-II overexpression, which may have substantial mechanistic and therapeutic impact.

MATERIALS AND METHODS

Patients and Sample Preparation. Tissues from 43 primary HCCs were obtained from 39 individuals, who underwent partial resection or total hepatectomy. The clinical data of the patients were collected from medical records and anonymized. Tissues were snap-frozen in liquid nitrogen and stored at −80°C. Tumor samples were cryoexcised and stained with hematoxylin and eosin, and morphology was evaluated independently by two pathologists. Criteria for inclusion into the array analyses were tumor content > 90% and absence of significant tumor necrosis (<10%), fibrosis (<20%), or tissue autolysis. According to Armed Forces Institute of Pathology criteria (27), 10 HCCs were classified as grade 1, 22 HCCs were classified as grade 2, 10 HCCs were classified as grade 3, and 1 HCC was classified as grade 4. Six patients were positive for HBV, and 11 were positive for HCV. Five HCCs were positive for HBV, and 11 were positive for HCV. Five HCCs were
Molecular Subtyping of HCCs

strongly associated with a history of chronic alcohol abuse, and one HCC was associated with genetic hemochromatosis (stage 3). A explaned liver from a healthy male not used for transplantation due to a hemangimia was used for reference RNA preparation. Also, male control liver RNAs were purchased from Ambion (Austin, TX) and Stratagene (La Jolla, CA) and hybridized in color-swapped replicates against normal liver RNA used in our study. Total RNA was isolated from ~0.125 cm² samples using a homogenizer (Mixier Mill MM 200; Retsch, Haan, Germany) and TRI Reagent (Sigma, Taufkirchen, Germany) according to the manufacturer’s instructions. RNA from cell lines (HepG2, Hep3B, HuH-7, Sk-Hep1, MCF7, T-47D, U-138 MG, and HT-29) was isolated using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol.

Complementary DNA Microarrays and Data Analyses. The cDNA microarrays were produced as described previously (28) with 21,632 different PCR products from the Research Genetics Human Clone set. The arrays were normalized using intensity-dependent normalization [LOWESS normalization (29)]. For hybridization 50 μg of total RNA were used in a reverse transcription reaction performed with Superscript II (Invitrogen, Karlsruhe, Germany) in the presence of Cy5-dCTP and Cy3-dCTP (Amersham, Freiburg, Germany) according to the manufacturer’s instructions. After probe purification using the QiAquick PCR purification kit (Qiagen), both reactions were combined in 35 μl of hybridization solution (3 × SSC, 0.2% SDS, and 0.2 μg/μl cot-1 DNA). Slides were hybridized at 65°C for 16 hours and washed at 42°C in 1 × SSC and 0.2% SDS, 0.2 × SSC and 1% SDS, and 0.2 × SSC twice for 5 minutes. After drying by snap centrifugation, the slides were scanned immediately using a confocal fluorescence scanner with lasers operating at wavelengths of 532 and 635 nm (ScanArray Lite; GSI Lumonics, Billerica, MA). Images were analyzed as described previously (15) using the program GenePix Pro 4.0 (Axon Instruments, Union City, CA). To test array consistency, 11 samples were hybridized in replicate, 9 of which were color-swapped. Pearson’s correlation coefficient of the replicate experiments was calculated based on the status of the reference RNA, we compared two commercially available normal human liver RNAs with “normal” liver RNA used in our study. Only 58 genes, often hormone-regulated (e.g., granulin, prostasin, or granulocyte macrophage colony-stimulating factor receptor (a chain precursor); not considered in subsequent cluster analyses), showed differences in expression (cutoff: 2-fold regulation), demonstrating that the RNA reference sample used in this study was representative of normal liver (data not shown). Pearson’s analyses exhibited a high correlation (r > 0.72) among 10 of 11 replicate experiments, indicating good reproducibility in the experiments (34). Furthermore, statistical analysis of different cDNA spots of representative genes revealed a highly significant correlation between single signals (r = 0.861; P < 0.001; the complete data set is available online*). Reevaluation of the array data using independent expression analyses (TaqMan analysis, Northern and Western hybridization, and immunohistochemistry) for SIAH-1, stathmin, β-catenin (data not shown), and IGF-II (see below) showed good correlation.

No significant clustering of HCCs with regard to tumor stage [tumor-node-metastasis (TNM) classification] or etiology (HBV-, HCV-, and alcohol-associated tumors) was established in our samples. Fifty-three genes discriminated well-differentiated (grade 1) from poorly differentiated (grade 3) HCCs (P < 0.075; Table 1). Among these were genes known to be involved in the regulation of mitosis (e.g., histone deacetylase 2 and replication factor C 4), apoptosis (e.g., nucleolin and desoxyribonuclease I-like 3), signal transduction (e.g., protein kinase C, ε type), and protein degradation (e.g., seven in absentia homologue-1, anaphase-promoting complex subunit 7, ankyrin repeat and SOCS box-containing protein 13).

Hepatocellular Carcinoma Subtyping. We next attempted molecular subtyping of HCCs based on the expression of highly discriminative genes and identified 118 cDNAs, which contained known genes (e.g., IGF-II, β-catenin, and apoptosis-inducing factor), hypothetical genes, and expressed sequence tags (P < 0.025; Fig. 1A; a complete list is available online*). Among these, a cluster of 26 genes known to be associated with apoptotic cell death and immune response was identified. Several IFN-regulated genes (e.g., IFN-induced

* http://www.medizin.uni-koeln.de/koelner/ki/Pathologie/supplementaldata.html.

RESULTS

General Complementary DNA Microarray Analyses. To verify the status of the reference RNA, we compared two commercially available normal human liver RNAs with “normal” liver RNA used in our study. Only 58 genes, often hormone-regulated (e.g., granulin, prostasin, or granulocyte macrophage colony-stimulating factor receptor (a chain precursor); not considered in subsequent cluster analyses), showed differences in expression (cutoff: 2-fold regulation), demonstrating that the RNA reference sample used in this study was representative of normal liver (data not shown). Pearson’s analyses exhibited a high correlation (r > 0.72) among 10 of 11 replicate experiments, indicating good reproducibility in the experiments (34). Furthermore, statistical analysis of different cDNA spots of representative genes revealed a highly significant correlation between single signals (r = 0.861; P < 0.001; the complete data set is available online*). Reevaluation of the array data using independent expression analyses (TaqMan analysis, Northern and Western hybridization, and immunohistochemistry) for SIAH-1, stathmin, β-catenin (data not shown), and IGF-II (see below) showed good correlation.

No significant clustering of HCCs with regard to tumor stage [tumor-node-metastasis (TNM) classification] or etiology (HBV-, HCV-, and alcohol-associated tumors) was established in our samples. Fifty-three genes discriminated well-differentiated (grade 1) from poorly differentiated (grade 3) HCCs (P < 0.075; Table 1). Among these were genes known to be involved in the regulation of mitosis (e.g., histone deacetylase 2 and replication factor C 4), apoptosis (e.g., nucleolin and desoxyribonuclease I-like 3), signal transduction (e.g., protein kinase C, ε type), and protein degradation (e.g., seven in absentia homologue-1, anaphase-promoting complex subunit 7, ankyrin repeat and SOCS box-containing protein 13).

Hepatocellular Carcinoma Subtyping. We next attempted molecular subtyping of HCCs based on the expression of highly discriminative genes and identified 118 cDNAs, which contained known genes (e.g., IGF-II, β-catenin, and apoptosis-inducing factor), hypothetical genes, and expressed sequence tags (P < 0.025; Fig. 1A; a complete list is available online*). Among these, a cluster of 26 genes known to be associated with apoptotic cell death and immune response was identified. Several IFN-regulated genes (e.g., IFN-induced

http://www.medizin.uni-koeln.de/koelner/ki/Pathologie/supplementaldata.html.

6059
Molecular Subtyping of HCCs

Table 1: List of 53 genes or expressed sequence tags that discriminate G1 from G3 HCCs (P < 0.075)

<table>
<thead>
<tr>
<th>Gene name</th>
<th>GenBank accession no.</th>
<th>Symbol</th>
<th>G1</th>
<th>G3</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2A histone family</td>
<td>AA488627</td>
<td>H2AFY</td>
<td>1</td>
<td>1.8</td>
</tr>
<tr>
<td>Expressed sequence tag</td>
<td>AA45944</td>
<td></td>
<td>1.6</td>
<td>2.6</td>
</tr>
<tr>
<td>Papillary renal cell carcinoma</td>
<td>AA88180</td>
<td>PRCC</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>Heterogeneous nuclear ribonucleoprotein M</td>
<td>AA504272</td>
<td>HNRPM</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Microphosphor protein 1</td>
<td>AA488979</td>
<td>MCR5</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Ribosomal protein S23</td>
<td>N73091</td>
<td>RPS23</td>
<td>1.3</td>
<td>1.6</td>
</tr>
<tr>
<td>Histone deacetylase 2</td>
<td>AA127093</td>
<td>HDAC2</td>
<td>1.2</td>
<td>1.8</td>
</tr>
<tr>
<td>CGI-69 protein</td>
<td>AA485441</td>
<td>CGI-69</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Replication factor C (activator 1) 4</td>
<td>N93924</td>
<td>RFC4</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Expressed sequence tag</td>
<td>N59870</td>
<td></td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Nucleolin</td>
<td>AA476294</td>
<td>NCL</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Bromodomain containing 4</td>
<td>R32788</td>
<td>BRD4</td>
<td>1.1</td>
<td>1.6</td>
</tr>
<tr>
<td>Sjogren syndrome antigen B (autoantigen La)</td>
<td>H24948</td>
<td></td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>CDW92 antigen</td>
<td>AA457261</td>
<td>CDW92</td>
<td>2.7</td>
<td>1.2</td>
</tr>
<tr>
<td>Aryl hydrocarbon receptor</td>
<td>AA461522</td>
<td>AHR</td>
<td>1.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Fatty acid desaturase 1</td>
<td>AA431773</td>
<td>FADS1</td>
<td>4</td>
<td>1.7</td>
</tr>
<tr>
<td>Vesicle docking protein p115</td>
<td>AA504342</td>
<td>VDP</td>
<td>1.2</td>
<td>1.8</td>
</tr>
<tr>
<td>Glyoxylate reductase/hydroxyypuvate reductase</td>
<td>R83908</td>
<td>GRHPR</td>
<td>3.9</td>
<td>3.9</td>
</tr>
<tr>
<td>Complement component 8, a polypeptide</td>
<td>H53665</td>
<td>C8A</td>
<td>7.6</td>
<td>7.6</td>
</tr>
<tr>
<td>Deoxyribonucleosine I-like 3</td>
<td>T73558</td>
<td>DNASEIL3</td>
<td>9.6</td>
<td>9.6</td>
</tr>
<tr>
<td>Liver-expressed antimicrobial peptide 2</td>
<td>R93723</td>
<td>LEAP-2</td>
<td>4.1</td>
<td>4.1</td>
</tr>
<tr>
<td>Liver-expressed antimicrobial peptide 2</td>
<td>T82264</td>
<td>LEAP-2</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Lutheran blood group (Anther b antigen included)</td>
<td>H24954</td>
<td>LU</td>
<td>3.6</td>
<td>3.6</td>
</tr>
<tr>
<td>Seven in absentia homologue</td>
<td>T71889</td>
<td>SIAB1</td>
<td>4.1</td>
<td>4.1</td>
</tr>
<tr>
<td>Expressed sequence tag</td>
<td>N58342</td>
<td></td>
<td>6.4</td>
<td>6.4</td>
</tr>
<tr>
<td>Mitochondrial ribosomal protein L46</td>
<td>AA446865</td>
<td>MRPL46</td>
<td>2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>CD8 antigen, a polypeptide (g32)</td>
<td>AA443584</td>
<td>CD8A</td>
<td>3.6</td>
<td>3.6</td>
</tr>
<tr>
<td>Protein kinase C, i</td>
<td>T75875</td>
<td>PRKCI</td>
<td>3.7</td>
<td>3.7</td>
</tr>
<tr>
<td>Expressed sequence tag</td>
<td>AA490120</td>
<td></td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Epithelial membrane protein 2</td>
<td>T88721</td>
<td>EMP2</td>
<td>2.6</td>
<td>2.6</td>
</tr>
<tr>
<td>Alcoholic dehydrogenase 6 (class V)</td>
<td>T87343</td>
<td>ADH6</td>
<td>4.7</td>
<td>4.7</td>
</tr>
<tr>
<td>Solute carrier family 38, member 2</td>
<td>A5989866</td>
<td>SLC38A2</td>
<td>3.8</td>
<td>3.8</td>
</tr>
<tr>
<td>Translocation protein 1</td>
<td>AA540205</td>
<td>TLOC1</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>Expressed sequence tag</td>
<td>R61700</td>
<td></td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Acyl-CoA synthetase long-chain family member 6</td>
<td>AA260887</td>
<td>FACEL6</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Expressed sequence tag</td>
<td>R60924</td>
<td></td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Hydroxysteroid (17-β) dehydrogenase 4</td>
<td>AA87914</td>
<td>HSD17B4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Betaine-homocysteine methyltransferase 2</td>
<td>N78012</td>
<td>BHCMT2</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>IFN-stimulated transcription factor 3, γ</td>
<td>AA291389</td>
<td>ISGF3G</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Cytochrome b5_6</td>
<td>R91950</td>
<td>CYB5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Formyltetrahydrofolate dehydrogenase</td>
<td>T52840</td>
<td>FTHFD</td>
<td>3.7</td>
<td>3.7</td>
</tr>
<tr>
<td>Anaphase-promoting complex subunit 7</td>
<td>AF191340</td>
<td>ANAPC7</td>
<td>3.1</td>
<td>3.1</td>
</tr>
<tr>
<td>Regucalcin</td>
<td>H05140</td>
<td>RGN</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Acetyl-coenzyme A acyltransferase 2</td>
<td>P42765</td>
<td>ACAT2</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Acetyl-coenzyme A dehydrogenase</td>
<td>P11310</td>
<td>ACADAM</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Hypothetical protein FLI13456</td>
<td>N25234</td>
<td>FLI13456</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Putative ethanolamine kinase</td>
<td>H77536</td>
<td>ETHK</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Expressed sequence tag</td>
<td>R02373</td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Solute carrier family 16</td>
<td>U59299</td>
<td>SLC16A2</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Sorbitol dehydrogenase</td>
<td>A54674</td>
<td>SORD</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Betaine-homocysteine methyltransferase</td>
<td>T58896</td>
<td>BHMT</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Ankyrin repeat and SOCS box-containing 13</td>
<td>T12477</td>
<td>ASB13</td>
<td>2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>Selenium-binding protein 1</td>
<td>T65736</td>
<td>SELENBP1</td>
<td>1.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

**NOTE.** Average fold changes are indicated.

Gene 35 IFI35, IFN-induced gene 27 IFI27, and IFN-stimulated protein 15k ISG15 were induced in 28 of 43 HCCs (65%, termed group A; Fig. 1B). In contrast, the remaining 35% (15 of 43; termed group B) showed reduced expression of some IFN-associated genes (IFN-induced transmembrane protein 1, IFN-related developmental regulator 2, and IFN-induced gene 27) as well as genes involved in apoptosis [e.g., tumor necrosis factor receptor superfamily, member 5 (CD40), apoptosis-inducing factor], which were in contrast up-regulated in group A. These differentiating expression patterns of genes involved in regulation of immune response and apoptosis suggested that HCCs of groups A and B may be distinguishable by the extent of tumor cell apoptosis and tumor-associated lymphocytic infiltration. This hypothesis was tested by apoptosis analysis (M30 cytodeath) and immunohistology for CD3-positive T lymphocytes using all available HCC tissues from group A (n = 17) and group B (n = 7). Indeed, the number of apoptotic tumor cells was significantly reduced in HCCs of group B (mean: 0.43 apoptotic cell per field of vision) in comparison with group A (mean: 0.98 apoptotic cell per field of vision; P = 0.039; Fig. 2A–C). Moreover, a significantly increased number of tumor-infiltrating T lymphocytes was observed in HCCs of group A (mean: 103.2 lymphocytes per field of vision) as compared with group B (mean: 46.6 lymphocytes per field of vision; P = 0.012; Fig. 2D–F). No significant differences between subgroup B1 and B2 tumors were observed concerning tumor cell apoptosis and lymphocyte infiltration (P > 0.05). HCV- and HBV-induced HCCs were both present in groups A and B.

**Differential Expression of Insulin-Like Growth Factor II in a Subgroup of Hepatocellular Carcinomas.** Based on the expression of the IFN-regulated genes, further examination showed that group B can be further subdivided into subgroups, termed B1 and B2, containing roughly equal numbers of cases (Fig. 1B). Subgroup B1 showed high-level overexpression of IGF-II (6 of 43; 14%), whereas IGF-II transcript levels in the remaining HCCs were down-regulated in 32 cases (74%) and were not significantly altered in 4 HCCs (9%). We confirmed the differential IGF-II expression using semiquantitative TaqMan analyses and Northern hybridization for the HCC cell
lines, nonhepatocellular cell lines, representative HCCs, and normal liver. In HCCs, two different large IGF-II transcripts (6.0 and 4.8 kb) were detectable by Northern hybridization, whereas in normal liver, only a single, faint but detectable band for IGF-II was observed at 6.0 kb (Fig. 3 A). A significant induction (up to 77-fold) of IGF-II transcript level was observed in HCCs of group B1 and a significant reduction (up to 88-fold) of IGF-II transcript level was observed in the HCCs of group A and B2 in comparison with normal liver (Fig. 3 B).

Moreover, IGF-II was highly expressed in all three HCC cell lines as compared with nonhepatocellular cell lines (Fig. 3 A and C).

In addition, the distinction of subgroups B1 and B2 (9 of 43; 21%) was supported by the down-regulation of genes involved in tumor

Fig. 1. Gene expression analyses of HCCs. A. Clustered image map of 118 highly discriminative genes for 43 HCC samples and 3 HCC cell lines (HuH-7, HepG2, and Hep3B). Clone identification number and standard abbreviations are indicated for each gene. B. Clustered image map of genes associated with apoptosis and immune response chosen from A. Genes found by discriminative analysis based on the IFN-induced cluster were annotated by LocusLink and their known activities in immune response or apoptosis (P < 0.025).

Fig. 2. Increased apoptosis of tumor cells and leukocytes infiltrating HCCs overexpressing IFN-related genes. A. Average values of apoptotic tumor cells per visual field from 17 group A (B) and 7 group B HCCs (C) is depicted after M30-cytodeath staining. D. The average number of infiltrating leukocytes per visual field from 17 group A (E) and 7 group B HCCs (F) is depicted after CD3 staining. For each section, the maximum numbers (14–65) of different fields of vision were counted.
A

![Image](image1)

**Fig. 3.** IGF-II mRNA expression in HCC cell lines and HCCs. A. Northern blot analysis was performed by separating 20 μg of total RNA isolated from hepatocellular cell lines (HuH-7, HepG2, and Hep3B), nonhepatocellular cell lines (Sk-Hep1, T-47D, MCF7, U-138MG, and HT29), normal liver, a group A HCC (R62), and subgroup B1 HCCs (R49 and R53). GAPDH expression served as a loading control. B. Semiquantitative IGF-II TaqMan analysis of group A HCCs (R30, R62, and R63) with low IGF expression and subgroup B1 HCCs (R37, R49, R53, and R9) with IGF-II overexpression. Normal healthy liver mRNA was used for calibration. C. Semiquantitative IGF-II TaqMan analysis of HuH-7 cell lines. HuH-7 mRNA was used for calibration.

Fig. 4. IFN-γ-dependent reduction of IGF-II expression in HCC cell lines. Semiquantitative IGF-II TaqMan analysis of HuH-7 cells treated with increasing concentrations of IFN-γ for 16 hours. Untreated cells were used for calibration.

Dissection was performed by separating 20 μg of total RNA isolated from hepatocellular cell lines (HuH-7, HepG2, and Hep3B), nonhepatocellular cell lines (Sk-Hep1, T-47D, MCF7, U-138MG, and HT29), normal liver, a group A HCC (R62), and subgroup B1 HCCs (R49 and R53). GAPDH expression served as a loading control.

B

![Image](image2)

C

![Image](image3)

necrosis factor signal transduction (fas-associated factor 1 and nef-associated factor 1) in subgroup B2, whereas these genes were not regulated or only mildly regulated in IGF-II-positive tumors (subgroup B1). The apparent exclusiveness with regard to the expression of IGF-II and IFN-induced genes was further investigated using the cDNA microarray data made public by the Stanford HCC study (16). Again the IFN cluster of genes (up-regulated in 52% of their HCCs) and the IGF-II cluster (in 29% of their HCCs) are mutually exclusive in their samples. Thus, although array and HCC composition were markedly different between the studies, the same subgrouping was reflected in the Stanford data. Comparably, in cDNA microarray data analysis of 65 human breast tumors (14), overexpression of IGF-II and IFN-regulated genes (e.g., signal transducer and activator of transcription 1, ISG15, IFN-induced gene 41, IFN-induced gene 30, and guanylate binding protein 1), in general, is mutually exclusive.

All three tested HCC cell lines (HuH-7, HepG2, and Hep3B) expressed elevated concentrations of IGF-II mRNA and also clustered together with the IGF-II-overexpressing subgroup B1 with regard to the other discriminating genes (Fig. 1A and B). Due to the mutual exclusivity of the IGF-II and IFN-regulated gene clusters, we tested whether IFN may directly suppress IGF-II expression in HCC cells. When HuH-7 cells were treated with increasing concentrations of recombinant IFN-γ, a significant reduction of IGF-II transcript levels was observed (Fig. 4). In silico analysis of the IGF-II gene demonstrated the presence of several potential binding sites for IFN-γ-dependent transcription factors [e.g., IFN-regulatory factors 1 (IRF-1; position 5523–5541), IRF-3 (positions 557–575 and 2548–2566)]. IRF-7 (positions 2640–2658 and 4170–4188), and STAT1 (positions 2711–2729 and 2969–2987)] in the P2-P4 promoter region (GenBank accession no. X03562) of the human IGF-II gene. Some of these factors (e.g., IRF-1) have been described to modulate up- or down-regulation of IFN-responsive target gene expression, depending on the biological context (35–37).

**DISCUSSION**

We have demonstrated that based on the expression pattern of genes with the most variation in expression between tumors and normal liver, human HCCs can be subdivided into two main groups: group A, characterized by high-level expression of IFN-regulated genes; and group B, which lacked induction of IFN-regulated genes and apoptosis-related genes. However, a few IFN-related genes did not show regulation at all, which may be because of cell type-specific requirements and general differences in the composition of the tumor-associated microenvironment.

A substantial subcluster of subgroup B was exemplified by high expression of IGF-II. The mutual exclusivity between IGF-II expression and IFN-induced genes detected in our analysis was also present in the data made public by the Stanford HCC study (16), which was completely independent with regard to sample and array composition. In addition, mutually exclusive overexpression of IGF-II and IFN-induced/regulated genes was, in general, also observed in breast cancer cDNA microarray analysis (14), suggesting a more general relationship of both pathways in oncogenesis.

The significant reduction of IGF-II expression in tumor cells by IFN-γ treatment, which was previously shown only for adrenal cells (38, 39), suggested that the mutual exclusivity of IGF-II and IFN-dependent clusters in HCCs was due in part to direct interference of IFN-induced transcription factors with IGF-II expression. Indeed,
several potential binding sites for IFN-dependent transcription factors are present in the P2-P4 promoter region of the IGF-II gene. P2-P4-derived IGF-II transcripts have been demonstrated as the main responsible source for IGF-II overexpression in human HCCs (40). The gene expression pattern in group A suggested correlation with elevated numbers of tumor-infiltrating T lymphocytes and tumor cell apoptosis. Indeed, we observed substantially more tumor-infiltrating lymphocytes and tumor cell apoptosis in HCCs of group A in comparison with group B. Similarly, in human mammary carcinoma, induction of IFN-related genes, which was mutually exclusive of IGF-II overexpression in a subset of tumors, correlated with increased numbers of tumor-infiltrating leukocytes and induction of nuclear STAT1 reactivity (13, 14). This demonstrates that group A represents HCCs with a more prominent inflammatory and apoptotic phenotype, suggesting a more efficient antitumor response. In this context, multivariate analyses with large numbers of patients have described a significantly better prognosis in HCCs with heavy lymphocyte infiltration (41), and case reports have attributed spontaneous HCC regression to severe inflammatory infiltration (42). Lymphocyte attraction may be due, in part, to secretion products of HCC cells such as chemokines, which are known to be IFN induced (43). Induction of HCC cell apoptosis by tumor-infiltrating lymphocytes is well established and has been attributed to the activation of the Fas/Fas ligand system (44). Because IGF-II acts as a survival factor by inhibiting apoptosis (45), it is tempting to speculate that reduced tumor cell apoptosis in group B is due to two different mechanisms, reduced lymphocyte-mediated apoptosis and modulation of apoptosis-regulating cytokine pathways (Fig. 5).

IGF-II is overexpressed in several animal models of HCC (46, 47), and it has been correlated with increased proliferation (48, 49), reduced apoptosis (45) and chemotherapeutic response of tumor cells (50), and neoangiogenesis after hypoxia (51). A correlation of IGF-II overexpression with HBV-associated hepatocarcinogenesis has been suggested previously (18, 52, 53) but was not confirmed by our results. However, the detection of an IGF-II-associated subcluster (subgroup B1) in HCCs with reduced expression of apoptosis-relevant genes (group B) is of biological and therapeutic significance. Reversible histone deacetylase inhibitors, such as trichostatin A, were shown to modulate the IGF axis (54, 55), potentially by induction of IGF-binding protein-3, which is the major binding protein for IGF-II and also induces apoptosis in a p53-independent manner (56). Trichostatin A analogs have already entered clinical trials for several carcinomas including breast, colon, prostate, and lung cancer (57, 58). Because dysregulation of the IGF-II transcription has been connected in some HCCs with genomic imprinting (40), re methylation strategies (e.g., by budesonide) also may decrease expression of IGF-II (59). Moreover, selective IGF-I receptor kinase inhibitors such as picropodophyllin (60) and NVP-AEW541 (61) have recently been shown to abrogate receptor autophosphorylation. Therefore, several HCC cell lines, such as HuH-7, Hep3B, and HepG2, that exhibit high-level expression of IGF-II and cluster together with HCCs of subgroup B1 are valid and representative in vitro models for functional testing of therapeutic substances modulating the IGF-II axis.

Our gene expression analyses provide a molecular framework for subtyping of HCCs that is independent of current morphological subtyping and may have therapeutic impact. Further analyses using larger cohort studies or meta-analyses will have to test whether this subtyping is also of prognostic impact.

REFERENCES


Molecular Profiling of Human Hepatocellular Carcinoma Defines Mutually Exclusive Interferon Regulation and Insulin-Like Growth Factor II Overexpression

Kai Breuhahn, Sebastian Vreden, Ramsi Haddad, et al.

Cancer Res 2004;64:6058-6064.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/17/6058

Cited articles
This article cites 57 articles, 21 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/17/6058.full.html#ref-list-1

Citing articles
This article has been cited by 15 HighWire-hosted articles. Access the articles at:
/content/64/17/6058.full.html#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.