

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

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## 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- $\gamma$  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 thermoablation, 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 p16<sup>INK4</sup> (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  $\alpha$  (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  $-80^{\circ}\text{C}$ . Tumor samples were cryosected 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

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strongly associated with a history of chronic alcohol abuse, and one HCC was associated with genetic hemochromatosis (stage 3). An explanted liver from a healthy male not used for transplantation due to a hemangioma 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  $\sim 0.125 \text{ cm}^3$  samples using a homogenizer (Mixer 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  $\mu\text{g}$  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  $\mu\text{l}$  of hybridization solution ( $3 \times \text{SSC}$ , 0.2% SDS, and 0.5  $\mu\text{g}/\mu\text{l}$  *cot-1* DNA). Slides were hybridized at 65°C for 16 hours and washed at 42°C in  $1 \times \text{SSC}$  and 0.2% SDS,  $0.2 \times \text{SSC}$  and 0.1% SDS, and  $0.2 \times \text{SSC}$  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 3,500 genes analyzed. These genes were filtered as those that had a pixel intensity of at least 150 in each channel in at least 75% of the arrays.

The HCC data were filtered for genes that changed 2-fold in 70% of tumors. In our study, 220 genes met this criteria and were used for hierarchical average linkage clustering, which was performed as described previously (30). Discriminative analysis was performed by using the Welch *t* test, which does not assume equal variance between different groups. Benjamini and Hochberg false discovery rate analysis (with a *P* cutoff of 0.05) was implemented to account for multiple testing errors. Based on these tests, highly discriminative genes were identified.

Wilcoxon's rank-sum test, analysis of variance, Mann-Whitney *U* test, and Spearman's rank correlation test were used for statistical data analyses. *In silico* promoter analysis of the human IGF-II gene (GenBank accession no. X03562) was performed using Genomatix Suite Software (MatInspector).

**Northern Hybridization.** Northern hybridization analyses of 20  $\mu\text{g}$  of total RNA were performed as described previously (31). Probes were generated by polymerase chain reaction (35 cycles of 94°C for 30 seconds, 58°C for 45 seconds, and 72°C for 60 seconds) with liver cDNA as the template using the following primers: IGF-II, IGF-II-1 (5'-GTGCTTCTCACCTTCTGGC-3'; position 583–603) and IGF-II-2 (5'-GTCTTGGGTGGGTAGAGCAA-3'; position 1047–1027; GenBank accession no. J03242); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), GAPDH-1 (5'-TTGTTGCCATCAATGACCC-3'; position 149–169) and GAPDH-2 (5'-TGACAAAGTG-GTCGTTGAGG-3'; position 986–966; GenBank accession no. M33197). Phospho screens (Amersham) and PhosphorImager (Storm 840; Molecular Dynamics, Sunnyvale, CA) were used for signal detection.

**TaqMan Analyses.** For cDNA syntheses, 1  $\mu\text{g}$  of total RNA was used for reverse transcription using Superscript II (Invitrogen). TaqMan Universal MasterMix (Applied Biosystems, Darmstadt, Germany) was used according to the manufacturer's protocol. The following primers and probes were applied for signal detection: (a) IGF-II, IGF probe (5'-Fam-TTCAGGGAGGC-CAAACGTACCG-Tamra-3'; position 997–1020), IGF primer-1 (5'-GTGCTTCTCACCTTCTGGC-3'; position 583–603), and IGF primer-2 (5'-GTCTTGGGTGGGTAGAGCAA-3'; position 1048–1028; GenBank accession no. J03242); and (b) 18S rRNA, rRNA probe (5'-Fam-AGCAG-GCGCGCAAATTACCC-Tamra-3'; position 473–493), rRNA primer-1 (5'-AAACGGCTACCACATCCAAG-3'; position 447–467), and rRNA primer-2 (5'-CCTCAATGGATCCTCGTTA-3'; position 602–582; GenBank acces-

sion no. X03205). The analysis was performed with a Prism 7700 Sequence Detection System (Applied Biosystems) using the following program: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

**Immunohistology.** Five-micrometer cryosections were fixed in 4% paraformaldehyde at room temperature for 10 minutes. Afterward, endogenous peroxidase was blocked (0.1%  $\text{H}_2\text{O}_2$  in methanol for 5 minutes), and slides were washed in PBS and incubated in 0.05% Triton/PBS for 5 minutes each. The blocking and signal detection procedures were performed as described previously (32). Primary antibodies were monoclonal anti-CD3 (1:200; Novocastra, Newcastle, United Kingdom) and monoclonal M30 cytodeath (1:100; Roche, Mannheim, Germany). Cytoplasmic (M30 cytodeath) and membranous staining (CD3) was quantified by counting the number of positive cells per fields of vision in all samples of group A ( $n = 17$ ) and group B ( $n = 7$ ) HCCs with sufficient remaining tissue for these analyses. The maximal number of different fields of vision (14–65) was counted for each tumor slide evaluated, and the mean values were analyzed by Mann-Whitney *U* test. The significance level was defined as  $P < 0.05$ .

**Interferon Treatment of Hepatocellular Carcinoma Cells.** Hepatoma cells (HuH-7) were treated with recombinant human IFN- $\gamma$  (600 and 1,000 units/ml; PeproTech, London, United Kingdom) according to Melen *et al.* (33) and collected after 16 hours for RNA isolation and TaqMan analysis as described above.

## 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.*, granulins, prostasin, or granulocyte macrophage colony-stimulating factor receptor  $\alpha$  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<sup>8</sup>). Reevaluation of the array data using independent expression analyses (TaqMan analysis, Northern and Western hybridization, and immunohistology) for *Slah-1*, *stathmin*,  $\beta$ -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 deoxyribonuclease I-like 3), signal transduction (*e.g.*, protein kinase C,  $\iota$  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,  $\beta$ -catenin, and apoptosis-inducing factor), hypothetical genes, and expressed sequence tags ( $P < 0.025$ ; Fig. 1A; a complete list is available online<sup>8</sup>). 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

<sup>8</sup> <http://www.medizin.uni-koeln.de/kliniken/pathologie/supplementaldata.html>.

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

Gene name	GenBank accession no.	Symbol	Regulation in HCCs	
			G1	G3
H2A histone family	AA488627	<i>H2AFY</i>	1	1.8
Expressed sequence tag	AA45944		1.6	2.6
Papillary renal cell carcinoma	AA488180	<i>PRCC</i>	1	1.7
Heterogeneous nuclear ribonucleoprotein M	AA504272	<i>HNRPM</i>	1	1.6
Microspherule protein 1	AA488979	<i>MCRS1</i>	-1.2	1.3
Ribosomal protein S23	N73091	<i>RPS23</i>	-1.3	1.6
Histone deacetylase 2	AA127093	<i>HDAC2</i>	-1.2	1.8
CGI-69 protein	AA485441	<i>CGI-69</i>	-1.5	1.5
Replication factor C (activator 1) 4	N93924	<i>RFC4</i>	-1.6	1.6
Expressed sequence tag	N59870		-1.3	1.3
Nucleolin	AA476294	<i>NCL</i>	-1.6	1.6
Bromodomain containing 4	R32788	<i>BRD4</i>	-1.1	1.6
Sjogren syndrome antigen B (autoantigen La)	H29485	<i>SSB</i>	-1.4	1.4
CDW92 antigen	AA457261	<i>CDw92</i>	-2.7	-1.2
Aryl hydrocarbon receptor	AA461522	<i>AHR</i>	-1.5	1.2
Fatty acid desaturase 1	AA431773	<i>FADS1</i>	-4	1.7
Vesicle docking protein p115	AA504342	<i>VDP</i>	1.2	-1.8
Glyoxylate reductase/hydroxypyruvate reductase	R83908	<i>GRHPR</i>	-1.3	-3.9
Complement component 8, $\alpha$ polypeptide	H53865	<i>C8A</i>	-1.6	-7.6
Deoxyribonuclease I-like 3	T73558	<i>DNASE1L3</i>	-2.5	-9.6
Liver-expressed antimicrobial peptide 2	R93723	<i>LEAP-2</i>	-1.5	-4.1
Liver-expressed antimicrobial peptide 2	T82264	<i>LEAP-2</i>	-1.6	-4.5
Lutheran blood group (Auberger b antigen included)	H24954	<i>LU</i>	-2.3	-3.6
Seven in absentia homologue	T71889	<i>SIAH1</i>	-1.4	-4.1
Expressed sequence tag	N58432		-1.6	-6.4
Mitochondrial ribosomal protein L46	AA446865	<i>MRPL46</i>	-1.3	-2.1
CD8 antigen, $\alpha$ polypeptide (p32)	AA443584	<i>CD8A</i>	-1.8	-3.6
Protein kinase C, $\iota$	T57875	<i>PRKCI</i>	1	-3.7
Expressed sequence tag	AA490120		1.1	-5
Epithelial membrane protein 2	T88721	<i>EMP2</i>	1.1	-2.6
Alcohol dehydrogenase 6 (class V)	T87343	<i>ADH6</i>	-1.2	-4.7
Solute carrier family 38, member 2	AA598996	<i>SLC38A2</i>	-1.3	-3.8
Translocation protein 1	AA450205	<i>TLOC1</i>	1	-1.7
Expressed sequence tag	R61700		1.1	-1.4
Acyl-CoA synthetase long-chain family member 6	AA426087	<i>FACL6</i>	2	-1.1
Expressed sequence tag	R60924		1.5	-1.8
Hydroxysteroid (17- $\beta$ ) dehydrogenase 4	AA487914	<i>HSD17B4</i>	2	-1.7
Betaine-homocysteine methyltransferase 2	N78012	<i>BHMT2</i>	2	-2.4
IFN-stimulated transcription factor 3, $\gamma$	AA291389	<i>ISGF3G</i>	2.5	-1.8
Cytochrome $b_5$	R91950	<i>CYB5</i>	2	-2.5
Formyltetrahydrofolate dehydrogenase	T52840	<i>FTHFD</i>	2.1	-3.7
Anaphase-promoting complex subunit 7	AF191340	<i>ANAPC7</i>	2.1	-3.1
Regucalcin	H05140	<i>RGN</i>	1.6	-2.2
Acetyl-coenzyme A acyltransferase 2	P42765	<i>ACAA2</i>	1.5	-2.4
Acyl-coenzyme A dehydrogenase	P11310	<i>ACADM</i>	2.3	-1.1
Hypothetical protein FLJ13456	N25234	<i>FLJ13456</i>	1.9	-1.1
Putative ethanolamine kinase	H77536	<i>FLJ10761</i>	4.7	-1.1
Expressed sequence tag	R02373		4.5	1
Solute carrier family 16	U59299	<i>SLC16A2</i>	2.4	-1.1
Sorbitol dehydrogenase	A54674	<i>SORD</i>	5.4	1.8
Betaine-homocysteine methyltransferase	T58896	<i>BHMT</i>	4	-1.9
Ankyrin repeat and SOCS box-containing 13	T12477	<i>ASB13</i>	1.5	-2.1
Selenium-binding protein 1	T65736	<i>SELENBP1</i>	2.8	-1.5

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



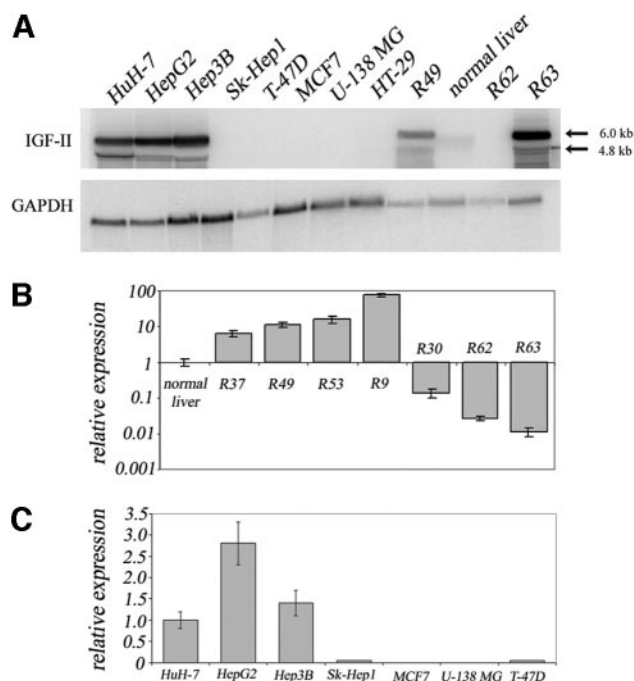


Fig. 3. IGF-II mRNA expression in HCC cell lines and HCCs. **A**, Northern blot analysis was performed by separating 20  $\mu$ g of total RNA isolated from hepatocellular cell lines (HuH-7, HepG2, and Hep3B), nonhepatocellular cell lines (Sk-Hep1, T-47D, MCF7, U-138MG, and HT29), normal liver, a group 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 hepatocellular (HuH-7, HepG2, and Hep3B) and nonhepatocellular (Sk-Hep1, MCF7, U-138MG, and T-47D) cell lines. HuH-7 mRNA was used for calibration.

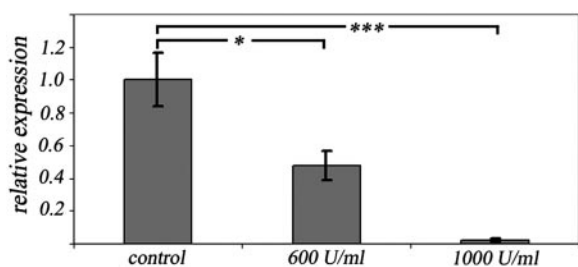


Fig. 4. IFN- $\gamma$ -dependent reduction of IGF-II expression in HCC cell lines. Semiquantitative IGF-II TaqMan analysis of HuH-7 cells treated with increasing concentrations of IFN- $\gamma$  for 16 hours. Untreated cells were used for calibration.

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.<sup>8</sup> 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.<sup>8</sup>

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- $\gamma$ , 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- $\gamma$ -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- $\gamma$  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,

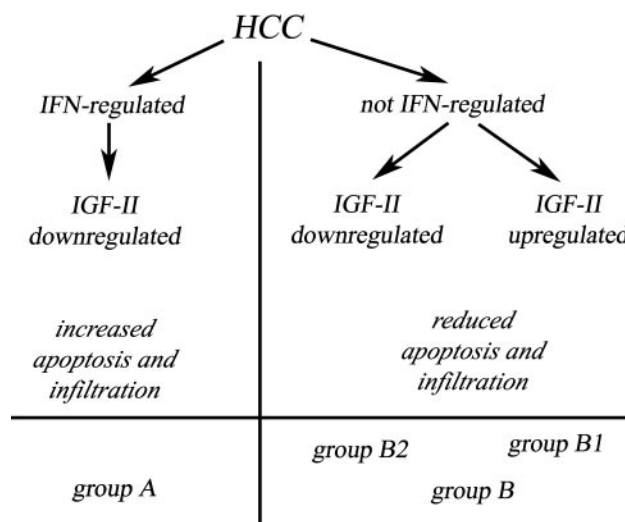


Fig. 5. Schematic display of HCC subtyping.

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 apoptoses 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 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), remethylation 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.

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