Human Cancer Biology

Insulin-like Growth Factor–Binding Protein-7 Functions as a Potential Tumor Suppressor in Hepatocellular Carcinoma

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Abstract

Purpose: Hepatocellular carcinoma (HCC) is a highly virulent malignancy with no effective treatment, thus requiring innovative and effective targeted therapies. The oncogene astrocyte-elevated gene-1 (AEG-1) plays a seminal role in hepatocarcinogenesis and profoundly downregulates insulin-like growth factor–binding protein-7 (IGFBP7). The present study focuses on analyzing potential tumor suppressor functions of IGFBP7 in HCC and the relevance of IGFBP7 downregulation in mediating AEG-1 function.

Experimental Design: IGFBP7 expression was detected by immunohistochemistry in HCC tissue microarray and real-time PCR and ELISA in human HCC cell lines. Dual FISH was done to detect LOH at IGFBP7 locus. Stable IGFBP7-overexpressing clones were established in the background of AEG-1–overexpressing human HCC cells and were analyzed for in vitro proliferation and senescence and in vivo tumorigenesis and angiogenesis.

Results: IGFBP7 expression is significantly downregulated in human HCC samples and cell lines compared with normal liver and hepatocytes, respectively, and inversely correlates with the stages and grades of HCC. Genomic deletion of IGFBP7 was identified in 26% of patients with HCC. Forced overexpression of IGFBP7 in AEG-1–overexpressing HCC cells inhibited in vitro growth and induced senescence, and profoundly suppressed in vivo growth in nude mice that might be an end result of inhibition of angiogenesis by IGFBP7.

Conclusion: The present findings provide evidence that IGFBP7 functions as a novel putative tumor suppressor for HCC and establish the corollary that IGFBP7 downregulation can effectively modify AEG-1 function. Accordingly, targeted overexpression of IGFBP7 might be a potential novel therapy for HCC.

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Introduction

Hepatocellular carcinoma (HCC) is among the 5 most common neoplasms worldwide with virtually no effective treatment of the advanced disease (1). This dire situation mandates better understanding of the molecular mechanism of hepatocarcinogenesis so that new targets might be identified for designing effective therapeutic intervention. Recent studies have emphasized the importance of the oncogene astrocyte elevated gene-1 (AEG-1) in tumor initiation, progression, angiogenesis, and metastasis in diverse cancer indications including HCC, where AEG-1 is overexpressed in more than 90% of patients with HCC (2, 3). Overexpression and knockdown studies have established a fundamental role of AEG-1 in the development and progression of HCC (3–5). AEG-1 exerts its pleiotropic tumorigenic effects by strongly modulating diverse intracellular signaling pathways as well as transcriptome and proteome profiles (3, 5–7). Microarray studies identified a plethora of AEG-1–modulated genes associated with growth, invasion, angiogenesis, metastasis, senescence, and chemoresistance (3, 6). In HCC cells, the most robustly AEG-1–downregulated gene was insulin-like growth factor–binding protein-7 (IGFBP7; ref. 3).

IGFBP7, also known as mac25 or IGFBP-related protein-1 (IGFBP-rP1), is a secreted protein belonging to the IGFBP family (8, 9). The IGF axis plays a key role in the growth, differentiation, and proliferation of mammalian cells and consists of 2 growth factors (IGF-I and IGF-II), their receptors (IGF-IR and IGF-IIR), and a group of IGFBPs (IGFBP1-7; ref. 8). IGFBPs regulate the bioavailability of IGFs by binding to IGFs with high affinity, thereby limiting IGF access to IGF-IR and inhibiting IGF activity (8, 10). However, IGFBPs also exert IGF-independent actions.

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Translational Relevance

This study identifies insulin-like growth factor–binding protein-7 (IGFBP7) as a novel tumor suppressor for hepatocellular carcinoma (HCC) and shows that down-regulation of IGFBP7 plays a central role in mediating the oncogenic functions of astrocyte elevated gene-1 (AEG-1). IGFBP7 expression is significantly down-regulated in human HCC samples compared with normal liver. Accordingly, IGFBP7 might represent a novel biomarker for human HCC and because it is a secreted protein it might also provide the basis for use as a serum biomarker for HCC, a hypothesis that needs to be experimentally validated. In vivo experiments document that moderate overexpression of IGFBP7 profoundly inhibits the growth of human HCC cells overexpressing AEG-1. As such, forced overexpression of IGFBP7 through a conditionally replication-competent adenovirus might provide an effective cancer gene therapy, particularly for more than 90% of patients with HCC in whom AEG-1 is overexpressed. Consequently, IGFBP7 has significant translational relevance in the context of HCC.

IGFBP7 differs from the other 6 members of this family by lacking the C-terminus and having 100 times lower affinity for IGF-I (9). IGFBP7 has been proposed to be a tumor suppressor protein for a variety of cancers. In breast cancer, there is an LOH at IGFBP7 locus in chromosome 4q, which is also a putative tumor suppressor locus in a variety of cancers including HCC (11). Although normal tissues express abundant IGFBP7, no or very weak IGFBP7 expression is detected in breast, prostate, and colorectal cancers, especially in the advanced stages (12–14). In breast cancers, low IGFBP7 denotes poor prognosis in estrogen receptor–negative invasive cases (12, 15). Interestingly, IGFBP7 expression is induced in senescent breast and prostate epithelial cells and IGFBP7 mediates oncogenic BRAF–induced senescence (16, 17). Overexpression of IGFBP7 results in G1 arrest and senescence in prostate cancer cells and inhibits growth of xenografts of human prostate, bladder, breast, and colorectal cancers in nude mice (18–21). In addition, IGFBP7 inhibits VEGF-induced angiogenesis (22).

In this article, we explore the role of IGFBP7 in HCC development and progression. We document that IGFBP7 expression gradually decreases with the stages and grades of HCC and a significant proportion of patients with HCC harbor IGFBP7 gene deletion. Overexpression of IGFBP7 significantly inhibited growth of human HCC cells both in vitro and in vivo. These findings identify IGFBP7 as a novel putative tumor suppressor for HCC.

Materials and Methods

Plasmids, cell lines, culture condition, viability assays, and chemical reagents

IGFBP7 (NM_001553) human cDNA clone was obtained from Origene Technologies, Inc., and cloned into pcDNA3.1(+)–zeo plasmid (Invitrogen). THLE-3 cells, normal human hepatocytes immortalized by SV40 T/t Ag, and human HCC cell lines, Hep3B, SK-Hep1, and FOCULIS, were obtained from the American Type Culture Collection and were cultured according to the instructions (3). The human HCC cell lines HepG3 and Huh7 were kindly provided by Dr. Paul Dent and were cultured as described (3). Generation of Hep-AEG-1-8, Hep-AEG-1-14, and Hep-AEG-1-20 clones, HepG3 cells stably expressing AEG-1, and Hep-pc-4, HepG3 cells stably transduced with empty vector, was as described (3). Hep-AEG-1-14 cells were stably transduced with IGFBP7 expression vector, using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The cells were then selected for 2 weeks in 200 μg/mL zeocin, and individual colonies were isolated and grown. Control zeocin-resistant clones of Hep-AEG-1-14 cells were similarly generated by transfection with empty pcDNA3.1 (+)-zeo vector. Cell viability was determined by standard MTT assay as described (3). For colony formation assays, 500 cells were plated in 6-cm dishes and colonies of more than 50 cells were counted after 2 weeks.

Patient samples and tissue microarray

Patient samples were obtained from the Liver Tissue Cell Distribution System (NIH contract #N01-DK-7-0004/ HHSN2672007000004C). The 18 matched normal liver and HCC samples include LTCD5 #1100, 1107, 1135, 1143, 1153, 1154, 1164, 1169, 1172, 1174, 1194, 1216, 1237, 1246, 1260, 1264, 1276, and 1282. Two human HCC tissue microarrays (TMA; Imgenex; IMH-360 and IMH-318), including 9 normal adjacent liver samples, were used for immunohistochemistry.

FISH

Dual-color FISH was done as described on HCC TMAs (23). Bacterial artificial chromosome (BAC)-derived test probe targeting IGFBP7 (4q12, RP11-313C13; BACPAC Resources Center) was labeled with Spectrum Orange; this was paired for dual-target hybridization with control probe CEP4 (probe targeting centromeric region of chromosome 4; Abbott Laboratories). The CEP4 probe provided enumeration of chromosome copy number for chromosome 4. Sections showing sufficient hybridization efficiency (majority of nuclei with signals) were considered informative and were scored by 2 reviewers. Nonneoplastic liver specimens served as the controls. Deletion for IGFBP7 was subsequently defined by an IGFBP7/CEP4 ratio of less than 0.73 (mean ± 3 SDs in nonneoplastic controls).

Immunohistochemistry

Immunohistochemistry in tumor sections and in TMA was done as described (3). The primary antibodies were anti-IGFBP7 (1:100, mouse monoclonal; R&D Systems), anti-Ki-67 (1:200, mouse monoclonal; BD Biosciences) and anti-CD31 (1:200, mouse monoclonal; Dako). Normal liver and matched HCC sections were also stained with anti-AEG-1 antibody (1:500, chicken polyclonal; in-house). The signals were developed by avidin–biotin–peroxidase
complexes with a 3,3′-diaminobenzidine substrate solution (Vector Laboratories). Images were analyzed with an Olympus microscope.

**Immunofluorescence and Western blot analyses**

Immunofluorescence and Western blot analyses were conducted as described (3). For immunofluorescence, the primary antibody was anti-γ-H2AX antibody (1:500, mouse monoclonal; Millipore) and the secondary antibody was Alexa Fluor 488–conjugated anti-mouse IgG (1:400; Molecular Probes). The slides were mounted in VECTASHIELD fluorescence mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Images were analyzed with a Zeiss confocal laser scanning microscope. For Western blotting, the primary antibodies were anti-p-IGF-IR (Tyr1161; 1:250, rabbit polyclonal; Cell Signaling), anti-IGF-IR (1:250, mouse monoclonal; Santa Cruz), anti-p-ERK (1:2,000, rabbit polyclonal; Cell Signaling Technology), anti-ERK (1:2,000, rabbit polyclonal; Cell Signaling), anti-p-AKT (Ser473; 1:1,000, rabbit polyclonal; Cell Signaling), anti-AKT (1:1,000, rabbit polyclonal; Cell Signaling), and EF1-α as loading control (1:1,000, mouse monoclonal; Millipore).

**ELISA**

The recombinant IGFBP7 protein used as a standard was obtained from R&D Systems (catalog #1334-B7). The wells of a 96-well plate were coated with the standard or with conditioned media (100 μL) overnight at 4 °C. The wells were washed 4 times with PBS and then blocked with 5% nonfat milk in PBS for 1 hour. Following another PBS washing, anti-IGFBP7 antibody (1:1,000) was added and incubated overnight at 4°C without shaking. After another 4 times with PBS washing, anti-mouse secondary antibody (1:1,000) was added and incubated at room temperature for 2 hours with gentle shaking. The wells were washed 4 times with PBS and 100 μL Glo substrate reagent (DY993; R&D Systems) was added to each well for 15 minutes and the reaction was stopped by adding 100 μL Stop Solution (DY994; R&D Systems). The plate was read at 560 nm with a multiplate reader (Turner Biosystems).

**Assay for senescence-associated β-galactosidase activity**

Cells were cultured for 6 days and senescence-associated β-galactosidase–positive cells were detected with a Senescence Detection kit (Biovision) according to the manufacturer’s protocol that follows the original method of detection (24).

**Total RNA extraction and real-time PCR assay**

Total RNA was extracted with a Qiagen miRNAeasy mini kit (Qiagen). Real-time PCR was carried out with an ABI 7900 fast real-time PCR system and by TaqMan gene expression assays for IGFBP7 and glyceraldehyde-3-phosphate dehydrogenase according to the manufacturer’s protocol (Applied Biosystems).

**Nude mice xenograft studies**

Cells (5 × 10⁶) were subcutaneously implanted in the flanks of athymic nude mice. Tumor volume was measured with calipers by the following formula: (width)² × length/2. Mice were followed for 3 weeks.

**Chicken chorioallantoic membrane assay**

Cells were seeded on the chorioallantoic membrane (CAM) surface of 9-day-old chick embryos according to established protocols (25). One week after inoculation, the neovascularure was examined and photographed. Angiogenesis was quantified by counting the blood vessel branch points under a stereomicroscope. The angiogenic index was calculated by subtracting the number of branch points from the branching in the control group.

**Capillary-like tube formation assay**

The formation of tube-like structures by human umbilical vein endothelial cells (HUVEC) on Matrigel (Chemicon) was achieved as previously described (26). HUVECs were cultured overnight in conditioned media from control-2, IGFBP7-11, and IGFBP7-12 clones. The degree of network formation was quantified with an Image analyzer (NIH Image).

**Statistical analysis**

Data were represented as the mean ± SEM and analyzed for statistical significance by one-way ANOVA followed by the Newman–Keuls test as a post hoc test. A value of P < 0.05 was considered as significant.

**Results**

The identification of IGFBP7 as a tumor suppressor for breast, prostate, and colorectal cancer prompted us to analyze IGFBP7 expression profile in patients with HCC by immunohistochemistry in TMAs containing 104 HCC samples and 9 normal adjacent liver samples. Strong IGFBP7 immunostaining was detected in 9 normal liver samples (Fig. 1A and Supplementary Table S1), whereas weak IGFBP7 staining was observed in the HCC samples (Fig. 1A and Supplementary Table S1). There was a gradual decrease in IGFBP7 expression, with the stages of HCC based on the Barcelona-Clinic Liver Cancer (BCLC) staging system (Fig. 1A and Supplementary Table S1). Moreover, in each stage, IGFBP7 expression was much lower in poorly differentiated grades than in moderately differentiated grades (Fig. 1A). To evaluate the negative correlation between IGFBP7 expression and stages of HCC, we conducted an ordinal logistic regression with the stage of HCC as the ordinal response and IGFBP7 expression as the independent variable in the proportional odds model. The hypothesis of association is highly significant (P < 0.001) by the Pearson χ² test (Supplementary Table S1).

LOH at the IGFBP7 locus (chromosome 4q12) has been reported in breast cancer (11). To examine the possibility that genomic deletion might be the underlying mechanism of IGFBP7 downregulation in human patients with HCC,
dual-color FISH was done on human HCC TMAs containing 9 normal liver samples and 50 HCC samples. BAC-derived test probe targeting IGFBP7 (orange color) was used along with a control probe that is specific for the pericentromeric region of chromosome 4 (CEP4; green color). The control probe (CEP4) provided information regarding the number of chromosome 4 present in the cell. While no normal liver samples showed IGFBP7 deletion, 13 of 50 (26%) HCC samples showed IGFBP7 deletion. Figure 1B shows a representative normal cell in which 2 orange (IGFBP7) and 2 green (CEP4) dots are observed. In the HCC cell, there are 2 signals for CEP4, but only one signal for IGFBP7 indicating deletion of one copy of the IGFBP7 gene.

We next evaluated IGFBP7 expression in THLE-3 cells, which are normal human hepatocytes immortalized by SV40 T/t Ag and several human HCC cell lines, namely, HepG3, Hep3B, Huh7, SK-Hep1, and FOCUS. Compared with THLE-3 cells, IGFBP7 mRNA expression was profoundly downregulated in all the HCC cell lines (Fig. 2A). These findings were confirmed by ELISA using conditioned media convincingly showing downregulation of secreted IGFBP7 protein in human HCC cell lines, compared with THLE-3 cells (Fig. 2B). IGFBP7 was initially identified as an AEG-1–downregulated gene by Affymetrix microarray. To corroborate this finding, we checked 3 different AEG-1–overexpressing clones of HepG3 cells, Hep-AEG-1-8, Hep-AEG-1-14, and Hep-AEG-1-20. As a control, we used Hep-pc-4 cells, which is a control hygromycin-resistant clone of HepG3 cells. In all 3 AEG-1–overexpressing clones, IGFBP7 mRNA expression was robustly downregulated compared with Hep-pc-4 cells (Fig. 2C). This finding was further verified by immunofluorescence analysis. IGFBP7 protein expression was significantly higher in Hep-pc-4 cells than in Hep-AEG-1-14 cells (Fig. 2D). These findings were further shown in matched normal liver and HCC samples from 18 patients by immunohistochemical analysis of AEG-1 and IGFBP7 expression. In 13 of these 18 patients, very low to undetectable levels of AEG-1 expression and high level of IGFBP7 expression were detected in normal liver, whereas significantly high levels of expression of AEG-1 and low levels of expression of IGFBP7 were detected in the matched HCC samples (Fig. 2E). No change in AEG-1 or IGFBP7 expression was evident in the remaining 5 patients. These findings further buttress the inverse relationship between AEG-1 and IGFBP7 expression.

HepG3 cells are nontumorigenic in nude mice and stable overexpression of AEG-1 in HepG3 cells (Hep-AEG-1-8 and Hep-AEG-1-14 clones) results in generation of highly aggressive, angiogenic, and metastatic tumors (3). The profound downregulation of IGFBP7 in Hep-AEG-1 clones prompted us to inquire whether IGFBP7 downregulation is obligatory for maintenance of the oncogenic phenotype conferred by AEG-1. For this reason, we generated stable clones of Hep-AEG1-14 cells expressing IGFBP7 by selection with zeocin. A zeocin-resistant clone of Hep-AEG1-14 cells (control-2) served as a control. Compared with the control-2 clone, IGFBP7-11 and IGFBP7-12 clones expressed significantly higher levels of IGFBP7 mRNA and protein, detected by real-time PCR and ELISA, respectively (Fig. 3A and B). It should be noted that the secreted IGFBP7 levels in IGFBP7-11 and IGFBP7-12 clones did not reach the levels observed in the parental HepG3 cells from which all these clones were generated (compare Figs. 2B and 3B). IGFBP7-11 and IGFBP7-12 clones showed significantly slower growth rate than control-2 clone analyzed by standard cell viability (MTT) and colony formation assays (Fig. 3C and D, respectively). However, this growth
inhibition was not profound in IGFBP7-overexpressing clones compared with control-2 clone, showing only about 27% inhibition by cell viability assay and about 23% inhibition by colony formation assay. The IGFBP7-induced growth inhibition might be mediated by the ability of IGFBP7 to interfere with IGF-I signaling. Indeed, we ob-

Figure 2. IGFBP7 is downregulated in human HCC cell lines and by AEG-1. A, determination of IGFBP7 mRNA expression by real-time PCR in the indicated cells. THLE-3 is normal immortal human hepatocytes. Glyceraldehyde-3-phosphate dehydrogenase was used as the normalization control. B, secreted IGFBP7 protein level in the conditioned media of the indicated cells determined by ELISA. C, determination of IGFBP7 mRNA expression by real-time PCR in Hep-pc-4 (pc-4) cells and 3 independent clones of HepG3 cells overexpressing AEG-1. D, immunofluorescence detection of IGFBP7 protein in Hep-pc-4 and Hep-AEG-1-14 cells. E, immunohistochemical analysis of AEG-1 and IGFBP7 expression in normal liver and matched HCC from the same patient. The figure represents data from one patient. Similar finding was observed in 13 of 18 patients with HCC. For A–C, data represent mean ± SEM of 3 independent experiments. *, P < 0.05.

Figure 3. Overexpression of IGFBP7 inhibits growth of AEG-1-overexpressing cells. Stable clones of Hep-AEG1-14 cells expressing IGFBP7 (IGFBP7-11 and IGFBP7-12) were generated by selection with zeocin. Zeocin-resistant clone of Hep-AEG1-14 cells (control-2) served as a control. A, IGFBP7 mRNA expression in the indicated cells detected by real-time PCR. B, secreted IGFBP7 protein level in the indicated cells detected by ELISA. C, cell viability (MTT) assay of the indicated cells. D, colony formation assay of the indicated cells. For A–D, data represent mean ± SEM of 3 independent experiments. *, P < 0.05. E, Western blot analysis conducted in the indicated cells with the indicated antibodies. EF1α was used as a loading control.
erved that the phospho-IGF-IR level was significantly downregulated, whereas total IGF-IR level was unchanged, in IGFBP7-11 and IGFBP7-12 clones compared with control-2 clone (Fig. 3E). It should be noted that the antibody used to detect p-IGF-IR might cross-react with p-IR (insulin receptor); however, the molecular weight of the detected band correlates strongly with p-IGF-IR rather than p-IR. The IGF-IR downstream signaling, such as activation of Akt and extracellular signal-regulated kinase (ERK), was also significantly inhibited in IGFBP7-11 and IGFBP7-12 clones compared with control-2 clone (Fig. 3E). We also observed that THLE-3 cells expressing higher level of IGFBP7 express lower level of phospho-IGF-IR and p-Akt than do several human HCC cell lines (Supplementary Fig. S1). Although p-ERK level in THLE-3 cells was lower than that in some of the human HCC cell lines, not all of them showed increased ERK activity, which might be explained by potential regulation of Akt and ERK signaling by a myriad of other signaling events (Supplementary Fig. S1).

IGFBP-7 induces senescence. As such, we evaluated induction of senescence in control-2, IGFBP7-11, and IGFBP7-12 clones by the SA-β-gal assay. There was a significant increase in the number of SA-β-gal-positive cells in IGFBP7-overexpressing clones compared with the control-2 clone following 1-week culture (Fig. 4A and B). These findings were confirmed by analyzing γ-H2AX foci, another marker for senescence. There was a significant increase in γ-H2AX foci in the nuclei of IGFBP7-11 and IGFBP7-12 clones when compared with the control-2 clone (Fig. 4C and D).

The in vitro growth-suppressing function of IGFBP7 was extended by in vivo assays. Control-2 and IGFBP7-11 and IGFBP7-12 cells were subcutaneously xenografted into the flanks of athymic nude mice and tumor development was monitored. Tumor growth was profoundly inhibited in the IGFBP7-11 and IGFBP7-12 clones as compared with the tumors generated from the control-2 clone (Fig. 5A and B). Immunohistochemical studies confirmed overexpression of IGFBP7 in IGFBP7-11 and IGFBP7-12 clones. In addition, there was marked downregulation of the angiogenesis marker CD31 (staining microvessels) and the proliferation marker Ki-67 in tumors derived from IGFBP7-11 and IGFBP7-12 clones as compared with those from the control-2 clone (Fig. 5C).

The in vivo growth suppression effect of IGFBP7 was significantly higher than observed by in vitro assays. We reasoned that in addition to direct inhibition of growth by the induction of senescence, IGFBP7 might interfere with tumor development indirectly by interference with tumor angiogenesis, a hypothesis supported by marked downregulation of CD31 staining in IGFBP7-overexpressing tumor sections. We confirmed the effect of IGFBP7 in angiogenesis by implanting control-2, IGFBP7-11, and IGFBP7-12 clones in the chicken CAM of 9-day-old chick embryos. After 1 week, neovascularization was examined, photographed, and quantified. Neovascularization was significantly inhibited in IGFBP7-11 and IGFBP7-12 clones, compared with the control-2 clone (Fig. 6A and B). These findings were extended further by endothelial cell tube formation assays. HUVECs were treated with conditioned media from control-2, IGFBP7-11, and IGFBP7-12 clones and endothelial cells tube formation was scored (Fig. 6C and D). Conditioned media from IGFBP7-11 and IGFBP7-12 clones significantly inhibited HUVEC tube formation as compared with conditioned media from the control-2 clone, further validating the antiangiogenic functions of IGFBP7.

Figure 4. IGFBP7 induces senescence. A, photomicrograph of control-2, IGFBP7-11, and IGFBP7-12 clones of Hep-AEG-1-14 cells stained for SA-β-gal after 1 week of culture. B, graphical representation of quantification of SA-β-gal-positive cells. At least 1,000 cells were counted for each group. Data represent mean ± SEM of 3 independent experiments. *, P < 0.05. C, photomicrograph of control-2, IGFBP7-11, and IGFBP7-12 clones of Hep-AEG-1-14 cells stained for γ-H2AX and counterstained with DAPI to stain the nucleus. D, graphical representation of quantification of γ-H2AX foci per cell. At least 100 cells were scored for each group. Data represent mean ± SEM of 3 independent experiments. *, P < 0.05.
Discussion

Although IGFBP7 has shown tumor suppressor functions in a number of epithelial cancers, very little is known about its anticancer role in HCC. In murine SV40-T/t Ag–induced liver cancer, IGFBP7 is silenced by methylation, although the mechanism and consequence of this silencing have not been dissected (27). A recent report showed that IGFBP7 is required for the sensitivity of HCC cells toward IFN-based anticancer therapy (28). The same report described the analysis of IGFBP7 in a small population of 30 patients showing a significantly better postoperative overall survival in patients with IGFBP7-positive HCC than in patients with IGFBP7-negative HCC (28). Ours is the first report with a comprehensive analysis of IGFBP7 expression in 104 patients with HCC in which we show a progressive decrease in IGFBP7 expression with advanced stages of HCC as well as with poorer grades of differentiation. We also document genomic deletion of IGFBP7 in 26% of patients with HCC analyzed. This is the first demonstration of IGFBP7 gene deletion in HCC as a potential mechanism of reduced IGFBP7 expression during the process of carcinogenesis. The markedly high expression of both IGFBP7 mRNA and protein in normal immortal hepatocytes, THLE-3 cells, compared with several HCC cell lines further reinforces a putative tumor suppressor role of IGFBP7 in HCC.

Affymetrix microarray analysis designed to probe the global gene expression changes induced by the oncogene AEG-1 revealed profound downregulation of IGFBP7.
mRNA expression by AEG-1 (26-fold downregulation by microarray and >10-fold downregulation by real-time PCR; ref. 3). Indeed, our in vitro and in vivo studies document that forced overexpression of IGFBP7 could effectively abrogate the tumor-promoting functions of AEG-1. Although AEG-1 is known to modulate the expression of a plethora of protumorogenic genes and proteins as well as altering signaling pathways, the profound inhibition of in vitro growth by forced IGFBP7 overexpression in AEG-1-overexpressing cells suggest a major role of IGFBP7 downregulation in mediating AEG-1 function. This notion is further strengthened by the observation that the significantly lower level of secreted IGFBP7 in IGFBP7-11 and IGFBP7-12 expressing clones of Hep-AEG-1-14 cells, compared with the parental nontumorogenic HepG3 cells (Figs. 2B and 3A), is sufficient to profoundly inhibit in vivo tumorigenesis induced by AEG-1 overexpression. The mechanism of IGFBP7 downregulation by AEG-1 remains to be determined. Does AEG-1 induce hypermethylation of the IGFBP7 promoter resulting in silencing of expression as observed in other cancer indications? Does AEG-1 interfere with transcription factor binding to the IGFBP7 promoter as is observed for the c-myc promoter where AEG-1 physically interacts with the transcription factor PLZF and prevents PLZF DNA binding (29)? Does AEG-1 enhance mRNA instability of IGFBP7?? These issues are currently being experimentally addressed to clarify the underlying molecular mechanism of IGFBP7 suppressor function in AEG-1-overexpressing cells.

The in vitro growth inhibitory effect of IGFBP7 might be mediated by its ability to interfere with IGF-I signaling (Fig. 3E). We observed that the in vivo inhibitory effect of IGFBP7 overexpression was more pronounced than the in vitro effect. We document that inhibition of angiogenesis might be one mechanism by which IGFBP7 indirectly abrogates tumor growth and progression. We show for the first time that IGFBP7 overexpression results in inhibition of neovascularization in the CAM model and microvessel density (CD31 expression) in a subcutaneous xenograft model in nude mice. We also document that conditioned media from IGFBP7-overexpressing cells inhibits HUVEC tube formation. Recombinant IGFBP7 inhibits VEGF-induced tube formation and VEGF-downstream signaling in HUVEC (22). IGFBP7 also downregulates VEGF expression in malignant melanoma cells (30). Thus, the combined effects of VEGF downregulation in HCC cells and direct inhibition of endothelial cell differentiation by IGFBP7 might mediate the antiangiogenic effects of IGFBP7. As a secreted protein, IGFBP7 might also modulate the function of the immune system generating an antitumor immune response, a hypothesis that remains to be experimentally tested.

In summary, we identify IGFBP7 as a unique and novel putative tumor suppressor for HCC. Our preliminary findings document that recombinant IGFBP7 efficiently inhibits growth of human HCC cells without affecting normal THLE-3 cells. In this context, IGFBP7 might be developed as a potential anti-HCC therapy. Studies are ongoing to develop targeted and effective delivery systems for administering IGFBP7 in patients with HCC.

Disclosure of Potential Conflicts of Interest

D. Sarkar is the Harrison Endowed Scholar in Cancer Research and a Bick scholar. P.B. Fisher holds the Thelma Newmeyer Corman Chair in Cancer Research and is a Samuel Waxman Cancer Research Foundation (SWCRF) investigator. No potential conflicts of interest were disclosed by other authors.

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