Insulin-like growth factor binding protein-7 (IGFBP7) functions as a potential tumor suppressor in hepatocellular carcinoma (HCC)

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Statement of Translational Relevance

The present study identifies insulin-like growth factor binding protein-7 (IGFBP7) as a novel tumor suppressor for hepatocellular carcinoma (HCC) and shows that downregulation of IGFBP7 plays a central role in mediating the oncogenic functions of Astrocyte elevated gene-1 (AEG-1). IGFBP7 expression is significantly downregulated in human HCC samples compared to normal liver. Accordingly, IGFBP7 might represent a novel biomarker for human HCC and since 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 the >90% of patients with HCC that overexpress AEG-1. Consequently, IGFBP7 has significant translational relevance in the context of HCC.
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 Fluorescence in situ hybridization was performed to detect loss of heterozygosity 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 to normal liver and hepatocytes, respectively, and inversely correlates with the stages and grades of HCC. Genomic deletion of IGFBP7 was identified in 26% of HCC patients. 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.
Introduction

Hepatocellular carcinoma (HCC) is among the five most common neoplasms world-wide with virtually no effective treatment for 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 >90% of HCC patients (2, 3). Overexpression and knock-down studies have established a fundamental role of AEG-1 in the development and progression of HCC (3-5). AEG-1 exerts its pleiotrophic 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) (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 two growth factors (IGF-I and IGF-II), their receptors (IGF-IR and IGF-IIR) and a group of IGFBPs (IGFBP1-7) (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. IGFBP7 differs from the other six 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 a loss of heterozygosity at IGFBP7 locus in chromosome 4q which is also a putative tumor suppressor
locus in a variety of cancers including HCC (11). While 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 ER 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). Additionally, IGFBP7 inhibits vascular endothelial growth factor (VEGF)-induced angiogenesis (22).

In the present manuscript, 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 HCC patients 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 (Rockville, MD) 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 FOCUS were obtained from the American Type Culture Collection (Manassas, VA) and were cultured according to the instructions (3). 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 up. 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, cells (500) were plated in 6-cm dishes and colonies > 50 cells were counted after 2 weeks.

**Patient samples and Tissue microarray (TMA):** Patient samples were obtained from the Liver Tissue Cell Distribution System (LTCDS) (NIH contract #N01-DK-7-0004 / HHSN267200700004C). The 18 matched normal liver and HCC samples include LTCDS #1100, 1107, 1135, 1143, 1153, 1154, 1164, 1169, 1172, 1174, 1194, 1216, 1237, 1246, 1260, 1264, 1276 and 1282. Two human HCC TMAs (Imgenex; IMH-360 and IMH-318), including 9 normal adjacent liver samples, were used for immunohistochemistry.

**Fluorescence in situ hybridization (FISH):** Dual color FISH was performed 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, Abbott Park, Illinois). 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 two reviewers. Non-neoplastic liver specimens served as the controls. Deletion for *IGFBP7* was subsequently defined by an *IGFBP7*: CEP4 ratio of <0.73 (mean ± 3 standard deviations in non-neoplastic controls).
**Immunohistochemistry:** Immunohistochemistry in tumor sections and in TMA was performed as described (3). The primary antibodies were anti-IGFBP7 (1:100; mouse monoclonal; R&D), 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 DAB substrate solution (Vector laboratories). Images were analyzed using an Olympus microscope.

**Immunofluorescence and Western blot analyses:** Immunofluorescence and Western blot analyses were performed as described (3). For immunofluorescence the primary antibody was anti-γ-H2AX antibody (mouse monoclonal; 1:500; Millipore) and the secondary antibody was Alexa488-conjugated anti-mouse IgG (1:400; Molecular Probes). The slides were mounted in VectaShield fluorescence mounting medium containing 4,6-diamidino-2-phenylindole (Vector Laboratories). Images were analyzed using a Zeiss confocal laser scanning microscope. For Western blot, the primary antibodies were anti-p-IGF-IR (Tyr1161; rabbit polyclonal; 1:250; Santa Cruz Biotech), anti-IGF-IR (mouse monoclonal; 1:250; Santa Cruz) anti-p-ERK (rabbit polyclonal; 1:2,000; Cell Signaling Technology), anti-ERK (rabbit polyclonal; 1:2,000; Cell Signaling), anti-p-AKT (Ser473; rabbit polyclonal; 1:1,000; Cell Signaling), anti-AKT (rabbit polyclonal; 1:1,000; Cell Signaling) and EF1-α as loading control (mouse monoclonal; 1:1000; Millipore).

**Enzyme-linked immunosorbent analysis (ELISA):** The recombinant IGFBP7 protein used as standard was obtained from R&D (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% non-fat milk in PBS for 1 h. Following another PBS wash anti-IGFBP7 antibody (1:1000) was added and incubated overnight at 4°C.
without shaking. After another 4 times PBS wash anti-mouse secondary antibody (1:1000) was added and incubated at room temperature for 2 h with gentle shaking. The wells were washed with PBS 4 times and 100 μl Glo Substrate Reagent (R&D, DY993) was added to each well for 15 min and the reaction was stopped by adding 100 μl Stop Solution (R&D, DY994). The plate was read at 560nm using a multiplate reader (Turner Biosystems).

**Assay for Senescence Associated β-galactosidase (SA-β-gal) activity:** Cells were cultured for six days and SA-β-gal-positive cells were detected using 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 using Qiagen miRNAeasy mini kit (Qiagen). Real time PCR was performed using an ABI 7900 fast real time PCR system and Taqman gene expression assays for IGFBP7 and GAPDH according to the manufacturer’s protocol (Applied Biosystems).

**Nude mice xenograft studies:** Cells (5X10⁵) were subcutaneously implanted in the flanks of athymic nude mice. Tumor volume was measured with calipers using the formula: (Width)² × length/2. Mice were followed for 3 weeks.

**Chicken chorioallantoic membrane (CAM) assay:** Cells were seeded on the CAM surface of 9-day-old chick embryos according to established protocols (25). One week after inoculation, the neovasculature 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 HUVECs on Matrigel (Chemicon) was performed 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 using an Image analyzer (National Institutes of Health Image).

**Statistical analysis:** Data were represented as the mean ± Standard Error of Mean (S.E.M) and analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by Newman-Keuls test as a post hoc test. A P value of < 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 HCC patients by immunohistochemistry in tissue microarrays (TMAs) containing 104 HCC samples and 9 normal adjacent liver samples. Strong IGFBP7 immunostaining was detected in the 9 normal liver samples (Fig. 1A and Table S1) while weak IGFBP7 staining was observed in the HCC samples (Fig. 1A and Table S1). There was a gradual decrease in IGFBP7 expression with the stages of HCC based on the BCLC staging system (Fig. 1A and Table S1). Moreover, in each stage IGFBP7 expression was much lower in poorly differentiated grades compared to 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 value < 0.001) by Pearson’s chi-square test (Table S1).

Loss of heterozygosity 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 HCC patients, dual color fluorescence *in situ* hybridization (FISH) was performed on human HCC TMAs containing 9 normal liver samples.
and 50 HCC samples. Bacterial artificial chromosome (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 out of 50 (26%) HCC samples showed
IGFBP7 deletion. Fig 1B shows a representative normal cell in which two orange (IGFBP7) and
two green (CEP4) dots are observed. In the HCC cell, there are two 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 to 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 demonstrating downregulation of secreted
IGFBP7 protein in human HCC cell lines, compared to THLE-3 cells (Fig. 2B). IGFBP7 was
initially identified as an AEG-1-downregulated gene by Affymetrix microarray. To corroborate
this finding, we checked three 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 three AEG-1-overexpressing clones,
IGFBP7 mRNA expression was robustly downregulated compared to 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 compared to Hep-AEG-1-14 cells (Fig. 2D). These
findings were further demonstrated in matched normal liver and HCC samples from 18 patients
by immunohistochemical analysis of AEG-1 and IGFBP7 expression. In 13 out of these 18
patients very low to undetectable levels of AEG-1 expression and high level of IGFBP7
expression were detected in normal liver while 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 non-tumorigenic 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 to 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 3B). 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 Fig. 2B and 3B). IGFBP7-11 and IGFBP7-12 clones showed significantly slower growth rate when compared to Control-2 clone analyzed by standard cell viability (MTT) and colony formation assays (Fig. 3C and 3D, respectively). However, this growth inhibition was not profound in IGFBP7 overexpressing clones compared to Control-2 clone, showing only ~27% inhibition by cell viability assay and ~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 observed that the phospho-IGF-IR level was significantly downregulated, while total IGF-IR level was unchanged, in IGFBP7-11 and IGFBP7-12 clones compared to 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 ERK, was also significantly inhibited in IGFBP7-11 and IGFBP7-12 clones compared to 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 compared to several human HCC cell lines (Fig. S1). Although p-ERK level in THLE-3 cells was lower than 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 (Fig. S1).

IGFBP-7 induces senescence. As such, we evaluated induction of senescence in Control-2, IGFBP7-11 and IGFBP7-12 clones using the senescence-associated β-galactosidase (SA-β-gal) assay. There was a significant increase in the number of SA-β-gal-positive cells in IGFBP7-overexpressing clones compared to the Control-2 clone following 1 week culture (Fig. 4A and 4B). 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 to the Control-2 clone (Fig. 4C and 4D).

The in vitro growth suppressing function of IGFBP7 was extended using 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 to the tumors generated from the Control-2 clone (Fig. 5A and 5B). 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 to those from the Control-2 clone (Fig. 5C).

The in vivo growth suppression effect of IGFBP7 was significantly higher than observed using 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 chorioallantoic membrane (CAM) of 9-day old chick embryos. After one week, neovascularization was examined, photographed and quantified. Neovascularization was significantly inhibited in IGFBP7-11 and IGFBP7-12 clones, compared to the Control-2 clone (Fig. 6A and 6B). These findings were extended further by endothelial cell tube formation assays. Human umbilical vein endothelial cells (HUVEC) were treated with conditioned media (CM) from Control-2, IGFBP7-11 and IGFBP7-12 clones and endothelial cells tube formation was scored (Fig. 6C and 6D). CM from IGFBP7-11 and IGFBP7-12 clones significantly inhibited HUVEC tube formation as compared to CM from the Control-2 clone, further validating the anti-angiogenic functions of IGFBP7.

**Discussion**

Although IGFBP7 has shown tumor suppressor functions in a number of epithelial cancers, very little is known about its anti-cancer 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 has not been dissected (27). A recent report showed that IGFBP7 is required for the sensitivity of HCC cells towards interferon-based anti-cancer therapy (28). The same report described the analysis of IGFBP7 in a small population of 30 patients demonstrating a significantly better post-operative overall survival in IGFBP7-positive HCC patients compared to IGFBP7-negative patients (28). Ours is the first report with a comprehensive analysis of IGFBP7 expression in 104 HCC patients in which we demonstrate 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 HCC patients 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 to 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) (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 pro-tumorigenic genes and proteins as well as altering signaling pathways, the profound inhibition of in vivo 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 to the parental non-tumorigenic HepG3 cells (Fig. 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 as well as 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 anti-angiogenic effects of IGFBP7. As a secreted protein, IGFBP7 might also modulate the function of the immune system generating an anti-tumor 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 HCC patients.

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Cancer Research and a Blick scholar. PBF holds the Thelma Newmeyer Corman Chair in Cancer Research and is a Samuel Waxman Cancer Research Foundation (SWCRF) Investigator.

References


cancer cell line alters tumor growth by a delay in G1 and cyclin A associated apoptosis.


Figure legends

Fig. 1. IGFBP7 is downregulated in human HCC samples. A. Analysis of IGFBP7 expression in tissue microarray by immunohistochemistry. C. Fluorescence In Situ Hybridization (FISH) was performed on human HCC samples for IGFBP7 and CEP4 (probe targeting pericentromeric region of chromosome 4). Red: IGFBP7; green: CEP4.

Fig. 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. GAPDH was used as 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 three independent clones of HepG3 cells overexpressing AEG-1. D. Immunofluorescence detection of IGFBP7 protein in Hep-pc-4 (pc-4) and Hep-AEG-1-14 (AEG1-14) 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 out of 18 HCC patients. For A-C, data represents mean ± SEM of three independent experiments. *: p<0.05.

Fig. 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 represents mean ± SEM of three independent experiments. *: p<0.05. E. Western blot analysis performed in the indicated cells with the indicated antibodies. EF1α was used as loading control.

Fig. 4. IGFBP7 induces senescence. A. Photomicrograph of Control-2, IGFBP7-11 and IGFBP7-12 clones of Hep-AEG-1-14 cells stained for senescence-associated β-galactosidase (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 represents mean ± SEM of three 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. B. Graphical representation of quantification of γ-H2AX foci/cell. At least 100 cells were scored for each group. Data represents mean ± SEM of three independent experiments. *: p<0.05.

Fig. 5. Overexpression of IGFBP7 inhibits AEG-1-mediated tumorigenesis in nude mice. Subcutaneous xenografts were established in athymic nude mice using Control-2, IGFBP7-11
and IGFBP7-12 clones of Hep-AEG-1-14 cells. A. A representative photograph of tumor-bearing mice at the end of the study. B. Measurement of tumor volume at the indicate time point. Data represents mean ± SEM. *: p<0.05. C. Tumor sections were immunostained for IGFBP7, CD31 and Ki-67.

Fig. 6. IGFBP7 inhibits angiogenesis. A. Control-2, IGFBP7-11 and IGFBP7-12 clones of Hep-AEG1-14 cells were implanted in chicken chorioallantoic membrane (CAM) and neovascularization was photographed. B. Graphical representation of new blood vessel formation in CAM when the indicated cells were implanted. The numbers indicate percentage of new blood vessels arising from the existing blood vessels in naïve CAM when VEGF-treated CAM was considered as 100%. Data represents mean ± SEM. *: p<0.05. C. HUVECs were treated with conditioned media from the indicated cells and tube formation was photographed. D. Graphical representation of tube formation by HUVEC treated with conditioned media from the indicated cells. The numbers indicate percentage of tube-like structures when VEGF-treated tube formation was considered as 100%. The data represents mean ± SEM. *: p<0.05.
Chen et al. Fig. 1

A. Normal Liver

Moderate Differentiation

Poor Differentiation

Stage I

Stage II

Stage III

Stage IV

B. Normal Liver

HCC
Chen et al. Fig. 3

A. IGFBP7 mRNA (A.U.)

<table>
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B. IGFBP7 (ng/ml)

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<td>IGFBP7-11</td>
<td>0.2</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>IGFBP7-12</td>
<td>0.1</td>
<td>11</td>
<td>10</td>
</tr>
</tbody>
</table>

C. Relative no. of viable cells

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-2</td>
<td>0.4</td>
<td>0.6</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>IGFBP7-11</td>
<td>0.2</td>
<td>0.3</td>
<td>0.5</td>
<td>0.7</td>
</tr>
<tr>
<td>IGFBP7-12</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
<td>0.4</td>
</tr>
</tbody>
</table>

D. Colony no./500 cells

<table>
<thead>
<tr>
<th></th>
<th>Control-2</th>
<th>IGFBP7-11</th>
<th>IGFBP7-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-2</td>
<td>100</td>
<td>120</td>
<td>140</td>
</tr>
<tr>
<td>IGFBP7-11</td>
<td>90</td>
<td>110</td>
<td>130</td>
</tr>
<tr>
<td>IGFBP7-12</td>
<td>80</td>
<td>100</td>
<td>120</td>
</tr>
</tbody>
</table>

E. Western Blot

- p-IGF-IR
- IGF-IR
- p-Akt
- Akt
- p-ERK
- ERK
- EF1α
Chen et al. Fig. 4

A.

B.

C.

D.
Chen et al. Fig. 5

A.

Control-2       IGFBP7-11       IGFBP7-12

B.

Tumor (mm³)

Wk 1       Wk 2       Wk 3       Wk 4

C.

Control-2       IGFBP7-11       IGFBP7-12

IGFBP7

CD31

Ki-67
Chen et al. Fig. 6

A. Control-2  IGFBP7-11  IGFBP7-12

B. 

<table>
<thead>
<tr>
<th></th>
<th>Control-2</th>
<th>IGFBP7-11</th>
<th>IGFBP7-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>New blood vessels (%)</td>
<td></td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

C. Control-2  IGFBP7-11  IGFBP7-12

D. 

<table>
<thead>
<tr>
<th></th>
<th>Control-2</th>
<th>IGFBP7-11</th>
<th>IGFBP7-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube formation (%)</td>
<td></td>
<td>*</td>
<td>*</td>
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</table>
Insulin-like growth factor binding protein-7 (IGFBP7) functions as a potential tumor suppressor in hepatocellular carcinoma (HCC)

Dong Chen, Byoung Kwon Yoo, Prasanna K. Santhekadur, et al.

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