RESEARCH ARTICLE

Induction of Apoptosis by IGFBP3 Overexpression in Hepatocellular Carcinoma Cells

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Abstract

<u>Background</u>: The insulin-like growth factor (IGF) system comprises a group of proteins that play key roles in regulating cell growth, differentiation, and apoptosis in a variety of cellular systems. The aim of this study was to investigate the role of insulin-like growth factor binding protein 3 (IGFBP3) in hepatocellular carcinoma. <u>Materials and Methods</u>: Expression of IGF2, IGFBP3, and PTEN was analyzed by qRT-PCR. Lentivirus vectors were used to overexpress IGFBP3 in hepatocellular carcinoma cell (HCC) lines. The effect of IGFBP3 on proliferation was investigated by MTT and colony formation assays. <u>Results</u>: Expression of IGF2, IGFBP3, and PTEN in several HCC cell lines was lower than in normal cell lines. After 5-aza-2'-deoxycytidine/trichostatin A treatment, significant demethylation of the promoter region of IGFBP3 was observed in HCC cells. Overexpression of IGFBP3 induced apoptosis and reduced colony formation in HUH7 cells. <u>Conclusions</u>: Expression of IGF2, IGFBP3, and PTEN in several HCC cell lines was lower than in normal cell lines. After 5-aza-2'-deoxycytidine/ trichostatin A treatment, significant demethylation of the promoter region of IGFBP3 was observed in HCC cells. Overexpression of IGFBP3 induced apoptosis and reduced colony formation in HUH7 cells.

Keywords: Hepatocellular carcinoma - IGF signaling pathway - IGFBP3 - proliferation

Introduction

Human hepatocellular carcinoma (HCC) is one of the most common solid tumors worldwide; it is the third most common cause of cancer-related mortality. Although much evidence has been collected about the heterogenicity of HCC, including chromosomal aberrations, gene mutations, epigenetic alterations, and activation of complex signaling pathways, the molecular mechanism of pathogenesis remains unclear.

Formation of HCC is usually sporadic, but an increased incidence of HCC is associated with familial disease syndromes such as familial adenomatous polyposis (FAP) and Beckwith-Wiedemann syndrome (BWS) (Buendia, 2002). In sporadic cases of HCC, loss of imprinting (LOI) or loss of heterozygosity (LOH) at the IGF2/H19 locus (Albrecht et al, 1994; Rainier et al., 1995) and biallelic inactivation of the APC gene (Oda et al., 1996) has been observed. In addition to LOH in the 11p15 region, there are more variations in copy numbers of chromosomes 1q, 8q, 17q, and 20q in HCC compared to normal cells. Furthermore, LOH is frequently detected at 1p and by trisomy of chromosome 2 in HCC (Kraus et al., 1996; Weber et al., 2000).

The IGF signaling pathway plays an important role in the normal development and growth of cells. However, development of tumors often lead to elevated expression of insulin-like growth factor 2 (IGF2) and thus to activation of the IGF signaling pathway (Foulstone et al., 2005). Activation of the mitogen-activated protein kinase MAPK is responsible for increased expression of the IGF gene (Kaneda and Feinberg, 2005). An inhibitor of this signaling pathway is insulin-like growth factor binding protein 3 (IGFBP3). IGFBP3 binds to IGF2 and reduces its bioavailability. IGFBP3 also suppresses another, IGF-independent, pathway; suppression of this pathway leads to apoptosis of tumor cells and thus prevents the growth of tumor cells (Grimberg and Cohen, 2000). Several studies have reported a role for the IGF signaling system in HCC. For example, increased levels of IGF2 and reduced expression of IGFBP3 and hypermethylation of its promoter were found in human HCC (Hanafusa et al., 2002; Breuhahn et al., 2006; Tang et al., 2006). In 2011, Rehem and El-Shikh reported that serum IGF-II levels can be used as a serological marker to discriminate HCC from cirrhosis, and IGFBP3 levels in patients with HCC are significantly lower than in healthy people and patients with liver cirrhosis (Rehem and El-Shikh, 2011). Thus,

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the IGF signaling pathway appears to play an important role in liver tumor development.

In the present study, we investigated the expression and methylation status of genes in the IGF signaling pathway in several HCC cell lines. We then further investigated the role of a key protein, IGFBP3, in the progression of HCC.

Materials and Methods

Cell lines and 5-aza-2'-deoxycytidine/trichostatin A treatment

HCC cell lines HUH7 and Hep1B were kept in our laboratory and cultured in RPMI 1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal calf serum (FCS; Gibco, Grand Island, NY, USA). Hep3B and HepG2 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Eagle's Minimum Essential Medium (MEM; Gibco, Grand Island, NY, USA) supplemented with 10% FCS. The cell lines were maintained in a humidified chamber with 5% CO_2 at 37°C. HUH7 and Hep3B were treated with 0.5 μM 5-aza-2'-deoxycytidine (5-Aza; Sigma, St. Louis, MO, USA) and 0.1 μM trichostatin A (TSA; Sigma). HepG2 and Hep1B were treated with 1.25μM 5-Aza and 0.5 μM TSA. The medium with demethylation agents was changed every 24h. For the 3 and 5 d treatments, cells were first treated for 24h with 5-Aza, then for 48 h with 5-Aza and TSA.

Lentivirus vectors for IGFBP3 and transfection of HCC cells

Human IGFBP3 cDNA was subcloned into plasmid pLenti6/V5. IGFBP3 and empty control recombinant lentivirus (Lv. IGFBP3 and Lv. Empty) were generated by Lipofectamine 2000 transfection reagent, according to the manufacturer's instructions. IGFBP3-pLenti6/V5 expression plasmid or pLenti6/v5 plasmid were cotransfected together with packaging vectors into 293T cells. Forty-eight hours after cotransfection, the viral supernatant was collected and the titer was determined. HCC cells were transiently transfected with control parent lentivirus or lentivirus expressing IGFBP3 (multiplicity of infection of 5-50). After infection for 4 h, the medium was changed and the HCC cells were further cultured in medium with 10% fetal bovine serum (FBS).

RNA extraction and quantitative real-time PCR

Total RNA was extracted with the TRI-Reagent kit (Molecular Research Center, Cincinnati, OH, USA)

according to the manufacturer's instructions. PCR primers used to amplify the region and PCR conditions are shown in Table 1. cDNA (40ng), 1 μM primer, and 1× iTaq SYBR Green Supermix with ROX (Bio-Rad) was used. The PCR program consisted of an initial denaturation of 2 min at 94°C followed by 40 cycles of 15 sec denaturation at 95°C, 15 sec annealing at 55°C, and 20 sec at 68°C. To confirm the specificity of the primers, a melting curve analysis was added at the end of the reaction process. All quantitative real-time PCR (qRT-PCR) was performed with the Mastercycler EP Realplex (Eppendorf AG, Hamburg, Germany).

MTT assay

Cell Proliferation Kit I (Roche Diagnostics) was used according to the manufacturer's protocol to determine the viability of cells. Viable cells metabolize the MTT compound to blue formazan dye; thus, the metabolic activity of cells correlates with the intensity of blue coloration. Approximately 5, 000 cells were added to each well of a 96-well plate and incubated for 24h. The test was then performed by adding 10μl of MTT labeling reagent to the cells and stopping the reaction after 4h with 100μl of solubization solution; afterwards, the cells were lysed. After the reaction had been stopped, absorbance was measured by photometric measurement at 595nm in a GENios Tecan microplate reader (Crailsheim). Measurements were made every 24h for a total test duration of 96-120h.

Colony formation assay

Colony formation assays with stably transfected cells were used to measure cell proliferation. Approximately 5, 000 cells were added to each well of a 6-well plate and cultured with selection medium. The antibiotic G418 (200mg/ml) or puromycin (1mg/ml) was used. After 14d, the medium was removed and the cells were washed with phosphate-buffered saline. The colonies were fixed with methanol and then stained with crystal violet for 5 min. The plates were gently washed and dried before colony evaluation with a microscope. Colonies that contained >30 cells were counted. Data are representative of three independent experiments.

Statistical analysis

All statistical analyses were carried out with SPSS statistical software package 13.0 (SPSS Inc., Chicago, IL, USA). Data were analyzed by ANOVA. p values<0.05 were considered statistically significant.

Table 1. PCR Primers and Conditions

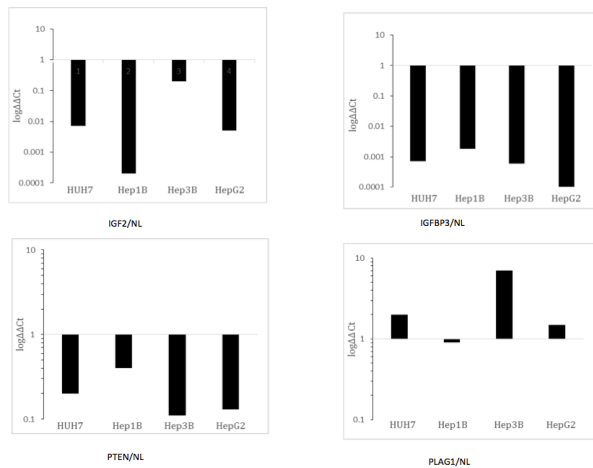|	Primer	Annealing temp	Product size
IGFBP3	5´-GTC CAA GCG GGA GAC AGA ATA T-3´ 5´-CCT GGG ACT CAG CAC ATT GA-3´	55°C	93 bp
PTEN	5´-ACC AGT GGC ACT GTT GTT TCA C-3´ 5´-TCA CCT TTA GCT GGC AGA CCA-3´	55°C	101 bp
IGF2	5´-CCT CCG ACC GTG CTT CC-3´ 5´-GGT GGA CTG CTT CCA GGT GT-3´	55°C	81 bp
PLAG1	5´-ACA AGT GCA TAC AAC AAG ACT GCA-3´ 5´-CAG GAG AAT GAG TAG CCA TGT GC-3´	55°C	81 bp

Figure 1. Expression of Proteins in the IGF Signaling System in HCC Cell Lines. RT-PCR analysis of IGF2, IGFBP3, PTEN, and PLAG1 expression in HUH7, Hep1B, Hep3B, and HepG2 cell lines compared with normal liver (NL) cells
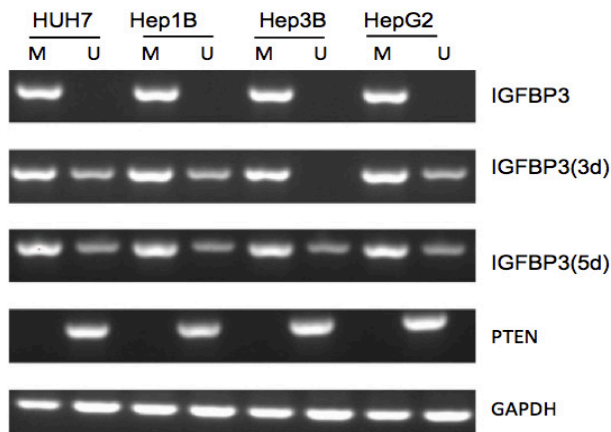

Figure 2. Demethylation of IGFBP3 and PTEN in 5-Aza/TSA–treated Cell Lines After 3 and 5 d. M, methylated; U, unmethylated

Figure 3. mRNA Expression of IGFBP3 (a) and PTEN (b) in Cell Lines After 3 and 5 d of 5-Aza/TSA treatment or without treatment. *Indicates treatment mean that is significantly different compared to the no-treatment group mean ($p<0.05$)

Figure 4. Expression of IGFBP3 in HCC Cells at 48 and 72h After Transfection. HCC cells were transiently transfected with control parent lentivirus or lentivirus expressing IGFBP3. mRNA expression of IGFBP3 in HCC cells was verified by RT-PCR

Results

Alteration in expression of genes in the IGF signaling pathway in HCC

Expression of IGF2, IGFBP3, and PTEN in HUH7, Hep1B, Hep3B, and HepG2 cells was analyzed by qRT-PCR. As shown in Figure 1, a significant decrease in the expression of IGF2 was observed in HCC cell lines compared to normal liver cells, even though the HCC cell lines had an LOH status. The lowest value of relative expression was 0.0002, which occurred in Hep1B cells. Expression of IGFBP3 and PTEN was also suppressed significantly in HCC cells compared to control cells. The lowest relative expression of IGFBP3 was 0.00001 in HepG2 cells, and PTEN relative expression was 0.12 in Hep3B cells. PLAG1 was overexpressed in HUH7, Hep3B, and HepG2 cells. The highest relative expression of PLAG1 was 5.9 in Hep3B cells. Slightly decreased expression of PLAG1 was observed in Hep1B cells, with a relative expression of 0.9. Because changes in expression could correlate with methylation status, we next investigated changes in the methylation of IGFBP3 and PTEN genes in HCC cell lines.
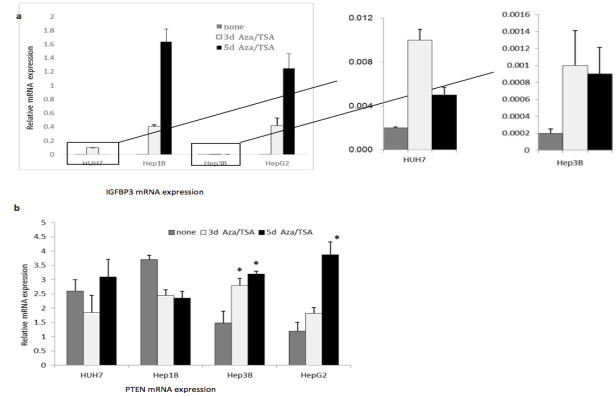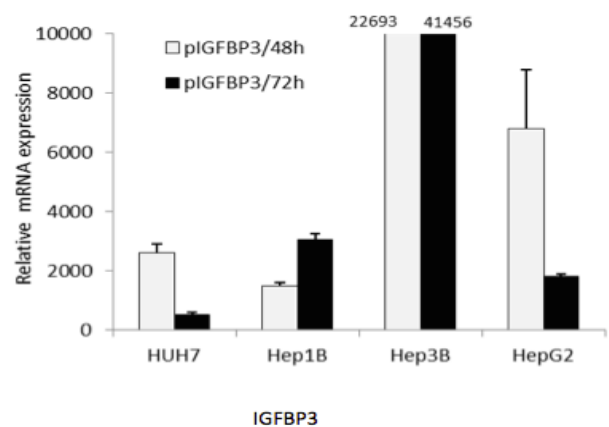
Methylation affects expression of IGFBP3 and PTEN in HCC

After 5-Aza/TSA treatment, significant demethylation of the promoter region of IGFBP3 was observed in HCC cell lines (Figure 2), and expression of IGFBP3 increased markedly after 3 and 5 d in all four cell lines ($p<0.05$; Figure 3a). The PTEN gene was unmethylated in all cell lines (Figure 2). A significant increase in PTEN expression was observed in HepG2 and Hep3B cells (Figure 3b).

Effect of IGFBP3 overexpression on proliferation of HCC cells

The HCC cells were transiently transfected with control parent lentivirus or lentivirus expressing IGFBP3. After 72h, mRNA expression of IGFBP3 in HCC cells was verified by RT-PCR. We observed significant overexpression of IGFBP3 in cells transfected with the lentivirus expressing IGFBP3 (Figure 4).

Next, the MTT assay was performed by standard methods in HCC cells. The proliferation rate of HUH7 cells transfected with lentivirus expressing IGFBP3 was markedly reduced compared to HUH7 cells transfected with the control parent lentivirus. Thus, overexpression
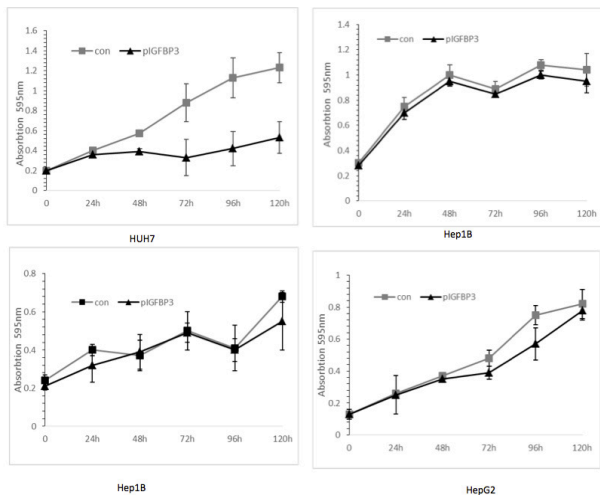
Figure 5. Effect of IGFBP3 on Proliferation of HCC Cells. Proliferation of HUH7, Hep1B, Hep3B, and HepG2 cells after transfection with lentivirus expressing IGFBP3 or control parent lentivirus was determined by MTT-based assay

Figure 6. Effect of IGFBP3 Expression on Colony Formation. a) Colony formation assay staining results for HUH7 cells transfected with lentivirus expressing IGFBP3 or control parent lentivirus. **b)** Number of cell colonies. **c)** Relative mRNA expression of IGFBP3 in HUH7 cells transfected with lentivirus expressing IGFBP3 or control parent lentivirus. **d)** mRNA expression of IGFBP3 in HUH7 cells was verified by RT-PCR. *Indicates mean that is significantly different between HUH7 cells transfected with lentivirus expressing IGFBP3 and HUH7 cells transfected with control parent lentivirus ($p<0.05$)

of IGFBP3 had a direct influence on the growth behavior of HUH7. Unexpectedly, as shown in Figure 5, cell proliferation in the other cell lines was not affected.

The HUH7 cell line was selected for use in the following *in vitro* experiments: we first transfected HUH7 cells with lentivirus expressing IGFBP3 or the lentivirus control. After selection with neomycin, stable expression of IGFBP3 in HUH7 cells was verified by RT-PCR (Figure 6). Then, for the colony formation assay, the stably transfected HUH7 cells were dispersed (5, 000 cells/well) and cultured in selection medium. Subsequently, the colonies were stained and counted. A significant difference was observed in the number of colonies formed by HUH7 cells transfected with lentivirus expressing IGFBP3 versus cells infected with control virus (Figure 6b). Overexpression of IGFBP3 reduced the growth behavior of HUH7 cells.

Discussion

Some growth defects, such as BWS, are associated with overexpression of IGF2 (Steenman et al., 2000). People with BWS and related syndromes have a higher incidence of HCC. Moreover, changes in the IGF2/H19 locus have been found in sporadic tumors (Albrecht et al., 1994). People with Wilms tumors and tumors that occur later in life, such as colon cancer, have also been found to have defects at the IGF2/H19 locus. Overexpression of IGF2 in HCC is regulated by the oncogene PLAG1. IGF2 expression also stimulates PLAG1 in neoplastic tumors of the salivary glands (Voz et al., 2000). Thus, both genetic and epigenetic mechanisms may be involved in the regulation of IGF2 in HCC.

Since epigenetic regulation of the IGF signaling pathway plays an important role in HCC pathogenesis, we analyzed the methylation status of both PTEN and IGFBP3. PTEN is a negative regulator of the IGF signaling pathway, which was analyzed for epigenetic decommissioning and can lead to a reduction in phosphatidylinositol-3, 4, 5-triphosphate (PIP3), an important neurotransmitter that
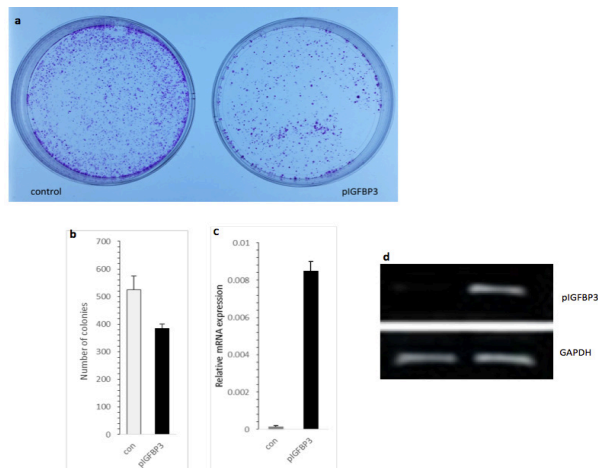
is involved in the regulation of cell growth (Maehama and Dixon, 1998). Decreased expression of PTEN activates growth-promoting signaling pathways and inhibits apoptosis, thus promoting the development of tumors. Deletions, protein instability, and epigenetic shutdown of PTEN have been found in various tumors. For example, PTEN expression is decreased in squamous tumors of the stomach (Shin et al., 2000). Similarly, loss of PTEN expression was detected in HCC (Ma et al., 2005). Our analysis of methylation of the PTEN promoter revealed that there was no promoter methylation in HCC cell lines. After 5-Aza/TSA treatment, expression of PTEN was significantly increased in HepG2 and Hep3B cells. Demethylation of CpGs could activate the expression of PTEN and inactivate the growth-promoting IGF signaling pathway.

Some studies have found that IGFBP3 is frequently methylated in kidney tumors, HCC, breast cancer, and gastric and colon carcinomas, and IGFBP3 methylation is an important factor in tumorigenesis (Ibanez de Caceres et al., 2006; Tomii et al., 2007). Prostate cancer risk is correlated with a single nucleotide polymorphism in IGFBP3 gene (Ye et al., 2013). In addition, IGFBP3 exerts an effect in cancer and displays functional interplay between the tumor microenvironment and IGFBP3 (Natsuizaka et al., 2014). CpGs were identified in our investigation of the IGFBP3 promoter in HCC cell lines. The promoter region has binding sites for the tumor suppressor p53. Thus, methylation of this region inhibits p53-mediated suppression by suppressing IGFBP3 expression. Subsequent expression analysis should be conducted to confirm the relevance of methylation. Consequently, there must be next to the decommissioning or other epigenetic mechanisms of IGFBP3 gene regulation. Oncogene E7 of human papillomavirus, which causes cancer of the cervis and uterus, is associated with

decreased expression of IGFBP3 (Mannhardt et al., 2000). In addition to decommissioning, epigenetic mechanisms may also play a role in reducing IGFBP3 expression in HCC. Up-regulation of IGFBP3 may play a role in the hepatotoxicity induced by doxorubicin in rat models (Ailin et al., 2013). In our study, methylation of the IGFBP3 promoter was found in all four HCC cell lines; after 5-Aza/TSA treatment, all cells showed significantly increased expression of IGFBP3. Thus, reactivation of demethylation of DNA in the promoter of IGFBP3 gene by 5-Aza/TSA treatment led to a reduction in cell growth in HCC cell lines. The decreased growth rate of HUH7 cells was due to overexpression of IGFBP3, and there was a significant reduction in the number of colonies. Protein levels of IGFBP3 were consistent with mRNA expression of IGFBP3; therefore, the activity of IGFBP3 alone was regulated at the transcriptional level (Chang et al., 2004). Increased IGFBP3 expression after 5-Aza/TSA treatment leads to demethylation of the promoter region in kidney tumors (Ibanez de Caceres et al., 2006), as we observed for HCC. Reactivation of IGFBP3 decreased cell proliferation and promoted apoptosis; therefore, it could constitute a potential strategy for reducing tumor growth.

In summary, our findings suggest that demethylation and overexpression of IGFBP3 leads to a reduction in cell proliferation. Epigenetic changes in the IGF signaling pathway may contribute to the emergence of HCC. Thus, demethylating agents that lead to the resumption of normal expression of IGFBP3, which negatively regulates the IGF signaling pathway, might be of use in the treatment of HCC.

Acknowledgements

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