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Expression of albumin, IGF-1, IGFBP-3 in tumor tissues and adjacent non-tumor tissues of hepatocellular carcinoma patients with cirrhosis

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Abstract

AIM: To explore the expression of albumin (ALB), insulin-like growth factor (IGF)-1, and insulin-like growth factor binding protein (IGFBP)-3 in tumor tissues and adjacent non-tumor tissues of hepatocellular carcinoma (HCC) patients with cirrhosis.

METHODS: Twenty-four HCC patients with cirrhosis who underwent hepatectomy were studied. ALB mRNA, IGF-1 mRNA, and IGFBP-3 mRNA in liver tissues (including tumor tissues and adjacent non-tumor tissues) were detected by reverse transcriptase-polymerase chain reaction (RT-PCR). Liver Ki67 immunohistochemistry staining was studied. At the same time, 12 patients with cholelithiasis or liver angioma who underwent operation were segregated as normal control.

RESULTS: In HCC patients with cirrhosis, hepatic ALB mRNA, IGF-1 mRNA, and IGFBP-3 mRNA of tumor tissues or adjacent non-tumor tissues were lower than the normal liver tissues, while in tumor tissues, hepatic ALB mRNA and IGFBP-3 mRNA were lower, hepatic IGF-1 mRNA was higher than in adjacent non-tumor tissues. Liver Ki67 labeling index (Ki67 LI) in tumor tissues or adjacent non-tumor tissues were higher than that in the normal liver tissues, while in tumor tissues it was higher than that in adjacent non-tumor tissues.

CONCLUSION: Imbalance of IGF-1 and IGFBP-3 may play a role in hepatocarcinogenesis and tumor development of liver cirrhosis patients.

Key words: Hepatocellular carcinoma; Insulin growth factor-1; Insulin-like growth factor binding protein-3

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INTRODUCTION

Extensive research on insulin-like growth factors (IGFs) during the last decade has greatly advanced our understanding of these peptides that are not only the endocrine mediators of growth hormone (GH)-induced metabolic and anabolic actions but also polypeptides that act in a paracrine and autocrine manner to regulate cell growth, differentiation, apoptosis, and transformation[1]. In biological fluids, IGFs are complexed with specific binding proteins (IGFBPs)[2-4]. The functions of IGFBPs are to modulate the biologic activity of IGFs. Imbalance of these diverse processes may preferentially favor uncontrolled cell proliferation leading to malignant transformation. Recent evidence from epidemiologic studies has confirmed an association between serum levels of IGFs and several malignancies at the population level and has resulted in a resurgence of scientific interest in this field.

IGF-1 and IGF-2 are single chain polypeptides, which have 62% homology with proinsulin. Serum concentrations of IGF-2 are higher than IGF-1 (400-600 ng/mL *vs* 100-200 ng/mL) in humans of all ages, are relatively stable after puberty and are not regulated by GH. IGF-2 has proliferative and antiapoptotic actions similar to IGF-1 since its effects are exerted by the IGF-1R[5]. However, IGF-2 plays a fundamental role in embryonic and fetal growth, whereas its role in the postnatal period is less important as it is substituted by IGF-1.

The liver is the central organ of the endocrine GH/IGF-1 axis. GH is secreted by the somatotrophic cells of the anterior pituitary, transported in the circulation by the high affinity growth hormone binding protein and acts through the hepatic GH receptor to regulate the production of the potent mitogenic growth factor IGF-1. The availability of IGF-1 to its tissue receptors is further regulated by the high affinity IGFBPs (IGFBP-1 to -6) of which the liver is also a significant source, with IGFBP-3 being most abundant[6]. Epidemiologic studies have found high circulating IGF-1 and low IGFBP-3 levels to be associated with an increased risk of developing breast, endometrial, lung, colorectal, and prostate cancer. IGFBP-3 was reported to be a growth suppressor in variable pathways[7,8].

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, with estimated 467 000 new cases per year[9]. HCC is one of the most common malignant tumors in the world. Since the 1990s HCC has become the second killer in various cancers in China. Most HCC cases arise in HBV and liver cirrhosis. In carcinogenesis induced by viral infection, the frequency of gene mutation is considered to increase as the mitotic activity of cells gets greater. Under conditions that increase the proliferative activity of hepatocytes such as chronic hepatitis and liver cirrhosis, the probability of mutation increases, resulting in increased occurrence of HCC.

Albumin (ALB) is the major protein produced in the liver, and its primary functions include transportation of a variety of substances in the blood and maintenance of osmotic pressure. In normal adults, ALB constitutes more than 65% of the total plasma protein content. The plasma concentration of ALB reflects its synthesis, degradation, and distribution. Thus, the expression of hepatic ALB mRNA was more useful in reflecting the degree of liver injury or liver functional reserve than serum ALB.

In this study, we used the reverse transcriptase-polymerase chain reaction (RT-PCR) technique to detect ALB mRNA, IGF-1 mRNA, IGFBP-3 mRNA in tumor tissues and adjacent non-tumor tissues of HCC patients with cirrhosis. At the same time, we have immunochemically detected Ki67 antigen which appears in all phases of the cell cycle of proliferating cells but is negative in G_0[10] on paraffinized section by microwave antigen retrieval method and compared the proliferative activity between adjacent non-tumor tissue as well as tumor tissues. We explored the roles of IGF-1 and IGFBP-3 in hepatocarcinogenesis and tumor development of liver cirrhosis patients.

MATERIALS AND METHODS

Patients and tissue samples

Of the patients at our department who underwent curative resection for HCC with liver cirrhosis between September 2002 and June 2003. Curative resection was defined as complete resection of all macroscopically detectable tumor with histological tumor clearance (the entire tumor mass was included in the surgical specimen without exposure of tumor cells on the cut edge). At the time of study entry, the inclusion criteria had no evidence of endocrine disease. Twenty-four HCC patients with liver cirrhosis who underwent hepatectomy were studied. At the same time, 12 patients with cholelithiasis or liver angioma who underwent operation were segregated as normal control and these patients had no liver cirrhosis and endocrine disease.

Liver samples (including HCCs and adjacent non-tumor tissues) were excised at the operation, immediately freeze clamped with liquid nitrogen, and stored at -70 ℃ for analysis of ALB mRNA, IGF-1 mRNA, and IGFBP-3 mRNA. For histological examination, some liver samples were fixed in 10% neutral-buffered formalin, embedded in paraffin.

Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was performed to measure the expression levels of ALB mRNA, IGF-1 mRNA, and IGFBP-3 mRNA in liver tissues. The primers used were deduced from the cDNA sequence. The sequences of the primers for ALB sense and antisense were 5'-CCCAAGTGTCAACTCCAACT-3' (sense) and 5'-GCAGGTCTCCTTATCGTCAG-3' (antisense), a 456 bp long fragment was amplified. The sequences of the primers for IGF-1 sense and antisense were 5'-AGCAGTCTTCCAACCCAATTA-3' (sense) and 5'-CACGGACAGAGCGAGCTG-3' (antisense), a 355 bp long fragment was amplified. The sequences of the primers for IGFBP-3 sense and antisense were 5'-ATATGGT-CCCTGCCGTAGA-3' (sense) and 5'-AAATCGAGG-CTGTAGCCAG-3' (antisense), a 345 bp long fragment was amplified. The sequences of the primers for β-actin sense and antisense were 5'-ACTCTTCCAGCCTTCCTTCCT-3' (sense) and 5'-TCACCTTCACCGTTCCAGTTT-3' (antisense), a 513 bp long fragment was amplified.

Total RNA was extracted from frozen liver specimens by the guanidinium isothiocyanate method. The RNA was quantified and checked for purity by spectrophotometry at 260 and 280 nm. Aliquots of total RNA were reverse transcribed using SperSriptII Reverse Transcriptase (Invitrogen Corp.) and subsequently amplified by PCR using the Taq DNA polymerase (Promega Corp.).

The PCR was carried out in 25 mL of reaction mixture containing 0.5 μL cDNA template, 2.5 μL 10×PCR-Buffer, 1.5 μL 25 mmol/L MgCl$_2$, 0.5 μL 10 mmol/L dNTPs, 0.5 μL 10 μmol/L ALB or IGF-1 or IGFBP-3 primers, 0.15 μL 10 μmol/L β-actin primers, 0.5 μL 5 IU/μL Taq DNA polymerase. The mixture was heated for 5 min at 94 ℃ for initial DNA denaturation, followed by 30 cycles of denaturation (at 94 ℃ for 45 s), annealing (ALB at 50 ℃ for 45 s, IGF-1 at 48 ℃ for 45 s, IGFBP-3 at 55 ℃ for 45 s), polymerization (at 72 ℃ for 1 min) and then a final extension of 10 min at 72 ℃. PCR reactions were stored frozen until analysis by agarose gel electrophoresis.

PCR reactions were electrophoresed on 1.5% agarose gel, stained with ethidium bromide and quantitated using the interactive build analysis system. The band intensity of the ALB or IGF-1 or IGFBP-3 was compared with the band intensity of the β-actin, and the amount of ALB mRNA, IGF-1 mRNA, and IGFBP-3 mRNA was estimated.

Immunohistochemistry

Two-step immunohistochemical staining technique was used. Main reagent included rabbit polyclonal antibody Ki67 Ab-4 (Neomarkers Corp.) and PV-6000 PicTure™ Kits. Briefly, sections were deparaffinized, rehydrated, and then immersed in 0.1 mol/L citric acid buffer (pH 6.0) and boiled for 5-10 min in a microwave oven. The slides were then rinsed gently with PBS at pH 7.2-7.4, and treated with 0.3% hydrogen peroxide in absolute methanol for 1 h at room temperature to remove endogenous peroxidase. The sections were then incubated with the primary antibody Ki67 Ab-4 (1:200 dilution) for 30 min at 37 ℃. After rinsing thrice with PBS, each for 2 min, the sections were incubated with PV-6000 for 30 min at 37 ℃. They were then rinsed thrice with PBS for 2 min, and visualized with DAB. Finally, the sections were then counterstained with hematoxylin.

On each slide, Ki67-positive nuclei were evaluated by

means of light microscopy at 400 magnification. A minimum of 1 000 cells, evaluated through a minimum of 200 cells per field in five different fields. The Ki67 labeling index (Ki67 LI) is the number (%) of positive cells.

Statistical analysis

Data are expressed as mean±SE. The statistical software SPSS 10.0 was used. Statistical significance was set at $P<0.05$.

RESULTS

In HCC patients with cirrhosis, hepatic ALB mRNA, IGF-1 mRNA, IGFBP-3 mRNA of tumor tissues or adjacent non-tumor tissues were lower than the normal liver tissues, while in tumor tissues, hepatic ALB mRNA and IGFBP-3 mRNA were lower, hepatic IGF-1 mRNA was higher than in adjacent non-tumor tissues. Liver Ki67 LI in tumor tissues was higher than that in adjacent non-tumor tissues, while in adjacent non-tumor tissues it was higher than that in the normal liver tissues (Table 1).

Table 1 Comparison of hepatic ALB mRNA, IGF-1 mRNA, IGFBP-3 mRNA and liver Ki67 LI

	n	ALB mRNA	IGF-1 mRNA	I GFBP-3 mRNA	Liver Ki67 LI (%)
Normal liver tissues	12	0.69±0.05	0.95±0.02	2.02±0.04	0±0
Tumor tissues	24	0.38±0.01[a]	0.76±0.03[a]	0.45±0.13[a]	17.3±5.9[a]
Adjacent non-tumor tissues	24	0.50±0.05[a,c]	0.43±0.06[a,c]	0.72±0.17[a,c]	0.2±0.1[a,c]

[a]$P<0.05$, tumor tissues or adjacent non-tumor tissues vs normal liver tissues; [c]$P<0.05$, adjacent non-tumor tissues vs tumor tissues.

DISCUSSION

The liver is probably the major source of circulating IGF-1 with GH confirmed as the dominant regulator of IGF-1 gene expression and serum levels in human disease[11-13]. IGF-1 is an important anabolic polypeptide with various effects. IGF-1 synthesis is disturbed in liver cirrhosis and reflects the severity of the clinical stage. It represents a good marker of hepatic function. The etiology of cirrhosis does not seem to influence its levels[14]. Serum IGFBP-3 proves to be a better marker for the hepatic synthetic capacity than serum ALB or cholinesterase[15]. Serum IGF-1, IGF-2, and IGFBP-3 may provide a new dimension in the assessment of liver dysfunction. Combined detection of serum IGF-1, IGF-2, and IGFBP-3 with Child-Pugh score is more effective in predicting prognosis than Child-Pugh score alone.

Giovannucci presented a model integrating nutrition, insulin and IGF-1 physiology ("bioactive" IGF-1), and carcinogenesis based on the following: (1) insulin and the IGF-1 axis function in an integrated fashion to promote cell growth and survival; (2) chronic exposure to these growth properties enhances carcinogenesis; and (3) factors that influence bioactive IGF-1 will affect cancer risk. The model presented here summarizes the data that chronic exposure to high levels of insulin and IGF-1 may mediate many of the risk factors for some cancers that are high in

Western populations. This hypothesis may help explain some of the epidemiologic patterns observed for these cancers, both from a cross-national perspective and within populations[16]. In HCC, HBV stimulates IGF-1R expression[17] and enhances IGF-2 gene expression[18]. Hepatitis C virus has also been related to increased IGF-2 transcription in HCC[19]. HCC is associated with a higher IGF-1:IGFBP-3 ratio than that found in patients with liver cirrhosis and a similar degree of liver failure[20].

IGF-1 and IGFBP-3 play a crucial role in the regulation of growth, cellular proliferation and transformation, and apoptosis. The local tissue expression of IGF-1 and IGFBP-3 has been associated with tumor grade, pathologic stage, and disease progression for patients with various malignancies. Epidemiologic studies have found high circulating IGF-1 and low IGFBP-3 levels to be associated with an increased risk of developing breast, endometrial, lung, colorectal, and prostate cancer. IGFBP-3 was reported to be a growth suppressor in variable pathways[7,8]. In the IGF receptor dependent pathway, IGFBP-3 binds to IGF-1 and 2 and suppresses its growth signal. In the IGF receptor independent pathways, the IGFBP-3 mediates wide varieties of growth suppression signal.

This study showed hepatic IGF-1 mRNA, IGFBP-3 mRNA of tumor tissues or adjacent non-tumor tissues were lower than the normal liver tissues, while in tumor tissues, hepatic IGFBP-3 mRNA was lower but hepatic IGF-1 mRNA was higher than in adjacent non-tumor tissues. Thus, imbalance of IGF-1 and IGFBP-3 are thought to be important in hepatocarcinogenesis and tumor development of liver cirrhosis patients. IGFBP-3 mRNA which decreased in local tissues may take part in important roles in carcinogenesis. As the IGFBP-3 is produced mainly in the liver, IGFBP-3 may play an important role in the carcinogenesis of HCC. IGFBP-3 functions as a general mediator of growth inhibitory and apoptosis-inducing pathways.

IGFBP-3 is the most abundant IGFBP in human serum and has been shown to be a growth inhibitory, apoptosis-inducing molecule, capable of acting via IGF-dependent and IGF-independent mechanisms. Over the last decade, several clinical studies have proposed that individuals with IGFBP-3 levels in the upper normal range may have a decreased risk for certain common cancers. This includes evidence of a protective effect against breast, prostate, colorectal, and lung cancer. In addition, a series of in vitro studies and animal experiments point towards an important role for IGFBP-3 in the regulation of cell growth and apoptosis. Epidemiologic and experimental evidence suggesting a role for IGFBP-3 as an anti-cancer molecule[21]. At the intracellular level this may either involve modulation of the bcl-2/bax ratio[22] or the p53 protein[23]. Kuemmerle et al., reported that endogenous IGFBP-3 directly inhibits proliferation of human intestinal smooth muscle cells by activation of TGF-betaRI and Smad 2, an effect which is independent of its effect on IGF-1 stimulated growth[24]. Interestingly, IGFBP-3 has been localized in the nucleus, implying a more direct transcriptional regulatory role, but it remains largely unknown how extracellular IGFBP-3 enters the cell[25].

The liver has the unique capacity to regulate its growth

and mass both in humans and in animals. This property is particularly remarkable because hepatocytes are cells which in their normal state rarely divide. However, their proliferative capacity and the ability of the liver to adapt to variable metabolic demands are not lost. To assess the proliferating cells, we have immunochemically detected Ki-67 nuclear antigen. Characterization of the Ki-67 antibody revealed an interesting staining pattern. The antibody was reactive with a nuclear structure present exclusively in proliferating cells. A detailed cell cycle analysis revealed that the antigen was present in the nuclei of cells in the G_1, S, and G_2 phases of the cell division cycle as well as in mitosis. Quiescent or resting cells in the G_0 phase did not express the Ki-67 antigen. Because the Ki-67 antigen was present in all proliferating cells (normal and tumor cells), it soon became evident that the presence of this structure is an excellent operational marker to determine the growth fraction of a given cell population. For this reason, antibodies against the Ki-67 protein were increasingly used as diagnostic tools in cell proliferation.

ALB is a ubiquitous protein that is synthesized only by hepatocytes. ALB is a polypeptide chain of 580 amino acids that is produced by hepatocytes[26]. The number of ALB molecules produced by a single hepatocyte has been estimated to be -40 000[27]. The expression of ALB gene is reduced in various liver diseases and the degree of reduction in the hepatic ALB mRNA level is generally correlated with the severity of the disease[28]. This study showed that liver Ki67 LI in tumor tissues was higher than that in adjacent non-tumor tissues, which was higher than that in normal liver tissues, but hepatic ALB mRNA in tumor tissues was lower than that in adjacent non-tumor tissues, which was higher than that in normal liver tissues. It represented that proliferating hepatocytes or tumor tissues had less function.

The IGF system performs a fundamental role in the regulation of cellular proliferation, differentiation, and apoptosis. Disruptions in the balance of IGF system components leading to excessive proliferation and survival signals have been implicated in the development of different tumor types. This study represented the imbalance of IGF-1 and IGFBP-3 which are thought to be important in hepatocarcinogenesis and tumor development of liver cirrhosis patients. Epidemiologic evidence indicates that increased levels of IGF-I, reduced levels of IGFBP-3 or an increased ratio of IGF-1 to IGFBP-3 in the circulation are associated with an increased risk for the development of several common cancers, including those of the breast, prostate, lung, and colon. The results of preclinical studies indicate that a diversity of interventions which antagonize IGF-1R signaling or augment IGFBP-3 function inhibit tumor cell growth in models of human cancers. A more comprehensive understanding of the interplay between cellular targets of the IGF system and antineoplastic agents will facilitate the development of novel strategies for the prevention and treatment of cancer[29]. Thus, the GH/IGF-I axis may be a target for cancer prevention and treatment.

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