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Insulin-Like Growth Factor-II: Molecular-Targeted Therapy for Hepatocellular Carcinoma

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1. Introduction

Hepatocellular carcinoma (HCC) is the third deadliest and 5th most common cancer world-wide. It ranks the second in China among all malignancies, and its mortality is almost equal to its morbidity [1-3]. Carcinogenesis of HCC is a multi-factor, multi-step and complex process, which is associated with a background of chronic and persistent infection of hepatitis B virus (HBV) or hepatitis C virus (HCV) [4-6]. Their infections along with alcohol and aflatoxin B1 intake are widely recognized etiological agents in HCC [7, 8]. Multiple genetic alterations, including the activation of oncogenes and inactivation of tumor suppressor genes, are required for malignancy in human cancers and are correlated with increased stages of carcinogenesis and further tumor progression with many characteristics, such as fast infiltrating growth, metastasis in early stage, high-grade malignancy, and poor therapeutic efficacy [9-11]. HCC prognoses are poor, and early detection is of the utmost importance [12, 13]. Most of HCC patients died quickly because of the rapid tumor progression, and hepatic resection or transplantation is the only potential curative treatment for HCC. Treatment options are severely limited by the frequent presence of metastases. Therefore, it is the 3rd leading cause of cancer-induced death worldwide with a very poor prognosis [14]. Growing understanding of the molecular mechanisms underlying the carcinogenesis of HCC is a multi-factor, multi-step, and complex process, involving chromosomal aberrations, gene mutations, epigenetic alterations, and activation of complex signaling pathways [15, 16].

Recently, studies have discovered changes in the insulin-like growth factor (IGF) axis that affect the molecular pathogenesis of HCC, and IGF-II is a polypeptide hormone secreted by many organs of the fetus [17, 18]. IGF axis (Figure.1) has emerged as an important pathway in the development and progression of HCC and as a potential therapeutic target. Human IGF-

II gene contains 9 exons (E_1~E_9) and 4 promoters (P_1~P_4, Figure1A). IGF system consists of the ligands, cell surface receptors, and the IGF binding proteins (IGFBPs) [19]. IGF receptors (IGF-1R and IGF-2R) are tyrosine kinase cell-surface receptor that binds either IGF-I or IGF-II. IGFBPs have key roles in regulating ligand bioavailability. IGF-II interacts with IGF-IR, IGF-IIR (lacks tyrosine kinase domain), and the exon 11-lacking (A) form of the insulin receptor (IR), and the IGFBPs. Hybrid receptors form from dimerization of IGF-IR and IR hemireceptors. These hybrid receptors retain high affinity for IGF-I, but have a significantly reduced affinity for insulin [20, 21].
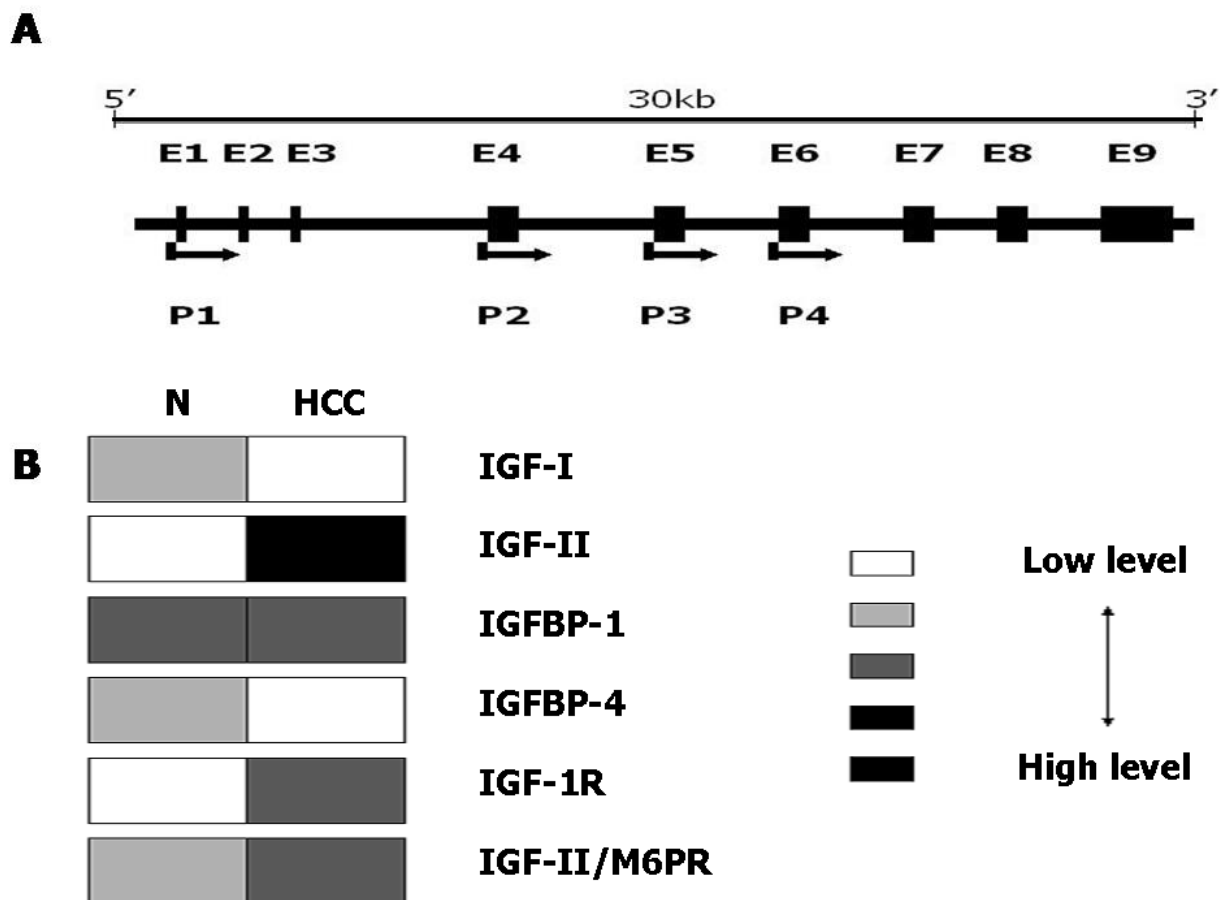

Figure 1. Schematic illustration demonstrating the structure of IGF-II gene (A) and the IGFs representative results (B) from immunohistochemical analysis or in situ hybridisation of antisense mRNA to normal liver (N) and HCC tissues. The intensity of gene expression is directly proportional to the darkness of the section. IGF, insulin-like growth factor; IGFBP, IGF binding protein; IGF-IR, IGF-I receptor; and IGF-II/M6PR, mannose 6-phosphate receptor.

IGFs, including IGF-I, IGF-II, IGF binding proteins, and their receptors (IGF-1R and IGF-2R) were well characterized in primary HCC tissue (Figure 1B) [22]. The IGFs system regulates many key aspects of cellular and whole- organism physiology and plays a crucial role in the regulation of cell growth, energy metabolism, differentiation, as well as key aspects of tumors such as transformation and anti-apoptotic signaling (Figure 2). Now, little is known of relationship between IGF-II gene's promoter methylation status and hepatocarcinogenesis.

IGF-II gene has complex regulation of transcription, resulting in multiple mRNA initiated by different promoters. Here, we review the complexity of IGF axis and focus on the expression of hepatic IGF-II and their gene during the malignant transformation of hepatocytes, the hepatic expression and circulating level of IGF-II in patients with liver diseases for prospectively elucidating the relationship between IGF-II level and the pathological features as well as the diagnosis and metastasis of HCC, and the effect of miRNA silencing IGF-II gene on inhibition of HepG2 cell proliferation with an urgent need to search for novel effective therapies for HCC. [23, 24]
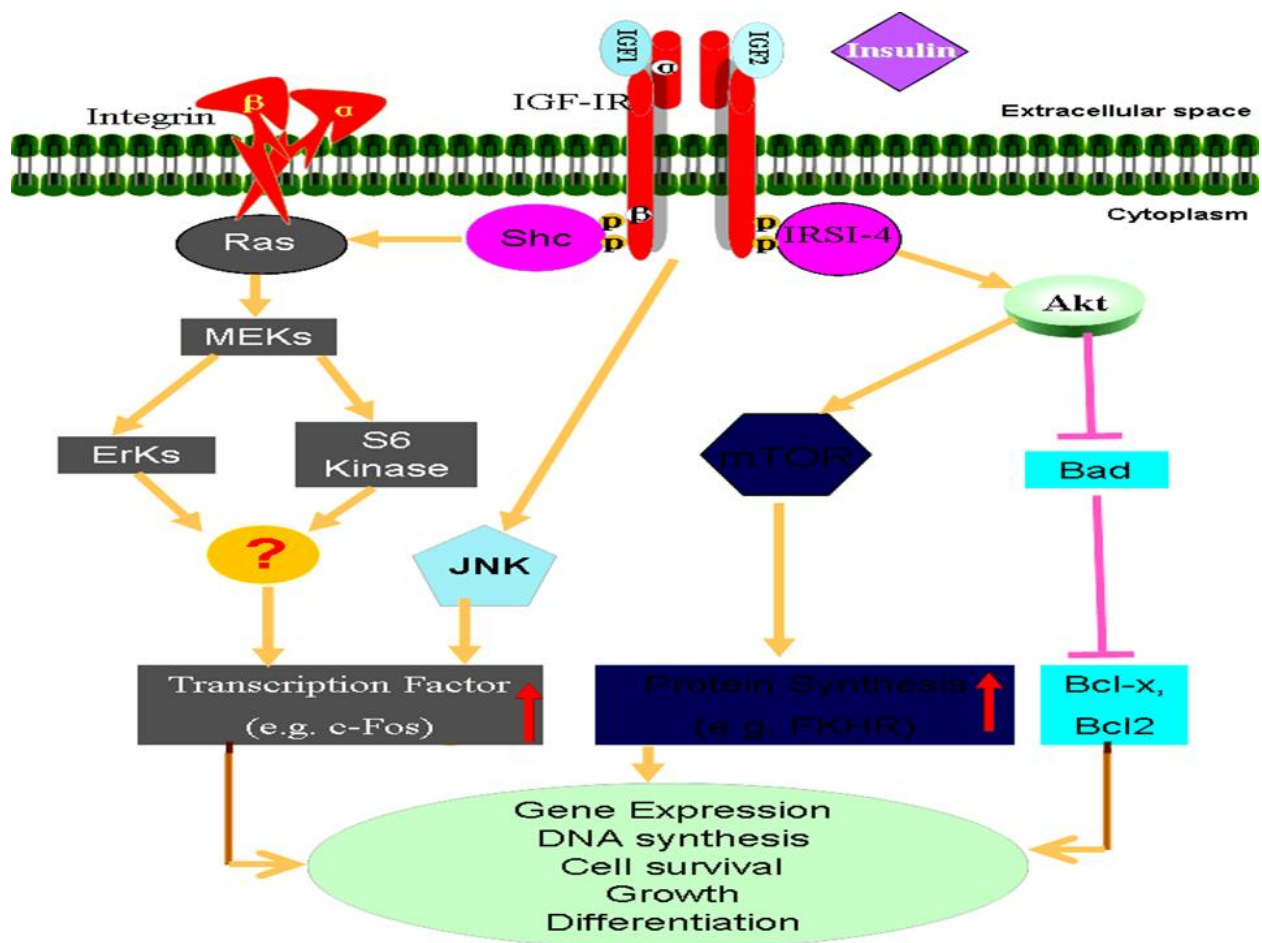

Figure 2. The IGF-II/IGF-1R intracellular pathway. Binding of the ligands IGF-I and IGF-II to IGF-IR activates its intrinsic tyrosine kinase activity resulting in signaling through cellular pathways that stimulates proliferation and inhibits apoptosis. The key downstream signaling pathways include PI3K-AKT-TOR and the RAF-MEK-ERK pathway. Therapeutic approaches that target the IGF-IR are being tested clinically and include antibodies directed at the extracellular portion of the receptor and small molecule tyrosine kinase inhibitors with specificity for IGF-IR. **ErK**, extracellular signal-related kinase; **IGF**, insulin-like growth factor; **IGF-IR**, IGF-I receptor; **mTOR**, mammalian TOR, target of rapamycin; **IRS**, insulin receptor substrate; **AKt**, Ak transforming; **Shc**, Src homology 2 domain-containing; **Ras**, rat sarcoma viral oncogene homolog.

2. Dynamic expression IGF-II during HCC development

IGF-II is a fetal growth peptide produced by the liver, which is over expressed in a wide variety of neoplasms including HCC and involved in experimental liver carcinogenesis [25-27]. 48 male Sprague-Dawley rats, 4~6 weeks old and weighing 120~160 g, were randomly divided into groups of 6 per cage, including control group and experimental groups. The control rats were given a standard diet, and the experimental rats were fed with 0.05 % 2-fluorenylacetamide (2-FAA, Sigma, USA) in an air-conditioned environment. One control rat and a group of experimental rats were sacrificed under mild ether anesthesia every 2 weeks(wk), blood was drawn from the heart and the serum was separated. The livers were washed free of blood, one part was used for pathological examination and immunohistochemical analysis, and the others were stored at -80 ℃. All procedures were conducted in accordance with the guidelines for experimental animals approved by the Animal Care and Use Committee of Nantong University. The morphological changes in rat liver cells were clearly seen during HCC development. The histological examination confirmed changes in hepatocytes from granule-like degeneration to atypical hyperplasia to HCC formation inducing with 2-FAA (Table 1).

Group		n	Histopathological change (HE staining)			
			Normal	Degeneration	Precancerous	HCC
Control		6	6	0	0	0
Experimental:	2nd wk	6	0	6	0	0
	4th wk	6	0	6	0	0
	6th wk	6	5	1	0	0
	8th wk	6	0	3	2	1
	10th wk	6	0	2	1	3
	12th wk	6	0	0	2	4
Total		42	6	22	6	8

Table 1. Histopathological Changes of Rat Liver during HCC Development

At the early stage during HCC induction process, the granular degeneration appeared in the cytoplasm and a large heterogeneous nucleus was seen (the degeneration group). At the intermediate stage, hepatic plate cell layers increased, focal cell layers surpassed three, the nuclear chromatin was denser, and the ratio of nucleus to cytoplasm increased (the precancerous group). At the later stage, the hepatic structure disappeared, the cells arranged into nido or funicular form, nuclei became middling large and the chromatin was denser, and the ratio of nucleus to cytoplasm increased. All of these were highly differentiated HCC (the HCC group).

At the same time, the expression levels of IGF-II in hepatic tissues and sera progressively increased (Table 2). *In vivo*, IGF-II is a growth factor that plays an important role during HCC development. It is synthesized and activated through tyrosine kinase and the IGF-I receptor. The process of IGF-II activation and expression has been confirmed in the liver tissues of HCC rats, transgenic rats and experimental animals infected with hepatitis virus. When the expression of IGF-II in the transgenic rat rise continuously, the risk of HCC increases. The reason for the high expression of IGF-II is the reactivation of the embryonic IGF-II gene. In the precancerous condition, IGF-II-mediated hepatocyte proliferation is mainly via IGF-IR by a paracrine mechanism. IGF-II was distributed in the cytoplasm of hepatocytes and over-expressed in the precancerous group, and the levels of IGF-II expression suggest that it may be secreted by hepatoma cells themselves and stimulate their proliferation via an autocrine mechanism. Although different levels of IGF-II expression were found in rat livers with different histopathological changes, the expression of IGF-II was detected in the hepatic cytoplasm of all rats fed with 2-FAA and none in the control group [28].

Group	n	IGF-II expression intensity Positive (%)	-	+	++	+++
Control	6	0 (0.0)	6	0	0	0
Degeneration	22	8 (36.36)	4	6	1	1
Precancerous	6	6 (100)*	0	3	2	1
HCC	8	8 (100)*	0	1	2	5

*$P<0.01$ vs. the control group.

Table 2. Dynamic Alteration of IGF-II Expression during Rat HCC Development

IGFs are potent autocrine and paracrine mitogens for liver cancer cell proliferation, and their bioactivity is reduced by IGFBP-3 [29]. Human embryonic liver cell lines also express IGF-II, suggesting that hepatoma cells may regain some embryonic characteristics like AFP secretion. A smaller proportion of IGF-II is associated with other IGFBPs, while less than 5% of IGF-II exists in the unbound or free form that is believed to be the biologically active fraction, capable of binding to the IGF-2R. IGF-II present in the ternary complex is not easily dissociated. However, IGF-II contained in low molecular weight binding complexes has a rapid turnover and may be the source of much of the free IGF-II detected. The levels of serum IGF-II protein were significantly higher in the HCC group than in the precancerous, degeneration and control groups (Table 3). Its main mechanism possibly is that the abnormal activation and over-expression cause precancerous cells in a high multiplication condition which transforms and finally induces HCC. The levels of IGF-II expression reflect the degree of pathological change in rat liver. Hepatic IGF-II may participate in liver cancer induction, and detection of IGF-II expression during HCC development could be a useful molecular marker for early diagnosis and prognosis of HCC [28].

Group	n	Serum IGF-II (ng/L)			Liver IGF-II (ng/mg protein)		
		Mean ± SD	t	P value*	Mean ± SD	t	P value*
Normal	6	149.7±19.1	7.40	<0.01	52.3±4.5	8.85	<0.01
Degeneration	22	174.2±43.4	6.75	<0.01	54.9±12.8	6.79	<0.01
Precancerous	6	274.1±24.1	4.30	<0.01	70.3±8.4	2.42	<0.05
HCC	8	450.3±112.6			80.7±7.4		

*$P<0.01$, vs. the HCC group.

Table 3. Dynamic Quantitative Analysis of Liver and Serum IGF-II Expression during Rat HCC Development

IGF-II is a kind of fetal growth factor, a mitogenic polypeptide closely related to insulin, highly expressed in hepatocarcinogenesis, and causes mitosis in different cell types. IGF-II may promote hepatocyte proliferation via a paracrine mechanism in the pre-cancerous stage. When hepatocytes are transformed into malignant cells, they may secrete IGF-II and promote malignant cell proliferation by an autocrine mechanism [30]. Identification of molecular abnormalities associated with an increased risk of HCC is particularly important to improve knowledge of both the pathways of liver carcinogenesis and the outcomes. Its gene has complex regulation of transcription, resulting in multiple mRNAs initiated by different promoters, which contribute to cell proliferation, differentiation, anti-apoptosis, and invasive behavior.

3. Difference of IGF-II expression in human HCC tissues

3.1. Alteration of fetal IGF-II promoter methylation status

Carcinogenesis of hepatocytes is a multi-factor, multi-step, and complex process. Genetic and epigenetic changes are the core biological processes in HCC. DNA cytosine methylation is a central epigenetic modification that has essential roles in cellular processes including genome regulation, development and disease [31-33]. Epigenetic changes include DNA methylation, histone modification, and DNA methylation is performed by DNA methyltransferase. The methylation status is closely associated with the development and progression of carcinoma. Hepatic IGF-II gene contains 4 promoters (P_1~P_4, Figure1A), P_1 for adult liver and P_2~P_4 for fetal liver [34-36].

The methylation status of IGF-II promoter and IGF-II expression were analyzed in HCC-, their surrounding-, and noncancerous- tissues, and the patterns by MPS were shown in Figure 3. The incidences of IGF-II P_3 methylation was 0% (0 of 40) in human HCC, 47.5% (19 of 40) in their surrounding, and 100% (40 of 40) in normal tissues, respectively; and the incidence was increased gradually (Table 4) from cancerous to non-cancerous parts of liver tissues, with significant differences among them (Table 4, χ^2 = 37.623, $P < 0.001$). The methylation

Figure 3. Methylation analysis of IGF-II promoter 3 region in human HCC tissues and IGF-II expression. A, Exon-intron and 4 promoter structure of human IGF-II gene. Exons are shown as numbered boxes (plain are coding). The 122 bp (P_3M) or 123 bp (P_3U) fragment of IGF-II promoter3 amplified for methylation analysis is enlarged below. Vertical lines indicate 13 CpG positions. **B,** The patterns of promoter methylation of hepatic IGF-II in different liver tissues. T-HCC; NT-Adjacent; N-Normal; U-Demethylation; M-Methylation; m, DNA marker.

rate of IGF-II P_3 in the adjacent tissues in poorly differentiated HCC were lower than well-differentiated ones ($P<0.001$), suggesting the positive correlation between demethylation status of IGF-II P_3 and hepatocarcinogenesis, suggesting aberrant methylation occurs before mutation and is an early event in the development of HCC. IGF-II is highly expressed in the fetal liver and early after birth, which is mainly based on activation of $P_2 \sim P_4$. But its expression is strongly reduced in adulthood, mainly based on activation of P_1. Several studies have shown elevated expression levels of IGF-II in preneoplastic lesions and very high levels in HCC, and so was the IGF-II $P_2 \sim P_4$, suggesting the correlation between IGF-II gene expression and promoter.

Group	n	M (%)	PM (%)	UM (%)	Z	P
HCC	40	0(0)	0(0)	40(100)	6.708	0.000
Adjacent	40	0(0)	19(47.5)	21(52.5)	4.290	0.000
Non-HCC	40	40 (100)	0(0)	0(0)		

$P<0.01$, compared with non-cancerous group; M, methylation of liver IGF-II gene P3 promoter; PM, part methylation of liver IGF-II gene P3 promoter; UM, unmethylation of of liver IGF-II gene P3 promoter.

Table 4. The Status of IGF-II Promoter 3 Methylation in Different Liver Tissues

Tumor differentiation and gene methylation while gene displayed different methylation profiles at different levels of differentiation, only two showed statistically significant differences. However, there was no correspondence with tumor progression toward poor differentiation. Only the gene displayed lower methylation in the normal progression of a tumor from well to moderate to poor differentiation. These findings might underscore the role of these genes in the tumor differentiation process. Tumor stage and gene methylation profile gene showed different methylation profiles at different tumor stages, with the gene displaying statistically significant differences with higher methylation at lower methylation at higher methylation at advanced stages [37].

3.2. Alteration of GF-II expression in different liver tissues

The expression of hepatocyte IGF-II was analyzed in HCC, their surrounding, and nocancerous tissues by immunohistochemistry were shown in Figure 4. Positive staining of hepatic IGF-II showed brown particles, located in cytoplasm with only a few in cellular nuclei but none in cell membrane. Positive cells were mostly located in the margin of portal area or near the central vein. The expression level of IGF-II went up with the histological changes. It was significantly higher in the HCC or the surrounding group than in the nocancerous group, and its levels in HCC group were obviously higher than in the surrounding group (*P*<0.05). There were 11 samples showed positive staining of IGF-II in degeneration group and 54.5 % of them were staining moderately and above. Five samples in precancerous lesion group showed positive staining of IGF-II and 80 % of them were staining moderately and above. Eight samples in cancerization group were positive for IGF-II staining and 87.5 % of them showed moderate and above. Thus we concluded that the expression intensities of IGF-II were associated with the morphological changes of hepatocytes [38, 39].
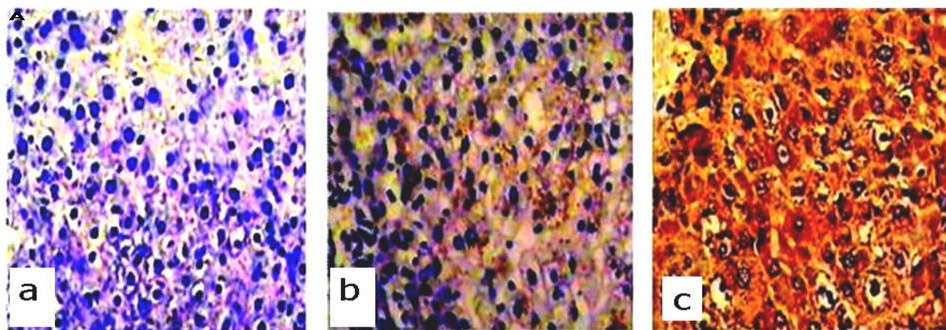

Figure 4. Immunohistochemical analysis with anti-human IGF-II in HCC tissues; a, the absence of cytoplamic staining for IGF-II (S-P, original magnification ×200) from non- cancerous tissues of human HCC; b, the IGF-II weakly positive staining in cytoplasm and cell membrane from the surrounding tissues; and c, the IGF-II strongly positive staining in cytoplasm and cell membrane (S-P, original magnification ×100) from human HCC tissues.

3.3. Expression of total RNA and IGF-II mRNA in HCC tissues

IGF-II may be a biological marker in the early diagnosis of HCC. The expression levels of IGF-II mRNA are different in different parts of HCC liver tissues. Different expression of hepatic

total RNA (µg/mg wet liver tissue) was found in the different parts of 36 HCC tissues. The total RNA levels were significantly lower in HCC tissues (17.9 ± 27.7 µg/mg wet liver tissue) than in self-control surrounding- (32.9 ± 31.2 µg/mg wet liver tissue, $P<0.05$) or non-cancerous liver tissues (41.4 ± 50.3 µg/mg wet liver tissue, $P < 0.01$), respectively [38].

Studies found that the amplified fragments of IGF-II mRNA by RT-PCR were identical to original designed ones with size of 170 bp and confirmed by sequencing analysis (Figure 5). The dilution experiments revealed that the lowest sensitivity of our system was 2 ng/L of total RNA (Figure 5A), the size of IGF-II DNA was identical to the original designed one, and confirmed by DNA sequencing analysis (Figure 5B). The incidence of positive IGF-II mRNA fragments was 100 % in HCC tissues, and significantly higher ($P<0.01$) than that in their surrounding (53.3 %) or in their non-cancerous (0%) liver tissues, respectively. [38, 39]
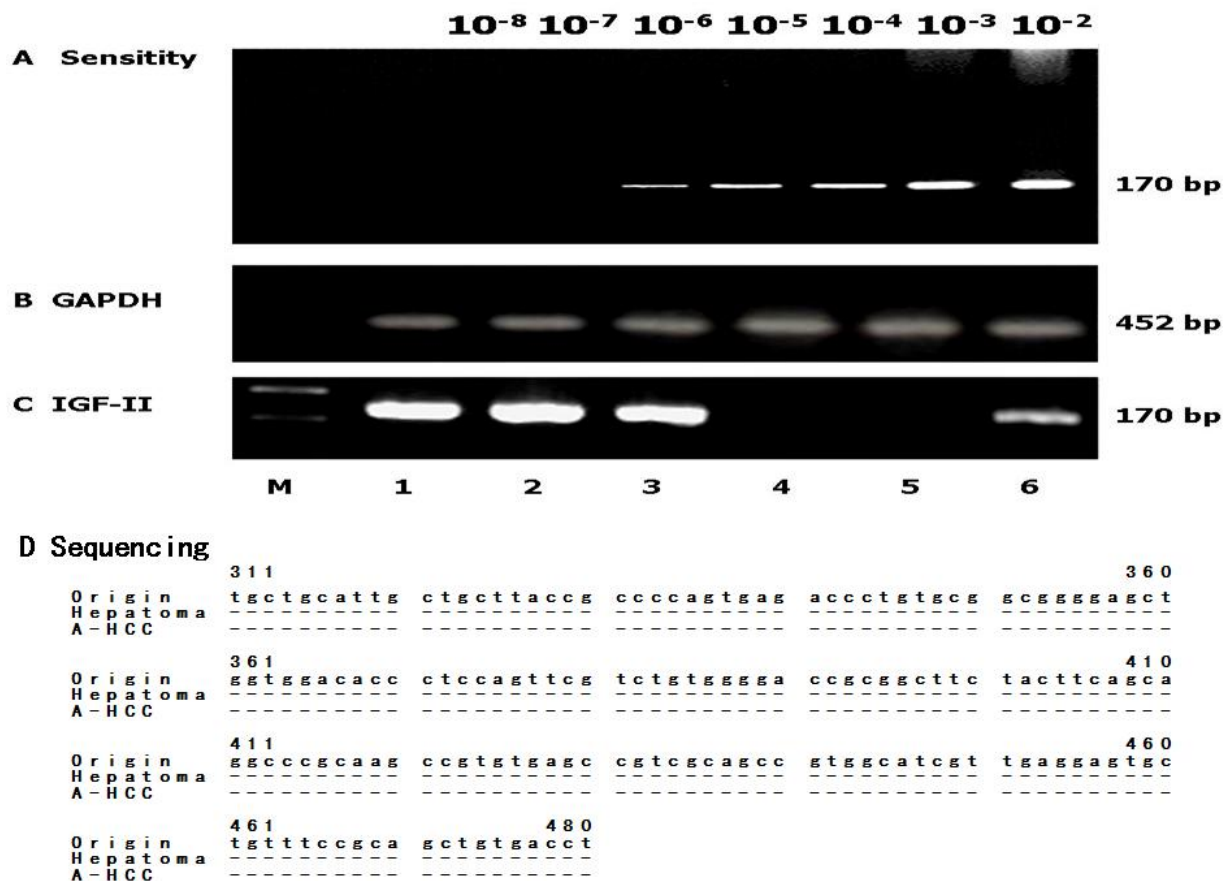
Figure 5. Amplification of IGF-II genomes from human liver tissues of HCC patients. IGF-II mRNAs were synthesized according to IGF-II cDNA with random hexamers and moloney murine leukemia virus reverse-transcriptase, and detected with different primer pairs by nested PCR (170 bp). The positive fragments of IGF-II genome were found distinctly in HCC or surrounding tissues. A, the sensitive limitation of the detection system (2 ng/L), using total RNA with $10^{-2} \sim 10^{-8}$ fold dilution and then amplified by nested PCR; B, the amplified fragments (452 bp) of glyceraldehyde-3-phosphate dehydrogenase genome from liver tissues; C, the amplification of IGF-II genomes in human liver tissues (No. 1~6). No. 1~3, the positively amplified fragments of IGF-II mRNA from cancerous tissues of HCC patients; No. 4~5, no positively amplified fragment from para-cancerous or non-cancerous tissue of HCC patients; No. 6, the positively

amplified fragments of IGF-II mRNA from para-cancerous tissue of HCC. GAPDH: glyceraldehyde-3 -phosphate dehy-drogenase. M: DNA molecular weight marker. D, Alignment of nucleotide sequences of the amplified fragments of IGF-II genome from different liver tissues in patients with HCC by sequence analysis. Origin: the cited sequence (170 bp, nt 311-480) of human IGF-II genome; Hepatoma: the amplification fragment of IGF-II genome from human HCC tissue; A-HCC: the amplified fragment of IGF-II genome from the surrounding tissue in HCC patients. HCC, hepatocel-lular carcinoma.

4. Expression of IGF-II associated with HBV or HCV infection

HCC is mainly associated with HBV and HCV infection. Activation of cell growth stimulator IGF-II gene is observed in tumor formation especially in viral associated HCC [5, 6, 11, 13]. IGF-II signaling is mediated through IGF-1R, up-regulation of IGF-II in some hepatocytes may lead to high focal IGF-II levels sufficient to saturate local IGF-II binding capacities, and may result in an increased susceptibility to cellular dedifferentiation and, ultimately, liver cancer. Elevated focal IGF-II transcript levels may therefore indicate an increased risk for HCC. SNPs sites (frequency ≥5%) of 376 HCC patients (312/21/43; HBV/HCV/NBNC) was found in angiogenic genes including VEGF, HIF-1α, and IGF-II (TT genotype more common). T allele (TT+CT genotype) at -13021C in IGF-II were independent risk factors in HCC recurrence. The SNPs in IGF-II genes may be important risk factors for the recurrence of HCC [40].

4.1. Expression of IGF-II and HBV Infection

In HBV, HBx protein promotes cell cycle progression, inactivates negative growth regulators, and binds to and inhibits the expression of p53 tumor suppressor gene and other tumor suppressor genes and senescence-related factors. During recent years evidence has accumu-lated that HBx protein modulates transcription of methyl transferases, causing regional DNA hypermethylation that results in silencing of tumor suppressor genes, or global hypomethy-lation that results in chromosomal instability, thereby playing a role in hepatocarcinogenesis. Particularly important among the anti- apoptotic properties is inhibition of p53 [41].

Recent experimental observations suggest that HBx protein may increase the expression of TERT and telomerase activity, prolonging the life-span of hepatocytes and contributing to malignant transformation. Carboxy-terminal truncated HBx protein loses its inhibitory effects on cell proliferation and pro-apoptotic properties, and it may enhance the protein's ability to transform oncogenes. Dysregulation of IGF-II enhances proliferation and anti-apoptotic effects of oncogenes, resulting in uncontrolled cell growth. Significant increase in fetal transcripts is associated with the p53 mutation and poor prognosis of the HCC patients and might serve as one of identification parameters of poor HCC prognosis. HBx product become transcriptional transactivators of cellular and viral genes, are known to play causative roles in HCC devel-opment [42, 43]

Using 240 different combinations of three one-base anchored oligo-dT primers and 80 arbitrary 13 mers, 16 genes were differentially expressed in the HBx- positive HCC. Unexpectedly,

upregulated genes in association with functional HBV proteins were different from those reportedly transactivated by HBV viral proteins in vitro. Ten genes were downregulated, including three novel genes. In contrast, 15 genes in HCC tissue negative for HBx-expression were preferentially expressed including IGF-II and 10 ribosomal proteins genes. Cellular genes involved in the viral protein-transactivation may generally differ from those not associated with transactivation in established HCC, and that the specific oncogenic coordination through the transactivation by viral proteins which works in experiments in vitro, may play only a potential role in hepatocarcinogenesis in vivo [43, 44].

HBV infection is one of the most important factors for HCC, especially HBV X gene (HBX) is closely related to hepatocarcinogenesis. And more than 80% of HCC cases are associated with HBV. Many studies showed HBV infection contributed to hypermethylation of tumour suppressor genes. The methylation rates of IGF-II P_3 in positive surface antigen HCC patients was significantly lower than those with negative surface antigen, suggesting DNA demethylation could related to HBV infection. DNA methylation may be the molecular-targeted therapies of HCC [41]. Further studies will allow us to investigate the changes of IGF-II gene promoter methylation status in patients' peripheral blood with HCC, and will elevate early diagnosis and monitor metastasis of HCC. These reference epigenomes provide a foundation for future studies exploring this key epigenetic modification in human disease and development. DNA cytosine methylation is a central epigenetic modification that has essential roles in cellular processes including genome regulation, development and disease [45, 46].

4.2. Expression IGF-II and HCV infection

In HCV, core protein is believed to transactivate host IGF-II receptor through PKC pathway and the inhibition of tumor cell growth can be achieved by blocking IGF-II pathway either at transcriptional level or increasing its binding with IGFBPs (IGF binding proteins) at C-terminal, so that it is not available in free form. IGFBP-6 is a specific inhibitor of IGF-II actions. Affinity of IGFBPs with IGFs is controlled by post- translational modifications. Phosphorylation of IGFBPs inhibits IGFs action on target cells while O-glycosylation prevents binding of IGFBP-6 to glycosamino- glycans and cell membranes and resulting in a 10-fold higher affinity for IGF-II. O-glycosylation and phosphorylation operate the functional expression of cellular proteins, this switching on and off the protein expression is difficult to monitor in vivo [44]. By using neural network based prediction methods, alternate O-β-GlcNAc modification and phosphorylation on Ser 204 control the binding of IGFBP-6 with IGF-II. This information may be used for developing new therapies by regulating IGFBP-6 assembly with IGF-II to minimize the risk of viral associated HCC [44].

During HCV/HBV infection, O-β-GlcNAc of IGFBP-6 at Ser 204 diminish their binding with IGF-II, increase IGF-II cellular expression and promote cancer progression which can lead to hepatocellular carcinoma. Furthermore, this site can be used for developing new therapies to control the IGF-II actions during viral infection to minimize the risk of hepatocellular carcinoma [46]. The possibility that HCV core gene product (HCV-core) acts as a transactivator in IGF-II gene transcription was tested. HCV-core protein increases endogenous IGF-II expression from promoter 4 (P_4) of the IGF-II gene through two cis-acting elements: Sp1 and Egr1

binding sites. Sp1 and Egr1 both bind to IGF-II P4 and functionally cooperate in mediating the maximal activity of IGF-II P4. HCV-core protein induced the binding of Sp1 and Egr1 on its binding sites on IGF-II P4. In addition, Sp1 and Egr1 were stimulated to phosphorylate by HCV-core, and its DNA binding activity was up-regulated upon HCV-core transfection [47].

Transfection with HCV-core in HepG2 cells stimulated the membrane translocation of protein kinase C (PKC) and the treatment of HCV-core transfected cells with calphostin C, a PKC inhibitor, blocked induction of Sp1 and Egr1 DNA binding activity, and eventually transcriptional transactivations of the IGF-II gene. Increasing the DNA binding activity of the phosphorylated form of Sp1 and Egr1 might be an important mechanism for regulating IGF-II gene expression and for promoting cell division during hepatic carcinogenesis. HCV-core functions as a positive regulator of IGF-II transcription through the PKC pathway and that Sp1 and Egr1 are direct targets of the transcriptional regulation of the IGF-II gene which plays an important role in HCV pathogenesis during the formation of HCC. [47]

5. Circulating IGF-II and clinicopathological features

There were 156 patients with HCC enrolled for this study at Affiliated Hospital of Nantong University, China. The patients' ages ranged from 26 to 74 years (median, 46 years). 134 patients (86 %) had a history of cirrhosis, and 22 (14%) had a history of chronic hepatitis [48]. All patients were diagnosed by blood biochemical tests, viral histology and B-ultrasonic examination. The incidence of hepatitis virus in these patients was 76 % (118 of 156) in HBsAg, and 10 % (16 of 156) in antibody to hepatitis C virus (ELISA, Beijing, China). The serum AFP level ranged from 33 to 2,500 ng/mL (median, 243 ng/mL). Serum AFP more than 50 ng/mL was taken as a positive result. Other cases included 39 patients with acute hepatitis, 72 patients with chronic hepatitis, 75 patients with decompensated cirrhosis, and 60 healthy subjects with negative-HBV markers (HBsAg, HBcAb, and HBV-DNA) and with normal serum ALT levels from the Nantong Central Blood Bank. All peripheral blood samples were collected in the morning, with anti-clot heparin, and peripheral blood mononuclear cells were separated immediately, according to the method as described previously. AFP-mRNA in peripheral blood was also detected in this study as described elsewhere. Ethics Statement, this study was approved by the Institutional Review Board, Affiliated Hospital of Nantong University, and written informed consent was obtained. The diagnosis of HCC and viral hepatitis was based on the criteria proposed by Chinese National Collaborative Cancer Research Group and the 2000 Prevention and Cure Scheme of Viral Hepatitis, respectively.

The levels of IGF-II expression in the serum of 224 patients with liver diseases was significantly higher ($P < 0.001$) in patients with HCC than in patients with liver cirrhosis or chronic hepatitis. Also, the level of serum IGF-II in patients with HCC was significantly higher ($P < 0.001$) than in patients with nonliver tumors or acute hepatitis. The evaluation of serum IGF-II and AFP levels for HCC diagnosis using the ROC curves is shown in Figure 6. The analysis of 2 markers for the whole range of sensitivities and specificities using the area (0.823 for AFP and 0.771 for IGF-II) under ROC curves indicated that the abnormality of serum IGF-II level could be a useful molecular marker for HCC diagnosis [48, 49].
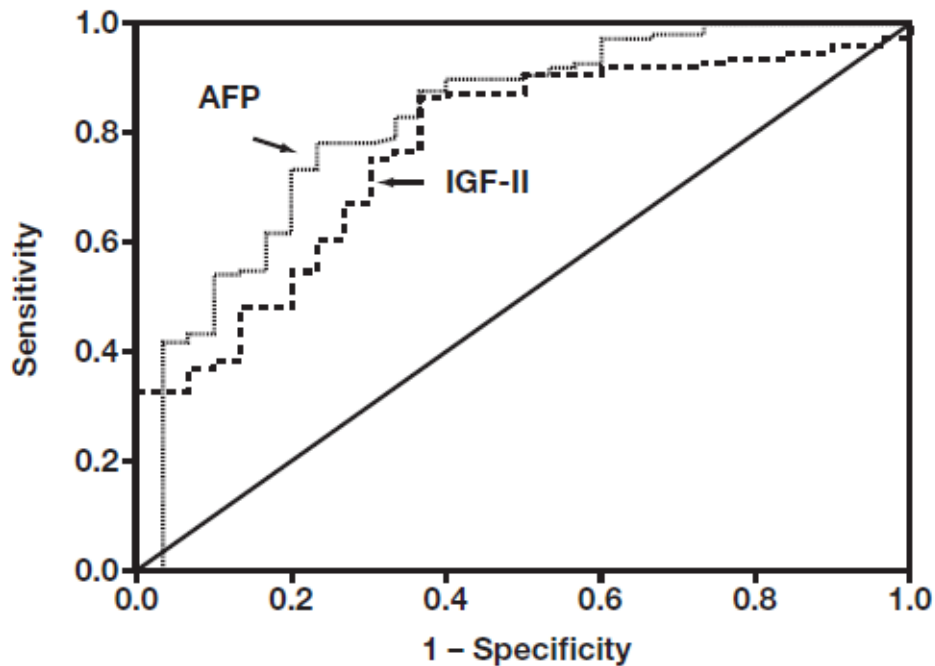
Figure 6. The diagnostic value of circulating IGF-II level for HCC. Receiver operating characteristic (ROC) curves for the serum insulin-like growth factor II (IGF-II) investigated marker for hepatocellular carcinoma. Sensitivity, true-positive rate; specificity, false-positive rate. The area under the ROC curves was 0.823 for α-fetoprotein (AFP) and 0.771 for IGF-II [50].

The positive frequency of circulating IGF-II mRNA was 34% in HCC, and no amplified fragment was found in other liver diseases, extrahepatic tumors, and normal controls. The circulating IGF-II mRNA was correlated with the stage of HCC, and its incidence was 100% in HCC with extrahepatic metastasis, and 35% in HCC with AFP-negative. No significant difference was found between tumor sizes and circulating IGF-II mRNA fragment and IGF-II mRNA can only be detected in the peripheral blood of HCC patients. The levels of circulating IGF-II mRNA and its diagnostic value increase with clinical stage of HCC and with distant metastases. Circulating IGF-II mRNA could be a useful molecular marker for HCC diagnosis, especially in monitoring extrahepatic metastases.

However, little is known about the diagnostic value of circulating IGF-II in the early stage of HCC. In the present study, we analyzed the localization and expression of IGF-II during the malignant transformation process in rat hepatic cells by immunohistochemistry, detected the dynamic changes of IGF-II expression in the liver and sera of HCC model rats, and discussed the possibility of circulating IGF-II becoming a marker for early diagnosis of HCC. Hence the abnormal expressions of IGF-II and IGF-II mRNA are useful tumor markers for HCC diagnosis, differentiation of extrahepatic metastasis, and monitoring postoperative recurrence. The pathologic characteristics of circulating IGF-II expression are shown in Table 5. The higher expression of hepatic IGF-II in patients with HCC was associated with HBV infection ($P<0.001$). However, no significant difference was found between IGF-II expression and patient sex, age, tumor size, extrahepatic metastasis, or AFP level ($P > 0.05$) [50, 51].

Group		No. of Cases	Mean ± SD IGF-II (ng/mL)	t	P
HCC		146	3.74 ± 0.67	—	—
Sex	Male	108	3.73 ± 0.65	0.224	0.823
	Female	38	3.77 ± 0.77		
Age (yr)	≥50	109	3.74 ± 0.64	0.117	0.907
	<50	37	3.75 ± 0.77		
Tumor size (cm)	≥5.0	66	3.77 ± 0.76	0.491	0.624
	<5.0	80	3.71 ± 0.59		
Extrahepatic metastasis	With	38	3.88 ± 0.69	2.013	0.058
	Without	108	3.62 ± 0.67		
α-Fetoprotein (ng/mL)	≥400.0	67	3.83 ± 0.67	1.564	0.120
	<400.0	79	3.66 ± 0.66		
Hepatitis B surface antigen	Positive	110	3.93 ± 0.50	5.390	0.000
	Negative	36	3.16 ± 0.80		

HCC, hepatocellular carcinoma; IGF-II, insulin-like growth factor II. *IGF-II values are given in conventional units; to convert to Système International (SI) units (nMol/L), multiply by 0.131. To convert the conventional units for alpha-fetoprotein to SI units (μg/L), multiply by 1.0.

Table 5. Pathologic Characteristics of Circulating IGF-II Expression in Patients with HCC*

6. Targeting IGF-1R

The IGF signaling axis is comprised of two receptors (IGF-1R and IGF-2R), the ligands IGF-1 and IGF-2, and a system of at least six binding proteins and attendant proteases that modulate ligand availability [52, 53]. Insulin also binds to the IGF-1R, but with 100- to 1000-fold lower affinity than that of the IGFs. The supporting preclinical and clinical data highlighting the significance of this pathway in HCC, and the early clinical trials of targeting this axis in advanced HCC. However, the underlying mechanisms that lead to malignant transformation of infected cells remain unclear. The efficacy profile seems to be promising [54, 55]. However, further studies are needed to define the exact role of IGF-1R inhibitors in clinical practice. IGF-1R with its ligands and intracellular pathway is involved in cell growth and survival control.

Many studies have shown how IGF-1R is over-expressed in HCC cell lines and histological samples [56, 57]. In recent years many trials have been conducted investigating IGF-1R as a

possible cancer therapy, with major efforts focusing on the use of monoclonal antibodies and small molecules directed against the IGF-1R-driven pathway. Several drugs are currently under intense investigation and in different experimental phases. Available data suggest that this class of drugs is well tolerated with mild to moderate side effects, when used alone or in combination with other therapeutic agents [58, 59].

It is speculated to serve as an autocrine growth factor in various cancers because they often co-express IGF-II and IGF-IR in hepatocarcinogenesis, and re-expression of IGF-II gene has recently been described in HCC. To date, several therapeutic strategies have been developed in order to specifically inhibit IGF-1R while sparing IR, Phase I/II studies have shown that a monotherapy with this class of drug seems to give stability of disease rather than responses. More recent studies are, in fact, investigating combination of anti-IGF-1R therapy with chemotherapy or other targeted agents, in order to give a wider tumor response through multiple blocking of intracellular pathways or DNA damages. Anti-IGF-1R drugs seem to be a very promising class of targeted agents for cancer therapy, although the real potential of this class of drugs, whether alone or in combination, needs to be further investigated in randomized studies. Another key point on which research should focus is to find a biological marker of potential efficacy of this class of drugs, in order to select which patients should really benefit from this treatment approach [60].

7. Inhibition of IGF-II on effect of HCC cell proliferation

Abnormal expression of IGF-II is associated with the hepatocyte malignant transformation and HCC progress [61-63]. Specific IGF-II miRNA plasmids were constructed and transfected to HepG2 cells to knockdown IGF-II expression for observing effects on the cell proliferation, survival, apoptosis, angiogenesis, and anchorage-independent colony formation [64-66]. IGF-II mRNA was evaluated by quantitative real-time polymerase chain reaction, and the level of IGF-II or VEGF was quantitatively analyzed by ELISA. Our data shown that down-regulation of IGF-II expression resulted in the viability alteration, proliferation inhibition, and apoptosis occurrence of HepG2 cells [67]. The level of VEGF expression in the supernatant of HepG2 cells in the IGF-II-miRNA-transfected group was significantly decreasing ($P<0.01$) than those in the untransfected group or the miRNA-neg-transfected group, with the susceptibility to anoikis and decreasing of anchorage-independent colony formation of HepG2 cells. Thus, concluding that IGF-II is a potential molecular target for HCC gene therapy [68, 69].

HepG2 cells transfected with four recombinant targeting IGF-II plasmids: pCMV-IGF-II-miR-1, pCMV-IGF-II-miR-2, pCMV-IGF-II-miR-3, and pCMV- IGF-II-miR-4, containing green fluorescent protein, are shown in Figure 7. After the HepG2 cells transfected with a high efficiency at 24 h (Figure 7a, b), the alterations of IGF-II gene expression at transcriptional level showed the four different silencing efficiencies, and one of the best plasmids was the pCMVIGF-II-miR-2. After the HepG2 cells were transfected with the pCMV-IGF-II-miR-2, the expression of IGF-II gene at transcriptional level at 48 h was significantly inhibited (down to 15 %, $P<0.001$, Figure 7c), and the same interference effects ($P<0.001$) were observed at protein

level (Figure 7d), with the IGF-II protein down to 25 % compared with the miR-neg group, indicating that the highly specific and efficient miRNA could suppress the activation of IGF-II expression in hepatoma cells at transcriptional or protein level.
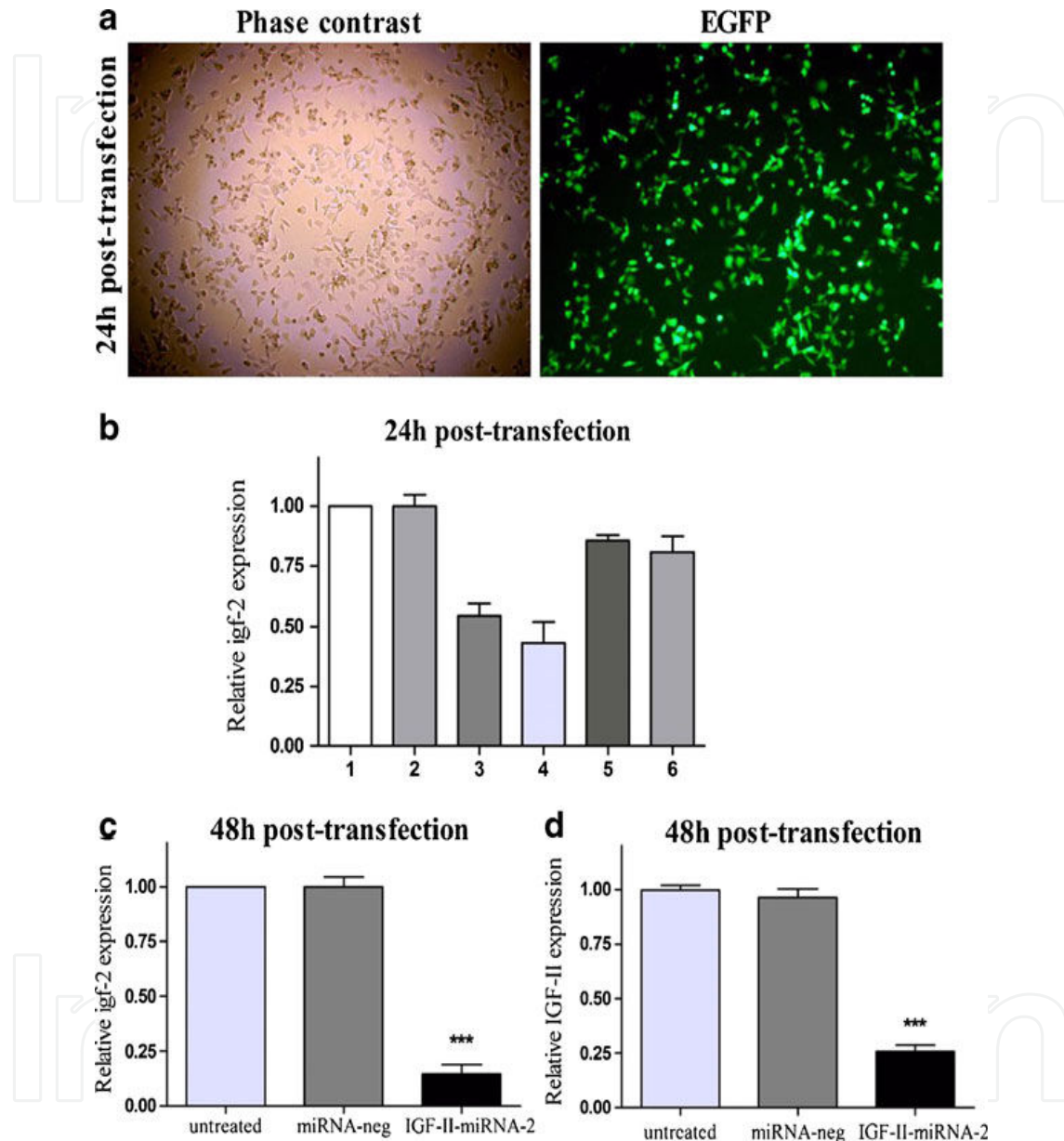

Figure 7. Suppression of IGF-II activation in HepG2 cells with different miRNAs. [67] HepG2 cells were transfected according to the preoptimized instructions. At 24 or 48 h after the HepG2 cells transfected with different IGF-II miRNAs, the cells were then harvested and checked. a The phase contrast and fluorescence photomicrographs (100× magnification) of the HepG2 cells transfected with different constructed pCMV-IGF-II-miR plasmid vectors containing green fluorescent protein at 24 h. b Analysis of IGF-II gene at transcriptional level in the HepG2 cells transfected with different pCMV-miR at 24 h. Lanes 1 the untreated HepG2 cells, 2 the HepG2 cells with IGF-II-miR-neg, 3 the HepG2 cells with IGF-II-miR-1, 4 the HepG2 cells with IGF-II-miR-2, 5 the HepG2 cells with IGF-II-miR-3, 6 the HepG2 cells with IGF-II-miR-4. c IGF-II gene transcriptional level at 48 h after the HepG2 cells were transfected with IGF-II-miR-2; d IGF-II protein level at 48 h after the HepG2 cells were transfected with IGF-II-miR-2 by ELISA. Data were expressed as mean ± SD from three independent experiments. ***$P<0.001$ vs. the IGF-II-miR-neg group.

The viability inhibition after the HepG2 cells transfected with miRNA determined by a trypan blue exclusion assay is shown in Figure 8. Compared with the control (miR-neg) group, the viability of the HepG2 cells transfected with IGF-II-miR-2 (Figure 8a) was notably inhibited at a time-dependent manner. The rapid growth of the HepG2 cells leads to insufficient blood supply, and solid cancer cells should evolve to tolerate nutrition starvation. The effect of IGF-II on the death of serum-deprived HepG2 cells was also evaluated by trypan blue staining. Compared with the miR-neg group, the IGF-II-miR-2-transfected group displayed higher death rates for both 48 h (31.3 % vs. 17.3 %, $P < 0.01$) and 72 h (68.7 % vs. 36.7 %, $P < 0.001$) after serum starvation (Figure 8b), whereas the miR-neg group showed a similar frequency of death as untreated group, indicating that highly IGF-II expression might increases the growth and survival of HepG2 cells *in vitro* [67]

The downregulation of IGF-II expression, which inhibited the proliferation of HepG2 cells by apoptosis mechanism, is shown in Figure 7c. The proliferation and apoptosis of the HepG2 cells were analyzed by EdU incorporation assay based on the measurement of DNA synthesis regarded as a gold standard for measuring the cell proliferation. Under common culture conditions (10 % FBS), the EdU incorporation assay displayed that DNA synthesis of the cells was significantly inhibited in the IGF-II-miR-2-transfected group as compared with the miR-neg-transfected group ($P < 0.01$; Figure 7c, d). Furthermore, the analysis of apoptosis revealed that the amount of apoptosis in the IGF-II-miR-2-transfected HepG2 group is increased as compared with the control group ($P < 0.01$; Figure 8c, e), indicating that downregulation of IGF-II expression might inhibit HepG2 cell proliferation. [67]

IGF-II is involved in the process of HCC development and has exhibited numerous genetic abnormalities as well as epigenetic alterations including modulation of DNA methylation. Targeted therapy to the IGF system is being studied in combination with chemotherapy as demonstrated by the phase II study. Other combination strategies are also being employed in breast cancer, given the important links between the ER and the IGF-IR pathway that have been discovered in experimental models. Constitutive activation of the IGF-signaling axis is frequently observed in human HCC. Especially the overexpression of the fetal growth factor IGF-II, IGF-IR, and cytoplasmic downstream effectors such as IRS contribute to proliferation, anti-apoptosis, and invasive behavior [70, 71].

8. Perspectives

The IGF system is emerging as a promising new target in cancer therapy and promises to revolutionize the way we select therapies in combination with chemotherapy, endocrine therapy, and other biological agents. The relevant alterations in this signaling pathway and independent in vivo models that support the central role IGF-II signaling during HCC development and progression. Since this pathway has become the center of interest as a target for potential anti-cancer therapy in many types of malignancies, various experimental strategies have been developed, including neutralizing antibodies and selective receptor kinase inhibitors, with respect to the specific and efficient reduction of oncogenic IGF-II/IGF-
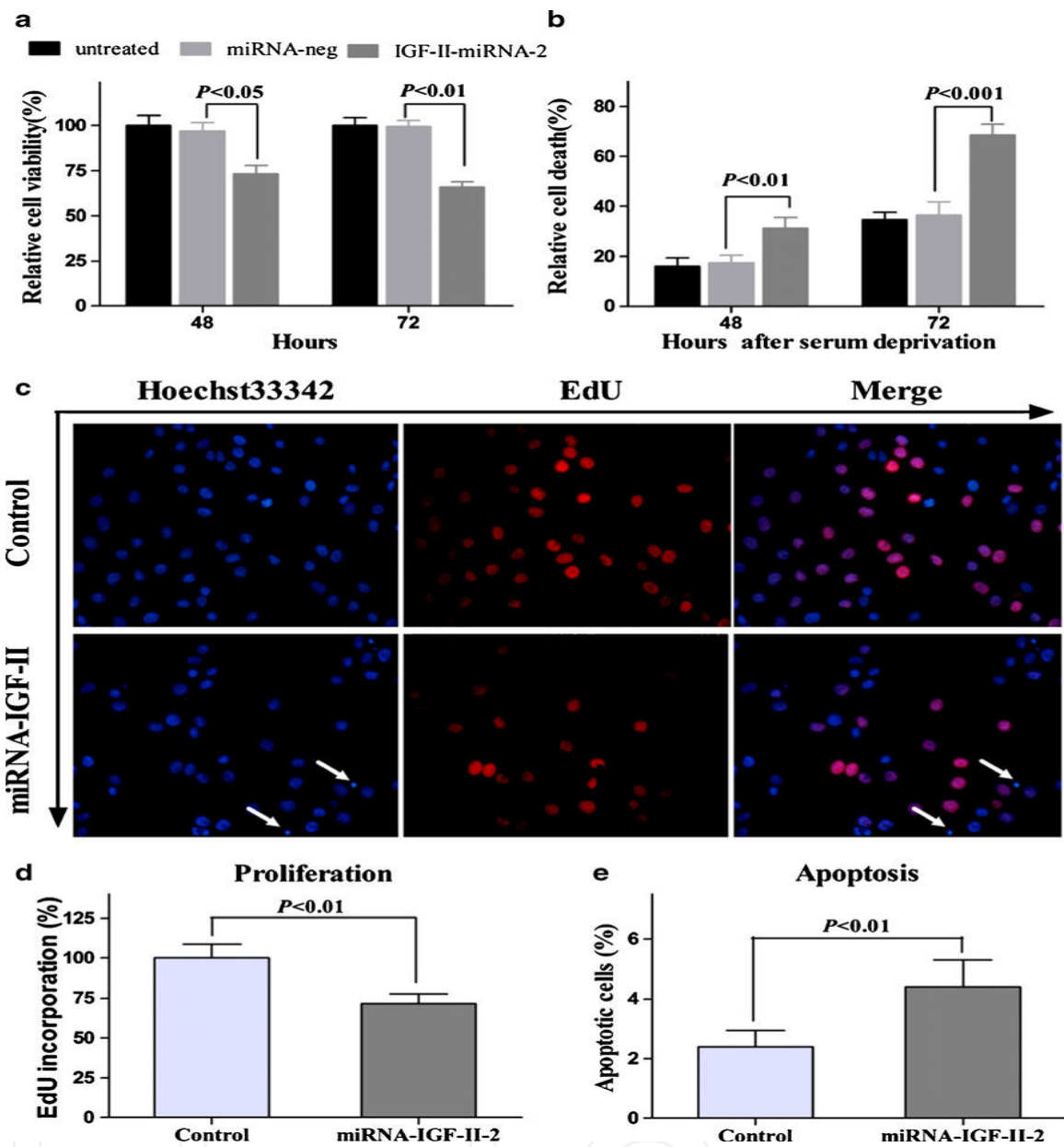
Figure 8. Silencing IGF-II expression on effect of HepG2 cell growth and survival. a The downregulation of IGF-II expression inhibition of HepG2 cell viability. At 48 h after transfection, the HepG2 cells were plated into 6-well plates (50×10⁴ cells per plate). After 48 and 72 h, cell viability was assayed with a trypan blue assay; b Suppression of IGF-II inhibits HepG2 cells survival. After transfection at 48 h, HepG2 cells were plated into 6-well plates (50×10⁴ cells per plate). After 4 h, the adherent cells were switched to serum-free medium and harvested for the trypan blue staining at different time points (48 or 72 h); then the trypan blue positive cells were counted and calculated as percent of total cell number. Data represented the mean ± SD from three independent experiments. c After the HepG2 cells (200×) transfected with miRNA were stained with the EdU incorporation and Hoechest 33342. At 48 h, the cells were plated in 96-well plates (2.0×103 cells per well) in triplicate for other 24 h, then exposed to EdU for 2 h, and visualized under a fluorescence microscopy. EdU (red), DNA synthesis; Hoechst 33342 (blue), nuclear staining; Apoptotic cells (white arrows) showed shrunken nuclei with a bright fluorescence appearance. d Quantitative analysis of the EdU incorporation assay. Data are represented from three independent experiments; e quantification of apoptotic events (white arrows) as determined in Figure 7c. The number of cells with nuclear morphological features of apoptosis was counted after staining with Hoechest 33342. Data are expressed as percentage of apoptotic cells based on counting 100 cells in randomly selected fields. Data are from three separate experiments, and mean values ± SD, from three independent experiments [67]

IR-signaling[72, 73]. Several laboratories have implicated constitutive activation of miRNA as one of the early key events involving in neoplastic progression of the liver. Further studies will permit us to analyze mechanism of human hepatocarcinogenesis and pay attention to these areas to be more practical up to present [74, 75].

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