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Mechanism of Hypoxia-Inducible Factor-1alpha Over- Expression and Molecular-Target Therapy for Hepatocellular Carcinoma

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common and rapidly fatal malignancies worldwide and has been ranked the second highest cancer killer in China since the 1990s, particularly in the eastern and southern areas, including the inshore area of the Yangtze River (1). Multiple risk factors are associated with HCC disease etiology, with the highest incidence in patients with chronic hepatitis B virus (HBV) and hepatitis C virus (HCV), although other factors such as genetic makeup and environmental exposure are involved (2~4). As a common malignant, solid tumor, HCC is characterized by fast infiltrating growth, early metastasis, high-grade malignancy, and poor therapeutic efficacy. It is a highly vascular tumor dependent on neovascularization and one of the most common and rapidly developing malignancies (5, 6). HCC treatment options are severely limited by the frequent presence of metastases (7~10, Fig.1).

Multistep malignance of HCC progression with multigene alterations mostly accompany with chronic hepatitis and liver cirrhosis (11, 12). Hypoxia inducible factor-1 (HIF-1) is a basic-Helix-Loop-Helix Per-Arnt-Sim protein (bHLH-PAS) consisting of α and β subunits and a key transcription factor regulating cellular responses to hypoxia (13, 14), and can regulate neovascularization, activate expressions of many hypoxia-response genes, leading to closely associate with HCC ecosystem for tumor growth, infiltration, metastasis and prognosis (15~17). HIF-1 α is an oxygen-dependent protein, which is degraded by poly ubiquitination and proteasomal degradation via the Von-Hippel-Lindau tumor suppressor protein under normoxic conditions (4, 18, 19). Here we briefly review the expression of rat hepatic HIF-1 α and its gene during the malignant transformation of hepatocytes, the hepatic expression and circulating level of HIF-1 α in patients with liver diseases for prospectively elucidating the relationship between HIF-1 α level and the pathological features as well as the diagnosis and metastasis of HCC, and the effect of miRNA silencing HIF-1 α gene on inhibition of HepG₂ cell proliferation.

2. HIF-1alpha expression and HCC development

Hepatocarcinogenesis is a complex process requiring multiple factors and multiple steps. Chemical carcinogens can induce cancer development in liver cells in a short time, with

Fig. 1. HCC development and the role of hepatic HIF-1 α . A, Model for the development of hepatocyte malignant transformation and the role of HIF-1 α during liver tumorigenesis. The gradual replacement of cells during normal physiologic turnover is accomplished by the proliferation of the differentiated liver cells, in response to various forms of liver injury to canceration. In contrast, when parenchymal cells are unable to proliferate (e.g., in response to hepatocyte toxins), rare cells associated with the bile ducts known as oval cells expand and then differentiate to restore liver mass. The alterations of many genes result in hepatocyte and oval cell proliferation and the development of HCC. B, The role of hepatic HIF-1 α . Regulation of the α -subunit is mediated by the oxygen dependent degradation domain (ODD), which contains two regulatory proline residues. Transcriptional activity of HIF-1 is facilitated by a N- vs. C-terminal transactivation domains (TAD-N vs. TAD-C) in HIF-1 α . B₁, Under normoxia O₂ is available and hydroxylation via FIH-1 and PHDs proceeds. FIH-1 hydroxylates Asn 803 in the C-TAD of HIF-1 α . This modification causes CBP/p300 to dissociate from HIF-1 α , thus repressing HIF-1 transcriptional activity. PHDs hydroxylate Pro 402 and Pro 564 within the ODD of HIF-1 α thereby making it available for the binding of pVHL. pVHL forms a E3-ubiquitin ligase complex with co-factors which subsequently facilitates poly-ubiquitination of HIF-1 α and thus degradation by the 26S proteasome. B₂, Scheme of the proposed role of HBx in the HIF-1-mediated angiogenesis of HCC. HBx interacts and stabilizes HIF-1 through inhibition of the interaction between pVHL and HIF-1 and the ubiquitin (Ub)-dependent degradation. Subsequently, HBx activates the HIF-1-dependent transcription and leads to angiogenesis. Under hypoxia O₂ is limited and PHDs as well as FIH are inactive. In turn HIF-1 α accumulates associates with the β -subunit and upon recruitment of the co-factor p300 forms the transcriptionally active HIF-1 complex. Activation of genes that contain HIF-responsive elements (HRE) in their promotor region follows

large-scale RNA transcription. The total RNA content gradually increases, and in the precancerous lesion, this can be significantly different from normal tissues. Angiogenesis is necessary for solid tumors larger than 1×1 mm, or the tumor remains dormant and does not metastasize. As soon as the angiogenesis stage arrives, potent metastasis is exhibited at once. The expression of HIF-1α plays important roles in angiogenesis, tumor growth, invasion, and metastasis in different cancers. We have investigated the expression of HIF-1α and its gene at the early stage of hepatocarcinogenesis, and provided the first evidence to show that HIF-1α is activated in preneoplastic hepatocytes during the early stage of carcinogenesis and long before the development of HCC.

Fig. 2. Appearance of rat liver and morphological changes of rat hepatocytes during 2-FAA-induced hepatocarcinogenesis. A-D: liver appearance of control rats, at the early, middle, and late stages during the malignant transformation of hepatocytes ; E-H: corresponding histopathological images confirming normal tissue, hepatocyte degeneration, precancerous, changes and HCC (original magnification ×100), respectively.

Forty-eight male Sprague-Dawley rats, 6 weeks old, weighing 150-160 g, were purchased from the Experimental Animal Center, Nantong University, China. All animals were treated according to the guidelines of Nantong University for the Care and Use of Laboratory Animals. The rats were randomly divided into 8 groups with 6 rats per cage. One group was selected as controls while the others made up the experimental groups. Among the 7 experimental groups one served as a substitute for accidental deaths. All rats were fed with general grain, except that the grain of the hepatoma model rats contained 0.05% 2-fluorenyl-acetamide (2-FAA, Sigma Chemical Co., USA). All rats were housed under bio-clean conditions. One control rat and one experimental rat were sacrificed every 2 weeks. All surgical procedures were conducted under deep ether anesthesia. Four ml of blood was drawn from the heart and anticoagulated with EDTA-K2. Plasma and karyocytes were separated and kept at -80 °C for further analysis. After washing off the blood, one liver

sample was fixed in 10% neutral buffered formalin and embedded in paraffin for pathological examination and immunohistochemical staining, and the rest were kept at -80 °C until use.

The changes of liver appearance and the pathohistology of hepatocytes during 2-FAA-induced rat HCC development are shown in Fig. 2. Apparent morphological changes (Fig. 2A-D) were confirmed pathohistologically examination (Fig. 2E-H). No pathological changes of rat hepatocytes were found in normal controls (Fig. 2E). During 2-FAA-induced hepatocarcinogenesis, granule-like degeneration in the cytoplasm of hepatocytes occurred at the early stage of hepatocarcinogenesis, with a few large and dysmorphic nuclei (degeneration group, $n=18$, Fig. 2F). An increase in the number of cell layers of hepatic plates was observed at the middle stage, at which there were more than 3 cell layers in some foci. Thickened chromatin in the nucleus was found, and the ratio of nucleus versus cytoplasm was elevated (precancerous group, $n=9$, Fig. 2G). At the late stage, the normal structure of liver tissue was completely destroyed, hepatocytes were rearranged to be nest-like and crudely cord-like, cellular nuclei were moderate in size and chromatin thickened, the ratio of nucleus versus cytoplasm was elevated, and the liver tissues were confirmed as highly differentiated (cancerous group, $n=9$, Fig. 2H).

Immunohistochemical staining confirmed positive expression of HIF-1 α as clear and brown particles, mainly located in the cytosol and nuclei, with no staining of the plasma membrane (Fig. 3). The positive staining was mostly located in the border of hepatic terminal portal venules or near the central veins (Fig. 2A). With carcinogenesis, the rate of HIF-1 α -positive expression increased and there was significantly higher intensity in the degeneration, precancerous, and cancerous groups than in the normal control (Table 1, $P < 0.05$), with a dynamically changing HIF-1 α expression intensity.

Fig. 3. Immunohistochemical staining with anti-HIF-1 α in rat HCC. A: HIF-1 α -positive expression in cytoplasm and cell membrane (S-P, original magnification $\times 200$) in HCC focus from rat hepatoma; B: HIF-1 α -negative expression, brown particles in cytoplasm and cell membrane (S-P, original magnification $\times 200$) in control rats.

Group	n	Positive (%)	Intensity of HIF-1α			
			-	+	++	+++
Control	6	0 (0.0)	6	0	0	0
Degeneration	18	14(77.8)	4	9	5	0
Precancerous	9	8(88.9)*	1	2	5	1
Cancerous	9	9(100)*	0	1	2	6

<0.01, compared with the control group.

Table 1. Comparative analysis of hepatic HIF-1α expression intensity at different stages of rat hepatocarcinogenesis

Reverse-transcribed HIF-1α cDNA from hepatic HIF-1α mRNA during the malignant alteration of rat hepatocytes was amplified by nested-PCR, and the sizes of amplified fragments were identical to the original designed ones, i.e., the size of the PCR product was 500 bp in the 1st PCR and 210 bp in the 2nd PCR (Fig. 4). The amplified fragments (210 bp) of the rat HIF-1α gene from the degeneration, the precancerous, and the cancerous livers were purified and confirmed by DNA sequencing. The alignments of their nucleotide sequences at the different stages of hepatocarcinogenesis by sequencing, and no alteration of the amplified gene fragment was found during the malignant alteration of rat hepatocytes.

Fig. 4. Amplification and alteration of HIF-1α gene from rat hepatoma tissues. In order to observe the alteration of HIF-1α at RNA level, HIF-1α mRNAs were synthesized to HIF-1α cDNA with random hexamers and moloney murine leukemia virus reverse-transcriptase, and detected with different primer pairs by nested PCR (210bp). The amplified positive fragments of HIF-1α gene were distinctly found in rat hepatoma tissues. HIF-1α mRNA in rat liver (Lanes. 1-4). Lane 1: control rat; lane 2: Degeneration rat; lane 3: precancerous rat; lane 4: HCC rat; marker: DNA molecular weight marker.

The levels of total RNA and HIF-1α mRNA expression in rat liver tissues during the malignant alteration of hepatocytes are shown in Table 2. The expression of total RNA and HIF-1α mRNA with histological alteration of hepatocytes was observed in the rats after treatment with 2-FAA, progressing from granule-like degeneration to precancerous lesions to HCC. The levels increased markedly in the cancerous group and the precancerous lesion group, and the incidence of amplified HIF-1α mRNA dynamically increased as hepatocytes

changed from normal to granule-like denaturation to precancerous and cancerous lesions. Induction of HIF-1α mRNA expression was detected in all the liver tissues of the cancerous group and parts of the precancerous lesion and degeneration group, i.e., 44.4% in the hepatocyte degeneration group, 77.8% in the precancerous lesion group, and 88.9% in the HCC group. It was significantly higher in the cancerous group than in the degeneration and the control groups, and the precancerous lesion group was also significantly higher than the normal control.

Group	n	Total RNA (μg/mg liver)	HIF-1α mRNA (%)
Control	6	1.58±0.49	0 (0.0)
Degeneration	18	1.91±0.60	8 (44.4)
Precancerous	9	2.00±0.21*	7 (77.8)*
Cancerous	9	2.86±0.60*	8 (88.9)*

*P<0.05, compared with the control group.

Table 2. Dynamic alterations of total RNA and amplification of HIF-1α mRNA in liver tissues at different stages of rat hepatocarcinogenesis

Nested PCR results revealed that HIF-1α mRNA was induced in hepatoma, precancerous and degenerative tissues, but was not expressed in normal tissues. During the course of cancer development, the levels of HIF1α mRNA in precancerous tissues were higher than in normal and degenerating tissues, and the levels of HIF-1α mRNA in HCC tissues were even higher than in precancerous tissues. The activation of HIF-1α gene transcription may participate in the signal transmission of cancer development. In the early stage, the expression of HIF-1α mRNA and HIF-1α was at low levels. In the advanced stage of cancer development, the expression of HIF-1α mRNA and HIF-1α was at high levels. The preneoplastic hepatic lesions showed increased levels of HIF-1α and HIF-1α mRNA compared with the normal liver. So the upregulation of hepatic HIF-1α protein synthesis and HIF-1α mRNA levels strongly suggest that HIF-1α participates in the development of HCC. The expression of HIF-1α gradually increased along with the histological changes, it was significantly higher in premalignant tissues than in the control group, and it may be related to the activation of signal pathways.

The quantitative data of hepatic and circulating HIF-1α expression are shown in Table 3. The HIF-1α levels showed a tendency to increase with the histopathological changes: cancerous group > precancerous lesion group > hepatocyte degeneration group > control group. The levels were markedly higher in the cancerous and precancerous lesion groups than in the hepatocyte degeneration and normal control groups. As a result of its low molecular weight, HIF-1α is easily released into the blood, leading to a higher concentration there. Blood levels in the precancerous lesion group were higher than in the hepatocyte degeneration and normal control groups (P<0.05). The HIF-1α blood levels in the cancerous group were markedly higher than in any other group (P<0.05). An apparent positive correlation between the levels in blood and liver samples was found (r=0.474, P = 0.030). The expression level of HIF-1α gradually increased both in liver cells and blood. The increasing tendency of hepatic and circulating HIF-1α was synchronized, suggesting that the increasing expression of HIF-1α is closely related to the malignant transformation of hepatocytes.

Group	n	HIF-1α in blood (μg/mg NP)	HIF-1α in liver (μg/mg)
Control	6	206.3±18.6	9.8±2.9
Degeneration	18	277.2±96.1	12.6±3.2
Precancerous	9	401.6±178.8*	16.9±2.2*
Cancerous	9	445.9±138.9*	23.5±8.7*

*P<0.05, compared with the control group.

Table 3. Quantitative analysis of HIF-1α dynamic expression in circulation and liver tissues at different stages of rat hepatocarcinogenesis

Increasing evidence suggests that HIF-1 activation occurs in the early stages of carcinogenesis. HIF-1α genes are overexpressed in morphologically normal single cells, forming multicellular foci or microcysts similar to overt HCC. HIF-1α was also shown to be expressed in a few cells in ductal hyperplastic areas adjacent to invasive cancer, and their malignant counterparts. Furthermore, HIF-1α genes were shown to be overexpressed in hyperplastic and dysplastic lesions during multistage carcinogenesis. Recent new findings from several laboratories have implicated constitutive activation of the transcription factor NF-κB as one of the early key events involved in neoplastic progression of chronic liver disease. Further studies will permit us to analyze mechanism of human hepatocarcinogenesis and to know how to target HIF-1α sites or RNA interference-mediated suppression of HIF-1α expression for HCC therapy. However, the combination of the pathological features of HIF-1α expression and some of the biomarkers with high sensitivity and specificity for early HCC seems to be more practical so far.

3. Expression difference in human HCC tissues

The self-controlled HCC and para-cancerous specimens (2 cm to cancer) were collected from 35 patients who underwent operations for liver cancer at the Affiliated Hospital of Nantong University. The specimens were immediately frozen in liquid nitrogen and kept at -85°C until required. The patients included 28 men and 7 women, ranging in age from 22 to 70 years. Prior written informed consent was obtained from all patients according to the World Medical Association Declaration of Helsinki, and the study received ethics board approval from the Affiliated Hospital of Nantong University. The histological types of all HCC specimens were graded in differentiation degrees as follows: well, 9; moderate, 12; and poor, 14. Of these specimens, 20 showed single tumor tubercles and the rest multiple; 14 were stage II, 13 were stage III, and 8 were stage IV. Each specimen was analyzed by total RNA abstraction and pathologic examination.

The expressions and cellular distribution of HIF-1α in HCC tissues and comparative analysis with their para-cancerous tissues are shown in Fig. 5. The positive HIF-1α was brown and granule-like, mainly presented in cytoplasm and few in nucleus, with obvious differences of HIF-1α positive expression intensity among different areas of tissues. HIF-1α staining in paracancerous tissues was showing significantly in the compressed hepatic cords and central veins. The intensity of HIF-1α expressions was significantly higher in paracancerous tissues than that in HCC, mainly due to more necrosis in the latter, representing that there is a very close relationship between high intensity of HIF-1α expressions and active proliferation or hypoxia microenvironment in paracancerous tissues. The distribution of positive cells was well-distributed and higher in adjacent areas of necrosis and tumor infiltration in HCC (Fig.5A), whereas it was showing significantly in the

compressed hepatic cords and the border of central veins in the para-cancerous tissues (Fig.5B). Moreover, the HIF-1 α positive staining was significantly higher ($P=0.017$) in the para- cancerous group (100%, 35 of 35) than in the corresponding HCC group (80%, 28 of 35). The intensity of hepatic HIF-1 α expression was also higher in the para-cancerous tissues than in the HCC tissues ($Z =4.728$, $P< 0.001$, Table 4).

Fig. 5. Immunohistochemical staining of HIF-1 α , total RNA levels and amplification of HIF-1 α mRNA in HCC or their paracancerous tissues. Hepatic HIF-1 α expression with brown particles in cytoplasm and cell membrane, A, the HCC tissue; B, the para-cancerous tissue (S-P, original magnification $\times 200$). C, the levels of total RNA expression in HCC or their paracancerous tissues; D, the HIF-1 α mRNA was synthesized to HIF-1 α cDNA and amplified by nested PCR (349 bp), Line 1, 2, the amplified fragment of HIF-1 α mRNA in HCC tissues; Line 3, 4, the amplified fragment of HIF-1 α mRNA in para-cancerous tissues; M, DNA marker with molecular weight standard. HCC, the hepatocellular carcinoma tissues; Para-HCC, the para-cancerous tissues.

HCC is mostly characterized by uncontrolled growth of tumor cells. Increasing oxygen consumption results in hypoxic microenvironment. HIF-1 α expression is significantly high in adjacent areas of necrosis and tumor infiltration. Many factors, such as hypoxia, oncogenes activation, inactivation of tumor suppressors, growth factors, inflammatory factors, can up-regulate HIF-1 α expressions, directly or indirectly promoting more than 2 %

human genes transcriptions, which are all related to oxygen and energy metabolism. Productive nucleic acids metabolism, abnormal gene expressions, development of HCC are closely associated with surrounding vessels state and hypoxic conditions (20).

Group	n	Positive (%)	P value*	HIF-1α intensity					
				Z value P value*					
				–	+	++	+++		
HCC	35	28 (80.0)	0.017	7	21	7	0	4.728	0.000
Para-HCC	35	35 (100)		0	10	18	7		

*P value vs the paracancerous tissue group; HCC, the hepatocellular carcinoma tissues.

Table 4. The comparative analysis of HIF-1α expression intensity in HCC or their paracancerous tissues

Fig. 6. Alignment of the amplified fragments of HIF-1α gene and homology analysis of their sequences. The HIF-1α mRNA from cancerous tissue and para-cancerous tissue of HCC patients was synthesized to HIF-1α cDNA and amplified by nested PCR (349 bp) and confirmed by sequencing. No mutation was found between HCC tissues and para-cancerous tissue. HIF-1α: the cited sequence (349 bp, nt 2452-2800) of human HIF-1α genome (NM_001530); HCC, the amplified fragment of HIF-1α genome from HCC tissues; Para-HCC, the amplified fragment of HIF-1α genome from their paracancerous tissues.

Hepatic total RNA was purified from human HCC or their para- cancerous tissues, the specific concentrations of total RNA were $12.4 \pm 7.3 \mu\text{g}/\text{mg}$ wet liver in the HCC group, and $53.8 \pm 52.0 \mu\text{g}/\text{mg}$ wet liver in the para-cancerous group (Fig.5C), with significant difference between them ($t = 3.05, P < 0.01$). The final amplified fragment of hepatic HIF-1 α gene was 349 bp (Fig.5D), and the incidence was 85.7% in the HCC group and 100 % in the para-cancerous group ($P > 0.05$). The amplified fragments of HIF-1 α gene were confirmed by sequencing, with consistent completely with the cited sequence of human HIF-1 α gene (Fig.6). The level of total RNA was obviously higher in paracancerous tissues than in HCC, indicating HIF-1 α mRNA involved in cell proliferation, neovascularization and metastasis and could be a prime target for gene therapy.

4. Expression of circulating HIF-1 α in HCC

One hundred thirty-one of HCC patients, 30 of chronic hepatitis, 22 of acute hepatitis, and 37 of cirrhosis were diagnosed at the Affiliated Hospital of Nantong University, Nantong, China, and 27 healthy people obtained from the Nantong Central Blood Bank as controls (Table 5). All cases were diagnosed by blood biochemical tests, with negative hepatitis viral markers (HBsAg, and anti-HCV antibody), normal alanine aminotransferase (ALT) levels, and B-ultrasonic examination. All samples (5 mL of peripheral blood) were collected in the morning and sera were separated at once. The serum AFP concentrations exceeded $50 \mu\text{g}/\text{L}$ were taken as a positive result. The diagnosis of HCC and viral hepatitis was based on the criteria proposed by Chinese National Collaborative Cancer Research Group (21) and at the Chinese National Viral Hepatitis Meeting (22), respectively.

Group	n	Sex	Age	HBsAg	AFP ($\mu\text{g}/\text{L}$)		
		M/F	(Year)	(+/-)	≤ 20	21-399	≥ 400
HCC	131	109/22	33-85	100/31	20	57	54
LC	37	16/21	20-82	23/14	24	11	2
CH	30	25/5	18-63	22/8	18	11	1
AH	22	17/5	24-80	10/12	18	4	0
NC	27	12/15	26-69	0/27	27	0	0

HCC, hepatocellular carcinoma; LC, liver cirrhosis; CH, chronic hepatitis; AH, acute hepatitis; NC, normal control.

Table 5. Patients’ data in the present study

The levels of circulating HIF-1 α expression in 220 patients with liver diseases are shown in Table 6. The circulating HIF-1 α level was increased, especially in patients with chronic liver diseases. If the cutoff value of serum HIF-1 α level was $>50 \mu\text{g}/\text{L}$, the incidence of HIF-1 α abnormality was 100 % in HCC, 89.2% in LC, 66.7 % in CH, none in AH or NC, respectively; And the cutoff values rise to $100 \mu\text{g}/\text{L}$, the abnormality of circulating HIF-1 α level was 90.8% in HCC and 27.0% in LC, none in CH or AH or NC, respectively. The level of serum

HIF-1α in HCC patients was significantly higher ($P < 0.001$) than those in cases with benign liver diseases.

The prognosis of HCC is poor, and early detection is of the utmost importance. Treatment options are severely limited by the frequent presence of metastases. Although the mechanisms of hepatocarcinogenesis have not been elucidated, a long-lasting inflammation induced by hepatitis virus infection is a definite risk for neoplastic degeneration and accumulation of genetic alterations. The fragments of circulating HIF-1α could be detected in all patients with HCC with extrahepatic metastasis; like circulating IGF-II, these results argue for growth factor-dependent HCC development and could provide novel markers of severity and prognosis for HCC. The present data indicate that the expression levels of serum HIF-1α, Ang-2, and VEGF could be detected only in the peripheral blood of patients with HCC.

Group	n	HIF-1α (μg/L)		>50 μg/L	>100 μg/L
		Ranges	Mean ± SD	n (%)	n (%)
HCC	131	57.5~208.5	136.3 ± 28.8	131(100)	119(90.8)
LC	37	39.1~123.4	84.6 ± 25.9*	33(89.2)	10(27.0)*
CH	30	38.0~96.4	58.8 ± 14.5*a	20(66.7)*	0(0)*
AH	22	33.1~48.8	37.6 ± 5.3*bc	0(0)*	0(0)*
NC	27	20.3~31.9	24.1 ± 3.3*de	0(0)*	0(0)*

* $P < 0.001$, *vs* the HCC group ; ^a $P < 0.001$, *vs* the liver cirrhosis group ($q = 4.39$) ; ^b $P < 0.01$ *vs* the chronic hepatitis group ($q = 3.17$) ; ^c $P < 0.001$, *vs* the liver cirrhosis group ($q = 7.31$) ; ^d $P < 0.001$, *vs* the chronic hepatitis group ($q = 5.47$) ; ^e $P < 0.001$, *vs* the liver cirrhosis group ($q = 9.99$) ; HCC, hepatocellular carcinoma; LC, liver cirrhosis; CH, chronic hepatitis; AH, acute hepatitis; NC, normal control.

Table 6. Quantitative analysis of circulating HIF-1α level (mean ± SD) in patients with liver diseases

The evaluation of serum HIF-1α and AFP levels for HCC diagnosis using the ROC curves is shown in Fig.7. The advantage of analyzing two markers over the whole range of sensitivities and specificities using the area (0.854 in AFP, 0.909 in HIF-1α) under ROC curves indicated that the abnormality of serum HIF-1α level could be a useful serological marker for HCC diagnosis.

5. Quantitative detection of VEGF and Ang-2

The levels of serum VEGF and Ang-2 were detected and the concentrations were calculated using a standard curve generated with specific standards. Inter and intra-assay variances were lower than 10%. The levels of circulating VEGF and Ang-2 expression in patients with chronic liver diseases are shown in Table 7. Like circulating HIF-1α expression, the circulating VEGF and Ang-2 levels were increased in patients with chronic liver diseases, especially in HCC patients. If the cutoff value with >280 μg/L for VEGF and >35 μg/L for

Ang-2, the incidence of VEGF and Ang-2 were 87.0 % and 94.7 % abnormality in HCC, 12.7 % and 2.9 % in LC, 13.3 % and 0% in CH, both none in NC, respectively.

Fig. 7. The relationship between circulating HIF-1α and AFP levels and receiver operating characteristic (ROC) curves. A, the scatter diagram of circulating HIF-1α and AFP levels in HCC patients, and no significant relationship was found between circulating HIF-1α and AFP levels; B, ROC curves for circulating HIF-1α and AFP investigated markers for HCC. Sensitivity = true-positive rate; specificity = false-positive rate; and the area under ROC curves was 0.854 for AFP and 0.909 for HIF-1α. Sensitivity and specificity were calculated according to the following formulas: Sensitivity = $a/(a+c)$; and Specificity = $d/(b+d)$, where a = true-positive cases, b = false-positive cases, c = false-negative cases, and d = true-negative cases. ROC curves were constructed by calculating the sensitivities and specificities at several cutoff points, and indicated that both of circulating HIF-1α and AFP level be useful molecular markers for HCC diagnosis.

HCC is known to contain aberrantly vascularized regions characterized by severe hypoxia. Hypoxia can stimulate cell proliferation, induce angiogenesis, accelerate invasion and is also responsible for treatment resistance in HCC. Activation of oncogenes or inactivation of tumor suppressors can change signaling pathway and up-regulate HIF-1α expression, leading to HIF-1α activation. Under hypoxic conditions it can be stabilized, binding to the specific sites of hypoxia-response target genes, regulating proliferation on transcriptional level and activating expression of many hypoxia-response genes, which are closely relevant with energy metabolism, angiogenesis, infiltration, metastasis and prognosis. The frequency of circulating HIF-1α and its diagnostic value increased with distal metastases of HCC hepatocytes. The pathological characteristics of serum HIF-1α associated with the levels of circulating VEGF and Ang-2 expression, the size of tumor and extra-hepatic metastasis, and but not to patients' gender, age, and AFP level.

Group	n	VEGF (μg/L) >280 μg/L		Ang-2 (μg/L) >35 μg/L	
		Mean ± SD	n (%)	Mean ± SD	n (%)
HCC	131	462.7±119.2	114(87.0)	40.8±3.5	124(94.7)
LC	37	216.3±54.5*	6(16.2)*	25.5±5.8*	1(2.7)*
CH	30	160.9±98.2*	4(13.3)*	20.9±7.1*	0(0)*
NC	27	140.9±54.5*	0(0)*	17.4±2.6*	0(0)*

*P < 0.001, *vs* the HCC group ; HCC, hepatocellular carcinoma; LC, liver cirrhosis; CH, chronic hepatitis; NC, normal control.

Table 7. The levels of circulating VEGF and Ang-2 expression in patients with chronic liver diseases

6. Clinicopathological features of HIF-1α expression

The clinicopathological characteristics of circulating HIF-1α expression in HCC patients are shown in Table 8. Significant difference was found between high HIF-1α expression and tumor size (P=0.007) or HCC with extra-hepatic metastasis (P< 0.001), but not with patients’ gender, age, or AFP level. There was a very close relationship between circulating HIF-1α level and VEGF (r=0.937, P <0.001) or Ang-2 (r= 0.933, P < 0.001), suggesting that high expression of HIF-1α associated with HCC metastasis and poor prognosis. Clinical pathological features of HIF-1α expression indicated that HIF-1α expression intensity and positive rate was lower in HCC than in paracancerous tissues, which were in accordance with total RNA. HIF-1α positive rate was associated with tumor diameter, because they were usually singles, enveloped, well-differentiated, more diplonts and less heteromorphism when tumors were small. With tumors swelling, the biological characteristics have changed, developed into the opposite. Therefore, the invasion is strengthened, and tumor blood supply can not satisfy growth demand. HBx and HIF-1α are presented in cytoplasm in HCC. Moreover, HBx can up-regulate HIF-1α under normoxia or hypoxia, reinforce HIF-1α transcriptional activity via MAPK pathway, increase HIF-1α protein levels, induce neovascularization and contribute to metastasis. No correlation was found between HIF-1α and HBsAg positive in HCC and further studies whether it associates with HBV replication are required.

7. Effect of miRNA silencing HIF-1α gene on HCC

In order to investigate the effect of miRNA silencing HIF-1α gene on inhibition of HepG₂ cell proliferation. Recently, we constructed the eukaryotic expression plasmids of HIF-1αmiRNA and report gene containing hypoxia-reponse element. After HepG₂ cells transfection with plasmid, the expression of HIF-1α gene and protein were determined by real time-PCR or Western blotting. The expressions of HIF-1α, VEGF, and Ang-2 were quantitatively detected by ELISA. The alterations of cell cycles and apoptosis rate were quantitatively measured by flow cytometry or Annexin V-FITC/PI double dyeing assay. At 72h After HepG₂ cell transfection with HIF-1αmiRNA, the down- regulation of HIF-1α was 87% at mRNA or 56% at protein level, and the decreasing of target gene was 46% in the

report gene, 54% in VEGF and 36% in Ang-2, respectively. The apoptotic ratio of HepG₂ cells was $22.46 \pm 0.61\%$ ($P < 0.01$), and the cell cycle changed greatly at the ratio of G₁ ($61.49 \pm 1.12\%$) and S phase ($22.40 \pm 0.58\%$, $P < 0.01$). After the cells combined with doxorubicin, the apoptotic ratio increased to $36.99 \pm 0.88\%$. The ratio of G₁ and S phase were upregulated to $65.68 \pm 0.91\%$ and $19.47 \pm 1.34\%$. HIF-1 α miRNA or / and doxorubicin can regulate the growth cycle, promote apoptotic and inhibit proliferation of HepG₂ cells.

Group		n	HIF-1 α (μ g/L)	t value	P value
HCC		131	136.3 $\pm$ 28.8		
Sex	Male	109	137.4 $\pm$ 28.7	1.009	0.315
	Female	22	130.7 $\pm$ 29.0		
Age	≥ 50 y	98	133.7 $\pm$ 30.1	1.702	0.091
	< 50 y	33	143.2 $\pm$ 24.0		
Tumor size	≥ 5.0 cm	53	144.4 $\pm$ 26.3*	2.721	0.007
	< 5.0 cm	78	130.8 $\pm$ 29.2		
AFP(μ g/L)	≥ 400.0	53	136.9 $\pm$ 25.8	0.201	0.841
	< 400.0	78	135.9 $\pm$ 30.7		
HBsAg	Positive	100	137.9 $\pm$ 29.6	1.712	0.089
	Negative	31	129.2 $\pm$ 25.5		
EHT	Yes	49	152.5 $\pm$ 21.5**	5.522	0.000
	No	82	126.6 $\pm$ 28.3		

* $P < 0.01$, *vs* the tumor size less than 5cm group; ** $P < 0.001$, *vs* the non- extrahepatic metastasis group; HCC, hepatocellular carcinoma; EHT, Extra- hepatic metastasis.

Table 8. The pathological characteristics of HIF-1 α levels (mean $\pm$ SD) in sera of HCC patients.

8. Perspectives

HCCs exhibit numerous genetic abnormalities as well as epigenetic alterations including modulation of DNA methylation (23, 24). Molecular factors are involved in the process of HCC development and metastasis (25~27). Recent findings from several laboratories have implicated constitutive activation of the transcription factor NFkappa B 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 (28~31). However, the hepatic HIF-1 α expression is associated with development and prognosis of HCC, and circulating HIF-1 α level is a useful molecular marker in HCC diagnosis, and monitor prognosis (32~36). HIF-1 α expression in hepatic tissues plays an important role in development and prognosis of HCC. HIF-1 α , as an initial hypoxia moderator, should be a promising molecular-target for the development of anti-HCC agents (37~40). The intensity of HIF-1 α expressions was significantly higher in paracancerous tissues than in HCC, mainly due to more necrosis in the latter, representing that there is a very close relationship between high intensity of HIF-1 α expressions and active metabolism or hypoxia microenvironment in paracancerous tissues and HIF-1 α could be a molecular-target for gene therapy (41, 42).

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