PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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LIVER CANCER

Prognostic significance of HIF-2α/EPAS1 expression in hepatocellular carcinoma

Gassimou Bangoura, Zhi-Su Liu, Qun Qian, Cong-Qing Jiang, Gui-Fan Yang, Sun Jing

Gassimou Bangoura, Zhi-Su Liu, Qun Qian, Cong-Qing Jiang, Department of General Surgery and Laboratory of Liver Cancer, Zhong Nan Hospital, Wuhan University School of Medicine, Wuhan 430071, Hubei Province, China
Gui-Fang Yang, Department of Pathology, Zhong Nan Hospital, Wuhan University School of Medicine, Wuhan 430071, Hubei Province, China
Sun Jin, Department of Health Statistics, School of Public Health, Wuhan University, Wuhan 430072, Hubei Province, China
Supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of China
Correspondence to: Zhi-Su Liu, Professor, Department of General Surgery and Liver Cancer Laboratory, Zhong Nan Hospital, Wuhan University School of Medicine, Wuhan 430071, Hubei Province, China. liuzs53@yahoo.com
Telephone: +86-27-67813007 Fax: +86-27-67812892
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Abstract

AIM: To evaluate the prognostic significance of HIF-2α/EPAS1 expression in hepatocellular carcinoma (HCC).

METHODS: Surgical specimens from 315 patients with HCC as well as 196 adjacent noncancerous lesions and 22 cases of normal liver tissue were investigated by immunohistochemistry (IHC) for HIF-2α/EPAS1 using a standard detection system. Correlations with clinicopathological factors, VEGF, microvessel density (MVD), and prognosis were analyzed.

RESULTS: Immunoreactivity of HIF-2α/EPAS1 was positive in 69.5% of HCC, 55.6% of adjacent noncancerous tissue, and 0% of normal liver tissue. And it was significantly correlated with tumor grade, venous invasion, intrahepatic metastasis, necrosis, and capsule infiltration. Correlation analysis of HIF-2α/EPAS1 with angiogenic factor VEGF ($P < 0.001$), and MVD ($P = 0.016$) was also noted. HIF-2α/EPAS1 protein was less frequently expressed in low MVD cases, whereas a high rate of expression was noted in cases with both medium and high MVD ($P = 0.042$). By Kaplan-Meier analysis, strong HIF-2α/EPAS1 staining (> 50% of tumor cells) in HCC correlated with a shortened survival in patients (Cox's regression, $P < 0.001$, $r = 3.699$).

CONCLUSION: We conclude that HIF-2α/EPAS1 expression may play an important role in tumor progression and prognosis of HCC. Assessment of HIF-2α/EPAS1 expression in HCC may be used as a diagnostic tool and possibly a target in the treatment of HCC.

Key words: Hepatocellular carcinoma; HIF-2α/EPAS1; Angiogenesis; IHC; Prognosis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a common neoplasm in oriental countries and South Africa. Although most HCCs are caused by HCV in Japan[1] and by intake of aflatoxin in Africa[2], the most common and dominant cause in China is HBV[3]. Over the past two years, great progress has been made in the diagnosis of HCC using such techniques as ultrasonography and computer tomography. With earlier diagnosis of this cancer, the number of resected cases has increased substantially. Consequently, the number of patients who survive for extended periods after hepatectomy recently has increased. Nonetheless, in spite of enormous efforts to improve clinical treatment, HCC remains a major carcinoma with high mortality. Poor differentiation, large tumor size, portal invasion and intrahepatic metastasis are known to shorten the disease free survival in this carcinoma.

Hypoxia has been demonstrated to be present in proliferative tumors[4]. The intratumoral hypoxia has been shown to correlate with tumor invasiveness, progression, and metastasis[5,6]. It is well-known that tumor cannot grow in the absence of angiogenesis, because of the lack of oxygen in the center of tumors[7], which results in apoptosis and necrosis. Many tumors grow in a hypoxic microenvironment, a condition associated with poor prognosis and resistance to clinical treatment. With angiogenesis, the tumor grows rapidly and can metastasize to remote sites[8]. This is a multistep process regulated by a balance between pro and antiangiogenic molecules produced by the tumor and host component cells[9]. To date, many factors promoting or inhibiting angiogenesis have been identified, including growth factors, cytokines

and proteases[10]. Among these factors, vascular endothelial growth factor (VEGF) is a vascular endothelium-specific growth factor and plays a central role in tumor angiogenesis, implicating in tumor-associated microvascular hyperpermeability and carcinogenesis. Over expression of VEGF mRNA and/or protein correlates with MVD, invasiveness, and poor prognosis in various cancers[11,12].

Recently, hypoxia inducible factors (HIF-2α/Endothelial PAS domain protein1 [EPAS1] and HIF-1α) have been identified. They are members of the basic helix-loop-helix/Per Arnt-Sim (PAS) transcription factors induced under hypoxia. When dimerized with the aryl hydrocarbon receptor nuclear translocator, both HIF-1α and HIF-2α/EPAS1 can induce the cascade of physiological response, including VEGF, glycolytic enzymes, tyrosine hydroxylase, and transferrin[13,14]. These genes are important for tumor adaptation to hypoxia, implicating the possible role of HIFs in tumor progression.

A study has recently found that the introduction of HIF-2α/EPAS1 cDNA led to up-regulation of endogenous VEGF in 293 human fetal kidney cell lines and HIF-2α/EPAS1 was involved in the angiogenesis of renal carcinoma[15]. In addition, overexpression of HIF-2α/EPAS1 was characterized in a number of human cancers and showed a close correlation with tumor metastatic activity[16,17]. Thus far, little is known about its expression in hepatic malignancies. Herein, we investigated the expression of HIF-2α/EPAS1 proteins in a series of patients with HCC, who were treated in our institution. In addition, adjacent noncancerous lesions and normal liver tissue were also detected.

MATERIALS AND METHODS

From January 1997 to December 2004, 590 consecutive patients with newly diagnosed HCC were managed at the Department of Surgery of Wuhan University Hospital. Because 197 patients were diagnosed as contraindication for resection of the liver mainly because of far-advanced tumor and/or poor hepatic functional reserve, they were excluded from this study. The other 479 consecutive patients underwent curative surgical resection. After discharged from the hospital, 78 patients were lost to follow up. The remaining 315 patients were enrolled in this study. There were 294 men and 21 women with age ranging from 46 to 79 years averaging 60.8. Serum samples obtained before surgery from all patients were assayed for HBV and HCV. One hundred ninety-six samples of paraneoplastic tissue were taken from adjacent noncancerous tissue 1 cm away from the tumor margin. Twenty-two samples of normal liver tissue were taken around the hepatic hemangioma. The diagnosis of all patients was confirmed histologically on resected specimens. None of the patients received preoperative chemotherapy or embolization therapy. Tumor recurrence and metastasis were clinically confirmed in 147 (46.7%) and 72 (22.9%) of 315 patients, respectively. The most popular treatment for recurrent HCC was percutaneous ethanol injection treatment (PEIT) in 79 patients (25.1%), followed by transhepatic arterial chemoembolization (TACE) in 46 (14.6%) or hepatic arterial infusion (HAI) chemotherapy in 22 patients (6.9%). The follow up of surviving patients at the time of this study was 715-2555 d (median 1616 d).

Immunohistochemical study

Immunohistochemical study was performed using the avidin-biotin-complex (ABC) method. Tissue sections of 4 μm thick were deparaffinized and rehydrated sections were treated with 0.3% hydrogen peroxide in methanol for 15 min at room temperature to block endogenous peroxidase activity, and then washed in phosphate-buffered saline (PBS) and incubated in 10% normal goat serum in PBS for 10 min to reduce nonspecific antibody binding. Mouse mAb anti-HIF-2α/EPAS1 (190b; Santa Cruz Biotechnology. Inc) was used as the primary antibody at a dilution of 1/80. Dilutions of mAb against VEGF (NeoMarker, Fremont, CA) and CD31 (JC70; DAKO, Glostrup, Denmark) were 1/25 and 1/50, separately. These slides were incubated with primary antibodies for 60 min at room temperature, followed by 3 washes with PBS. Sections were then incubated with biotinylated IgG for 20 min followed by 3 washes. The avidin-biotin complex (strept ABC complex kit, DAKO, Mississauga, Ontario, Canada) technique and 3, 3-diaminobenzidine/hydrogen peroxide were used to detect antigen-antibody binding. Negative controls were included by replacement of the primary antibody with PBS. All IHC slides were counterstained with hematoxylin.

Scoring of HIF-2α/EPAS1 expression

Immunoreactivity was scored as negative to intensive according to the percentage of positive cells and the intensity of staining. The cases in which more than 65% of cells were stained intensely (+++), or moderately (++) or weakly (+) were considered positive; and those stained equivocally (±) or not stained were considered negative (-). Only strong, moderate and weak reactions were judged to be true positive because equivocal staining might include false-positive results. According to this grading criterion, three independent pathologists examined all the immunostained specimens blinded to the clinical data of patients. In cases of disagreement, which occurred in 10% of the cases, a fourth pathologist was recruited for final evaluation.

VEGF and MVD detection and assessment

A smaller isoform ($VEGF_{165}$) of VEGF was used for identification and evaluation of VEGF expression in this study. In brief, paraffin-fixed slides were autoclaved for 7 min in 10 mmol/L citrate buffer pH 6.0 before deparaffinization and rehydration. Evaluation of the staining was semi-quantitatively graded based on scores determined by intensity distribution as: strong +++ (dark brown), moderate ++ (brown), weak + (light brown), or negative - (no staining). The scores were determined by three pathologists independently blinded to patients' data.

For microvessel quantification, the immunostained tissue sections were assessed simultaneously by two pathologists using a confocal microscope. Each pathologist gave each section a score, and discrepancies were resolved

Table 1 Expression of HIF-2α/EPAS1 in HCC

	Less than paraneoplastic tissue	Equal to paraneoplastic tissue	Greater than paraneoplastic tissue
HIF2α/EPAS1	9 (4.8%)	59 (31.6%)	119 (63.6%)

through discussion. Both pathologists were blinded to the clinical data of the patients. Briefly, slides were first observed at low magnification (× 40, and × 100). Areas with the highest vascularization within the tumor tissue (adjacent to the normal liver tissue) were chosen, and microvessels were counted in three chosen fields (× 200). Microvessels adjacent to necrotic areas were excluded from appraisal. The final MVD was the mean of the vessel counts obtained in the fields.

Statistical analysis

SPSS software (version 11.5) was used for statistical analysis. The correlations between expression of HIF-2α/EPAS1 and clinicopathologic parameters, VEGF, or MVD were assessed with the Chi-square test or the Spearman correlation test as appropriate. The Kaplan-Meier method was used to estimate survival as a function of time, and the log-rank test was used to determine survival differences. The Cox regression model was used for multivariate analysis of prognostic factors. A P value < 0.05 was considered to be significant.

RESULTS

Expression of HIF-2α/EPAS1 in HCC and non-cancerous liver tissue

Of the 196 cases of non-cancerous tissue, 109 cases (55.6%) were positive, and 87 cases (44.4) were negative for HIF-2α/EPAS1. Of the 315 HCC cases, 219 and 96 were classified as positive and negative, respectively. In 119 cases (63.6%), HIF-2α/EPAS1 in HCC was higher than in non-cancerous liver tissue, whereas 59 cases (31.6%) and 9 cases (4.8%) expressed this protein in HCC at a level equal to or less than that in cancerous liver tissue (Table 1). The HIF-2α/EPAS1 protein was localized mainly in the cytoplasm of the cells in the cancerous tissue as well as in the surrounding noncancerous tissue with similar intensity (Figure 1A and B). Overexpression of HIF-2α/EPAS1 was found in perinecrotic areas (Figure 1C), and also in the cytoplasm of macrophages (Figure 1D).

Correlation between HIF-2α/EPAS1 expression and clinicopathological features

As shown in Table 2, the positive expression of HIF-2α/EPAS1 was significantly correlated with tumor grade (P = 0.0541), venous invasion (P = 0.001), necrosis (P = 0.037), intrahepatic metastasis (P = 0.045), and capsule infiltration (P = 0.011). In contrast, no statistically significant correlation was found with age, gender, tumor size, virus status, cirrhosis, capsule formation, and Child's classification.

Table 2 Correlation of clinicopathologic parameters with HIF-2α/ EPAS1 expression

		HIF-2α/EPAS1			
Clinicopathologic parameters	*n*	Positive	Negative	χ^2	*P*
Age (yr)					
⩾ 65	214	143	71	2.299	0.129
< 65	101	76	25		
Gender					
Male	260	175	85	0.452	0.063
Female	55	44	11		
Liver cirrhosis					
Present	142	101	41	0.314	0.576
Absent	173	118	55		
Hepatitis B					
Present	212	144	68	0.783	0.376
Absent	103	75	28		
Hepatitis C					
Present	22	17	5	0.67	0.413
Absent	293	202	91		
Child's classification					
A	182	129	53	0.371	0.541
B	133	90	43		
Tumor size (cm)					
⩽ 2	62	20	42		
2-5	97	52	45	3.417	0.065
> 5	156	147	9		
Tumor capsule					
Present	118	87	31	1.575	0.21
Absent	197	132	65		
Capsule infiltration					
Present	75	61	14	6.479	0.011
Absent	240	158	82		
Vascular invasion					
Present	73	62	11	10.646	0.001
Absent	242	157	85		
Edmondson-Steiner class					
I	78	29	49		
II	100	53	47	5.081	0.024
III	137	137	0		
Intrahepatic metastasis					
Present	97	75	22	4.02	0.045
Absent	218	144	74		
Necrosis					
Present	108	67	41	4.348	0.037
Absent	207	152	55		

Correlation between HIF-2α/EPAS1 and angiogenic markers

HIF-2α/EPAS1 expression was examined by both VEGF and tumor vascularity in all 315 cases. Positive expression of VEGF was found mostly in the cytoplasm of tumor cells (Figure 1E). VEGF expression and high MVD (Figure 1F) were observed at significantly higher levels in tumors with positive HIF-2α/EPAS1 than in tumors with negative HIF-2α/EPAS1 (P < 0.001 and P = 0.016); respectively (Table 3). Similar correlation with tumor grade (P = 0.007), tumor size (P = 0.044), venous invasion (P = 0.006), capsule infiltration (P = 0.049), and intrahepatic metastasis (P = 0.018) were observed for VEGF expression (data not shown).

HIF-2α/EPAS1 and patient survival

In the entire cohort, the overall survival rates of patients with HIF-2α/EPAS1-negative tumors were significantly

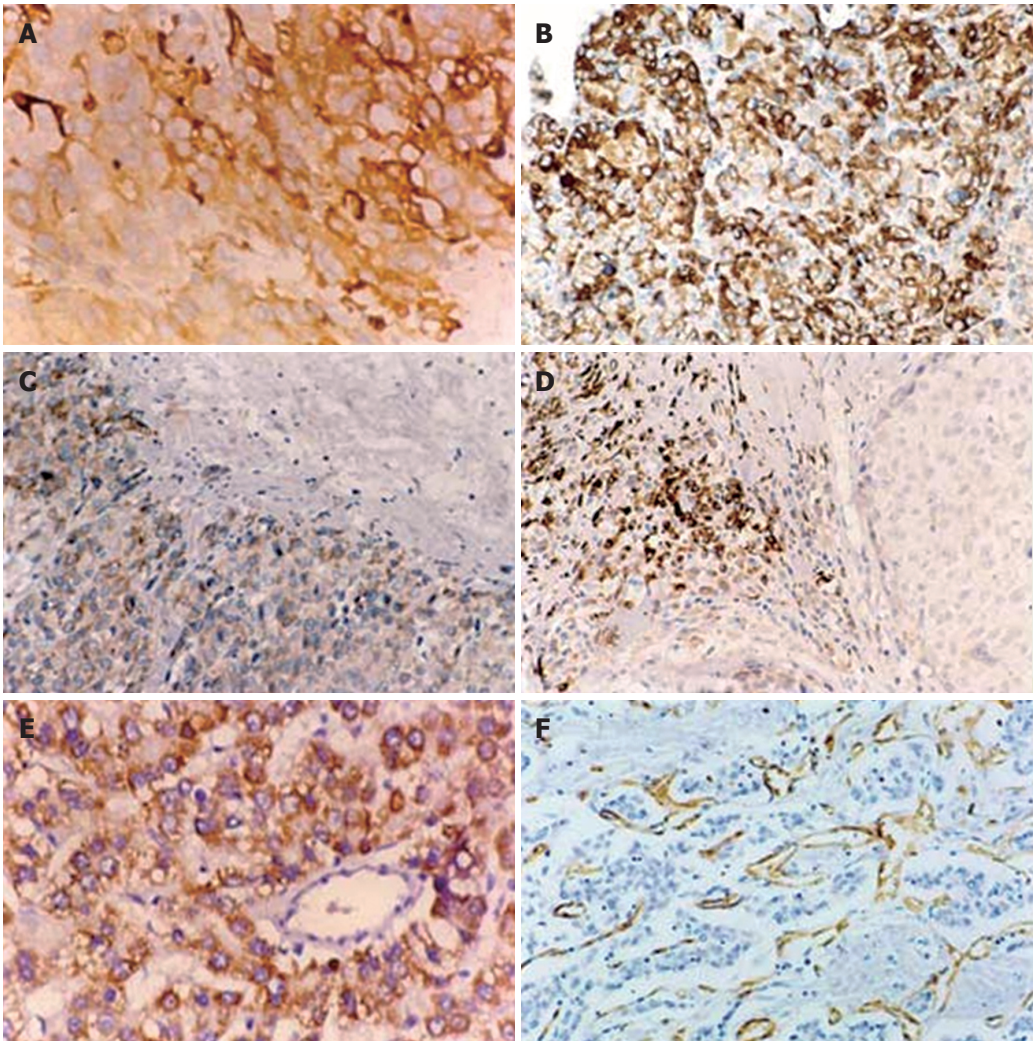

Figure 1 Representative examples of immunohistochemical staining for HIF-2α/EPASE1, VEGF, and CD31 in HCC. **A**: Strong cytoplasmic immunoreactivity of HIF-2α/EPASE1 is observed in cancers cells (× 400). **B**: Strong staining in the cytoplasm of noncancerous cirrhotic tissue (× 400). **C**: HIF-2α/EPAS1-positive staining in perinecrotic area (N) and **D**: In the cytoplasm of macrophages (× 400). **E**: Parallel studies of VEGF protein (cytoplasmic staining) and **F**: CD31 (for microvessels) immunohistochemistry performed on HCC (× 200).

Table 3 Correlation of HIF-2α/EPAS1 expression with VEGF expression and MVD in HCC

Parameters	*n*	HIF-2α/EPAS1 expression		*P*
		Positive	Negative	
VEGF				
Weak	56	33	23	
Moderate	70	40	30	
Strong	114	82	32	0
Negative	75	64	11	
MVD				
Low	99	57	42	
Medium	103	71	32	0.016
High	113	91	22	

Table 4 Multivariate survival analysis (Cox's regression)

Parameters	β	SE	Relative risk	*P*	95% CI
Tumor size (cm) (≤ 2-5 *vs* ≥ 5)	0.341	0.183	1.659	0.766	0.762-0.456
Edmondson-Steiner class (I, II *vs* III)	0.157	0.095	1.17	0.91	0.986-1.083
Necrosis (no *vs* yes)	0.252	0.158	1.287	0.881	0.425-5.820
Venous invasion (no *vs* yes)	0.079	0.13	1.521	0.001	2.564-17.617
Tumor capsule (no *vs* yes)	0.366	0.149	1.269	0.633	0.824-1.544
Capsule infiltration (no *vs* yes)	0.552	0.148	1.737	0.832	0.562-5.234
Intrahepatic metastasis (no *vs* yes)	0.477	0.153	1.611	0.002	1.081-3.872
Child's class (A *vs* B)	0.592	0.173	1.646	0.373	0.195-1.873
HIF-2α/EPAS1 (no *vs* yes)	1.308	0.176	3.699	0	1.385-10.236

higher when compared with those of the HIF-2α/EPAS1-positive group ($P = 0.003$; log-rank test; Figure 2A). A significant difference in survival between the two groups of patients with and without venous invasion was also observed ($P = 0.014$; Figure 2B). The 5 year survival rate of HIF-2α/EPAS1 positive and negative cases was 48% and 78%, respectively.

Furthermore, in multivariate analysis, most of the potential prognostic factors representing tumor aggressiveness such as tumor size, tumor grade, venous invasion, intrahepatic metastasis, necrosis, Child's classification, tumor capsule, capsule invasion, and HIF-2α/EPAS1 expression were included in the Cox's regression model. The results indicated that, in addition to venous invasion and intrahepatic metastasis, HIF-2α/EPAS1 expression was an independent significant prognostic factor ($P < 0.001$; Table 4).

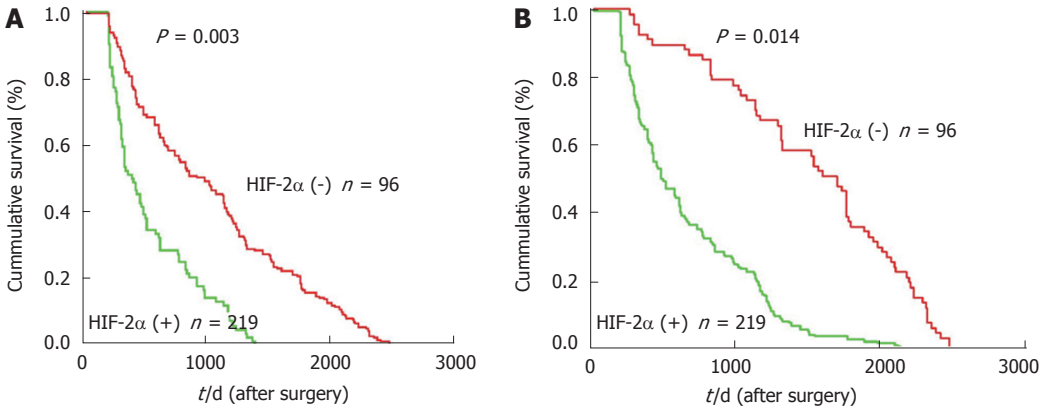

Figure 2 The overall 7-year survival curves of patients with positive HIF-2α/EPAS1 and negative HIF-2α/EPAS1 are shown for the entire cohort, $P = 0.003$ (**A**), as well as patients with venous invasion and without venous invasion, $P = 0.014$ (**B**).

DISCUSSION

It is becoming increasingly apparent that hypoxia is central to tumor angiogenesis and that high expression of HIF-α family is predictive of cancer progression and associated with a poor prognosis in human tumors. To further characterize the angiogenic effect of HIF-2α/EPAS1 protein in HCC, a parallel IHC study of VEGF was performed, in which VEGF was assessed as a major marker of angiogenesis. In addition, to study the relationship between HIF-2α/EPAS1 expression and vascularization, we investigated the distribution of MVD in areas of HIF-2α/EPAS1 expression. It is well-known that new blood vessels can be stimulated to grow when factors promoting angiogenesis are up-regulated or those inhibiting angiogenesis are down-regulated[18,19]. Hypoxia in the tumor microenvironment is sufficient to activate HIF-dependent gene expression[4,20]. However, tumor growth rate may not always be associated with hypoxic conditions, whereas HIF-2α/EPAS1 expression may be influenced by factors other than hypoxia. Similar to other HIF-α (C-fos and cyclic AMP-responsive element binding protein), HIF-2α/EPAS1 protein is assumed to be accumulated upon exposure of cells to hypoxia. Evidence is accumulating that HIF-2α/EPAS1 may activate a series of gene expressions essential for angiogenesis such as VEGF[10,21,22], and many subsequent studies have shown that high levels of VEGF are produced by various types of tumors. In addition, the importance of VEGF as a mediator of tumor angiogenesis is suggested by studies showing that tumor angiogenesis and subsequent tumor growth are inhibited *in vitro* by antibodies directed against VEGF receptors[23,24], by expression of dominant negative VEGF receptors[25], and by antisense VEGF[26]. Oxygen deprivation is a potent stimulus to induce VEGF expression[27,28]. This process is partly mediated through the post-transcriptional modification of the mRNA; however, several studies have shown that HIF-2α/EPAS1 plays an important role in the activation of VEGF transcription[28]. Numerous studies have demonstrated that the expression of VEGF is a significant predictor of increased risk of metastatic disease and overall survival by stimulating angiogenesis in HCC[29,30] and other carcinomas[31]. In the present study, we found that VEGF expression and MVD counts were significantly higher in tumors with positive HIF-2α/EPAS1 than in tumors with negative HIF-2α/EPAS1, indicating a major role of HIF-2α/EPAS1 in tumor growth and progression of HCC through regulation of VEGF, which is associated with increased tumor neovascularization.

To our knowledge, this is the first study of the expression of HIF-2α/EPAS1 in HCC in a large number of cases. HIF-2α/EPAS1 overexpression as compared to noncancerous lesions was observed in 63.6% of the cases. Previously Beasley *et al*[32] demonstrated that HIF-2α/EPAS1 protein was expressed in both head and neck squamous cell carcinoma and adjacent normal tissue. However, the expression level was usually higher in the carcinoma. Talk *et al*[17] reported that human malignant tissues expressed higher levels of HIF-2α/EPAS1 protein in cancers compared with noncancerous tissue. Koukourakis *et al*[33] showed that the normal epithelium of esophagus did not express HIF-2α/EPAS1, whereas carcinoma tissue expressed this protein at a high incidence. These results including ours may provide evidence that ubiquitination and degradation of HIF-2α/EPAS1 may occur throughout the entire process of tumor carcinogenesis and there was a significantly increasing frequency of HIF-2α/EPAS1 abnormalities from noncancerous cells to cancer.

Survival analysis showed that HIF-2α/EPAS1 expression in HCC was significantly correlated with potential prognostic factors, which was dependent on MVD, such as tumor grade, venous invasion, intrahepatic metastasis, necrosis and capsule infiltration. Furthermore, HIF-2α/EPAS1 itself, to some extent, affects patient survival. Indeed, highly vascularized HCC with HIF-2α/EPAS1 expression showed a poorer prognosis. These findings are consistent with a number of previous clinical studies of breast[34], nasopharyngeal[35], colorectal[36] cancers and renal cell carcinoma[15]. Taken together, these demonstrated that HIF-2α/EPAS1 expression was related to an unfavorable prognosis.

In this study, we found HIF-2α/EPAS1 expression in macrophages. Several studies have found elevated levels of HIF-2α/EPAS1 in tumor cells and macrophages located in avascular and perinecrotic areas of human tumor, with a poor prognosis in the latter condition[17,37-39]. As macrophages have been shown to be one of terminally differentiated cells that can produce a number of potent chemoattractants such as VEGF[40,41], their chemotaxis, infiltration, degranulation may promote tumor angiogenesis and progression.

Interestingly, we found higher HIF-2α/EPAS1 protein expression in perinecrotic regions, though the differences did not reach statistical significance. This, when taken with the fact that macrophages appeared to be more potently pro-angiogenic at these sites[37,38], may help explain our findings. As HIF-2α/EPAS1 has been shown to be accumulated by hypoxic macrophages in human tumors[17,42], our finding may indicate that HIF-2α/EPAS1 protein may be released by macrophages, which is part of the mechanism by which this protein is most expressed in perinecrotic regions. On the other hand, direct evidence supporting microenvironmental mechanisms of HIF-α activation in diverse types of human tumor could offer an alternative explanation.

The clinical significance of HIF-2α/EPAS1 expression in tumors remains largely unexplored as monoclonal antibodies available for immunohistochemistry have been developed only recently. Talks *et al*[17] recently reported the expression of HIF-α in a panel of normal human tissues and benign or malignant tumors and first showed the expression of the molecule in a good percentage of human carcinomas. However, studies of the relation between HIF-2α/EPAS1 expression with angiogenic factors and receptors, microvessel density or other molecular markers or with prognosis of human carcinomas are limited. Investigations of these angiogenic factors in endothelial carcinoma[15,35,43] and in relation to signal transduction via HIF-2α/EPAS1 when the receptors do and do not bind to this protein and when dimerization with aryl hydrocarbon receptor nuclear translocator occurs between HIF-2α/EPAS1 and other HIF-α protein family, should help clarify the significance of HIF-2α/EPAS1 in human cancers, including HCC.

In the present study, we found cytoplasmic immunoreactivity of HIF-2α/EPAS1. However, equivocal staining was sometimes observed in the nuclei, which we did not consider as positive. Although nuclear HIF was assumed to be the active form, clearly it was synthesized and also degraded in the cytoplasm[35,44]. These findings, at least in part, could explain the cytoplasmic location of HIF-2α/EPAS1, which was a tumor specific finding and could better reflect the HIF up-regulation pathways in paraffin embedded material. This is in accordance with the scoring system proposed by Zhong *et al*[44].

In conclusion, the present study shows that, HIF-2α/EPAS1 has a role in progression of HCC, which occurs early in the development of the disease as evidenced by its expression in noncancerous lesions. HIF-2α/EPAS1 overexpression is significantly correlated with reduced survival in patients with HCC. Overexpression of HIF-2α/EPAS1 is related to up-regulation of angiogenic factors. We believe that assessment of HIF-2α/EPAS1 expression in HCC may be useful as a diagnostic tool and possibly a therapeutic target in the treatment of HCC.

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