

Expression of HIF-2 α /EPAS1 in hepatocellular carcinoma

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Abstract

AIM: To investigate the expression of hypoxia-inducible factor (HIF)-2 α /endothelial PAS domain protein1 (EPAS1) in hepatocellular carcinoma (HCC).

METHODS: Expression of HIF-2 α /EPAS1 was investigated immunohistochemically on paraffin-embedded sections from 97 patients with HCC. To further confirm that HIF-2 α /EPAS1 in HCC tissues also correlated with angiogenesis, a parallel immunohistochemistry study of vascular endothelial growth factor (VEGF) was performed on these 97 cases.

RESULTS: HIF-2 α /EPAS1 could be detected in 50 of 97 cases (51.6%), including 19 weakly positive (19.8%), and 31 strongly positive (31.1%), the other 47 cases were negative (48.4%). The expression of HIF-2 α /EPAS1 was significantly correlated with tumor size, capsule infiltration, portal vein invasion, and necrosis. A parallel immunohistochemical analysis of VEGF demonstrated its positive correlation with capsule infiltration, portal vein invasion, and HIF-2 α /EPAS1 overexpression, which supported the correlation of HIF-2 α /EPAS1 up-regulation with tumor angiogenesis. No apparent correlation was observed between HIF-2 α /EPAS1 and capsular formation, presence of cirrhosis, and histological grade.

CONCLUSION: HIF-2 α /EPAS1 is expressed in most of HCC with capsular infiltration and portal vein invasion, which indicates a possible role of HIF-2 α /EPAS1 in HCC metastasis.

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INTRODUCTION

Hypoxia is an essential development and physiological stimulus and plays a key role in the physiology of cancer, heart attack, stroke, and other major causes of mortality^[1]. Recently, hypoxia-inducible factors HIF-1 α and HIF-2 α /endothelial PAS domain protein1 (EPAS1) have been identified. They are basic helix-loop-helix/Per-Arnt-Sim (PAS) transcription factors induced under hypoxia^[2,3]. When dimerized with aryl hydrocarbon nuclear translocator (also known as HIF-3 α , another basic helix-loop-helix family member), both HIF-1 α

and HIF-2 α can bind to the hypoxia response element in a battery of genes and transactivate their expression, including vascular endothelial growth factor (VEGF)^[3,4]. These genes are important for tumor adaptation to hypoxia, implicating the possible role of HIFs in tumor progression. Tumor can not grow larger than 1-2 mm³ in the absence of angiogenesis, because of the lack of oxygen in the center of tumors, which result in apoptosis and necrosis^[5]. Many tumors contain hypoxic microenvironments, a condition associated with poor prognosis and resistant to clinical treatment. Along with HIF-1 α , EPAS1 is also known as HIF-2 α ^[6,7], HIF-like factor^[8], and HIF-related factor^[9]. The expression of HIF-2 α /EPAS1 has been reported in many human malignancies^[10,11]. We chose to study HIF-2 α /EPAS1 expression in order to evaluate its clinical significance and identify the relation with some important markers of carcinoma progression, which are also predictors of metastasis.

MATERIALS AND METHODS

Clinical material

We examined 97 surgical resection specimens from patients (76 males, 21 females) with HCC, who underwent surgery at Xiangya hospital between January 1994 and December 2001. The age of patients ranged from 34 to 78 years (61.4 \pm 8.9 years). The clinical features of the patients were noted with reference to clinical reports and pathology reports, including Edmondson-Steiner classification. In principle, grades I, II and III-IV and the characteristics of these patients are summarized in Table 1.

Immunohistochemical determination

Tissues were fixed with 10% formaldehyde in phosphate-buffered saline, embedded in paraffin, and cut into 5 μ m-thick tissue sections. The sections were deparaffinized in xylene and rehydrated in grade ethanol. Endogenous peroxidase was blocked by immersing the sections in 3% H₂O₂ in 100% methanol for 20 minutes at room temperature. Antigen retrieval was achieved by micro-waving sections at 95 $^{\circ}$ C for 10 minutes in 0.001 M citrate buffer (pH 6.7). For immunohistochemical detection of diluted mouse monoclonal antibodies against HIF-2 α /EPAS1 (190b) (DAKO, Glostrup, Denmark) and 1:200 diluted mAb against VEGF (Ab3) (Fremont, CA). Substitution of the primary antibodies with normal mouse IgG was used as a negative control. After washing, specimens were incubated with peroxidase-conjugated goat anti-mouse IgG for 30 min at room temperature. The color reaction was developed by incubating the sections with 0.5 mg/ml 3,3'-diaminobenzidine and 3% (vol/vol) H₂O₂ in phosphate-buffer saline for 7 min. The sections were counterstained slightly with Meyer hematoxylin and mounted.

VEGF determination and assessment

Mouse monoclonal antibody Ab3, neomarker (Fremont, CA) for detecting a smaller isoform (VEGF₁₂₁) of VEGF were used for the identification and evaluation of VEGF expression in this study. Staining with VEGF monoclonal antibody required antigen retrieval, which was best done by microwave pretreatment. In brief, paraffin-fixed slides were autoclaved for 7 min in pretreatment before deparaffinization and

rehydration. Evaluation of staining was semiquantitatively graded based on score determination by intensity distribution as strong ++ (dark brown), weak + (brown), or negative - (no staining). The score was determined independently by at least three of four observers.

Statistical analysis

A computer software SPSS 10.0 program was used for statistical analysis. Spearman's correlation coefficient test was used to assess the relationship between HIF-2 α /EPAS1, VEGF expression versus histological grade and tumor size. χ^2 test was used to assess the correlation between HIF-2 α /EPAS1, VEGF, versus existence of necrosis, cirrhosis, capsular formation, capsular infiltration and portal vein invasion. $P < 0.05$ was considered to be statistically significant.

Table 1 Correlation between HIF-2 α /EPAS1 and clinicopathological features of HCC patients

Variant	HIF-2 α /EPAS1 expression (No of cases)			No of cases 97	Significance
	-	+	++		
Pathological grade					
Grade I	9	4	3	16	NS
Grade II	23	8	7	38	
Grade III-IV	15	7	221	43	
Tumor size					
≤ 2	22	3	8	33	0.001 ^a
2-5	16	14	13	43	
> 5	9	2	10	21	
Cirrhosis					
With	27	9	17	53	NS
Without	20	10	14	44	
Capsule formation					
With	8	1	9	18	NS
Without	39	18	22	79	
Capsule infiltration					
With	3	3	10	16	0.011 ^a
Without	44	16	21	81	
Portal vein invasion					
With	15	15	18	48	0.001 ^a
Without	32	4	13	49	
Necrosis					
With	19	12	23	54	0.010 ^a
Without	28	7	8	43	

^a $P < 0.05$ vs the expression of HIF-2 α /EPAS1 was significant in HCC tissues with capsule infiltration, portal vein invasion, necrosis, and tumor size. NS, not significant.

RESULTS

In the 97 cases studied, positive staining of HIF-2 α /EPAS1 protein was localized mainly in the cytoplasm, and occasionally and faintly in the membrane of HCC cells (Figures 1A and 1B). In general, most positive stains were observed in the perinecrotic regions near the tumor foci (Figure 1C). There was expression of HIF-2 α /EPAS1 in cancer cells, whereas no positive staining was seen in the stroma cells of HCC (Figure 1D). Immunoreactivity of HIF-2 α /EPAS1 was also observed in macrophages, as previously reported^[10]. In addition, we also found positive staining in the intraluminal surfaces of hepatic vessels. All the positive immunoreactivity in macrophages was in the cytoplasm. The immunoreactivity was even stronger in macrophages than that in tumor cells (Figure 1E). All the tumor cluster infiltrating to the tissue showed moderate to strong positive staining of HIF-2 α /EPAS1 (Figure 1F). Fifty of 97 cases (51.5%) were positive, including 31 strongly positive (31.9%), 19 weakly positive (19.8%). In the 50 HIF-2 α /EPAS1-positive cases, 35 cases (64.8%) had necrosis, and 15 cases

(16.27%) had no necrosis. Positive staining was seen in 33.3% of the small-sized HCC (≤ 2 cm), 62.7% of the medium-sized (2-5 cm), and 57.1% of the HCCs larger than 5 cm. HCC larger than 5 cm in diameter though not significantly different between the two groups, had a relatively lower HIF-2 α /EPAS1 expression compared to medium-sized tumors. HIF-2 α /EPAS1 staining was seen in 81.2% of HCC patients with capsule infiltration, and 45.6% in those without them. Positive staining was also seen in 68.7% in HCC patients with portal vein invasion, and 34.6% in those without them (Table 1). HIF-2 α /EPAS1 positivity was significantly correlated with tumor size, capsule infiltration, portal vein invasion, and existence of necrosis ($P < 0.05$ respectively). To further confirm whether HIF-2 α /EPAS1 expression in HCC tissues also correlated with angiogenesis, a parallel immunohistochemical study of VEGF was performed on these 97 cases, in which VEGF expression was assessed as a major marker for angiogenesis (Table 2). Positive staining was found mostly in the cytoplasm of tumor cells (Figures 2A and 2B), and only weaker staining in stroma areas was detectable. VEGF expression in tumors had strong correlation with capsule infiltration, portal vein invasion, and necrosis ($P < 0.05$ respectively). The overexpression of VEGF in capsule infiltration and portal vein invasion was found to correlate positively with HIF-2 α /EPAS1 expression ($P < 0.05$, Table 3), supporting the correlation of HIF-2 α /EPAS1 up-regulation with tumor metastasis and angiogenesis in HCC.

Table 2 Correlation between VEGF protein and clinicopathological features of HCC patients

Variants	NO of cases 97	VEGF expression			Significance
		-	+	++	
Pathological grade					
Grade I	6	6	0	0	NS
Grade II	76	28	22	26	
Grade III-IV	15	8	3	4	
Tumor size					
≤ 2	13	11	0	2	NS
2-5	49	16	14	19	
> 5	35	15	11	9	
Cirrhosis					
With	53	20	10	23	NS
Without	44	22	15	7	
Capsule formation					
With	18	7	5	6	NS
Without	79	35	20	24	
Capsule infiltration					
With	16	4	2	10	0.011 ^a
Without	81	38	23	20	
Portal vein infiltration					
With	48	14	16	18	0.020 ^a
Without	49	20	9	12	
Necrosis					
With	54	15	17	22	0.011 ^a
Without	43	27	8	8	

^a $P < 0.05$ vs the expression of VEGF was significant in HCC with capsule infiltration, portal vein invasion, and with necrosis. NS, not significant.

Table 3 Correlation between HIF-2 α /EPAS1 and VEGF protein expression in HCC tissues

Variants	NO of cases	Staining score			Significance
		-	+	++	
HIF-2 α /EPAS1	97	47	19	31	0.017
VEGF	97	42	25	30	

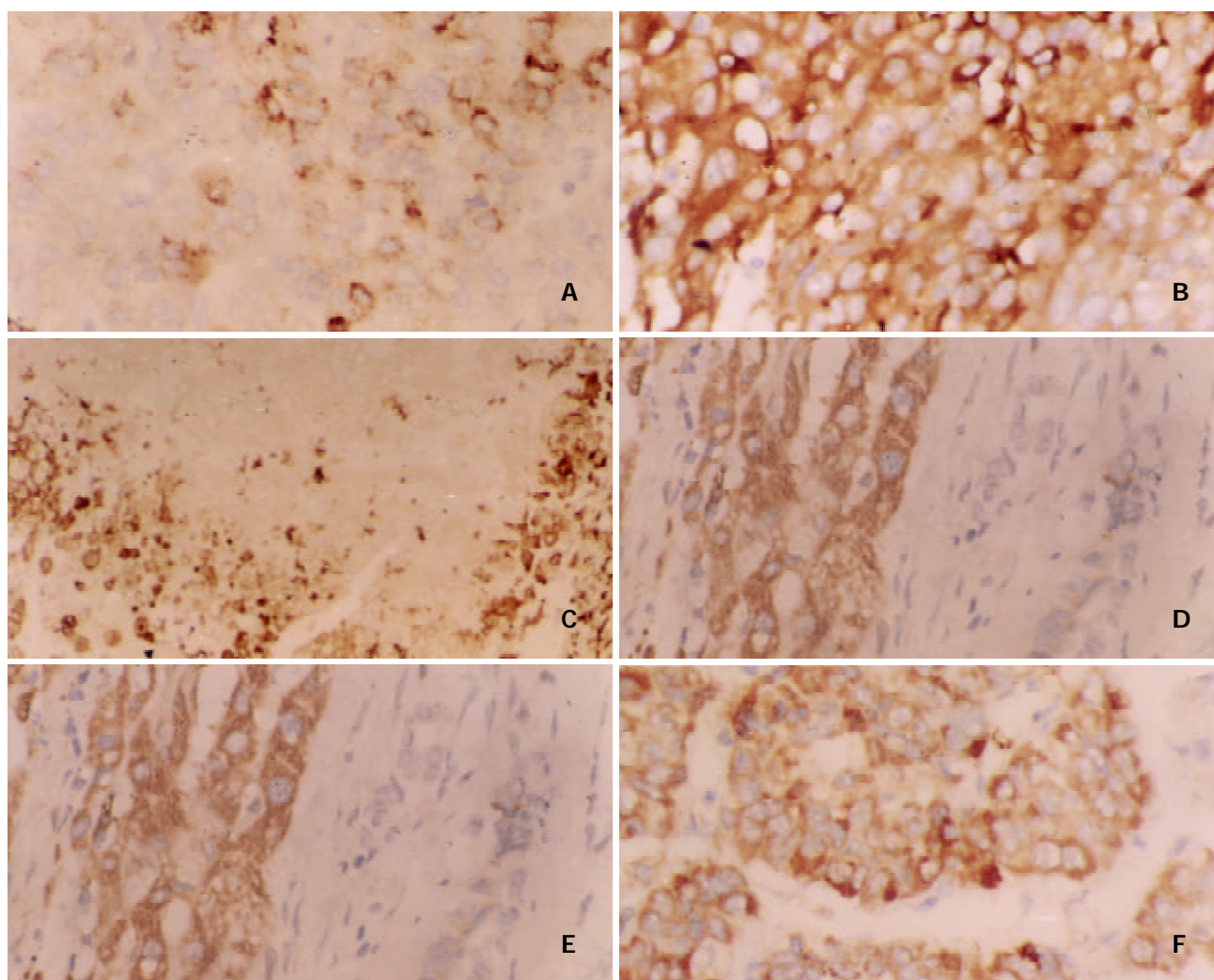


Figure 1 Characterization of HIF-2 α /EPAS1 expression in human hepatocellular carcinoma tissues by IHC technique. A: Weak expression of HIF-2 α /EPAS1 in membranes and cytoplasm of HCC cells ($\times 400$). B: Strong cytoplasmic immunoreactivity of HIF-2 α /EPAS1 in HCC cells ($\times 400$). C: HIF-2 α /EPAS1 positive staining (arrows) in perinecrotic region near tumor in a HCC sample (with capsular infiltration and portal vein invasion) ($\times 400$). D: Strong HIF-2 α /EPAS1 expression in HCC tissues whereas no staining in adjacent stroma cells ($\times 400$). E: Strong staining in the cytoplasm of macrophages compared with cancer cells, showing weak staining for HIF-2 α /EPAS1 ($\times 400$). F: Moderate to strong positive staining of HIF-2 α /EPAS1 in tumor clusters infiltrating to the tissue ($\times 400$).

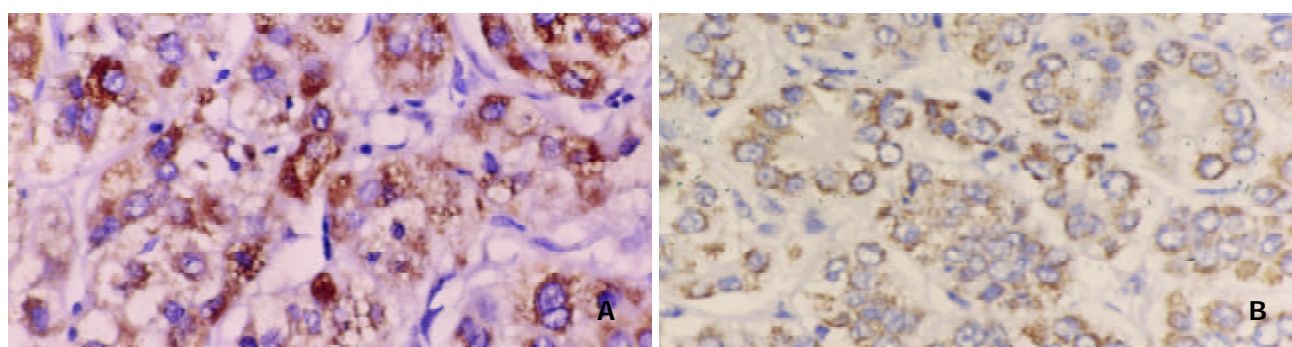


Figure 2 Parallel study of VEGF protein expression in HCC samples. A mAb against VEGF was used for immunostaining of slides from HCC patients. A: HCC with capsular infiltration and portal vein invasion showing strong staining (++) in the cytoplasm of tumor cells. B: HCC without capsular infiltration and portal vein invasion showing weak staining (+) ($\times 200$).

DISCUSSION

Angiogenesis appeared to be one of the crucial steps in tumor’s transition from small, harmless cluster of mutated cells to a large, malignant growth, capable of spreading to other organs throughout the body^[12]. HCC is a typical hypervascular tumor of the digestive organs. It seems likely that the formation of

tumor vessels precedes tumor growth and is indispensable in maintaining tumor viability, because hepatic arterial embolization frequently causes necrosis and induces a marked reduction in tumor size. In the present study, we investigated the expression of HIF-2 α /EPAS1 in HCC tissues. To further confirm whether HIF-2 α /EPAS1 in HCC tissues also correlated

with angiogenesis, we performed an immunohistochemistry study of VEGF protein. We also examined the correlation between HIF-2 α /EPAS1, VEGF protein expression and clinicopathological features.

Our data showed that tumor size was correlated with HIF-2 α /EPAS1. Small HCC had significantly lower HIF-2 α /EPAS1 expression compared with medium-sized tumors. What was contrary to previous finding^[13,14] was that tumors with large sizes had higher expression than smaller and moderate sizes, even these tumors were relatively less vascular compared with the large-sized ones. However, it has been reported that the intercapillary distance increased as the tumor size or weight increased, possibly because of different rates of endothelial cells and neoplastic cell turnover^[15,16]. The turnover time of endothelial cells was 50 to 60 hours while that of the neoplastic cells was 22 hours, and significantly shorter^[17]. On the other hand, the characteristics of tumor microcirculation could offer another explanation for the reduction of HIF-2 α /EPAS1 expression as the tumor became smaller or larger. Generally, blood flow, oxygen pressure, and pH values were less in tumors than in the counterpart normal tissues^[18], and because of the absence of lymphatic vessels, the interstitial pressure was often high in tumors, leading further transport problems^[19]. As a result, hypoxia and necrosis were a general phenomenon of tumors, especially large ones^[20]. It was assumed that rapid cell proliferation at the center of tumor could lead to increased interstitial pressure, which may lead to compression closure of capillaries and consecutive tumor necrosis^[21]. The current results showed that HCCs of 2 to 5 cm in diameter had the highest HIF-2 α /EPAS1 expression compared with larger and smaller tumors. This observation could be considered important for regional chemotherapy, because intuitively, tumor, tumor metastasis, and tumor death should be closely correlated with tumor-induced hypoxia and necrosis. Cells in hypoxic regions have been thought to be more resistant to the effects of radiotherapy and many conventional chemotherapeutic agents than their normoxic counterparts^[22,23].

We also found strong immunoreactivity in macrophages. The significance of HIF-2 α /EPAS1 in these cells warrants further study. As they have been shown to be one of the terminally differentiated cells that can produce a number of potent angiogenic cytokines such as VEGF^[24,25], their chemotaxis, infiltration, degranulation may promote tumor angiogenesis and progression. A parallel IHC study of angiogenesis marker demonstrated that in HCC tissues, overexpression of both VEGF and HIF-2 α /EPAS1 was coincidentally found, supporting the notion that HIF-2 α /EPAS1 expression is correlated with angiogenesis in HCC.

In the current work, significantly more HIF-2 α /EPAS1 protein expression was present in perinecrotic regions. This, when taken with the fact that macrophages appeared to be more pro-angiogenic at these sites^[26] may help to explain our observation. As HIF-2 α /EPAS1 has been shown to be accumulated by hypoxic macrophages in human tumor^[11,27], our finding may indicate that HIF-2 α /EPAS1 protein may be released by macrophages and is part of the mechanism by which this protein is most expressed in perinecrotic regions. On the other hand, direct support for microenvironmental mechanisms of HIF- α activation in diverse types of human tumor could offer an alternative explanation.

Results from our analysis of HIF-2 α /EPAS1 expression in perinecrotic areas were consistent with a number of reports from clinical studies on breast^[28-30], ovarian^[31], and lung^[32,33] cancers and in hemangioblastomas^[10]. These reports all demonstrated that macrophage hotspots were remotely located from the vascular hotspots of tumors, suggesting that macrophages may preferentially migrate toward areas of relative hypoxia^[29]. This in turn might attract macrophages into

tumor, which then contribute to angiogenic process, giving rise to association between high levels of angiogenesis and extensive necrosis^[29]. Macrophages might be attracted to necrotic tumors by chemotactic factors, such as VEGF^[26,34,35].

As a potent pro-angiogenic cytokine, VEGF has been reported to be overexpressed in both malignant tumors^[30,36] and stroma cells^[30,34] and macrophages^[26,34,37]. Expression of VEGF was up-regulated in poorly vascularized areas of breast carcinomas^[28,29,38]. VEGF positive macrophages were restricted to areas of VEGF production^[26,28]. Evidence is accumulating that VEGF might be activated in stroma cells, especially in macrophages, with the process mediated by the VEGF receptor ftl-1^[35]. Thus, the subcellular mechanisms mediating hypoxia on VEGF gene by macrophages are not known at present. This most likely involved one or more of the pathways activated by hypoxia in transformed cells^[37,40], including the activation of such transcription factors as hypoxia-inducible factors (HIFs) 1, 2 (otherwise known as EPAS1). In this study, we observed the overexpression of VEGF protein in tumor and VEGF expression positively correlated with HIF-2 α /EPAS1 expression. We found that the highest VEGF expression was detectable mostly in tumor areas and only weaker staining in necrotic and stroma areas was detectable.

It is widely accepted that angiogenesis necessitates the degradation of extracellular matrix, this process requires protease activation and release. Plasmin was thought to be one of the key proteases involved in this process^[39]. Angiogenesis also appear to be involved in the invasion of tumors into surrounding tissues, because this invasion requires concomitant neovascularization through the sprouting of endothelial cells in the tumor stroma. It has been reported that VEGF induced both urokinase-type plasminogen activator (PA) and tissue type PA in endothelial cells^[40], and hypoxia might promote cellular invasion by stimulating the expression of urokinase type plasminogen activator (uPAR)^[41]. Therefore, enhanced expression of these angiogenic factors would likely indicate the ability of tumors to invade the tumor stroma as well as the ability to promote the development of new blood vessels. Based on these considerations, we examined both portal involvement and capsule infiltration. These two clinicopathological features have been thought to be the most important clinical factors in assessing liver tumor, and HCC in particular, as they were strongly correlated with the metastasis of HCC^[42-44]. Our results were in agreement with this concept. We found that the portal vein involvement and capsular infiltration were correlated with the expression of both HIF-2 α /EPAS1 and VEGF proteins. HCC patients with capsular infiltration and portal vein invasion had more HIF-2 α /EPAS1 and VEGF expression than those without them, indicating that HIF-2 α /EPAS1 and VEGF expression may be associated with a poor prognosis of patients with HCCs.

The clinical significance of HIF-2 α /EPAS1 expression in tumors remains largely unexplored as monoclonal antibodies available for immunohistochemistry have been recently developed. Talks *et al* 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^[11]. However, studies on the HIF-2 α /EPAS1 expression with angiogenic factors and receptors, with microvessel density or with other molecular markers or with prognosis of human carcinomas are few. Investigations regarding these angiogenic factors which have been partially done for endothelial carcinoma^[3,33,45], and regarding the status of signal transduction via HIF-2 α /EPAS1 when the receptors do and do not bind to this protein and when dimerization with aryl hydrocarbon receptor nuclear translocator occurs between HIF-2 α /EPAS1 and other HIF- α protein family, should help clarify the significance

of HIF-2 α /EPAS1 in human cancers, including HCC.

In this study, we found cytoplasmic immunoreactivity of HIF-2 α /EPAS1, but equivocal staining was sometimes observed in the nuclear which we did not regard as positive. Although it was assumed that nuclear HIF was the active form, clearly it was synthesized and also degraded in the cytoplasm^[45,46]. These findings, at least in part, could explain the cytoplasmic location of HIF-2 α /EPAS1, which was a tumor specific finding and could better reflect the HIF up-regulation pathways in paraffin embedded material. This suggestion was in accordance with the scoring system proposed by Zhong *et al*^[46].

In conclusion, HIF-2 α /EPAS1 expression in HCC and its clinical association with necrosis seem to be a good predictive tool and possibly a target therapy for metastasis of liver cancer, especially HCC. The finding that medium-sized HCCs had the highest expression of HIF-2 α /EPAS1 compared with smaller and larger HCCs can be used, after further evaluation, as a therapeutic guide during the selection of cases for chemotherapy.

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