

Defective Beclin-1 and elevated hypoxia-inducible factor (HIF)-1 α expression are closely linked to tumorigenesis, differentiation, and progression of hepatocellular carcinoma

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Abstract Hepatocellular carcinoma (HCC) is an aggressive cancer with a poor prognosis. Autophagy and hypoxia have been involved in HCC tumorigenesis. In the present study, we examined the relationship between Beclin-1 expression and hypoxia-inducible factor (HIF)-1 α expression in HCC by immunohistochemistry on 65 tumor specimens. Their correlations with clinicopathological features were also explored. There was a loss of Beclin-1 protein expression in 49.2 % of HCC. Beclin-1 expression was only significantly correlated with virus infection status ($p=0.025$) and marginally associated with HCC grade ($p=0.057$). Forty-two tumors (64.6 %) showed high HIF-1 α expression, and it was significantly associated with large tumor size ($p=0.003$), multifocal tumors ($p=0.038$), and advanced stage ($p=0.043$). Beclin-1 expression was significantly associated with HIF-1 α expression ($p=0.001$). HCC cases were further stratified according to their hypoxia status into hypoxic and normoxic groups. In the hypoxic group, Beclin-1 expression was negatively correlated with HCC high tumor grade ($p<0.001$), advanced stage ($p=0.013$), large size ($p=0.002$), and multifocal tumors ($p=0.047$). In the normoxic group, no significant relations between Beclin-1 expression and any of the clinicopathological parameters were identified. Our findings that reduced Beclin-1 and high HIF-1 α expression are associated with the development and progression of HCC may provide molecular therapeutic targets toward inhibiting HCC development and progression.

Keywords Beclin-1 · Hypoxia-inducible factor · Hepatocellular carcinoma · Immunohistochemistry · Clinicopathological factors

Introduction

Hepatocellular carcinoma (HCC), a global health problem, is the sixth most common malignancy and the third most common cause of cancer-related deaths worldwide and is commonly secondary to hepatitis B and C infections [1]. In Egypt, HCC ranks as the first cancer in males and the second in females constituting 33.63 and 13.54 % of cancer incidence, respectively [2]. Over the last decade, a considerable increase, from 4.0 to 7.2 %, was observed in the proportion of chronic liver disease Egyptian patients with HCC [3]. HCC is characterized by poor prognosis despite improved diagnostic and treatment strategies [4]. Although several clinicopathological parameters and molecular factors have been reported to be associated with the prognosis of HCC [5], more effective biomarkers are necessary to predict the clinical course and outcome of patients with HCC. Recently, autophagy has become a critical focus in cancer research. Autophagy has emerged as a homeostatic mechanism recycling intracellular constituents. Autophagy is believed to be a nonapoptotic form of programmed cell death; most of the evidences support autophagy as a survival pathway required for cellular viability. Simultaneous defects in autophagy and apoptosis activate the DNA damage response-promoted gene amplification and accelerate tumorigenesis in various organs [6]. Beclin-1 (*BECN1*), the mammalian orthologue of yeast *Atg6/Vps30*, has been mapped to a tumor susceptibility locus ~150 kb centromeric to *BRCA1* on human chromosome 17q21 [7]. It is considered as a haploinsufficient tumor suppressor gene [8]. Loss of *BECN1* leads to reduction in autophagic vacuole formation and an unpredicted increase in several malignancies, including

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HCC [9]. HCC as a solid tumor shows the character of tissue hypoxia, especially when the tumor grows rapidly and angiogenesis fails to catch up with the speed of tumor growth. Hypoxia induces tumor necrosis which limits the size of HCC. However, severe hypoxia leads to pro-survival reactions, elevated angiogenesis, tumor invasion, and metastasis [4]. Hypoxia-inducible factor (HIF)-1 is the first identified mediator of cell response to hypoxia in mammalian cells cultured under reduced oxygen tension. This transcription factor is a heterodimer composed of α and β subunits [10]. There was a growing body of evidence that linked HIFs and pathogenesis of HCC [11]. Under hypoxic conditions, HIF-1 α induces the autophagy cascade, removes damaged mitochondria, and limits reactive oxygen species (ROS) damage in order to protect the cell from apoptotic death [12]. However, the role of autophagy and hypoxia is still controversial in cancer, especially in tumor progression. Recent studies had confirmed that hypoxia-induced autophagy-related gene Beclin-1 expression might be important for disease progression and be correlated with patient outcome in several tumors [13]. The present study performed immunohistochemical analyses to determine the expression of the Beclin-1 and HIF-1 α proteins in HCC cases and non-neoplastic hepatic tissues, to evaluate a potential association between Beclin-1 and HIF-1 α expression, and to correlate the expressions of both markers to the clinicopathological factors.

Material and methods

Sixty-five cases of primary hepatocellular carcinoma specimens were collected from the files of the Department of Pathology, Minia University Hospital, and Minia Oncology Center, El-Minia, Egypt, during the period from 2009 to 2014. Clinical and pathological characteristics (Table 1), including age, sex, tumor size, focality, histological grade, stage, and hepatitis virus-associated infection, were obtained from the patients' files. All hematoxylin and eosin (H&E)-stained slides were reviewed to revise histological grade. The clinicopathological stage was determined according to the International Union Against Cancer (UICC) TNM classification system [14]. The patients were 52 males and 14 females with age ranges from 40 to 74 years (median 55 years).

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues (4 μ m thick) were dewaxed in xylene and rehydrated in gradient ethanol solutions. Antigenic retrieval was performed in sodium citrate (pH=6.0) with a microwave. Endogenous peroxidase activity was blocked by hydrogen peroxide 0.3 % for 30 min. Sections were then incubated with primary rabbit monoclonal antibody for Beclin-1 (1:100, Abcam, EPR1733Y) and mouse monoclonal antibodies for HIF-1 α (1:200, Abcam, 1A3) at 4 °C

Table 1 Clinicopathological features of primary HCC cases

Clinicopathological features	Sample (Percentage)
Age (years)	
\leq 50	21 (32.3)
$>$ 50	44 (67.7)
Sex	
Female	14 (21.5)
Male	51 (78.5)
Hepatitis status	
Negative	10 (15.4)
HCV	42 (64.6)
HBV	13 (20)
Cirrhosis	
No	12 (18.5)
Yes	53 (81.5)
Tumor size (cm)	
\leq 5	44 (67.7)
$>$ 5	21 (32.3)
Focality	
Solitary	43 (66.2)
Multifocal	22 (33.8)
Grade	
1	8 (12.3)
2	32 (49.2)
3	25 (38.5)
Stage	
I	18 (27.7)
II	22 (33.8)
III	14 (21.5)
IV	11 (16.9)

HCV hepatitis C virus, HBV hepatitis B virus

overnight, followed with secondary antibody for 30 min. Finally, the sections were processed to develop color with diaminobenzidine (DAB) and were counterstained with Mayer's hematoxylin. Sections of normal breast and lung carcinoma tissues were served as a positive control for Beclin-1 and HIF-1 α , respectively. For a negative control, the specific primary antibody was replaced with phosphate-buffered saline (PBS).

Evaluation of IHC staining

Immunohistochemical staining level was assessed and scored by two independent pathologists, who were blind to the clinicopathological and follow-up information. Expression of Beclin-1 was defined as positive if distinct moderate or strong immunoreactivity was present in $>$ 10 % of the cells [15]. Regarding HIF-1 α scoring, low expression was defined as

<10 % of cells exhibiting nuclear staining and/or cytoplasmic staining. High expression was determined when ≥ 10 % of cells exhibited nuclear staining and/or distinct cytoplasmic staining [16].

Statistical analysis

Statistical evaluation was performed using the SPSS software package (SPSS 16.0). The chi-square test was used to analyze the association between Beclin-1 and HIF-1 α expression and their association with clinicopathological features. The level of statistical significance was set at $p \leq 0.05$.

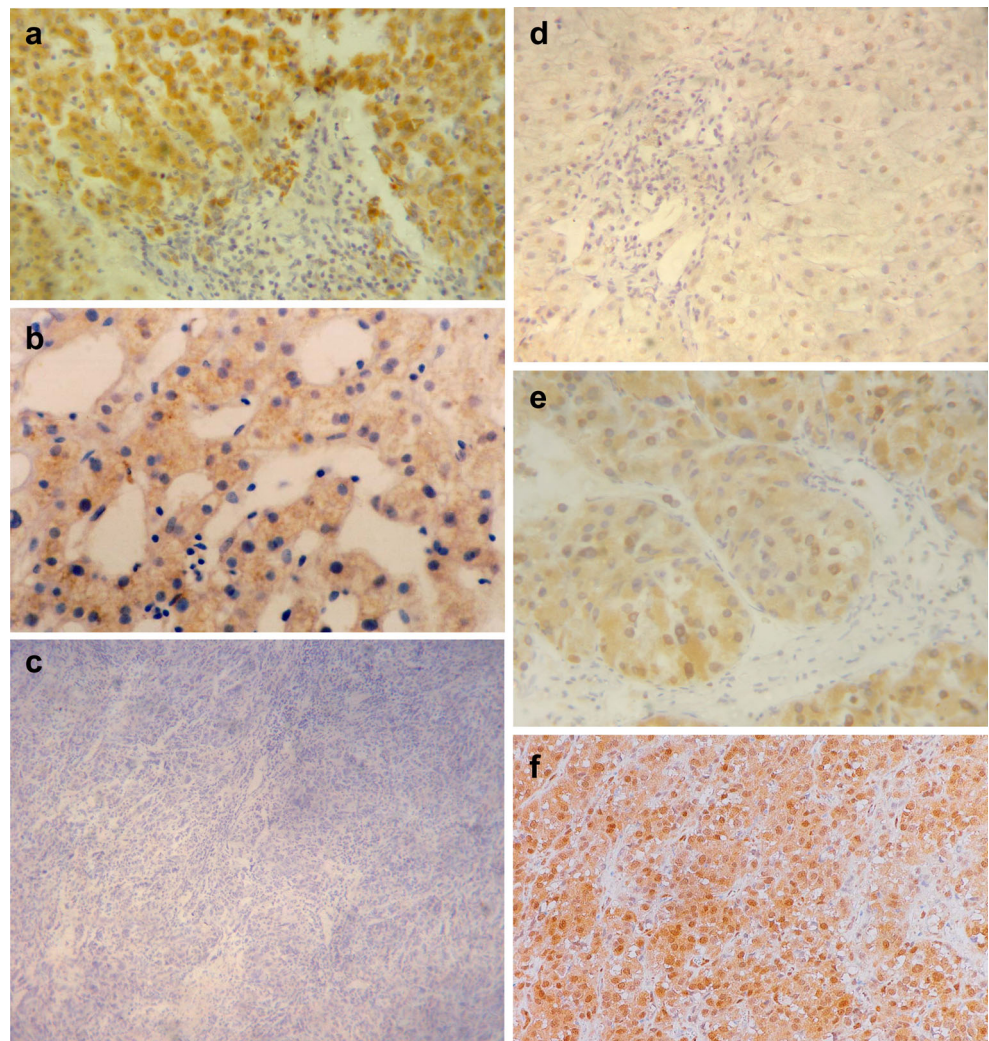
Results

Positive cytoplasmic Beclin-1 expression was found in 18/21 (85.7 %) of adjacent non-neoplastic hepatic tissues and in 33/65 (50.8 %) of HCC cases (Fig. 1a, b), while it was negative in 32/65 (49.2 %) of HCC cases (Fig. 1c). The expression of the

Beclin-1 protein in the HCC tissue was significantly lower than that of the adjacent non-neoplastic tissues ($p=0.005$). Conversely, HIF-1 α staining was observed in the nucleus as well as cytoplasm of tumor cells in 64.6 % of HCC (Fig. 1e, f), compared to the negligible expression in the non-neoplastic hepatic tissues (Fig. 1d) ($p<0.001$). Among HCC cases, 42/65 tumors displayed hypoxia (defined by HIF-1 α high expression) compared to 23/65 tumors exhibiting normoxia (defined as HIF-1 α low or deficient expression).

The correlation of Beclin-1 expression with the clinicopathological characteristics of HCC is shown in Table 2. Reduced Beclin-1 expression was marginally associated with HCC grade ($p=0.057$). A significant positive association was found between Beclin-1 expression and virus infection status ($p=0.025$). Among HBV-associated tumors and HCV-associated tumors, 76.9 and 50 % were Beclin-1 positive, respectively, while 80 % of HCC negative for hepatitis virus infection showed negative Beclin-1 expression. No significant differences in Beclin-1 expression with respect to other characteristics were found.

Fig. 1 a–f Immunohistochemical expression of Beclin-1 and HIF-1 α in primary HCC and adjacent nonneoplastic hepatic tissues. **a** Positive Beclin-1 expression in adjacent nonneoplastic hepatic tissue with hepatitis. **b** Positive Beclin-1 expression in low-grade HCC. **c** Negative Beclin-1 expression in high-grade HCC. **d** HIF-1 α expression in adjacent nonneoplastic hepatic tissue with hepatitis. **e** HIF-1 α expression in low-grade HCC. **f** HIF-1 α expression in high-grade HCC. Immunohistochemistry: diaminobenzidine (DAB) chromogen and hematoxylin counterstaining. Original magnification $\times 400$ for all figures except for **c** $\times 200$



HCC cases were further stratified according to their hypoxia status into hypoxic and normoxic groups. The relationship between Beclin-1 expression and different clinicopathological parameters was separately studied in these groups (Table 2). In the hypoxic group, Beclin-1 expression was negatively correlated with HCC high tumor grade ($p<0.001$), advanced stage ($p=0.013$), large size ($p=0.002$), and multifocal tumors ($p=0.047$). In the normoxic group, no significant relations between Beclin-1 expression and any of the clinicopathological parameters were identified.

Table 3 demonstrates the association of HIF-1 α expression and clinicopathological features. High HIF-1 α immunoreactivity was significantly associated with large tumor size ($p=$

0.003), multifocal tumors ($p=0.038$), and advanced stage ($p=0.043$), while no statistically significant associations were detected in relation to other clinicopathological variables.

Relationship between Beclin-1 and HIF-1 α expression in HCC cases

The present work found that Beclin-1 expression rates were positively associated with hypoxic conditions ($p=0.001$). A significant positive association was found between Beclin-1 and HIF-1 α expression (Table 4). In the 33 HCC cases marked as Beclin-1-positive tumors, 5/33 (15.2 %) had low HIF-1 α and 28/33 (84.8 %) had high HIF-1 α . Whereas, in the

Table 2 Relations between Beclin-1 expression and clinicopathological features in the whole study group as well as in normoxic and hypoxic groups in primary HCC cases

Features	Whole study group			HIF-1 α low (normoxic group)			HIF-1 α high (hypoxic group)		
	Beclin negative	Beclin positive	<i>p</i>	Beclin negative	Beclin positive	<i>p</i>	Beclin negative	Beclin positive	<i>p</i>
Age (years)									
≤50	12 (57.1 %)	9 (42.9 %)	0.378	7 (100 %)	–	0.095	5 (35.7 %)	9 (64.3 %)	0.817
>50	20 (45.5 %)	24 (54.5 %)		11 (68.8 %)	5 (31.2 %)		9 (32.1 %)	19 (67.9 %)	
Sex									
Female	5 (35.7 %)	9 (64.3 %)	0.253	3 (60 %)	2 (40 %)	0.263	2 (22.2 %)	7 (77.8 %)	0.425
Male	27 (52.9 %)	24 (47.1 %)		15 (83.3 %)	3 (16.7 %)		12 (36.4 %)	21 (63.6 %)	
Hepatitis									
Negative	8 (80 %)	2 (20 %)	0.025 ^a	4 (100 %)	–	0.412	4 (66.7 %)	2 (33.3 %)	0.105
HCV	21 (50 %)	21 (50 %)		13 (72.2 %)	5 (27.8 %)		8 (33.3 %)	16 (66.7 %)	
HBV	3 (23.1 %)	10 (76.9 %)		1 (100 %)	–		2 (16.7 %)	10 (83.3 %)	
Cirrhosis									
No	3 (25 %)	9 (75 %)	0.063	2 (100 %)	–	0.435	1 (10 %)	9 (90 %)	0.073
Yes	29 (54.7 %)	24 (45.3 %)		16 (76.2 %)	5 (23.8 %)		13 (40.6 %)	19 (59.4 %)	
Tumor size (cm)									
≤5	19 (43.2 %)	25 (56.8 %)	0.158	16 (76.2 %)	5 (23.8 %)	0.435	3 (13 %)	20 (87 %)	0.002 ^a
>5	13 (61.9 %)	8 (38.1 %)		2 (100 %)	–		11 (57.9 %)	8 (42.1 %)	
Focality									
Solitary	21 (48.8 %)	22 (51.2 %)	0.929	16 (84.2 %)	3 (15.8 %)	0.132	5 (20.8 %)	19 (79.2 %)	0.047 ^a
Multifocal	11 (50 %)	11 (50 %)		2 (50 %)	2 (50 %)		9 (50 %)	9 (50 %)	
Grade									
1	3 (37.5 %)	5 (62.5 %)	0.057	3 (100 %)	–	0.590	–	5 (100 %)	<0.001 ^a
2	12 (37.5 %)	20 (62.5 %)		11 (73.3 %)	4 (26.7 %)		1 (5.9 %)	16 (94.1 %)	
3	17 (68 %)	8 (32 %)		4 (80 %)	1 (20 %)		13 (65 %)	7 (35 %)	
Stage									
I	7 (38.9 %)	11 (61.1 %)	0.639	6 (85.7 %)	1 (14.3 %)	0.236	1 (9.1 %)	10 (90.9 %)	0.013 ^a
II	11 (50 %)	11 (50 %)		10 (83.3 %)	2 (16.7 %)		1 (10 %)	9 (90 %)	
III	7 (50 %)	7 (50 %)		1 (33.3 %)	2 (66.7 %)		6 (54.5 %)	5 (45.5 %)	
IV	7 (63.6 %)	4 (36.4 %)		1 (100 %)	–		6 (60 %)	4 (40 %)	

Test of significance: chi-square test and Fisher's exact test. A *p* value ≤0.05 is considered significant

HCV hepatitis C virus, HBV hepatitis B virus

^a Significant association

Table 3 Relation between HIF-1 α expression and clinicopathological features in primary HCC cases

Features	HIF-1 α low expression	HIF-1 α high expression	<i>p</i> value
Age (years)			
≤50	7 (33.3 %)	14 (66.7 %)	0.811
>50	16 (36.4 %)	28 (63.6 %)	
Sex			
Female	5 (35.7 %)	9 (64.3 %)	0.977
Male	18 (35.3 %)	33 (64.7 %)	
Hepatitis			
Negative	4 (40 %)	6 (60 %)	0.065
HCV	18 (42.9 %)	24 (57.1 %)	
HBV	1 (7.7 %)	12 (92.3 %)	
Cirrhosis			
No	2 (16.7 %)	10 (83.3 %)	0.133
Yes	21 (39.6 %)	32 (60.4 %)	
Tumor size (cm)			
≤5	21 (47.7 %)	23 (52.3 %)	0.003 ^a
>5	2 (9.5 %)	19 (90.5 %)	
Focality			
Solitary	19 (44.2 %)	24 (55.8 %)	0.038 ^a
Multifocal	4 (18.2 %)	18 (81.8 %)	
Grade			
1	3 (37.5 %)	5 (62.5 %)	0.108
2	15 (46.9 %)	17 (53.1 %)	
3	5 (20 %)	20 (80 %)	
Stage			
I	7 (38.9 %)	11 (61.1 %)	0.043 ^a
II	12 (54.5 %)	10 (45.5 %)	
III	3 (21.4 %)	11 (78.6 %)	
IV	1 (9.1 %)	10 (90.9 %)	

Test of significance: chi-square test and Fisher's exact test. A *p* value ≤0.05 is considered significant

HCV hepatitis C virus, HBV hepatitis B virus

^a Significant association

32 HCC cases classified as Beclin-1-negative tumors, the low HIF-1 α and high HIF-1 α tumors were 18/32 (56.3 %) and 14/32 (43.8 %), respectively.

Table 4 The association between Beclin-1 and HIF-1 α expression in primary HCC cases

	Low HIF-1 α	High HIF-1 α	<i>p</i> value
Negative Beclin-1	18 (56.3 %)	14 (43.7 %)	0.001 ^a
Positive Beclin-1	5 (15.2 %)	28 (84.8 %)	

Test of significance: chi-square test and Fisher's exact test. A *p* value ≤0.05 is considered significant

^a Significant association

Discussion

The role of autophagy in carcinogenesis is double sided and dependent on the cellular context. Autophagy exerts an inhibitory role in the initial stage of tumorigenesis, via suppression of inflammation and preserving genome stability. On the other hand, autophagy acts as a prosurvival mechanism to protect cancer cells against cell death induced by cellular stress. However, as cellular stress continues to result in excessive and sustained autophagy, tumor cell death would follow [17, 18]. Direct evidence linking autophagy to tumor suppression comes from the fact that one of its key regulators, Beclin-1, exhibits a tumor suppressor function and has been found to be monoallelically deleted in several tumors [17, 19]. The present study demonstrated deficient Beclin-1 expression in 49.2 % of HCC. As previously reported in various tumors [13, 15, 20, 21], a significantly decreased Beclin-1 expression in HCC tissues compared to the adjacent nonneoplastic tissues was found in this series. These findings support the hypothesis that a defective autophagy can contribute to carcinogenesis of various tumors.

In this study, Beclin-1 expression was more frequently associated with well-differentiated HCC than poorly differentiated tumors, although this difference was marginally significant. Recently, several studies reported that Beclin-1 expression was inversely correlated with poor clinicopathological features and unfavorable prognosis in HCC [15, 21] as well as in other tumors [20, 22–24], suggesting that Beclin-1 expression might reverse the aggressive phenotypes as a tumor suppressor in several types of cancers. Several mechanisms underlying the tumor suppressor role of Beclin-1 could be considered. Beclin-1 delays cell cycle progression [25], suppresses tumor angiogenesis and proliferation [26], limits chromosomal instability and reduces the frequency of additional mutation in tumors [27]. By contrast, other studies demonstrated its association with aggressive features and poor outcome in nasopharyngeal carcinoma [28] and oral squamous cell carcinoma [29]. This may be due to variations in intrinsic properties of these tumor types.

Here, a significant positive association was found between Beclin-1 expression and hepatitis virus infection status, where 76.9 % of HBV-associated tumors and 50 % of HCV-associated tumors were Beclin-1 positive while the great majority of HCC negative for hepatitis virus infection showed negative Beclin-1 expression. Consistent with this finding, previous studies demonstrated that hepatitis B virus x protein (HBx) upregulates endogenous Beclin-1 mRNA and protein levels in the liver and hepatoma cell lines via transactivation of Beclin-1 promoter activity [30]. Also, HCV induces autophagy by upregulation of Beclin-1 at the transcriptional level [31].

Recent studies highlighted the role of HIFs and hepatic oxygen levels in the pathogenesis of chronic liver disease and HCC that may provide new therapeutic opportunities for

treatment [11, 32–34]. This study demonstrated high HIF-1 α expression in 64.6 % of HCC cases with deficient expression in adjacent nonneoplastic tissue. High HIF-1 α was significantly associated with large tumor size, multifocal tumors, and advanced stage. These findings were in agreement with other studies that considered HIF-1 α as a negative prognostic indicator in HCC [35–37]. High HIF-1 α expression was also noted in 57.1 and 92.3 % of hepatitis C- and hepatitis B-positive HCC cases, respectively. Previous studies suggested that both HBV and HCV induce a cellular hypoxic response and stabilize HIF-1 α [38, 39]. Moreover, hypoxia promotes HBV and HCV replication, suggesting a role of HIF-1 α in hepatitis virus pathogenesis and oncogenicity that may provide a novel therapeutic target to limit viral replication and associated pathologies [34].

In order to investigate whether a hypoxic status influences Beclin-1 expression and function, cases were stratified according to HIF-1 α expression into hypoxic and normoxic groups. Beclin-1 expression was investigated in relation to clinicopathological features in both groups separately. Interestingly, the association between reduced Beclin-1 and the aggressive clinicopathological features including high tumor grade, large size, advanced stage, and multifocal tumors was only significant in the hypoxic group. On the contrary, Beclin-1 expression failed to demonstrate any correlation with clinicopathological features in the normoxic group of HCC. Based on this finding, we propose that the autophagic Beclin-1-induced clinicopathological effect was driven by hypoxia in HCC and that the cumulative effects induced by reduced Beclin-1 and high HIF-1 α expression may promote a high-grade malignant phenotype and aggressive behavior in HCC. A previous experimental study, using a primary mouse melanoma tumor model, demonstrated that Beclin-1 deficiency was significantly associated with aggressive growth phenotype and increased angiogenesis in tumors under a hypoxic condition compared to those with a normoxic condition [26]. Another study demonstrated that reduced Beclin-1 expression predicted worse overall survival among patients with HIF-1 α -overexpressing breast cancers [13]. This raises a concern regarding the role of autophagy and hypoxia in the progression of malignant tumors.

Furthermore, this study demonstrated a significant association between the autophagic Beclin-1 expression and high HIF-1 α in this series where tumors with high HIF-1 α expression more frequently developed Beclin-1 upregulation than those with low HIF-1 α expression. This finding was similar to that found by previous studies for squamous cell carcinoma of the esophagus [20] nasopharyngeal [39] and breast [13] carcinomas, supporting the critical role of hypoxia in the activation of autophagy. On the contrary, Wu et al. [24] failed to find any correlation between HIF-1 α and Beclin-1 expression.

In conclusion, the close association between Beclin-1 and HIF-1 α identified in the current study confirms the critical

role of hypoxia in the activation of autophagy. Moreover, reduced Beclin-1 and elevated HIF-1 α expression were significantly associated with aggressive clinicopathological features, supporting their pivotal role in the development and progression of HCC. Therefore, both markers could be considered as useful targets for new molecular therapies besides conventional treatments against HCC.

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