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Research on correlations of magnetic resonance imaging features and pathological changes in liver cancer with Beclin1 expression

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Abstract. – OBJECTIVE: Our study aims to explore the correlations of magnetic resonance imaging (MRI) features and pathological changes in liver cancer with the messenger ribonucleic acid (mRNA) and protein expression of Beclin1.

PATIENTS AND METHODS: The mRNA and protein expression levels of Beclin1 in 42 cases of liver cancer and para-carcinoma tissues from liver cancer patients were detected via fluorescence quantitative polymerase chain reaction (FQ-PCR) and immunohistochemistry, and its correlations with the clinicopathological characteristics and MRI features were analyzed.

RESULTS: The mRNA expression of Beclin1 in liver cancer tissues was significantly higher than that in para-carcinoma tissues, and statistical correlation between pathological grade and mass size of liver cancer was found ($p < 0.05$). The immunohistochemical results revealed that the Beclin1 positive expression was mainly located in the cytoplasm, and the level was significantly increased compared with that in corresponding para-carcinoma tissues ($p < 0.05$). The MRI showed that both Min-apparent diffusion coefficient (ADC) and Mean-ADC in Beclin1 high-expression group were significantly lower than those in Beclin1 low-expression group.

CONCLUSIONS: The Beclin1 expression plays an important role in the occurrence and development of liver cancer. MRI shows significant correlation with the level of Beclin1 in liver cancer, which provides certain value in the evaluation of the biological behaviors and prognosis of liver cancer.

Key Words:
Liver cancer, Beclin1, MRI features, Clinicopathological.

Introduction

Primary liver cancer represents a type of severe malignant tumors in the digestive system, which often indicates a poor prognosis. Hepatocellular carcinoma (HCC) accounts for about 90% of all types of primary liver cancers, and it is the third most common cause of cancer death[1]. The morbidity rate of liver cancer is comparably high in the Asian-Pacific region, and the amount of new cases of liver cancer in China accounts for more than 50% in the world. Currently, the treatment of liver cancer is mainly dominated by the operation, transarterial chemoembolization, chemotherapy, sorafenib-targeted drug therapy and radiofrequency ablation. These comprehensive therapies greatly increase the cure rate and effectively extend the life of patients. However, the therapeutic effect is still unsatisfactory due to the lack of specific methods of early detection and timely treatment of liver cancer[2,3]. Autophagy is a catabolic process in cells, through which the cytoplasmic proteins and organelles are transferred to lysosomes, and then degraded and recycled[4]. Increasing studies have demonstrated that autophagy is closely related to the occurrence and progression of tumors[5]. However, a dispute over the role of autophagy in these processes still exists. According to previous studies, autophagy can relieve metabolic stress, improve genomic stability and inhibit occurrence of tumors. However, recent evidence also contends that autophagy enhances the resistance of glioblastoma to chemotherapy[6]. In 1998, mammalian homologous gene Beclin1 of the yeast Atg6/Vps30 was discovered and cloned. Beclin1 is located on human chromosome 17q21, functions as a key regulator of autophagy, and induces autophagy maturation through participating in the formation of autophagosome and endosome[7]. On the other hand, it is reported that the abnormal expression of Beclin1 is correlated with the occurrence and prognosis of breast cancer, gastric cancer and lymphoma[8]. However, controversial result in HCC was observed. Beclin1$^{+/-}$

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mice are more likely to suffer from malignant tumors compared to wild-type mice, including liver cancer[9]. Moreover, Beclin1 expression is correlated with cirrhosis, Edmundson grade and vascular infiltration[10]. Interestingly, it has been revealed that the Beclin1 expression is positively correlated with the prognosis of HCC patients[11], although its expression related with prognosis and clinicopathological factors of HCC is still a debatable point[12]. In this study, the protein and messenger ribonucleic acid (mRNA) levels of Beclin1 in patients with liver cancer were measured, and its correlations with the magnetic resonance imaging (MRI) and pathological features of liver cancer were analyzed, so as to evaluate their clinical value in liver cancer.

Patients and Methods

Clinical Data

The tissue specimens and clinical data of a total of 109 liver cancer patients admitted to the Weihai Municipal Hospital from January 1, 2016 to January 1, 2018 were enrolled, and the postoperative cancer tissues and para-carcinoma normal tissues were also collected from the liver cancer patients admitted to our hospital from January 1, 2016 to June 1, 2018. Finally, 42 cases of cancer tissues and para-carcinoma tissues were obtained. The freshly collected tissue specimens were prepared into frozen sections, followed by hematoxylin-eosin (HE) staining. The paraffin sections were made to analyze the semi-quantitative expression of Beclin1 protein via immunohistochemistry (IHC). The RNA was extracted to detect the Beclin1 mRNA expression level. All human tissues were obtained according to the Human Subject Protocol approved by the Review Committee of our hospital.

Quantitative Polymerase Chain Reaction (qPCR)

The total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The tissue specimens (mung bean- and soybean-sized) were taken into the mortar and ground into powder with the liquid nitrogen poured in. Next, each specimen was added with 1 mL TRIzol and fully lysed on ice. cDNA was synthesized using the TaKaRa PrimeScript™ Kit (Bio Inc., Otsu, Shiga, Japan). The gene sequences of the target gene and internal reference GAPDH were obtained in GenBank, the primers were designed using Primer-Blast software of the NCBI website, and the primer sequences were synthesized by Sangon (Shanghai, China). Beclin1 forward primer 5' TGGATGTGGAGAAAGGCAAG 3', reverse primer 5' TCGTCAGCATGAACTTGAGAG 3'. GAPDH forward primer 5' AATCCCATCACCATCTTCCAG 3', reverse primer 5' AAATGAGCCCCAGCCTTC 3'. The reaction system was 10 μL, and the reaction conditions are as follows: pre-denaturation at 95°C for 2 min, followed by 40 cycles, including 95°C for 15 s, and 60°C for 60 s. After DNA denaturation, annealing and primer extension, the single strand complementary to the template strand was synthesized, DNA replicated in the semi-conservative manner and the gene to be detected was amplified by several million times. After the reaction, the dissolution curve showed single peak, indicating the specificity of qPCR product. The relative mRNA expression level was calculated as follows: $2^{-\Delta Ct}$ [ΔCt=Ct (target gene)-Ct (β-actin)], and the fold change between different treatments was calculated using 2-ΔΔCT, where ΔΔCT = ΔCT (experimental group) - ΔCT (control group).

Immunohistochemistry

The liver cancer and corresponding para-carcinoma normal tissues were fixed with formaldehyde and embedded in paraffin, followed by immunohistochemical SP staining using the Beclin1 polyclonal antibody (purchased from Abcam, 1:500, Cambridge, MA, USA). Positive control: the tissue containing the test antigen was detected according to the official website of Abcam, and there was strongly positive expression. Negative control: the primary antibody was replaced with PBS, and the results were all negative. The positive signals showed yellow, brown yellow or dark brown color. 5 high-power fields (10×40) were randomly observed under an electron microscope, and the proportion of positive cells containing yellow, brown yellow or dark brown signals and the signal intensity were used as criteria.

Western Blotting

The protein assay kit (Beyotime, Shanghai, China) was used in this experiment. In brief, after the protein denaturation, the protein was carefully added into the well, 3-5 μL Marker was also added and an appropriate amount of 1 × electrophoresis solution was added into the outer tank. Electrophoresis was performed at a constant voltage of 80 V. When the protein band in the correspond-

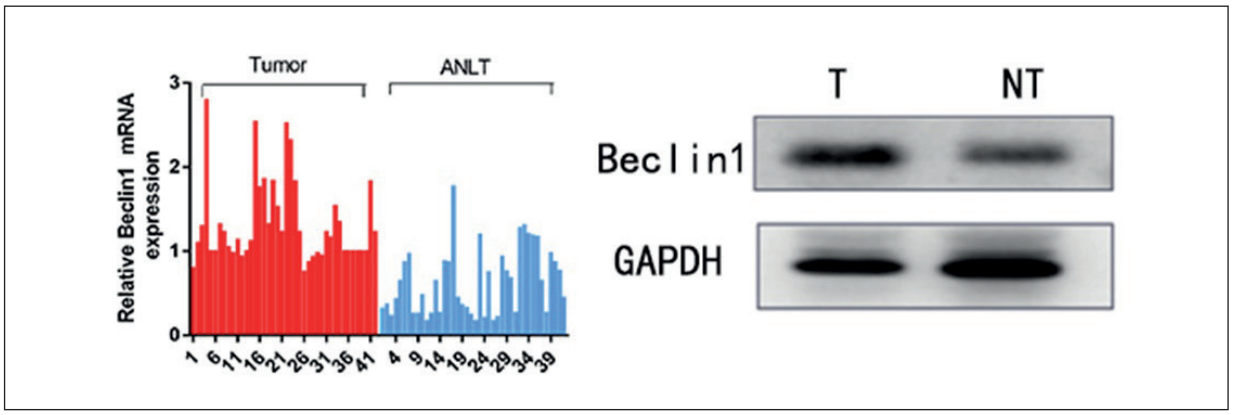

Figure 1. Beclin1 expression level in liver cancer and para-carcinoma tissues detected via PCR and Western blotting.

ing position of the bromophenol blue (Marker) was separated, the voltage was raised to 120 V, and the electrophoresis was terminated when the bromophenol blue reached the bottom of the separation gel. The polyvinylidene difluoride (PVDF) membrane with the same size of the gel was cut, activated with methanol and placed into the transfer solution for equilibrium for 5 min. The sponge mat, filter paper, gel, PVDF membrane, filter paper and sponge mat were placed in order into the vertical transfer tank according to the direction of the electrode, the bubbles between the gel and the PVDF membrane were excluded using the test tube, and the transfer solution was fully poured into the tank, followed by membrane transfer in an ice box under a constant current of 300 mA for 60-90 min. Then, the PVDF membrane was blocked in TBST containing 5% skim milk on a shaking table at 37°C for 1 h, incubated with primary antibody and secondary antibody and washed. After exposure, the color was developed using the Image Lab gel imaging system (Bio-Rad, Hercules, CA, USA) and the gray value was analyzed.

Statistical Analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. The correlation between Beclin1 expression level and clinicopathological characteristics was analyzed via chi-square test. *t*-test was used for the comparison of measurement data between two groups, and continuous data from multiple groups were analyzed by using one-way ANOVA, with the Tukey's post-hoc test. All tests were two-sided tests and $p < 0.05$ suggested that the difference was statistically significant.

Results

Expression of Beclin1 in Liver Cancer and Para-Carcinoma Tissues

Both the mRNA and protein levels of Beclin1 in 42 pairs of liver cancer and para-carcinoma tissues were detected via RT-PCR and Western blotting. As shown in Figure 1 and Table I, the expression of Beclin1 in cancer tissues was significantly higher than those in para-carcinoma tissues ($p < 0.05$), and there was a significant correlation between the mRNA and protein expression levels of Beclin1 ($p < 0.05$).

Immunohistochemical Analysis of Beclin1 expression in Liver Cancer and Para-Carcinoma Tissues

The Beclin1 protein expression was then detected in liver cancer and para-carcinoma tissues via immunohistochemistry. The Beclin1 protein was found positively expressed in cytoplasm, indicating that the positive expression rate of Beclin1 protein in liver cancer was higher than that in para-carcinoma tissues (Figure 2).

Table I. Correlation between Beclin1 mRNA and protein expressions.

	Beclin1 mRNA	
Beclin1 protein	Positive	Negative
Positive	34	3
Negative	2	7

Note: $p < 0.05$.

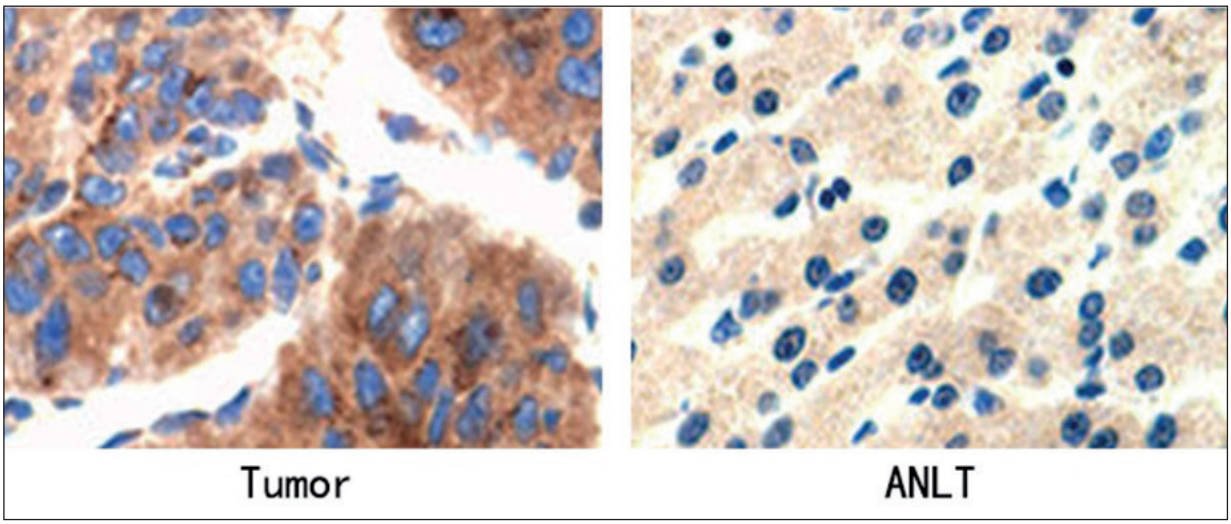

Figure 2. Beclin1 expression detected via immunohistochemistry. High expression of Beclin1 in cancer tissues was shown compared to that in para-carcinoma tissues.

MRI Manifestations of Liver Cancer

The analysis of MRI features of liver cancer with different Beclin1 expression levels was further compared. Mixed edge and internal enhancement were observed in liver cancer patients with low expression of Beclin1, while large mass, tumor rupture hemorrhage and necrosis were indicated in those with high expression of Beclin1 (Figure 3, 4). The MRI parameters, including Max-apparent diffusion coefficient (ADC), Min-ADC, Mean-ADC, D, f and D*, in different groups were analyzed. The results revealed that the Min-ADC and Mean-ADC in Beclin1 high-expression group were significantly lower than those in Beclin1 low-expression group ($p < 0.05$), and there was no statistically significant difference of Max-ADC between the two groups. By contrast, the D* value was higher in low-expression group ($p < 0.05$) (Table II).

Correlation Between Beclin1 Expression and Clinicopathological Characteristics

The correlation between Beclin1 expression and clinicopathological characteristics was analyzed, and the results manifested that the Beclin1 expression was correlated with the tumor size, alpha fetoprotein (AFP), pathological grade and bile duct tumor thrombus ($p < 0.05$), but no significant correlation was observed between Beclin1 and the patient's gender and age, tumor stage, lymph

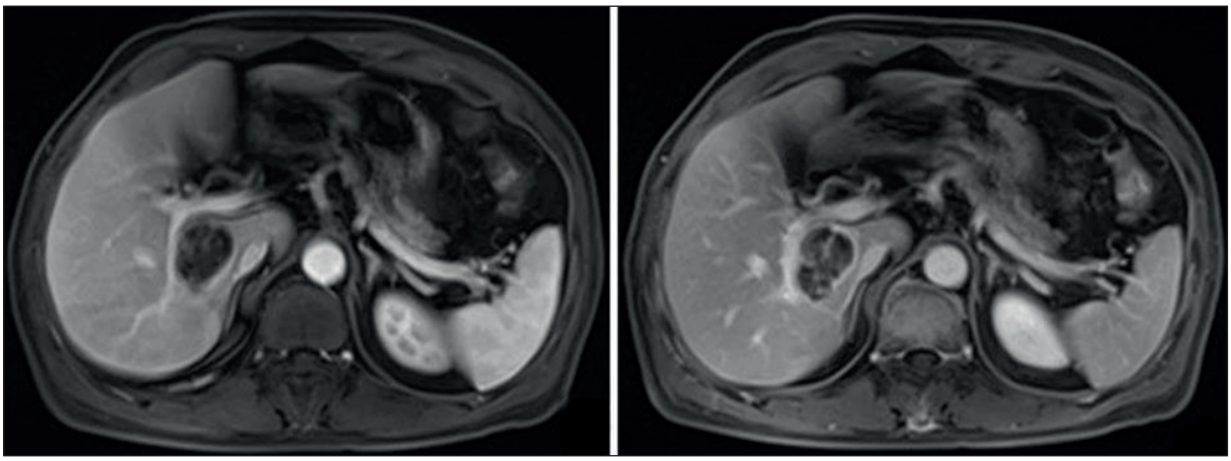

Figure 3. MRI of liver cancer with low expression of Beclin1. Mixed edge and internal enhancement were observed.

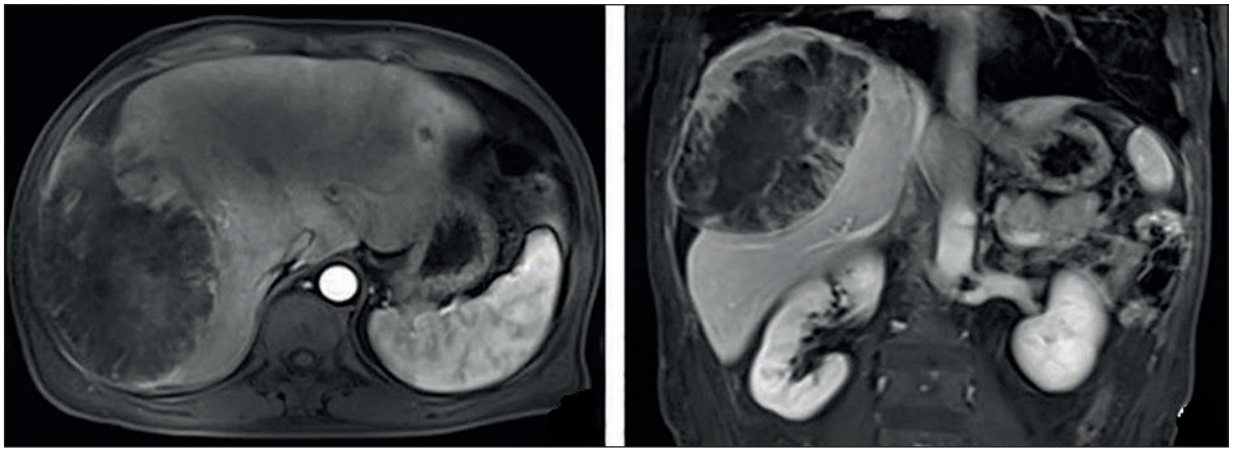

Figure 4. MRI of liver cancer with high expression of Beclin1. Large mass, tumor rupture hemorrhage and necrosis were found.

node metastasis. The level of Beclin1 in patients showed positive relation with tumor diameter and the AFP level. In addition, the expression rate of Beclin1 in tumor in pathological grade III-IV (47.8%) was higher than that in grade I-II (19.0%) ($p < 0.001$), while its expression rate in bile duct tumor thrombus group (71.4%) was also higher than that in non-bile duct tumor thrombus group (36.7%) ($p < 0.01$) (Table III).

Discussion

Autophagy is a normal physiological process that maintains the cell homeostasis[13]. It is considered as a natural, regulatory and destructive mechanism in cells that enables the cell recovery from the undesired or broken formats. Beclin1, as a component of the hVps34/III PI3K complex, plays a crucial role in autophagy[14]. The PI3K complex contributes to mediating the positioning process of other autophagy cyanates in the precursor cell membrane. The excessive self-loss of cells during sustained stress and autophagy leads to death, usually characterized by autophagy. During this process, Beclin1 and a large number of autophagosomes are produced by cells[15]. The aberrant expression of Beclin1 protein in different tumors indicates the close relation of tumorigenesis with Beclin1-induced autophagic cell death. Previous finding showed that the Beclin1 expression in breast cancer MCF-7 cell lines significantly declined or even cannot be detected[16]. The stable overexpression with Beclin1 can, however, significantly improved autophagy and reduced tumorigenic ability. The decrease of the autophagy gene Beclin1 can significantly reduce autophagy, protect tumor cells from autophagic cell death, and play a promoting role in the development of tumor cells. Genomic instability becomes one of the characteristics of tumor, as well as one of the essential mechanisms of cell canceration. Genomic instability is frequently increased due to the environment and metabolic stress in the body. The monoallelic deletion of the autophagy gene Beclin1 damages the cell autophagy, harms the survival of normal cells during metabolic stress and promotes tumorigenesis. There are a large number of damaged mitochondria, p62 and ubiquitin protein polymers in the mouse model with autopha-

Table II. MRI parameters of liver cancer with different Beclin1 expression levels.

MRI parameter	Beclin1 high-expression	Beclin1 low-expression	z	p
Min-ADC ($\times 10^{-3}$ mm^2/s)	0.69 ± 0.22	0.76 ± 0.28	2.28	0.03
Max-ADC ($\times 10^{-3}$ mm^2/s)	1.08 ± 0.49	1.45 ± 0.52	1.30	0.16
Mean-ADC ($\times 10^{-3}$ mm^2/s)	1.06 ± 0.56	1.13 ± 0.16	2.72	0.01
D ($\times 10^{-3}$ mm^2/s)	0.96 ± 0.17	1.33 ± 0.43	223	0.03
f (%)	17.97 ± 10.25	14.78 ± 11.12	-1.07	0.28
D* ($\times 10^{-3}$ mm^2/s)	76.77 ± 59.78	36.98 ± 39.74	2.29	0.03

Table III. Correlation between Beclin1 expression and clinicopathological characteristics.

Characteristic	Case (n)	Beclin1 high-expression	Beclin1 low-expression	p
Gender				
Male	61	28	33	0.456
Female	48	18	30	
Age				
> 50	51	35	16	0.623
≤ 50	58	31	27	
Diameter of tumor				
> 5 cm	47	40	7	0.003
≤ 5 cm	62	20	42	
AFP (μg/L)				
> 400	60	49	11	0.021
≤ 400	49	24	25	
TNM stage				0.234
I-II	67	32	34	
III-IV	42	8	34	
Pathological grade				
I-II	45	11	34	
III-IV	64	52	12	0.000
Bile duct tumor thrombus				
Yes	49	35	14	0.023
No	60	22	38	
Lymph node metastasis				
Yes	50	38	12	0.107
No	59	26	33	

gy defects[17]. The massive accumulation of these substances will further induce the production of reactive oxygen species, lead to chromosomal instability and obviously raise the morbidity rate of cancer. Beclin1 can effectively prevent gene mutation, improve genomic stability, and ultimately reduce the incidence rate of cancer. It is proposed that genomic stability is maintained by reducing harmful proteins, removing damaged organelles and reducing DNA damage[18]. In the development of tumor, autophagy also exerts pivotal function and it can promote or inhibit the growth of tumor cells in distinct states of tumor. It has been found in recent studies that Beclin1 is abnormally expressed in HCC, but the results are still controversial. Previous finding showed that Beclin1 has no correlation with any clinicopathological characteristic, such as TNM stage and tumor size[19]. The Beclin1 expression is not associated with the clinical features and prognosis of HCC[20]. However, accumulative studies have also demonstrated that the Beclin1 expression in cancer tissues is significantly lower than that in normal tissues and liver cirrhosis tissues[21,22]. Beclin1 expression is correlated with cirrhosis, Edmundson grade and vascular infiltration. Moreover, the expression of Beclin1 indicates the overall survival. In this study, similarly, it was found that the positive expression of Beclin1 was correlated with AFP, tumor size, pathological grade and bile duct tumor thrombus, which is partially consistent with results of previous studies. All the results suggest that Beclin1 is related to the development of HCC, so it may be a potential prognostic biomarker for HCC. Of note, recent studies have demonstrated that the clinical value of Beclin1 expression can be greatly increased combined with other related genes, such as HIF-1α and Bcl-xL[23]. Beclin1 expression is significantly correlated with the tumor differentiation in Bcl-xL$^+$ patients, but not correlated with that in Bcl-xL$^-$ patients. In addition, the positive expression of Beclin1 indicates that both overall survival and disease-free survival are increased in Bcl-xL$^+$ HCC[24]. Similar markers are found between Beclin1 and apoptosis, such as Bcl-2 and Bax. Kotsafti et al[25] found that the Beclin1 expression has a close correlation with tumor differentiation and size and number of tumor, but has no correlation with TNM stage in HIF-1α high-expression group and HIF-1α low-expression group. Therefore, Beclin1 combined with other molecules can be used in the diagnosis and prognostic prediction of HCC. Our data for the first time revealed that the Beclin1 expression was correlated with MRI features of liver cancer. Min-ADC and Mean-ADC were significantly lower

in Beclin1 high-expression group than those in low-expression group. The possible reason is that there is less free water and abundant blood supply in liver cancer tissues. The apoptosis and necrosis of liver cancer tissues will lead to the increase in free water, therefore the ADC value is higher than that in solid tumor.

Conclusions

We preliminarily clarified the expression of autophagy-related gene Beclin1 in liver cancer, providing a theoretical basis for the further study on the molecular mechanism of regulating tumor autophagy activity and the occurrence and development of tumor.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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