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*Original Article*

# UNC51-like kinase 1 as a potential prognostic biomarker for hepatocellular carcinoma

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**Abstract:** Autophagy is a fundamental cell biological process that confers stress tolerance, limits damage, and sustains viability under adverse conditions. Defective autophagy is associated with diverse diseases. The study aimed to investigate the relationship between UNC51-like kinase 1 (ULK1) expression and clinicopathological characteristics as well as survival in patients with hepatocellular carcinoma (HCC). Expression levels of ULK1 in 55 paired HCC and paracancerous tissues were examined using immunohistochemistry. Although not statistically significant, the expression of ULK1 in adjacent peritumoural tissue was lower than those in HCC tissues ($P$ = 0.113). Expression level of ULK1 was significantly associated with tumor size ($P$ = 0.015) after adjusted for age, sex, histologic grade, cirrhosis and TNM. Survival analysis showed that patients with high ULK1 expression had worse survival time than those with low ULK1 expression (hazard rate = 2.684, 95% CI 1.029–7.006, $P$ = 0.044). The findings of the present study provide evidence that ULK1 represents a potential novel prognostic biomarker for HCC patients and may play an important role during the progression of HCC.

**Keywords:** Hepatocellular carcinoma, autophagy, UNC51-like kinase 1, survival, biomarker

## Introduction

Liver cancer is the sixth most common cancer and the third leading cause of cancer deaths in the world [1]. Liver cancer incidence rates are increasing in several low-risk developed countries and decreasing in some high-risk developing countries [2]. It was estimated that there were about 748,300 new liver cancer cases and 695,900 cancer deaths worldwide in 2008 [3]. China accounts for more than half of these cases and deaths [1]. Hepatocellular carcinoma (HCC) is the major histological subtype, accounting for 70% to 85% of the total liver cancer cases [4].

Salvage chemotherapy is not commonly to treat HCC patient due to a low response rate. Disturbance of cell-death signaling pathways is the main reason for treatment failure [5]. Apoptosis, the major form of cell death, plays an essential role in the maintenance of tissue homeostasis by eliminating unnecessary, damaged and neoplastic cells and defending against pathogenic infections [6, 7]. Dysregulation of apoptosis is usually associated with HCC resistance for treatment [8, 9]. Therefore, it is essentially important for HCC treatment to find an alternative process that induces cancer cell death.

Recently, scientists have become more interested in autophagy, called type II cell death. Autophagy is a fundamental cell biological process that confers stress tolerance, limits damage, and sustains viability under adverse conditions. Defective autophagy is associated with diverse diseases, including metabolic disor-

ders, idiopathic inflammatory diseases, and cancer [10-14]. Autophagy can either promote or inhibit the growth of cancer cells depending on the cellular context [15]. However, in most cases, autophagy promotes cancer cell survival and sustains cell viability [16, 17]. UNC51-like kinase 1 (ULK1), a mammalian serine/threonine protein kinase, plays an essential role in the initiation of autophagy, though the exact molecular mechanisms remain unknown. Aberrant expression of ULK1 was associated with poor prognosis in breast cancer and esophageal squamous cell carcinoma [18-20]. Therefore, in the present study, we investigated the expression of ULK1 in HCC and adjacent papacancrous tissues, and evaluated the relationship between expression of ULK1 and clinicopatholgoical features and survival outcome in HCC patients.

## Material and methods

### *Patients*

The study was approved by the ethical committees of National Engineering Center for Biochip at Shanghai and Taizhou People's Hospital. HCC and paracancerous tissues with informed consent were collected between 2006 and 2008 from 55 patients who underwent surgery. Tissue samples were stored at the Biobank Center of National Engineering Center for Biochip at Shanghai. All patients received no anticancer therapy before tumour resection. All patients were pathologically diagnosed with primary HCC.

### *Tissue microarray (TMA) construction*

The formalin-fixed, paraffin-embedded specimens were provided by the Biobank Center of National Engineering Center for Biochip at Shanghai. TMA containing 55 paired HCC and matched paracancerous tissues was constructed according to a method described previously [21]. Hematoxylin and Eosin (H&E)-stained standard slides from each patient were reviewed by a senior pathologist to determine the locations from which cores should be taken for TMA construction. Two cores of each sample were taken from each region using an automated tissue arrayer (Beecher Instruments, Sun Prarie, WI) described previously [22]. TMA sections (5μm) were cut from the tissue array block. All arrayed sample were stained with H&E to confirm the presence of tumor and paracancerous tissues.

### *Immunohistochemistry (IHC)*

The IHC staining was performed using TMA slides that were deparaffinized in xylene, rehydrated through a graded alcohol series, washed with Tris-buffered saline, and processed using a streptavidin–biotin–peroxidase complex method. For antigen retrieval, TMA slides were boiled by a pressure cooker in 10 mM sodium citrate buffer (pH 6) for 10 minutes. After quenching of endogenous peroxidase activity and blocking of antibody nonspecific binding, the TMA slides were incubated with anti-ULK1 antibody at a dilution of 1:100 (Abgent, CA, USA) at 4°C overnight in a moist chamber. The slides were treated with corresponding secondary biotinylated rabbit antibody (Dako, Glostrup, Denmark) for 30 minutes at 37°C and then washed with Tris-buffered saline. The slides were incubated with StrepAB Complex/horseradish peroxidase (1:100) (Dako, Glostrup, Denmark) for 30 minutes at 37°C. Chromogenic immunolocalization was performed by exposure to 0.05% 3,3-diaminobenzidine tetrahydrochloride. The negative control was performed by replacing the primary antibody with normal rabbit IgG. Other cores containing HCC were used as positive controls. Sigma hematoxylin was used as counterstain for IHC.

Sections of each specimen were analyzed by two independent pathologists blinded to the clinicopathological information. In cases of disagreement, a consensus was reached by joint review of slide. ULK1 expression was scored based on the following criteria: 1, 0 points for no staining; 2, 1 point for < 20%; 3, 2 points for 20-75%; 4, 3 points for > 75% of tumor tissue stained, as described previously [23]. The intensity of ULK1 immunoreactivity was graded on a scale of 0 to 3 (0, none; 1, weak, 2, intermediate; and 3 strong). The total score was determined by the following formula: Staining index = intensity X positive rate. In the present study, staining index $\leq 4$ was considered low expression, and staining index > 4 was considered as high expression.

### *Statistical analysis*

The association between individual clinicopathological variables and ULK1 expression was

**Table 1.** Clinicopathological characteristics of HCC patients

| Characteristics | No. of Patients | % |
|---|---|---|
| Age (years) | | |
| median | 54 | |
| range | 38-72 | |
| Sex | | |
| female | 6 | 10.9 |
| male | 49 | 89.1 |
| Cirrhosis | | |
| Yes | 21 | 38.2 |
| No | 34 | 61.8 |
| Histologic grade | | |
| I | 5 | 9.1 |
| II | 34 | 61.8 |
| III | 11 | 20.0 |
| unknown | 4 | 9.1 |
| TNM | | |
| I | 40 | 72.7 |
| II | 7 | 12.7 |
| III | 7 | 12.7 |
| IV | 1 | 1.8 |
| Tumor size (cm) | | |
| median | 5.5 | |
| range (cm) | 1-30 | |

statistically analyzed using the $X^2$-test. Kaplan-Meier method and log-rank test were used to investigate the relationship between ULK1 expression and over survival (OS) in HCC patients. Cox regression model was used to test the independence of risk factors for HCC. Since only one patient was TNM stage IV, we combined TNM stage III and IV groups into one. A $P$ value < 0.05 was considered statistically significant. Statistical analyses were carried out using the SPSS software package (version 17.0). All statistics were two-tailed.

## Results

### *Characteristics of HCC patients*

The characteristics of HCC patients enrolled in this study were summarized in **Table 1**. Among 55 HCC patients, 49 cases are male (89.1%), and 6 cases are female (10.9%). The median age of 55 patients was 54 years (range 38 to 72 years). Histologic grade of HCC was 1, 2, and 3 in 5, 34, and 11 cases, respectively. Forty (72.7%) of them, were stage as TNM stage I, 7 (12.7%) as TNM stage II, 7 (12.7%) as TNM stage III, and 1 (1.8%) were TNM stage IV. Twenty-one patients (38.2%) were also suffering from liver cirrhosis. The median tumor size was 5.5 cm (range 1 to 30 cm).

### *The expression of ULK1 in HCC and paracanerous tissues*

IHC staining for ULK1 was found in all HCC and adjacent tissues (**Figure 1**). The localization of the ULK1 protein was cytoplasm in all of hepatocytes and HCC cells. However, nuclear localization of ULK1 was rarely observed in HCC cells. Although not statistically significant, the expression of ULK1 in adjacent peritumoural tissues was lower than those in HCC tissues ($P$ = 0.113). Furthermore, no difference was observed in the cirrhotic tissues adjacent to HCC compared with inflammatory tissues adjacent to HCC.

### *The relationship between expression of ULK1 and clinicopathological characteristics of HCC patients*

To investigate the association between clinicopathological characteristics and ULK1 staining in cancer tissue, immunohistochemical staining index was analyzed to examine the relationship. The ULK1 was highly expressed in 39 HCC tissues. The analysis showed that expression level of ULK1 was significantly associated with tumor size ($P$ = 0.017) (**Table 2**). The difference remain significant even after adjusted for age, sex, histologic grade, cirrhosis and TNM ($P$ = 0.015). No other significant difference was observed between expression of ULK1 and clinicopathological features.

### *Survival analysis*

The median survival time was 32 months. Kaplan Meier curve showed a significant correlation between low expression levels of ULK1 in HCC and overall survival (OS). Patients with low ULK1 expression had longer survival time than those with high ULK1 expression ($P$ = 0.033) (**Figure 2**). Cox regression was used to analyze the relationship between ULK1 expression and OS. At invariable Cox regression, age, sex, histologic grade, cirrhosis, long diameter of tumor and TNM were not associated with survival time. As shown in **Table 3**, patients with high ULK1 expression showed a hazard rate (HR) of 2.684 (95% CI 1.029–7.006, $P$ = 0.044).

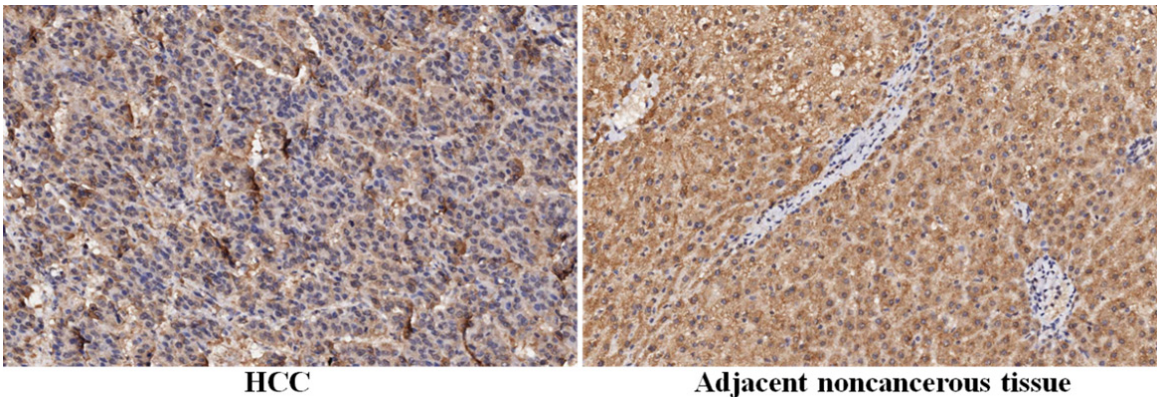


**Figure 1.** Differential expression of ULK1 in HCC and adjacent noncancerous tissues.

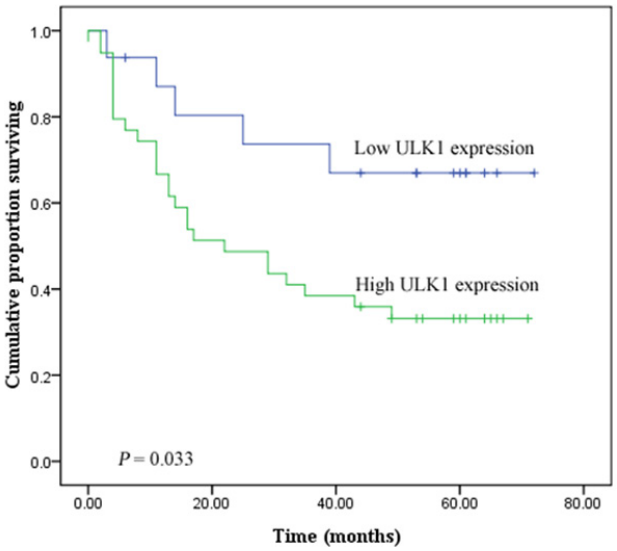


**Figure 2.** Kaplan-Meier analysis of overall survival for patients with HCC.

## Discussion

In the present study, we demonstrated that the expression of ULK1 was overexpressed in HCC and cancerous tissues. We found that high expression level of ULK1 was related to tumor size and worse survival time in patients with HCC. To the best of our knowledge, this is the first report that has revealed the relationship between ULK1 expression and prognosis of HCC patients.

The newly described links between autophagy and macromolecules metabolism in liver suggests that alteration in autophagic function may play a role in the pathophysiology of liver disorders. The prototypic functions of autophagy are to recycle essential nutrients and provide energy for survival during nutrient and oxygen deprivation and other stressful conditions. In the present study, ULK1 was expressed in HCC, as well as cirrhotic and inflammatory tissues adjacent to HCC. The expression levels of ULK1 in HCC was weakly higher than those in paracancerous tissue, indicating that HCC cells had a high demand for nutrients and oxygen to maintain their high proliferation rate, especially if the tumor was large. This means that although liver has a very rich blood supply, there is also a certain degree of deprivation of nutrient and energy in liver cancer cell. Therefore, cancer cells have a high level of ULK1 expression to effectively promote autophagy initiation and protect them against both apoptosis and necrosis. This might provide a better understanding of why patients with larger tumor have a high expression of ULK1.

For cancer therapy, either conventional radiotherapy and chemotherapy, or recent target therapy, the main purpose is to reduce the energy supply of cancer cell. Therefore, under metabolic stress, the residual cancer cells become fragile. However, even if there is a huge metabolic stress, cancer cells rely on autophagy to survive and maintain their activity. Since almost every therapeutic regimen can induce autophagy in cancer cells, many studies support the role of autophagy in cancer cell resistance to treatment [24, 25]. Therefore, if the protective effect of autophagy is inhibited, cancer cells will not be able to effectively respond

**Table 2.** Association between ULK1 expression and clinicopathological features

| Variables | ULK1 expression | | |
|---|---|---|---|
| | Low | High | *P* value |
| Age (years) | | | |
| < 50 | 9 | 8 | 0.011 |
| ≥ 50 | 7 | 30 | |
| Gender | | | |
| female | 3 | 3 | 0.232 |
| male | 13 | 36 | |
| Size | | | |
| < 4 | 9 | 9 | 0.017 |
| ≥ 4 | 7 | 30 | |
| Cirrhosis | | | |
| Yes | 7 | 14 | 0.586 |
| No | 9 | 25 | |
| Histologic grade | | | |
| I | 2 | 3 | 0.225 |
| II | 12 | 22 | |
| III | 1 | 10 | |
| TNM | | | |
| I | 12 | 28 | 0.422 |
| II | 3 | 4 | |
| III + IV | 1 | 7 | |

**Table 3.** Cox regression analysis of overall survival

| Features | HR (95% CI) | *P* value |
|---|---|---|
| age | 1.031 (0.474-2.240) | 0.939 |
| sex | 1.404 (0.491-4.020) | 0.527 |
| tumor size | 1.045 (0.999-1.094) | 0.054 |
| cirrhosis | 0.852 (0.413-1.757) | 0.665 |
| histologic grade | 1.950 (0.950-4.001) | 0.069 |
| TNM | 1.551 (0.996-2.416) | 0.052 |
| ULK1 | 2.684 (1.029-7.006) | 0.044 |

to metabolic stress and then die. This is particularly effective for cancer cells with defective apoptosis. In vitro and in vivo studies have shown that inhibiting the activity of autophagy with autophagic inhibitor increased the anticancer effect of standard therapy in a variety of cancer types [14, 25]. Several aberrant expressions of autophagy-related proteins were reported to be associated with prognosis for some cancers [26-30]. In the present study, we found that high ULK1 expression means worse survival time for patients with HCC. Jiang et al. also found that the expression level of ULK1 is inversely related to survival time of patient with esophageal squamous cell carcinoma [20]. However, breast cancer patients with high ULK1 expressions have longer survival time [31]. These results indicate that autophagy may play different roles in different types of cancer. In the present study, ULK1 was highly expressed in HCC, indicated high level of autophagy activity in HCC. Autophagy may a main cause of higher resistance to treatment in HCC and thus represent a novel therapeutic target. A more detailed investigation of overexpression of ULK1 in the progression of HCC may eventually lead to the development of improved strategies/therapies for HCC.

In conclusion, our results, for the first time, revealed that ULK1 represents a potential novel prognostic biomarker for HCC. However, further studies are needed to clarify the exact role of ULK1 in carcinogenesis and progression of HCC.

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