

Association of Autophagy Defect with a Malignant Phenotype and Poor Prognosis of Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is an aggressive cancer with a poor prognosis. The role of autophagy and the prognostic value of autophagic genes are largely unknown in HCC. Here, we showed decreased expression of autophagic genes and their corresponding autophagic activity and increased expression of the antiapoptotic gene *Bcl-xL* in HCC cell lines compared with a normal hepatic cell line. We also found decreased expression of the autophagic gene *Beclin 1* in 44 HCC tissue samples compared with adjacent nontumor tissues. In addition, we found that the most aggressive malignant HCC cell lines and HCC tissues with recurrent disease displayed much lower autophagic levels, especially when *Bcl-xL* was overexpressed. Interestingly, in a tissue microarray study consisting of 300 HCC patients who underwent curative resection, the expression of *Beclin 1* was only significantly correlated with disease-free survival (DFS; $P < 0.0001$) and overall survival (OS; $P < 0.0001$) in the *Bcl-xL*⁺ group. Multivariate and univariate analyses also revealed that *Beclin 1* expression was an independent predictor for DFS and OS in *Bcl-xL*⁺ patients. In addition, we found a significant correlation between *Beclin 1* expression and tumor differentiation in *Bcl-xL*⁺ but not in *Bcl-xL*⁻ HCC patients. In conclusion, our data showed expression of autophagic genes and their corresponding autophagic activities were suppressed in HCC. The autophagy defects synergized with altered apoptotic activity might facilitate tumor malignant differentiation, which results in a more aggressive cancer cell phenotype and poor prognosis of HCC. [Cancer Res 2008; 68(22):9167–75]

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent malignant tumors in southern China, Taiwan, southeastern Asia, and sub-Saharan Africa (1). The overall survival (OS) of HCC patients remains poor despite improved diagnostic and treatment strategies. Indeed, HCC is a type of cancer highly resistant to conventional antineoplastic medicines (2), which is partially attributed to the property of insensitivity to cell death induced by cytotoxic agents. It is well known that the avoidance of apoptosis is one of the hallmarks of cancer cells (3) and that failure

to induce apoptosis by anticancer treatments contributes to chemotherapeutic failure and tumor progression. However, the role of autophagy, an alternative caspase-independent cell death program (4, 5), and its underlying molecular mechanism, is still controversial in cancer, especially in tumor progression.

Autophagy is an evolutionarily conserved process that involves lysosomal degradation of cytoplasmic and cellular organelles. Autophagy has emerged as a homeostatic mechanism regulating the turnover of long-lived or damaged proteins and organelles, and buffering metabolic stress induced under starvation conditions by recycling intracellular constituents (6). Although cell death resulting from progressive cellular consumption has been attributed to unrestrained autophagy, which led to the belief that autophagy is a nonapoptotic form of programmed cell death, most of the evidences support autophagy as a survival pathway required for cellular viability (7, 8). Interestingly, defects in autophagy also play a role in tumorigenesis. For example, the essential autophagy regulator *Beclin 1* is monoallelically deleted in human ovarian, breast, and prostate cancers (9, 10). In addition, *Beclin 1*^{+/-} or *Atg4C*^{-/-} mice are prone to tumors (11–13). Paradoxically, these findings suggest that the loss of a survival pathway enhances tumor growth. Recent studies have shown that simultaneous defects in autophagy and apoptosis activate the DNA damage response *in vitro*, promote gene amplification and aneuploidy, and then accelerate mammary tumorigenesis (14, 15). Thus, loss of the prosurvival role of autophagy is likely to contribute to tumor progression by promoting genome damage and instability in an apoptosis-deficient background. Furthermore, stimulation of necrotic cell death and inflammation caused by defects in both autophagy and apoptosis provides a cell with nonautonomous means of tumor promotion through induction of a chronic wound-healing response (16). In fact, the protective role of autophagy that involves mitigation of genome damage and inflammation in tumors limits chronic tumor necrosis in response to metabolic stress and ultimately suppresses tumor carcinogenesis and progression. However, to date, the related clinical significance of this hypothesis has not been investigated.

Several molecules involved in the control and execution steps of autophagy have highlighted the close link among the autophagy, tumorigenesis, and tumor progression. Although *Beclin 1*, an important autophagy regulator, has been found to be monoallelically deleted or express at decreased levels in some human cancers, its expression pattern in HCC and the role of such molecules in clinical prognosis are largely unknown.

To study the role of autophagy in HCC under different apoptotic conditions, we examined the expression of autophagic genes, their corresponding autophagic activity, and expression of antiapoptotic genes in HCC cell lines and tissue samples. Our results suggest that in HCC with compromised apoptosis, autophagy defects are not only associated with malignant phenotype and poor differentiation

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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of HCC cells but also represent poor survival, which can be independently predicted by the autophagic gene *Beclin 1*.

Materials and Methods

Patient samples. Patient samples were collected after obtaining informed consent according to an established protocol approved by the Ethics Committee of Fudan University. The data do not contain any information that may lead to the identification of the patients.

Samples used in real-time PCR studies were randomly collected from the patients undergoing curative resection at the Liver Cancer Institute, Zhongshan Hospital, Fudan University, in March 2006. Samples were collected immediately after resection, transported in liquid nitrogen, and stored at -80°C . Sixteen frozen tissue samples used in Western blotting studies were also obtained from above patients.

Tumor specimens used in tissue microarray (TMA) studies were obtained from 300 consecutive HCC patients who underwent curative resection without preoperative treatment at the Liver Cancer Institute, Zhongshan Hospital, Fudan University, between 1997 and 2000. For each patient, complete follow-up data were available and the diagnosis of HCC was confirmed by pathologic examination.

Cell lines. Human HCC cell lines, including HepG2, Hep3B, SMMC-7721, MHCC97-L (17–19), MHCC97-H (19), HCCLM3 (17, 20), HCCLM6 (21), and a human normal hepatic cell line, L-02 (22, 23), were routinely maintained in high-glucose DMEM or RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin. All cell lines were cultured at 37°C in a humidified incubator in an atmosphere of 5% CO_2 .

Autophagy analysis. For starvation studies, cells were cultured on 0.1% gelatin-coated dishes or coverslips, washed twice with PBS, once with Earle's Balanced Salt Solution (EBSS; Sigma-Aldrich), then incubated in EBSS at 37°C for the indicated times. Autophagy was assessed by GFP-microtubule-associated protein light chain 3 (LC3) redistribution and electron microscopy (24).

For GFP-LC3 redistribution, L-02, SMMC-7721, and HCCLM3 cells were transfected with a GFP-LC3 expression plasmid using Lipofectamine 2000 (Invitrogen). Redistribution was detected 24 h after transfection using an inverted fluorescence microscope. The fraction of GFP-LC3-positive (>3 punctate staining sites per cell) cells was determined in three independent experiments. Eight random fields representing 200 cells were counted.

For electron microscopy, cells were immediately fixed with 2.5% glutaraldehyde with 0.1 mol/L sodium cacodylate and stored at 4°C until embedding. Samples were postfixed with 1% osmium tetroxide, followed by an increasing gradient dehydration step using ethanol and propylene oxide. Samples were then embedded and ultrathin (50–60 nm) sections were cut using an ultramicrotome (LKB-I). Images were examined with a JEM-1200 electron microscope at 80 kV after samples were stained with 3% uranyl acetate and lead citrate. For quantitative analysis of autophagy, the number of autophagic vesicles per viable cell was scored. For quantification of viable cells using electron micrographs, high-powered micrographs ($\times 8,000$ – $10,000$) of 20 single cells from multiple distinct low-powered fields were obtained from each specimen.

Real-time PCR. Total RNA was extracted from cell lines and frozen tumor specimens using Trizol Reagent (Invitrogen). Total RNA (2 μg) was reverse transcribed using a RevertAid first-strand cDNA synthesis kit (Fermentas). Reverse transcription-PCR was performed before quantitative real-time PCR. *Atg5*, *Beclin 1*, *Atg7*, and *Bcl-xL* mRNA expressions were determined by real-time PCR using SYBR Premix Ex Taq (TaKaRa). PCR amplification cycles were programmed for 10 s at 95°C , followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Data were collected after each annealing step. Actin was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. Relative expression of genes was calculated and expressed as $2^{-\Delta\text{Ct}}$, as previously described (25). The following primers were used: actin 5'-CAACTGGGACGACATG-GAGAAAAT-3' and 5'-CCAGAGGCGTACAGGGATAGCAC-3'; *Atg5* 5'-TG-GCCATCAATCGAAACTC-3' and 5'-TGCAGCCACAGGACGAAACAG-3'; *Beclin 1* 5'-AGTGCCTTACTACTGTCTG-3' and 5'-ACTGCCTCT-

GTGTCTTCAATCTT-3'; *Atg7* 5'-GCAAGCCCGCAGAGATGTGGA-3' and 5'-GCAGCAATGACGGCAGGAAGC-3'; *Bcl-xL* 5'-GGGTAAACTGGGTCG-CATTGTG-3' and 5'-AAAGTATCCAGCCGCCGTTCTC-3'.

Western blot analysis. Western blot analysis was performed as previously described (26). Briefly, the proteins from total cell lysates were separated by standard SDS-PAGE and then transferred to polyvinylidene difluoride membranes. The membranes were washed, blocked, and incubated with the specific primary antihuman antibodies against *Atg5* (1:200; Abgent), *Beclin 1* (1:1,000; EPR1733Y, Abcam), *Atg7* (1:200; Abgent), *Bcl-xL* (1:1,000; 54H6, Cell Signaling), or glyceraldehyde-3-phosphate dehydrogenase (1:5,000; Millipore), followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The reactions were detected by enhanced chemiluminescence assay.

TMA and immunohistochemistry. A TMA was constructed as previously described (27). Briefly, all the HCC tissues were reviewed by two histopathologists, and representative areas free from necrotic and hemorrhagic materials were premarked in the paraffin blocks. Two core biopsies (1 mm in diameter) were taken from the donor blocks and transferred to the recipient paraffin block at defined array positions. Three different TMA blocks were constructed. Each contained 200 cylinders. Consecutive sections (4 μm in thickness) were placed on 3-aminopropyltriethoxysilane-coated slides (Shanghai Biochip Co., Ltd.).

Monoclonal rabbit antibodies against human *Beclin 1* (1:50, Abcam) and *Bcl-xL* (1:50, Cell Signaling) were used. Immunohistochemistry was performed using a two-step protocol (Novolink Polymer Detection System) as previously described (27). Briefly, after microwave antigen retrieval, tissues were incubated with primary antibodies for 60 min at room temperature, followed by incubation for 30 min with the secondary antibody (RE7112, Novolink Polymer). The sections were developed in 3,3'-diaminobenzidine solution under microscopic observation and counterstained with hematoxylin. Negative control slides in which the primary antibodies were omitted were included in all assays.

Evaluation of immunohistochemical variables. Three independent pathologists without knowledge of the patient characteristics evaluated the immunohistochemical staining. Scores were assigned to the intensity and percentage of positive staining of the cytoplasm in the whole cylinder. Discrepancies were resolved by consensus between the three pathologists with a multithread microscope. The criteria for achieving a positive score of *Beclin 1* included moderate or strong immunoreactivity present in >10% of the cells. The immunohistochemical results for *Bcl-xL* were scored as previously described (28). The sample was regarded as positive when either the intensity of staining was moderate or strong immunoreactivity was observed in >60% of cancer cells. The higher score was considered to be the final score in cases where a difference between duplicate tissue cores was observed.

Statistical analyses. Comparisons of quantitative data were analyzed using Student's *t* test between two groups or by one-way ANOVA for multiple groups. Categorical data were analyzed using the χ^2 or Fisher's exact tests. The Kaplan-Meier method was used to determine survival probability and differences were assessed by the log-rank test. Cox univariate and multivariate regression analyses were used to determine independent prognostic factors. Statistical significance was set at $P < 0.05$. All analyses were performed using SPSS software (v.15.0).

Results

Autophagic genes expression, autophagic activity, and antiapoptotic genes expression in HCC cell lines. The mRNA and protein expression of autophagic genes (*Atg5*, *Beclin 1*, *Atg7*) was evaluated in several established HCC cell lines. Almost all autophagic genes were observed in lower mRNA expression levels compared with a normal hepatic cell line L-02 (Fig. 1A), which was paralleled by Western blotting at the protein level (Fig. 1B).

In those HCC cell lines, HepG2, Hep3B, SMMC-7721, and MHCC97-L cells have relatively normal morphologies and capability to secrete plasma proteins (18) and relatively low invasiveness

and metastatic potentials (17). MHCC97-H, HCCLM3, and HCCLM6 have been generated from a poor differentiated human HCC and have extremely high invasiveness and metastatic activities (17, 21). Therefore, they were separated into two groups consisting of either low-grade malignant HCC cells (HepG2, Hep3B, SMMC-7721, MHCC97-L) or high-grade malignant HCC cells (MHCC97-H, HCCLM3, HCCLM6).

Real-time PCR analysis showed that the mRNA expression levels of Atg5, Beclin 1, and Atg7 were lower in the high-grade malignant group than in the low-grade malignant group ($P = 0.0003$, $P < 0.0001$ and $P = 0.096$, respectively; Fig. 1A). Western blotting analysis also revealed decreased protein expression in the high-grade malignant group (Fig. 1B). These results suggested that decreased expression of autophagic genes might correlate with the malignant phenotype of HCC.

To examine the expression of antiapoptotic genes in HCC cell lines, the antiapoptotic Bcl-2 family proteins Bcl-2 and Bcl-xL were

evaluated. As described previously (29), Bcl-2 expression was extremely low in the HCC cell lines (data not shown). In contrast, overexpression of Bcl-xL was observed in all HCC cell lines (Fig. 1B). Moreover, HCCLM3 was resistant to the apoptotic stimulator staurosporine, which can be reversed partially by Bcl-xL knockdown (Supplementary Fig. S1), suggesting that apoptosis resistance is a general characteristic of those HCC cell lines.

These initial observations indicated that autophagy may be down-regulated and antiapoptotic capability may be up-regulated in HCC cell lines.

To further confirm the relationship between autophagic activity and malignant phenotype in HCC, the autophagic activities of a normal hepatic cell line (L-02), a low-grade malignant HCC cell line (SMMC-7721), and a high-grade malignant HCC cell line (HCCLM3) were examined under starvation conditions. Electron microscopy revealed a low level of baseline autophagosome formation that was increased minimally in response to starvation treatment in

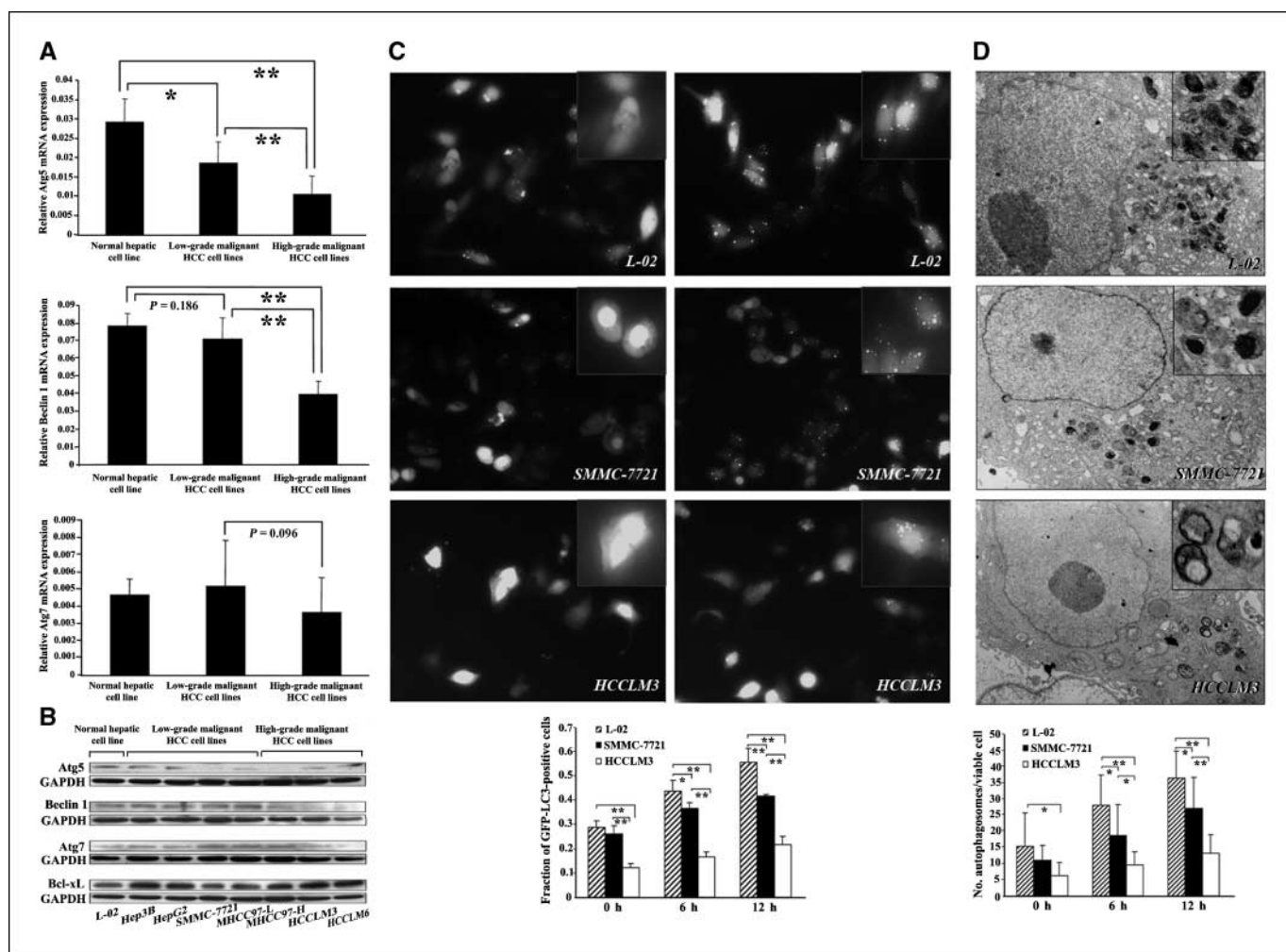
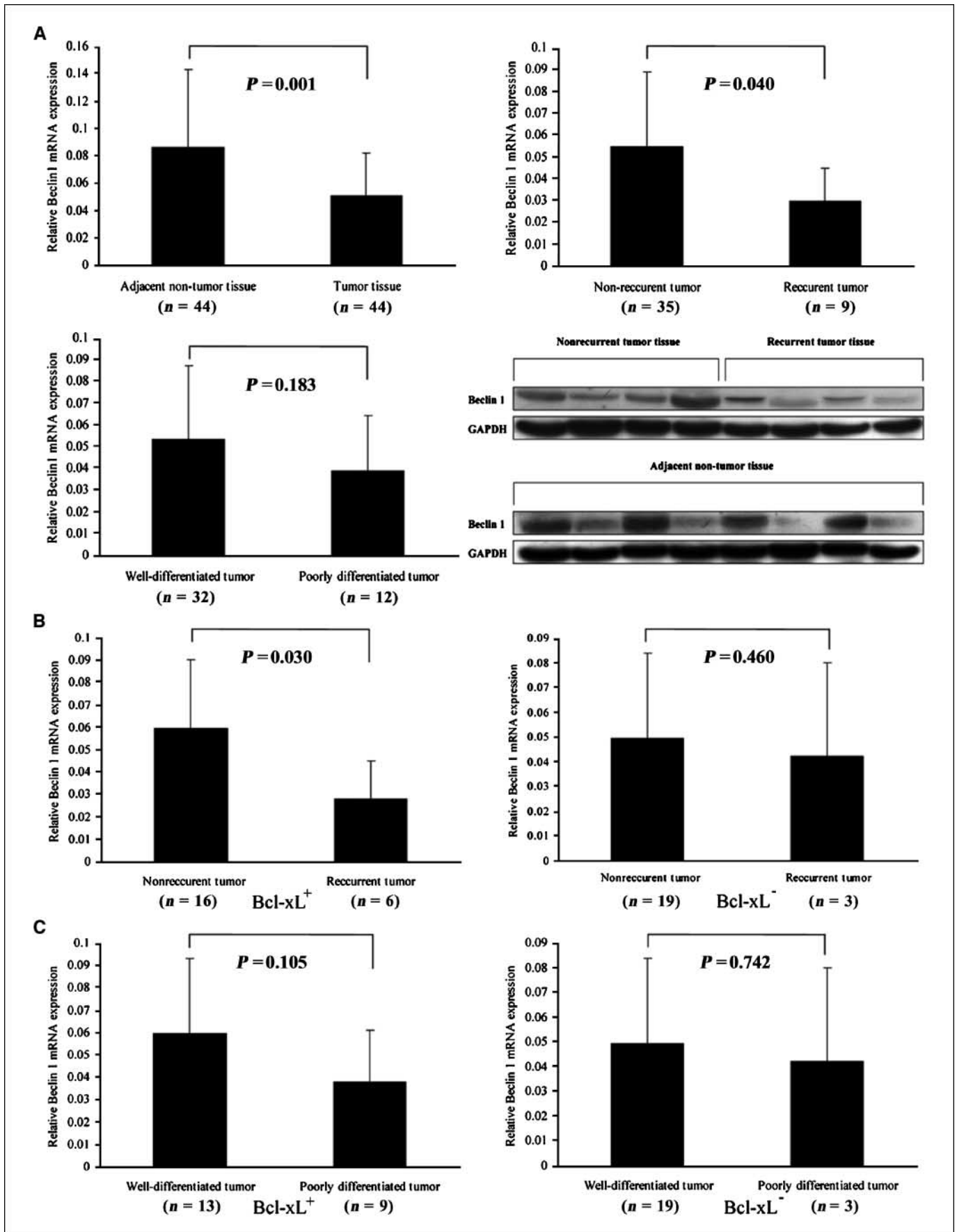


Figure 1. Autophagic gene expression, autophagic activity, and antiapoptotic gene expression in HCC cell lines. **A**, seven established HCC cell lines were separated into the low-grade malignant and high-grade malignant HCC group depending on the extent of differentiation, invasiveness, and metastatic ability. Relative Atg5, Beclin 1, and Atg7 mRNA expressions were compared among a normal hepatic cell line and those two groups of HCC cell lines. **B**, Western blotting analysis on Atg5, Beclin 1, Atg7, Bcl-xL in L-02, and seven HCC cell lines. **C**, a normal hepatic cell line (L-02), a low-grade malignant HCC cell line (SMMC-7721), and a highly malignant HCC cell line (HCCLM3) were transfected with GFP-LC3, cultured under normal (left) or starvation conditions (incubated in EBSS for 6 and 12 h; right), then analyzed by fluorescent microscopy. The fraction of GFP-LC3-positive (>3 punctuate staining sites per cell) cells was determined in three independent experiments. Eight random fields representing 200 cells were counted. Magnification $\times 200$, $\times 400$. **D**, L-02, SMMC-7721, and HCCLM3 cultured under normal or starvation conditions (incubated in EBSS for 6 and 12 h) were examined by electron microscopy. High-powered micrographs ($\times 8,000$ - $10,000$) of 20 single viable cells were obtained for quantification of autophagic vesicles. The number of autophagic vesicles per cell was scored ($*P < 0.05$, $**P < 0.001$).



HCCLM3 cells (Fig. 1D). In contrast, autophagosome formation was induced to a significantly higher level in L-02 than SMMC-7721 and HCCLM3 cells under starvation conditions (Fig. 1D).

To further quantify the level of autophagy, GFP-LC3 redistribution, which indicates autophagosome formation, was examined. Under normal conditions, the basal number of GFP-LC3-positive (>3 punctuate staining sites per cell) cells was significantly higher in L-02 and SMMC-7721 than in HCCLM3 cells. Starvation markedly increased the number of autophagic vacuoles in L-02 cells. The number of GFP-LC3-positive cells was moderately increased in SMMC-7721 cells, whereas only a slight increase in HCCLM3 cells was observed (Fig. 1C).

Taken together, these results indicated that autophagic activity was suppressed in some HCC cell lines by various degrees and maybe the more aggressive HCC cell lines like HCCLM3 exhibited much lower autophagic levels.

Autophagic protein Beclin 1 and antiapoptotic protein Bcl-xL expression in HCC tissues. Because Beclin 1 is a very important and well-documented autophagy-related protein (4, 9, 12, 24, 30, 31), we compared the mRNA expression of Beclin 1 in 44 HCC tissue samples with adjacent nontumor tissues. A significant decrease in Beclin 1 mRNA expression was observed in the tumor tissue compared with adjacent nontumor tissue ($P = 0.001$; Fig. 2A). The lower expression of Beclin 1 was found in ~81.8% (36 of 44) tumor samples, compared with matched adjacent nontumor tissue samples. Among above tumor samples, 9 cases decreased over 3-fold, and 11 cases exhibited 2- to 3-fold reductions. These findings were confirmed by Western blot analysis on 8 HCC samples selected from the 44 HCC cases. Decreased Beclin 1 protein levels were observed in most cases (6 of 8) especially in recurrent HCC compared with the nontumor tissues (Fig. 2A). Moreover, the lower Beclin 1 mRNA expression of HCC correlated with recurrent disease ($P = 0.040$), and poor differentiation of tumor was not significant ($P = 0.183$; Fig. 2A). These findings, combined with the autophagic activity studies in cells, indicated that decreased Beclin 1 expression in HCC tissues might not only contribute to the autophagy defect but also influence the clinical prognosis.

As described earlier, the antiapoptotic protein Bcl-xL was overexpressed in a variety of HCC cell lines. However, no significant difference in Bcl-xL mRNA levels was observed between the recurrent and nonrecurrent groups in 44 HCC tissue samples ($P = 0.708$; data not shown), suggesting that decreased Beclin 1 expression rather than Bcl-xL overexpression might correlate with the poor prognosis of HCC.

To determine whether defects in apoptosis influence the autophagy-related prognosis, we divided the HCC cases into low Bcl-xL expression (Bcl-xL⁻) and high Bcl-xL expression (Bcl-xL⁺) groups by using the median Bcl-xL expression level as the cutoff. As shown in Fig. 2B, in the Bcl-xL⁺ group, the expression of Beclin 1 was significantly decreased in recurrent cases compared with nonrecurrent cases ($P = 0.030$). The mean relative Beclin 1 mRNA expression levels in recurrent and nonrecurrent cases were 0.028 and 0.060, respectively, in the Bcl-xL⁺ group, and 0.050 and 0.042,

respectively, in the Bcl-xL⁻ group. Thus, we hypothesized that the autophagy-related prognosis of HCC might require antiapoptotic conditions.

We also examined the relationship between tumor differentiation and Beclin 1 expression. In the Bcl-xL⁻ group, the level of Beclin 1 expression was very similar in well-differentiated (0.049) and poorly differentiated (0.042) tumors ($P = 0.742$; Fig. 2C). However, in the Bcl-xL⁺ group, the mean relative Beclin 1 expression levels were 0.060 and 0.038, respectively ($P = 0.105$; Fig. 2C). These results indicated that autophagy might be correlated with tumor differentiation, especially under conditions in which apoptosis is compromised.

Autophagy defect is associated with poor prognosis of HCC in Bcl-xL-positive background. To further validate our proposed hypothesis, we used high-throughput TMA technology to assess the relationship between autophagy and prognosis of HCC in a group of 300 HCC patients who underwent curative resections. We found that 32% (95 of 300) and 52% (157 of 300) of the HCC patients exhibited positive Beclin 1 and Bcl-xL expression, respectively. Expression of both positive Beclin 1 and Bcl-xL was observed in 55 HCC cases (Fig. 3A).

The 3-, 5-, and 7-year disease-free survival (DFS) and OS rates of these HCC patients were 57.2% and 71.7%, 46.2% and 55.7%, and 41.5% and 43.9%, respectively. Patients with negative Beclin 1 expression had a significantly poorer prognosis than Beclin 1-positive patients (DFS, $P = 0.0002$; OS, $P = 0.022$; Fig. 3B). The 3-, 5- and 7-year DFS and OS rates for Beclin 1⁻ and Beclin 1⁺ patients were 51.6% and 69.8% versus 69.3% and 75.8%, 37.4% and 51.2% versus 65.8% and 65.3%, and 32.8% and 39.9% versus 60.6% and 52.5%, respectively.

When all HCC patients were stratified by Bcl-xL expression, we found that the prognosis of Beclin 1⁻ patients was much worse than for Beclin 1⁺ patients in the Bcl-xL⁺ group ($P < 0.0001$; Fig. 3B). However, no significant difference in the survival rates of Beclin 1⁻ and Beclin 1⁺ patients was observed in the Bcl-xL⁻ group (DFS, $P = 0.978$; OS, $P = 0.233$; Fig. 3B). In the Bcl-xL⁺ group, the 3-, 5- and 7-year DFS and OS rates for Beclin 1⁻ and Beclin 1⁺ patients were 44.7% and 66.7% versus 81.7% and 85.5%, 25.8% and 43.1% versus 77.7% and 78.2%, and 22.3% and 30.2% versus 71.4% and 63.5%, respectively.

The prognostic value of Beclin 1 expression in HCC patients with a Bcl-xL-positive background. The correlation between Beclin 1 expression and clinicopathologic parameters in all HCC patients was statistically analyzed (Table 1). A significant correlation between the absence of Beclin 1 expression and high serum α -fetoprotein (AFP) levels was observed ($P = 0.008$). An absence of Beclin 1 expression occurred more frequently in poorly differentiated HCC than in well-differentiated HCC, although this difference was not statistically significant ($P = 0.185$). No significant correlation was found between Beclin 1 expression and other variables, including age, sex, tumor size, and tumor number.

Importantly, in the Bcl-xL⁺ group, a significant correlation between the absence of Beclin 1 and poor tumor differentiation

Figure 2. Autophagic level in HCC tissue samples. A, relative Beclin 1 mRNA expression in nontumor and tumor tissue (top left), nonrecurrent and recurrent tumor tissue (top right), and well-differentiated and poorly differentiated tumor tissue (bottom left). Columns, mean from three independent experiments; bars, SD. Beclin 1 expression in eight tumor tissue samples (four nonrecurrent and four recurrent cases) and corresponding nontumor tissue samples were confirmed by Western blotting analysis (bottom right). B, a total of 44 HCC cases were divided into two groups—low Bcl-xL expression group (Bcl-xL⁻) and high Bcl-xL expression group (Bcl-xL⁺) by using the median of relative Bcl-xL mRNA expression as the cutoff. Relative Beclin 1 mRNA expression of nonrecurrent and recurrent tumor tissue in Bcl-xL⁺ group and Bcl-xL⁻ group. C, relative Beclin 1 mRNA expression of well-differentiated and poorly differentiated tumor tissue in the Bcl-xL⁺ and Bcl-xL⁻ groups.

was observed ($P = 0.033$). In contrast, this correlation was not statistically significant in the Bcl-xL⁻ group ($P = 0.693$; Table 1).

The prognostic value of Beclin 1 expression was evaluated in HCC patients using univariate analysis, which showed that Bcl-xL expression, age, sex, HBV infection, liver cirrhosis, AFP levels, alanine aminotransferase levels, and tumor differentiation had no prognostic significance for DFS and OS. However, the tumor-node-metastasis stage, vascular invasion, tumor size, and tumor number were predictors for DFS and OS. Beclin 1 expression was also a significant predictor for tumor recurrence and OS ($P = 0.0003$ and $P = 0.023$, respectively) in all of the study population (Table 2). We further assessed the prognostic value of Beclin 1 in Bcl-xL⁺ and Bcl-xL⁻ HCC patients. Beclin 1 expression was a predictor for both DFS and OS in the Bcl-xL⁺ group but not in the Bcl-xL⁻ group (Supplementary Tables S1 and S2).

Multivariate analysis was conducted with four of the variables (Beclin 1 expression, vascular invasion, tumor size, and tumor number), which was shown to be significant in the univariate analysis and no obvious correlation between each other. Negative Beclin 1 expression was still the independent variable for predicting poor DFS and OS, especially in Bcl-xL⁺ patients ($P < 0.0001$ and $P = 0.0002$, respectively; Supplementary Table S3).

Collectively, these results showed that in HCC with positive Bcl-xL expression, an autophagy defect (i.e., negative Beclin 1 expression) was associated with poor tumor differentiation and poor survival that could be independently predicted by the autophagic gene *Beclin 1*.

Discussion

Inactivation of autophagy-specific genes such as *Beclin 1* has been shown to lead to increased tumorigenesis in mice. Enforced expression of such genes (*Beclin 1* and *Atg5*) inhibits the formation of human breast tumors in mouse models (32). Furthermore, net deletions of several autophagy-specific genes are commonly found in human malignancies (32). Thus, autophagy may be a tumor-suppressor pathway and its decreased activity may contribute to the development of human cancer (33).

In the present study, we found that the expression of autophagic genes was extremely low in HCC cell lines especially in highly malignant HCC cell lines. Because mounting evidence shows that monoallelic deletion or decreased protein expression of Beclin 1 could lead to compromised autophagic activities both *in vitro* and *in vivo* (12, 14–16, 24, 30, 31, 34, 35), we

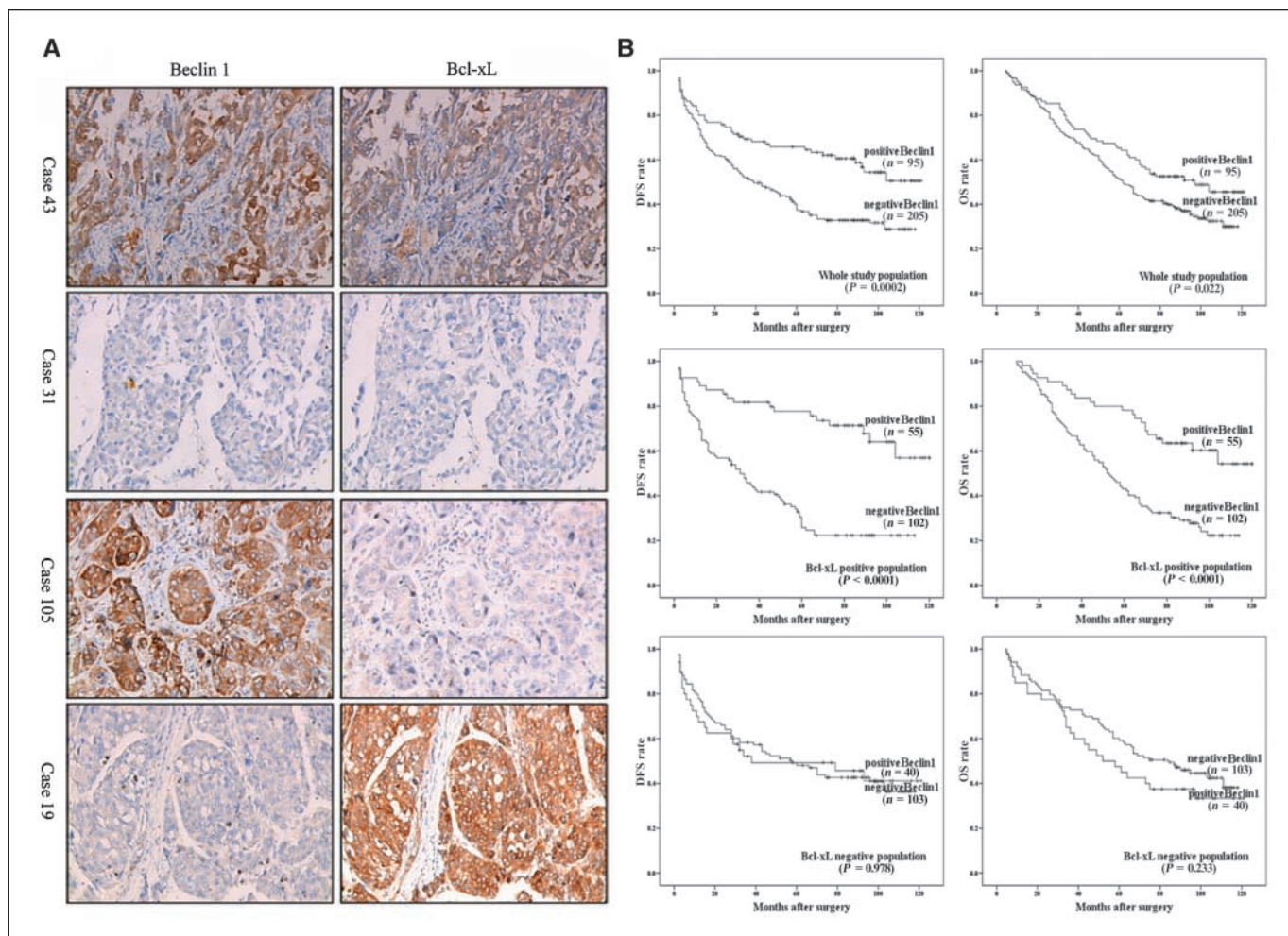


Figure 3. The expression patterns of Beclin 1 and Bcl-xL in HCC TMA and survival analysis. A, consecutive sections were used for immunohistochemical study on Beclin 1⁺, Beclin 1⁻, Bcl-xL⁺, and Bcl-xL⁻ cells: both positive case (Case 43); both negative case (Case 31); Beclin 1⁺ and Bcl-xL⁻ case (Case 105); Beclin 1⁻ and Bcl-xL⁺ case (Case 19). Positive cells were stained brown. Magnification, $\times 400$. B, Kaplan-Meier analysis of DFS and OS for Beclin 1 expression in whole study population (top), Bcl-xL-positive population (middle), and Bcl-xL-negative population (bottom).

Table 1. Correlation between Beclin 1 expression and clinicopathologic characteristics in the whole study group and in the Bcl-xL–positive and Bcl-xL–negative groups

	Whole study group			Bcl-xL–positive group			Bcl-xL–negative group		
	Beclin1 expression		<i>P</i>	Beclin1 expression		<i>P</i>	Beclin1 expression		<i>P</i>
	Negative (<i>n</i> = 205)	Positive (<i>n</i> = 95)		Negative (<i>n</i> = 102)	Positive (<i>n</i> = 55)		Negative (<i>n</i> = 103)	Positive (<i>n</i> = 40)	
Sex									
Female	32	16	0.787	13	7	0.997	19	9	0.583
Male	173	79		89	48		84	31	
Age, y									
≤52	103	53	0.371	43	29	0.205	60	24	0.849
>52	102	42		59	26		43	16	
Preoperative AFP (ng/mL)									
≤20	89	57	0.008	55	38	0.065	34	19	0.107
>20	116	38		47	17		69	21	
HBsAg									
Negative	31	16	0.703	20	10	0.828	11	6	0.474
Positive	174	79		82	45		92	34	
Liver cirrhosis									
No	14	11	0.166	3	7	0.034*	11	4	1*
Yes	191	84		99	48		92	36	
ALT (units/L)									
≤75	179	88	0.171	89	52	0.150	90	36	0.780*
>75	26	7		13	3		13	4	
Tumor size (cm)									
≤5	110	53	0.730	60	32	0.938	50	21	0.671
>5	95	42		42	23		53	19	
Tumor number									
Single	173	82	0.664	83	47	0.518	90	35	0.984
Multiple	32	13		19	8		13	5	
Vascular invasion									
No	176	83	0.722	92	50	0.885	84	33	0.895
Yes	29	12		10	5		19	7	
TNM stage									
I	149	70	0.856	75	42	0.697	74	28	0.827
II/III	56	25		27	13		29	12	
Tumor differentiation									
I-II	140	72	0.185	67	45	0.033	73	27	0.693
III-IV	65	23		35	10		30	13	

Abbreviations: ALT, alanine aminotransferase; TNM, tumor-node-metastasis; HBsAg, hepatitis B surface antigen.

*Fisher's exact tests, and χ^2 tests for all the other analysis.

focused on the autophagic gene *Beclin 1*. As a consequence, we examined its expression by real-time PCR in 44 HCC and adjacent nontumor tissues, and found that, consistent with breast cancer (9, 10), a significant decrease in Beclin 1 expression occurred in HCC tissues compared with nontumor tissues. These findings suggested that HCC might possess defective autophagy.

Antiapoptosis is also a characteristic of cancers that is regulated by a series of molecular cellular events (36). Members of the Bcl-2 protein family, key regulators of apoptosis, include antiapoptotic proteins such as Bcl-2 and Bcl-xL and proapoptotic proteins such as Bax and Bak (37). Deficiencies in proapoptotic proteins or expression of antiapoptotic proteins block apoptosis. Previous studies (29, 38, 39) have reported that in HCC, endogenous Bcl-xL

inhibits apoptosis produced by various stress-inducing conditions such as staurosporine treatment, serum starvation, and p53 activation. The HCC cell lines established at our institute (such as MHCC97-L and HCCLM3) also exhibit resistance to a variety of apoptotic stimuli (data not shown). Here, we found that Bcl-xL is highly expressed in various HCC cells as previously reported (29) and the knockdown Bcl-xL can reverse the apoptotic resistance induced by staurosporine in HCCLM3 cells. Thus, we proposed that Bcl-xL may, at least partially, contribute to the antiapoptotic properties of HCC.

The functional relationship between autophagy and apoptosis within the tumor is complex. Initially, autophagy was considered to be an alternative pathway to cellular demise that was termed autophagic cell death (or type II cell death; ref. 40). However,

Table 2. Univariate analyses of factors associated with recurrence and survival

Variable	DFS		OS	
	Hazard ratio (95% CI)	P	Hazard ratio (95% CI)	P
Sex (female vs male)	1.265 (0.823-1.946)	0.284	1.025 (0.685-1.534)	0.903
Age, y (≤ 52 vs > 52)	0.887 (0.660-1.192)	0.427	0.877 (0.655-1.174)	0.377
AFP (ng/mL; ≤ 20 vs > 20)	1.172 (0.872-1.576)	0.291	1.268 (0.947-1.699)	0.111
HBsAg (negative vs positive)	1.295 (0.842-1.992)	0.239	1.385 (0.901-2.128)	0.138
Liver cirrhosis (no vs yes)	1.476 (0.821-2.653)	0.193	1.844 (0.975-3.490)	0.060
ALT (units/L; ≤ 75 vs > 75)	1.516 (0.993-2.316)	0.054	1.262 (0.808-1.972)	0.306
Tumor size (cm; ≤ 5 vs > 5)	1.374 (1.023-1.846)	0.035	1.427 (1.067-1.909)	0.017
Tumor number (single vs multiple)	1.625 (1.107-2.386)	0.013	1.758 (1.219-2.534)	0.003
Vascular invasion (no vs yes)	1.938 (1.314-2.859)	0.001	1.589 (1.074-2.352)	0.021
TNM stage (I vs II/III)	1.963 (1.436-2.685)	< 0.0001	1.813 (1.333-2.465)	0.0002
Tumor differentiation (I-II vs III-IV)	1.333 (0.973-1.826)	0.073	1.347 (0.987-1.839)	0.060
Beclin 1 (positive vs negative)	1.920 (1.349-2.733)	0.0003	1.465 (1.053-2.038)	0.023
Bcl-xL (negative vs positive)	1.057 (0.787-1.421)	0.713	1.064 (0.795-1.425)	0.675

NOTE: Univariate analysis, Cox proportional hazards regression model. Abbreviation: 95% CI, 95% confidence interval.

increasing evidence suggests that autophagy constitutes a stress adaptation that avoids tumor death (and de facto suppresses apoptosis; refs. 41, 42). Recent studies have shown that defective autophagy synergized with defective apoptosis results in increased DNA damage and genomic instability that ultimately facilitates tumor progression (14, 15). Thus, coordination of autophagy and apoptosis may play an important role in tumor carcinogenesis and tumor progression.

In our study, we observed lower expression levels of autophagic genes in high-grade malignant HCC cells compared with low-grade malignant ones. GFP-LC3 redistribution and electron microscopy analyses revealed that autophagic activity was suppressed in some HCC cell lines, especially in the more aggressive HCC cell lines. Our findings also confirmed that those specific HCC cells possessed compromised autophagic activity consistent with their decreased expression of autophagic genes. Moreover, those HCC cell lines all exhibited high Bcl-xL expression level, but we did not find a close correlation between Bcl-xL expression and HCC malignancy in these cells. Interestingly, when we evaluated Beclin 1 expression in HCC tissue samples grouped according to their Bcl-xL expression levels, we found that in Bcl-xL⁺ HCC samples, the relative Beclin 1 expression level in well-differentiated tumors was higher than in poorly differentiated tumors. In contrast, in Bcl-xL⁻ HCC samples, the expression of Beclin 1 in poorly differentiated and well-differentiated cells did not differ significantly. Immunohistochemical analysis of 300 HCC cases also revealed the significant correlation between Beclin 1 expression and tumor differentiation in the Bcl-xL⁺ group. Thus, our data suggest that the malignant phenotype or differentiation of HCC is only correlated closely with Beclin 1 expression in a Bcl-xL⁻ positive background. These findings may indicate that an autophagy defect increases the accumulation of genome damage and mutation rate, ultimately promoting malignancy and invasive differentiation in HCC cells as described in previous studies (14, 15).

An aggressive cancer cell phenotype always results in recurrence or a poor prognosis (43). Thus, this study, which focuses on the combined role of autophagy and apoptosis in the prognosis of

HCC, is extremely important. We found that the relative Beclin 1 mRNA expression in a series of HCC patients with recurrent disease was significantly reduced compared with nonrecurrent cases in the Bcl-xL⁺ group. Immunohistochemical analysis on Bcl-xL⁺ HCC cases revealed that the 3-, 5-, and 7-year OS and DFS rates in Beclin 1⁻ patients were significantly lower than in Beclin 1⁺ patients. Moreover, Beclin 1 was the strongest independent predictor for OS and DFS in Bcl-xL⁺ HCC patients by both multivariate and univariate analyses. To our knowledge, this is the first report that describes an autophagy-related prognosis and shows the prognostic value of autophagic gene expression in human cancers.

Our study raises the concern of why an autophagy defect was not associated with malignancy, invasive differentiation, and poor prognosis in Bcl-xL⁻ negative HCC cells. We speculate that in most circumstances, tumor cells could be eliminated by apoptosis regardless of the functional status of autophagy (44). Moreover, several studies have shown that autophagic death only occurred when apoptosis was inhibited (4, 40). Autophagy has previously been shown to play a survival role in the carcinogenesis and progression of tumors with intact apoptosis (8, 45). Recently, it has been shown that concurrent inactivation of autophagy and apoptosis led to tumor inflammation and chronic necrosis that would promote tumorigenesis (16). On the other hand, complex mechanism is involved in regulating the malignant phenotype of HCC and there would be some alternative pathways independent of apoptosis or autophagy, or independent of being regulated by Bcl-xL and Beclin 1.

In conclusion, our study shows that the expression of autophagic genes and their corresponding autophagic activities are suppressed in some HCC cells. Autophagy defects may be associated with malignant phenotype and poor prognosis of HCC in an apoptosis-compromised background.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

- Bosch FX, Ribes J, Diaz M, Cleries R. Primary liver cancer: worldwide incidence and trends. *Gastroenterology* 2004;127:S5-16.
- Llovet JM, Beaugrand M. Hepatocellular carcinoma: present status and future prospects. *J Hepatol* 2003;38 Suppl 1:S136-49.
- Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57-70.
- Shimizu S, Kanaseki T, Mizushima N, et al. Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. *Nat Cell Biol* 2004;6:1221-8.
- Rashmi R, Pillai SG, Vijayalingam S, Ryerse J, Chinnadurai G. BH3-only protein BIK induces caspase-independent cell death with autophagic features in Bcl-2 null cells. *Oncogene* 2008;27:1366-75.
- Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. *Science* 2000;290:1717-21.
- Lum JJ, Bauer DE, Kong M, et al. Growth factor regulation of autophagy and cell survival in the absence of apoptosis. *Cell* 2005;120:237-48.
- Colell A, Ricci JE, Tait S, et al. GAPDH and autophagy preserve survival after apoptotic cytochrome *c* release in the absence of caspase activation. *Cell* 2007;129:983-97.
- Liang XH, Jackson S, Seaman M, et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* 1999;402:672-6.
- Aita VM, Liang XH, Murty VV, et al. Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21. *Genomics* 1999;59:59-65.
- Qu X, Yu J, Bhagat G, et al. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest* 2003;112:1809-20.
- Yue Z, Jin S, Yang C, Levine AJ, Heintz N. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc Natl Acad Sci U S A* 2003;100:15077-82.
- Marino G, Salvador-Montoliu N, Fueyo A, et al. Tissue-specific autophagy alterations and increased tumorigenesis in mice deficient in Atg4C/autophagin-3. *J Biol Chem* 2007;282:18573-83.
- Karantza-Wadsworth V, Patel S, Kravchuk O, et al. Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. *Genes Dev* 2007;21:1621-35.
- Mathew R, Kongara S, Beaudoin B, et al. Autophagy suppresses tumor progression by limiting chromosomal instability. *Genes Dev* 2007;21:1367-81.
- Deegenhardt K, Mathew R, Beaudoin B, et al. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell* 2006;10:51-64.
- Ye QH, Qin LX, Forgues M, et al. Predicting hepatitis B virus-positive metastatic hepatocellular carcinomas using gene expression profiling and supervised machine learning. *Nat Med* 2003;9:416-23.
- Liu TZ, Chen PY, Chiu DT, et al. Detection of a novel lactate dehydrogenase isozyme and an apparent differentiation-associated shift in isozyme profile in hepatoma cell lines. *Cancer Lett* 1994;87:193-8.
- Lee TK, Man K, Poon RT, et al. Signal transducers and activators of transcription 5b activation enhances hepatocellular carcinoma aggressiveness through induction of epithelial-mesenchymal transition. *Cancer Res* 2006;66:9948-56.
- Li Y, Tang Y, Ye L, et al. Establishment of a hepatocellular carcinoma cell line with unique metastatic characteristics through *in vivo* selection and screening for metastasis-related genes through cDNA microarray. *J Cancer Res Clin Oncol* 2003;129:43-51.
- Li Y, Tian B, Yang J, et al. Stepwise metastatic human hepatocellular carcinoma cell model system with multiple metastatic potentials established through consecutive *in vivo* selection and studies on metastatic characteristics. *J Cancer Res Clin Oncol* 2004;130:460-8.
- Peng DJ, Sun J, Wang YZ, et al. Inhibition of hepatocarcinoma by systemic delivery of Apoptin gene via the hepatic asialoglycoprotein receptor. *Cancer Gene Ther* 2007;14:66-73.
- Xu ZG, Du JJ, Zhang X, et al. A novel liver-specific zona pellucida domain containing protein that is expressed rarely in hepatocellular carcinoma. *Hepatology* 2003;38:735-44.
- Liang C, Feng P, Ku B, et al. Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. *Nat Cell Biol* 2006;8:688-99.
- Nanjundan M, Nakayama Y, Cheng KW, et al. Amplification of MDS1/EVI1 and EVI1, located in the 3q26.2 amplicon, is associated with favorable patient prognosis in ovarian cancer. *Cancer Res* 2007;67:3074-84.
- Herman-Antosiewicz A, Johnson DE, Singh SV. Sulforaphane causes autophagy to inhibit release of cytochrome *c* and apoptosis in human prostate cancer cells. *Cancer Res* 2006;66:5828-35.
- Gao Q, Qiu SJ, Fan J, et al. Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. *J Clin Oncol* 2007;25:2586-93.
- Miyamoto Y, Hosotani R, Wada M, et al. Immunohistochemical analysis of Bcl-2, Bax, Bcl-X, and Mcl-1 expression in pancreatic cancers. *Oncology* 1999;56:73-82.
- Takehara T, Liu X, Fujimoto J, Friedman SL, Takahashi H. Expression and role of Bcl-xL in human hepatocellular carcinomas. *Hepatology* 2001;34:55-61.
- Takahashi Y, Coppola D, Matsushita N, et al. Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. *Nat Cell Biol* 2007;9:1142-51.
- Furiya N, Yu J, Byfield M, Pattingre S, Levine B. The evolutionarily conserved domain of Beclin 1 is required for Vps34 binding, autophagy and tumor suppressor function. *Autophagy* 2005;1:46-52.
- Levine B. Cell biology: autophagy and cancer. *Nature* 2007;446:745-7.
- Mathew R, Karantza-Wadsworth V, White E. Role of autophagy in cancer. *Nat Rev Cancer* 2007;7:961-7.
- Zhu H, Tannous P, Johnstone JL, et al. Cardiac autophagy is a maladaptive response to hemodynamic stress. *J Clin Invest* 2007;117:1782-93.
- Kunchithapautham K, Rohrer B. Apoptosis and autophagy in photoreceptors exposed to oxidative stress. *Autophagy* 2007;3:433-41.
- Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. *Nature* 2001;411:342-8.
- Galonek HL, Hardwick JM. Upgrading the BCL-2 network. *Nat Cell Biol* 2006;8:1317-9.
- Chun E, Lee KY. Bcl-2 and Bcl-xL are important for the induction of paclitaxel resistance in human hepatocellular carcinoma cells. *Biochem Biophys Res Commun* 2004;315:771-9.
- Nishikawa T, Nakajima T, Moriguchi M, et al. A green tea polyphenol, epigallocatechin-3-gallate, induces apoptosis of human hepatocellular carcinoma, possibly through inhibition of Bcl-2 family proteins. *J Hepatol* 2006;44:1074-82.
- Yu L, Alva A, Su H, et al. Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. *Science* 2004;304:1500-2.
- Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat Rev Mol Cell Biol* 2007;8:741-52.
- Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. *Nature* 2008;451:1069-75.
- Tung-Ping Poon R, Fan ST, Wong J. Risk factors, prevention, and management of postoperative recurrence after resection of hepatocellular carcinoma. *Ann Surg* 2000;232:10-24.
- Jin S, White E. Role of autophagy in cancer: management of metabolic stress. *Autophagy* 2007;3:28-31.
- Amaravadi RK, Yu D, Lum JJ, et al. Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. *J Clin Invest* 2007;117:326-36.