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ORIGINAL ARTICLE

Decreased expression of Beclin-1 in patients with hepatocellular carcinoma

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Summary

Purpose: *As a conserved cellular stress response, autophagy has recently been demonstrated to be involved in the pathogenesis of several human cancers. Beclin-1 is an important autophagy gene that is abnormally expressed in a variety of human cancers. In this study, we investigated the expression of Beclin-1 in Hepatocellular Carcinoma (HCC).*

Methods: *A total of 83 patients with primary HCC were enrolled in this study. The expression of Beclin-1, PCNA, NET-1, Bcl-2, and Bax was measured in tissue microarray, including 83 cases of HCC and 46 adjacent non-tumor liver tissues. The association of the expression of Beclin-1 with clinicopathological features as well as PCNA, NET-1, Bcl-2, and Bax were analyzed.*

Results: *The positive rate of Beclin-1 in HCC tissues was significantly lower than that in adjacent tissues (x^2=4.013, p=0.012). Beclin-1 expression in HCC tissues was negatively correlated with the expression of PCNA, NET-1, and anti-apoptotic protein Bcl-2, but positively correlated with pro-apoptotic protein Bax expression. Meanwhile, Beclin-1 expression was negatively correlated with HCC Edmondson grading (p=0.0058). Furthermore, Beclin-1 expression was significantly lower in HCC patients with liver cirrhosis (p=0.029) or vascular invasion (p=0.011) than those in HCC patients without cirrhosis or vascular invasion.*

Conclusion: *Decreased expression of Beclin-1 was observed in HCC tissues and negatively correlated with HCC Edmondson grading, suggesting that Beclin-1 might be a valuable prognostic indicator for HCC.*

Key words: *Beclin-1, hepatocellular carcinoma, clinicopathological features*

Introduction

Liver cancer (LC) is the sixth most common cancer and the third leading cause of cancer-related death in the world. In 2008, there were 749,000 new cases of LC, with an estimated 696,000 deaths around the world [1]. Hepatocellular carcinoma (HCC) is one of the most common liver cancers, as well as one of the most common malignant tumors in the world [2]. Currently, hepatectomy is the most common treatment for HCC and one of the most effective surgical intervention to achieve a long-term survival [3]. Most HCC patients are diagnosed with potential hepatic dysfunction in advanced stage [4], with a very poor prognoses due to a low survival rate. Although it is reported that several numerator elements and histological features are associated with the prognosis of HCC [5], more effective biomarkers are necessary for predicting the clinical outcomes of HCC patients.

Autophagy is a genetic programming process that can recycle the long-lived proteins or damaged organelles [5]. Several studies have shown that autophagy plays an important role in the occurrence and development of cancer [6]. However, the role of autophagy in the growth and metastasis of human

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cancers is not fully clear. Therefore, autophagy has become a focus in cancer research [7,8]. The loss of autophagy may promote the tumor progression through promoting the genomic damage and instability. About 30 specific genes that regulate autophagy have been identified in yeast, 16 of which are homologous genes [9]. Among these genes, Beclin-1 plays a key role in autophagy in mammals [10], which is a counterpart of the yeast Atg6 gene in mammals, and a part of the type III phosphatidylinositol 3-kinase complex required for the formation of autophagic vesicles [11,12]. Beclin-1 is found to be abnormally expressed in human melanoma [13], colon cancer [14], ovarian cancer [15], and brain cancer [16].

This study aimed to investigate the expression of Beclin-1 in LC tissues and its correlations with clinicopathological features and prognosis.

Methods

Patients and follow-up

A total of 83 HCC patients followed up at the Sixth People's Hospital of Qingdao from January 2010 to December 2011 were selected. They all suffered from primary HCC and had not received prior treatment for HCC but had undergone surgery as initial therapy. All patients were followed up regularly. HCC diagnosis was confirmed via histopathology. A total of 46 non-cancer liver tissues from 83 HCC patients were used as negative controls. This study was approved by the ethics committee at the Sixth People's Hospital of Qingdao and all the enrolled objects had signed informed consent.

Test reagents

Envision + peroxidase blocking solution was purchased from DakoCytomation (Glostrup, Denmark). Mouse anti-human monoclonal antibodies [proliferating cell nuclear antigen (PCNA), NET-1, B-cell lymphoma-2 (Bcl-2), Bcl-2 associated X protein (Bax), and Survivin] were purchased from Zymed Laboratories (Carlsbad, CA, USA). DAKO Envision + /horseradish peroxidase (HRP) system was bought from DAKO (DAKO, Carpinteria, CA, USA). Diaminobenzidine (DAB) kit interleukin (IL)-1-9032 was bought from Fuzhou Maxim Biotech Co., Ltd (Fujian, Fuzhou, China).

Tissue microarray (TMA)

The tissue microarray was constructed according to the method of Qun (patent number: ZL 2008 10022 170.4) and the specific method is as follows: All HCC tissues were stained with Hematoxylin-Eosin (HE) and then examined by two pathologists. Representative areas without necrotic and hemorrhagic materials were marked on the paraffin block. Two cylindrical tissue cores with a diameter of 1.6 mm were removed from the donor block and transferred into the recipient paraffin block and the planar array locations were recorded. A total of three different TMAs was constructed, each containing more than 100 cylinders. Finally, TMA consisted of 83 HCC tissues and 46 adjacent non-cancerous tissues (ANT). Serial sections with a thickness of 4 μm were cut from ANT and placed on a microscope slide for immunohistochemical staining.

Immunohistochemical staining

After 5 μm-thick sections were fixed by formalin and embedded into paraffin, Beclin-1, PCNA, NET-1, Bcl-2, Bax, and surviving cancer cells were detected by Envision + /DAB analytical method. A total of 10 TMA serial sections were prepared from each TMA and stained. Paraffin sections were dewaxed in xylene and treated with microwave. Then the slide was placed into 10 mM sodium citrate buffer (pH 6.0), heated in boiling water for 10 min, cooled at room temperature for 20 min, incubated with Envision + peroxidase blocking solution for 5 min and washed with 0.05% Tris Buffered Saline Tween-20 (TBST) (pH 7.4). Then the primary antibody was added onto the slide for incubation at room temperature for 30 min. Following this, the slide was washed again with TBST (pH 7.4), followed by detection using DAKO Envision + /HRP system. Sections were cultured with DAB for 15 min and slightly counterstained with hematoxylin. PCNA and Bcl-2, and Beclin-1, NET-1, and Bax in HCC and cytoplasm were used as positive controls, while TBS in the same concentration, instead of primary antibody, was used as negative control.

Staining pattern and evaluation

Positive staining intensity and percentage of cytoplasm /nucleus in the whole cylinder were scored blindly by two independent investigators, who did not previously know the clinical information . The immunocompetence distribution was graded as follows: grade 0 (<5%), grade 1 (5-25%), grade 2 (25-50%), grade 3 (50-75%), and grade 4 (>75%), respectively. The positive staining intensity was divided into 4 grades as follows: grade 0 (no staining), grade 1 (weak), grade 2 (moderate), and grade 3 (severe), respectively. The two grades were multiplied with each other. The final expression scores of Beclin-1, PCNA, NET-1, Bcl-2, Bax, and Survivin were calculated and the positive percentage integral value was multiplied with the staining intensity score (0-12 points). The intratumoral protein expression is defined as follows: negative (-) (0 points), low expression (+) (1-6 points), and high expression (++) (> 6 points).

Western blotting analysis

TMA HCC tissues and ANT were taken, lysed with lysis buffer, and centrifuged at 12000 g and 4°C for 10 min. The supernatant was collected to extract the total protein. The concentration of protein was measured using the Bicinchoninic Acid (BCA) method. Total protein extracting solution and 5 × protein loading buffer were mixed evenly at a volume ratio of 1:5, treated with boiling water bath for 15 min and naturally cooled at 4°C. The corresponding Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) separation gel was prepared according to the molecular weight of target protein. Denatured protein samples were added

into the loading wells for loading based on the protein concentration and the total protein content in each well was kept the same. The electrophoresis was performed under constant pressure of 220 V until the bromophenol blue reached the bottom of gel. According to the molecular weight of target protein, the gel was cut and placed into transfer buffer. A layer of Polyvinylidene Fluoride (PVDF) membrane and six layers of filter paper were cut according to the size of gel. PVDF membrane was immersed into the methanol for 10 s first and then PVDF membrane and filter paper were placed into the transfer buffer. Then the positive pole - three layers of filter paper - PVDF membrane – gel - three layers of filter paper - negative pole were placed on the membrane transfer instrument in order. Their edges should be aligned to prevent blistering. After the membrane transfer under constant pressure of 60 V for 1 h, the PVDF membrane attached with protein was sealed using 5% skim milk powder on a shaking table at 4°C overnight. The sealed membrane was washed with TBST for 10 min and incubated in the corresponding primary antibody at 4°C overnight. After the membrane was washed with TBST for 3 times (10 min/time), it was incubated using the corresponding secondary antibody on a shaking table at room temperature for 3 h and it was washed again with TBST for 3 times (10 min/time). Reagent A and B in Electrochemiluminescence (ECL) kit were evenly mixed at a volume ratio of 1:1 and added dropwise onto the PVDF membrane, followed by color development in a dark place for 1 min. The excess liquid around the membrane was sucked dry with the filter paper. Then the membrane was placed into the gel imager, followed by photography under the dynamic integral mode and observation of results. Lab Works 4.6 professional image analysis software was used to analyze the images.

Statistics

Data were analyzed using GraphPad Prism 5 software and shown as mean ± standard deviation (SD). Follow-up time was from the first postoperative day to death. The follow-up period ranged from 12 to 60 months (median 48.1). The last follow-up date was January 2016. Overall survival (OS) referred to survival between surgery and date of death. The correlations of Beclin-1 expression with cell proliferation-related proteins (PCNA and NET-1), apoptosis-related proteins (Bcl-2 and Bax), and clinicopathological characteristics (Edmondson grading) were analyzed by Spearman rank correlation test. $P < 0.05$ was considered statistically significant.

Results

Beclin-1 expressions in HCC and ANT

Among the 83 patients there were 66 (79.5%) males and 17 (20.5%) aged from 22 to 76 years (median 47). The expression of Beclin-1 in 83 LC tissues and 46 non-cancer tissues was detected by immunohistochemical staining, showing a

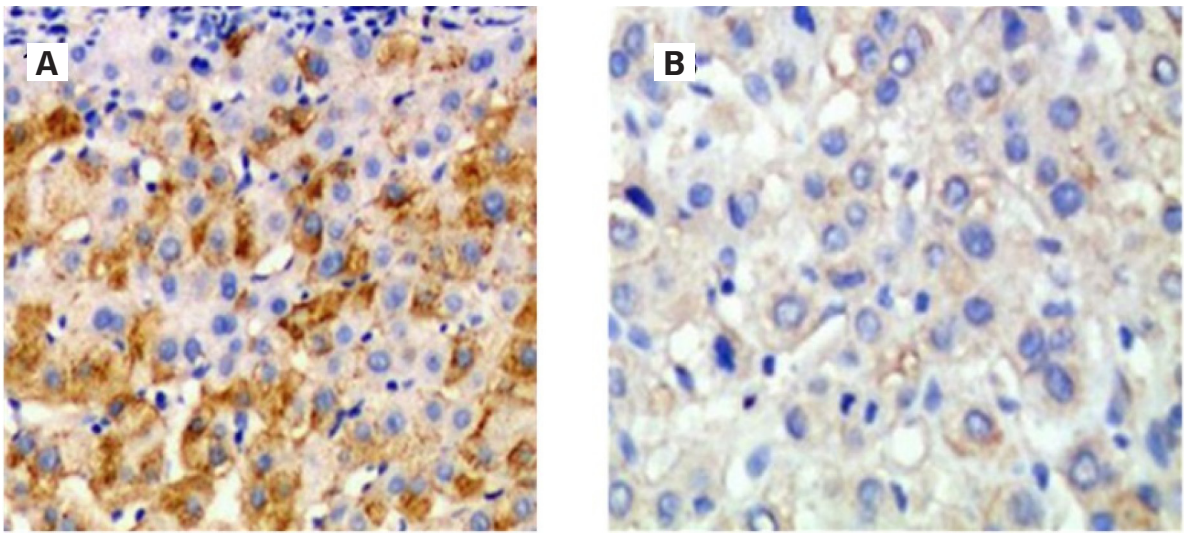

Figure 1. Immunohistochemical staining analysis of the expression of Beclin-1 in liver cancer tissues **(A)** and non-cancer tissues **(B)**. (original magnification×400). The Figure shows that the level of Beclin-1 in liver cancer tissues was significantly increased compared to that in non-cancer tissues.

Table 1. Expression of Beclin-1 in liver cancer tissues and non-cancer tissues

Sample	*n*	*Beclin-1 expression*			
		-(%)	*+(%)*	*++(%)*	
Liver cancer tissues	83	21	46	15	x^2=4.013
Non-cancer tissues	46	4	27	15	p=0.012

significantly lower positive rate of Beclin-1 expression in LC tissues than that in non-cancer tissues (x^2=4.013, p=0.012) (Table 1 and Figure 1).

Association of Beclin-1 expression with clinicopathological features of HCC

To investigate whether the expression of Beclin-1 was associated with the clinicopathological features of HCC, we performed correlative analysis and showed that the expression of Beclin-1 in tumor tissues was negatively correlated with HCC Edmondson grading (p=0.0058) (Figure 2A) as demonstrated by significantly higher positive expression of Beclin-1 in Edmondson grade I-II (79.5%, 31/39) than that in grade III-IV in HCC (54.5%, 24/44). Similarly, the positive expression rate of Beclin-1 in HCC patients with liver cirrhosis (66.7%, 48/72) was significantly lower than that in patients without liver cirrhosis (81.8%, 9/11) (p=0.0028) (Figure 2B). Moreover, the expression of Beclin-1 was negatively correlated with HCC in patients with liver cirrhosis (p=0.029). The positive expression rate of Beclin-1 in HCC patients with vascular invasion displayed a significantly lower positive rate of Beclin-1 (39.1%, 9/23) compared with that in HCC patients without vascular invasion (75.0%, 45/60) (p=0.011) (Figure 2C). Taken together, these data suggest that abnormal expression of Beclin-1 was associated with the clinicopathological features of HCC.

Expressions of Beclin-1, and proliferation- and apoptosis-related proteins

Western blot analysis showed that the expression levels of Beclin-1 and Bax in HCC were significantly lower than those in ANT (p=0.038 and 0.014) (Figure 3A and 3B). However, the expression levels of proliferation- and apoptosis-related proteins (PNCA, NET-1 and Bcl-2) in HCC were significantly higher than those in ANT (p=0.033, 0.024 and 0.009) (Figure 3C-E).

Correlations of Beclin-1 expression with proliferation- and apoptosis-related proteins in HCC

Correlation analyses showed that Beclin-1 expression was negatively correlated with the expressions of PCNA, NET-1, and Bcl-2 (r=-0.559, -0.548 and -0.456; p<0.001) (Figure 4A-C), but positively correlated with the expression of Bax (r=0.568, p=0.027) (Figure 4D).

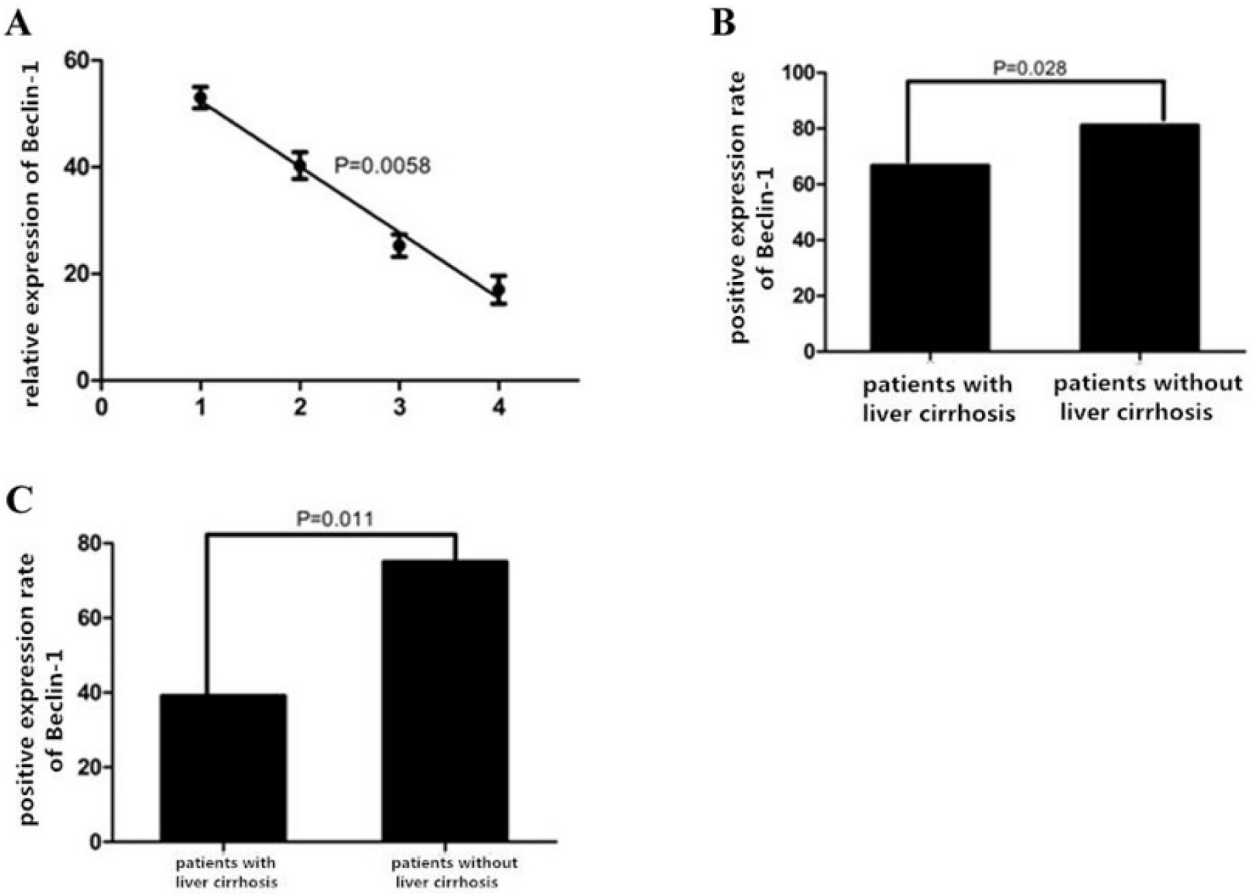

Figure 2. Associations of Beclin-1 expression with clinicopathological features of HCC. **(A):** HCC Edmondson grading; **(B):** HCC with or without liver cirrhosis; **(C):** HCC with or without vascular invasion.

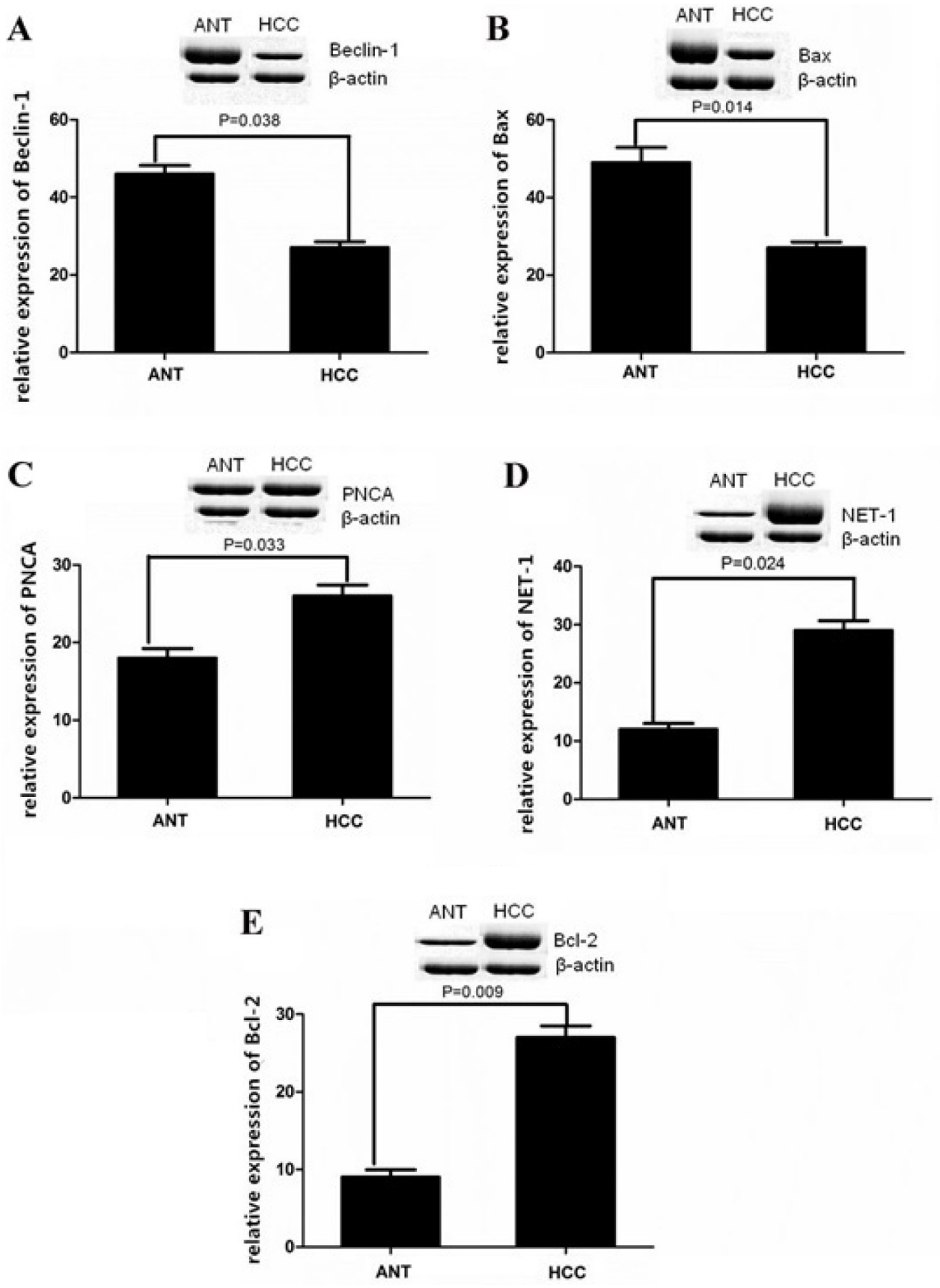

Figure 3. Expression of Beclin-1 **(A)**, Bax **(B)**, PCNA **(C)**, NET-1 **(D)** and Bcl-2 **(E)** in HCC and ANT. HCC tumor tissues and adjacent non-tumor tissues (ANT) were isolated followed by extraction of protein for measuring the expression of Beclin-1, Bax, PCNA, NET-1 and Bcl-2 by western blot. The Figure shows that Beclin-1 and Bax protein were remarkably reduced in HCC tumor tissues compared to the adjacent non-tumor tissues, with evident increase of PNCA, NET-1 and Bcl-2 expressions.

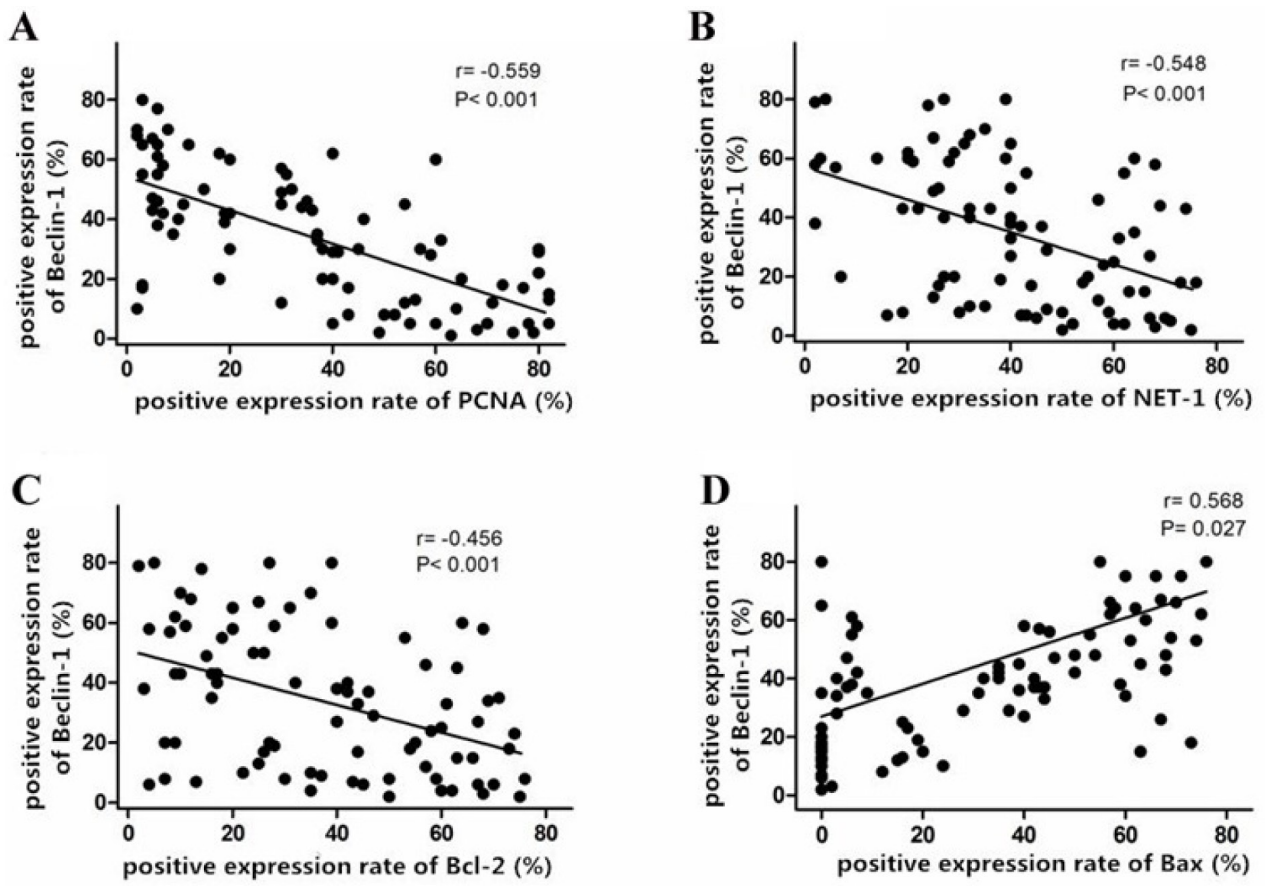

Figure 4. Correlations of Beclin-1 expression with PCNA **(A)**, NET-1 (B) and Bcl-2 **(C)** and Bax **(D)** in HCC. The relationship of Beclin-1 expression with PCNA, BET-1, Bcl-2 or Bax were analyzed by Spearman rank correlation test. Beclin-1 expression was negatively correlated with the levels of PCNA, NET-1 and Bcl-2, but positively associated with the expression of Bax.

Discussion

Autophagy is a strictly-regulated catabolic process that involves the degradation of intracellular components by lysosomes during cell growth, development, and tumorigenesis [17]. The effect of autophagy in tumorigenesis is bi-directional. In the tumor microenvironment, autophagy acts as a means of temporary survival responding to the metabolic stress. However, as cell stress continues to lead to sustained or progressive autophagy, cell death will ensue. In addition, new evidence shows the autophagy characteristics in cells treated with chemotherapeutic agents. Whether the high-level autophagy induced by cytotoxic drugs is considered as a direct cell-death pathway or garbage disposal mechanism remains unclear [18]. At present, the molecular mechanism of autophagy has been examined in many studies to elucidate the roles of autophagy in the occurrence and development of cancer. Beclin-1, an orthologous gene of Atg6 in mammals, is a proximal step in autophagy [19], which recruits proteins from the cytoplasm for autophagy degradation or provision of membrane components for autophagy pathway. Zou et al. found that the natural flavone A extracted from Scutellaria root can induce the Beclin-1-mediated autophagy in human HCC HepG2 cells [20]. Inactivation of Beclin-1 has been shown to lead to an increase in the incidence rate of tumor in mice. Beclin-1 has high percentages in human breast cancer, ovarian cancer, and prostate cancer and its protein level has been found to be decreased in human breast cancer, ovarian cancer, and brain tumor [21]. In addition, the high expression of Beclin-1 has been shown to inhibit the formation of human breast tumors in mouse model [22]. Therefore, Beclin-1 may play a role as a tumor suppressor and its down-regulation may contribute to the occurrence of human cancer. In this study, Beclin-1 was mainly localized in the cytoplasm of HCC tissues and its expression level in HCC tissues was significantly decreased compared with that in ANT. These results are similar to those obtained by Liang et al. [23], among which Beclin-1 is lowly expressed in human breast epithelial cancer cell lines and tissues, and highly expressed everywhere in normal breast epithelium. Another study [24] showed that trans-

fection of Beclin-1 into transformed breast cancer cell lines can reduce its tumorigenic potential in nude mice. These findings suggest that defective autophagy may exist in these cancers. Moreover, it has been shown that autophagy enhances tumor development and protects tumor cells from stimuli causing cell death [22]. In HCC xenograft tumor *in vivo*, inducing autophagy can inhibit the tumor growth and support the autophagy-mediated anti-tumor activity. These different results also show that Beclin-1 may play a role in a tissue-specific manner.

In this study, the expression level of Beclin-1 was negatively correlated with Edmondson grading, background of liver cirrhosis, and vascular invasion of HCC. It was found in this study that Beclin-1 expression in Edmondson grade I-II was higher than that in grade III-IV in HCC. Similarly, Beclin-1 expressions in HCC with vascular invasion and liver cirrhosis were significantly lower than those in HCC without vascular invasion and liver cirrhosis. These results indicate that Beclin-1 may be an immediate early response gene in tumorigenesis. The absence of Beclin-1 may be an early event in HCC, so it plays a more pivotal role in HCC progression. In order to identify the potential mechanism of Beclin-1 in affecting the malignant transformation of HCC, the correlations of Beclin-1 expression in HCC with proliferation-associated proteins, such as PCNA and NET-1, and apoptosis-related proteins, such as Bcl-2 and Bax, were studied. PCNA is a nuclear antigen that is expressed in proliferating cells at all stages of cell cycle, except G0 phase, and is used as a marker of proliferation. As a member of the transmembrane 4 superfamily (TM4SF), NET-1 serves as a molecular service protein to enhance the formation and stability of functional signal transduction complexes through connecting specific cell surface proteins, such as lineage-specific proteins, integrins, and other TM4SFs. Therefore, NET-1 plays an important role in cell signal transduction, regulation, adhesion, migration, proliferation, and differentiation [5,6]. Previous studies have shown that NET-1 expression is closely related to HCC proliferation, which is significantly up-regulated during cancer genesis [14]. Bcl-2 is one of the major anti-apoptotic proteins commonly found in mitochondrial membrane and the cytoplasm. When cells are deprived of survival signals or stressed, Bcl-2 is released from the mitochondrial membrane and replaced with pro-apoptotic factors, such as Bax. When the Bcl-2 level is decreased, the permeability of mitochondrial membrane will be increased and several proteins that can activate the Caspase cascade are released. In this study, Spearman correlation analyses showed that Beclin-1 had significantly negative correlations with PCNA, NET-1 and Bcl-2. There was a significantly positive correlation between Beclin-1 and Bax. Since Bax is a pro-apoptotic factor and Bcl-2 is an apoptosis-inhibiting factor, data in this study confirmed the correlation between Beclin-1 expression and Bax overexpression, suggesting that autophagy may be positively correlated with HCC apoptosis. A new function of autophagy in promoting HCC cell death was proposed, involving the inhibition of cell proliferation and promotion of apoptosis. However, the mechanism of this new function is still unclear.

Cell proliferation and apoptosis play important roles in the occurrence, development and progression of cancer. The expressions of PCNA and NET-1, as tumor-related factors, are closely related to cell proliferation. The increased expression of Bax and decreased expression of Bcl-2 lead to a higher activation level of apoptosis. Therefore, it is speculated that the co-expression of these genes in HCC has a synergistic effect, which greatly reduces the progression of cancer by inhibiting proliferation and promoting apoptosis. However, future experiments are required to confirm this hypothesis both *in vitro* and *in vivo*.

In conclusion, decreased expression of Beclin-1 was observed in HCC tissues and was negatively correlated with Edmondson grading, suggesting Beclin-1 might be a marker for evaluating the prognosis of HCC.

Conflict of interests

The authors declare no conflict of interests.

References

1. Cancer Incidence, Mortality and Prevalence Worldwide in 2008, Section of Cancer Information, GLOBOCAN 2008 [http://globocan.iarc.fr]
2. Lin XD, Lin LW. Local injection therapy for hepatocellular carcinoma. Hepatobiliary Pancreat Dis Int 2006;5:16-21.

3. Wang Q, Tan YX, Ren YB et al. Zinc finger protein ZBTB20 expression is increased in hepatocellular carcinoma and associated with poor prognosis. BMC Cancer 2011;11:271.
4. Kundu M, Thompson CB. Autophagy: basic principles and relevance to disease. Annu Rev Pathol 2008;3:427-55.
5. Qiu J, Huang P, Liu Q et al. Identification of MACC1 as a novel prognostic marker in hepatocellular carcinoma. J Transl Med 2011;9:166.
6. 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.
7. Livesey KM, Tang D, Zeh HJ, Lotze MT. Autophagy inhibition in combination cancer treatment. Curr Opin Investig Drugs 2009;10:1269-79.
8. Turcotte S, Giaccia AJ. Targeting cancer cells through autophagy for anticancer therapy. Curr Opin Cell Biol 2010;22:246-51.
9. Cheong H, Klionsky DJ. Biochemical methods to monitor autophagy-related processes in yeast. Methods Enzymol 2008;451:1-26.
10. Eskelinen EL, Saftig P. Autophagy: a lysosomal degradation pathway with a central role in health and disease. Biochim Biophys Acta 2009;1793:664-73.
11. Sun Q, Fan W, Zhong Q. Regulation of Beclin 1 in autophagy. Autophagy 2009;5:713-16.
12. Wang J. Beclin 1 bridges autophagy, apoptosis and differentiation. Autophagy 2008;4:947-8.
13. Pirtoli L, Cevenini G, Tini P et al. The prognostic role of Beclin 1 protein expression in high-grade gliomas. Autophagy 2009;5:930-6.
14. Li BX, Li CY, Peng RQ et al. The expression of beclin 1 is associated with favorable prognosis in stage IIIB colon cancers. Autophagy 2009;5:303-6.
15. Shen Y, Li DD, Wang LL, Deng R, Zhu XF. Decreased expression of autophagy-related proteins in malignant epithelial ovarian cancer. Autophagy 2008;4:1067-8.
16. Miracco C, Cosci E, Oliveri G et al. Protein and mRNA expression of autophagy gene Beclin 1 in human brain tumours. Int J Oncol 2007;30:429-36.
17. Lomonaco SL, Finniss S, Xiang C et al. The induction of autophagy by gamma-radiation contributes to the radioresistance of glioma stem cells. Int J Cancer 2009;125:717-22.
18. Pan B, Yi J, Song H. MicroRNA-mediated autophagic signaling networks and cancer chemoresistance. Cancer Biother Radiopharm 2013;28:573-8.
19. Edinger AL, Thompson CB. Defective autophagy leads to cancer. Cancer Cell 2003;4:422-24.
20. Mathew R, Karantza-Wadsworth V, White E. Role of autophagy in cancer. Nat Rev Cancer 2007;7:961-7.
21. Levine B. Cell biology: autophagy and cancer. Nature 2007;446:745-7.
22. Fujii S, Mitsunaga S, Yamazaki M et al. Autophagy is activated in pancreatic cancer cells and correlates with poor patient outcome. Cancer Sci 2008;99:1813-9.
23. Liang XH, Jackson S, Seaman M et al. Induction of autophagy and inhibition of tumorigenesis by Beclin 1. Nature 1999;402:672-6.
24. Oh S, Xiaofei E, Pirooz SD et al. Downregulation of autophagy by Bcl-2 promotes MCF7 breast cancer cell growth independent of its inhibition of apoptosis. Cell Death Differ 2011;18:452-64.