Original Articles

Expression of beclin 1, an autophagy-related protein, in human cervical carcinoma and its clinical significance

H.Y. Cheng[1], Y.N. Zhang[2], Q.L. Wu[3], X.M. Sun[2], J.R. Sun[2], X. Huang[2]

[1]Department of Obstetrics and Gynecology, Qingdao Central Hospital, Qingdao, Shandong
[2]Department of Gynecology, Cancer Center, Sun Yat-Sen University, Guangzhou, Guangdong
[3]Department of Pathology, Cancer Center, Sun Yat-Sen University, Guangzhou, Guangdong (P. R. China)

Summary

Purpose: To investigate the impact of beclin 1 on prognosis of cervical cancer, we determined the expression of beclin 1 in cervical cancer, cervical intraepithelial neoplasia (CIN) and normal cervical tissues. *Methods:* A total of 122 cases of cervical cancer, 35 cases with CIN and 31 cases with uterine fibroids were collected at the Cancer Center of Sun Yat University to determine the expression of beclin 1. *Results:* Beclin 1 positive rate in normal cervical tissues, CIN tissues and cervical cancers was 83.9%, 74.3% and 53.3%, respectively, and it was significantly different between the three groups ($p < 0.01$). Beclin 1 expression was negatively correlated with cervical cancer differentiation, lymph node metastasis, recurrence and death ($p < 0.05$). The negative expression is the risk factor affecting overall survival ($p < 0.05$) and progression-free survival (PFS) ($p < 0.05$). Multivariate analysis showed that beclin 1 negative expression was an independent risk factor of PFS time. *Conclusions:* Beclin 1 may play a role in the occurrence and development of cervical cancer. Beclin 1 positive expression in patients indicates a better prognosis.

Key words: Cervical cancer; Autophagy; Beclin 1; Overall survival (OS); Progression-free survival (PFS).

Introduction

Cervical cancer is one of the common malignant tumors in the female reproductive system. According to the statistics, there are approximately 51 million new cases of cervical cancer every year and 80% of the cases are from developing countries with annual deaths of about 28 million [1]. With the extensive cancer screening and continuous improvement of cancer treatment, morbidity and mortality have decreased. However, uncontrolled local tumor, recurrence and metastasis are the leading causes of death. Therefore, it has been an urgent requirement to identify effective treatments and examination indexes in order to reduce relapse and improve survival rate.

It was found that programmed cell death and autophagy inhibition were associated with the occurrence of cancer. Most scholars have divided cell death into apoptosis, autophagy and necrosis [2, 3]. Autophagy is known as type II programmed cell death, which was discovered in recent years as a new type of programmed cell death, and is independent of the non-procaspase [3, 4]. Autophagy is a normal function of cells. It can be induced immediately when the self-steady-state is changed (such as developmental differentiation and tissue reconstruction), or the body is subjected to a variety of stimuli (such as injury, hypoxia, high temperature, infection, hormones, etc.). Through autophagy, the cells can remove excessive or damaged organelles and intracellular components [5, 6].

The occurrence of autophagy is associated with ATG (autophagy-related gene) family genes. Beclin 1 and yeast ATG6, the same series of substances, is a kind of diallelic tumor suppressor gene related to autophagy; it is also a specific gene involved in autophagy in mammals. In 1999, Aita *et al*., [7] found that the gene encoding beclin 1 is located in the human chromosome 17q21, and successfully cloned the gene beclin 1.

Although the role of beclin 1 has been investigated in tumors such as liver, lung, breast, ovarian, endometrial, esophageal and colon cancers, few studies are available with regard to the role of beclin 1 in cervical cancer. This study was designed to detect the expression of beclin 1 in cervical cancer by immunohistochemical staining to explore the relationship between beclin 1 expression and pathological features, and to elucidate the effect of beclin 1 on prognosis.

Materials and Methods

Clinical information

A total of 188 cases studied were divided into three groups: cervical cancer, cervical intraepithelial neoplasia (CIN) and normal cervix. The experimental group included 122 cervical cancer cases (median age: 39 years) collected from the Cancer Center of Sun Yat-Sen University between January 1998 and December 2003. The criteria for the cases in the experimental group were: 1) patients were diagnosed with cervical squamous cell carcinoma by pathological analysis and initial treatment; 2) IB1~ IIA stages were obtained by FIGO clinical staging; 3) patients received radical hysterectomy and pelvic lymphadenectomy (Wertheim-Meigs operation) in the hospital; 4) radiotherapy, chemotherapy, biological and immune therapy had not been conducted before operation; 5) complicated or secondary cancer did not occur; and, 6) complete clinical data and pathological specimens were available and postoperative follow-up

Revised manuscript accepted for publication April 11, 2011

Fig. 1a

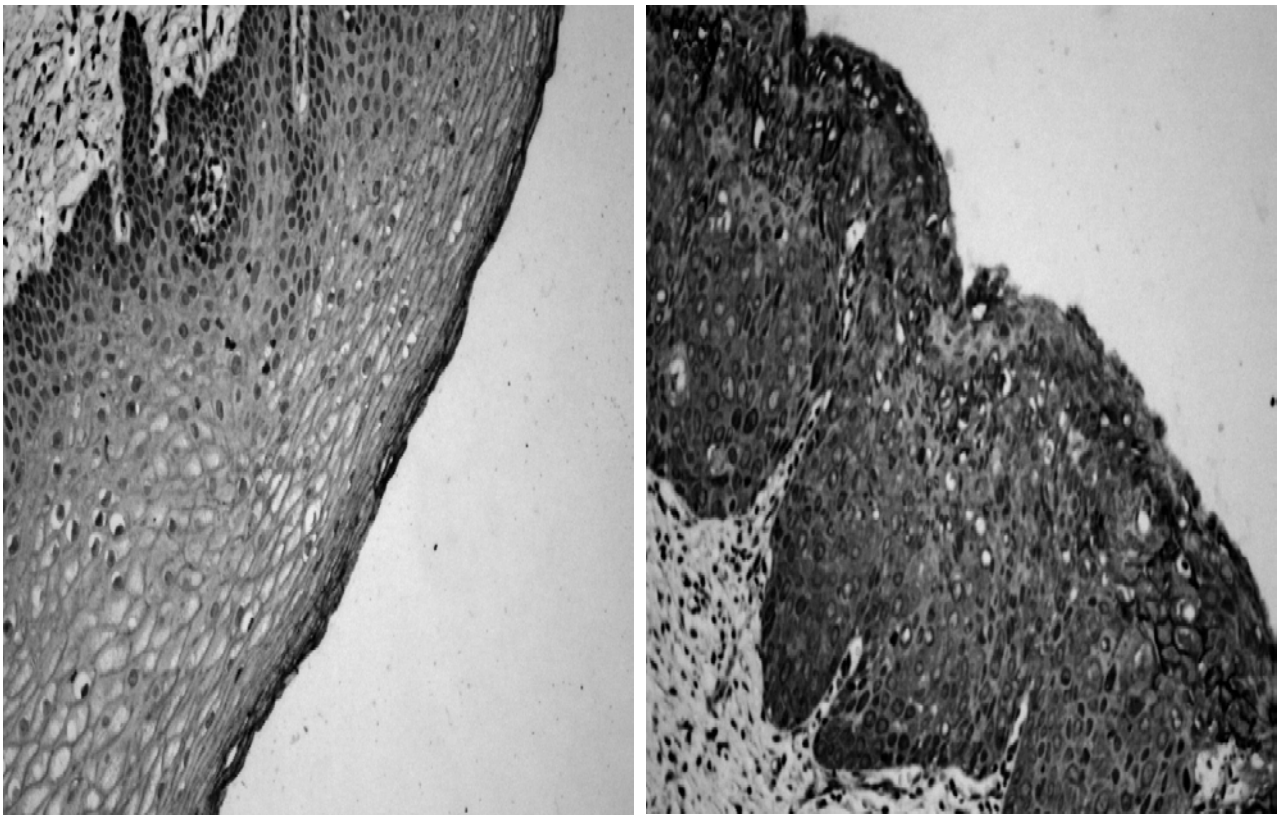

Fig. 1b

Figure 1. — High expression of beclin 1 in normal tissue (a) and in CIN tissue (b) (10 × 20).

information was complete. A second group included 35 cases (median age: 39 years) with CINII-III, and the third group included 31 cases (median age: 40 years) with normal cervix. Clinical staging established by the International Federation of Gynecology and Obstetrics (FIGO) in 1995 was adopted for the patients in this study. Detailed information on the cases is shown in Table 1. There was no significant difference in the age among the three groups.

Pathological analysis

Paraffin-embedded tissue samples from the 188 cases were examined and serial sections (4 μm thick) were cut from the wax block. Monoclonal antibody against beclin 1 (Abcam, USA) was used for immunohistochemical staining. A polymer immunohistochemistry kit containing HRP-conjugated goat anti-rabbit IgG antibody was purchased from Jinqiao Biotechnology Co., Ltd (Beijing, China); 0.01M PBS buffer at pH 7.4 and 3,3-diamine benzidine tetrahydrochloride (DAB) solution were prepared immediately before use. Immunohistochemistry was performed by using a two-step non-biotin labeling method. Briefly, the slides were baked and dewaxed. High-pressure antigen restoration was performed using citric acid solution (pH 6.0), and the slides were rinsed successively with distilled water and PBS buffer (pH 7.4). After endogenous peroxidase activities were inhibited by peroxidase blocker, the slides were washed and then incubated with non-immune goat serum, anti-beclin 1 antibody and washed with PBS. Subsequently, the slides were incubated with HRP-conjugated goat anti-rabbit IgG antibody polymer and washed with PBS. Hematoxylin counterstaining was performed subsequent to DAB colorimetric detection. After treated with 0.1% saline alcohol, the slides were rinsed with tap water, dehydrated with 95% alcohol, dried and sealed with neutral resin. Beclin 1 positive brain sections were used for positive control, and PBS instead of primary antibodies for negative control.

Grading of the pathological observations

All the slides were blindly and independently examined by two pathologists. The cells with brown granules in cytoplasm were recognized as positive cells. Beclin 1 was localized in the cell membrane, cytoplasm and Golgi apparatus. Ten fields with 100 cells in one field were randomly selected and examined with a high magnification lens. The slides were evaluated according to the ratio of positive cells and intensity of the staining. The grading for the ratio of positive cells is based on the following criteria: “0” indicates that the positive cells account for less than 10% of the total cells, “1” indicates that the positive cells account for 10%-25% of the total cells, “2” indicates the positive cells account for 26%-50% of the total cells and “3” indicates that the positive cells account for more than 50% of the total cells. Grading for the staining intensity is based on the following criteria: “0” indicates no staining, “1” indicates light yellow staining, “3” indicates yellow to brown yellow staining, and “2” indicates the staining intensity between “1” and “3”. We used the formula: total grade=grade for positive cell ratio+grade for staining intensity to evaluate each sample. Total grade 0-1 indicates negative, 2-3 weakly positive, 4-5 medium strongly positive, and ≥ 6 strongly positive.

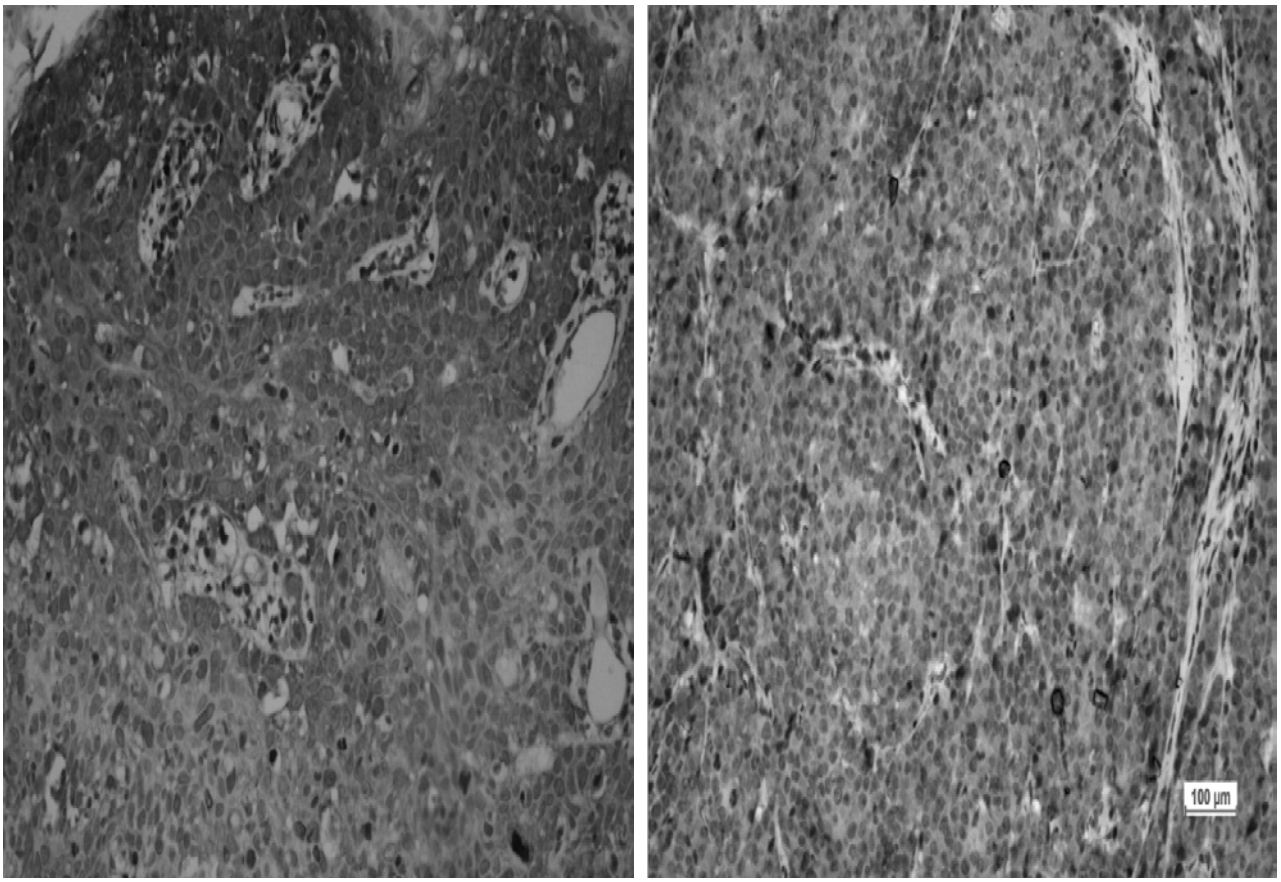

Fig. 2a Fig. 2b

Figure 2. — Positive (a) and negative (b) expression of beclin 1 in cervical cancer tissues (10 × 20).

Statistical analysis

The SPSS15.0 package was used for statistical analysis. Chi-square test or Fisher's exact test were used for categorical data, and rank test for the ranked data. Continuous variables were subjected initially to a normal distribution test. If the continuous variables were in accord with the normal distribution, Student's *t*-test was used. Otherwise, the rank test was performed. Spearman rank correlation analysis was used for rank correlation. Life table methodology was used to calculate the survival rate. The Kaplan-Meier method was used to draw survival curves. The log-rank test and Cox proportional hazard model were used for multivariate prognostic analysis; $p < 0.05$ was considered statistically significant.

Prognostic evaluation

Two indicators, overall survival (OS) and progression-free survival (PFS), were used to evaluate prognosis. OS refers to the time from surgery to the last time of follow-up or death. PFS indicates the time from surgery to clinical or pathological recurrence.

Results

Expression of beclin 1 in normal cervical, CIN and cervical squamous carcinoma tissues

The positive expression rates of beclin 1 in normal cervical, CIN and cervical cancer tissues were 83.9% (26/31), 74.3% (26/35) and 53.3% (65/122), respectively. Chi square analysis showed that the positive rates of beclin 1 were statistically different between the three groups ($p < 0.01$). There was no significant difference between the positive rate in the normal cervical group and that in the CIN group ($\chi^2 = 1.099$, $p = 0.295$). The positive rate was significantly different between the normal cervical group and cervical cancer group ($\chi^2 = 25.270$, $p < 0.01$), and between the CIN group and cervical cancer group ($\chi^2 = 13.009$, $p < 0.01$) (Figures 1 and 2).

Relationship between beclin 1 expression and the factors associated with cervical squamous cell carcinoma

Statistical analysis showed that beclin 1 expression was negatively correlated with tumor differentiation, lymph node metastasis, recurrence and death ($p < 0.05$), while was not significantly correlated with age, tumor diameter, FIGO staging, SccAg, depth of cervix invasion, or surgical margin (Table 1).

Effect of beclin 1 and other clinical and pathological factors on the prognosis of cervical cancer: univariate analysis

Statistical analysis showed that tumor differentiation, lymph node metastasis and the negative expression of

Table 1. — *Relationship between beclin 1 expression and clinicopathological factors in cervical squamous cell carcinoma.*

Clinicopathological factors	Number of cases	Beclin negative	Beclin positive	Test value	*p* value
Age					
≤ 35 years-old	35	20	15	2.141	0.143
>35 years-old	87	37	50		
Tumor diameter					
≤ 4 cm	103	48	55	0.004	0.951
> 4 cm	19	9	10		
FIGO staging					
IB	103	50	53	0.882	0.348
IIA	19	7	12		
SccAg					
≤ 1.5 ng/ml	76	33	43	0.882	0.348
> 1.5 ng/ml	46	24	22		
Tumor differentiation					
Highly differentiated	52	14	38	14.271	0.000
Poorly differentiated	70	43	27		
Depth of infiltration					
Upper or medium muscle layer	60	28	34	0.123	0.726
Deep muscle layer	62	29	31		
Lymph node metastasis					
Negative	99	42	57	3.895	0.048
Positive	23	15	8		
Surgical margin					
Negative	114	52	62	-0.039 *	0.355
Positive	8	5	3		
Recurrence					
No	97	40	58	8.006	0.005
Yes	25	17	7		
Death					
No	108	46	62	-0.139 *	0.011
Yes	14	11	3		

Table 2. — *Multivariate analysis of prognosis of cervical cancer.*

Survival	Factors	B	SE	Wald	df	sig	Exp (B)	95% CI for Exp (B) Lower	Upper
OS	Lymph node metastasis	1.636	0.535	9.339	1	0.002	5.136	1.798	14.669
PFS	Lymph node metastasis	1.016	0.417	5.938	1	0.015	2.763	1.220	6.256
	Beclin 1	-1.042	0.454	5.269	1	0.022	0.353	0.145	0.859

beclin 1 were the risk factors for OS ($p < 0.05$), while age, tumor diameter, FIGO staging, SccAg, depth of cervix invasion, surgical margin were not risk factors for OS (Figures 3 and 4). SccAg, tumor differentiation, lymph node metastasis, surgical margin, depth of cervix invasion and the negative expression of beclin 1 were the risk factors for PFS ($p < 0.05$), while age, tumor diameter, FIGO staging were not risk factors for PFS (Figures 3-4).

Effect of beclin 1 and other clinical and pathological factors on the prognosis of cervical cancer: multivariate analysis

We included data from the *univariate* analysis into the Cox regression model and the results showed that lymph node metastasis was the independent prognostic factor for OS, while lymph node metastasis and negative expression of beclin 1 were the independent risk factors for PFS (Table 2).

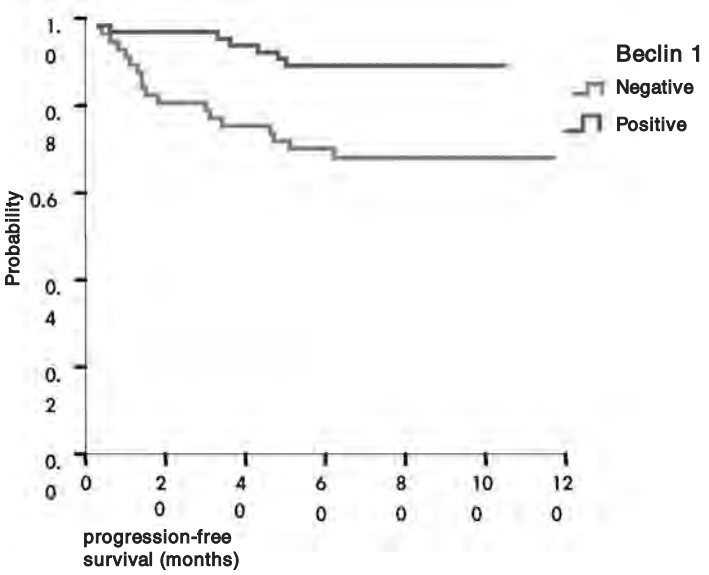

Figure 3. — Relationship between Beclin 1 expression and PFS in cervical cancer ($p = 0.004$).

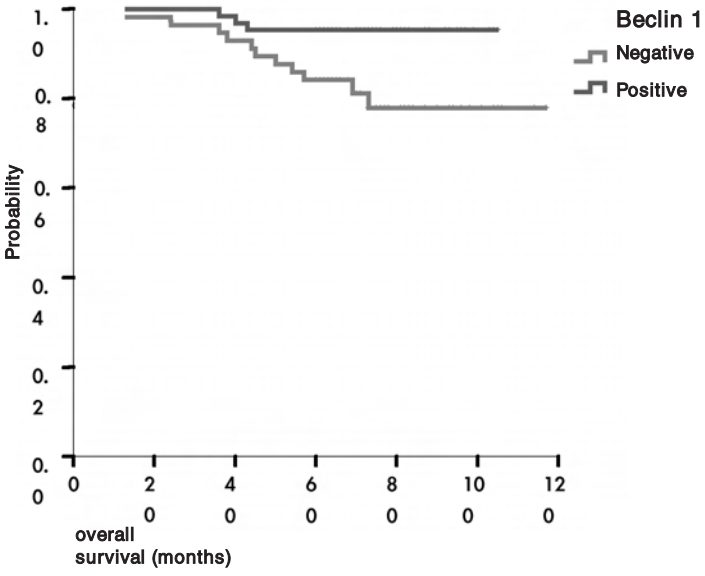

Figure 4. — Relationship between Beclin 1 expression and OS in cervical cancer ($p = 0.013$).

Discussion

At present, tumor research has reached the molecular level. Many studies have confirmed that the occurrence of tumors is related to the activation of oncogenes and inactivation of tumor suppressor genes. Recently, the role of autophagic genes in cancer has become the new research focus. Autophagic genes contain double alleles and are the specific genes involved in autophagy in mammals. The autophagic gene, beclin l, is frequently absent in a number of malignant tumors, which causes scholars to be attentive. Beclin 1 is the key factor mediating the localization of other autophagic proteins in pre-autophagic

bodies. Beclin 1 is an important gene involved in the regulation of autophagic bodies in mammals. It can inhibit the growth of tumors by enhancing autophagy. In many tumor cell lines, beclin 1 is expressed at a low level [8, 9-13]. The key roles of autophagy during the process of tumor development are embodied in the following two aspects. Firstly, autophagy changes the protein metabolism disorder, maintains internal stability and inhibits the occurrence of tumors by adjusting the intracellular peroxide concentration. Secondly, reduction in the function of autophagy increases oxidative stress and accumulation of tumorigenic mutations [14]. In this study, we found that beclin 1 had a positive expression rate of 83.9%, 74.3% and 53.3% in normal cervical, CIN, and cervical cancer tissues, respectively. There was no significant difference between the positive expression rate in normal cervical groups and that in the CIN group. In contrast, the positive expression rate of beclin 1 was significantly different between normal cervical and cervical cancer tissues and between CIN tissues and cervical cancer tissues ($p < 0.01$). Although the positive expression rate of beclin 1 in the CIN group was not significantly different from that in the normal cervical group, the positive expression of beclin 1 had a declined trend from the normal cervical to CIN group and from the CIN to cervical cancer group. These results indicate that lack of beclin 1 expression may be an early molecular event in cervical precancerous lesions. Decreasing expression of beclin 1 from normal cervix to CIN and from CIN to cervical cancer also suggests that beclin 1 may play a certain role in the occurrence and development of cervical cancer.

The key feature of tumor growth *in vivo* is its aggressive growth and metastasis, which is the main reason why a tumor is difficult to treat. There are generally the following steps during the process of metastasis of malignant tumors: 1) growth of an early primary tumor; 2) tumor angiogenesis; 3) shedding and invasion of tumor cells into the interstitial tissue; 4) invasion of tumor cells into the vascular system; 5) formation of tumor thrombus; 6) localized growth of secondary tissues and organs; and, 7) continuous spread of metastatic carcinoma [15]. Thus, the invasion of tumor cells into the lymphatic vascular system is the basis for metastasis. Our study showed that lymph node metastasis is not only an independent risk factor for PFS, but also the independent prognostic factor affecting OS. The beclin 1 expression was significantly correlated to tumor differentiation ($p < 0.01$), lymph node metastasis ($p = 0.048$), relapse ($p = 0.005$) and death ($p = 0.011$). We hypothesized that downregulation of beclin 1, a haplo-insufficient tumor suppressor gene, results in the deficiency of autophagy and apoptosis, genome mutation and increases in the malignant phenotype, thereby increasing the corrosive nature of tumor cells and promoting tumor metastasis. This study also found that 5-year PFS rate and survival rate in beclin 1-negative patients were 68.1% and 81.2%, respectively. In contrast, 5-year PFS rate and survival rate in beclin 1-positive patients were 89.2%, 95.4%, respectively, which was significantly different to those in beclin 1-negative patients. Multivariate analysis showed that beclin 1 is an independent factor affecting the PFS time. Based on these results, we concluded that beclin 1-positive patients had a better prognosis. Improving autophagic activity in cervical cancer cells may become a new target for biological therapy of cervical cancer.

It has been demonstrated that improving autophagic activity in cervical cancer cells can be used for the treatment of cervical cancer in animal models. Qu *et al*. [9] and Yue *et al*. [10] established beclin 1-deficient mice and embryonic stem cells, and showed that Beclin l defects significantly increased the incidence of liver cancer, lung cancer and lymphoma. Liang *et al*., [8] transfected the beclin l gene into the human breast cancer cell line MCF-7 (deficient in the expression of beclin 1) and found that the number of autophagic vacuoles was increased, the *in vitro* proliferation ability of cancer cells and the malignant phenotype was decreased, and the tumor-forming ability of MCF-7 was reduced in nude mice. It has been demonstrated that a variety of anti-tumor drugs exert an anti-tumor effect through increasing the expression of beclin 1. Identification of an anti-tumor drug that can target the molecular mechanism of autophagy is of important significance for the treatment of tumors. Treatment of MCF-7 cells with tamoxifen can cause cell death with typical features of autophagy. Activation of autophagic death in breast cancer cells by tamoxifen is caused by ceramide-mediated upregulation of beclin 1 [16, 17]. With the progress in the molecular biology of cancer, introduction of autophagic death into the treatment of tumors will become a new target for cancer therapy.

In conclusion, beclin 1 may play a role in the occurrence and development of cervical cancer, and patients with beclin 1 positive expression have a better prognosis.

Acknowledgement

The work was supported by Guangdong Provincial Science and Technology Planning Project (No. 2006B35901005).

References

[1] Valdespino V.M., Valdespino V.E.: "Cervical cancer screening: state of the art". *Curr. Opin. Obstet. Gynecol.,* 2006, *18,* 35.
[2] Abraham M.C., Shaham S.: "Death without caspases caspases without death". *Trends Cell. Biol.,* 2004, *14*, 184.
[3] Lockshin R.A., Zakeri Z.: "Apoptosis, autophagy and more". *Int. J. Biochem. Cell. Biol*., 2004, *36*, 2405.
[4] Shintani T., Klionsky D.J.: "Autophagy in health and disease: a double-edged sword". *Science*, 2004, *306,* 990.
[5] Yoshimori T.: "Autophagy. a regulated bulk degradation process inside cells". *Biochem. Biophys Res. Commun.,* 2004*, 313,* 453.
[6] Levine B., Yuan J.: "Autophagy in cell death: an innocent convict?". *J. Clin. Invest.,* 2005, *115*, 2679.
[7] Aita V.M., Liang X.H., Murty V.V., Pincus D.L., Yu W., Cayanis E. *et al*.: "Cloning and genomic organization of beclinl a candidate tumor Suppressor gene on chromosome 17q21". *Genomics,* 1999, *59*, 59.
[8] Liang X.H., Jackson S., Seaman M., Brown K., Kempkes B., Hibshoosh H. *et al*.: "Induction of autophagy and inhibition of tumorigenesis by beclin 1". *Natura1,* 1999, *402*, 672.
[9] Qu X., Yu J., Bhagat G., Furuya N., Hibshoosh H., Troxel A. *et al*.: "Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene". *J. Clin. Invest.,* 2003, *112*, 1809.

[10] Yue Z., Jin S., Yang C., Levine A.J., 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.
[11] Miracco C., Cosci E., Oliveri G., Luzi P., Pacenti L., Monciatti I. *et al.*: "Protein and mRNA expression of autophagy gene Beclin 1 in human brain tumours". *Int. J. Oncol.,* 2007, *30,* 429.
[12] Wang Z.H., Xu L., Duan Z.L., Zeng L.Q., Yan N.H., Peng Z.L.: "Beclin 1-mediated macroautophagy involves regulation of caspase-9 expression in cervical cancer HeLa cells". *Gynecol. Oncol.*, 2007, *107*, 107.
[13] Duan Z.L., Peng Z.L., Wang Z.H.: "Expression and involved signal transduction pathway of autophagy gene beclin 1 in epithelial ovarian cancer". *Sichuan Da Xue Xue Bao Yi Xue Ban,* 2007, *38,* 239.
[14] Holm E., Hildebrandt W., Kinscherf R., Droge W.: "Low postabsorptive net protein degradation in male cancer patients: lack of sensitivity to regulatory amino acids?". *Oncol. Rep.,* 2007, *17,* 695.
[15] Ma D.: "Tumor and tumor metestasis". In: Zeng Y.X., Lv Y.Y., Zhu M.H. (eds.). Bei Jing, P.R. China, The people's Hygienic Publication Society, 2003, 242.
[16] Bursch W., Ellinger A., Kienzl H., Torok L., Pandey S., Sikorska M. *et al.*: "Active cell death induced by the anti-estrogens tamoxifen and ICI 164 384 in human mammary carcinoma cells (MCF-7) in culture: the role of autophagy". *Carcinogenesis,* 1996, *17,* 1595.
[17] Scarlatti F., Bauvy C., Ventruti A., Sala G., Cluzeaud F., Vandewalle A. *et al.*: "Ceramide-mediated macroautophagy involves inhibition of protein kinase B and up-regulation of beclin 1". *J. Biol. Chem.,* 2004, *279,* 18384.

Address reprint requests to:
Y.N. ZHANG, M.D.
Department of Gynecology
Cancer Center Sun Yat-Sen University
651 Dongfeng Road East
Guangzhou
Guangdong Province (China)
e-mail: zhangyannapds@163.com