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Research Paper

Expression of Beclin1 in non small cell lung cancer and its clinical significance

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Abstract

Objective: To investigate the expression of autophagy related gene; Beclin1 in human non-small cell lung cancer(NSCLC) tissues. **Methods:**Protein expression of Beclin1 was determined by immunofluorescence staining and Western Blot, mRNA expression was analyzed by RT-PCR. **Results:**Immunofluorescence staining revealed that the level of Beclin1 expression in lung cancer was significantly lower than that in adjacent noncancerous tissues and normal tissues(expression rate 8.3%, P=0.000). The Beclin1 mRNA expression in lung cancer, adjacent noncancerous tissues and normal tissues was 1.372 ($\pm$ 0.475) 1.721 ($\pm$ 0.521) and 1.553($\pm$ 0.554) when F = 15.0, $P < 0.01$. Beclin1 protein expression in lung cancer, adjacent noncancerous tissues and normal tissues was 3.453($\pm$ 0.852) 5.423($\pm$ 1.351) and 6.878($\pm$0.997) F = 11.2, $P < 0.01$. **Conclusion:**The protein and mRNA expression of Beclin1 in lung cancer was much lower than those in and around cancer tissues and normal tissues, those differences having statistical significance. However in adjacent noncancerous tissues and normal tissues, the expression showed no difference.

Key words: autophagy; Beclin1; lung cancer

INTRODUCTION

During the formation and advancement of NSCLC, many factors were involved, such as the inactivation of anti-oncogene, perturbation of Cell homeostasis, barriers of damaged cellular organelle elimination and hindrance of apoptosis and autophagy. Autophagy is an intracellular process that results in cytosolic component's degradation, the malfunction of which is associated with such ailments as neurodegenerative diseases, myopathy, tumor, aging and invasion by microbial pathogens. Beclin1 is an anti-oncogene (closely related to autophagy) which encodes autophagy associated protein. Beclin 1 has been found down-regulated in breast cancer, ovarian cancer and prostatic carcinoma[1]. However research has been seldom reported about the Beclin1 in lung cancer. In order to analyze the relationship of Beclin1 and lung cancer, we tested the expression of Beclin1 protein and mRNA in lung cancer, and in adjucent noncancerous tissues and normal tissues, and investigated their relationship to lung cancer.

MATERIALS AND METHODS

Patients and samples

Fresh 54 NSCLC tissues (including 33 adenocarcinoma. AD, 17 squamous cancers SC, 2 adenosquamous carcinomaADC , 1 large cell carcinoma.LC and 1 metastatic carcinoma.MC.)and adjacent non-cancerous tissues(1-3 cm away from tumor) and normal tissues (>5 cm away from tumor) were obtained immediately from the resected speciments of NSCLC patients who underwent a pulmonary lobectomy at the department of Thoracic Surgery, Union Hospital,Wuhan between June 2006 and Oct 2006. 5 normal human lung tissues were collected from bunamiodyl excision at the same time which served as the control. All tissue samples were snap-frozen in liquid nitrogen and stored at -196℃ in EP tube immediately after being rinsed with 0.9% saline. 54 patients included 39 males and 15 females, aged 38 to 77 years, average 55.4 $\pm$ 8.7 years. Based on the

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UICC staging status(NSCLC 1997) 11 patients were classified as stage Ⅰ,27 stage Ⅱ and 16 stage ⅢⅠa. No patient received chemotherapy or radiotherapy before the operation.

Immunofluorescence

All samples were frozen and sliced(0.1 mm) at the department of Pathology, Union Hospital and fixed by 50% acetone and 50% alcohol(1:1) for 5 min, then the ruptured membrane was fixed by 0.3% Triton. After blocking by BSA, sections were incubated with primary antibody polyclonal goat Beclin1(Santa cruz, USA) and diluted to 1:200 at 4℃ overnight. After being washed by PBS, sections were incubated with rabbit anti-goat IgG FICT(diluted to 1:100) at 37℃ for 0.5 h in darkness. Immunostaining was visualized using laser confocal microscopy at 353 nm WL. The negative control was performed by PBS substitution for Beclin1 antibody staining.

RT-PCR for mRNA detecting

Total RNA of each treatment was extracted by Trizol Reagent(TakaRa.Japan) and the synthesis cDNA was performed following manufacturer's protocol, respectively. For reverse transcription, 4 mg of total RNA was transcribed. First-strand Synthesis System (Invitrogen,USA) was used for RT-PCR. PCR reaction used 2 ml of complementary DNA with 0.2 mM dNTP mix,2 mM MgCl2,10×PCR buffer,1U Taq polymerase, and 100 mM primers. The PCR reaction was performed as follows:94℃(1 min), 55℃(1.5 min) and 72℃ (0.5 min) for 30 cycles(25 cycles for GAPDH control). The primers used for RT-PCR were as follows; GAPDH: 5′-ACCACAGTCCATGCCATCAC-3′(sence), 5′-TCCACC ACCCT GTTGCTGTA-3′(antisence), 452 bp;Beclin 1:5′-ATCCTGGACCGTGTCACCA-TCCAGG-3′(sence),5′-GTTGAGCTGAGTG-TCCAGCTGG-3′(antisence), 363 bp.

Western blot for Beclin1 protein detecting

Cells were lysated by 200 μl of RIPA buffer, and protein level was measured by Coomassie Brilliant Blue staining. Protein was separated by vertical electrophoresis and transferred onto the membrane. After blocking, the membrane was incubated in the primary antibody Beclin 1(1:1000) at 4℃ overnight, and then was incubated with a secondary antibody labeled by alkaline phosphatase (1:3000, Zhong San Jing Qiao Biotechnology, Beijing) at room temperature for 2 h. After alkaline phosphatase staining for 5 min, images of labeled protein bands were examined by film exposure. Integral absorbency(A) of each strap was analyzed with Quantity One v4.52(band analysis) software. The staining intensities of the positive band and β-actin signals were determined respectively and the ratio of them was used to indicate the relative expression amount of Beclin in each group.

Statistical analysis

Data sets were analyzed by SSPS11.5. Immunofluorescence results were analyzed by χ^2 Test. Results of RT-PCR and Western blot were tested by ANOVA F. A value of $P < 0.05$ was considered to be significant.

RESULTS

Immunofluorescence results

The Beclin1 was found distributed in the whole cells. The expression positive rate was 100% in both adjacent noncancerous tissues and normal tissues, while the expression of Beclin1 was detectable in 5 cases of lung cancer displaying a vacuole-like state(positive rate was 8.3%).The difference was statistically significant. $\chi^2 = 60.9$, $P = 0.00$(***Fig 1***).

mRNA and protein expression

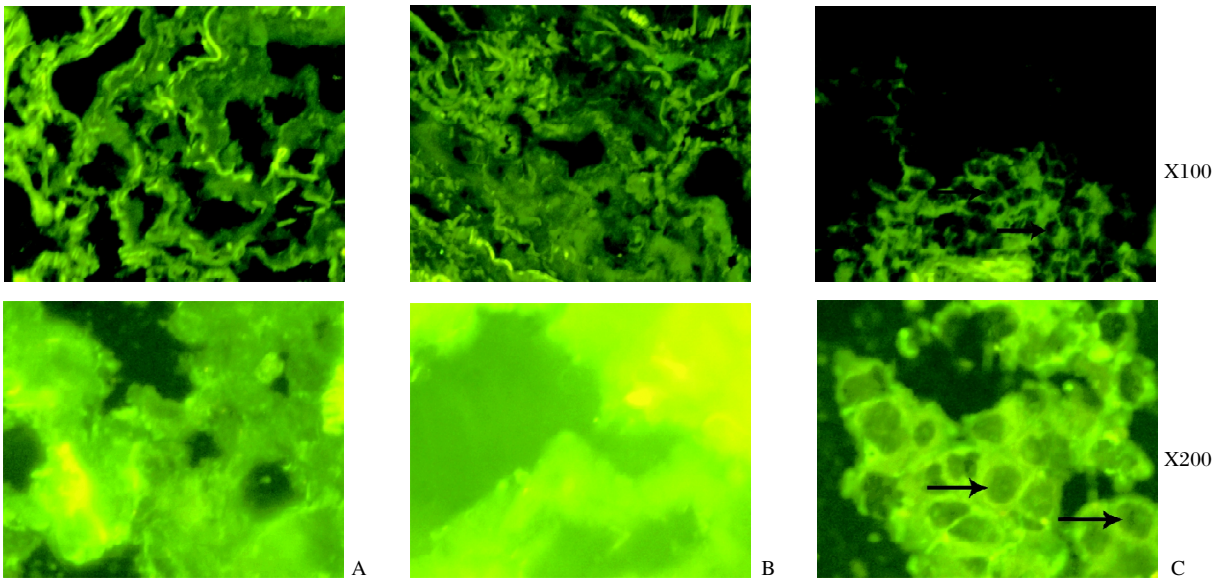
RT-RCR showed that Beclin1 mRNA was detected in all tissues, while the expression of Beclin1 mRNA in lung cancer(1.372 ± 0.475) was significantly weaker than that of the adjacent noncancerous tissues(1.721 ± 0.521) and normal tissues(1.553 ± 0.554) $F = 15.0$, $P = 0.00$(***Tab 1,Fig 2***). Western blot demonstrated the expression of lung cancer(3.453 ± 0.852) Beclin1 protein was significantly downregulated compared with adjacent noncancerous tissues(5.423 ± 1.351) and normal tissues(6.878 ± 0.997) $F = 11.2$, $P < 0.01$(***Tab 1, Fig 3***). No significant difference in the expression was detected between the adjacent noncancerous tissues and normal tissues.

Beclin1 expression and pathological type or clinical stage of NSCLC

Statistical analysis demonstrated there was no relationship between Beclin1 gene expression and pathological type or clinical stage of lung cancer(***Tab 2***).

DISCUSSION

Beclin1 gene is located at chromosomes 17q21 with a length of 150 kb. Beclin1 was considered as a haploinsufficient tumor suppressor, and was responsible for the deletion of one allete which was one of the malignant transformation agents[2]. The monoalletical deletion and mutation of Beclin1 were detected in 75% of ovarian cancer patients[3,4], 50% of breast cancers[5], and 40% of prostate cancers[6], with different levels of down-regulation of Beclin1[7]. However, biallelic deletion of beclin 1 had not been demonstrated in human cancers, and little was reported about the relationship between Beclin1 and human lung cancer. Our study showed that the expression of Beclin1 gene, was significantly lower



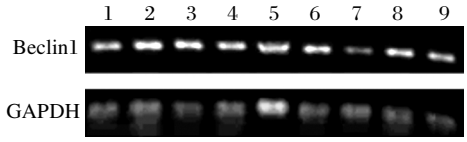
A: Beclin1 expression in normal lung tissues; B: Beclin1 expression in adjacent noncancerous tissues; C:Beclin1 expression in lung cancer(vacuole-like state)

Fig 1 Immunofluorescence analysis of the expression of Beclin1

Tab 1 **Beclin1 mRNA and protein expression in different tissues**

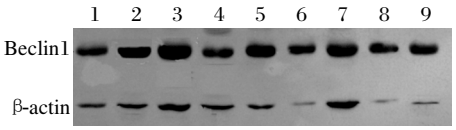
N=54	lung cancer	adjucent noncancerous	normal	control	*P*
Beclin1(mRNA)	1.372 ± 0.475**	1.721 ± 0.521	1.553 ± 0.554	1.523 ± 0.635	0.00
Beclin1(Western)	3.453 ± 0.852**	5.423 ± 1.351	6.878 ± 0.997	5.336 ± 1.174	0.00

**$P < 0.01$



Lane 1,4,7:NSCLC tissues; Lane2,5,8:adjacent noncancerous tissues; Lane3,6,9:normal tissues.

Fig 2 Analysis of the RT-PCR product of Beclin1 mRNA in lung tissues, lane1,4,7 weakly expressed while others expressed well.



Lane 1,4,7:NSCLC tissues; Lane 2,5,8:adjacent noncancerous tissues; Lane 3,6,9:normal tissues.

Fig 3 Analysis of the Western blot product of Beclin1 protein in lung issues, lane1,4,7 weakly expressed while others expressed well.

Tab 2 **Beclin1 expression in different pathological types or clinical stages of lung cancer**

TYPE	CASE	mRNA		*P*	Protein		*P*
Pathotype							
AD	33	1.421 ± 0.461	*t* = 1.28	31	3.404 ± 0.431	*t* = 0.395	0.696
SC	17	1.220 ± 0.333			3.672 ± 0.425		
ADC	2	……					
OTHERS	2	……					
Clinical stage							
Ⅰ	11	1.329 ± 0.362	*F* = 0.138	0.871	3.312 ± 0.303	*F* = 0.334	0.718
Ⅱ	27	1.327 ± 0.500			3.318 ± 0.494		
ⅢⅠa	16	1.241 ± 0.433			3.611 ± 0.293		

in lung cancer tissues than in normal tissues, but that no significant difference was detected in adjacent noncancerous tissues or normal tissues. In immunofluorescence photos, lung cancer tissues exhibited the bulk of negative stain; RT-PCR also showed lower Beclin1 gene transcription in lung cancer. We found that there was no relationship between the Beclin1 expression and pathology or clinic stage of lung cancer. The association between cancer biology and Beclin1 needs further exploration. As a specific gene participate autophagy in mammals, Beclin1gene participates in tumorigenesis through autophagy modulation[2].

Autophagy was the major intracellular degradation/recycling system, ubiquitous in eukaryotic cells[7]. It

contributes to the turnover of cellular components (including long-lived protein and injured organelle) by delivering portions of the cytoplasm and organelles to lysosomes where they were digested[8]. Autophagy played a major role in maintaining homeosis and it was the major form of long-lived protein and organelle degradation[9]. ATG(AuTophaGy-related gene) participated in autophagy, (the mammalian homologue to ATG6 is Beclin1). Beclin1 regulates the autophagy activity by modulating the allocation of other ATG proteins in autophagosome,as a complex with PI3K (phosphatidylinositol 3-kinase)[10]. Upregulation of Beclin1 stimulated autophagy in mammalian cells[11].

Beclin1 gene was closely related to tumor:it's the homologue of Bcl-2, and regulates autophagy or apoptosis by interacting with Bcl-2[12]. In mice with Beclin1 knockout, the spontaneous malignancy rate was much higher(including breast tumor, lung cancer and liver cancer) and the mice were more susceptible to HBV-induced premalignant lesions. Other studies indicated there's increased malignancy incidence of epithelium tissue and hematopoietic system in Beclin1 defected mice[2,13]. The expression of Beclin1 protein was low in breast cancer while much higher in normal breast cells, human breast carcinoma cells MCF7 leads to the loss of malignant morphologic properties after beclin 1 gene is transfered, those changes including a decreased rate of cell proliferation[11]; impaired clonogenicity(in vitro); and reduced ability to form tumors in nude mice. All such findings implicated the tumor suppression effects of Beclin1 gene, and provided new avenues for tumor therapy. Arico S. found Beclin1 gene was closely associated with certain signal pathways, for example: activation of PTEN reduced Beclin1 expression[14]; Rb was perturbed in many tumors, one recent study demonstrated Beclin1 was the objective gene of E2F which was the important ingredient in Rb[15,16]. Beclin1 could also enhance the anti-tumor effect in co-operation with some drugs(for example:cisplatin). In Furuya D's study, up-regulation of Beclin1 could increase apoptosis rate induced by cisplatin in gastric cancer cell line MKN28, while apoptosis rate reduced when Beclin1 was inhibited[17].

Beclin1 is a new kind of anti-oncogene which is distinct from any orthodox concept, it works out only when expressed in both chromosome. Little data is known about the value of Beclin1 as an aid to early diagnosis and prevention of lung cancer, and it's effect on malignant cells of different period, and it's therapeutic efficacy in co-operation with other anti-tumor methods also need to be explored[18-20]. Further research about Beclin1 should provide evidence about the mechanism in the occurrence and advance of lung cancer, and help to develop new therapeutic methods.

References

[1] Aita VM, Liang XH, Murty VV, Pincus DL, Yu W, Cavanis E, *et al.* Cloning and genomic organization of *beclin 1*, a candidate tumor suppressor gene on chromosome 17q21. *Genomics* 1999;59:59-65.

[2] Yue Z,Jin S,Yang C,Levine AJ, Heintz N. Beclin1,an autophagy gene essential for early embryonic development,is a haploinsufficient tumor suppressor. *Proc Natl Acad Sci USA* 2003; 100: 15077-82

[3] Tangir J,Muto MG, Bercowitze RS,Welch WR, Bell DA, Mok SC. A 400 kb novel deletion unit centromeric to the BRCA1 gene in sporadic epithelial ovarian cancer. *Oncogene* 1996; 12: 735-40.

[4] Futreal PA ,Marks JR ,Marks JR, Iglehart JD, Zimmerman W, Barret JC. Detection of frequent allelic loss on proximal chromosome 17q in sporadic breast carcinoma using microsatellite length polymorphisms. *Cancer Res* 1992; 52: 2624.

[5] H Saito, J Inazawa, S Saito, Koi S, Kasumi F, Sagae S, *et al.* Detailed deletion mapping of chromosome 17q in ovarian and breast cancers: 2-cM region on 17q21.3 often and commonly deleted in tumors. *Cancer Res* 1993; 53: 3382-85

[6] Gao X, Zacharek A, Salkowski A, Grignon DJ, Sakr W, Porter AT, *et al.* Loss of heterozygosity of the BRCA1 and other loci on chromosome 17q in human prostate cancer. *Cancer Res* 1995; 55: 1002-05.

[7] Cuervo AM, Dice JF. A receptor for the selective uptake and degradation of proteins by lysosomes. *Science* 1996; 273: 501-03.

[8] Klionsky DJ , Emr SD. Autophagy as a regulated pathway of cellular degradation. *Science* 2000; 290: 1717-21.

[9] Takahiro Shintani , Daniel J. Klionsky. Autophagy in Health and Disease:A Double Edged Sword. *Science* 2004; 306: 990-95.

[10] Edinger A L,Thompson C B.Defective autophagy leads to cancer. *Cancer Cell* 2003; 4: 422-24.

[11] Liang X H, Jackson S, Seaman M, Brown K, Kempkes B, Hiboshoosh H, *et al.* Induction of autophagy and inhibition of tumorigenesis by Beclin1. *Nature* 1999; 402: 672-76

[12] Liang XH, Kleeman LK, Jiang HH, Gorden G, Goldman JE, Berry G,*et al.* Protection against fetal Sindbis virus encephalitis by Beclin, a novel Bcl-2 interacting protein. *J Virol* 1998; 72: 8586-96.

[13] Qu X, Yu J,Bhagat G,Furuva N, Hiboshoosh H, Troxel A, *et al.* Promotion of tumorgenesis by heterozygous disruption of the beclin1 autopahgy gene. *J Clin Inves* 2003; 112: 1809-20.

[14] Arico S, Petiot A, Bauvy C, Dubbelhuis PF, Meijer AJ, Codogno P, *et al.* The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. *J Biol Chem* 2001; 276: 35243-46.

[15] Weinmann AS, Bartley SM, Zhang T, Zhang MQ, Farnham PJ. Use of Chromatin Immunoprecipitation To Clone Novel E2F Target Promoters. *Mol Cell Biol* 2001; 21: 6820-32.

[16] Nevins JR. The Rb/E2F pathway and cancer. *Hum Mol Genet* 2001; 10: 699-703.

[17] Furuya D,Tsuji N,Yagihashi A, Watanbe N.Beclin 1 augmented cis-diamminedichloroplatinum induced apoptosis via enhancing caspase-9 activity. *Exp Cell Res* 2005; 307: 26-40.

[18] Klionsky D J . Autophagy. *Curr Biol* 2005; 15: 282-83.

[19] Marino G, Lopez-Otin C. Autophagy: molecular mechanisms, physiological functions and relevance in human pathology. *Cell Mol Life Sci* 2004; 61: 1439-54.

[20] Eskelinen E L. Maturation of autophagic vacuoles in mammalian cells. *Autophagy* 2005; 1: 1-10