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Expression of autophagy-related protein LC3B, p62, and cytoplasmic p53 in human retinoblastoma tissues

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Abstract. – OBJECTIVE: Dysfunction of autophagy have been implicated in development and progression of diverse human cancers. However, the exact role and mechanism of autophagy has not been fully understood in human cancers, especially in retinoblastoma (Rb).

PATIENTS AND METHODS: We determined the autophagy activity in human Rb tissues by assessing the autophagy markers microtubule-associated protein light chain 3B (LC3) and p62 (SQSTM1) in formalin fixed and paraffin embedded human tissue by immunohistochemistry and then associated their expression with patient clinicopathological features. We further explored the correlation between the expression of LC3B and p62 and the expression of cytoplasmic p53, a newly identified autophagy suppressor, in Rb tissues.

RESULTS: Our data revealed that the expression of LC3B and p62, was significantly associated with disease progression and tumor invasion of Rb. Furthermore, we also revealed that cytoplasmic expression of p53 was inversely associated with the behavior of tumor invasion. Finally, Spearman correlation analysis demonstrated that cytoplasmic expression of p53 was significantly and inversely correlated to the expression of both LC3B and p62.

CONCLUSIONS: Autophagy might play an important role in human Rb progression, and LC3B and p62 may be useful predictors of disease progression in patients with Rb.

Key Words: Autophagy, LC3B, p62, p53, Retinoblastoma.

Introduction

Retinoblastoma (Rb) is a common primary intraocular malignant tumor of infancy and childhood, especially in children under the age of 5 years[1]. In recent years, the incidence of Rb is increasing which has been estimated between 1:30000 and 1:15000 live births every year in the world[2]. Rb can result in a serious damage to the vision and eyes of children patients, even a life endangerment in advanced stages[3]. In the early stages, enucleation is a good way for the treatment of Rb patients with a cure rate approach 95%[4-6]. For advanced diseases, radiotherapy and chemotherapy are required in preserving treatment or in addition to enucleation. Chemotherapy can reduce tumor volume and increase the efficacy of local therapies. Moreover, chemotherapy has been used effectively in prophylaxis of Rb patients with high-grade pathological features and in treatment of extraocular and metastatic tumors. However, drug resistance and relapses are major problems to chemotherapy for Rb patients. A variety of factors with different mechanisms contribute to the chemoresistance, including host factors, specific genetic or epigenetic alterations in the tumor cells and so on.

Autophagy is an evolutionarily conserved eukaryotic process for maintaining homeostasis by degrading their unnecessary cellular components and utilizing its breakdown components or removing impaired or accumulated cellular organelles and molecules, which enables cells to survive in less favorable situations such as starvation[7]. Autophagy plays important roles not only in physiological processes, including adaptation to hypoxia and antigen presentation, but also in a number of pathological processes, including tumor initiation and progression[8,9]. However, the exact role of autophagy in regulating human tumor cell death or survival remains controversial. On one side, some reports demonstrated that autophagy can act as a

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tumor suppressor to eliminate damaged organelles and recycle macromolecules, thus protecting against human normal cell malignant transformation and carcinogenesis. On the other side, recent studies also revealed that autophagy can function as a prosurvival pathway to protect tumor cells in response to metabolic stresses such as nutrient deprivation, hypoxia and absence of growth factors, especially in the presence of chemotherapy or some targeted therapies, which suggested that autophagy plays a role of oncogene[10-12]. To date, the role and functions of autophagy in human Rb development and progression is not clear. In this study, we investigated the clinical significance of autophagy activity in human Rb by assessing the autophagy markers microtubule-associated protein light chain 3 (LC3B) and p62 (SQSTM1) in formalin fixed and paraffin embedded human RB tissues by immunohistochemistry. We further explored the correlation between LC3B and p62 and the expression of cytoplasmic p53, a newly identified autophagy suppressor, in Rb tissues.

Patients and Methods

Patients and Specimens

41 cases of tissue samples were collected from 41 children with Rb (23 males and 18 females; aged from 1 year to 8 years) who underwent primary enucleation in the Anhui Provincial Hospital and the First affiliated Hospital of Anhui Medical University (Hefei, China) between 2003 and 2010. In these patients, 32 had unilateral tumor and 9 had bilateral tumors. None of the patients was treated with chemotherapy or radiotherapy before surgical resection. Hematoxylin and eosin (H&E) stained slides were reviewed for necrosis, calcification and tumor differentiation. Behavior of tumor invasion was assessed on the basis of histopathological high-risk factors including invasion of choroid, anterior chamber, sclera, iris and ciliary body, and optic nerve[13]. The patients were grouped according to the International Intraocular Retinoblastoma Classification[14]. The Institutional Ethics Committee's approval for the project was granted and was in compliance with the Helsinki Declaration.

Immunohistochemical Analysis

Immunohistochemical stain of LC3B, p62 and p53 in tissue sections (3 μm) was performed by using peroxidase-conjugated streptavidin complex method with monoclonal antibodies against LC3B (1:100, Sigma-Aldrich, St. Louis, MO, USA), p62 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and p53 (1:100, Santa Cruz Biotechnology) as reported previously[15]. In brief, tissue sections were firstly deparaffinized in xylene, rehydrated in a graded series of ethanol solutions and then heated in a microwave oven in 0.01 M sodium citrate buffer (pH 6.0) for 10 min for antigen retrieval. After that, the tissue sections were immersed in 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity. After being incubated in 10% non-immune goat serum for 10 min, the tissue sections were then incubated with certain primary antibody in moist chamber for 1 hr at ambient temperature. After being washed in phosphate-buffered saline (PBS) for 3 times, the tissue sections were incubated with biotinylated rabbit anti-mouse immunoglobulin G for 15 min and then incubated with peroxidase-conjugated streptavidin for 15 min. To visualize the positive signals, the sections were incubated with 3, 3′-diaminobenzidine (DAB) solution for 2-5 min and then counterstained with hematoxylin solution, dehydrated, and mounted. All experiments included separately known positive and negative controls. For the negative control, the primary antibody was just replaced by PBS only.

Scoring of Stained Sections

The stained slides were reviewed and scored under an Olympus microscope (Olympus America Inc., Melville, NY, USA) independently by two experienced pathologists who had no knowledge of the patients' identities or clinical status. LC3B and p62 stains were classified in two tier grading system, low expression and high expression, as in previous studies[16,17]. The percentage of cytoplasm p53 stained cells were calculated in three 100× fields. When the mean of the percentage was 25% or more, the case was graded as p53 high.

Statistical Analysis

All statistical analyses were performed using SPSS software system for Windows (version 13.0; SPSS, Chicago, IL, USA). Pearson's X-square test was used to test statistical significance of association between clinicopathological parameters and the expression of LC3B and p62. Spearman's coefficient was calculated to determine the association between the expression levels of LC3B, p62 and p53. p values < 0.05 were considered statistically significant.

Results

Expression of LC3B and p62 in Rb Tissues

LC3B and p62 immunostains showed diffuse cytoplasmic stain on Rb tissues. The expression of LC3B and p62 revealed a variegated pattern in tumor cells of Rb with a diffuse, radiating or scattered granular pattern from the center of florets and rosettes (Figure 1). In general, 23 out of 41 (56.1%) Rb were high expressed for LC3B protein and 26 out of 41 (63.4%) were high expressed for p62 protein.

To investigate the clinical significance of LC3B and p62 expression in Rb, we further correlated their expression to the clinicopathological parameters in patients with Rb. As shown in Table I, expression of LC3B was significantly associated with the status of high risk of Group E ($p = 0.035$), late TNM stage ($p = 0.001$) and tumor optic nerve invasion ($p = 0.001$), while no significant association was observed between LC3B expression and patient age, gender, tumor laterality, tumor differentiation, necrosis, calcification, tumor choroidal invasion, anterior chamber invasion, sclera invasion or iris and ciliary body invasion (all $p > 0.05$). Similarly, expression of the other autophagy marker, p62, was significantly associated with late tumor TNM stage ($p = 0.009$) and tumor optic nerve invasion ($p = 0.033$). However, no significant association was observed between p62 expression and any other clinicopathological parameters (all $p > 0.05$).

Expression of Cytoplasmic p53 in Rb Tissues

Positive signal of p53 protein was located in the nuclear and/or cytoplasm of Rb tumor cells (Figure 2). Since only cytoplasmic p53 was demonstrated to be involved in the regulation of cell autophagy[18,19], we just took the expression of cytoplasmic p53 into account in the current study. In general, 18 out of 41 (43.9%) Rb cases had a positive expression of cytoplasmic p53. Moreover, as Table II shown, low expression of cytoplasmic p53 was significantly associated with tumor calcification ($p = 0.001$) and tumor optic nerve invasion ($p = 0.001$). However, no significant association was observed between p53 cytoplasmic expression and any other clinicopathological parameters (all $p > 0.05$).

Correlation between the expression of LC3B, p62 and cytoplasmic p53

To investigate the relationship among LC3B, p62 and cytoplasmic p53 in Rb, we performed Pearson correlation analysis to explore the corre-

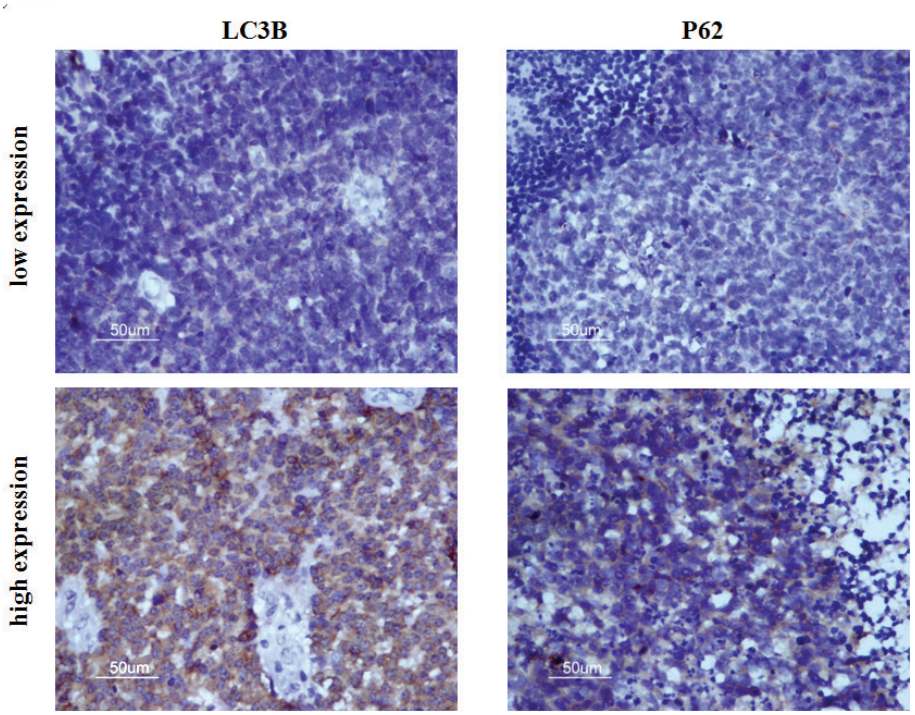

Figure 1. LC3B and p62 expression in retinoblastoma tissues. Low expression of LC3B and p62 was present in retinoblastoma tissues (top). High expression of LC3B and p62 was present in retinoblastoma tissues (bottom). Magnification: x200.

Table I. Association of LC3B and p63 expression with clinicopathological parameters from retinoblastoma patients.

		LC3B expression (n (%))		P63 expression (n (%))	
Parameter	**n**	**high**	***p***	**high**	***p***
Age (years)					
≤ 5	33	19 (57.6)	0.698	20 (60.6)	0.448
> 5	8	4 (50.0)		6 (75.0)	
Gender					
Male	23	15 (65.2)	0.183	15 (65.2)	0.786
Female	18	8 (44.4)		11 (61.1)	
Laterality					
Unilateral	32	18 (56.3)	0.970	18 (56.3)	0.073
Bilateral	9	5 (55.6)		8 (88.9)	
Grouping					
Group E	35	22 (62.9)	0.035	23 (65.7)	0.460
Group D-A	6	1 (16.7)		3 (50.0)	
Staging					
$T2aN_0M_0$- $T4bN_0M_0$	28	11 (39.3)	0.001	14 (50.0)	0.009
$T1N_0M_0$	13	12 (92.3)		12 (92.3)	
Differentiation					
Poor	27	14 (51.9)	0.447	16 (59.3)	0.443
Well	14	9 (64.3)		10 (71.4)	
Necrosis					
Yes	21	11 (52.4)	0.623	11 (52.4)	0.133
No	20	12 (60.0)		15 (75.0)	
Calcification					
Yes	17	7 (41.2)	0.105	9 (52.9)	0.241
No	24	16 (66.7)		17 (70.8)	
Choroidal invasion					
Massive	20	11 (55.5)	0.890	12 (60.0)	0.658
Focal	21	12 (57.1)		14 (66.7)	
Anterior chamber invasion					
Yes	5	2 (40.0)	0.439	3 (60.0)	0.866
No	36	21 (58.3)		23 (63.9)	
Scleral invasion					
Yes	6	2 (33.3)	0.224	4 (66.7)	0.858
No	35	21 (60.0)		22 (62.9)	
Iris and ciliary body invasion					
Yes	7	6 (85.7)	0.083	6 (85.7)	0.179
No	34	17 (50.0)		20 (58.8)	
Optic nerve invasion					
Yes	14	13 (92.9)	0.001	12 (85.7)	0.033
No	27	10 (37.0)		14 (51.9)	

lation of their expression. As expected, the cell autophagy markers, LC3B and p62 was positively and significantly correlated to each other ($p = 0.003$) and the Pearson's correlation coefficient for them was 0.450. Of note, cytoplasmic expression of p53 was inversely and significantly correlated to both LC3B ($p = 0.003$, $r_s = -0.652$) and p62 ($p = 0.012$, $r_s = -0.389$).

Discussion

The principal tumor suppressor p53, a nuclear transcriptional regulatory protein, is involved in DNA damage, cell cycle, apoptosis and oncogene activation[20]. Basically, the expression of p53 protein in normal cells is low so that it can be hardly detected by immunohistochemistry[21]. On the other hand, p53 can increase protein quantities and subsequently immunoreactivity, which could be detectable when the wildtype TP53 gene for the p53 protein become mutated during cancer development to display non-sense point mutations[22,23]. Since the accumulation of different mutated tumor suppressors and oncogenes is the main cause for cancer development, inactivation of p53 play an important role in human tumorigenesis by many mechanism. An emerging area of

research unravels the additional activities and functions of p53 in the cytoplasm, where p53 can induce cell apoptosis and inhibit autophagy in human cancers[18].

Herein we firstly demonstrated that high level of tumor autophagy activity, labeled by LC3B and p62, was significantly associated with disease progression and tumor invasion of Rb. Furthermore, we also revealed that cytoplasmic expression of p53, an inhibitor of cell autophagy, was inversely associated with the behavior of tumor invasion. Finally, Spearman correlation analysis confirmed that cytoplasmic expression of p53 was significantly and inversely correlated to LC3B and p62. In brief, these results suggested that increased capacity of tumor cell autophagy played an important role in the progression of human Rb and cytoplasmic p53, which plays anti-autophagic role, might be involved in that process.

Several studies[24-26] suggest that dysfunction of cell autophagic acitivity plays an important role in various human diseases, including neurodegenerative diseases, ageing, and cancer. However, the exact role of autophagy in tumor development and progression is not fully understood, even be controversial. Some researches demonstrated that autophagy plays an oncogene role in

Table II. Association of cytoplasmic expression of p53 with clinicopathological parameters from retinoblastoma patients.

		p53 expression (n (%))		
Parameter	**n**	**low**	**high**	***p***
Age (years)				
≤ 5	33	20 (60.6)	13 (39.4)	0.585
> 5	8	4 (50.0)	4 (50.0)	
Gender				
Male	23	13 (56.5)	10 (43.5)	0.767
Female	18	11 (61.1)	7 (38.9)	
Laterality				
Unilateral	32	18 (56.3)	14 (43.8)	0.575
Bilateral	9	6 (66.7)	3 (33.3)	
Grouping				
Group E	35	20 (57.1)	15 (42.9)	
Group D-A	6	4 (66.7)	2 (33.3)	0.662
Staging				
$T2aN_0M_0$- $T4bN_0M_0$	28	14 (50.0)	14 (50.0)	0.103
$T1N_0M_0$	13	10 (76.9)	3 (23.1)	
Differentiation				
Poor	27	15 (55.6)	12 (44.4)	0.591
Well	14	9 (64.3)	5 (35.7)	
Necrosis				
Yes	21	13 (61.9)	8 (38.1)	0.654
No	20	11 (55.0)	9 (45.0)	
Calcification				
Yes	17	5 (29.4)	12 (70.6)	0.001
No	24	19 (79.2)	5 (20.8)	
Choroidal invasion				
Massive	20	10 (50.0)	10 (50.0)	0.279
Focal	21	14 (66.7)	7 (33.3)	
Anterior chamber invasion				
Yes	5	2 (40.0)	3 (60.0)	0.369
No	36	22 (61.1)	14 (38.9)	
Scleral invasion				
Yes	6	4 (66.7)	2 (33.3)	0.662
No	35	20 (57.1)	15 (42.9)	
Iris and ciliary body invasion				
Yes	7	6 (85.7)	1 (14.3)	0.109
No	34	18 (52.9)	16 (47.1)	
Optic nerve invasion				
Yes	14	13 (92.9)	1 (7.1)	0.001
No	27	11 (40.7)	16 (59.3)	

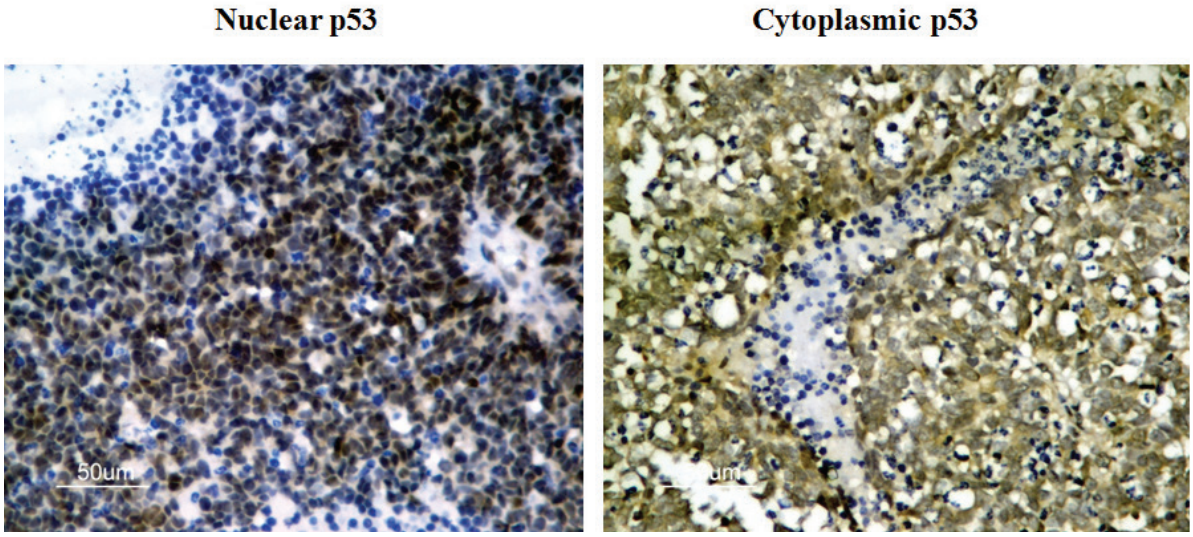

Figure 2. P53 expression in retinoblastoma tissues. High expression of p53 was located in the nucleus of tumor cells in retinoblastoma tissues (left). High expression of p53 was located in the cytoplasm of tumor cells in retinoblastoma (right). Magnification: x200.

diverse cancers for its pro-survival capacity of cancer cells[27-29]. This hypothesis is that autophagy can help cancer cells survive and proliferate under stressful conditions such as insufficient energy and resources[30]. On the contrary, other studies revealed that autophagy plays a tumor suppressor role in cancer initiation. For example, allelic loss of the essential autophagy gene, Beclin-1, was significantly associated with high frequency of diverse human malignant tumors including ovarian, breast, and prostate cancers[9,31]. Furthermore, Beclin-1 heterozygous mice which have impaired capacity of autophagy were more predisposed to the development of spontaneous cancers, such as lymphoma, lung cancer, hepatocellular cancer and breast cancer[32,33]. Therefore, more and more researcher accepted the hypothesis that autophagy might be a double-edged sword in cancer, because they may act as a tumor suppressor in cancer development and as a promoter in cancer progression and treatment[34]. To investigate the role of autophagy in Rb progression, we performed immunohistochemistry to detect the expression of LC3B and p62, two biomarkers of autophagy level, in human Rb tissues. The process of cell autophagy can be regulated by many autophagy-related genes (ATGs). The ATG12-ATG5-ATG16 and the ATG8 conjugation systems, two ubiquitin-like conjugation systems, are essential for autophagosome formation and cargo recruitment[35]. The ATG8 conjugation system is mediating the lipidation of ATG8 family members including the human homologoues LC3A, LC3B, LC3C and GABARAPs[35]. LC3B is one of the most widely used markers to monitor autophagy[36]. The other one of the best-studied autophagy regulators is p62, also named sequestosome1 (SQSTM1). P62 participates in autophagy-dependent elimination of many different cargos, such as ubiqutinated protein aggregates and bacteria[37]. Due to interact with LC3, p62 is constantly degraded via autophagy and autophagy inhibition results in the accumulation of p62 positive aggregates[37]. Therefore, monitoring of p62 degradation is used to measure autophagic flux under different conditions[36]. Detection of LC3B and p62 to monitor the level of autophagy can be performed by many techniques, such as western blotting and GFP-LC3 fluorescence microscopy, and so on[36]. Of note, a recent study by Schläfli et al[38] demonstrated that immunohistochemistry staining of LC3B and p62 on formalin fixed and paraffin embedded human tissues is specific and reliable to measure the level of cell autophagy. To this end, we employed immunohistochemistry to detect the expression of LC3B and p62 protein in 41 cases of human Rb tissues to evaluate the level of autophagy and found that more than half of the cases showed high expression of these two autophagy markers. Furthermore, high expression of LC3B and p62 was significantly associated with disease progression and tumor invasion of Rb, suggesting autophagy might play a pro-

tection role for tumor cells in Rb progression. Wu et al[39] detected the expression of LC3B in 150 human hepatocellar cancers and found high expression of LC3B was significantly associated with large tumor size, advanced stages, worse relapse free and overall survival rates. Similarly, Liu et al[17] discovered that expression of LC3B and p62 was increased in oral squamous cell carcinoma, compared with the normal oral mucosa, and high level of LC3B and p62 expression in oral squamous cell carcinoma was associated with aggressive clinicopathologic features and unfavorable prognosis. Furthermore, Burdelski et al[40] analyzed the p62 protein levels by immunohistochemistry on a tissue microarray containing 12,427 prostate cancers and found that strong staining of p62 was significantly linked to high Gleason grade, advanced pathologic tumor stage, positive nodal status, positive resection margin, and early PSA recurrence. These results are in line with ours, suggesting autophagy plays an important role in cancer progression and expression of LC3B and p62 might be served as biomarkers for monitoring disease progression and patient survival.

To determine whether p53 is involved in the autophagy process of Rb, we employed the immunohistochemistry to detect the cytoplasmic expression of p53. We found that 43.9% Rb tissues had cytoplasmic expression of p53 and low expression of cytoplasmic p53 was significantly associated with tumor optic nerve invasion which is just contrast to the clinical significance of LC3B and p62. Moreover, Spearman correlation analysis confirmed that cytoplasmic expression of p53 was significantly and inversely correlated to the expression of both LC3B and p62 in Rb, suggesting cytoplasmic expression of p53 plays an anti-autophagy role in Rb. X-linked inhibitor of apoptosis (XIAP) is an important member of inhibitors of apoptosis (IAP) family and has been well characterized to have the most potent antiapoptotic ability by directly binding to caspases[41,42]. Huang et al[43] revealed that XIAP could suppress autophagy via MDM2-p53 (cytoplasmic) pathway in diverse tumor cells. An et al[44] demonstrated that mimulone induced autophagy of human lung adenocarcinoma cells by decreasing the levels of p53 and p-mTOR and increasing of p-AMPK, and inhibition of p53 transactivation by pifithrin-α and knockdown of p53 enhanced induction of autophagy. These reports imply that p53-regulated autophagy plays an important role in human cancer progression.

Conclusions

We evaluated the level of autophagy by detecting the autophagy markers of LC3B and p62 and explored their clinical significance. Moreover, we investigated the expression of cytoplasmic p53 by immunohistochemistry and correlated to the level of autophagy. Our study, for the first time, revealed that expression of LC3B and p62 was significantly associated with the disease progression and was also significantly correlated to cytoplasmic p53, suggesting autophagy might play an important role in Rb progression. Further investigations are necessary to validate and explore the mechanisms of autophagy-related genes involved in Rb.

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Conflicts of interest

The authors declare no conflicts of interest.

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