Int J Clin Exp Pathol 2013;6(10):2145-2156
www.ijcep.com /ISSN:1936-2625/IJCEP1308029

Original Article

Expression of autophagy-related proteins in phyllodes tumor

Sang Kyum Kim, Woo Hee Jung, Ja Seung Koo

Department of Pathology, Yonsei University College of Medicine, Severance Hospital, Seoul, South Korea

Received August 13, 2013; Accepted September 5, 2013; Epub September 15, 2013; Published October 1, 2013

Abstract: Phyllodes tumors (PTs) are classified as fibroepithelial tumors and their histologic grade is determined primarily by the features of the stromal component. In this study, we examined the expression profiles of autophagy-related proteins in the stromal component of PTs and analyzed their clinical implications. We selected 204 human PT samples which were excised and diagnosed at Severance Hospital from 2000 to 2008 and created tissue microarray (TMA) blocks. Immunohistochemical assays for autophagy-related proteins (beclin-1, LC3A, LC3B, and p62) were then performed on these samples. The surgical specimens from higher grade PTs less frequently displayed cytoplasmic expression of beclin-1, LC3A, LC3B, and p62 in the stromal component ($p<0.001$). In univariate analysis, the following profiles were associated with shorter disease-free survival and overall survival: nuclear beclin-1 positivity in the stromal component ($p=0.013$ and $p=0.044$, respectively), LC3A positivity in the stromal component ($p<0.001$ and $p<0.001$, respectively), and p62 positivity in the stromal component ($p=0.012$ and $p=0.004$, respectively). In conclusion, we determined that increased activity of autophagy-related proteins correlated with a higher histologic grade and poorer prognosis in PTs. These results lead us to conclude that the autophagy activity of the stromal cells plays a key role in the progression of PTs.

Keywords: Breast, phyllodes tumor, autophagy

Introduction

Phyllodes tumor (PT) is a relatively uncommon fibroepithelial tumor, comprising only 0.3-1.5% of all breast tumors [1]. However, it is hard to distinguish PT from other fibroepithelial tumors because of its heterogeneous histologic features [1, 2]. Although PT contains both epithelial and stromal components which could be neoplastic, on histological grading, it is classified primarily by the features of the stromal components as follows: cytologic atypia of stromal cells, stromal hypercellularity and overgrowth, sarcomatous change, and mitotic activity [3, 4]. Clinically, high-grade PTs can present with malignant behaviors such as local recurrence or distant metastasis. Therefore, it is necessary to discover reliable markers for the malignant features of the stromal component to accurately predict tumor progression.

Autophagy is defined as a catabolic pathway of lysosomal degradation of the cellular components. Among the three types of autophagy, macroautophagy particularly involves the stress-response pathway to maintain cellular homeostasis by removal of dysfunctional or damaged cellular components, as well as by recycling useful cellular components [5-9]. In this study, autophagy is referred to as macroautophagy to explain the cellular process within the cancer cells.

Cancer cells thrive in harsh environments, such as hypoxic or low nutrient states, surviving through angiogenesis and/or aerobic glycolysis. However, in the case of aggressive malignant tumors, it is hard for cancer cells to meet the high metabolic demand so that they cannot fully recover using the classical pathways. Autophagy as an alternative metabolic pathway conserves energy within the cancer cells by recycling cytoplasmic components [10, 11]. In contrast, unrestrained autophagy could induce progressive consumption of cellular constituents and ultimately lead to cell death [12, 13]. Interestingly, autophagy has a profound effect on both tumor suppression and tumor progres-

sion. However, not much was known about the expression profiles of autophagy-related proteins in PTs until recently.

In this study, we explored the relevance of autophagy-related protein expression patterns and histologic grade in human PTs. On the basis of this observation, we evaluated the ability of autophagy-related proteins to predict prognosis.

Methods and materials

Patient selection and clinicopathologic analysis

This retrospective study was approved by the institutional review board of Yonsei University Severance Hospital. Our inclusion criteria defined a study population of 204 patients who had been histologically diagnosed with PT after having tumors excised at Yonsei University Severance Hospital from 2000 to 2008. All tissues were fixed in 10% buffered formalin and embedded in paraffin. All archival hematoxylin and eosin (H&E)–stained slides were reviewed by three pathologists and histologic grading was performed based on the criteria of the WHO Blue Book [1]. Histologic parameters such as stromal cellularity (mild, moderate, and severe), stromal atypia (mild, moderate, and severe), stromal mitosis (10 HPFs), stromal overgrowth, and tumor margin (expanding or infiltrative) were evaluated on H&E–stained slides. Included clinical parameters were patient age at initial diagnosis, sex, tumor recurrence, and tumor metastasis.

Tissue microarray

We selected formalin-fixed paraffin-embedded (FFPE) tumor tissue samples after retrospective review of H&E–stained slides of human PTs. The most representative areas of each tumor sample were assembled in a 5x4 array after extraction of tumor cores as small as 5 mm in diameter. We attempted to include all of the epithelial and stromal components of PTs in the recipient blocks. Each PT sample had two tissue cores in TMA and each separate tissue core was assigned a unique tissue microarray location number that was linked to a database including other clinicopathologic data.

Immunohistochemistry

All immunostainings were performed using FFPE tissue sections. Five μm-thick sections were obtained with a microtome, transferred onto adhesive slides, and dried at 62°C for 30 minutes. After incubation with primary antibodies for beclin-1 (polyclonal, 1:100, Abcam, Cambridge, UK), LC3A (EP1528Y, 1:100, Abcam, Cambridge, UK), LC3B (polyclonal, 1:100, Abcam, Cambridge, UK), and p62 (SQSTM1, 1:100, Abcam, Cambridge, UK), immunodetection was performed with biotinylated anti-mouse immunoglobulin, followed by peroxidase-labeled streptavidin using a labeled streptavidin biotin kit with 3,3′-diaminobenzidine chromogen as substrate. Optimal primary antibody incubation time and concentration were determined via serial dilution for each immunohistochemical assay with an identically fixed and embedded tissue block. The primary antibody incubation step was omitted in the negative control. A positive control was included for each experiment; Beclin-1: normal breast tissue, LC3A and LC3B: brain tissue, and p62: spleen tissue. Slides were counterstained with Harris hematoxylin. The staining was interpreted by two pathologists on a multiview microscope.

For measurement of immunostaining intensity, we divided PTs in four groups as follows: 0 (negative), 1 (weakly positive), 2 (moderately positive), and 3 (strongly positive). For measurement of proportion of stained cells, we divided PTs in three groups as follows: 1 (negative), 2 (positive less than 30%), and 3 (positive more than 30%). Our indication standard for estimation of immunohistochemical results for beclin-1, LC3A, LC3B, and p62 was immunostaining intensity multiplied by the proportion of stained cells. The total score after multiplication was divided as follows: 0 to 1 as negative and 2 to 9 as positive [14].

Statistical analysis

Data were processed using SPSS for Windows, version 12.0 (SPSS Inc., Chicago, IL, USA). Student's *t* and Fisher's exact tests were used to examine any difference in continuous and categorical variables, respectively. When analyzing data with multiple comparisons, a corrected *p*-value with application of Bonferroni multiple comparison procedure was used. Significance was assumed when $P<0.05$. Kaplan-Meier survival curves and log-rank statistics were employed to evaluate time to tumor metastasis and time to survival. Multivariate

Table 1. Clinicopathologic characteristics of patients with phyllodes tumor

Parameter	Number of Patients N=204 (%)	Phyllodes tumor (%)			*P*-value
		Benign N=156	Borderline N=32	Malignant N=16	
Age (years, mean±SD)	40.2±12.3	38.9±12.2	43.1±11.0	47.6±12.9	0.009
Tumor size (Cm, mean±SD)	4.0±2.6	3.6±2.1	4.2±2.5	6.7±4.6	<0.001
Stromal cellularity					<0.001
Mild	123 (60.3)	122 (78.2)	1 (3.1)	0 (0.0)	
Moderate	68 (33.3)	34 (21.8)	27 (84.4)	7 (43.8)	
Marked	13 (6.4)	0 (0.0)	4 (12.5)	9 (56.3)	
Stromal atypia					<0.001
Mild	161 (78.9)	154 (98.7)	7 (21.9)	0 (0.0)	
Moderate	33 (16.2)	2 (1.3)	23 (71.9)	8 (50.0)	
Marked	10 (4.9)	0 (0.0)	2 (6.3)	8 (50.0)	
Stromal mitosis					<0.001
0–4 / 10 HPFs	159 (77.9)	156 (100.0)	3 (9.4)	0 (0.0)	
5–9 / 10 HPFs	34 (16.7)	0 (0.0)	29 (90.6)	5 (31.3)	
≥10 / 10 HPFs	11 (5.4)	0 (0.0)	0 (0.0)	11 (68.8)	
Stromal overgrowth					<0.001
Absent	187 (91.7)	156 (100.0)	29 (90.6)	2 (12.5)	
Present	17 (8.3)	0 (0.0)	3 (9.4)	14 (87.5)	
Tumor margin					<0.001
Circumscribed	183 (89.7)	153 (98.1)	24 (75.0)	6 (37.5)	
Infiltrative	21 (10.3)	3 (1.9)	8 (25.0)	10 (62.5)	
Tumor local recurrence	18 (8.8)	5 (3.2)	6 (18.8)	7 (43.8)	<0.001
Distance metastasis	8 (3.9)	0 (0.0)	1 (3.1)	7 (43.8)	<0.001

SD, standard deviation; HPFs, high-power fields.

regression analysis was performed using the Cox proportional hazards model.

Results

Expression of autophagy-related proteins according to the histologic grade of PT

In **Table 1**, we compared the histologic grade of 204 PTs to the other parameters, and found that a higher histologic grade was associated with increased stromal cellularity, atypia, mitosis, overgrowth, infiltrative tumor margin, and frequent tumor recurrence and metastasis ($p<0.001$). To examine autophagy activity in the PTs, we performed immunohistochemistry of autophagy-related proteins (**Figure 1**). Proteins used to assess autophagy activities were as follows: beclin-1 as a participant in nucleation [15-18], LC3 as a participant in autophagosome formation [19-21], and p62 as a scaffold protein that conveys ubiquitinated proteins to the autophagosome [22, 23]. **Figure 2** is a representative heatmap of the expression of autophagy-related proteins according to the histologic grade of PTs. On immunohistochemical analysis, a higher histologic grade correlated with increased expression of autophagy-related proteins in the stromal component ($p<0.001$), although the epithelial component had nothing distinctive about it (**Tables 2**-**4**). We assumed that the autophagy activity in the stromal component had a major impact on tumor progression in PTs.

When looking at the effect of autophagy activity on the progression of PT, we examined the correlation between expressions of autophagy-related proteins with clinicopathologic parameters (**Tables 2**-**4**). As a result, expression of beclin-1, LC3A, LC3B, and p62 in the stromal cells, but not in the epithelial cells, was associated with increased stromal cellularity, increased stromal atypia, increased stromal mitosis, and stromal overgrowth ($p<0.05$). Particularly, LC3A expression of the stromal component

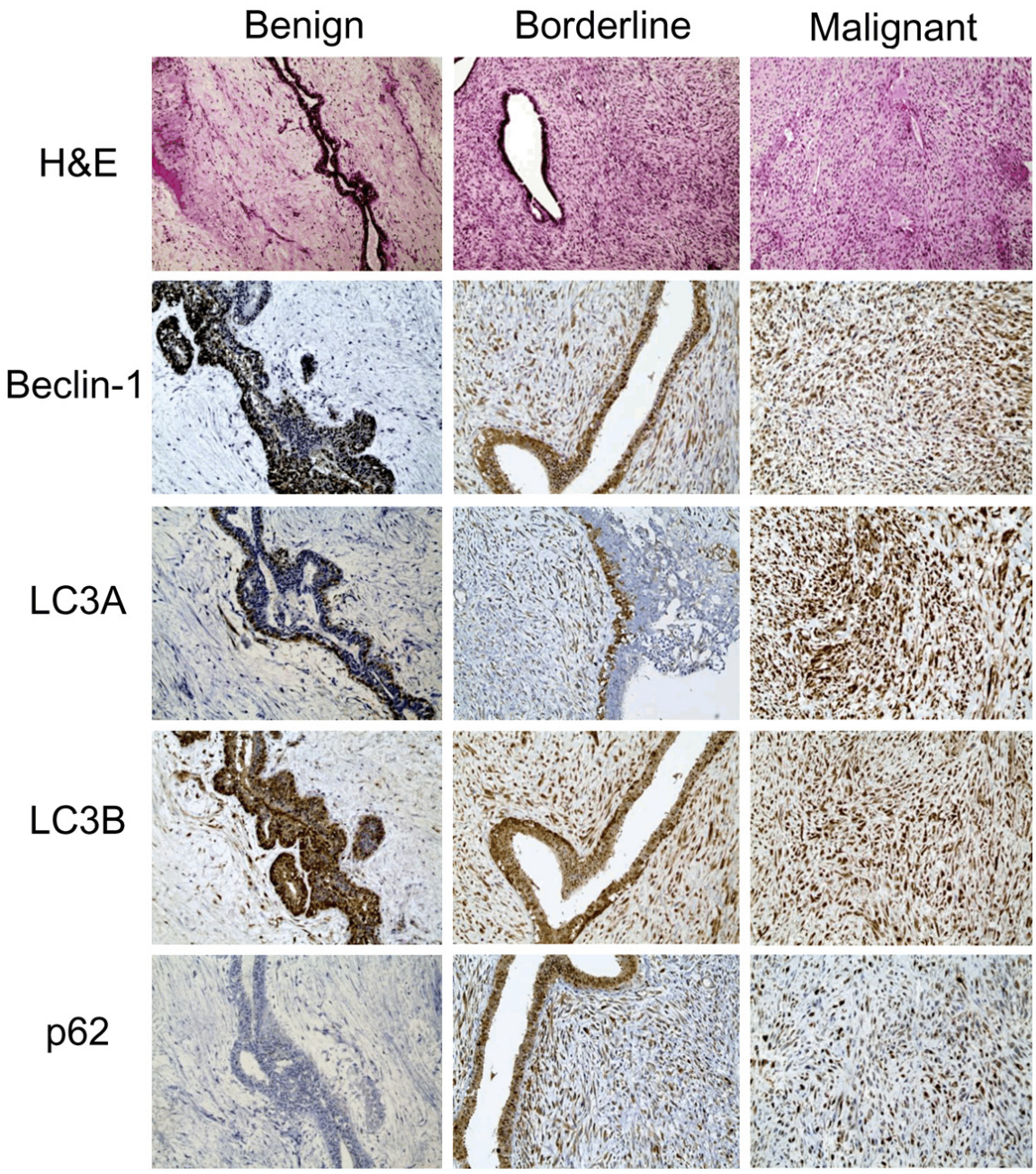

Figure 1. Immunohistochemical stains of autophagy-related proteins in phyllodes tumors. Expression of cytoplasmic beclin-1, LC3A, LC3B, and p62 in the stromal component increases as the histologic grade of phyllodes tumor increases.

was significantly associated with tumor recurrence and distant metastasis (p=0.010, and <0.001, respectively).

Impact of autophagy-related proteins on patient prognosis

Based on the above observation, we investigated the effect of each autophagy-related protein on the prognosis of the PT patients (**Figure 3**). We performed univariate analysis (**Table 5**) and found that factors associated with shorter disease-free survival and shorter overall survival were nuclear beclin-1 positivity in the stromal component (p=0.013, and p=0.044, respectively), LC3A positivity in the stromal component (p<0.001, and p<0.001, respectively), and p62 positivity in the stromal component

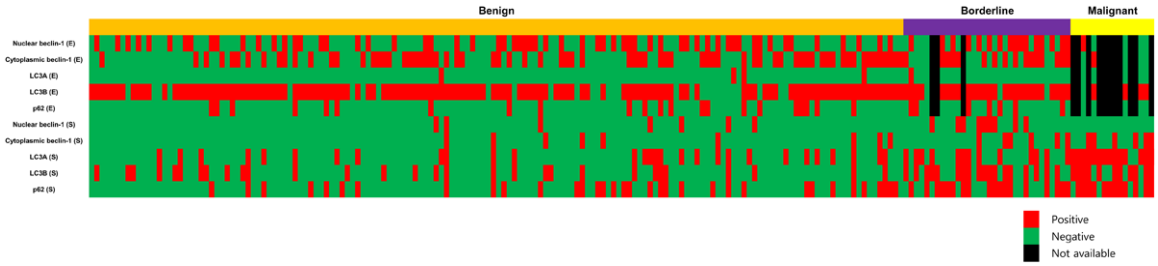

Figure 2. A heatmap of the expression of autophagy-related proteins according to the histologic grade of phyllodes tumor.

Table 2. Expression of autophagy-related proteins according to the histologic grade of phyllodes tumor

Parameter	Number of Patients N=204 (%)	Phyllodes tumor (%)			*P*-value
		Benign N=156	Borderline N=32	Malignant N=16	
Nuclear beclin-1 (E)*					0.232
Negative	111 (58.4)	95 (60.9)	13 (44.8)	3 (60.0)	
Positive	79 (41.6)	61 (39.1)	16 (55.2)	2 (40.0)	
Cytoplasmic beclin-1 (E)*					0.933
Negative	101 (53.2)	84 (53.8)	13 (44.8)	4 (80.0)	
Positive	89 (46.8)	72 (46.2)	16 (55.2)	1 (20.0)	
Nuclear beclin-1 (S)					0.090
Negative	188 (92.2)	149 (95.5)	23 (71.9)	16 (100.0)	
Positive	16 (7.8)	7 (4.5)	9 (28.1)	0 (0.0)	
Cytoplasmic beclin-1 (S)					<0.001
Negative	183 (89.7)	147 (94.2)	26 (81.3)	10 (62.5)	
Positive	21 (10.3)	9 (5.8)	6 (18.8)	6 (37.5)	
LC3A (E)*					0.980
Negative	185 (97.4)	152 (97.4)	28 (96.6)	5 (100.0)	
Positive	5 (2.6)	4 (2.6)	1 (3.4)	0 (0.0)	
LC3A (S)					<0.001
Negative	148 (72.5)	127 (81.4)	19 (59.4)	2 (12.5)	
Positive	56 (27.5)	29 (18.6)	13 (40.6)	14 (87.5)	
LC3B (E)*					0.421
Negative	34 (17.9)	30 (19.2)	3 (10.3)	1 (20.0)	
Positive	156 (82.1)	126 (80.8)	26 (89.7)	4 (80.0)	
LC3B (S)					<0.001
Negative	145 (71.1)	126 (80.8)	14 (43.8)	5 (31.3)	
Positive	59 (28.9)	30 (19.2)	18 (56.3)	11 (68.8)	
p62 (E)*					0.650
Negative	161 (84.7)	134 (85.9)	22 (75.9)	5 (100.0)	
Positive	29 (15.3)	22 (14.1)	7 (24.1)	0 (0.0)	
p62 (S)					<0.001
Negative	140 (68.6)	122 (78.2)	14 (43.8)	4 (25.0)	
Positive	64 (31.4)	34 (21.8)	18 (56.3)	12 (75.0)	

*14 cases without an epithelial component were excluded. E, epithelial component. S, stromal component.

(p=0.012, and p=0.004, respectively). Next, we performed Cox multivariate analysis (parameters included were stromal cellularity, stromal atypia, stromal mitosis, stromal overgrowth,

Table 3. Correlation between clinicopathologic factors with expression of autophagy-related proteins in epithelial component*

Parameters	Nuclear beclin-1			Cytoplasmic beclin-1			LC3A			LC3B			P62		
	(-) n=111 (%)	(+) n=79 (%)	*p*-value	(-) n=101 (%)	(+) n=89 (%)	*p*-value	(-) n=185 (%)	(+) n=5 (%)	*p*-value	(-) n=34 (%)	(+) n=156 (%)	*p*-value	(-) n=161 (%)	(+) n=29 (%)	*p*-value
Stromal cellularity			1.065			4.155			1.865			2.385			1.470
Mild	78 (70.3)	45 (57.0)		66 (65.3)	57 (64.0)		121 (65.4)	2 (40.0)		25 (73.5)	98 (62.8)		107 (66.5)	16 (55.2)	
Moderate	28 (25.2)	33 (41.8)		32 (31.7)	29 (32.6)		58 (31.4)	3 (60.0)		7 (20.6)	54 (34.8)		49 (30.4)	12 (41.4)	
Marked	5 (4.5)	1 (1.3)		3 (3.0)	3 (3.4)		6 (3.2)	0 (0.0)		2 (5.9)	4 (2.6)		5 (3.1)	1 (3.4)	
Stromal atypia			0.220			3.620			4.315			2.160			0.230
Mild	99 (89.2)	62 (78.5)		87 (86.1)	74 (83.1)		157 (84.9)	4 (80.0)		31 (91.2)	130 (83.3)		140 (87.0)	21 (72.4)	
Moderate	11 (9.9)	15 (19.0)		12 (11.9)	14 (15.7)		25 (13.5)	1 (20.0)		2 (5.9)	24 (15.4)		19 (11.8)	7 (24.1)	
Marked	1 (0.9)	2 (2.5)		2 (2.0)	1 (1.1)		3 (1.6)	0 (0.0)		1 (2.9)	2 (1.3)		2 (1.2)	1 (3.4)	
Stromal mitosis			1.175			2.905			4.415			3.370			3.165
0–4 / 10 HPFs	96 (86.5)	63 (79.7)		86 (85.1)	73 (82.0)		155 (83.8)	4 (80.0)		30 (88.2)	129 (82.7)		136 (84.5)	23 (79.3)	
5–9 / 10 HPFs	14 (12.6)	15 (19.0)		14 (13.9)	15 (16.9)		28 (15.1)	1 (20.0)		3 (8.8)	26 (16.7)		23 (14.3)	6 (20.7)	
≥10 / 10 HPFs	1 (0.9)	1 (1.3)		1 (1.0)	1 (1.1)		2 (1.1)	0 (0.0)		1 (2.9)	1 (0.6)		2 (1.2)	0 (0.0)	
Stromal overgrowth			2.855			5.000			5.000			2.240			5.000
Absent	110 (99.1)	77 (97.5)		99 (98.0)	88 (98.9)		182 (98.4)	5 (100.0)		33 (97.1)	154 (98.7)		158 (98.1)	29 (100.0)	
Present	1 (0.9)	2 (2.5)		2 (2.0)	1 (1.1)		3 (1.6)	0 (0.0)		1 (2.9)	2 (1.3)		3 (1.9)	0 (0.0)	
Tumor margin			5.000			3.945			0.220			5.000			3.495
Circumscribed	103 (92.8)	73 (92.4)		93 (92.1)	83 (93.3)		173 (93.5)	3 (60.0)		32 (94.1)	144 (92.3)		148 (91.9)	28 (96.6)	
Infiltrative	8 (7.2)	6 (7.6)		8 (7.9)	6 (6.7)		12 (6.5)	2 (40.0)		2 (5.9)	12 (7.7)		13 (8.1)	1 (3.4)	
Tumor recurrence	9 (8.1)	3 (3.8)	1.825	5 (5.0)	7 (7.9)	2.765	12 (6.5)	0 (0.0)	5.000	3 (8.8)	9 (5.8)	2.265	12 (7.5)	0 (0.0)	1.095
Distance metastasis	2 (1.8)	0 (0.0)	2.560	1 (1.0)	1 (1.1)	5.000	2 (1.1)	0 (0.0)	5.000	0 (0.0)	2 (1.3)	5.000	2 (1.2)	0 (0.0)	5.000

*14 cases without an epithelial component were excluded.

Table 4. Correlation between clinicopathologic factors with expression of autophagy-related proteins in stromal component

Parameters	Nuclear beclin-1			Cytoplasmic beclin-1			LC3A			LC3B			P62		
	(-) n=188 (%)	(+) n=16 (%)	*p*-value*	(-) n=183 (%)	(+) n=21 (%)	*p*-value*	(-) n=148 (%)	(+) n=56 (%)	*p*-value*	(-) n=145 (%)	(+) n=59 (%)	*p*-value*	(-) n=140 (%)	(+) n=64 (%)	*p*-value*
Stromal cellularity			0.250			0.010			<0.001			<0.001			<0.001
Mild	118 (62.8)	5 (31.3)		116 (63.4)	7 (33.3)		107 (72.3)	16 (28.6)		103 (71.0)	20 (33.9)		100 (71.4)	23 (35.9)	
Moderate	58 (30.9)	10 (62.5)		58 (31.7)	10 (47.6)		37 (25.0)	31 (55.4)		40 (27.6)	28 (47.5)		34 (24.3)	34 (53.1)	
Marked	12 (6.4)	1 (6.3)		9 (4.9)	4 (19.0)		4 (2.7)	9 (16.1)		2 (1.4)	11 (18.6)		6 (4.3)	7 (10.9)	
Stromal atypia			0.850			<0.001			<0.001			<0.001			<0.001
Mild	152 (80.9)	9 (56.3)		152 (83.1)	9 (42.9)		131 (88.5)	30 (53.6)		128 (88.3)	33 (55.9)		126 (90.0)	35 (54.7)	
Moderate	26 (13.8)	7 (43.8)		25 (13.7)	8 (38.1)		15 (10.1)	18 (32.1)		13 (9.0)	20 (33.9)		13 (9.3)	20 (31.3)	
Marked	10 (5.3)	0 (0.0)		6 (3.3)	4 (19.0)		2 (1.4)	8 (14.3)		4 (2.8)	6 (10.2)		1 (0.7)	9 (14.1)	
Stromal mitosis			0.155			<0.001			<0.001			<0.001			<0.001
0–4 / 10 HPFs	152 (80.9)	7 (43.8)		150 (82.0)	9 (42.9)		130 (87.8)	29 (51.8)		129 (89.0)	30 (50.8)		124 (88.6)	35 (54.7)	
5–9 / 10 HPFs	25 (13.3)	9 (56.3)		25 (13.7)	9 (42.9)		16 (10.8)	18 (32.1)		13 (9.0)	21 (35.6)		13 (9.3)	21 (32.8)	
≥10 / 10 HPFs	11 (5.9)	0 (0.0)		8 (4.4)	3 (14.3)		2 (1.4)	9 (16.1)		3 (2.1)	8 (13.6)		3 (2.1)	8 (12.5)	
Stromal overgrowth			3.145			0.015			<0.001			<0.001			<0.001
Absent	173 (92.0)	14 (87.5)		172 (94.0)	15 (71.4)		145 (98.0)	42 (75.0)		142 (97.9)	45 (76.3)		136 (97.1)	51 (79.7)	
Present	15 (8.0)	2 (12.5)		11 (6.0)	6 (28.6)		3 (2.0)	14 (25.0)		3 (2.1)	14 (23.7)		4 (2.9)	13 (20.3)	
Tumor margin			3.365			1.215			<0.001			0.005			0.060
Circumscribed	169 (89.9)	14 (87.5)		166 (90.7)	17 (81.0)		141 (95.3)	42 (75.0)		137 (94.5)	46 (78.0)		131 (93.6)	52 (81.3)	
Infiltrative	19 (10.1)	2 (12.5)		17 (9.3)	4 (19.0)		7 (4.7)	14 (25.0)		8 (5.5)	13 (22.0)		9 (6.4)	12 (18.8)	
Tumor recurrence	14 (7.4)	4 (25.0)	0.200	16 (8.7)	2 (9.5)	5.000	7 (4.7)	11 (19.6)	0.010	10 (6.9)	8 (13.6)	0.855	8 (5.7)	10 (15.6)	0.155
Distance metastasis	7 (3.7)	1 (6.3)	2.440	6 (3.3)	2 (9.5)	0.970	0 (0.0)	8 (14.3)	<0.001	4 (2.8)	4 (6.8)	1.160	2 (1.4)	6 (9.4)	0.065

*p-values are corrected for multiple testing using the Bonferroni correction.

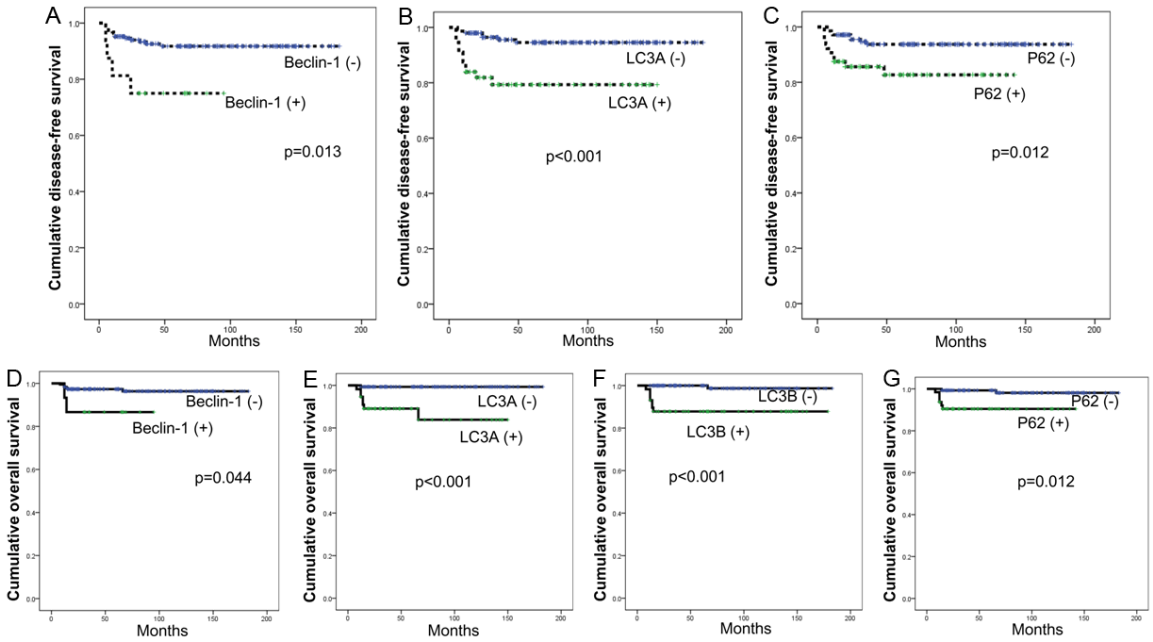

Figure 3. Disease-free survival and overall survival curves according to the status of autophagy-related proteins.

tumor margin, nuclear beclin-1 in the stromal component, LC3A in the stromal component, and p62 in the stromal component); the results are shown in **Table 6**. Stromal overgrowth (hazard ratio: 12.381, 95% CI: 1.991-76.978, P=0.007), and nuclear beclin-1 positivity in the stromal component (hazard ratio: 3.358, 95% CI: 1.023-12.371, P=0.046) were correlated with shorter disease-free survival and stromal overgrowth (hazard ratio: 111.262, 95% CI: 6.175-2004.642, P=0.007) was associated with shorter overall survival.

Finally, we found that the expression of autophagy-related proteins adversely affected the disease-free survival and the overall survival of patients with PTs.

Discussion

It is known that autophagy is involved in both tumor suppression and tumor progression [10-13]. In this study, we investigated the expression profiles of autophagy-related proteins, beclin-1, LC3A, LC3B, and p62, in human PTs. We found that a higher histologic grade of PT was correlated with greater expression of beclin-1, LC3A, LC3B, and p62 proteins in the stromal component (p<0.001). These results support those of previous studies which have been performed in tumors from several other organs [15, 18, 20, 24-31]. In terms of breast tumors, it is also known that expression of autophagy-related markers are associated with the histologic grade and the molecular subtype of breast cancer, although there is still no valid research evaluating the autophagy status in PTs [25].

Autophagy induction in cancer cells can occur due to hypoxia [10], and it has been previously demonstrated that expression of hypoxia-inducible factor 1 alpha (HIF-1α) and its downstream targets are associated with a higher histologic grade in fibroepithelial tumors of the breast [32]. Higher-grade PTs have been found to have relatively more stromal overgrowth than the lower-grade PTs, which is attributed to the fact that they frequently experience more hypoxia in the tumor microenvironment. It is thought that autophagy provides the nutrients needed for cancer survival and expression of HIF-1α allows higher-grade PTs to adapt to hypoxia. Furthermore, the metabolic demand has been shown to be elevated in higher-grade PTs based on the report that the expression of glycolysis-related proteins is associated with the histologic grade of PTs [33]. Therefore, we suggested that autophagy activity could compensate for the metabolic demand required for tumor survival up to a point. However, the activation of autophagy has a flip-side: tumor sup-

Table 5. Univariate analysis of the impact of expression of autophagy-related proteins on prognosis by the log-rank test

Parameter	Disease-free survival		Overall survival	
	Median survival (95% CI) months	*P*-value	Median survival (95% CI) months	*P*-value
Nuclear beclin-1 (E)*		0.247		0.750
Negative	168 (159-177)		181 (177-184)	
Positive	171 (164-179)		176 (172-181)	
Cytoplasmic beclin-1 (E)*		0.319		n/a
Negative	174 (167-181)		n/a	
Positive	161 (150-172)		n/a	
Nuclear beclin-1 (S)		0.013		0.044
Negative	169 (162-176)		177 (172-181)	
Positive	74 (56-91)		84 (69-98)	
Cytoplasmic beclin-1 (S)		0.748		0.107
Negative	167 (160-174)		177 (172-181)	
Positive	86 (75-97)		86 (76-97)	
LC3A (E)*		n/a		n/a
Negative	n/a		n/a	
Positive	n/a		n/a	
LC3A (S)		<0.001		<0.001
Negative	174 (168-180)		181 (179-184)	
Positive	121 (106-136)		130 (116-144)	
LC3B (E)*		0.515		0.173
Negative	146 (132-160)		154 (146-163)	
Positive	172 (165-179)		181 (179-184)	
LC3B (S)		0.119		<0.001
Negative	170 (162-177)		181 (178-184)	
Positive	155 (140-170)		158 (144-172)	
p62 (E)*		n/a		n/a
Negative	n/a		n/a	
Positive	n/a		n/a	
p62 (S)		0.012		0.004
Negative	172 (165-179)		180 (176-183)	
Positive	120 (107-132)		129 (120-139)	

*14 cases without an epithelial component were excluded. E, epithelial component. S, stromal component.

pression may occur in the form of cell death induced by excessive consumption of the cellular components [12, 13].

In terms of our results, the expression of nuclear beclin-1, LC3B, and p62 in the stromal component was related to poor prognosis in PT patients. Particularly, nuclear expression of beclin-1 in the stromal component was associated with shorter disease-free survival in multivariate analysis. We ascertained that beclin-1 could be expressed both in the nucleus and the cytoplasm as seen in the results of preceding studies [25, 34]. In a study on brain tumors, beclin-1 protein shuttled between the nucleus and the cytoplasm and this expression shift was explained by loss of beclin-1 gene function [34]. In addition, there is a report that mutant beclin-1 is located in the nucleus [35]. Therefore, we suspect that the nuclear expression of beclin-1 is not related to autophagy regulation. Previous investigators have studied the correlation between beclin-1 expression and prognosis; some tumors with beclin-1 expression were associated with poor prognosis, while others showed a favorable prognosis [18, 27, 31, 36]. Meanwhile, both loss of and overexpression of beclin-1 has been associated with poor prognosis in colon cancer patients [37]. Thus, we cannot determine whether the expression of beclin-1 is associated with prognosis in cancer patients unless further study on the correlation of the expression and the localization of beclin-1 and prognosis of tumors is performed. Otherwise, the expression of LC3B and p62 is known to be associated with poor prognosis in tumors of other organs, consistent with our results [38, 39].

We performed immunohistochemical analysis, a static method, for evaluation of autophagy activity. However, autophagy is a multi-step dynamic process and thus, autophagy flux should be measured to evaluate the variations of autophagy activity over time [23]. Accordingly,

Table 6. Independent prognostic factors for disease-free survival and overall survival by multivariate analysis

Parameter	Disease-free survival			Overall survival		
	Hazard ratio	95% CI	*p*-value	Hazard ratio	95% CI	*p*-value
Stromal cellularity			0.922			0.208
Mild vs. moderate/marked	0.905	0.123-6.659		10.054	0.278-364.219	
Stromal atypia			0.951			0.614
Mild vs. moderate/marked	0.935	0.107-8.163		0.519	0.041-6.631	
Stromal mitosis			0.972			0.447
0-4 / 10 HPFs vs. ≥5 / 10 HPFs	1.057	0.046-24.176		0.172	0.002-16.017	
Stromal overgrowth			0.007			0.001
Absent vs. present	12.381	1.991-76.978		111.262	6.175-2004.642	
Tumor margin			0.693			0.514
Circumscribed vs. Infiltrative	0.752	0.183-3.091		0.575	0.109-3.038	
Nuclear beclin-1 (S)			0.046			0.321
Negative vs. Positive	3.558	1.023-12.371		3.380	0.305-37.446	
LC3A (S)			0.263			0.296
Negative vs. Positive	1.905	0.616-5.890		3.776	0.312-45.721	
p62 (S)			0.457			0.249
Negative vs. Positive	1.500	0.516-4.364		3.056	0.458-20.406	

S, stromal component.

our study is limited in that we used paraffin blocks of tumors and therefore could not verify that the changes resulted from autophagy activity.

We confirmed that the cytoplasmic beclin-1, LC3A, LC3B, and p62 expression in the stromal component increases as the histologic grade of PTs increases. Therefore, autophagy inhibitors might be candidates for anti-tumor agents for PTs and they have been reported to suppress tumor growth in other organs [40-43].

In conclusion, we demonstrated that the expression of cytoplasmic beclin-1, LC3A, LC3B, and p62 in the stromal component is associated with a higher histologic grade and poorer prognosis in PTs, and these results suggest the correlation of increased autophagy activity and tumor progression.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology (2012R1A1A1002886).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Ja Seung Koo, Department of Pathology, Yonsei University College of Medicine, Severance Hospital, 50 Yonsei-ro, Seodaemun-gu, Seoul, 120-752, South Korea. Tel: 82-2-2228-1772; Fax: 82-2-362-0860; E-mail: kjs1976@yuhs.ac

References

[1] Tavassoli FA, Devilee P, (Eds.). World Heath Organization Classification of Tumors. Lyon: IARC Press, 2003.

[2] Anderson B, Lawton T, Lehman C and Moe R. Phyllodes tumor. In: Morrow M, Osborne C, editors. Disease of the Breast. Philadelphia: Lippincott & Wilkins; 2004. pp: 991-1006.

[3] Tan PH, Jayabaskar T, Chuah KL, Lee HY, Tan Y, Hilmy M, Hung H, Selvarajan S and Bay BH. Phyllodes tumors of the breast: the role of pathologic parameters. Am J Clin Pathol 2005; 123: 529-540.

[4] Sawyer EJ, Hanby AM, Ellis P, Lakhani SR, Ellis IO, Boyle S and Tomlinson IP. Molecular analysis of phyllodes tumors reveals distinct changes in the epithelial and stromal components. Am J Pathol 2000; 156: 1093-1098.

[5] Levine B and Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev Cell 2004; 6: 463-477.

[6] Mizushima N. Autophagy: process and function. Genes Dev 2007; 21: 2861-2873.

[7] Mizushima N, Levine B, Cuervo AM and Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature 2008; 451: 1069-1075.
[8] Yang Z and Klionsky DJ. Eaten alive: a history of macroautophagy. Nat Cell Biol 2010; 12: 814-822.
[9] Murrow L and Debnath J. Autophagy as a stress-response and quality-control mechanism: implications for cell injury and human disease. Annu Rev Pathol 2013; 8: 105-137.
[10] Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, Mukherjee C, Shi Y, Gelinas C, Fan Y, Nelson DA, Jin S and White E. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell 2006; 10: 51-64.
[11] Roy S and Debnath J. Autophagy and tumorigenesis. Semin Immunopathol 2010; 32: 383-396.
[12] Baehrecke EH. Autophagy: dual roles in life and death? Nat Rev Mol Cell Biol 2005; 6: 505-510.
[13] Debnath J, Baehrecke EH and Kroemer G. Does autophagy contribute to cell death? Autophagy 2005; 1: 66-74.
[14] Won KY, Kim GY, Kim YW, Song JY and Lim SJ. Clinicopathologic correlation of beclin-1 and bcl-2 expression in human breast cancer. Hum Pathol 2010; 41: 107-112.
[15] Chen Y, Lu Y, Lu C and Zhang L. Beclin-1 expression is a predictor of clinical outcome in patients with esophageal squamous cell carcinoma and correlated to hypoxia-inducible factor (HIF)-1alpha expression. Pathol Oncol Res 2009; 15: 487-493.
[16] Li BX, Li CY, Peng RQ, Wu XJ, Wang HY, Wan DS, Zhu XF and Zhang XS. The expression of beclin 1 is associated with favorable prognosis in stage IIIB colon cancers. Autophagy 2009; 5: 303-306.
[17] Pirtoli L, Cevenini G, Tini P, Vannini M, Oliveri G, Marsili S, Mourmouras V, Rubino G and Miracco C. The prognostic role of Beclin 1 protein expression in high-grade gliomas. Autophagy 2009; 5: 930-936.
[18] Wan XB, Fan XJ, Chen MY, Xiang J, Huang PY, Guo L, Wu XY, Xu J, Long ZJ, Zhao Y, Zhou WH, Mai HQ, Liu Q and Hong MH. Elevated Beclin 1 expression is correlated with HIF-1alpha in predicting poor prognosis of nasopharyngeal carcinoma. Autophagy 2010; 6: 395-404.
[19] Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y and Yoshimori T. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 2000; 19: 5720-5728.
[20] Sivridis E, Koukourakis MI, Zois CE, Ledaki I, Ferguson DJ, Harris AL, Gatter KC and Giatromanolaki A. LC3A-positive light microscopy detected patterns of autophagy and prognosis in operable breast carcinomas. Am J Pathol 2010; 176: 2477-2489.
[21] Yoshioka A, Miyata H, Doki Y, Yamasaki M, Sohma I, Gotoh K, Takiguchi S, Fujiwara Y, Uchiyama Y and Monden M. LC3, an autophagosome marker, is highly expressed in gastrointestinal cancers. Int J Oncol 2008; 33: 461-468.
[22] Komatsu M, Waguri S, Koike M, Sou YS, Ueno T, Hara T, Mizushima N, Iwata J, Ezaki J, Murata S, Hamazaki J, Nishito Y, Iemura S, Natsume T, Yanagawa T, Uwayama J, Warabi E, Yoshida H, Ishii T, Kobayashi A, Yamamoto M, Yue Z, Uchiyama Y, Kominami E and Tanaka K. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. Cell 2007; 131: 1149-1163.
[23] Mizushima N, Yoshimori T and Levine B. Methods in mammalian autophagy research. Cell 2010; 140: 313-326.
[24] Ahn CH, Jeong EG, Lee JW, Kim MS, Kim SH, Kim SS, Yoo NJ and Lee SH. Expression of beclin-1, an autophagy-related protein, in gastric and colorectal cancers. APMIS 2007; 115: 1344-1349.
[25] Choi J, Jung W and Koo JS. Expression of autophagy-related markers beclin-1, light chain 3A, light chain 3B and p62 according to the molecular subtype of breast cancer. Histopathology 2013; 62: 275-286.
[26] Giatromanolaki A, Koukourakis MI, Harris AL, Polychronidis A, Gatter KC and Sivridis E. Prognostic relevance of light chain 3 (LC3A) autophagy patterns in colorectal adenocarcinomas. J Clin Pathol 2010; 63: 867-872.
[27] Giatromanolaki A, Koukourakis MI, Koutsopoulos A, Chloropoulou P, Liberis V and Sivridis E. High Beclin 1 expression defines a poor prognosis in endometrial adenocarcinomas. Gynecol Oncol 2011; 123: 147-151.
[28] Giatromanolaki AN, Charitoudis GS, Bechrakis NE, Kozobolis VP, Koukourakis MI, Foerster MH and Sivridis EL. Autophagy patterns and prognosis in uveal melanomas. Mod Pathol 2011; 24: 1036-1045.
[29] Sivridis E, Koukourakis MI, Mendrinos SE, Karpouzis A, Fiska A, Kouskoukis C and Giatromanolaki A. Beclin-1 and LC3A expression in cutaneous malignant melanomas: a biphasic survival pattern for beclin-1. Melanoma Res 2011; 21: 188-195.
[30] Thompson HG, Harris JW, Wold BJ, Lin F and Brody JP. p62 overexpression in breast tumors and regulation by prostate-derived Ets factor in breast cancer cells. Oncogene 2003; 22: 2322-2333.
[31] Yu M, Gou WF, Zhao S, Xiao LJ, Mao XY, Xing YN, Takahashi H, Takano Y and Zheng HC. Be-

clin 1 expression is an independent prognostic factor for gastric carcinomas. Tumour Biol 2013; 34: 1071-83.
[32] Kuijper A, van der Groep P, van der Wall E and van Diest PJ. Expression of hypoxia-inducible factor 1 alpha and its downstream targets in fibroepithelial tumors of the breast. Breast Cancer Res 2005; 7: R808-818.
[33] Kwon JE, Jung WH and Koo JS. The expression of metabolism-related proteins in phyllodes tumors. Tumour Biol 2013; 34: 115-124.
[34] Miracco C, Cosci E, Oliveri G, Luzi P, Pacenti L, Monciatti I, Mannucci S, De Nisi MC, Toscano M, Malagnino V, Falzarano SM, Pirtoli L and Tosi P. Protein and mRNA expression of autophagy gene Beclin 1 in human brain tumours. Int J Oncol 2007; 30: 429-436.
[35] Negri T, Tarantino E, Orsenigo M, Reid JF, Gariboldi M, Zambetti M, Pierotti MA and Pilotti S. Chromosome band 17q21 in breast cancer: significant association between beclin 1 loss and HER2/NEU amplification. Genes Chromosomes Cancer 2010; 49: 901-909.
[36] Huang JJ, Zhu YJ, Lin TY, Jiang WQ, Huang HQ and Li ZM. Beclin 1 expression predicts favorable clinical outcome in patients with diffuse large B-cell lymphoma treated with R-CHOP. Hum Pathol 2011; 42: 1459-1466.
[37] Koukourakis MI, Giatromanolaki A, Sivridis E, Pitiakoudis M, Gatter KC and Harris AL. Beclin 1 over- and underexpression in colorectal cancer: distinct patterns relate to prognosis and tumour hypoxia. Br J Cancer 2010; 103: 1209-1214.
[38] Rolland P, Madjd Z, Durrant L, Ellis IO, Layfield R and Spendlove I. The ubiquitin-binding protein p62 is expressed in breast cancers showing features of aggressive disease. Endocr Relat Cancer 2007; 14: 73-80.
[39] Zhao H, Yang M, Zhao J, Wang J, Zhang Y and Zhang Q. High expression of LC3B is associated with progression and poor outcome in triple-negative breast cancer. Med Oncol 2013; 30: 475.
[40] Amaravadi RK, Yu D, Lum JJ, Bui T, Christophorou MA, Evan GI, Thomas-Tikhonenko A and Thompson CB. Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. J Clin Invest 2007; 117: 326-336.
[41] Carew JS, Medina EC, Esquivel JA 2nd, Mahalingam D, Swords R, Kelly K, Zhang H, Huang P, Mita AC, Mita MM, Giles FJ and Nawrocki ST. Autophagy inhibition enhances vorinostat-induced apoptosis via ubiquitinated protein accumulation. J Cell Mol Med 2010; 14: 2448-2459.
[42] Carew JS, Nawrocki ST, Kahue CN, Zhang H, Yang C, Chung L, Houghton JA, Huang P, Giles FJ and Cleveland JL. Targeting autophagy augments the anticancer activity of the histone deacetylase inhibitor SAHA to overcome Bcr-Abl-mediated drug resistance. Blood 2007; 110: 313-322.
[43] Gupta A, Roy S, Lazar AJ, Wang WL, McAuliffe JC, Reynoso D, McMahon J, Taguchi T, Floris G, Debiec-Rychter M, Schoffski P, Trent JA, Debnath J and Rubin BP. Autophagy inhibition and antimalarials promote cell death in gastrointestinal stromal tumor (GIST). Proc Natl Acad Sci U S A 2010; 107: 14333-14338.