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Autophagy patterns and prognosis in uveal melanomas

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Autophagy is a self-degradation mechanism by which cells recycle their own cytoplasmic constituents. It has been claimed that, under certain conditions, such a process may be associated with tumor progression. In this study, the autophagic activity was investigated in a series of 99 uveal melanomas after immunohistochemical staining for the autophagy-associated proteins *MAP1LC3A* and *BECN1*, most commonly known as LC3A and Beclin 1, respectively. These were assessed in parallel with the hypoxia-inducible factor 1α (*HIF1A*) and its downstream protein lactate dehydrogenase 5 (composed by five *LDHA* subunits). Increased autophagic reactivity, detected by *MAP1LC3A* or *BECN1*, was associated with intense pigmentation and tumor hypoxia. Uveal melanomas with extensive overexpression of *BECN1* or those with underexpression of this protein were associated with the worst prognosis, but the former manifested metastases much earlier than the latter; only 58% of patients with extensive *BECN1* overexpression were alive at 4 years, compared with 80% of patients with underexpressed patterns. It is concluded that autophagy is commonly upregulated in uveal melanomas, and may be associated with hypoxia and intense pigmentation. There is a strong association between extensive *BECN1* overexpression and early metastases/poor prognosis, and between underexpression of this protein and late metastases/better prognosis.
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Autophagy is a self-degradation mechanism by which cells recycle their long-lived proteins and defective organelles under conditions of microenvironmental stress, such as oxygen depletion and nutrient deprivation.[1] By disposing excess or defective cytoplasmic components into the lysosomes, cells renew themselves acquiring, at the same time, energy through metabolic consumption of the digested material. Although autophagy is a major cell survival pathway, its excessive activation leads to massive degradation of cellular components, shifting the balance to self-destruction and autophagic cell death. Several studies suggest that autophagy is intensified in neoplastic cells, but its role in growth and progression of human malignancies remains poorly understood and even more complex is its significance in the response of neoplastic cells to various cytotoxic agents and radiation.[2]

Autophagy is characterized by the formation of double membrane vacuoles containing cytoplasmic constituents, the autophagosomes; these are fused with lysosomes to form the autolysosomes and which subsequently degrade and recycle the sequestered material. An essential component of the autophagic vacuoles is the microtubule-associated protein 1 light chain 3, that is *MAP1LC3A* (LC3A)—a homolog of yeast autophagy-related (Atg) protein 8. *MAP1LC3A* exists in two forms, the *MAP1LC3A-I* (cytosolic) and the *MAP1LC3A-II* (membrane bound).[3–5] *MAP1LC3A-I* (18 kDa) is derived from a proLC3 (30 kDa) protein after cleavage by Atg4.

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Following activation by Atg7 and Atg3, *MAP1LC3A-I* is converted into the membrane-bound form *MAP1LC3A-II* (16 kDa).[5] The latter binds tightly to preautophagosomal, autophagosomal and autolysosomal membranes forming a suitable marker of autophagic activity.[3–5]

BECN1 (Beclin 1) is the mammalian ortholog of yeast Atg6 gene, which is essential for autophagosome formation.[6,7] The human Beclin 1 gene resides on chromosome 17q21 and is monoallelicaly deleted in many human breast, ovarian and prostatic cancer cell lines.[6–9] Furthermore, Beclin $1^{+/-}$ mutant mice suffer from high incidence of spontaneous tumors, suggesting that *BECN1* has a tumor suppressor function[6,7,9–11] with a regulatory role in autophagy.[11]

Hypoxia is a prevailing feature in malignant tissues mainly because of an insufficient, and often immature, vascular network that prevents adequate blood and oxygen perfusion.[12,13] Experimental studies suggest that hypoxia is a potent stress stimulus triggering autophagy in normal and malignant cells.[14] The hypoxia-inducible factor *HIF1A*, a key transcription factor regulating the expression of a variety of genes involved in angiogenesis and anaerobic metabolism,[15] is also involved in the regulation of autophagy in neoplastic cells under hypoxic conditions.[16–18]

In the present study, we investigated the autophagic process and its relationship to prognosis in a series of 99 uveal melanomas after immunohistochemical staining for the Atg proteins *MAP1LC3A* and *BECN1*. The possible relationship between autophagy and intratumoral hypoxia, assessed by the expression of *HIF1A* and its downstream protein lactate dehydrogenase 5,[15] was also sought. Note that LDH5 is composed of five *LDHA* subunits.

Table 1 Clinical characteristics and histopathological features in patients with uveal melanomas

	No. cases
Number of patients	99
Age (years)	
Median (range)	62 (26–89)
Sex	
Female/male	53/46
Melanoma cell type	
Spindle cells	27
Mixed, with spindle cells prevailing	54
Mixed, with epithelioid cells prevailing	12
Epithelioid cells	6
Localization	
Ciliary body	2
Ciliary body and choroid	31
Choroid anteriorly to equator	20
Posterior pole	46
Pigmentation	
Low (absent and minimal)	36
High	63
Necrosis	
Limited (absent and minimal)	76
Extensive	23
Tumor size	
Diameter at the base	
≤ 15 mm	56
>15 mm	43
Height	
≤ 8 mm	45
>8 mm	44

Materials and methods

Tissue Specimens

A total of 99 uveal melanomas treated with enucleation at the Department of Ophthalmology, Charité Medical University, Campus Benjamin Franklin, Berlin were included in this study. None of the patients had been treated with radiotherapy before or after the operation. The median follow-up was 79 months (range 6–154 months). Informed consent was obtained from the patients. The study was also approved by the Research Committee of the Democritus University of Thrace/University General Hospital of Alexandroupolis, where it was conducted.

The specimens had been fixed in 10% formalin and processed routinely to paraffin wax. Following histopathological examination, the melanoma cells were assigned to cell types (spindle cell, epithelioid, and mixed-cell type with spindle or epithelioid cell predominance) and assessed for localization, pigmentation, and necrosis, as indicated in Tables 1 and 2. Tumor thickness was assessed by ultrasonography.

The Primary Antibodies and the Immunohistochemical Techniques

Autophagy was detected by a standard immunohistochemical technique using two different primary antibodies: the purified rabbit polyclonal antibody MAP1LC3A (AP1805a; Abgent, San Diego, CA, USA) at 1:20 dilution;[19–21] and the rabbit monoclonal antibody EPR1733Y to *BECN1* (ab51031; Abcam, Cambridge, UK), used at a dilution 1:50.[22]

The detection of hypoxia-related proteins *HIF1A* and *LDHA* (LDH5 isoenzyme) was achieved after employing a further two markers: the ESEE122 monoclonal antibody (Oxford University, UK) at dilution 1:20 and overnight incubation, for identification of the *HIF1A* protein;[23] and the polyclonal antibody ab9002 (Abcam) at dilution 25 μg/ml and 75 min incubation, for revealing *LDHA* (LDH5 isoenzyme).[24,25]

Table 2 Association of *MAP1LC3A* expression with pathological parameters in 99 uveal melanomas

	MAP1LC3A cytoplasmic			*MAP1LC3A perinuclear*		
	Low	*High*	P-*value*	*Low*	*High*	P-*value*
Cell type						
Spindle cells	17	10		20	7	
Mixed/spindle cells prevailing	29	25	0.46	35	19	0.42
Mixed/epitheliod cells prevailing	8	4		7	5	
Epithelioid cells	5	1		6	0	
Location						
Ciliary body	2	0		2	0	
Ciliary body and choroid	16	15	0.25	15	16	0.005[a]
Choroid anteriorly to equator	10	10		13	7	
Posterior pole	31	15		38	8	
Pigmentation						
Low (absent and minimal)	26	10	0.05	33	3	0.0002
High	33	30		35	28	
Tumor necrosis						
Limited (absent and minimal)	47	29	0.40	54	22	0.35
Extensive	12	11		14	9	
Tumor size						
Diameter at the base						
≤ 15 mm	37	19	0.13	41	15	0.26
>15 mm	22	21		27	16	
Height						
≤ 8 mm	30	15	0.19	29	16	0.40
>8 mm	29	25		39	15	

[a]Posterior pole *vs* other.

Tissue sections from uveal melanomas were cut at 3 μm and stained immunohistochemically as previously described.[19–25]

Evaluation of Autophagic Activity and Hypoxia

The patterns of *MAP1LC3A* expression in uveal melanomas were diffuse cytoplasmic and cytoplasmic/juxta-nuclear (Figure 1a and b). These were evaluated as follows. The proportion of tumor cells showing a diffuse cytoplasmic reactivity per section was determined at $\times 200$ magnification. Using the 50% as a cutoff point, cases were divided into groups of low ($\leq 50\%$) and high ($>50\%$) cytoplasmic *MAP1LC3A* reactivity. The proportion of tumor cells expressing the perinuclear *MAP1LC3A* pattern was assessed in a similar manner. These tumors were meant to be separated into groups of low, intermediate, and high reactivity using the 33rd and 67th percentiles. However, as the 67th percentile was 0%, two groups of perinuclear reactivity were eventually formed, the low and the high (see Results).

The reactivity of *BECN1* in uveal melanomas was in all cases diffuse cytoplasmic and was evaluated, on the basis of the extent and intensity of staining, at $\times 200$ magnification, as previously indicated:[22]

1. Low level: when cytoplasmic reactivity expressed strongly in $<10\%$ of tumor cells, and/or weakly in $>90\%$ of tumor cells (Figure 1c);
2. Overexpression: when $>10\%$ of tumor cells showed a strong cytoplasmic reactivity, and no $>50\%$ were negative; overexpression was considered as being (2a) extensive, when $>50\%$ of neoplastic cells expressed strong *BECN1* reactivity or (2b) limited, when 10–50% of tumor cells showed a similar reaction (Figure 1d);
3. Underexpression: if $>50\%$ of tumor cells in a section were negative.

HIF1A and *LDHA* (LDH5 isoenzyme) reactivity was mixed cytoplasmic and nuclear. The percentage of melanoma cells with nuclear and/or strong cytoplasmic *HIF1A* expression was assessed separately in all optical fields at $\times 200$ magnification and the mean value of all fields was used to score each case. Melanomas with nuclear *HIF1A* expression in $>10\%$ of tumor cells and/or strong cytoplasmic expression in $>50\%$ of tumor cells were considered as being of high reactivity.[26] *LDHA* (LDH5 isoenzyme) was evaluated in a similar way. The assessment was performed blindly by two independent observers (AG and ES).

Western Blotting

In order to validate the specificity of *MAP1LC3A* antibody, a western blot analysis of mouse liver supernatant and pellet extracts was performed using the AP1805a antibody.

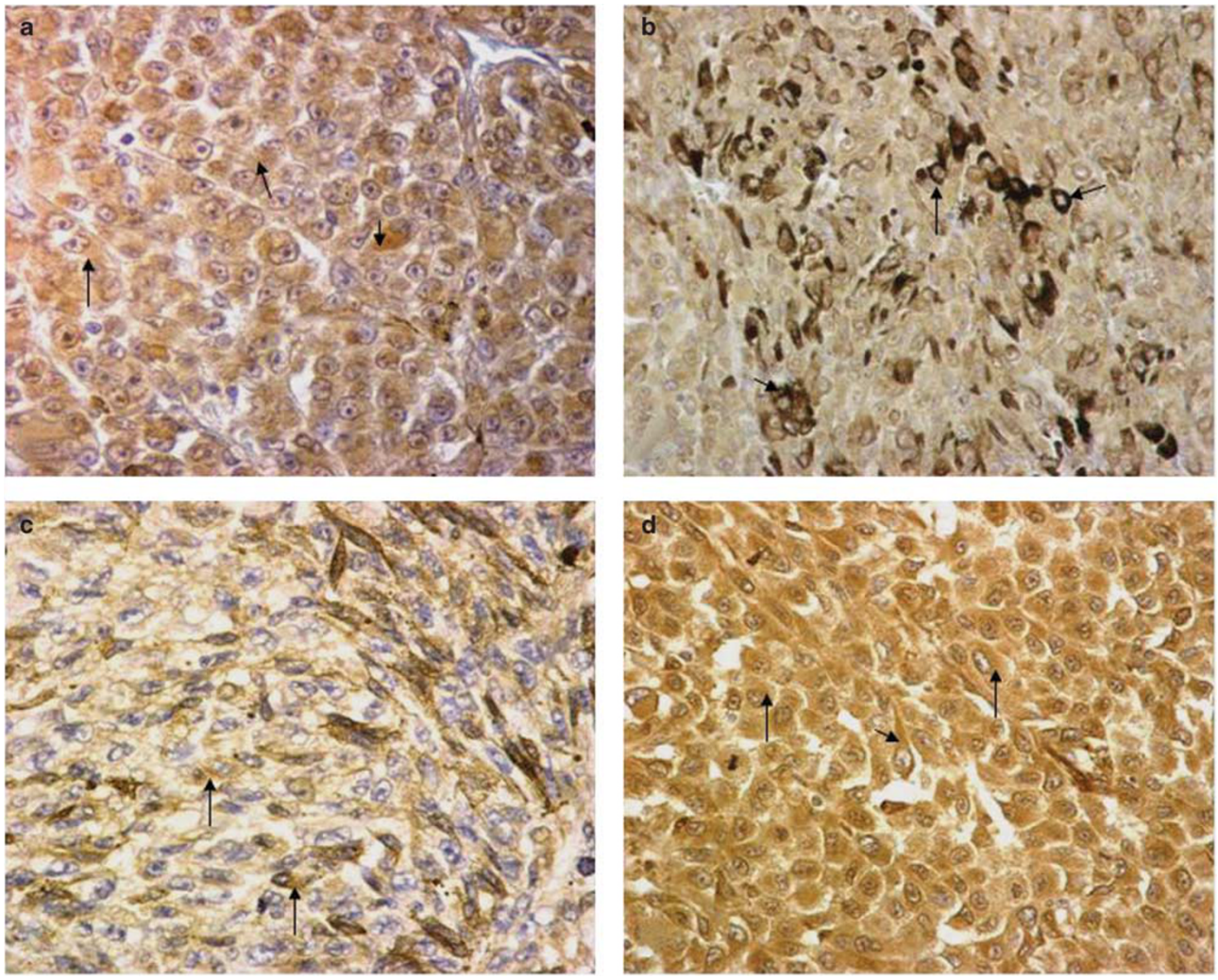

Figure 1 (**a**) Diffuse cytoplasmic pattern of *MAP1LC3A* in a uveal melanoma. (**b**) Cytoplasmic/perinuclear pattern of *MAP1LC3A* in a uveal melanoma. (**c**) Weak cytoplasmic reactivity of *BECN1* in a uveal melanoma. (**d**) Strong cytoplasmic reactivity of *BECN1* in a uveal melanoma.

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 5.0 package (GraphPad, San Diego, CA, USA; http://www.graphpad.com). The χ^2, the Fisher's exact *t*-test, or the unpaired two-tailed *t*-test was used for testing relationships between categorical variables. Linear regression analysis was used to compare groups of continues variables. A *P*-value ≤ 0.05 was considered significant.

Results

Table 1 shows the patient characteristics and the histopathological features of all uveal melanomas in the series. The diameter of the tumors, assessed by ultrasonography, ranged from 4 to 23 mm (median 15 mm) at the base of the lesions, and was from 1 to 22 mm (median 9 mm) height.

MAP1LC3A Antibody Validation

Validation of MAP1LC3A antibody's specificity (AP1805a; Abgent), performed by western blot analysis of mouse liver extracts, gave two bands corresponding to the *MAP1LC3A-I* and *II* proteins (Figure 2).

MAP1LC3A and BECN1 Expression Patterns

After immunohistochemical staining for *MAP1LC3A*, two distinct patterns of cytoplasmic expression were consistently recognized in uveal melanomas: the diffuse cytoplasmic, and the cytoplasmic/juxta-nuclear occurring in a crescentic or ring-like (perinuclear) manner. A third pattern, the so-called 'stone-like structures', reported earlier in a number of epithelial tumors by our group, was not detected in uveal melanomas.[19,27]

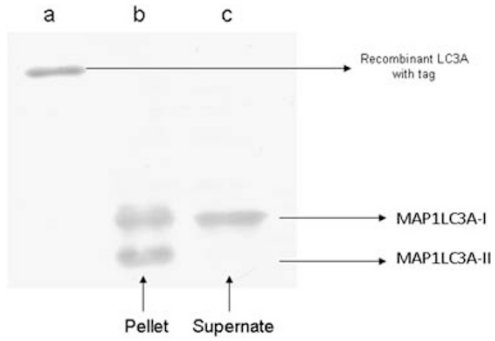

Figure 2 Western blotting showing (**a**) the band of recombinant *MAP1LC3A* protein with tag, (**b**) the two bands of *MAP1LC3A*, the *MAP1LC3A-I* (soluble), and the *MAP1LC3A-II* (membrane bound) form, in the pellet from mouse liver, and (**c**) *MAP1LC3A-II*, but not *MAP1LC3A-I*, in the supernate of the tissue.

The diffuse cytoplasmic *MAP1LC3A* pattern varied considerably between cases, ranging from 0 to 100% of melanoma cells (median 40%). Using the 50th (50%) percentile, the cases were divided into groups of low (0–50%, 59 cases), and high (51–100%, 40 cases) cytoplasmic reactivity.

The perinuclear *MAP1LC3A* pattern also varied between cases, ranging from 0 to 90% of tumor cells (median 0%). The 33rd and 67th percentiles were 0%, so cases were grouped in two categories of low (0%, 68 cases) and high (10–90%, 31 cases) perinuclear reactivity.

BECN1 was diffusely expressed in the cytoplasm of melanoma cells. Overexpression was evident in 48/99 cases (48.4%), of which 22/99 (22.2%) were extensive (>50% of cells), and 26/99 (26.2%) were limited. Underexpression was noted in 29/99 (29.3%) cases, while a basal level of BECN1 activity was seen in 22/99 (22.2%) cases.

Both high cytoplasmic and perinuclear *MAP1LC3A* expression were related with extensive beclin 1 overexpression ($P=0.02$ and $P=0.03$, respectively) (Table 4).

Intra- and Inter-observer Variability

The immunohistochemical expression patterns for MAP1LC3A (cytoplasmic and perinuclear) and *BECN1* (cytoplasmic) were examined for intra- and inter-observer variability. Two experienced pathologists assessed the slides separately and repeated the assessment 30 days later. The second assessment was highly correlated with the first for all observers ($r>0.97$, $P<0.0001$ for all three parameters). Similarly, the two investigator's scoring correlated to each other for all three variables ($r>0.92$, $P<0.0001$). The final decision was taken on the conference microscope.

MAP1LC3A and BECN1 in Relation to Pathological Features

Both high cytoplasmic and perinuclear *MAP1LC3A* patterns were linked with intense pigmentation of uveal melanomas ($P=0.01$ and $P=0.0002$, respectively).

Uveal melanomas composed exclusively of spindle cells were declining to express *BECN1* ($P=0.005$) (Table 3). Overexpression of *BECN1*, whether limited or extensive, was linked with intense pigmentation ($P=0.01$).

There was a significant association between high cytoplasmic *MAP1LC3A* expression and extensive overexpression of *BECN1* ($P=0.03$), and between high perinuclear *MAP1LC3A* expression and the overall *BECN1* overexpression ($P=0.03$) (Table 4).

MAP1LC3A and Hypoxia-Related Proteins

Linear regression analysis showed that perinuclear, but not the cytoplasmic, *MAP1LC3A* pattern expression was directly linked with the hypoxia-related proteins *HIF1A* and *LDHA* (LDH5 isoenzyme) (Table 5). The same applied to *BECN1*. No association was noted between cytoplasmic *MAP1LC3A* pattern and the hypoxia-linked proteins.

In group analysis, a high perinuclear *MAP1LC3A* expression was associated with a high *LDHA* (LDH5 isoenzyme) expression, both cytoplasmic ($P<0.0001$) and nuclear ($P<0.0001$). Similarly, an increased perinuclear *MAP1LC3A* expression was connected with *HIF1A* expression, whether cytoplasmic or nuclear, but the difference was not significant ($P=0.15$ and 0.12, respectively) (data not shown).

Survival Analysis

Table 6 shows the univariate and multivariate analysis of metastatic and death events. The presence of an epithelioid component in uveal melanomas was linked with a poor metastasis-free and overall survival ($P=0.02$ and 0.02, respectively). At univariate analysis, an extensive overexpression of *BECN1* as well as an underexpression of this protein were linked with poor metastasis-free interval and disease-specific overall survival ($P=0.01$).

Figure 3 shows that patients being almost at the two extremes of *BECN1* expression, that is, patients with extensive overexpression of *BECN1* and those with underexpression of this protein had the worse prognosis. Nevertheless, the former manifested metastases much earlier than the latter. In fact, in the presence of extensive overexpression of *BECN1*, only 58% of the patients were alive at 4 years. By contrast, underexpression patterns correlated with delayed appearance of metastases and death events, with over 80% of patients being alive at 4 years ($P=0.02$ for metastasis and $P=0.05$ for death events).

In multivariate analysis, the patterns of *BECN1* expression and the presence of an epithelioid component were the only prognostic variables that maintained an independent significance (Table 6).

Table 3 Association of *BECN1* expression with pathological parameters in 99 uveal melanomas

	Underexpression	*Low level*	*Overexpression*		P-*value*
			Limited	*Extensive*	
Cell type					
Spindle cells	14	3	4	6	
Mixed/spindle cells prevailing	12	8	13	21	0.005[a]
Mixed/epitheliod cells prevailing	1	4	4	3	
Epithelioid cells	2	0	1	3	
Location					
Ciliary body	0	0	1	1	
Ciliary body and choroid	7	4	10	10	0.20
Choroid anteriorly to equator	7	1	2	10	
Posterior pole	15	10	9	12	
Pigmentation					
Low (absent and minimal)	16	6	7	7	0.01[b]
High	13	9	15	26	
Tumor necrosis					
Limited (absent and minimal)	23	12	16	25	0.93
Extensive	6	3	6	8	
Tumor size					
Diameter at the base					
$\leq$15 mm	19	9	8	19	0.20
$>$15 mm	10	6	14	14	
Height					
$\leq$8 mm	15	8	7	15	0.47
$>$8 mm	14	7	15	18	

[a]Pure spindle cell *vs* presence of epithelioid component/underexpression Beclin 1 *vs* all other.
[b]Underexpression/low level *vs* limited/extensive overexpression.

Table 4 Association between *MAP1LC3A* and *BECN1* expression

	MAP1LC3A cytoplasmic			*MAP1LC3A perinuclear*		
	Low	*High*	P-*value*	*Low*	*High*	P-*value*
MAP1LC3A perinuclear						
High	18	13		—	—	—
Low	41	27	0.82	—	—	—
BECN1						
Overexpression, extensive	15	18		21	12	
Overexpression, limited	15	7		12	10	
Low level	9	6		14	1	
Underexpression	20	9	0.04[a]	21	8	0.03[a]

[a]Extensive overexpression *vs* all other.

Discussion

Hypoxia is a major microenvironmental condition in solid tumors that defines tumor aggressiveness either by selecting resistant sub-populations of cancer cells or by triggering specific molecular pathways that facilitate tumor growth, invasiveness, and metastasis.[28,29] Hypoxic cells have been detected in human melanoma xenografts by pimonidazole immunohistochemistry[30] and, indeed, were shown to promote metastatic spread.[31,32] *HIF1A*, in particular, is largely expressed in human neoplasia, including melanomas, and is linked with metastasis[33,34] and melanocyte transformation, in conjunction with the Akt gene.[35]

Furthermore, *HIF1A* is actively involved in triggering autophagy, a major intracellular pathway promoting the survival of tumor cells under oxygen and nutrient deprivation.[16–18] This autophagic process has been documented in human melanomas as early as 1982 by Horikoshi *et al*,[36] and melanoma cells are known to induce autophagic activity with

Table 5 Linear regression analysis of the percentage of melanoma cells expressing strongly *BECN1* and cytoplasmic or perinuclear *MAP1LC3A* patterns according to cytoplasmic or nuclear expression of *HIF1A* and *LDHA* (LDH5 isoenzyme)

	MAP1LC3A cytoplasmic		*MAP1LC3A perinuclear*		*BECN1*	
	P-*value*	r-*value*	P-*value*	r-*value*	P-*value*	r-*value*
HIF1A						
Cytoplasmic	0.53	0.07	0.02	0.24	0.008	0.30
Nuclear	0.55	0.06	0.0002	0.41	0.14	0.17
LDHA (LDH5 isoenzyme)						
Cytoplasmic	0.34	0.10	<0.0001	0.68	<0.0001	0.55
Nuclear	0.72	0.03	<0.0001	0.61	0.001	0.36

Table 6 Univariate and multivariate analysis in metastatic and death events in 99 uveal melanomas

Variable	*Univariate*		*Multivariate*	
	H-*ratio*	P-*value*	t-*ratio*	P-*value*
Metastatic events				
LC3 cytoplasmic[a]	0.85	0.66	0.45	0.64
LC3 perinuclear[b]	0.61	0.18	1.07	0.28
BECN1[c]	3.60	<0.0001	4.62	<0.0001
Histology[d]	0.45	0.02	2.96	0.004
Diameter[e]	1.14	0.67	0.35	0.72
Height[f]	1.35	0.34	0.27	0.78
Pigmentation[g]	0.67	0.23	0.29	0.76
Necrosis[h]	0.67	0.33	0.54	0.58
Death events				
LC3 cytoplasmic[a]	0.96	0.92	0.48	0.62
LC3 perinuclear[b]	0.60	0.18	0.90	0.36
BECN1[c]	3.83	0.0002	4.71	<0.0001
Histology[d]	0.44	0.02	3.18	0.002
Diameter[e]	1.04	0.90	1.17	0.24
Height[f]	1.45	0.26	0.93	0.35
Pigmentation[g]	0.70	0.29	0.37	0.71
Necrosis[h]	0.64	0.30	0.28	0.77

[a]Low *vs* high.
[b]Low *vs* high.
[c]Under and overexpression *vs* underexpression/limited expression.
[d]Absence *vs* presence of epithelioid component.
[e]<15 mm *vs* >15 mm.
[f]<8 mm *vs* >8 mm.
[g]Low pigmentation *vs* high.
[h]Limited necrosis *vs* extensive necrosis.

perinuclear expression after being irradiated with UV light.[37] Yet, the role of autophagy and its links with hypoxia and the autophagy/hypoxia interaction in human melanomas remains obscure.

In this study, the autophagy-related proteins Atg8 (*MAP1LC3A*) and Atg6 gene (*BECN1*) were used as immunohistochemical targets to detect autophagic activity in a series of 99 uveal melanomas.

Miracco *et al*[38] investigating the expression of autophagic genes in cutaneous melanocytic lesions found a gradual decrease in these proteins with tumor progression; the proportion of cases with high cytoplasmic expression of LC3 declined from benign through dysplastic to malignant melanocytic lesions, being at lowest in metastatic melanomas. While a similar decrease in *MAP1LC3A* expression was reported in cerebral and ovarian cancer,[39,40] in other malignancies, such as esophageal and gastrointestinal carcinomas, an increased LC3 expression was prevailing.[41] There were also reports connecting an increased LC3 activity with poor outcome in pancreatic tumors and with a better survival in glioblastoma patients with poor performance score.[40,42]

In most of these studies, however, the expression of LC3 protein was reported as being consistently cytoplasmic, a staining pattern that was also seen with a frequency of 40% in our series of uveal melanomas, and which, presumably, corresponds to the soluble non-membrane-bound form of *MAP1LC3A* (*MAP1LC3A-I*). It should be mentioned, however, that a substantial proportion of cases amounting to 31% in our material, also showed a distinct perinuclear *MAP1LC3A* expression. This is not entirely unexpected, since an analogous 'paranuclear pinpoint stain' was described by Miracco *et al*[38] in their series of cutaneous malignant melanomas. Rieber and Rieber[37] investigating human C8161 melanoma cells, also noted that 30% of UV-irradiated melanomas showed this juxta- or perinuclear pattern of expression. This is interesting as lysosomes usually accumulate to the perinuclear region where they fuse with cytoplasmic autophagosomes (a process also known as autophagocytosis) to form the autolysosomes. It appears, therefore, that the perinuclear *MAP1LC3A* expression may reflect an active autophagic function, as indicated by the accumulation of *MAP1LC3A-II*-positive autophagosomes to the perinuclear region of the lysosomal domain.[43,44]

The question, of course, remains why melanomas, whether uveal or cutaneous, are lacking 'stone-like' structures, despite being aggressive tumors. Obviously, there is no easy answer to that, but it is presumed that malignant melanocytic tumors, being endowed with a rich vascular supply, either need not require an accelerated form of autophagy to survive the adverse conditions of the microenvironment or that these tumors are more resistant to

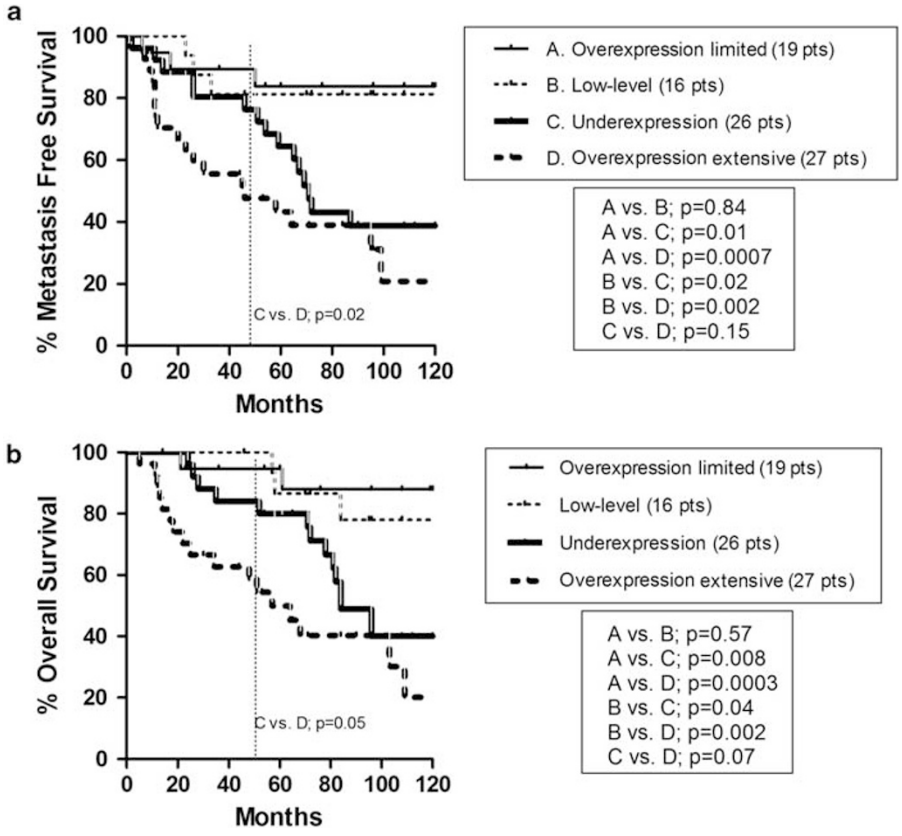

Figure 3 Kaplan–Meier survival curves of (**a**) metastasis-free survival, and (**b**) overall disease-specific survival, stratified by *BECN1* expression.

nutrient deprivation—a phenomenon known as austerity.[45] Alternatively, there may be another, yet unsuspected, mechanism operative in malignant melanomas.

With regard to *BECN1*, an autophagy-related gene with tumor suppressor activity, Miracco *et al*[38] reported comparable results to those shown for *MAP1LC3A*, that is gradual decline of cytoplasmic expression of *BECN1* in cutaneous melanocytic lesions from 100% in benign nevi to 86.4% in dysplastic nevi, 54.3% in melanomas, and 26.7% in melanoma metastases. Such decreased cytoplasmic expression of *BECN1* has been correlated with tumor progression in breast, ovarian, and cerebral neoplasms[39,46,47] and with a reduced survival rate in neoplasms of esophageal, colorectal, hepatocellular, and cerebral origin.[48–51] Liang *et al*[46] explained this immunohistochemical decline in cytoplasmic expression of the protein in terms of *BECN1* inactivation and loss of its tumor suppressor functions. There have been, however, other tumors, including colorectal and gastric adenocarcinomas, in which progression appeared to be dependent upon an increased *BECN1* expression.[52]

The above conflicting results, although may suggest different roles of *BECN1* expression depending on cancer cell types,[47,52] they may also imply the existence of various patterns of *BECN1* expression up to date unrecognized. In this context, we hypothesized that there are four patterns of BECN1 expression, the low level of *BECN1* expression, which may reflect a basal level of autophagic function; the overexpression of *BECN1* (two grades), which apparently represent an intensification of the autophagic machinery under the adverse intratumoral conditions of hypoxia and acidity, as indicated in this and in other studies;[50,53] and, of course, there may be a reduction or loss of *BECN1* expression, a probable result of allelic gene deletions[8] or a consequence of deregulated microRNA function, such as miT-30a.[54] As *BECN1* interacts with members of the bcl-2 protein acting as a tumor suppressor,[55] potentiation of the anti-apoptotic machinery may account, at least in part, for the poor outcome of patients with reduced Beclin 1 expression.

Indeed, in the present study, both the underexpression and the excessive overexpression of *BECN1* were linked with metastases and an overall poor disease-specific survival. However, the temporal patterns of metastases and death were distinct, as overexpression was correlated with a rapid onset of metastases, while the underexpression of *BECN1* was linked with delayed onset of metastatic disease and delayed death rates after 4–5 years from surgery. As only overexpression was linked with hypoxia/acidity features (*HIF1A* and *LDHA*, LDH5

isoenzyme), it is postulated that the reasons of disease progression largely differ between the two groups. *MAP1LC3A* expression, on the other hand, did not show any association of clinical importance, given that the association between high cytoplasmic or perinuclear expression and intense pigmentation does not seem to affect the outcome.

It is concluded that autophagy is upregulated in over 40–50% of uveal melanomas, and these cases were correlated with tumor hypoxia; the latter was inferred by the intimate relationship of *MAP1LC3A* and *BECN1* with *HIF1A* and *LDHA* (LDH5 isoenzyme), the major LDH isoenzyme involved in anaerobic metabolism. The overexpression of *BECN1*, being linked with tumor hypoxia and acidity, was associated with poor prognosis. A low *BECN1* expression, probably representing gene silencing mechanism in a subset of tumor, was also linked with death events but these were of delayed metastasis. Uveal melanomas with the presence of an epithelioid component showed a clear tendency for metastases and a reduced overall survival, but this has been known for long[56] and needs no further comment. It will be of interest to investigate in the future the relation of autophagy with tumor-infiltrating macrophages in uveal melanomas.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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