

## Protein and mRNA expression of autophagy gene *Beclin 1* in human brain tumours

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**Abstract.** *Beclin 1* is an autophagy gene, the role of which as a tumour suppressor has recently been recognized in a few studies. We examined the expression of Beclin 1 protein in 212 primary human brain tumours, including 97 high-grade glial tumours, 29 low-grade glial tumours, 4 grade III meningiomas, 19 grade II meningiomas, 52 grade I meningiomas, and 11 medulloblastomas. In 94 cases, including 56 glial tumours, 35 meningiomas, and 3 medulloblastomas we also assessed *Beclin 1* mRNA expression by real-time RT-PCR. In most high-grade astrocytic, ependymal neoplasms and atypical meningiomas we found a decrease of cytoplasmic protein expression that was, instead, high in the majority of low-grade tumours and in medulloblastomas. The expression level of *Beclin 1* mRNA was significantly lower in glioblastomas than in grade II ( $p=0.04$ ) and grade I ( $p=0.01$ ) astrocytomas; in grade III than in grade I astrocytomas ( $p=0.01$ ); in grade II than in grade I meningiomas ( $p=0.03$ ); and in all glial tumours when compared to all meningiomas ( $p<0.0001$ ). Cytoplasmic expression is thought to be linked to the functional protein. Our observations are in line with studies that demonstrated decreased expression of Beclin 1 in human breast, ovarian, prostate and ovarian cancer and furtherly support its involvement also in brain tumours, which had previously been demonstrated in a few experimental studies, both in spontaneous and in therapy-induced autophagy. Furthermore, our study suggests possible differences of Beclin 1 involvement and its role among the different histotypes of brain neoplasms. Further studies are needed

to highlight *Beclin 1* function in tumour suppression and/or in tumour survival through autophagy and other related programmed cell death processes in brain tumours.

### Introduction

*Beclin 1*, the mammalian orthologue of the yeast *Apg6/Vps30* gene, is a major determinant in the initiation of autophagy (1-3). Autophagy, comprising microautophagy, chaperone-mediated autophagy, and macroautophagy is the lysosomal route for cellular protein degradation and organelle turnover, likewise used for recycling of materials (4-6). It allows for normal mammalian cell survival under conditions of nitrogen starvation or stress, in order to obtain amino acids and other small molecules needed for the maintenance of essential cell functions. In extreme cases, stress situations and/or external agents can lead to autophagic cell death (5,7-10), although this event in cells with intact apoptotic machinery has been recently regarded with criticism (11). Autophagy deregulation occurs in various pathological processes, such as cardiomyopathy (12), neurodegenerative disease (13), and cancer (14,15). Autophagy seems a novel mechanism in cell growth control and tumour suppression, therefore interest is growing on this process and its regulators in cancer, and their possible therapeutical implications (16,17). Usually, autophagy is reduced in cancer cells when compared to their normal counterparts (14). Autophagy is regulated by many genes, that are referred to as 'autophagy genes' (*ATG*) (18,19). Furthermore, a great variety of stimuli are able to modulate autophagy under normal and/or pathological conditions (1-20). The *Beclin 1* gene induces macroautophagy (hereafter called 'autophagy'). The relevance of *Beclin 1* as an autophagic gene has been evidenced by both experimental studies and human cancer observations. In mice, heterozygous disruption/mutation of the *Beclin 1* gene caused increased cell proliferation and development of cancer, that expressed Beclin 1 protein from the remaining wild-type allele, leading to the conclusion that *Beclin 1* is an aploinsufficient tumour suppressor gene (2,21).

Beclin 1 forms a multimeric complex with Atg14, Vps34/class 3 phosphatidylinositol 3-kinase (PI3k), and Vps15, that is necessary for the formation of autophagosome, during the

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autophagic sequestration process (22,23). The complex class 3 PI3k-Beclin 1 was found to be relevant in the regulation of autophagy (23), and PI3k was recognized as the major physiological partner for Beclin 1 in a human glioblastoma cell line (3).

In humans, the *Beclin-1* gene is monoallelically deleted/underexpressed in 40-75% of cases of breast, ovarian, and prostate cancer (1,24,25). Its expression was found decreased also in gastric cancer (15).

In human glioblastoma (GBM), spontaneous autophagy was found to be reduced, and some autophagy regulators altered (1,25-27). However, there are very few studies that evaluated the expression of ATG products at the tissue level in human cancer and, to our knowledge, Beclin 1 expression has not yet been extensively investigated in human brain neoplasms at the tumour tissue level. In this study, we analysed the expression of Beclin 1 protein and its mRNA in a series of human brain tumours including glial and non-glial neoplasms, by immunohistochemistry and quantitative real-time RT-PCR.

## Materials and methods

**Samples.** Two-hundred and twelve brain tumours surgically removed from January 2001 to December 2005 entered our study. Sample selection was restricted to cases where an extensive tumour resection had been performed. In 207 cases surgery was performed prior to radiation or chemotherapy; in 5 cases of glioblastomas, we examined the recurrences in patients that had received radiotherapy. Tumour tissue had been fixed in 10% buffered formalin, and then embedded in paraffin for routine studies and immunohistochemistry. Tumours were diagnosed by three expert pathologists (Drs Clelia Miracco, Pietro Luzi and Piero Tosi) according to the World Health Organization international histologic classification (28). There were 77 glioblastomas (72 primary tumours and 5 recurrences); 26 astrocytomas (11 grade III, 6 grade II, and 9 grade I, pilocytic astrocytomas); 10 oligodendrogliomas (6 grade III, and 4 grade II); 3 grade III, 7 grade II, and 3 grade I ependymomas; 75 meningiomas (52 grade I, 19 grade II, and 4 grade III); and 11 medulloblastomas. At the time of surgery, in 94 cases, including 56 glial neoplasms, 35 meningiomas, and 3 medulloblastomas, small tumour fragments were taken from unfixed tissue, avoiding necrotic areas, immediately frozen and stored at -80°C for biological molecular studies. Fragments of normal cerebral and cerebellar cortex of 4 autaptic brains and of three arterovenous malformations and one cavernous haemangioma served as the normal control. We also examined cancer and normal breast tissue in 3 cases of human NOS infiltrating ductal breast carcinomas.

**Immunohistochemistry.** For each tumour, 4- $\mu$ m thick sections were cut from at least two different paraffin blocks and processed for immunohistochemistry. Paraffin sections were pretreated with WCAP citrate buffer pH 6.0 (Bio-Optica, Milan, Italy), for 40 min at 98.5°C, cooled and washed in distilled water. Anti-human Beclin 1 antibody BECN1 (Santa Cruz, DBA-Italia, Milan, Italy; diluted 1:50) was then applied. Sections were washed in distilled water, developed with a streptavidin-biotin kit (Biogenex, Menarini, Florence, Italy),

using diaminobenzidine as the chromogen, and counterstained with Meyer's hematoxylin.

For each case, a negative control was obtained by replacing the specific antibody with non-immune serum immunoglobulins at the same concentration of the primary antibody.

**Assessment of immunostaining.** Both nuclear and cytoplasmic staining were evaluated by two independent pathologists as previously described. Briefly, cytoplasmic expression was 0 if negative; and, when positive, it was scored from 2 to 5, on the basis of both the stain intensity (1-3) and the percentage of cells stained (1,  $\leq$ 50%; 2, >50%). We considered score 2 as a low cytoplasmic protein expression, and scores 4 and 5 as high protein expression. Nuclear expression was considered negative if <10% of nuclei were stained by the antibody; independently on the degree of positivity, if >10-50% of nuclei were positive, it was given a score of 1 and, if present in >50% of nuclei, the case was scored as 2.

**Gene expression analysis.** RNA isolation and cDNA preparation. Total RNA was extracted from frozen fragments, using TRIzol reagent (Invitrogen, Milan, Italy), as previously described (29). Briefly, for cDNA synthesis, RNA (500 ng) was reverse transcribed in a final volume of 20  $\mu$ l containing 4  $\mu$ l of 5X First-Strand Buffer; 1  $\mu$ l of dNTPs 10 mM; 0.6  $\mu$ l (24 U) of Rnase RNase inhibitor (Promega, Florence, Italy); 2  $\mu$ l of DDT 0.1 M; 1  $\mu$ l (200 U) of M-MLV Reverse Transcriptase (Invitrogen); 0.5  $\mu$ l (200 ng) random examers (Pharmacia, Milan, Italy). The samples were incubated at 25°C for 10 min, and 37°C for 50 min. Reverse transcriptase was inactivated by heating at 70°C for 15 min. No-RT controls were performed by omitting the addition of the reverse transcriptase enzyme, and no-template controls were performed by the addition of nuclease-free water. All products were stored at -20°C for future use.

**Quantitative real-time RT-PCR.** Quantitative real-time RT-PCR was performed as previously described (29), with slight modification. A relative quantitation of Beclin 1 mRNA expression normalized to the endogenous reference gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), was performed by real-time reverse transcription PCR (RT-PCR), using the DNA Engine Opticon 2 System (MJ Research, Celbio, Milan, Italy) and the Brilliant® SYBR®-Green QPCR Master mix (Stratagene®). A fluorescent signal is generated which increases in direct proportion to the amount of PCR product. Primers, and the size of amplification products of tested genes are listed in Table I.

The PCR reaction mixture (final volume, 20  $\mu$ l) contained 10  $\mu$ l of 2X Master mix (Invitrogen), 5 pmol of Beclin 1 primers (or 6 pmol of GAPDH primers), and 1  $\mu$ l (25 ng) of cDNA.

Thermocycling conditions were 10 min at 95°C, followed by 40 cycles of denaturation (95°C, 20 sec), annealing (56°C, 25 sec), extension (72°C for 30 sec). At the end of the 40 PCR cycles, the temperature (TM), at which the cDNA amplicon and intercalated SYBR-Green complex was denaturated, was determined by continuously recording the fluorescence during progressive heating up to 95°C with a ramp rate of 0.1°C/sec. Gene products of some samples are shown in Fig. 1. All

Table I. PCR primers pairs used for the quantification of *Beclin 1* mRNA.

Gene	Sequence	Product (bp)
Bec forward primer <sup>a</sup>	5'-CAA GAT CCT GGA	191
Bec reverse primer	CCG TGT CA-3'	
GAPDH forward primer	5'-TGG CAC TTT CTG TGG	87
GAPDH reverse primer	ACA TCA-3'	
	5'-TGC ACC ACC AAC TGC TTA-3'	
	5'-GAG GGC ATG GAC TGT GGT CAT-3'	

<sup>a</sup>Furuya *et al*, 2005 (15).

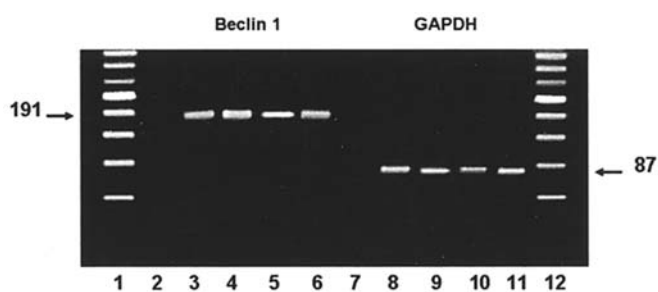


Figure 1. RT-PCR products electrophoresed on 2% agarose gel. Glycer-aldehyde-3-phosphate-dehydrogenase (GAPDH), 87 bp; *Beclin 1*, 191 bp. Lanes 1 and 12, the molecular size marker; lanes 3 and 8, a glioblastoma; lanes 4 and 9, a grade I meningioma; lanes 5 and 10, a grade II meningioma; lanes 6 and 11, a medulloblastoma.

samples were analyzed in duplicate wells of a 96-well plate. The results of real-time RT-PCR were represented by the parameter Ct (threshold cycle number): the lowest Ct values correspond to the highest nucleic acid starting copy number. Gene expression value was calculated as previously reported (29) and expressed by the parameter  $2^{\Delta Ct}$ . The highest  $2^{\Delta Ct}$  values correspond to the lowest *Beclin 1* nucleic acid copy number in the sample.

**Data analysis.** Median values of  $2^{\Delta Ct}$  were compared by using the Mann-Whitney U test. Statistical significance was set at  $p < 0.05$ . Groups of tumours including less than 5 cases were not statistically analysed.

## Results

***Beclin 1* protein.** We found differences in protein expression and its subcellular localization between non-neoplastic and neoplastic tissues, among different tumour types and in low-grade vs high-grade brain tumours (Table II).

In normal cerebral and cerebellar cortex, the protein was highly expressed in the cytoplasm of different types of neurones, including cerebellar granules and, to a lesser extent, in glial cells. In normal cells, we usually observed a perinuclear cytoplasmic positivity and, in some neurones and oligodendrocytes, a nucleocytoplasmic positivity.

**Glial neoplasms.** Among astrocytic and ependymal tumours, cytoplasmic protein expression usually was higher in low-grade tumours, whereas the highest percentages of tumour cells showing nuclear protein expression were registered in high-grade tumours (Table II; Figs. 2 and 3). Oligodendrogliomas, instead, showed a high nucleocytoplasmic positivity also in high-grade tumours. High-grade glial tumours usually showed a more heterogeneous pattern of positivity. Grade I and grade II astrocytomas showed a high cytoplasmic protein expression (scores 4 and 5) in 77.8 and 66.7% of cases, respectively, whereas 43% of GBM were either negative or showed a low cytoplasmic protein expression (score 2). A high score of nuclear positivity was found in 66.7% of grade I astrocytomas, in 33.3% of grade II astrocytomas, in 81.8% of grade III astrocytomas and in 85.7% of glioblastomas. One hundred percent of grade I and II ependymomas showed a high score of cytoplasmic protein expression, whereas 66.6% of grade III ependymomas were either negative or showed a low cytoplasmic score. A high nuclear score was found in 28.6% of grade II, and in 100% of grade III ependymomas. A high cytoplasmic protein expression was observed in all grade II oligodendrogliomas, and in most grade III cases (83.3%); all oligodendrogliomas showed a high nuclear score.

**Meningiomas.** Cytoplasmic staining tended to be higher in low-grade tumours, vice versa, nuclear staining was higher and more heterogeneous in high-grade neoplasms (Table II; Figs. 4 and 5). As for the cytoplasmic positivity, a high score was assigned to most (73%) grade I meningiomas; percentage of highly scored cases dropped to 36.8% in grade II, and to 25% in grade III meningiomas. A high score of nuclear positivity was registered in 5.8% of grade I, in 42.1% of grade II, and in 50% of grade III meningiomas.

**Medulloblastomas.** A high score of cytoplasmic protein expression was observed in a high percentage (63.6%) of medulloblastomas, whereas 18.2% of cases were negative (Table II; Fig. 6). Vice versa nuclei were negative in most (63.6%) cases of medulloblastoma.

Many cases of brain tumours also showed a nucleolar positivity to *Beclin 1*, that was usually associated with nuclear positivity in glial tumours and in medulloblastomas. The nucleolar pattern of positivity was quite different in various tumour types. In grade I-II astrocytomas, nucleoli were usually not evident; in grade III astrocytomas and glioblastomas, nucleoli were often numerous, small and/or medium-sized. In both grade II and III oligodendrogliomas, as well as in medulloblastomas, nucleoli were either not evident or, if positive, very small. In grade I meningiomas we observed usually 1-2 large, *Beclin 1*-positive nucleoli, usually inside negative nuclei.

Intratumoural lymphocytes were usually negative to *Beclin 1*. Some endothelial cells of normal vessels showed

Table II. Cytoplasmic (score: 0-5) and nuclear (score: 0-2) expression of Beclin 1 protein in brain tumours.

	Cs					Ns		
	0	2	3 no. (%)	4	5	0	1 no. (%)	2
GBM (no. 77)	10 (13)	23 (30)	29 (38)	15 (19)	0	2 (2.6)	9 (11.7)	66 (85.7)
AIII (no. 11)	0	4 (36.4)	4 (36.4)	3 (27.2)	0	0	2 (18.2)	9 (81.8)
AII (no. 6)	0	0	2 (33.3)	4 (66.7)	0	2 (33.3)	2 (33.3)	2 (33.3)
AI (no. 9)	0	0	2 (22.2)	1 (11.1)	6 (66.7)	1 (11.1)	2 (22.2)	6 (66.7)
OIII (no. 6)	0	0	1 (16.7)	5 (83.3)	0	0	1 (16.7)	5 (83.3)
OII (no. 4)	0	0	0	4 (100)	0	0	0	4 (100)
EpIII (no. 3)	1 (33.3)	1 (33.3)	1 (33.3)	0	0	0	0	3 (100)
EpII (no. 7)	0	0	0	4 (57.1)	3 (42.9)	4 (57.1)	1 (14.3)	2 (28.6)
EpI (no. 3)	0	0	0	2 (66.7)	1 (33.3)	2 (66.7)	1 (33.3)	0
Me III (no. 4)	2 (50)	0	1 (25)	0	1 (25)	1 (25)	1 (25)	2 (50)
Me II (no. 19)	1 (5.3)	7 (36.8)	6 (31.6)	5 (26.3)	0	5 (26.3)	6 (31.6)	8 (42.1)
Me I (no. 52)	1 (2)	3 (5.8)	10 (19.2)	28 (53.8)	10 (19.2)	25 (48)	24 (46.2)	3 (5.8)
Md (no. 11)	2 (18.2)	0	2 (18.2)	5 (45.4)	2 (18.2)	7 (63.6)	2 (18.2)	2 (18.2)
Nbt (no. 5)	0	0	0	5 (100)		4 (80)	1 (20)	
NBC (no. 7)	0	0	0	2 (28)	5 (72)	5 (72)	2 (28)	
IDBc (no. 3)	0	1 (33.3)	2 (66.7)	0	0	0	3 (100)	0
NBrt (no. 3)	0	0	0	0	3 (100)	3 (100)	0	0

Cs, cytoplasmic positivity score; Ns, nuclear positivity score. no., number of cases. (%), percentage of cases. GBM, glioblastomas. AIII, AII, AI, astrocytomas grade III, II, I WHO. OIII, OII, oligodendrogliomas grade III, II WHO. EpIII, EpII, EpI, ependymomas grade III, II, I WHO. MeIII, MeII, MeI, meningiomas grade III-II-I WHO. Md, medulloblastomas. Nbt, non-cancerous brain tissue adjacent to GBM. NBC, normal brain cortex adjacent to angiomas/autoptic. IDBc, infiltrating ductal breast cancer. NBrt, normal breast tissue.



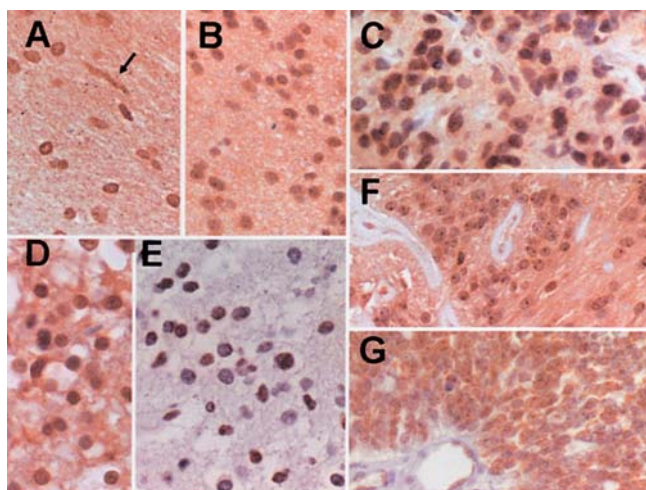


Figure 2. Nucleo-cytoplasmic positivity to Beclin 1 in a grade I pilocytic astrocytoma (A, the arrow indicates a Rosenthal fiber, aspecifically stained by the antibody), in a grade II astrocytoma (B), in a grade II oligodendroglioma (D), and in a grade I ependymoma (F). Negative and/or weakly-stained cytoplasm, and positive nuclei in a glioblastoma (C), in a grade III oligodendroglioma (E), and in a grade III ependymoma (G). Streptavidin-biotin, substrate; diaminobenzidine, original magnification, x400.

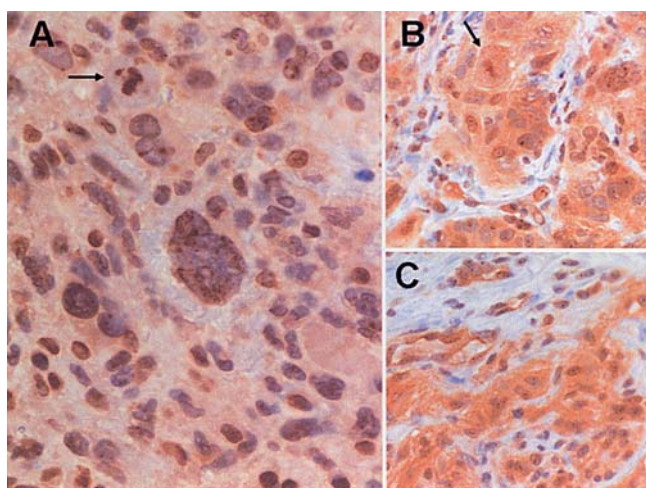


Figure 3. Predominantly nuclear staining in most tumour cells, including a mitotic figure (arrow) in a glioblastoma (A). Nucleo-cytoplasmic staining in most tumour cells, including a mitotic figure (arrow), in another 2 cases of glioblastoma (B and C). Notice the strong cytoplasmic and/or nucleo-cytoplasmic positivity of tumour vessel endothelial cells. Streptavidin-biotin, substrate; diaminobenzidine, original magnification, x400.

a cytoplasmic positivity, whereas, especially in high-grade tumours, endothelial cells of neofomed vessels usually showed a strong cytoplasmic and/or nucleo-cytoplasmic positivity.

*Beclin 1 mRNA*.  $2^{\Delta Ct}$  values in each type and grade of tumours and significant p-values are reported in Table III. A significantly lower mRNA Beclin 1 expression was found in glioblastomas when compared to grade II ( $p=0.04$ ), and grade I ( $p=0.01$ ) astrocytomas; and in grade III astrocytomas when compared to grade I ( $p=0.01$ ) astrocytomas. Five cases of glioblastomas also showed a significantly lower mRNA Beclin 1 expression level than the adjacent apparently normal brain tissue ( $p=0.01$ ). Grade II meningiomas showed a significantly lower mRNA

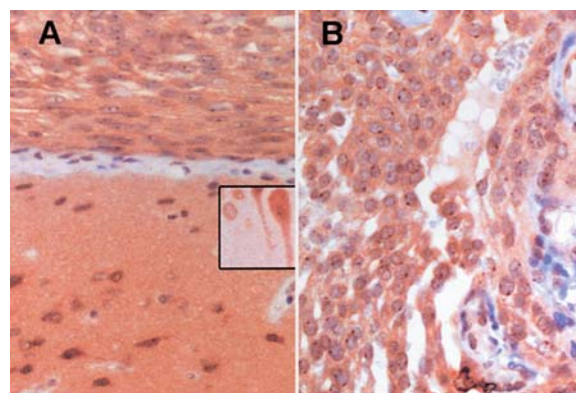


Figure 4. Moderate-to-strong cytoplasmic and nucleolar staining in 2 cases of grade I meningioma (A and B). Positivity to Beclin 1 is also observable in the underlying brain cortex, both in cells and in the neuropilus (A). Strongly-stained pyramidal neurons stand out against the almost negative background of the neuropilus in an autaptic, normal brain (A, inset). Streptavidin-biotin, substrate; diaminobenzidine, original magnification, x400.

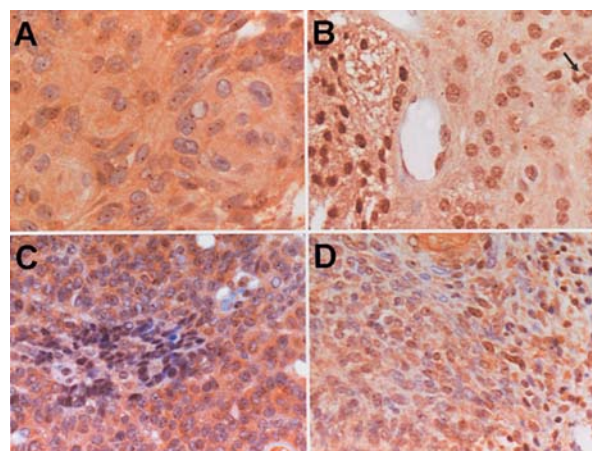


Figure 5. Beclin 1 staining of tumour cell cytoplasm in a grade I meningioma (A) and of tumour cell nuclei in a grade II atypical meningioma (B, the arrow indicates a positively-stained mitotic cell). Heterogeneous nucleo-cytoplasmic staining in a grade III papillary meningioma (C), and in a grade III anaplastic meningioma (D). Streptavidin-biotin, substrate; diaminobenzidine, original magnification, x400.

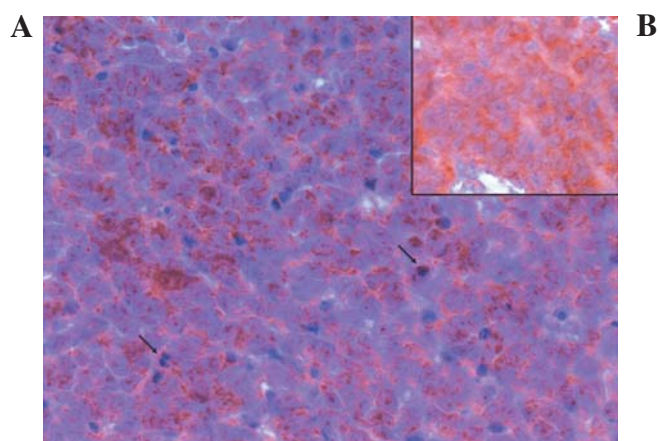


Figure 6. Medulloblastoma showing cytoplasmic positivity to Beclin 1 in the majority of tumour cells, nucleo-cytoplasmic positivity in few cells (A), and in cells exhibiting morphological features of programmed cell death (A, arrows). Another case of medulloblastoma with a strong tumour cell cytoplasmic positivity is depicted in the inset (B). Streptavidin-biotin, substrate; diaminobenzidine, original magnification, x400.

Table III. The median and the range of  $2^{\Delta Ct}$  values are reported in each type and grade of brain tumours and in the other analysed tissues.<sup>a</sup>

	Median	Range	p-value
GBM (no. 35)	132.61	37.61-480.87	GBM vs Nbt = 0.01 <sup>b</sup>
AIII (no. 5)	72.40	46.3-110.13	GBM vs A II = 0.04
AII (no. 5)	43.20	38.6-105.49	GBM vs A I = 0.01
AI (no. 5)	42.58	19.60-53.33	A III vs A I = 0.01
EpIII (no. 3)	255.30	106.01-437.0	GBM + A III vs A I = 0.02
EpII (no. 3)	41.72	35.72-44.16	Me II vs Me I = 0.03
MeIII (no. 3)	41.36	8.47-49.99	AGt vs AMe = 0.0001
MeII (no. 9)	53.67	36.57-196.65	
MeI (no. 23)	41.01	14.57-110.93	
Md (no. 3)	30.10	26.59-33.85	
Nbt (no. 5)	50.10	40.64-57.88	
NBC (no. 3)	40.20	30.6-52.54	
IDBC (no. 3)	233.05	218.22-521.2	
NBrt (no. 3)	79.62	65.81-160.7	

<sup>a</sup>The highest  $2^{\Delta Ct}$  values correspond to the lowest mRNA Beclin 1 gene expression. p-values are also reported. no., number of cases. GBM, glioblastomas. Nbt, non-cancerous brain tissue adjacent to GBM. AIII, AII, AI, astrocytomas grade III, II, I WHO. EpIII, EpII, ependymomas grade III, II WHO. MeIII, MeII, MeI, meningiomas grade III-II-I WHO. Md, medulloblastomas. NBC, normal brain cortex adjacent to angiomas. IDBC, infiltrating ductal breast cancer. NBrt, normal breast tissue. AGt, all glial tumours. AMe, all meningiomas.

<sup>b</sup>Comparison made between GBM and the adjacent NBt in only 5 cases.

beclin 1 expression level than grade I meningiomas ( $p=0.03$ ). A significantly lower mRNA beclin 1 expression was found in all astrocytic glial tumours (grade IV-III-II-I) when compared to all meningiomas (grade III-II-I) ( $p<0.0001$ ). Differences among the other groups of tumours were either not statistically analysed, due to the small number of cases, or not significant.

## Discussion

Defects in self-destruction processes are hallmarks of neoplastic cells. Among the five types to date known of programmed cell death (7,30), type I or apoptosis has been for decades the main focus of numerous studies that led to the discovery of many defective mechanisms in neoplasms. However, the type II of programmed cell death or autophagy has only recently met the interest of researchers in human cancer. There is no clear separation between the two types of programmed cell death and molecular switching points between apoptosis and autophagy can occur, mostly related to the nature of external death signals (31-34). Furthermore, morphologic features of autophagic and apoptotic cell death can be observed in the same cell (35). Induction of autophagy is usually accompanied by increase of Beclin 1 and microtubule-associated protein I light-chain 3 cellular content, the latter being considered a measure of autophagosome formation, i.e. of the double-membrane structure containing undigested cytoplasmic materials including organelles, that fuses with the lysosomal membrane eventually forming the degrading structure termed the autolysosome/autophagolysosome during the autophagic process (36). *Beclin 1* is a tumour suppressor gene that is thought to be involved in both apoptosis and autophagy (1,37,38), and probably constitutes a link between these two

processes (15,39). Its product, a 60-kDa protein, was able to interact with the prototypic apoptosis inhibitor Bcl2 (1,40), and there is a large body of evidence that it plays a fundamental role in autophagy. It has been ascertained that autophagy is positively regulated by the PTEN tumour suppressor gene and by class 3 PI3k products, and negatively regulated by the oncogenic class I PI3k signaling pathway (41). Beclin 1 complexes with Vps34/class 3 PI3k and interacts with some regulators of autophagy that are recognized to be involved in routes leading to tumour progression and possible targets of novel therapies in glioblastoma (1,22,25-27).

In some neoplasms, including glioblastoma, it is becoming evident that induction of autophagic death rather than of apoptosis and/or enhancing the autophagy-dependent death potential of apoptosis-resistant tumour cells would be a novel approach to reduce tumour resistance to therapies (17,42). On the other hand, autophagy provides a mechanism of self-defense for cancer cells, and the use of autophagy inhibitors may be hypothesized to hamper the defensive autophagy in cancer cells induced by therapy (27). In human glioblastoma, both spontaneous and therapy-induced autophagic cell death occur, and temozolomide and rapamycin inhibitors, that are used as chemotherapeutics in glial tumours, are powerful inducers of autophagy (43). Temozolomide, as well as radiation at low dosage, have been found to induce protective autophagy (16,27,43). Autophagy has been found to occur in normal brain (13,14). One of the autophagy regulators, the gene *Beclin 1* was first cloned from a normalized human infant brain cDNA library, and a widespread expression was thereafter demonstrated in various human tissues by Northern blot analysis (24,44). It has also been investigated in some human cancer cell lines, including glioblastoma, in a few studies



(1,3,25). To date, in only a few cancer types its expression has been evaluated at the tissue level (1,15,41). In the nervous system, beclin 1 was found to be involved in processes of autophagic cell death and, in human brain, an increased Beclin 1 expression has been observed in some pathological conditions including injury (36,45,46). To our knowledge, there are not, to date, studies that examined Beclin 1 protein and gene expression at the tissue level in human brain neoplasms. The large majority of brain tumours we studied were glial (astrocytic) tumours and meningiomas. In accordance with findings reported in other types of cancer in the literature (1,15,41), also in glioblastoma we observed a lower Beclin 1 mRNA expression when compared to the apparently normal brain tissue fragment removed from the same subject. However, our observations are limited, because of the small number of cases we examined, due to the difficulty of obtaining noncancerous tissue in brain tumours. Concerning the protein expression, we observed positivity to Beclin 1 also in normal cells, especially in pyramidal neurons and oligodendrocytes. We usually observed a trend towards a decrease of Beclin 1 mRNA and cytoplasmic protein expression with increasing tumour grade in glial neoplasms as well as in meningiomas, usually with a shift towards a nuclear protein expression. It seems that the protein Beclin 1 is able to shuttle between the nucleus and the cytoplasm, and that the nuclear protein is not involved in autophagy regulation and tumour growth inhibition (44). Mutant Beclin 1 was found to localize in the nucleus (44). The association of a prevalently nuclear expression in high-grade tumours with a decreased cytoplasmic expression in the present study suggests a loss of gene function. *Beclin 1* is today considered a tumour suppressor gene (2,21), although its role in tumour suppression has been related to inhibition of cellular proliferation, rather than to cell death induction (11). We noticed that, in glial and meningeal tumours positive to Beclin 1, mitotic cells maintained protein expression, either nuclear or cytoplasmic, whereas most cells showing features of programmed cell death were negative; the latter were, instead, positive to Beclin 1 in medulloblastomas. These observations need further investigation focusing on possible correlation between gene expression and the extent of tumour cell death and proliferation on tissue sections of different tumour types. In 4 out of 5 cases of glioblastoma recurrences that we analysed, Beclin 1 cytoplasmic protein expression was higher than in the primary tumours (data not shown), whereas nuclear expression was unaltered (it scored 2 both in the primary and in the recurrences); mRNA was not examined. Although the limited number of cases does not allow us to draw any reliable conclusion, we can hypothesize that radiation might have contributed to the increase of Beclin 1 cytoplasmic protein expression in these cases, directly or through the action of intermediate factors. In a study on two cell lines, radiation was proved to stimulate the production of ceramide that, in turn, was found to promote autophagy and tumour cell death both by inhibiting the class I PI3k negative signaling pathway and by stimulating the expression of Beclin 1 (47).

Interestingly, we found a significantly higher ( $p < 0.0001$ ) mRNA Beclin 1 expression level in meningiomas when compared to glial tumours, which suggests differences of gene involvement in different brain tumour histotypes. Accordingly, in medulloblastomas, although our study was limited to a few

cases, we noticed that there was a relatively high mRNA and cytoplasmic protein expression of Beclin 1, which prompts us to hypothesize a possible involvement of Beclin 1 in medulloblastoma tumour cell survival and/or death. Apoptosis is thought to be the main type of cell death in medulloblastoma, however, it is worth studying the importance of autophagy and regulator genes in this type of tumour, that shows some analogies with neuroblastoma, in which the occurrence of autophagic processes has been ascertained, although their role has not been clarified (48,49). The present study underlines the relevance of Beclin 1 in the pathobiology of brain tumours. The decrease of its cytoplasmic expression, thought to be linked to the functional protein, that we observed in most high-grade glial tumours and atypical meningiomas, furtherly supports its role as a tumour suppressor in these neoplasms. In medulloblastomas and in the other high-grade brain tumours that showed a strong cytoplasmic expression, we hypothesize that the protein is involved also in other mechanisms, allowing for tumour cell-survival. However its role, whether tumour-defensive or cancer-suppressive via the elimination of cancer cells by triggering cell death, and its possible therapeutic implication, warrant further investigation. Correlation with other biological parameters, such as tumour cell turnover, vascularization, including comparison between the primary tumours and recurrences, and the patient status, could add to the knowledge of the role of this gene in brain neoplasms.

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