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Review

The prognostic role of Beclin 1 protein expression in high-grade gliomas

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High-grade gliomas (HGG) have a poor outcome, however, prognostic subgroups of patients may be individuated by some clinico-biological parameters. It was recently demonstrated that the main response of HGG to therapy is autophagic death. Autophagy is involved in tumor suppression, and is defective in HGG, in which we previously found an underexpression of *beclin 1* autophagic gene protein product. Underexpression of Beclin 1 protein has been correlated to poor patient outcome in other tumor types. In this paper, the prognostic role of Beclin 1 expression in HGG patients was investigated. We first evaluated the tumor cell cytoplasmic expression of Beclin 1 protein (BPCE), in a sample of 76 HGG by immunohistochemistry, and compared it with cell proliferation and apoptosis. We found high BPCE score positively correlated with apoptosis, and negatively with cell proliferation ($p < 0.05$). We then correlated BPCE score with survival and other prognostic parameters (histological grading, MGMT gene methylation status, age, patient performance status according to the Karnofski classification (KPS), extent of surgery, radiation therapy (RT) modality, temozolomide chemotherapy (TMZ CHT), and optimal/suboptimal post-surgical treatment). Forty-seven (61.8%) and twenty-nine (38.2%) patients showed high and low BPCE scores, respectively. BPCE showed statistically significant correlations with survival both at the univariate ($p = 0.03$) and multivariate analysis ($p = 0.037$). High BPCE was also positively correlated with high KPS values ($p = 0.023$), and with the accomplishment of an optimal postoperative therapy ($p = 0.037$). Furthermore, among patients showing a MGMT methylated gene, survival was significantly higher in cases with a higher BPCE score. BPCE score might be added to pathological evaluation of HGG for prognostic purposes.

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Introduction

High-grade gliomas (HGG) have a poor prognosis, although a classification (*Recursive Partitioning Analysis*) into various categories of patients with different life expectancies was made by Curran et al. several years ago.[1] This study demonstrated statistical correlations of the survival time with many factors, such as: the histological grading of the tumor (grade III, anaplastic astrocytoma, AA, vs. grade IV, glioblastoma, GB), some characteristics of the patients (age, Karnofski performance status [KPS], neurological status), and treatment modalities (extent of surgical resection, dose of postoperative RT). More recently, the methylation status of the O6-methylguanine-DNA methyltransferase (MGMT) gene promoter revealed to be an independent prognostic factor and a powerful predictor of response to therapy, to be added in the patho-biological characterization of HGG.[2] Biological profiling of HGG is under continuous investigation, with the purpose of further improving the accuracy of prognosis and therapy selection. Standard treatment for HGG patients, i.e., maximal surgical removal of the tumor and postoperative radiation therapy (RT) combined with temozolomide chemotherapy (TMZ CHT), still yields unsatisfactory results; this is mainly due to high resistance of infiltrative tumor cells to programmed cell death (PCD).[3] Apoptotic PCD is limited in GB (i.e., the vast majority of HGG), and much research is addressed to both increase it, as well as to discover novel targetable pathways and molecules involved in other types of noncaspase dependent PCD.[4,5] Type II (autophagic) PCD is emerging as a main target in HGG, both in experimental and clinical studies.[4-7] Macroautophagy (hereafter called "autophagy") is a major self-digesting pathway that allows for cell survival under stress conditions.[8] It has recently been found deregulated in human cancer, and most studies indicate its involvement in cell growth control and tumor suppression.[9] Extreme autophagy may determine cell death, and autophagy is indispensable for the execution of apoptosis.[10] In humans, about 18 autophagic genes (Atg) have been identified, so far.[9] They are distinct from genes involved in apoptosis, although the two processes are strictly interconnected.[11] *Beclin 1* (Atg 6 in yeasts), a gene indispensable for the first phases

Table 1 **Frequency counts and percentages (in round brackets) of main features of patient sample at presentation and treatment modality**

Variable	Description	Low BPCE score	High BPCE score	Overall	p
Age	≤50 y	4 (23.5%)	13 (76.5%)	17 (22.4%)	>0.05
	>50 y	25 (42.4%)	34 (57.6%)	59 (77.6%)	
KPS	80–100%	18 (31.0%)	40 (69.0%)	58 (76.3%)	0.023
	≤70%	11 (61.1%)	7 (38.9%)	18 (23.7%)	
Grading	Grade III (AA)	1 (14.3%)	6 (85.7%)	7 (9.2%)	>0.05
	Grade IV (GB)	28 (40.6%)	41 (59.4%)	69 (90.8%)	
Surgery	Gross radical resection	7 (33.3%)	14 (66.7%)	21 (27.6%)	>0.05
	Partial resection/Biopsy	22 (40.0%)	33 (60.0%)	55 (72.4%)	
RT	Radical, PBI, D = 60–70 Gy	19 (38.0%)	31 (62.0%)	50 (65.8%)	>0.05
	Palliative, WBI, D ≤50 Gy	10 (38.5%)	16 (61.5%)	26 (34.2%)	
TMZ CHT	Yes	11(26.2%)	31 (73.8%)	42 (55.2%)	0.020
	No	18 (52.9%)	16 (47.1%)	34 (44.7%)	
Treatment	Optimal	8 (24.2%)	25 (75.8%)	33 (42.1%)	0.037
	Sub-optimal	21 (47.7%)	22 (52.3%)	43 (57.9%)	
Ki67	Number of cases (%)	29 (38.2%)	47 (61.8%)	76 (100%)	<0.001
	Median/range	50/10–80	25/8–70	30/8–80	
Apoptosis	Number of cases (%)	29 (38.2%)	47 (61.8%)	76 (100%)	0.002
	Median/range	2/0–5	3.5/0–13	3/0–13	
MGMT	Unmethylated	12 (52.2%)	11 (47.8%)	23 (44.2%)	>0.05
	Methylated	14 (48.3%)	15 (51.7%)	29 (55.8%)	

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For quantitative variables, median and range of variation are also underneath added. p-values in last column refer to Fisher exact and Mann-Whitney tests, respectively for correlation analysis of 2 x 2 contingency tables and comparison of quantitative variables. KPS = Karnofski Performance Status; AA = Anaplastic Astrocytoma; GB = Glioblastoma; RT = Radiotherapy; D = Dose; PBI = Partial Brain Irradiation; WBI = Whole Brain Irradiation; TMZ = Temozolomide; CHT = Chemotherapy.

of autophagy, was found deregulated in cancer.[9,12] Its protein product is reduced during gliomagenesis, being progressively less expressed through Grade I to Grade IV gliomas.[13]

In this study we matched the cytoplasmic expression of Beclin 1 protein (BPCE), which is thought to be linked to its autophagic function, with tumor cell proliferation and apoptotic death in tumor sections, as well as with patient survival and with prognostic parameters of clinico-biological relevance, in a series of 76 HGG patients.

Results

Patients. There were 76 HGG, including 7 AA and 69 GB. Main conventional, clinical prognostic factors, i.e., age, KPS, extent of surgical resection, RT modality and dose, and TMZ CHT administration are listed in Table 1. Age and KPS were binarily categorized using respectively the values of 50 years and 70% as cut-off levels.

In Table 1, the distinction of patients based on "optimal" vs. "sub-optimal" postoperative therapeutic management is also given as an additional either favorable or unfavorable prognostic factor, respectively, according to a previous experience.[14] We considered as an "optimal" management both the high-dose (60–70 Gy) partial brain irradiation (PBI) delivered with a conformal technique after a three-dimensional planning (3D CRT), and the TMZ CHT administration at least during the phase concomitant to RT.

Beclin 1 immunohistochemistry and BPCE score. Beclin 1 variably stained tumor cell cytoplasm, and a score was given in each case (Fig. 1). A nuclear positivity, which was also scored, was observed in tumor cells. A high and a low BPCE score was assigned to 47 (61.8%; high BPCE score group), and 29 (38.2%; low BPCE score group) cases, respectively.

BPCE score and clinical prognostic parameters. A statistically significant correlation was found between BPCE score and KPS, TMZ CHT, and treatment modality (Table 1).

BPCE score, tumor cell proliferation and apoptosis. Statistically significant differences in proliferating tumor cells, as identified by Ki-67 nuclear expression, and apoptosis numbers between high and low BPCE score groups were found (Fig. 1; Table 1). A negative correlation was found for the percentage of proliferating cells, and a positive correlation for apoptosis number (Table 1).

MGMT gene promoter methylation status. MGMT status was assessed in 52 (68.4%) out of 76 HGG. There were 29/52 (55.8%) MGMT methylated, and 23/52 (44.2%) unmethylated cases (Table 1).

BPCE score and MGMT. MGMT methylated and unmethylated cases were almost equally distributed among the two BPCE score groups. Among methylated cases, 15 (51.7%) belonged to high BPCE score group, and 14 (48.3%), to low BPCE score group; among unmethylated cases, 11 (47.8%) belonged to high BPCE score group, and 12 (52.2%) to low BPCE score group.

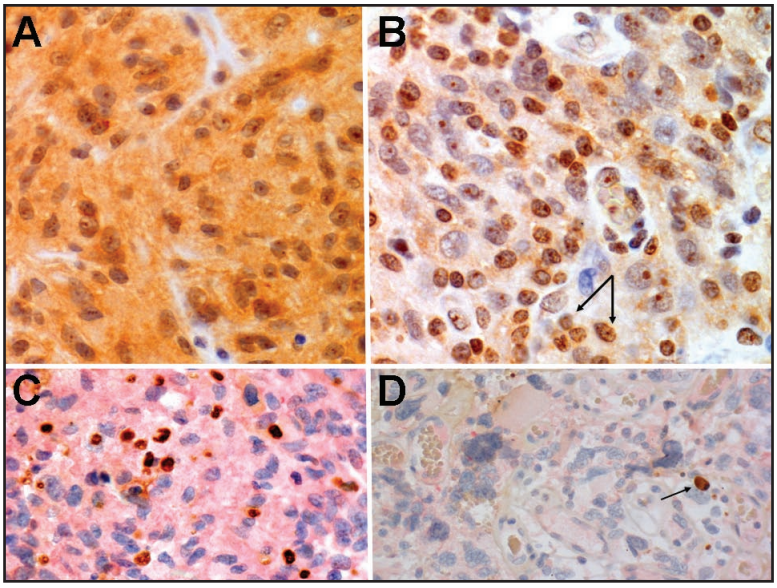

Figure 1. Beclin 1 immunohistochemistry. Representative cases of high (A), and low (B) BPCE score groups. Strongly positive, brownish tumor cell cytoplasms in (A); negative or weakly-stained cytoplasms in (B). Positive nuclei are also shown (B, arrows). TUNEL-positive apoptotic nuclei in a GB with high (C), compared to a GB with low (D) BPCE score (D, arrow: an isolated apoptotic cell) (A and B) Beclin 1 immunohistochemistry; Avidin-biotin method; chromogen: diaminobenzidine; original magnification, x400 (C and D) Double stain for apoptoses (TUNEL; chromogen: diaminobenzidine), and beclin 1 (immunohistochemistry; chromogen: new fuchsin); original magnification, x200.

There were no significant differences between MGMT methylation status and BPCE score (Table 1).

Nuclear positivity, BPCE score and prognostic parameters. Nuclear Beclin 1 positivity did not correlate with BPCE score nor with any other prognostic parameters.

Statistical survival analysis and correlations. Table 2 gives survival sample data descriptive statistics as median and patient survival percentage evaluated at 1 and 2 years. In the whole data sample median, 1- and 2-year survival values were 12 months, 50% and 25%, respectively. All the considered parameters, that is, grading, age, KPS, BPCE score, extent of surgery, RT modality and dose, TMZ CHT administration, and the administration of an "optimal" postoperative treatment, showed statistical significance at the monovariate analysis (log-rank test, $p < 0.05$) as prognostic variables for survival (Table 2). The Kaplan-Meier survival analysis, distinguished for low and high BPCE score in all patients, and stratified for KPS, is also shown in Figure 2. No significant correlation was found between nuclear positivity and survival. In the group of 52 patients analyzed for MGMT methylation status, the latter was a significant survival predictor ($p < 0.0001$).

Among patients with methylated MGMT gene, survival time was significantly higher in patients showing high BPCE (median survival 36 months vs. 15 months in high and low BPCE score groups, respectively). BPCE, instead, did not affect survival in the unmethylated MGMT, unfavorable prognostic group of patients.

The results of Cox regression model of multivariate survival analysis are reported in Table 3. All the clinical prognostic variables, as well as BPCE score, were entered in the Cox model. The odds ratio, OR, expresses the increased risk for death for each unfavorable parameter considered. All the prognostic variables showed statistical significance at the multivariate analysis. Lower bounds of 95% confidence interval of all ORs were greater than 1, enforcing significance of OR estimates. In particular, the highest OR, equal to 6.10 is associated to KPS ≤70%, whose risk is estimated more than six (three, in the worst condition of lower 95% CI bound) times higher than KPS >70%.

Table 2 **Survival results and monovariate analysis (log-rank test)**

		Median survival (months)	1 y	2 y	p
Whole series		12 m	50%	25%	
Age	≤50 y	18 m	70%	47%	0.035
	≥50 y	11 m	42%	18%	
Grading	AA	29 m	71%	71%	0.014
	GB	11 m	46%	19%	
KPS	80–100%	17 m	62%	32%	0.000
	≤70%	4 m	6%	0%	
BPCE	High score 47 (61.75%)	15 m	56%	32%	0.030
	Low score 29 (38.25%)	10 m	37%	11%	
Surgery	Gross total resection	25 m	85%	55%	0.000
	Partial resection/Biopsy	8 m	36%	13%	
RT	Radical, PBI, D = 60–70 Gy	16 m	64%	34%	0.000
	Palliative, WBI, D ≤50 Gy	4 m	19%	5%	
TMZ CHT	Yes	17 m	64%	32%	0.001
	No	5 m	27%	15%	
Treatment	Optimal	20 m	73%	39%	0.030
	Sub-optimal	6 m	30%	13%	
MGMT	Unmethylated	4 m	13%	8%	0.000
	Methylated	25 m	72%	51%	

MGMT methylation status was analyzed in 52 out of 76 patients. AA = Anaplastic Astrocytoma; GB = Glioblastoma; KPS = Karnofski Performance Status; BPCE = Beclin1 Protein Cytoplasmic Expression; RT = Radiotherapy; PBI, = Partial Brain Irradiation; WBI = Whole Brain Irradiation; TMZ = Temozolomide; CHT = Chemotherapy; MGMT = Gene Promoter Methylation Status.

Discussion

The overall survival values obtained are similar to those reported by others.[15] Moreover the impact on survival of standard prognostic variables, such as age, histology grade, KPS, extent of surgery, RT, TMZ CHT administration, and MGMT methylation status is in line with literature data.[1,2,15]

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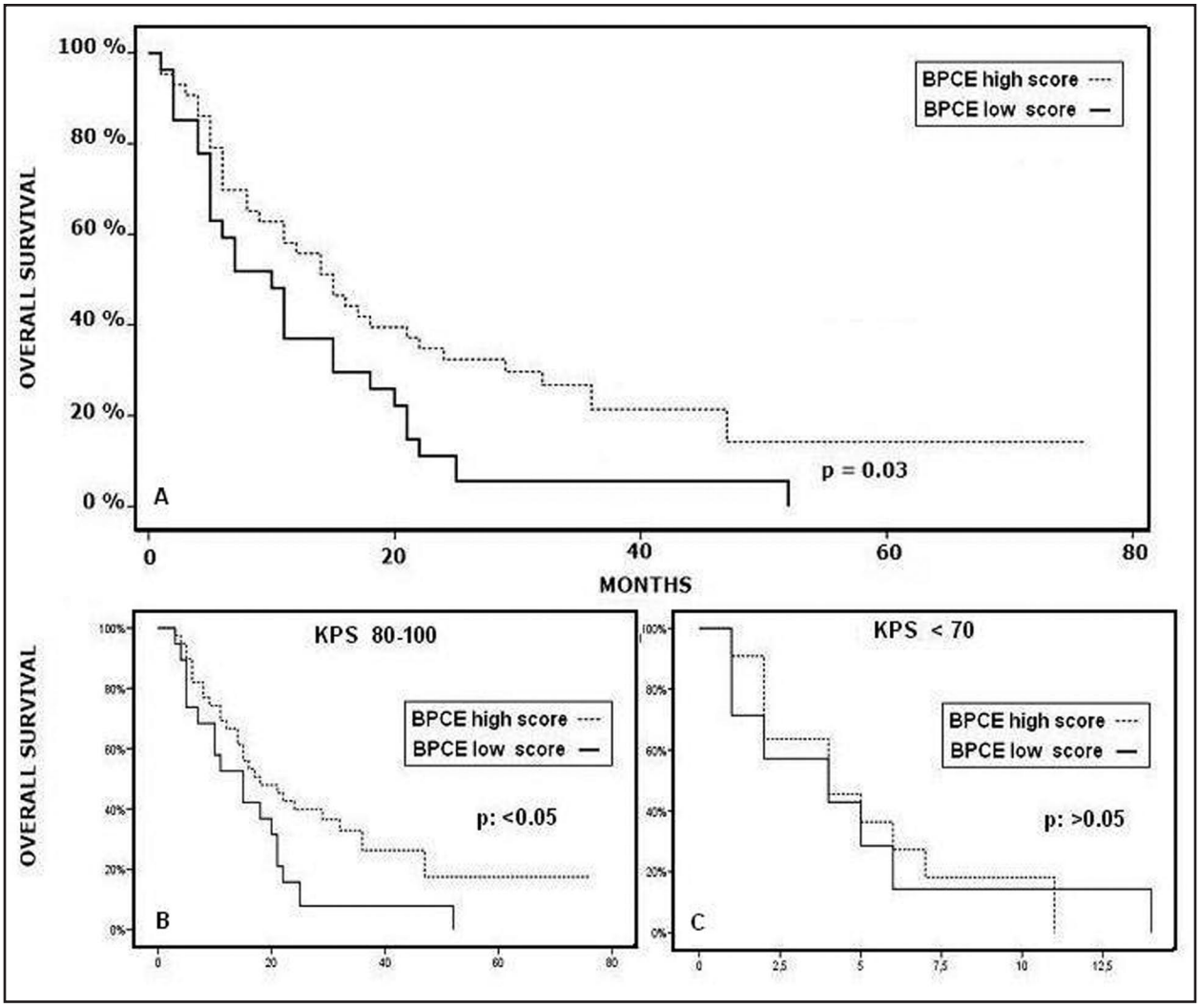

Figure 2. Comparison of Kaplan-Meier survival curves between high and low BPCE score (log-rank test, p = 0.03) in all patients (A). Stratification analysis for high (p < 0.05) (B) and low (p > 0.05) (C) KPS values.

The novelty of the present study is the prognostic relevance of a biological parameter, the cytoplasmic expression of Beclin 1 protein (BPCE), which is easily viewable on tumor tissue section. Beclin 1 is a main actor of autophagy, a major process for cell proteins and organelles degradation and recycling, recently found altered in human cancer, and most studies indicate its role as tumor suppressor.[8,9] Increasing data from the literature support the relevance of autophagy in glial neoplasms. Autophagic cell death, rather than apoptosis, has been induced in glioblastoma cells by radiation and chemotherapeutic molecules, including TMZ and mTOR inhibitors.[4,6,7,17,18] mTOR (the mammalian target of rapamycin and of other new rapamycin analogs), involved in one of the most frequently activated pathways in GB, besides promoting tumor cell proliferation, inhibits autophagy.[4] Investigation into autophagy machinery in cancer is indeed growing, aiming at both finding novel therapeutic targets, and improving the effect of current therapies.[4,5] Autophagic pathways are guaranteed by the functionality of related genes and their products. Allelic loss and/or reduced cytoplasmic cell expression of the

Table 3 **Multivariate analysis (Cox regression test)**

	OR	p	CI (95%)
Age >50	1.97	0.044	1.02–3.81
Grading: GB	3.86	0.025	1.19–12.59
KPS ≤70%	6.10	0.000	3.22–11.55
BPCE: Low score	1.74	0.037	1.03–2.94
Surgery: Partial resection/Biopsy	3.48	0.000	1.78–6.81
RT: Palliative, WBI, D ≤50 Gy	3.50	0.000	2.02–5.95
TMZ CHT: No	2.24	0.002	1.36–3.71
Treatment: Sub-optimal	2.57	0.000	1.52–4.33

Odds ratio (OR), p values, and confidence interval (CI) are reported. GB = Glioblastoma; KPS = Karnofski Performance Status; BPCE = Beclin1 Protein Cytoplasmic Expression; RT = Radiotherapy; WBI = Whole Brain Irradiation; TMZ = Temozolomide; CHT = Chemotherapy.

autophagic gene *beclin 1* correlates with impaired autophagy and development and/or progression of neoplasms, both at the experimental and human clinical level.[9,12] Defective *beclin 1* and tumor progression are found in ovarian, breast and prostatic human cancer.[9] High levels of *beclin 1* expression are strongly associated with longer survival in esophageal and stage IIIB colon cancer patients, and low protein levels positively correlate with malignant phenotype and poor prognosis in hepatocellular carcinoma.[9,19-21] Furthermore, *beclin 1*-dependent autophagy is either decreased or enhanced by oncogenes and tumor suppressors, respectively.[9] Altogether, these data support an involvement of *beclin 1* in tumor suppression. *beclin 1*-dependent autophagic death seems to occur in case of defective apoptosis, and *beclin 1* appears to govern both processes.[10,11] In a previous study,[13] we found that the cytoplasmic protein expression of *beclin 1* (which mirrors its autophagic functionality) is decreased in high-grade, vs. low-grade glial neoplasms, suggesting a loss of gene function. We observed that, whereas all low grade (Grade I and II) gliomas highly express the protein in the cytoplasm, 54.5% of high grade (Grade III and IV) gliomas are either negative or weakly positive to Beclin 1; conversely, the percentage of cases highly expressing the protein in the nucleus increase from 53.3% in low grade, to 85.2% in high grade gliomas. Beclin 1 expression is, therefore, heterogeneous among high grade gliomas. These results prompted us to investigate whether Beclin 1 protein expression could individuate subgroups of high grade gliomas bearing a different prognosis. In the present study, we report a positive correlation of a high BPCE score with patient survival and with high KPS values. Beclin 1 nuclear positivity, which is associated with a loss of gene functionality,[22,23] instead, did not differ between subgroups of patients. In our experience, a decrease of BPCE score accompanied an increase of tumor cell proliferation, and a decrease of apoptosis, in line with previous observations.[24] Defective autophagy might synergize with altered apoptosis and increased tumor cell proliferation, inducing tumor progression and therapy resistance, partly justifying a higher tumor aggressiveness and poorer prognosis in patients with low BPCE score. The impact of Beclin 1 pathways on apoptosis is still a matter of fervent investigation and debate. There is evidence that Beclin 1 is involved in both PCD types, that one type of PCD may switch to another type, and that both PCD pathways may be triggered under certain circumstances.[10,11,25-28] Therefore, there is a considerable crosstalk between apoptosis and autophagy, and the autophagy machinery might be used to promote apoptosis.

We cannot assess whether the association we found between decreased BPCE score, increased proliferation, and decreased apoptosis directly depend on an impaired Beclin 1 functionality or on a more complex *beclin 1* and PCD negative regulation by other genes. Differences in BPCE score, however, which mirror differences of tumor cell context and biological profiles, might allow for selection of patients destined to distinctive postoperative therapies.

The involvement of autophagy in GB is also supported by investigation on another gene, *LC3*, which is so far considered the most reliable marker of autophagy. High *LC3* expression was recently associated with an improved survival in GB patients with poor performance score, whereas in patients with normal performance its low expression correlates with better survival.[29] In our study, high *beclin 1* expression positively correlated with a higher performance score, whereas it did not affect survival in patients with poor KPS. These apparently contrasting data might mirror differences in the two genes and in related pathways, as well as the dual nature of autophagy, which might lead either to cell survival or death depending on tumor cell context and on tumor histotype, according to some authors.[30] These remarks also support the need of further investigation on autophagy and related processes in individual patients.

Tumor tissue is a precious source of information for assessing a biological profile of the disease in each patient: therapeutic strategies might be envisaged either to potentiate or decrease autophagy, depending on tumor cell context and/or on histotype, in order to improve clinical response.[5] Based on our results, we suggest that a high expression of the cytoplasmic protein product of *beclin 1* might represent a positive prognostic indicator in HGG, which is in line with investigations in other tumors.[19-21] Both the monovariate (median survival of 15 months, 56% survival value at 1 y and 32% at 2 y for high score, vs. 10 months, 37% and 11% for low score, p = 0.03) and the multivariate analysis (OR 1.743 for low score, p = 0.037, CI 1.033–2.940) confirm that high BPCE scores are associated with a relatively better survival in HGG patients (Tables 2 and 3, Fig. 2). Furthermore, high BPCE scores are significantly associated with patients with good KPS values, indicating suitability for optimal postoperative therapy (Table 1). Due to the small size of the study, we cannot infer whether these correlations are related to an inherently, relatively favorable clinical course of the cases showing high BPCE score, or these tumors more effectively respond to RT or TMZ CHT, in a comparison with the other cases. In fact, the possible role of BPCE as an independent predictor of prognosis should be verified by considering other biological parameters. In our study, the MGMT status, which is so far considered one of the most reliable biological prognosis predictor,[2] was assessed in 68.4% of the evaluated cases. It was a powerful prognosis indicator also in our cases; however, among MGMT methylated gene patients, high BPCE score was an additional favorable prognosis factor, with a 36 month-median survival for patients showing a high score vs. 15 month-median survival for patients showing a low score. This suggests a possible independent prognostic value of BPCE.

In conclusion, the results of the present study seem encouraging. Extending investigation on a larger series of patients to validate the association of BPCE with patient outcome and exploiting other autophagy-related pathways and players in tumor tissue might give further insight into the relevance of autophagy in HGG patients.

Patients and Methods

Patients. A sample of 76 HGG, representative of patient population, referred after surgery to the Section of Radiological Sciences (Unit of Radiotherapy) of the Department of Human Pathology and Oncology of the University of Siena from January 2001 to December 2007, entered the study. Tumor sample analyses as well as diagnoses were performed at the Section of Pathological

Anatomy of the same department. The informed consent was obtained from all patients for all therapeutic procedures. The present study was performed in accordance with the ethical standards of the Helsinki Declaration in 1975, as revised in 1983, after approval of the local ethics committee.

Post-surgical management. All the patients were submitted to a postoperative treatment schedule including RT and CHT, after a protocol-driven selection, as described.[14] Based on this treatment protocol, "optimal" postoperative therapy could not be accomplished in some patients. High-dose RT was in fact delivered only to tumors not exceeding 6.5 cm in diameter, and TMZ CHT was not administered to patients with low KPS. All patients underwent computed tomography (CT) and/or magnetic resonance (MR) with contrast enhancement for treatment planning.

Molecular characterization of tumor samples for the methylation status of the MGMT gene promoter. MGMT gene promoter methylation was assessed in 52 out of the 76 (68.4%) cases, by using methylation-specific Polymerase Chain Reaction (PCR). Briefly, genomic DNA was extracted from paraffin tumor sections and treated with sodium bisulfite using the EZ DNA Methylation-Gold kit (HISS Diagnostics, GmbH, Freiburg, Germany). Primer sequences were used to detect methylated and unmethylated MGMT promoter sequences. PCR products were separated on 2% agarose gel. A glioma cell line with a completely methylated MGMT promoter, and peripheral blood mononucleated cells, served as positive and negative control samples, respectively.

Immunohistochemistry and assessment of BPCE and cell proliferation in tumor samples. Immunohistochemistry was performed as described[13] on 4-μm thick sections of the most representative tumor areas. The anti-human Beclin 1 rabbit polyclonal antibody BECN1 (M-300) (Santa Cruz, DBA-Italia, Milan, Italy; catalog number SC-11427, diluted 1:50), and the anti-human Ki67 rabbit monoclonal MIB1 antibody (clone SP6; Bio Optica; catalog number RM.9106-5, diluted 1:200), were applied, after pretreatment with WCAP citrate buffer pH 6.0 (Bio-Optica, Milan, Italy) for 40 min at 98.5°C. We used a streptavidin-biotin kit (Biogenex, Menarini, Florence, Italy), and diaminobenzidine, which developed a brownish stain in positive cells. Meyer's hematoxylin served as counterstaining. For each case and for each antibody, a negative control was obtained by replacing the specific antibody with non-immune serum immunoglobulins at the same concentration of the primary antibody. Staining was independently evaluated by two of the authors (CM, VM), as described.[13] BPCE was scored 0 if negative, and from 2 to 5, if positive, on the basis of both the stain intensity (1–3) and the percentage of positive cells (1: ≤50%, 2: >50%). We considered score 0–2 as a low, and scores 3–5 as a high protein expression, respectively. For nuclear positivity, cases with <10% of positive nuclei were considered negative (score 0); cases with >10–50% of positive nuclei were scored 1, and cases with >50% positive nuclei were scored 2. Cell proliferation was assessed by counting Ki67-positive tumor cell nuclei in at least 10 high power (x400) fields; counts were expressed as the percentage among tumor cell nuclei.

TUNEL assay and apoptosis assessment. The TUNEL reaction was carried out with the ApopTag Plus Peroxidase In Situ Apoptosis Detection kit (Chemicon International, S7100 catalogue number) according to the supplier's instructions. Briefly, after dewaxing, 5-μm-thick tumor sections were incubated with Proteinase K (20 μg/mL) in phosphate-buffered saline (PBS) for 15 minutes at room temperature and then with 3% hydrogen peroxide in PBS for 5 minutes. After rinsing with PBS and distilled water, sections were incubated with TdT Enzyme and Reaction Buffer (1:3 μL) in a humidified chamber at 37°C for 1 hour, washed, incubated with anti-digoxigenin peroxidase conjugate, then washed again with PBS. Diaminobenzidine served as the peroxidase substrate; the TUNEL$^+$ stained nuclei were dark brown. Counterstain was performed with methyl green. Positive controls were included in the kit. Negative controls were obtained by omitting the TdT enzyme in the TUNEL reactions.

TUNEL positive nuclei were counted by two of the authors (VM, CM) in at least 10 fields (x200), and counts were expressed as the median number per field.

TUNEL-Beclin 1 double stain. In 10 representative cases of both low and high BPCE score samples, TUNEL and Beclin 1 co-staining was also performed to better visualize apoptoses inside beclin 1-immunostained tumor areas. For double staining, after processing with TUNEL, sections were washed, and immunohistochemistry for Beclin 1 was performed, as described above. New Fuchsin Substrate System (Bio-Optica, Milan, Italy), instead of diaminobenzidine, was used as chromogen, which stained red the cytoplasm of Beclin 1-positive cells.

Follow-up. After treatment, all the patients were invited to follow-up visits every three months. The compliance to the follow-up program was poor, due to the dismal prognosis of malignant gliomas, and many patients deceased during the intervals between the controls. Therapeutic results are reported, therefore, only in terms of overall survival after the beginning of the postoperative treatment, on the basis of a registry research carried out on December, 2008. The follow-up period was, therefore, 1–8 years.

Statistical survival analysis and correlations. Overall survival was evaluated by the Kaplan-Meier method and curves. The log-rank test was used for the statistical comparison of survival between binary categories of each prognostic variable and treatment options (monovariate survival analysis). The multivariate survival analysis was executed using the Cox regression model; odds ratio (OR) and corresponding 95% confidence interval (CI) was also estimated. Correlation of frequency rate between low/high BPCE score and binary categories of all other considered prognostic and therapeutic factors was statistically investigated using the Fisher exact test applied to 2 x 2 contingency tables.

All statistical computations were performed using the SPSS-12 software.

A significance level of 95% ($p < 0.05$) was always chosen to verify statistical hypotheses.

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References

1. Curran WJ Jr, Scott CB, Horton J, Nelson J, Weinstein A, Fischbach J, et al. Recursive partitioning analysis of prognostic factors in three Radiation Therapy Oncology Group malignant glioma trials. J Natl Cancer Inst 1993; 85:704-10.
2. Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. N Eng J Med 2005; 352:997-1003.
3. Sathornsumetee S, Rich JN. Designer therapies for glioblastoma multiforme. Ann N Y Acad Sci 2008; 1142:108-32.
4. Lefranc F, Facchini V, Kiss R. Proautophagic drugs: a novel means to combat apoptosis-resistant cancers, with a special emphasis on glioblastomas. Oncologist 2007; 12:1395-403.
5. de Bruin EC, Medema JP. Apoptosis and non-apoptotic deaths in cancer development and treatment response. Cancer Treatment Rev 2008; 34:737-49.
6. Yao KC, Komata T, Kondo Y, Kanzawa T, Kondo S, Germano IM. Molecular response of human glioblastoma multiforme cells to ionizing radiation: cell cycle arrest, modulation of the expression of cyclin-dependent kinase inhibitors, and autophagy. J Neurosurg 2003; 98:378-84.
7. Takeuchi H, Kondo Y, Fujiwara K, Kanzawa T, Aoki H, Mills GB, Kondo S. Synergistic augmentation of rapamycin-induced autophagy in malignant glioma cells by phosphatidylinositol 3-kinase/protein kinase B inhibitors. Cancer Res 2005; 65:3336-46.
8. Xie Z, Klionsky DJ. Autophagosome formation: machinery and adaptions. Nat Cell Biol 2007; 9:1102-9.
9. Chen N, Karantza-Wadsworth V. Role and regulation of autophagy in cancer. Biochim Biophys Acta 2009; doi:10.1016/j.bbamcr.2008.12.013.
10. Gozuacik D, Kimchi A. Autophagy and cell death. Curr Topics Dev Biol 2007; 78:217-45.
11. Moretti L, Cha YI, Niermann KJ, Lu B. Switch between apoptosis and autophagy. Radiation-induced endoplasmic reticulum stress? Cell Cycle 2007; 6:793-8.
12. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, et al. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J Clin Invest 2003; 112:1809-20.
13. Miracco C, Cosci E, Oliveri G, Luzi P, Pacenti L, Monciatti L, et al. Protein and mRNA expression of autophagy gene Beclin 1 in human brain tumors. Int J Oncol 2007; 30:429-36.
14. Pirtoli L, Rubino G, Marsili S, Oliveri G, Vannini M, Tini P, et al. Three-dimensional conformal radiotherapy, temozolomide chemotherapy, and high-dose fractionated stereotactic boost in a protocol-driven, postoperative treatment schedule for high-grade gliomas. Tumori 2009; 95:329-37.
15. Magrini SM, Ricardi U, Santoni R, Krengli M, Luppatelli M, Cafaro I, et al. Patterns of practice and survival in a retrospective analysis of 1722 adult astrocytoma patients treated between 1985 and 2001 in 12 italian radiation oncology centers. Int J Radiat Oncol Biol Phys 2006; 65:788-99.
16. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. European Organisation for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups; National Cancer Institute of Canada Clinical Trials Group. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Eng J Med 2005; 352:987-96.
17. Kanzawa T, Kondo Y, Ito H, Kondo S, Germano I. Induction of autophagic cell death in malignant glioma cells by arsenic trioxide. Cancer Res 2003; 63:2103-8.
18. Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S. Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. Cell Death Differ 2004; 11:448-57.
19. Chen Y, Lu Y, Lu C, 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; 9.
20. Li BX, Li CY, Peng RQ. The expression of beclin 1 is associated with favourable prognosis in stage IIIB colon cancers. Autophagy 2009; 5:303-6.
21. Shi YH, Ding ZB, Zhou J, Qiu SJ, Fan J. Prognostic significance of Beclin 1-dependent apoptotic activity in hepatocellular carcinoma. Autophagy 2009; 5:380-2.
22. Liang XH, Yu J, Brown K, Levine B. Beclin 1 contains a leucine-rich nuclear export signal that is required for its autophagy and tumor suppressor function. Cancer Res 2001; 61:3443-9.
23. Kato K, Hasui K, Wang J, Kawano Y, Aikou T, Murata F. Homeostatic mass control in gastric non-neoplastic epithelia under infection of *helicobacter pylori*: an immunohistochemical analysis of cell growth, stem cells and programmed cell death. Acta Histochem Cytochem 2008; 41:23-38.
24. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, Levine B. Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 1999; 402:672-6.
25. Kim HP, Wang X, Chen ZH, Lee SJ, Huang MH, Wang Y, et al. Autophagic proteins regulate cigarette smoke-induced apoptosis: protective role of heme oxygenase-1. Autophagy 2008; 4:887-95.
26. Cho DH, Jo YK, Hwang JJ, Lee YM, Roh SA, Kim JC. Caspase-mediated cleavage of ATG6/Beclin-1 links apoptosis to autophagy in HeLa cells. Cancer Letters 2009; 274:95-100.
27. Park K-J, Lee S-H, Lee CH, Jang JY, Chung J, Kwon MH, Kim YS. Upregulation of Beclin-1 expression and phosphorylation of Bcl-2 and p53 are involved in the JNK-mediated autophagic cell death. Biochem Biophys Res Commun 2009; 382:726-9.
28. Zhang H, Kong X, Kang J, Su J, Li Y, Zhong J, Sun L. Oxidative stress induces parallel autophagy and mitochondria dysfunction in human glioma U251 cells. Toxicol Sci 2009.
29. Aoki H, Kondo Y, Aldape K, Yamamoto A, Iwado E, Yokoyama T, et al. Monitoring autophagy in glioblastoma with antibody against isoform B of human microtubule-associated protein 1 light chain 3. Autophagy 2008; 4:467-75.
30. Yu L, Strandberg L, Lenardo MJ. The selectivity of autophagy and its role in cell death and survival. Autophagy 2008; 4:567-73.

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