Cyclin D1 Co-localizes with Beclin-1 in Glioblastoma Recurrences: A Clue to a Therapy-induced, Autophagy-mediated Degradative Mechanism?

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Abstract. *Background: Glioblastoma (GB) recurrences are rarely removed, therefore, tissue modifications induced by radiotherapy, and temozolomide chemotherapy are scarcely known. Nuclear cyclin D1 is associated with GB progression and resistance to therapy. We previously found that the expression of autophagic protein beclin-1 is a major determinant of prognosis in GB. Patients and Methods: In 31 patients with primary GB and their recurrences, we investigated the protein expression of cyclin D1 and beclin-1, before and after radiotherapy-temozolomide therapy by immunohistochemistry. Results: Most (20/31) primary GBs were negative for nuclear cyclin D1, and highly expressed beclin-1. In their recurrences, cytoplasmic cyclin D1 positivity was observable, which co-localized with beclin-1. Eleven primary GBs instead exhibited low beclin-1 expression and were positive for nuclear cyclin D1; three of their recurrences exhibited an increase of beclin-1, which co-localized with cyclin D1 in the cytoplasm. Conclusion: Our results suggest therapy-induced degradation of cyclin D1 via autophagy.*

Glioblastoma (GB) is the most common and lethal primary brain tumor in adults (1). Despite current multimodal treatment, which consists of maximal resection, radiation (RT), and temozolomide chemotherapy, the median survival is only about 15 months at most (2). It easily acquires resistance to RT/temozolomide, and relapses, usually within 9-12 months (3). Only about a quarter of relapsed tumors are surgically removed (4, 5), consequently investigations on the effects of RT and temozolomide therapy in GB tissue from patients are rare.

Cyclin D1, required in the G_0-G_1 transition of the normal cell cycle, is a proto-oncogene, which has a relevant role in the initiation and progression of cancer [for review, see (6)]. Its expression correlates with malignancy grade of glioma (7). Cyclin D1 overexpression predicts a poor clinical prognosis in GB, and its inhibition attenuates the invasiveness of human GB cells (8). In actively cycling cells, cyclin D1 shuttles in and out of the nucleus, finally undergoing proteasomal degradation (9). Irradiation is known to increase degradation of cyclin D1 through the ubiquitin–proteasome system (6).

Beclin 1 is a key player of autophagy, the other cornerstone of cellular catabolism, which is involved in multiplex physiological and pathological processes (10). The impact of autophagy in cancer is complex and depends on the cell context (11). In previous studies, in patients with GB, we found that beclin-1 protein expression was positively correlated with survival (12, 13). In GB cells, we found that both RT and temozolomide induce autophagy, and that autophagy modulation sensitizes GB cells to radiation (14, 15), promoting autophagy-related tumor cell death, which has been recognized as the most common type of cell death in GB [for review, see (16)]. An interplay between cyclins and autophagy, as well as between the two major protein degradation systems, autophagy and the proteasome, has recently been shown (17-19).

In this preliminary study, we investigated the protein expression of cyclin D1 and beclin-1 in a series of primary GBs (pGBs), and in their recurrences (rGBs), aiming at assessing therapy-induced effects on these proteins in matched pGB-rGB tissues.

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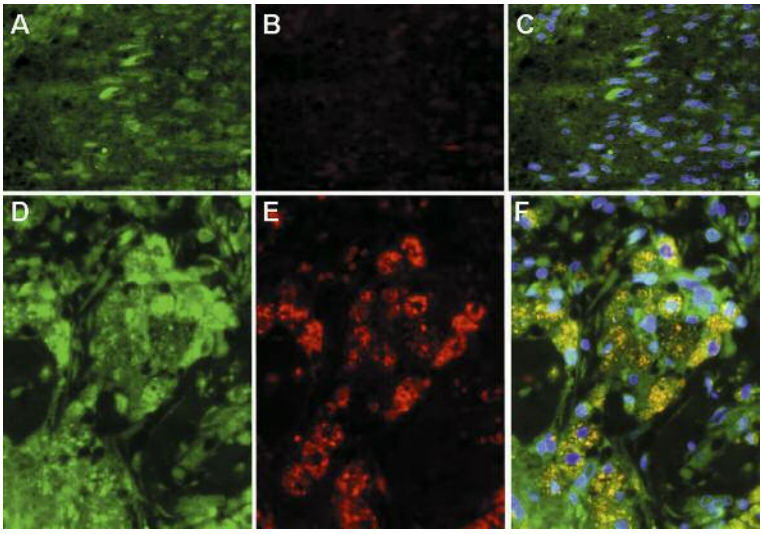

Figure 1. *Double immunofluorescence staining for beclin-1 (A, D; green stain) and cyclin D1 (B, E, red stain). A representative case of a primary glioblastoma highly expressing beclin-1 (A), and negative for cyclin D1 (B) is shown. No cytoplasmic co-localization of the two proteins is evident in the merged image (C). In the recurrent tumor, high beclin-1 (D) and cyclin D1 (E) cytoplasmic positivity is apparent; co-localization of the two proteins is shown in the merged image in the cytoplasm of many tumor cells (F, yellow). Nuclei are marked by staining with 4,6-diamidino-2-phenylindole (merged images C, F). Original magnification x650.*

Materials and Methods

Patients. The study was performed according to the Declaration of Helsinki (1964), and was approved by both the Institutional Review Board and Ethics Committee of the University Hospital of Siena. All patients gave informed consent to the use of their excised tissue.

We retrospectively reviewed the medical records of patients affected by GB [grade IV glioma, according to the WHO Classification (1)] who completed postoperative combined RT-temozolomide treatment from February 2008 to July 2013. According to the standard European Organisation for Research and Treatment of Cancer protocol (2), temozolomide was administered concurrently with RT (continuous daily dose of 75 mg/m^2 during the RT course), followed by the sequential temozolomide schedule (150-200 mg/m^2 for 5 days, q28th day). RT was delivered by a 6-10 MeV linear accelerator X-ray beam, to partial brain volumes with 3D-conformalor intensity-modulated techniques, up to a planned total dose of at least 59.4 Gy, with standard fractionation (1.8-2 Gy/day for 5 days per week). All patients (31 cases) who had a surgically removed recurrence within 1 year (9-12 months) entered the present study. In all cases, O^6-methylguanine-DNA-methyltransferase (*MGMT*) promoter methylation status had been assessed in primary GBs; it was not assessed in the recurrent tumors.

Methylation status of the MGMT promoter. MGMT gene-promoter methylation status was assessed by methylation-specific polymerase chain reaction, as previously described (12, 13). Briefly, genomic DNA was extracted from paraffin-embedded tumor sections and treated with sodium bisulfite using the EZ DNA Methylation-Gold kit (HISS Diagnostics, GmbH, Freiburg, Germany). A glioma cell line with a completely methylated MGMT promoter, and peripheral blood mononucleated cells, served as positive and negative control samples, respectively.

Cyclin D1 and beclin-1 immunohistochemistry. Immunohistochemistry was performed as described elsewhere (12, 13). Briefly, 3-μm thick paraffin sections of 10% formalin-fixed tumor fragments were dewaxed and rehydrated. Before applying anti-cyclin D1 mouse monoclonal (NCL-L-CYCLIN D1-GM clone P2D11F11, diluted 1:50; Novocastra, Milan, Italy), or anti-beclin-1 rabbit polyclonal (B6186, diluted 1:200; Sigma-Aldrich Milan, Italy) primary antibodies, sections were pre-treated either with Pronase XIV of *Streptomyces griseus* (Bio-Optica, Milan, Italy) at 37˚C for 10 minutes, or with WCAP citrate buffer (pH 6.0) (Bio-Optica), for 40 min at 98.5˚C, respectively.

The evaluation of the signal was performed by UltraVision LP Large Volume Detection System HRP Polymer (Bio-Optica) with O^6-methylguanine-DNA-methyltransferase diaminobenzidine as chromogen (Dako, Milan, Italy) for 8 min. Sections were then counterstained with Meyer's hematoxylin. In all cases, negative controls were performed by repeating the procedure and omitting the primary antibody.

Assessment of immunostaining. Staining was independently evaluated by two of the authors (CM, MT) at medium resolution (×20 objective) throughout all tumor sections. Cyclin D1-positive nuclei were counted, and expressed as a percentage of all tumor cell nuclei. We considered cases with fewer than 10% cyclin D1-positive nuclei as negative, and those with 10% or more cyclin D1-positive nuclei as positive. Beclin-1 cytoplasmic positivity was scored 0 if negative, and from 2 to 5, if positive, on the basis of both the staining intensity and the percentage of positive cells, as described elsewhere (12, 13). We considered scores 0-2 as low (L-Becl), and scores 3-5 as high beclin-1 protein expression, respectively.

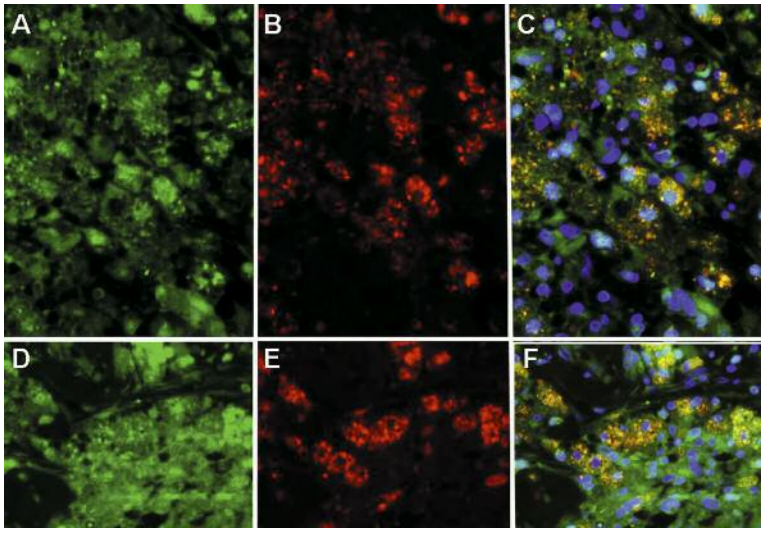

Figure 2. Double immunofluorescence staining for beclin-1 (A, D; green stain) and cyclin D1 (B, E, red stain). Another two recurrences of glioblastoma showing high beclin-1 (A, D) and cyclin D1 (B, E) cytoplasmic expression; co-localization of the two proteins is shown (C, F, yellow). Nuclei are marked by staining with 4,6-diamidino-2-phenylindole (merged images C, F). Original magnification ×650.

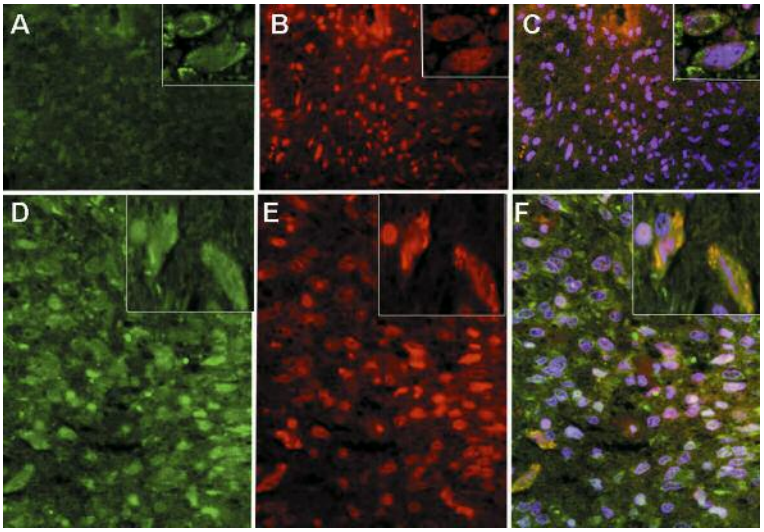

Figure 3. *Double immunofluorescence staining for beclin-1 (A, D; green stain) and cyclin D1 (B, E, red stain). A low beclin-1-expressing primary GB (A), positive for cyclin D1 (B) is shown; no co-localization of the two proteins is apparent in rare beclin-1-positive tumor cells (A, B, C; C, inset). High beclin-1 expression (D) is evident in the recurrence, which is positive for cyclin D1 (E). Co-localization of the two proteins can be seen in several tumor cells (D, E, F; F, inset). Nuclei are marked by staining with 4,6-diamidino-2-phenylindole (merged images C, F). Original magnification: ×650, inset: ×1,000.*

Double cyclin D1/beclin-1 immunofluorescence stain. Double immunofluorescence was performed as described elsewhere (13). Briefly, 4-μm-thick sections were dewaxed in xylene and rehydrated in graded ethanol solutions (100%, 95%, 80% and 70%), 5 min each, and washed in dH_2O. Antigen retrieval was obtained by incubation with 10 mM sodium citrate buffer (pH 6.0) at a sub-boiling temperature for 20 min. Sections were then cooled for 10 minutes, washed in phosphate-buffered saline (PBS), and incubated overnight at 4˚C with anti-cyclin D1 and anti-beclin-1 antibodies. After washing three times with PBS, sections were incubated with the secondary fluorochrome-conjugated antibody (goat anti-rabbit Alexa Fluor 488, goat anti-mouse Alexa Fluor

568) for 1 h at room temperature in the dark. The nuclei were counterstained by incubating the sections for 10 min with 4',6-diamidino-2-phenylindole. Slides were washed in PBS and mounted with Antifade. In each case, a negative control was obtained by omitting the primary antibody. Images were acquired and analyzed with a microscope Leica AF CTR6500HS (Microsystems).

Results

Patients. Out of a series of 129 patients newly diagnosed with GB, 102 (79.1%) experienced recurrence after a median follow-up of 13 months (range=6-67 months). Thirty-one of them underwent a second surgery within 1 year (range=9-12 months).

All patients [19 males; 12 females; median age=51 years (range=36-62)] operated upon for GB recurrence were positive for *MGMT* methylation in their primary GB. In no case had temozolomide been discontinued until recurrence.

Cyclin D1 and beclin-1 immunohistochemistry. Primary GBs: Most primary GBs (20 cases) were negative for nuclear cyclin D1 and exhibited a high cytoplasmic expression of beclin-1. Eleven primary GBs were positive for nuclear cyclin D1 and exhibited low cytoplasmic beclin-1 protein expression. In cyclin D1-positive cases, the percentage of positive tumor cells was high, ranging from about 40% to 60% (median=55%). Cytoplasmic positivity for cyclin D1 was virtually absent in all cases. In no case was cyclin D1 positivity associated with high beclin-1 protein expression.

Recurrent GBs: In the 20 patients with cyclin D1-negative/high beclin-1 expression in their primary GBs, this pattern of protein expression did not change in the recurrent tumors, but, in addition, we observed cytoplasmic positivity for cyclin D1 in large tumor areas, although its distribution was heterogenous. After therapy, in eight recurrences out of the 11 cases of primary GB that were cyclin D1-positive with low beclin-1 expression, this pattern of protein expression did not change, whereas in the three remaining cases, cytoplasmic beclin-1 positivity increased and a cyclin D1-positive/high beclin-1 pattern of protein expression was observed. In these latter three cases, cyclin D1 cytoplasmic positivity was also detectable in tumor cells.

Double cyclin D1/beclin-1 immunofluorescence staining. The pattern of protein expression was better evident in double cyclin D1/beclin-1 immunofluorescence-stained sections. In recurrences of cyclin D1-negative/high beclin-1 primary GBs, after therapy, cytoplasmic positivity for cyclin D1 was observed, although heterogenous, which co-localized with beclin-1 in many cells in the recurrent tumor (Figures 1 and 2). Cytoplasmic cyclin D1/beclin-1 co-localization was also observable in the three cyclin D1-positive/high beclin-1 recurrences of primary GB, which exhibited a cyclin D1-positive/low beclin-1 pattern of protein expression before therapy (Figure 3).

Discussion

Only about a quarter of patients experiencing relapse after primary therapy undergo surgery of GB recurrence, according both to other authors (4, 5) and our present experience, therefore, the analysis of matched primary-recurrrent GB tissue is rarely reported in the available literature (20-22). Comparative profiling of GB tissue before and after therapy are unraveling differences in their molecular signatures, providing evidence for the choice of alternative tailored therapies (20, 22). However, much information on the effect of RT, temozolomide and other therapies still derives from *in vitro* studies on GB cells and, more recently, from interesting *in vitro* models of recurrent GB (21).

All our patients underwent surgery and postoperative RT with concurrent and sequential administration of temozolomide, which is presently the gold standard for GB patients (2).

Both RT and temozolomide have a key role in the fate of GB tumor cells, which are resistant to apoptotic stimuli, their death mainly occurring through autophagy [for review, see (16)]. Ionizing radiation induces various types of DNA damage, both directly (DNA base damage, and DNA single-strand and double-strand breaks), and indirectly, *via* production of reactive oxygen species (6). Cyclin D1 degradation is one of the DNA damage responses induced after radiation in order to maintain genomic stability (6). Radiation-induced cyclin D1 degradation is known to occur through the proteasomal system, and a DNA damage response has been identified which induces tumor cell radioresistance by down-regulating the ubiquitine-proteasome degradation of cyclin D1 (6).

It was recently observed that cyclin A2 is degraded through autophagy, and that liver tumorigenesis is regulated by autophagic degradation of cyclin D1 (17, 18). It was also recently demonstrated that the proteasome and autophagic systems of degradation are not mutually exclusive, but can act in a complementary manner (8).

In our hands, RT/temozolomide induced beclin-1 overexpression, albeit in a limited number of cases (3/11; 27.7%), and promoted cyclin D1 degradation in GBs highly expressing beclin-1. Our results suggest that the autophagic route of cyclin D1 degradation could be induced by therapy, since cyclin D1 and beclin-1 cytoplasmic co-localization was virtually absent before therapy. We are aware that experimental studies on GB cells exposed to

radiation, temozolomide, and combined treatments, are mandatory to support this hypothesis, to assess whether the autophagy and proteasome systems are alternative or complementary route of cyclin D1 degradation, and to identify the role of each type of treatment, for further therapeutic developments. Furthermore, investigations on other proteins of the autophagic machinery could better highlight the involvement of autophagy in cyclin D1 degradation.

Reoperation is not routinely recommended in most patients, which largely depends on the location of the neoplasm and patient functional status (5). In previous studies, we found that beclin-1 expression in GB correlated with a better prognosis (12, 13). The concurrence of methylated MGMT and high beclin-1 expression could be a relatively favorable molecular signature that identifies subgroups of reoperable patients.

In GBs overexpressing beclin-1, autophagic degradation of cyclin D1 could impact on therapy efficacy. This hypothesis deserves further investigation, also taking into account the extreme heterogeneity of GBs, and their subgrouping in molecular subtypes (20).

Conclusion

An effect of RT/temozolomide therapy in inducing autophagy-mediated cyclin D1 degradation in highly beclin-1-expressing GBs could be hypothesized, although this possibility must be supported by mechanistics studies and analysis on a larger series of patients. Due to the small number of cases investigated, statistical analysis was not performed, and we consider our data preliminary observations. We aim to extend our study to a larger number of patients, with parallel experimental investigation on GB cells, to support our observation and to establish, as much as possible, the role of RT and temozolomide alone, and in combined treatments.

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