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Different Involvement of Autophagy in Human Malignant Glioma Cell Lines Undergoing Irradiation and Temozolomide Combined Treatments

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ABSTRACT

Glioblastoma (GB) has a poor prognosis, despite current multimodality treatment. Beside surgical resection, adjuvant ionizing radiation (IR) combined with Temozolomide (TMZ) drug administration is the standard therapy for GB. This currently combined radio-chemotherapy treatment resulted in glial tumor cell death induction, whose main molecular death pathways are still not completely deciphered. In this study, the autophagy process was investigated, and in vitro modulated, in two different GB cell lines, T98G and U373MG (known to differ in their radiosensitivity), after IR or combined IR/TMZ treatments. T98G cells showed a high radiosensitivity (especially at low and intermediate doses), associated with autophagy activation, assessed by Beclin-1 and Atg-5 expression increase, LC3-I to LC3-II conversion and LC3B-GFP accumulation in autophagosomes of irradiated cells; differently, U373MG cells resulted less radiosensitive. Autophagy inhibition, using siRNA against *BECN1* or *ATG-7* genes, totally prevented decrease in viability after both IR and IR/TMZ treatments in the radiosensitive T98G cells, confirming the autophagy involvement in the cytotoxicity of these cells after the current GB treatment, contrary to U373MG cells. However, rapamycin-mediated autophagy, that further radiosensitized T98G, was able to promote radiosensitivty also in U373MG cells, suggesting a role of autophagy process in enhancing radiosensitivity. Taken together, these results might enforce the concept that autophagy-associated cell death might constitute a possible adjuvant therapeutic strategy to enhance the conventional GB treatment. J. Cell. Biochem. 113: 2308–2318, 2012.

KEY WORDS: RADIOSENSITIVITY; GLIOBLASTOMA; AUTOPHAGY

New therapies that provide highly specific glioblastoma (GB) tumor cell killing and complete eradication of cancer cells are urgently needed. Although ionizing radiation (IR) and concomitant chemotherapy agents are widely employed after surgical resection, GB patients very often have a poor clinical course, with a median survival of 12–15 months. During the past two decades, new putative cell death modalities have been investigated in order to overcome cell death resistance to the common GB therapies. Interest of oncologists was focused on autophagy, a mechanism responsible for degrading long-lived proteins and cytoplasmic organelles [Levine and Klionsky, 2004]. This process involves a membranous organelle, the autophagosome [Maiuri et al., 2007], constituted of double-membrane vesicles that progressively engulf cytoplasmic constituents and deliver them to lysosomes for degradation. This ability makes autophagy a good candidate for a survival mechanism in response to several stresses, such as damaged mitochondria, protein aggregation, pathogens, and nutrient starvation [Levine and Klionsky, 2004; Levine and Yuan, 2005]. However, recent studies suggested that autophagy could also contribute to another cell death pathway under certain circumstances, the

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so-called Type II programmed cell-death ("autophagy-associated cell death"), that is morphologically and biochemically distinct from Type I, known as apoptosis [Qu et al., 2003]. Moreover, studies on apoptosis defective cells are nowadays suggesting that autophagy as well as lysosomal membrane permeabilization might emerge as a cell death mechanism upon treatment with various anti-cancer drugs, once the primary cell death pathway is inhibited [Fehrenbacher et al., 2004]. To this regard, a number of studies have reported that in response to various anticancer therapies, autophagic cell death is activated in cancer cells derived from tissues such as breast, colon, prostate, and brain [Bursch et al., 1996; Komata et al., 2004; Scarlatti et al., 2004; Kanzawa et al., 2005]. Furthermore, recent in vitro and in vivo studies on GB identified autophagy as the major non-apoptotic cell death type, also manifested following IR [Zhuang et al., 2009] and TMZ [Kanzawa et al., 2004]. It has been demonstrated the evidence of a major susceptibility of GB cells to autophagy-associated cell death after IR, rather than to apoptosis [Zhuang et al., 2011]. It was recently reported also that autophagy induction in glioma-initiating cells, having stem-like properties, increased their radiosensitivity [Lefranc and Kiss, 2007].

These premises prompted us to explore how autophagy affects different human GB cells, T98G and U373MG, in response to IR, alone or combined with TMZ. In T98G, more radiosensitive than U373MG cells, autophagy was strongly activated, and its inhibition prevented IR effects, also when radiation was combined with TMZ. In addition, autophagy induction by rapamycin promoted radiosensitivity also in U373MG cells. Thus, this study further provided evidence of the role of autophagy in radiosensitizing human GB cell lines, highlighting autophagy modulation as an adjuvant strategy beside the current therapies.

MATERIALS AND METHODS

CELL CULTURES AND CHEMICALS

T98G and U373MG established human malignant glioma cell lines (provided by ECACC) were cultivated in D-MEM medium supplemented with 10% FBS, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 1% L-glutamine (Invitrogen), at 37°C and 5% CO_2 atmosphere. Cells were cultured 2 days before treatments.

Bafilomycin A1 was used as a lysosomal inhibitor, added 6 h before cell harvesting at 10 nM concentration. Temozolomide (TMZ, 3,4-dihydro-3-methyl-4-oxoimidazo [5,1-d]-as-tetrazine-8-carboxamide) was used as a chemotherapeutic agent. Rapamycin and Staurosporine were employed as autophagy and apoptosis inducers, respectively. All these reagents, provided by Sigma–Aldrich, were dissolved in dimethyl sulfoxide (DMSO; Sigma) and diluted in culture medium to the appropriate concentration.

IR, TMZ, AND COMBINED IR/TMZ TREATMENTS

For IR treatment, 2×10^5 cells were seeded onto 25 cm^2 culture flasks, which were placed in a water phantom for dose homogeneity. Cells were irradiated with a 6-MV X-ray linear accelerator (Clinac 600) at a dose-rate of 244.5 cGy/min, with different doses (0.35; 1.2; 2; 3; 5; and 7 Gy).

For experiments using only TMZ, cells were seeded onto a culture substrate according to the assay, before addition of TMZ at different concentrations (100; 200; 400; 500; 600; 800; and 1,000 μM).

For combined IR/TMZ cells treatment, immediately after IR administration as above, TMZ was added to the cells at 300 μM concentration.

RAPAMYCIN-MEDIATED AUTOPHAGY INDUCTION

Rapamycin-mediated autophagy induction was carried out seeding 2×10^5 cells onto 25 cm^2 culture flasks 12 h before rapamycin addition. Cells were incubated for 24 h with different rapamycin concentrations (0.1; 0.3; 0.5; 0.75; 1 μM) to obtain autophagy induction, as previously described [Takeuchi et al., 2005].

CELL VIABILITY ANALYSIS

To evaluate the cytotoxic effects of IR and TMZ, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine cell viability, as previously reported [Fu et al., 2009; Hur et al., 2009]. Cell viability was measured 48 h after treatments.

CLONOGENIC SURVIVAL ASSAY

To evaluate re-plating efficiency, clonogenic survival assays were performed as previously described [Barbieri et al., 2011]. Colonies that contained more than 50 cells were counted using a clono-counter software (http://www.ansci.wisc.edu/equine/parrish/index.html); the surviving fraction (SF) was calculated, dividing counted clones by plated cells and normalizing to the corresponding control sample. Each experiment was performed in triplicate.

WESTERN BLOT ANALYSIS

To evaluate autophagy and apoptosis markers activation, Western blot analyses were performed. Before loading in SDS–PAGE, protein extracts were boiled in Laemmli sample buffer (2% SDS, 6% glycerol, 150 mM B-mercaptoethanol, 0.02% bromophenol blue and Tris–HCl pH 6.8, 62.5 mM) and denatured for 5' at 95°C. SDS–PAGE gels were used to separate proteins by size in the presence of electric current. Gels were run in running buffer (TrisOH pH 8.3, 25mM, glycine 192 mM, SDS 1%) at 90 V until the dye front overcame the stacking gel and then at 120 V until Bromophenol blue exited the gel. After electrophoresis, proteins were transferred onto nitrocellulose membrane Hybond-C Extra (GE Healthcare), using the semi-dry blotters TE70 PWR (GE Healthcare). Transfer was performed at 60 mA for 1 h and 15', in presence of transfer buffer (25 mM TrisOH pH 8.3, 192 mM Glycine, Methanol 20%, v/v). Membranes were blocked 1 h with 8% non-fat milk in TBS (138 mM NaCl, 20 mM TrisOH pH 7.6) containing 0.1% Tween 20 and incubated over-night at 4°C with primary antibodies. Species-specific peroxidase-labeled ECL secondary antibodies (GE Healthcare) were employed. Protein signals were revealed using the "ECL Advance Western Blotting Detection Kit" (GE Healthcare). The following primary antibodies were employed: Beclin-1, Atg-7, Atg-5, LC3B, Phospho-p70 S6 Kinase, p70 S6 Kinase, Caspase-3 and -7 (Cell Signalling Technology).

LC3B-GFP EXPRESSION ANALYSIS

For autophagy detection, GB cells were transduced immediately after IR with BacMam LC3B-GFP or BacMam LC3B(G120A)-GFP viral vectors, accordingly with the Premo Autophagy Sensor Kit (Invitrogen) instructions. After 24 h, analysis was performed evaluating the amount of fluorescence vesicular dots, using an inverted fluorescence microscope Eclipse Nikon TS100. The average number of LC3B-GFP puncta per cell was calculated using a custom macro for NIH ImageJ software.

NUCLEAR MORPHOLOGY INVESTIGATION

Nuclear morphology was investigated through DAPI staining of irradiated cells. Specifically, after irradiation, 10^5 cells were seeded into 30 mm^2 culture dishes, fixed with 4% formaldehyde (AppliChem) and stained with ProLong Gold antifade reagent with DAPI (Invitrogen). Nuclear morphology was evaluated with inverted fluorescence microscope Eclipse Nikon TS100.

FACS ANALYSIS OF SUB-G1 APOPTOTIC POPULATION

To analyze sub-G1 apoptotic population, a cell cycle analysis with a FACS-Calibur (Partec PASII) using propidium iodide was carried out. In detail, 48 h after each IR treatment, a cell suspension of 3×10^5 cells was fixed with 70% ethanol (AppliChem), stained with propidium iodide (500 g/ml H2O + Igepan 0.5% + RNAse 100 U/ml), and analyzed by FACS. For each measurement at least 2×10^4 events were acquired.

AUTOPHAGY INHIBITION USING siRNA TRANSFECTION STRATEGY

To knockdown *BECN1* and *ATG-7* autophagic genes, specific siRNA transfections were carried out using NEON Transfection System (Invitrogen). In details, 3×10^5 cells were transfected using a 100 μl-tip with 4 μM of Silencer Select Negative Control #1 (siRNA NC), *BECN1* Silencer Select Pre-designed siRNA pool (siRNA BECN1) or *ATG-7* Silencer Select Pre-designed siRNA pool (siRNA ATG-7), all provided by Ambion, or with mock R buffer alone. After electroporation, cells were seeded into 25 cm^2 culture flasks and incubated 48 h (to obtain Beclin-1 and Atg-7 protein silencing) before IR or IR/TMZ administration. Beclin-1 and Atg-7 protein down-regulation was assessed by immunoblotting.

STATISTICAL ANALYSIS

Statistical analyses were performed by MANOVA for repeated measures, using SPSS 14.0 software. All *P*-values lower than 0.05 were considered statistically significant.

RESULTS

IR AND TMZ ADMINISTRATION DIFFERENTLY AFFECT T98G AND U373MG CELLS

To evaluate IR effects in terms of cell viability and re-plating efficiency, respectively MTT-based assays and clonogenic tests were performed in both T98G and U373MG cell lines, irradiated or not at different doses (0.35; 1.2; 2; 5; and 7 Gy), as reported in Table I. As shown (Fig. 1A, left panel), cell viability of T98G resulted more affected than that rated in U373MG cells, especially after employing lower IR doses. Similarly, SF (estimated by clonogenic test) of T98G showed a major sensitivity to IR low doses than U373MG cells, mainly at 1.2 and 2 Gy, (Fig. 1A, right panel). In U373MG cells, a substantial SF reduction was obtained only at the highest doses employed. A kinetic study using TMZ alone (from 100 to 1,000 μM) was carried out, before combining IR and TMZ, to evaluate its cytotoxicity. In T98G cells, cell viability decreased only at the highest TMZ concentrations (800–1,000 μM), while, in U373MG cells, viability was dose-dependently affected (Fig. 1B, left panel). The results of clonogenic tests (Fig. 1B, right panel) indicated that the TMZ concentration able to induce similar effects on SF in both cell lines was lower than that observed in cell viability, and confirmed the different sensitivity to TMZ of the two cell lines at low concentrations. Combining IR with TMZ administration (300 μM), viability of T98G was more affected than that rated in U373MG cells, especially after low IR dosages (Fig. 1C, left panel). However, SF evaluation showed reduced values in U373MG compared to T98G cells, but a decrease of U373MG SF values was also highlighted in not irradiated control cells, suggesting TMZ as one of the factors involved in SF decrease.

TABLE I. Glioma Cell Viability After IR and Combined IR/TMZ Treatments

	% cell viability				SF			
	T98G		U373MG		T98G		U373MG	
	IR	IR + TMZ	IR	IR + TMZ	IR	IR + TMZ	IR	IR + TMZ
0 Gy	100.0	93.7	100.0	90.8	1.00	0.76	1.00	0.33
0.35 Gy	43.5	38.0	73.5	66.7	0.58	0.44	0.88	0.31
1.2 Gy	45.6	27.4	76.0	64.1	0.15	0.10	0.85	0.28
2 Gy	45.6	32.0	78.1	65.5	0.08	0.08	0.59	0.19
5 Gy	48.5	41.0	77.5	68.7	0.05	0.04	0.45	0.06
7 Gy	63.3	50.8	67.7	50.2	0.04	0.01	0.07	0.03

IR ADMINISTRATION INDUCES AUTOPHAGY IN T98G CELLS

To assess the cell death pathways involved in radiosensitivity, the major autophagic and apoptotic markers were comparatively investigated (Fig. 2). Beclin-1 and Atg-5 autophagic markers showed the highest expression levels at low and intermediate IR doses in T98G cells; at the same dosages, a weak Caspase-3/7 cleavage indicated early apoptosis activation. In U373MG cells, Beclin-1 expression poorly increased after high dose of IR, while Atg-5 expression increased at intermediate IR doses. However, in this line, the autophagy induction resulted lower than that rated in T98G cells and Caspase-3 and -7 did not show any cleavage, indicating no apoptosis activation.

To deeper investigate the cell death pathways involved in T98G cells (affected by IR more than U373MG), LC3-I to LC3-II conversion, BacMam LC3B-GFP transduction, DAPI staining and sub-G1 apoptotic population analysis were carried out (Fig. 3). As highlighted, in irradiated T98G cells, LC3-I to LC3-II conversion (rated by LC3-II/LC3-I ratio) was higher than that observed in not irradiated control cells (Fig. 3A). Therefore, to discern whether the increase in LC3-II, following IR treatment, was due to autophagy stimulation or to inhibition of LC3-II degradation, the lysosomal

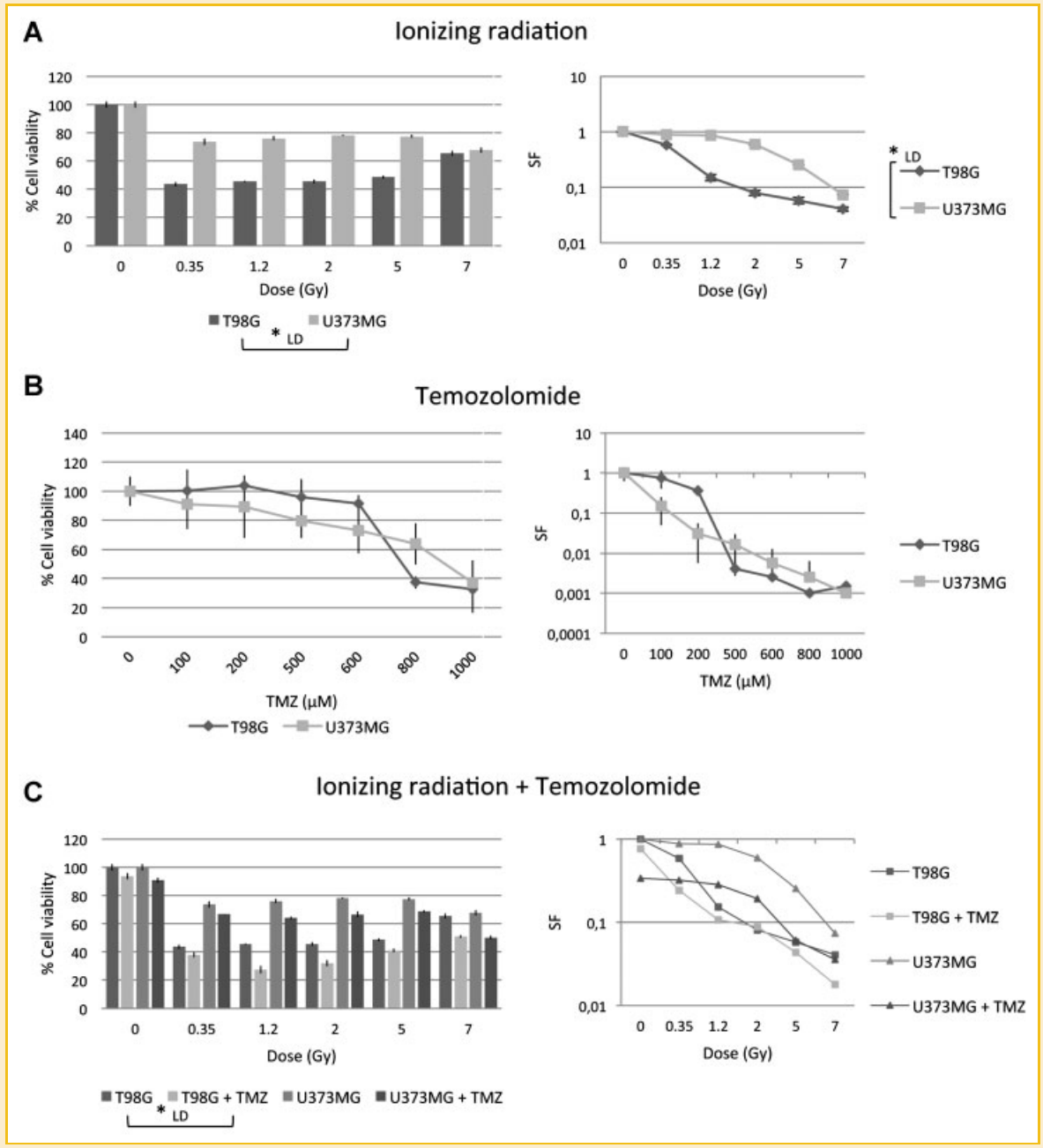


Fig. 1. Cytotoxic effects of IR (A), TMZ (B) and combined IR/TMZ (300 μM) (C) treatments. Cell viability, reported in the left side, was measured 48 h after treatments. SF (surviving fraction), reported in the right side, was obtained by clonogenic test. Radiations were employed at different doses of Gray -Gy- (0.35; 1.2; 2; 5; and 7 Gy). TMZ concentrations ranging from 100 to 1,000 μM were added to the cultured cells; in combined treatment, TMZ was added to the culture immediately after IR administration at 300 μM concentration. Results of three independent experiments were reported with error bars. *$P < 0.05$; *LD referred to low doses of 1.2–2 Gy.

inhibitor Bafilomycin A1 (BafA1, 10 nM concentration) was added to the cells, as originally reported [Tanida et al., 2005]. As a result, the presence of BafA1 further increased LC3-II levels at each IR dose, as compared to IR treatment alone; thus, according to these results, low and intermediate doses of IR induced an enhancement of the autophagic flux in T98G cells. LC3-II expression was also evaluated after irradiation by adding the Promo Autophagy Sensor LC3B-GFP (BacMam 2.0) viral vector, able to transduce and express fluorescent LC3B protein. As a control, the BacMam LC3B(G120A)-GFP was also employed; this mutation prevents LC3 cleavage and subsequent lipidation during normal autophagy, and thus protein localization remains cytosolic and diffuse. While untreated and BacMam LC3B(G120A)-GFP transduced T98G cells showed a diffuse expression pattern, irradiated cells presented a punctate fluorescent distribution, suggesting LC3B-GFP accumulation in autophagosome-like vesicles (Fig. 3B). To quantify the autophagic forming vesicles, the average number of LC3B-GFP puncta per cell was measured using a custom macro for NIH ImageJ software. As

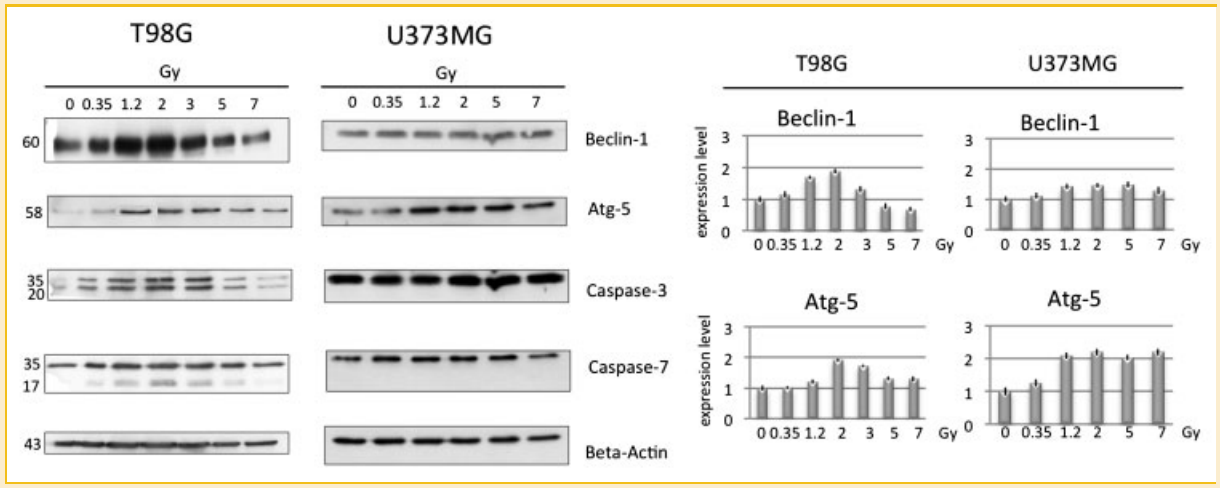


Fig. 2. Cell-death molecular investigations. Protein expression evaluation in T98G and U373MG cells 48 h after IR, by immunoblotting with specific antibodies against the autophagic Beclin-1 and Atg-5 and the apoptotic markers Caspase-3 and Caspase-7. For densitometric analysis, protein expression is normalized with Beta-Actin (using ImageJ software) and referred to the expression of untreated cells.

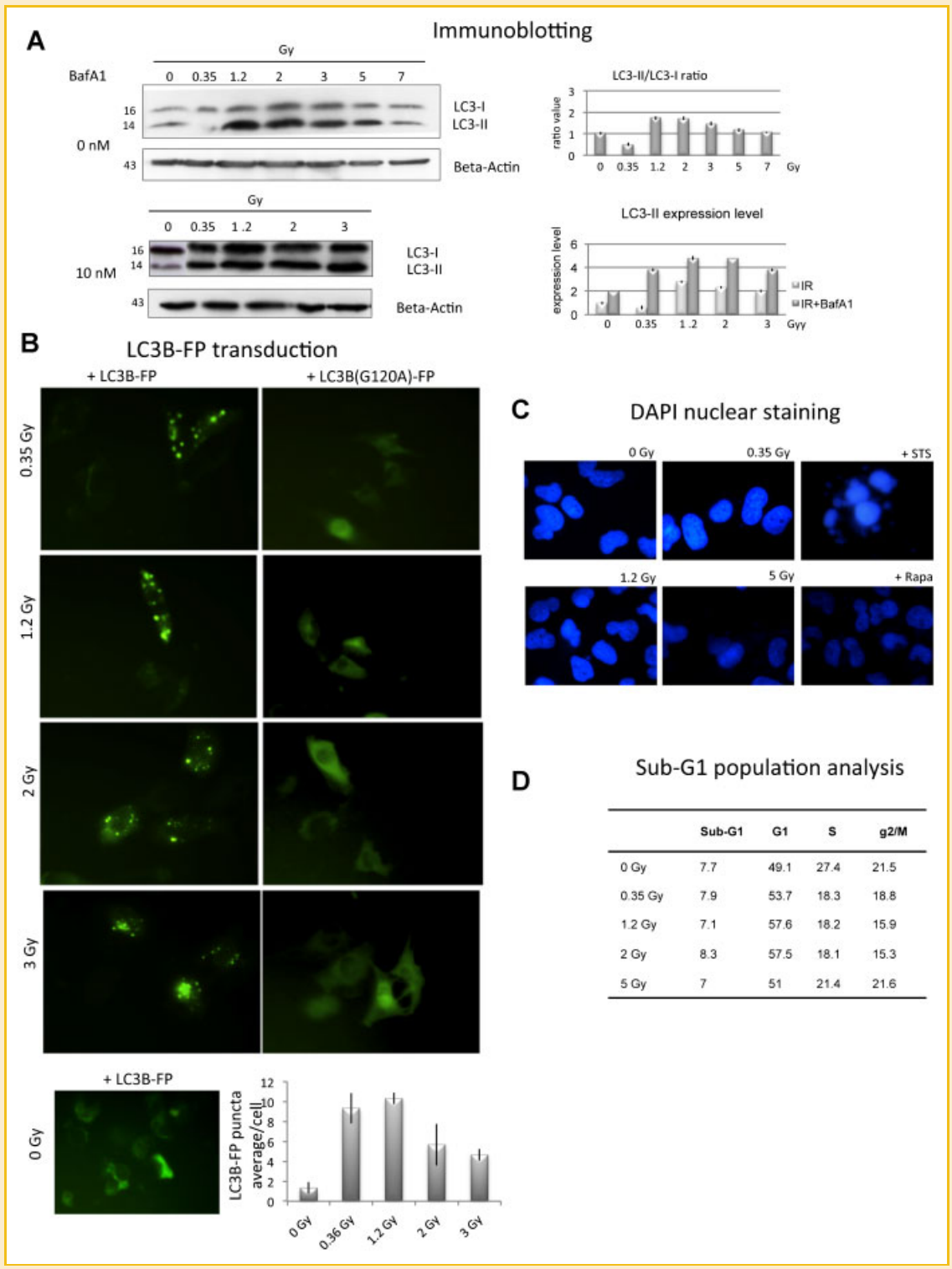
reported, IR induced a significant increase in the average number of LC3B-GFP dots per cell. Differently, nuclear morphology analysis by fluorescence microscope after DAPI-staining of irradiated cells, did not highlight typical features of apoptosis induction like nuclear fragmentation, chromatin condensation and apoptotic-bodies formation (Fig. 3C); comparable absence of apoptosis induction processes was obtained in T98G cells treated with the autophagy-inducer rapamycin (100 nM). In the same panel, as expected, the treatment with the apoptotic-inducer Staurosporine (100 nM) highlighted apoptotic features in T98G cells. Finally, after propidium iodide cell-staining, an estimation of the sub-G1 apoptotic population was calculated in T98G cells by cell cycle FACS analysis. As reported (Fig. 3D), no appreciable variations were observed comparing sub-G1 populations of not-irradiated and irradiated cells, suggesting no significant apoptosis induction.

INDUCTION OF RAPAMYCIN-MEDIATED AUTOPHAGY PROMOTES RADIOSENSITIVITY

To better assess the involvement of autophagy-associated cell-death activation in radiosensitizing GB cell lines, a rapamycin-mediated autophagy induction was performed in T98G and U373MG cells before IR treatment. mTOR inhibition by rapamycin was evaluated by immunoblotting using ph-p70S6K and p70S6K antibodies, and assessing LC3-I to LC3-II conversion. Rapamycin treatment blocked mTOR kinase activity (Fig. 4), decreasing the ph-p70S6K/p70S6K ratio and induced autophagy, increasing LC3-I to LC3-II conversion in both cell lines at the highest rapamycin concentration employed. To evaluate the effects of IR on viability and clonogenic capabilities, rapamycin (1 μM) was added to the cultured cells 24 h before IR exposure. T98G cell viability further decreased at low doses of IR, when treated with rapamycin, as shown (Fig. 5, left upper graph), as compared to the irradiated only cells. In the same cells, SF values drastically decreased after IR of rapamycin-treated (1 μM) cells, in which re-plating efficiency was almost completely absent. To better evaluate the IR contribution in sensitizing autophagy-induced T98G cells, concentrations lower than 1 μM were employed. In details, rapamycin concentrations of 0.5 and 0.75 μM were used in T98G cells, 24 h before IR and, then, viability was assessed. In terms of cell viability, no differences were observed (data not shown) between treated and untreated cells, as expected [Takeuchi et al., 2005]. On the contrary, as shown (upper right graph of Fig. 5), significant variations in SF values were observed especially in correspondence of low IR doses treatment. In U373MG cells, rapamycin treatment before IR did not induce significant cell viability changes compared to IR alone (lower left graph of Fig. 4), while a significant decrease of SF (lower right graph of Fig. 4) was observed. Remarkably (Table II), after irradiation of rapamycin-treated U373MG, the SF trend became similar to that of irradiated T98G (as reported in Fig. 1A).

AUTOPHAGY INHIBITION PREVENTED IR AND IR/TMZ EFFECTS IN T98G CELLS

To confirm the involvement of autophagy in enhancing GB cell radiosensitivity, two major autophagy-correlated genes, *BECN1* and *ATG-7*, were knocked down by siRNA technology. Expression levels of Beclin-1 and Atg-7 proteins were reported almost absent from 48 to 120 h after siRNA transfection, while no differences were observed between siRNA Negative Control (NC) and not transfected cells (data not shown). Thus, T98G and U373MG cells were transfected with siRNA (as above), 48 h before IR or IR/TMZ treatments. As reported, Beclin-1 and Atg-7 expression levels clearly decreased 48 h after siRNA *BECN1* or *ATG-7* transfection in both cell lines (Fig. 6A); no cytotoxicity was detected after siRNA transfection in both cell lines (data not shown). Autophagy inhibition totally prevented effects of 1.2 Gy, alone and combined with TMZ in T98G, both in term of cell viability and SF (Fig. 6B,C, Table III). On the contrary, autophagy inhibition in U373MG did not show any significant difference between control and transfected cells.



	Sub-G1	G1	S	g2/M
0 Gy	7.7	49.1	27.4	21.5
0.35 Gy	7.9	53.7	18.3	18.8
1.2 Gy	7.1	57.6	18.2	15.9
2 Gy	8.3	57.5	18.1	15.3
5 Gy	7	51	21.4	21.6

Fig. 3. Cell-death investigation in T98G cells. A: Evaluation of LC3-I to LC3-II conversion 48 h after IR in presence/absence of Bafilomycin A1 (BafA1, 10 nM), by immunoblotting. BafA1 was added 6 h before cell harvesting. For densitometric analysis, protein expression is normalized with Beta-Actin (using ImageJ software) and referred to the expression of untreated cells. B: Calculation of LC3B-GFP puncta average per cell, after BacMam LC3B-GFP transduction in irradiated T98G cells. As a control, BacMam LC3B(G120A)-GFP was also transduced. Fluorescent puncta were observed by fluorescence-microscope at 40X magnification 24 h after transduction. C: Nuclear morphology of DAPI-stained cells was evaluated by fluorescence-microscope at 40× magnification after 24 h of each treatment. Staurosporine (STS) and rapamycin (Rapa) were employed at 100 nM. D: Sub-G1 population was estimated by cell cycle FACS analysis, 48 h after irradiation.

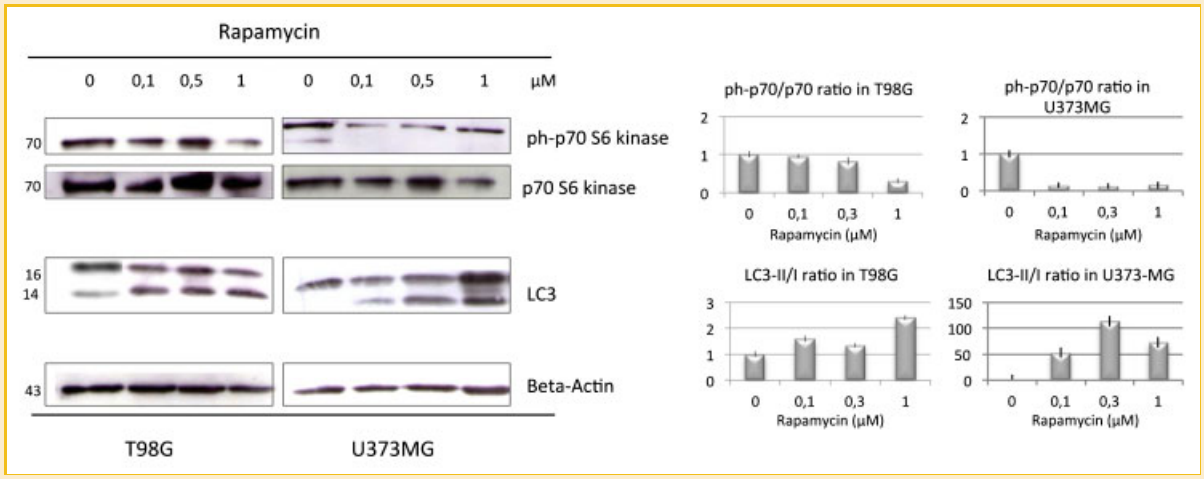


Fig. 4. Rapamycin-mediated autophagy induction in T98G and U373MG cells. Evaluation of ph-p70S6K, p70S6K, and LC3 expression by immunoblotting, after 24 h of rapamycin incubation (0.1, 0.5, and 1 μM). For densitometric analysis, protein expression is normalized with Beta-Actin (using ImageJ software) and referred to the expression of untreated cells.

DISCUSSION

Recent significant advances in understanding the biology of GB have not lead to substantial improvement, both in the therapeutic response and in the clinical management; a small but significant therapeutic gain was obtained by concurrent TMZ and radiotherapy followed by adjuvant TMZ. This is currently the standard of care for GB, as extensively argued [Omar and Mason, 2010]. Beside radiation-induced apoptosis, type II of programmed cell-death or autophagy-associated death has been found activated in different

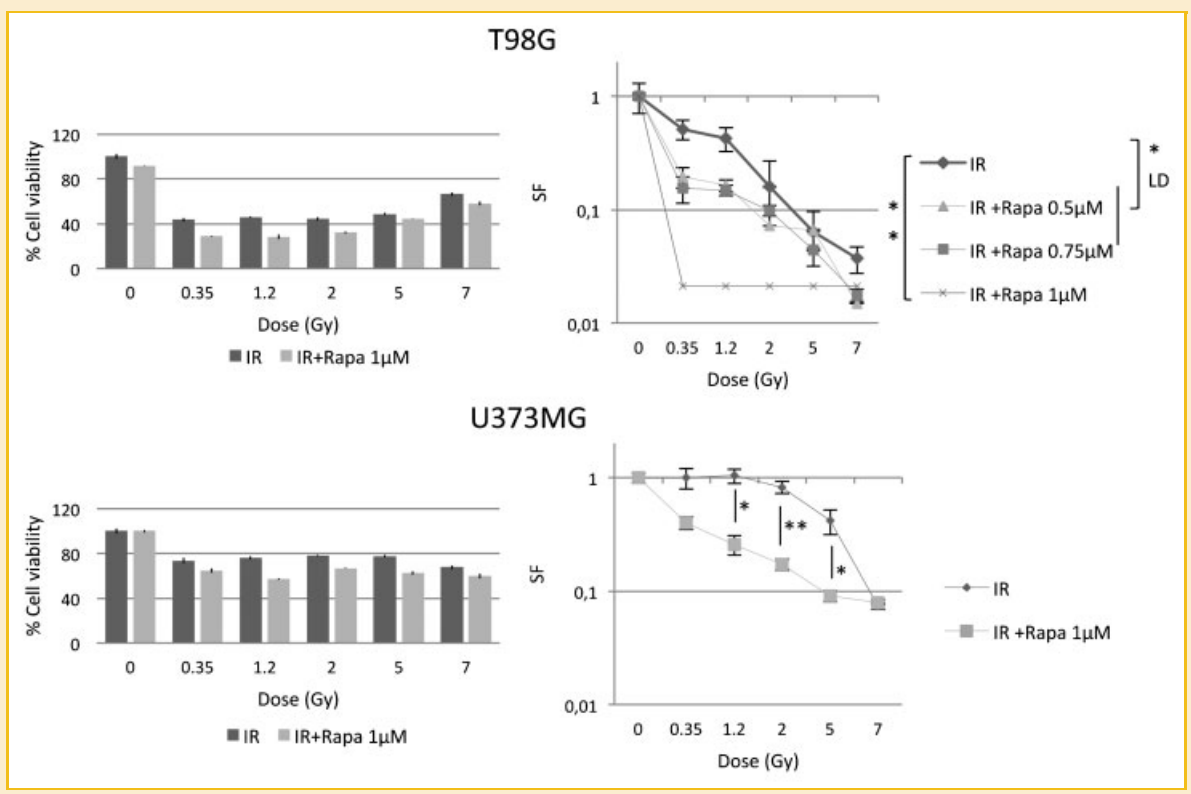


Fig. 5. Cell viability of autophagy-induced irradiated cells. Rapamycin was added to the cultured cells 24 h before IR, to induce autophagy. Radiations were employed at different doses (0.35; 1.2; 2; 5; and 7 Gy). Cell viability, reported in the left side, was measured 48 h after IR. SF (surviving fraction), reported in the right side, was obtained by clonogenic test. Results of three independent experiments were reported with error bars. $^{*}P < 0.05$, $^{**}P < 0.01$; *LD referred to low doses of 0.35–1.2 Gy.

TABLE II. SF Values of Autophagy-Induced Irradiated Cells

	T98G			U373MG		
	IR	IR + Rapa 0.5 μM	IR + Rapa 0.75 μM	IR + Rapa 1 μM	IR	IR + Rapa 1 μM
0 Gy	1.00	1.00	1.00	1.00	1.00	1.00
0.35 Gy	0.51	0.90	0.15	0.01	0.99	0.39
1.2 Gy	0.42	0.16	0.14	0.01	1.00	0.25
2 Gy	0.15	0.07	0.09	0.01	0.82	0.17
5 Gy	0.06	0.06	0.04	0.01	0.41	0.09
7 Gy	0.03	0.01	0.01	0.01	0.07	0.07

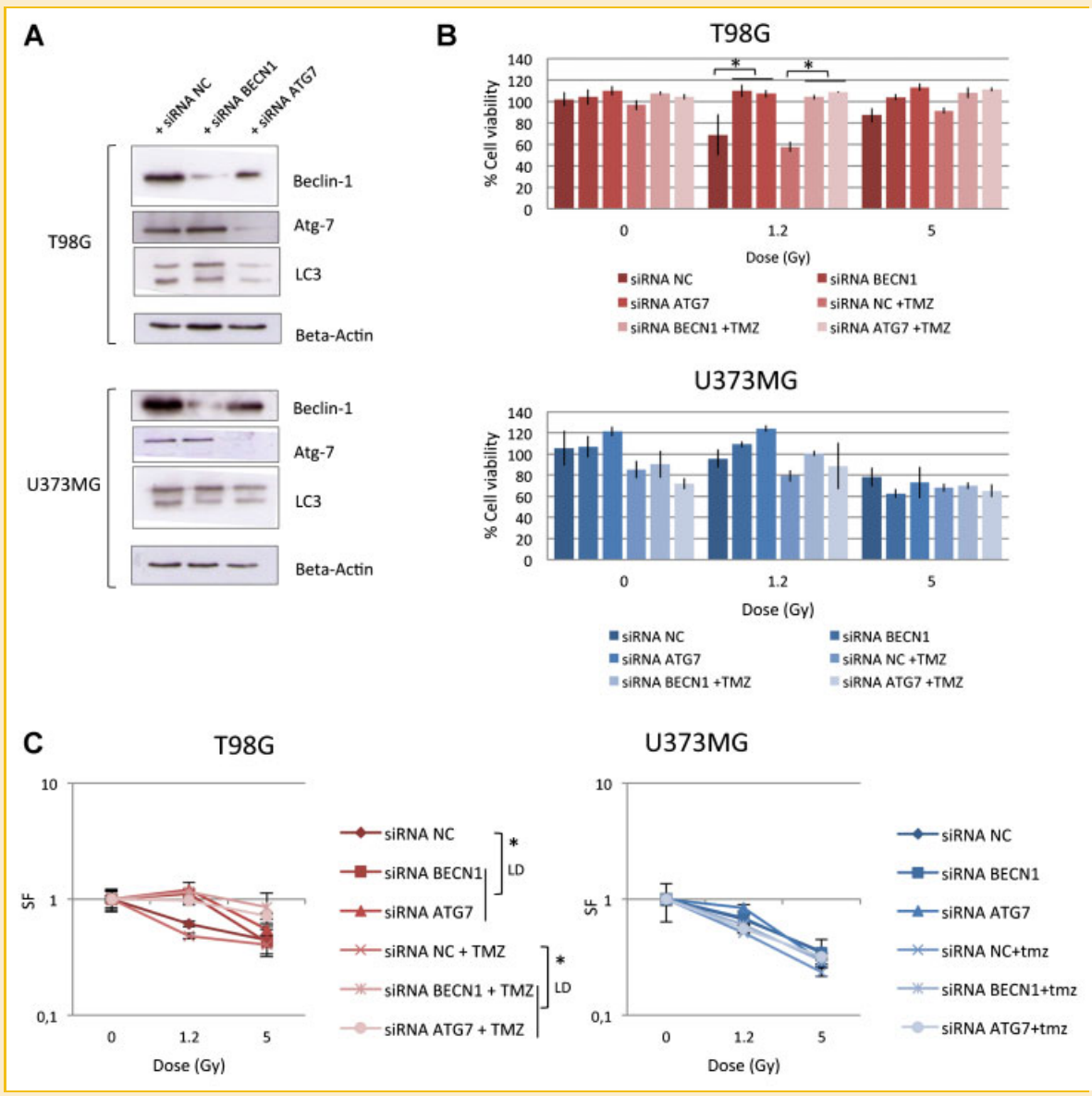


Fig. 6. Effects of IR and IR/TMZ on GB cells after autophagy inhibition. A: Expression of Beclin-1 and Atg-7 proteins was detected by immunoblotting, 48 h after transfection of specific siRNA (4 μM each); NC (negative control) siRNA was similarly transfected, as a negative internal control. B: Cell viability was measured 48 h after treatments. C: SF (surviving fraction) was obtained by clonogenic test. IR were employed at different doses (1.2 and 5 Gy), 48 h after siRNA transfection; in combined treatment, TMZ was added to the culture immediately after IR administration at 300 μM concentration Results of three independent experiments were indicated with error bars. $^{*}P < 0.05$; *LD referred to low dose of 1.2 Gy.

TABLE III. Viability of IR and IR/TMZ-Treated Cells After Autophagy Inhibition

	% Cell viability						SF					
	T98G			U373MG			T98G			U373MG		
	0 Gy	1.2 Gy	5 Gy	0 Gy	1.2 Gy	5 Gy	0 Gy	1.2 Gy	5 Gy	0 Gy	1.2 Gy	5 Gy
+ siRNA NC	100.0	68.9	87.1	105.4	95.6	78.2	1.00	0.61	0.44	1.00	0.67	0.35
+ siRNA BECN1	104.1	109.6	103.7	106.8	109.1	62.4	1.00	1.11	0.42	1.00	0.69	0.34
+ siRNA ATG7	109.8	107.0	113.2	121.2	124.1	73.0	1.00	1.21	0.55	1.00	0.84	0.29
+ siRNA NC/TMZ	96.6	75.1	94.3	85.3	79.2	68.0	1.00	0.48	0.40	1.00	0.51	0.23
+ siRNA BECN1/TMZ	107.5	104.1	101.8	90.0	100.4	69.9	1.00	1.16	0.85	1.00	0.60	0.29
+ siRNA ATG7/TMZ	104.2	108.8	110.9	71.8	88.6	65.1	1.00	0.97	0.71	1.00	0.55	0.30

tumor cell lines after IR, including GB [Zhuang et al., 2009]. In fact, apoptosis is believed to account for approximately 20% of radiation-induced death [Verheij and Bartelink, 2000], and other types of non-apoptotic cell death, including mitotic catastrophe, necrosis, and autophagy, mostly occur after radiation. Autophagy occurs spontaneously and induced by both radio- and chemotherapy (including TMZ) in GB, in vitro, as well as in vivo studies [Kanzawa et al., 2004; Zhuang et al., 2009]. In this study, T98G cells showed autophagy activation after low and intermediate IR doses, documented by the increase of Beclin-1 and Atg-5 expression and LC3-I to LC3-II conversion, closely correlated with the number of autophagosomes, quantified as fluorescent puncta in LC3B-GFP transduced cells. Although a weak activation of Caspase-3 [also described by Krueger et al., 2007], and Caspase-7 was detected after low doses of IR, no significant apoptosis induction was highlighted by nuclear morphology observation and cell cycle analysis of sub-G1 population. Similarly to T98G, in U373MG cells no apoptosis induction was observed after IR treatment, and the expression of the autophagy-related markers Beclin-1 and Atg-5 was poorly increased, this probably accounts for the minor radiosensitivity of this cell line. Accordingly, after administration of low dosages of IR, T98G appeared much more radiosensitive than U373MG cells [Joiner et al., 2001]; this radiosensitivity to low doses, higher than expected, is known as ''hyper-radiosensitivity'' (HRS) [Joiner et al., 2001]. The mechanism underlying this phenomenon is not fully elucidated: HRS was supposed to occur mainly via apoptosis [Krueger et al., 2007], but other cell-death pathways, including autophagy, might be involved [Verheij and Bartelink, 2000]. Although autophagy process was already described after IR [Yao et al., 2003; Ito et al., 2005; Fujiwara et al., 2007; Tsuboi et al., 2009], to date the autophagic process after HRS in mammalian cells was poorly investigated; however, in Drosophila, autophagic death has recently been described after HRS [Moskalev et al., 2011].

To enforce the role of autophagy in radiosensitizing malignant glioma cells, rapamycin was employed in this work, to activate autophagy before IR treatment. In particular, rapamycin inhibits the anti-autophagic regulator pathway PI3K/Akt/mTOR, inducing autophagy in human GB cells [Iwamaru et al., 2007]. The inhibition of this pathway and the corresponding autophagy induction were confirmed in our cells both by the decrease of the ph-p70S6K/p70S6K ratio and the increase of LC3-I to LC3-II conversion, respectively. In line with Zhuang et al. [2011, 2009], rapamycin-induced autophagy enhanced the radiosensitizing of T98G and U373MG cells. Importantly, whereas in T98G rapamycin-mediated autophagy further decreased cell viability, in U373MG the induction of autophagy manifested HRS, enhancing the radiosensitivity of this cell line. To note, in T98G, more radiosensitive than U373MG, the employed concentration of rapamycin (1 μM) was crucial, and the re-plating efficacy assessed by clonogenic assay was completely null. In order to prevent the complete inhibition of clonogenic capabilities, and to discriminate a possible different contribution among the employed IR doses, rapamycin concentrations lower than 1 μM, just regarding T98G cells, were used. However, using these concentrations, radiosensitizing was limited to low IR dosages probably due to a not significant rapamycin action or caused by HRS phenomenon manifested by this cell line.

As previously reported, concurrent TMZ and radiotherapy followed by adjuvant TMZ is the standard of care for GB [Omar and Mason, 2010]. Although involvement of autophagy after TMZ administration was extensively reported in human malignant glioma cells [Kanzawa et al., 2004; Rhee et al., 2009; Natsumeda et al., 2011], evidences documenting the role of autophagy in combined IR/TMZ treatments might be better addressed. Thus, to discern the possible role of autophagy in response to the agents employed in the current GB patient therapy, a combined radio/chemo-treatment (IR and TMZ) was then performed. Firstly, TMZ alone was directly in vitro administered. The drug induced a decrease in T98G cell viability only at the highest TMZ concentrations, while in U373MG cells viability decreased starting from the lowest concentrations, and in a dose-dependent manner. U373MG cells appeared more sensitive to TMZ than T98G cells. It is well established that MGMT (methylated-DNA-protein-cysteine methyltransferase) effectively reverses the cytotoxic action of TMZ; thus, high MGMT expression levels are associated with TMZ resistance. Differently from T98G cells, the expression level of MGMT was found to be low in U373MG [Chalmers et al., 2009]. In our experience, accordingly, *MGMT* mRNA expression was assessed by quantitative Real Time PCR and confirmed to be significantly lower in U373MG compared to T98G cells (data not shown). This kinetic study using TMZ alone was preliminary to combined IR/TMZ treatments; in order to evaluate the effective IR contribution over period of 2 weeks in cells undergoing IR/TMZ treatment, a TMZ concentration able to induce similar effect in T98G and U373MG after clonogenic tests was identified. To this regard, TMZ concentration of 300 μM was used for combined IR/TMZ treatment. After this treatment, the greatest variation in viability, compared to

that measured after IR alone, was observed in T98G cells subjected to low and intermediate IR doses. In U373MG cells, the SF decrease appeared also in correspondence of not irradiated, TMZ-treated cells, suggesting TMZ alone as a critical factor in SF decrease.

Finally, the autophagy process was inhibited using specific siRNAs directed against the autophagic genes *BECN1* and *ATG-7*, as suggested by current guidelines for autophagy investigations [Klionsky et al., 2008], instead of the common inhibitor 3-Methyladenine. In T98G cells, the inhibition of the process totally prevented the cytotoxic effects of IR and IR/TMZ, suggesting a crucial role of autophagy in the sensitivity of these cells not only to IR, but also to the currently used IR/TMZ treatment. On the contrary, in U373MG cells, less radiosensitive and not significantly autophagy-activated after IR, the inhibition of this process did not importantly alter the overall cell viability.

In conclusion, our results enforce the role of autophagy to enhance the efficacy of IR: autophagy activation was able to further sensitize T98G cells, and increase the radiosensitivity of U373MG cells. Therefore, in the more radiosensitive T98G cells, autophagy inhibition completely annuls the effects of IR and IR/TMZ, suggesting a role of this process also in the current GB treatment. Finally, autophagy is here indicated as a possible cell-death mechanism involved in sensitivity to low doses of IR in T98G cells.

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REFERENCES

Barbieri G, Palumbo S, Gabrusiewicz K, Azzalin A, Marchesi N, Spedito A, Biggiogera M, Sbalchiero E, Mazzini G, Miracco C, Pirtoli L, Kaminska B, Comincini S. 2011. Silencing of cellular prion protein (PrPC) expression by DNA-antisense oligonucleotides induces autophagy-dependent cell death in glioma cells. Autophagy 7:840–853.

Bursch W, Ellinger A, Kienzl H, Torok L, Pandey S, Sikorska M, Walker R, Hermann RS. 1996. Active cell death induced by the anti-estrogens tamoxifen and ICI 164 384 in human mammary carcinoma cells (MCF-7) in culture: The role of autophagy. Carcinogenesis 17:1595–1607.

Chalmers AJ, Ruff EM, Martindale C, Lovegrove N, Short SC. 2009. Cytotoxic effects of temozolomide and radiation are additive- and schedule-dependent. Int J Radiat Oncol Biol Phys 75:1511–1519.

Fehrenbacher N, Gyrd-Hansen M, Poulsen B, Felbor U, Kallunki T, Boes M, Weber E, Leist M, Aattela M. 2004. Sensitization to the lysosomal cell death pathway upon immortalization and transformation. Cancer Res 64:5301–5310.

Fu J, Liu ZG, Liu XM, Chen FR, Shi HL, Pangjesse CS, Ng HK, Chen ZP. 2009. Glioblastoma stem cells resistant to temozolomide-induced autophagy. Chin Med J (Engl) 122:1255–1259.

Fujiwara K, Iwado E, Mills GB, Sawaya R, Kondo S, Kondo Y. 2007. Akt inhibitor shows anticancer and radiosensitizing effects in malignant glioma cells by inducing autophagy. Int J Oncol 31:753–760.

Hur JM, Hyun MS, Lim SY, Lee WY, Kim D. 2009. The combination of berberine and irradiation enhances anti-cancer effects via activation of p38 MAPK pathway and ROS generation in human hepatoma cells. J Cell Biochem 107:955–964.

Ito H, Daido S, Kanzawa T, Kondo S, Kondo Y. 2005. Radiation-induced autophagy is associated with LC3 and its inhibition sensitizes malignant glioma cells. Int J Oncol 26:1401–1410.

Iwamaru A, Kondo Y, Iwado E, Aoki H, Fujiwara K, Yokoyama T, Mills GB, Kondo S. 2007. Silencing mammalian target of rapamycin signaling by small interfering RNA enhances rapamycin-induced autophagy in malignant glioma cells. Oncogene 26:1840–1851.

Joiner MC, Marples B, Lambin P, Short SC, Turesson I. 2001. Low-dose hypersensitivity: Current status and possible mechanisms. Int J Radiat Oncol Biol Phys 49:379–389.

Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S. 2004. Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. Cell Death Differ 11:448–457.

Kanzawa T, Zhang L, Xiao L, Germano IM, Kondo Y, Kondo S. 2005. Arsenic trioxide induces autophagic cell death in malignant glioma cells by upregulation of mitochondrial cell death protein BNIP3. Oncogene 24:980–991.

Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS, Baba M, Baehrecke EH, Bahr BA, Ballabio A, Bamber BA, Bassham DC, Bergamini E, Bi X, Biard-Piechaczyk M, Blum JS, Bredesen DE, Brodsky JL, Brumell JH, Brunk UT, Bursch W, Camougrand N, Cebollero E, Cecconi F, Chen Y, Chin LS, Choi A, Chu CT, Chung J, Clarke PG, Clark RS, Clarke SG, Clavé C, Cleveland JL, Codogno P, Colombo MI, Coto-Montes A, Cregg JM, Cuervo AM, Debnath J, Demarchi F, Dennis PB, Dennis PA, Deretic V, Devenish RJ, Di Sano F, Dice JF, Difiglia M, Dinesh-Kumar S, Distelhorst CW, Djavaheri-Mergny M, Dorsey FC, Dröge W, Dron M, Dunn WA Jr, Duszenko M, Eissa NT, Elazar Z, Esclatine A, Eskelinen EL, Fésüs L, Finley KD, Fuentes JM, Fueyo J, Fujisaki K, Galliot B, Gao FB, Gewirtz DA, Gibson SB, Gohla A, Goldberg AL, Gonzalez R, González-Estévez C, Gorski S, Gottlieb RA, Häussinger D, He YW, Heidenreich K, Hill JA, Høyer-Hansen M, Hu X, Huang WP, Iwasaki A, Jäättelä M, Jackson WT, Jiang X, Jin S, Johansen T, Jung JU, Kadowaki M, Kang C, Kelekar A, Kessel DH, Kiel JA, Kim HP, Kimchi A, Kinsella TJ, Kiselyov K, Kitamoto K, Knecht E, Komatsu M, Kominami E, Kondo S, Kovács AL, Kroemer G, Kuan CY, Kumar R, Kundu M, Landry J, Laporte M, Le W, Lei HY, Lenardo MJ, Levine B, Lieberman A, Lim KL, Lin FC, Liou W, Liu LF, Lopez-Berestein G, López-Otín C, Lu B, Macleod KF, Malorni W, Martinet W, Matsuoka K, Mautner J, Meijer AJ, Meléndez A, Michels P, Miotto G, Mistiaen WP, Mizushima N, Mograbi B, Monastyrska I, Moore MN, Moreira PI, Moriyasu Y, Motyl T, Münz C, Murphy LO, Naqvi NI, Neufeld TP, Nishino I, Nixon RA, Noda T, Nürnberg B, Ogawa M, Oleinick NL, Olsen LJ, Ozpolat B, Paglin S, Palmer GE, Papassideri I, Parkes M, Perlmutter DH, Perry G, Piacentini M, Pinkas-Kramarski R, Prescott M, Proikas-Cezanne T, Raben N, Rami A, Reggiori F, Rohrer B, Rubinsztein DC, Ryan KM, Sadoshima J, Sakagami H, Sakai Y, Sandri M, Sasakawa C, Sass M, Schneider C, Seglen PO, Seleverstov O, Settleman J, Shacka JJ, Shapiro IM, Sibirny A, Silva-Zacarin EC, Simon HU, Simone C, Simonsen A, Smith MA, Spanel-Borowski K, Srinivas V, Steeves M, Stenmark H, Stromhaug PE, Subauste CS, Sugimoto S, Sulzer D, Suzuki T, Swanson MS, Tabas I, Takeshita F, Talbot NJ, Tallóczy Z, Tanaka K, Tanaka K, Tanida I, Taylor GS, Taylor JP, Terman A, Tettamanti G, Thompson CB, Thumm M, Tolkovsky AM, Tooze SA, Truant R, Tumanovska LV, Uchiyama Y, Ueno T, Uzcátegui NL, van der Klei I, Vaquero EC, Vellai T, Vogel MW, Wang HG, Webster P, Wiley JW, Xi Z, Xiao G, Yahalom J, Yang JM, Yap G, Yin XM, Yoshimori T, Yu L, Yue Z, Yuzaki M, Zabirnyk O, Zheng X, Zhu X, Deter RL. 2008. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy 4:151–175.

Komata T, Kanzawa T, Nashimoto T, Aoki H, Endo S, Nameta M, Takahashi H, Yamamoto T, Kondo S, Tanaka R. 2004. Mild heat shock induces autophagic growth arrest, but not apoptosis in U251-MG and U87-MG human malignant glioma cells. J Neurooncol 68:101–111.

Krueger SA, Joiner MC, Weinfeld M, Piasentin E, Marples B. 2007. Role of apoptosis in low-dose hyper-radiosensitivity. Radiat Res 167:260–267.

Lefranc F, Kiss R. 2007. Autophagy, the Trojan horse to combat glioblastomas. Neurosurg Focus 20:E7.

Levine B, Klionsky DJ. 2004. Development by self-digestion: Molecular mechanisms and biological functions of autophagy. Dev Cell 6:463–477.

Levine B, Yuan J. 2005. Autophagy in cell death: An innocent convict? J Clin Invest 115:2679–2688.

Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. 2007. Self-eating and self-killing: Crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol 8:741–752.

Moskalev AA, Plyusnina EN, Shaposhnikov MV. 2011. Radiation hormesis and radioadaptive response in Drosophila melanogaster flies with different genetic backgrounds: the role of cellular stress-resistance mechanisms. Biogerontology 12:253–263.

Natsumeda M, Aoki H, Miyahara H, Yajima N, Uzuka T, Toyoshima Y, Kakita A, Takahashi H, Fujii Y. 2011. Induction of autophagy in temozolomide treated malignant gliomas. Neuropathology 31:486–493.

Omar AI, Mason WP. 2010. Temozolomide: The evidence for its therapeutic efficacy in malignant astrocytomas. Core Evid 4:93–111.

Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen EL, Mizushima N, Ohsumi Y, Cattoretti G, Levine B. 2003. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J Clin Invest 112:1809–1820.

Rhee DJ, Kong DS, Kim WS, Park KB, Lee JI, Suh YL, Song SY, Kim ST, Lim DH, Park K, Kim JH, Nam DH. 2009. Efficacy of temozolomide as adjuvant chemotherapy after postsurgical radiotherapy alone for glioblastomas. Clin Neurol Neurosurg 111:748–751.

Scarlatti F, Bauvy C, Ventruti A, Sala G, Cluzeaud F, Vandewalle A, Ghidoni R, Codogno P. 2004. Ceramide-mediated macroautophagy involves inhibition of protein kinase B and up-regulation of beclin 1. J Biol Chem 279: 18384–18391.

Takeuchi H, Kondo Y, Fujiwara K, Kanzawa T, Aoki H, Mills GB, Kondo S. 2005. Synergistic augmentation of rapamycin-induced autophagy in malignant glioma cells by phosphatidylinositol 3-kinase/protein B inhibitors. Cancer Res 65:3336–3346.

Tanida I, Minematsu-Ikeguchi N, Ueno T, Kominami E. 2005. Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. Autophagy 1:84–91.

Tsuboi Y, Kurimoto M, Nagai S, Hayakawa Y, Kamiyama H, Hayashi N, Kitajima I, Endo S. 2009. Induction of autophagic cell death and radiosensitization by the pharmacological inhibition of nuclear factor-kappa B activation in human glioma cell lines. J Neurosurg 110:594–604.

Verheij M, Bartelink H. 2000. Radiation-induced apoptosis. Cell Tissue Res 301:133–142.

Yao KC, Komata T, Kondo Y, Kanzawa T, Kondo S, Germano IM. 2003. 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 98:378–384.

Zhuang W, Qin Z, Liang Z. 2009. The role of autophagy in sensitizing malignant glioma cells to radiation therapy. Acta Biochim Biophys Sin 41:341–351.

Zhuang W, Li B, Long L, Chen L, Huang Q, Liang Z. 2011. Induction of autophagy promotes differentiation of glioma-initiating cells and their radiosensitivity. Int J Cancer 129:2720–2731.