ABSTRACT

The WHO definition of high-grade gliomas relies on the presence of microvascular proliferation and/or tumor necrosis. New genetic data provides deeper understanding of different cell death processes and thus strengthen the link between the mechanism governing the establishment and progression to highly aggressive tumors such as glioblastoma. Apoptosis, necrosis and autophagy are important in the development of cancer but how they take place or how they are regulated is poorly understood. Here we show that there is a profound alteration of the genes of mitophagy in glioma. Specifically, the lack of BNIP3 (BCL2 Interacting Protein 3) copies correlates with increasing glioma degree. In addition, these alterations in BNIP3 show a distribution associated to cell death processes and p53 mutations. By using a mitochondrial stress inducing system, we have observed that BNIP3 regulates mitochondrial potential loss therefore triggering cell death with apoptotic or necrotic properties depending on p53 status. P53 mutant cells show necrosis characteristics lacking mitophagy activation. On the contrary, p53 wild-type cells demonstrate mitophagy activation, mitochondrial potential loss and apoptotic hallmarks. Chemical inhibition of PTEN-PI3K-AKT pathway also leads to apoptosis activation similar to the p53 wild-type condition. In conclusion, the type of death generated by the mitochondrial and oxidative stress based system depends on p53 and PTEN-PI3K-AKT status, which can lead to cell death with apoptotic or necrotic traits, being the first associated with less aggressive gliomas.

Keywords: glioma, BNIP3, p53, PTEN, apoptosis, necrosis, mitophagy

INTRODUCTION

Glioblastoma (GB) is the most aggressive brain tumor. A high percentage of GB (<90%) develop rapidly in elderly patients, without clinical or histologic evidence of a less malignant precursor lesion (primary GB) (1). On the other hand, secondary GB progress from low-grade diffuse astrocytoma (1). Histologically, primary and secondary GB are largely indistinguishable, but they differ in their genetic and epigenetic landscapes (2, 3). Histopathology, both type of GB have a high...
cellular density, frequent nuclear atypia, microvascular proliferation and necrotic areas, which distinguish them from low-grade gliomas (4). Compelling functional studies, conducted over the last two decades, have established that apoptotic programmed cell death serves as a natural barrier to cancer development (5-7). In contrast to apoptosis, in which dying cells shrink into an almost-invisible corpse that is rapidly phagocytized by neighboring cells, necrotic cells become bloated and explode, releasing their contents into the surrounding tissue microenvironment (8, 9). Therefore, necrosis stimulates inflammatory processes that ultimately favor the progression of cancer (9, 10). Autophagy is the cellular mechanism that disassembles unnecessary of dysfunctional components. Although some groups suggest that it can be concomitant and execute certain steps of cell death, autophagy is generally thought of as a survival mechanism controlling both necrosis and apoptosis processes (11-13). In addition, autophagy has been shown to control mitochondrial status by a recycling mechanism called mitophagy. This process is able to degrade damaged mitochondria, harboring low mitochondrial potential and thus maintain mitochondrial homeostasis. One of the best-described mitophagy mechanisms depends on PINK1/Parkin, although other proteins such as BNIP3/BNIP3L or FUNDC1 could also be of importance. Mitophagy is a process that deeply affects mitochondrial function, ROS (reactive oxygen species) production and MOMP (mitochondrial outer membrane permeabilization), which can control death with apoptotic or necrotic characteristics. Furthermore, mitophagy has a fundamental role in the elimination of damaged mitochondria, which relieves metabolic cell stress. Moreover, mitochondrial is the central organelle in the execution of apoptotic death (14). Despite all these, little is known about the control of mitophagy during the processes of necrosis and apoptosis. Mitophagy has been implicated in energy metabolism, cell cycle, and senescence. Moreover, it is also related to tumor progression and metastasis, although its role is not fully understood. BNIP3, for example, functions as a tumor suppressor in the progression of breast cancer (15). We have shown that autophagy and mitophagy are key in the development and treatment of gliomas because they are essential for the survival of these tumors (16-19).

Here, we show that there are alterations in multiple genes involved in the induction of mitophagy in gliomas. Those include copy number loss (CNL) of PINK1, Parkin (PARK2), FUNDC1 and VCP/p97. It is noteworthy the pronounced CNL observed in BNIP3, which positively correlates with the histological progression of gliomas. Our in silico analysis also reflects that lower expression of BNIP3 is associated with cell death-related processes and with the presence of p53 mutations and PTEN genetic loss. Using a death induction system that combines cyanide and hydrogen peroxide (lis/lin/GO), we demonstrate here that these mitochondrial stressors induce mitochondrial potential loss, mitophagy and apoptosis in wild-type p53 GB cells, whereas they induce necrosis in p53 mutant GB cells. Chemical inhibition of PTEN-P13K-AKT pathway also leads to apoptosis activation similar to the p53 wild-type condition. In conclusion, the type of death generated by the mitochondrial and oxidative stress based system depends on the status of p53 and PTEN-P13K-AKT, which can lead to cell death with apoptotic or necrotic traits, being the first associated with less aggressive gliomas.

MATERIALS AND METHODS

Special reagents
Non-commercial antibody recognizing the following proteins was used: ATG12-ATG5. It was kindly provided by Noboru Mizushima (Department of Cell Physiology, Tokyo Medical and Dental University, Japan).

The commercial primary antibodies used were: caspase-3 and p-AKT (473) (Cell Signaling); BNIP3, LC-3 and actin (Sigma); Hsp70 and MnSOD (Stressgen); and CD-63, cytochrome c and BAX (BD Bioscience); BNIP3, p53 and TOMM20 (Santa Cruz Biotechnology). The secondary antibodies for western blot studies were horseradish peroxidase conjugated to anti-rabbit or anti-mouse IgGs (DAKO, P0448 and P0161) and were anti-mouse or anti-rabbit IgGs alexa-488 or -594 labeled (Invitrogen, A21206 and A21203) for immunofluorescence.

Some special reagents used were: linamarin (lin, 50, 200 or 500 μg/ml; L466000 from Toronto Research
Chemicals); glucose oxidase (GO, 5.5 mEU/ml), N-acetyl-L-cysteine (NAC, 10 mM), 3-methyladenine (3MA, 10 mM), 1,5-isoquinolinediol (I138, DIQ, 300 μM), puromycin (P7255), and 3-[4,5-dimethylthiazolo-2- yl]-2,5-diphenyltetrazolium bromide (M2128, MTT, 200 μg/ml) all of them from Sigma; and bafilomycin A1, (SCBT, 2163, 100 nM).

Glialoma database and bioinformatic analyses
Classification into histological types was retrieved from The Cancer Genome Atlas (TCGA). Low and high grade glioma datasets (https://www.ncbi.nlm.nih.gov/pubmed/27157931) were analyzed together with mitophagy genes values (https://xenabrowser.net/; https://www.ncbi.nlm.nih.gov/pubmed/24120142). Other genetic alterations of the mitophagy or other signaling genes were analyzed from the TCGA datasets, which were downloaded respectively from cBioPortal (http://www.cbioportal.org/) and TCGA databases (http://tcga-data.nci.nih.gov/docs/publications/1gggbm_2015) using the UCSC cancer browser. The REMBRANDT datasets were downloaded from (http://www.betastasis.com/glioma/rembrandt/) and (https://gdoc.georgetown.edu/gdoc/). BNIP3 expression and copy number variation in histological grades and subtype were retrieved from the TCGA glioblastoma dataset (https://www.ncbi.nlm.nih.gov/pubmed/20129251) together with BNIP3 expression values. For the analysis of gene ontology we used the 500 genes that positively correlate with BNIP3 chosen using the Spearman correlation coefficient and this gene cluster were analyzed with the DAVID program 6.8. Statistically significant differences in BNIP3 copy number deletion between histological grades of glioblastomas were calculated using a Student’s t test.

Viral vector production
Pseudotyped lentivectors were produced using reagents and protocols from Didier Trono with the following modifications: HEK 293T cells were transiently co-transfected with 10 μg of the correspondent lentiviral vector, 5 μg of the packaging plasmid pCMVdr8.74 (Addgene plasmid 8455) and 2 μg of the VSV G envelope protein plasmid pMD2G (Addgene plasmid 11514) using Lipofectamine and Plus reagent following instructions of the supplier (Invitrogen, 18324 and 11514, respectively). Lentivector directing expression of shRNA specific to p53 [24] was purchased from Sigma and non-targeting PLKO.1 scramble shRNA from Addgene (plasmid 1864). The sequences encoding shcontrol and shBNIP3 were cloned in the plasmid pShSuper.neo (Oligoengine™) following commercial indications as previously described (17).

Cell culture
U-373 MG and U-87 MG were obtained from the ATCC and were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 10 U/ml penicillin and 10 μg/ml streptomycin at 37ºC, 7% CO2 and 97% relative humidity. Cells were passaged after enzymatic disaggregation using trypsin. Cell survival was determined using MTT assay (MTT, 200 μmol/L) as previously described (17).

Western blot analysis
Proteins were extracted from cells by using lysis buffer (50 mM pH 7.5 HCl-Tris 300 mM NaCl, 0.5% SDS and 1% Triton X-100) and 10-30 μg of these proteins were separated by SDS-PAGE and transferred to nitrocellulosa membranes. Blots were developed by peroxidase-conjugated secondary antibody and proteins were made visible by enhanced chemiluminescence with ECL (Pierce).

Flow cytometry
U-87 MG and U-373 MG were disaggregated into individual cells with Accumax (5 min, room temperature) and next they were stained with annexin-V-PE diluted in PBS-1% BSA and 1% Triton X-100) and 10-30 μg of these proteins were separated by SDS-PAGE and transfered to nitrocellulosa membranes. Blots were developed by peroxidase-conjugated secondary antibody and proteins were made visible by enhanced chemiluminescence with ECL (Pierce).

Immunofluorescent staining
U-87 MG and U-373 MG cells were grown in DMEM 10% FBS over coverslips and then fixed in 4% paraformaldehyde for 10 min. Cells were blocked for 1 hour in 2% BSA and 1% Triton X-100 in PBS and
incubated over-night with the primary antibody. Specific fluorescence-labeled secondary antibodies were used and DNA was stained with DAPI.

**Tumor samples and RT-PCR assays**

For qRT-PCR, using Roche RNA kit we isolated total RNA from patients’ derived xenografts of brain tumors provided by Hospital Universitario 12 de Octubre (Madrid, Spain) and cDNAs were synthetized with RT-PCR kit (Takara). qRT-PCR was performed with the Light Cycler 1.5 (Roche) using SYBR Premix Ex Taq quantifying by the delta delta Ct method. The following specific primers were used: BNIP3 (F: 5′-GCTCCAGACACACAAAGAT-3′ R: 5′-TGAGATGCTGCGCTTC-3′) and HPRT-I (F:5′-TGACACTGGCAAAAACATGCA-3′ R: 5′-GGTCCCTTTACCCAGCAAGCT-3′).

**RESULTS**

**Genetic alterations in mitophagy related genes and their involvement in the pathology of gliomas**

The main tumor-suppressor gene alterations in the development of the pathology of GB are mutations or deletion in p53, p16 and PTEN, which are associated with the progression of the pathology. However, little is known about the underlying mechanisms driving the aggressiveness of these tumors, which is mainly associated to an over-boost of pro-inflammatory signals where mitochondria and cell death play a central role. To address this issue, we used TCGA glioma database to analyze the copy number variations of several mitophagy-associated genes (20). Thus, we could observe a profound CNL of several genes such as BNIP3, FUNDC1, PARK2, PINK1 and VCP (p97) (Fig.1A). Some of these alterations have been already documented in GB, as is the case of Parkin (PARK2) (21, 22). The data showed that the BNIP3 gene presents a high frequency of CNL in glioma, which correlates with the progression of the histological grade (Fig.1B). Moreover, we observed that loss copy number and low expression of BNIP3 correlates with worse clinical outcome in glioma patients (Fig. 1C-E). For this reason, we subdivided the cohort of gliomas into low or high expression of BNIP3 using levels of RNA-seq on the low and high-grade glioma dataset. Similar to the results obtained in other studies, where the loss of copies of Parkin is associated with mutations in p53 (23), our volcano plot analysis showed that low BNIP3 expression is associated with mutations in p53 and PTEN (Fig. 1F). In addition, we confirmed in samples from human GB patients that tumors with mutant p53 have lower BNIP3 levels in comparison with p53 wild-type gliomas (Fig. 1G). At this point, it is important to note that gliomas that harbor BNIP3 CNL show low levels of other regulators of its function such as Bcl2 (data not shown). Then, we evaluated the genes that are co-expressed with BNIP3 in the glioma merge dataset to determine which biological processes are associated with the function of this gene in glioma. With this aim, we used 1000 genes that are positively co-expressed with BNIP3 in a significant way by Spearman’s correlation and we analyzed the associated processes using DAVID gene ontology. As shown in Figure 1H, these include processes related to organelles and regulation of cell death.

**Differences on the cell death morphology induced by the lis/lin/GO system depending on the status of p53**

Based on our previous data, we wondered whether BNIP3 might be regulating cell death in gliomas and if this could contribute to the progression of the pathology (7, 17, 18). To answer this question, we used a mitochondrial stress mediated cell death-inducer system, called lis/lin/GO (linamarse/linamarin/glucose oxidase). This therapeutic strategy is based on the combination of cyanide and oxidative stress to abrogate tumor growth (16-19). We have previously observed that this system induces cell death on a BNIP3 dependent manner and regulated by mitophagy (17).

Thus, by using system lis/lin/GO we evaluated the efficiency and characteristics of the induced cell death as a function of p53 status. To drive this analysis we used three cellular models of GB: Hs 683 (p53 mutant/PTEN wild-type), U-87 MG (p53 wild-type/PTEN null) and U-373 MG (p53 mutant/PTEN null) (24), which shown different level of BNIP3 (Fig. 2A). Then, these cells were treated with the lis/lin/GO.
system. In the Hs 683 and U-87 MG cells, *lis*/lin/GO system produced cell death (Annexin-V+/IP-), without breaking plasma membrane (Fig. 2B, top and middle row), typical of apoptosis. In contrast, in U-373 MG, cell death occurred upon plasma membrane rupture and Annexin-V+/IP+ profile (Figure 2A, bottom row),
Figure 2: Cell death morphology caused by the lis/lin/GO system in human GB cells. (A) Western blot analysis of the expression of PTEN, p53, BNIP3 and actin in human glioma cells. (B) Representative dot plot of the staining with annexin-V PE and 7-AAD by flow cytometric of U-87 MGlis and U-373 MGlis cells treated with lin (0.5 mg/ml) and GO (5 mUE/ml) for 72 h. The U-87 MGlis and U-373 MGlis cells were generated by infection with a retrovirus encoding the lis gene (linamarase) followed by puromycin selection (1 mg/ml). (C) Kinetics of the survival percentage measured by MTT of the same cell lines treated as mentioned in the absence or presence of NAC (10 mM), * p value <0.05, n=3 by group. (D) Representative Western blot analysis of the expression of BNIP3 and Actin in U-87 MGlis and U-373 MGlis in absence or presence of lin (0.5 mg/ml) and GO (5 mUE /ml) for 48 h and (E) quantification of the data, * p value <0.05, n=3 by group.

typical of necrosis or late apoptosis. Induction of the later cell death produces a delayed cytotoxicity of lis/lin/GO system, which is shown in the MTT analysis, Figure 2C. To corroborate that the lis/lin/GO system generates cell death due to oxidative stress production we used the antioxidant NAC (N-acetylcysteine) and we could observe the rescue of lis/lin/GO mediated cytotoxic effect. In both GB cells, the lis/lin/GO system was able to induce expression of BNIP3, although U-87 MG presented a higher basal amount and generated a greater BNIP3 induction (Fig 2D and E).
Differential response at the mitochondrial level under the induction of death by lis/lin/GO system is dependent on p53/PTEN status

Different steps occur during cell death. In apoptosis, for example, one of the first events is the loss of mitochondrial potential and the subsequent activation of the caspases pathway, which governs the dismantling of the cellular organization. To study the effect of system lis/lin/GO on the cell death characteristics, we studied the mitochondrial status, in both p53 wild-type and mutant GB models. As shown in Figure 3 A-C, we measured mitochondrial potential and plasma membrane integrity by staining with DiOC$_6$(3) and propidium iodide, respectively. This staining indicates that, when the cell loses mitochondrial potential and maintains plasmatic membrane integrity, they die with apoptosis characteristics. However, when cells die loosing...
plasmatic membrane integrity and maintaining mitochondrial potential, they die with necrosis features. Hs 683 and U-87 MG cells, expressing wild-type PTEN or p53, showed a stronger loss of mitochondrial potential than U-373 MG cells, with null PTEN and mutant p53 (Fig. 3A-B). In addition, the inability of U-373 MG cells to induce the mitochondrial outer membrane permeabilization

Figure 4: Crosstalk between mitophagy and apoptosis in death induced by the lis/lincRG system. (A) Representative image of immunofluorescence against TOMM20 (mitochondrial marker, red) and CD63 (lysosome marker, green) in U-87 MG/lis and U-373 MG/lis in control condition or treated with lin (0.5 mg/ml) and GO (5.5 mEU/ml) for 24 h. The arrows indicate co-localization and (B) Quantification of the immunofluorescence shown in (A), * p value <0.05, n=3 by group. (C) Representative immunofluorescence images of U-87 MG/lis cells after treatment with lin and GO for 48 h showing the cellular localization of Bax (green) and MnSOD (red) in the absence or presence of bafilomycin A1 (100 nM) and nuclei were stained with DAPI; bars 10 μm. (D) Quantification of the immunofluorescence shown in (C), * p value <0.05, n=3 by group. (E) Western blot analysis showing the presence of Bax and cytochrome c in mitochondrial extracts or cytoplasm of U-87 MG/lis cells similarly treated with lin and GO and in the presence of bafilomycin A1 during 48 h.
(MOMP), seemed to favor the loss of plasmatic membrane integrity (Fig. 3C).

We then analyzed the mitochondrial characteristics at structural level by the immuno-detection of proteins involved in the induction of apoptosis, such as cytochrome c and BAX. In this situation, the system lis/lin/GO induced loss of cytochrome c and accumulation of BAX in the mitochondria in U-87 MG cells, with wild-type p53 (Fig. 3D-E). On the contrary, these events did not occur in the cells with mutant p53 (U-373 MG) (Fig. 3F), where cytochrome c was maintained in mitochondria and no translocation of BAX was observed. Therefore, the mitochondrial stress generated by the lis/lin/GO system induces a classical activation of p53 through BAX and generates the release of cytochrome c and MOMP. These events do not occur in cells expressing mutant p53.

Mitophagy is induced by mitochondrial stress in p53 wild-type but not in p53 mutant glioma cells

One of the main mechanisms of the organelles homeostasis is autophagy, which allows the recycling of these organelles and leads to the elimination of its toxic effect and the generation of energy (25, 26). In particular, our interest focused on mitophagy due to its ability to eliminate damaged mitochondria. Therefore, we analyzed the participation of mitophagy on the cell death induced by the lis/lin/GO system. For that, we stained GB cells with CD63 as a lysosomal marker and TOMM20 as a mitochondrial marker. We observed that the lis/lin/GO system induced a significant co-localization of mitochondrial markers with lysosomes, which is a hallmark of mitophagy in cells wild-type p53 cells (U-87 MG), although this process was not observed in p53 mutant cells (U-373 MG) (Fig. 4A and B). These results suggest that the lis/lin/GO system induces mitochondrial stress and mitophagy in wild-type p53, but not in p53 mutant GB cells. However, autophagy was induced in both cell types as we observed autophagic vacuoles in response to the lis/lin/GO system in the U-373 MG cells (mutant p53) (Fig S1A and B). Moreover, the generated death could be rescued with 3-methyl-adenine (3MA) (an autophagy inhibitor) or the shRNA against ATG5 (a gene necessary for autophagy) (Fig S1C and D). These results were similar to those obtained in U-87 MG cells (p53 wild-type) (data not shown) as we had previously shown (17).

Next, we observed that the induction of BAX and the loss of cytochrome c from the mitochondria in U-87 MG cell (Fig. 3C) could be inhibited in the presence of bafilomycin A1, a potent inhibitor of the vacuolar ATPase, which impaired autophagy flux and mitophagy (Fig. 4C-D). These results were also confirmed by subcellular fractionation and western blot analysis where the lis/lin/GO system induced BAX expression and cytochrome c release to the cytosol, and this process was reversed by the addition of bafilomycin A1 (Fig. 4E). Altogether, these results show that the lis/lin/GO system is capable of inducing apoptosis and mitophagy features in wild-type p53 GB cells.

Down-regulation of BNIP3, p53 mutant or PTEN-PI3K-AKT signal produces a shift towards necrotic cell death characteristics

The regulation of cell death and the relationship between different death types can determine the development of multiple pathologies. One of the most intricate crosstalk occurs in cancer development. On one hand, the impairment of apoptosis is key in the initiation of tumorigenesis, on the other hand, necrosis has been shown to be one of the important processes in the development and progression of aggressive phenotypes in gliomas.

To shed light on the interrelationship between different death types and apoptotic or necrotic characteristics, we set out to modify the levels of BNIP3 or mutant p53, as well as the regulation of PTEN/PI3K/AKT pathways. First, using a specific shRNA for BNIP3 (Fig. 5A-B), we observed that LC3II levels decrease in U-87 MG cells. We also simultaneously analyzed three features associated with cell death, the loss of mitochondrial potential (DiOC_{6}(3)), the integrity of the plasma membrane (propidium iodide) and the induction of autophagic vacuoles (acridine orange). The downregulation of BNIP3 induced a switch in the response of U-87 MG cells to the lis/lin/GO, reducing the increase in the number of autophagic vacuoles, favoring a necrotic profile of cell death (Fig. 5C and D).
In order to study what is the implication of key genes in the development of gliomas such as p53 and PTEN, we analyzed the inhibition of PTEN/PI3K/AKT, using the inhibitor Ly 29004, in the model U-373 MG (p53 mutant), after the lis/ling/GO treatment. We could observe that Ly29004 induced the loss of mitochondrial membrane potential in such conditions (Fig. 6A). Then we studied the effect of the downregulation of mutant p53 gene expression combined it with the inhibition of PI3K by Ly29004 in U-373 MG (Fig. 6B). The downregulation of p53 generated an increase of BNIP3 and mitochondrial potential loss after lis/ling/GO treatment and this effect was strongly enhanced by PI3K inhibition (Figure 6 B-D).

In summary, these data indicate that BNIP3 favors the apoptotic profile in wild-type p53 GB cells and the
elimination of this gene generates necrotic characteristics. In the opposite situation, absence of mutant p53 or lack of activation of the PTEN/PI3K/AKT pathway favors apoptotic characteristics.

DISCUSSION

Deciphering the involvement of cell death in pathology development has been one of the main contributions for the majority of the disease treatments, being cancer one of the most benefited, from diagnosis to therapeutic approaches. Exhaustive analyses during last decades have demonstrated that different cell death types play distinct roles depending on cancer stage (7). Remarkably, gliomas show strong apoptosis resistance and necrosis is fundamental for their progression to high-grade gliomas and the establishment of aggressive phenotype, known as GB (3, 4).

It had been recently demonstrated that several mitophagy genes are deregulated at genomic level in gliomas; among them, it is important to note Parkin (PARK2) alterations such as mutations and deletions in GB (22). Here, we have reevaluated a mitophagy gene
set (previously validated), analyzing their modification in a large glioma patient TCGA dataset (n=1122). We have observed that BNIP3 exhibits higher frequency of CNL than Parkin and shows important involvement in high and low grade glioma patients. This lead us to hypothesize that BNIP3 might be a strong candidate to be involved in gliomagenesis. Another remarkable aspect of Parkin is its function as a tumor suppressor and its loss of function associated to p53 mutations. Similarly, we have observed that BNIP3 low expression levels are associated to mutations in two key tumor suppressor genes in glioma development as are PTEN and p53. Additional to these analyses at the genetic level, several mitochondrial protein functions have demonstrated an involvement in different crucial pathways for cancer development. It is important to note that mitochondrial alterations, as well as apoptosis, necrosis and mitophagy, play a central role in the majority of processes that drive tumorigenesis (7). One of the main pathways studied in cancer is autophagy, due to the fact that tumors strongly depend on this mechanism to avoid metabolic stress to which these cells are subjected during cancer development (27, 28).

To unravel how the mechanisms of apoptosis, necrosis and mitophagy may take place in gliomas we have used a mitochondrial stress-inducer system (lis/lin/GO) that causes cell death (16-19). The use of this system helped us to understand several aspects of gliomas such as their strong sensitivity to mitochondrial stress (17, 18). We could observe that BNIP3 and mitophagy are central in the execution of glioma cell death. Accordingly, several groups have demonstrated that BNIP3 plays a central role in gliomas as well as in other cancer types (15, 29-33).

We have deepened in the regulation of different death types and mitophagy and we could observe that mitophagy operates at the same level of apoptosis and may condition necrosis characteristics. These data are in agreement with other works where association of BNIP3 to HIF1 hypoxia factor induction has been observed. This factor is induced in tumor necrotic areas as a response for the generation of vascular recovery in order to change tumor microenvironment, allowing tumor growth what it is promoted in BNIP3 KO mice (15, 33-35). It is especially important to note that our results indicate that p53 wild-type GB, which are sensitive to apoptosis, may be susceptible to progress to more aggressive tumors by BNIP3 loss and the subsequent necrosis induction. On the other hand, putative BNIP3 function as tumor suppressor is a hot topic because, as we demonstrated here, its absence in the tumor may favor necrosis induction, which is an evident demonstration of the acquisition of more aggressive phenotypes in gliomas (20, 33).

Additionally, we observed that BNIP3 CNL correlates with tumor suppressors PTEN and p53 loss of function. This direct relation lead as to hypothesize that both cooperate in the generation of tumor cells with high adaptation capacity. To understand these aspects is necessary to consider tumor as a tissue in continuous evolution where apoptosis resistance favors progression and allows specific microenvironment control (9). Thus, we think that the study of these processes regulation in cancer development is crucial, mainly in GB, where microenvironment and specially necrosis show a narrow relationship. In fact, other authors have demonstrated diverse mechanisms by which BNIP3 expression is inhibited, leading to an important tumor profile change (36-38). However, more studies are necessary to reinforce the idea that p53 and PTEN mutations may be conductors of gliomas progression from low to high grade associated to BNIP3 loss.

The relevance of understanding cell death regulation in cancer and the alterations of these mechanisms lays on the fact that numerous targets have been found to induce cell death. Moreover, many therapeutic strategies rely on perturbations of these processes in order to generate an efficient GB therapy, which remains as a pending issue in modern medicine (20). Nowadays, multiple works show a source of possible therapeutic targets in mitochondria for specific treatments in particular cancer types (39, 40). In conclusion, the screening of additional and more specific mitochondrial stress inductors will provide new and more efficient therapies for GB treatment.

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ABBREVIATIONS:
3MA: 3-methyl-adenine
Astro: Astrocytoma
BAFI: Bafilomycin A1
AO: Acidine orange
BNIP3: BCL2 Interacting Protein 3
CNL: copy number loss
DiOC6(3): 3,3′-Dihexyloxacarbocyanine Iodide
DMSO: Dimetilsulfoxido
GB: Glioblastoma
Ly: LY294002
lis/lin/GO: linamarase/linamarin/glucose oxidase
MnSOD: manganese-dependent superoxide dismutase
MOMP: mitochondrial outer membrane permeabilization
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC: N-acetyl-L-cysteine
OA: Olygoastrocytoma
OD: Olygodendroglioma
PI: Propidium Ioduro
PM: Mitochondrial potential
ROS: Reactive oxygen species
WHO: World Health Organization

REFERENCES


Figure S1: Induction of autophagy by the lis/lin/GO system in U-373 MG.

(A) Representative confocal images of U373-MGlis cells transfected with GFP-LC3 treated with \( \text{As}_2\text{O}_3 \) (6 mM), lin (0.5 mg/ml) and GO (5 mUE/ml), and bafilomycin (100 nM) as positive control for the formation of vacuoles, for 24 h. (B) Quantification of the percentage of cells exhibiting a dotted LC3 pattern with respect to the total number GFP-cells. Graph represents the mean values and standard deviations per field counting a total of 150-200 cells per condition. (C) Survival percentage measured by MTT of U-373 MGlis cells treated for 48 with lin and GO as in (A) and in the presence of 3-methyladenine (3MeA, 10 mM). (D) Survival percentage measured by MTT of U373MGlis cells infected with a lentivirus encoding a shRNA control (shcontrol) or against ATG5 (shATG5) treated for 48 h with lin and GO. The graphs (C-D) show mean values and standard deviations of 4 independent samples.