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TET1 regulates DNA repair in human glial cells

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Abstract

Glioblastomas are the most aggressive of malignant brain cancers with a median patient survival of approximately 18 months. We recently demonstrated that Tet methylcytosine dioxygenase 1 (TET1) is involved in cellular responses to ionizing radiation (IR) in glial-, glioblastoma-, and non-tumor-derived cells. This study used a lentiviral-mediated knockdown of TET1 to further dissect the contribution of TET1 to the DNA damage response in glial cell lines by evaluating its role in DNA repair. TET1-deficient glial cell lines displayed attenuated cytotoxicity compared to non-targeted knockdown after treatment with IR but these differences were not observed between control and TET1 deficient in response to inhibitors of Na⁺/K⁺-ATPase. Additionally, the percentage of glial cells displaying γ H2A.x foci was greatly reduced in TET1-deficient glial cells compared to non-targeted knockdown conditions in response to IR and topoisomerase inhibitors. We also observed a lower percentage and a delay in 53BP1 foci formation, a marker of non-homologous end-joining, in response to IR and topoisomerase inhibitors in TET1-deficient glial cells. DNA-PK, another marker of non-homologous end-joining, was also lower in TET1-deficient glial cell lines. Interestingly, TET1-deficient glial cells displayed higher numbers of DNA strand breaks compared to control cells and repaired DNA breaks less efficiently in Comet assays. We suggest that attenuated DNA repair in TET1 deficient gliomas leads to genomic instability, which underlies poor patient survival.

Keywords

Human glioma; TET1; Non homologous DNA repair; Ionizing radiation; DNA-PK

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Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

1. Introduction

The DNA damage response (DDR) is a protective mechanism that is evoked when cells are exposed to agents that damage DNA. It involves a delay in the cell cycle to provide time to repair DNA, protein-protein interactions to localize DNA repair enzymes to the site of damage, and apoptosis if the DNA damage cannot be repaired (Li et al., 2016), (Hanawalt, 2015), (Blackford and Jackson, 2017), (He et al., 2016). DDR has evolved to preserve genomic integrity, which is a prerequisite for proper cell function and faithful transmission of the genome to progeny. Most agents used in cancer therapy are effective by causing sufficient DNA damage to induce apoptosis. Resistance to these chemotherapeutic drugs is very often due to mutations in the genes in the DDR pathway (O'Connor, 2015; Nilles and Fahrenkrog, 2017). To initiate the DDR, serine/threonine kinases in the Phosphatidylinositol 3-kinase-related kinase family, Ataxia Telangiectasia Mutated (ATM), Ataxia Telangiectasia and Rad3 Related (ATR), and DNA Dependent Protein Kinase catalytic subunit (DNA-PKcs) are activated by DNA strands breaks (Blackford and Jackson, 2017). An early event in DNA repair is the phosphorylation of serine-139 in variant histone H2A.x (γ H2A.x), which serves as an activating signal for recruitment of the different complexes that will initiate and execute DNA repair (Podhorecka et al., 2010). ATM and DNA-PK activation results in different DNA repair mechanisms, homologous recombination (HR) and non-homologous end joining (NHEJ), respectively. NHEJ is a faster and more error-prone form of DNA repair than HR and utilizes 53BP1 to promote the end-joining of distal DNA ends (Rothkamm et al., 2015).

Patient survival very much depends on sensitivity of the tumor to therapy. Because IR is the primary agent for treating gliomas and Tet methylcytosine dioxygenase 1 (TET1) responds to reactive oxygen conditions (Coulter et al., 2013), we recently investigated potential roles for TET1 in glioma cell lines in response to ionizing radiation (IR) and found that knockdown of TET1 led to greater resistance to IR in colony forming assays (Coulter et al., 2017). We also found greater numbers of apoptotic cells and greater induction of γ H2A.x when TET1 was expressed. TET family members catalyze the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) (Wu and Zhang, 2017), suggesting that this enzymatic process could be involved in the DDR. Interestingly, better survival has been reported in patients with gliomas that have higher levels of hydroxymethylcytosine (5hmC) (Orr et al., 2012) (Johnson et al., 2016). It is possible that higher levels of 5hmC result from higher levels of TET1 activity and an unexplored role for TET1 in the DDR yields greater sensitivity to IR.

In this study, we further characterize the involvement of TET1 in survival and the DDR in glial cell lines. We report that the effects of TET1 deficiency on cell survival are relatively specific to agents that target DNA. TET1 deficiency attenuated the effects of IR on survival but not the effects of ouabain, which targets Na, K, ATPase and kills cells by disrupting ion homeostasis (McConkey et al., 2000; Winnicka et al., 2007). We also report that TET1-deficient glial cells displayed an impaired DDR that involves NHEJ.

2. Methods

2.1. Chemicals and reagents

Transfections were accomplished using Lipofectamine 2000 (Thermo Fisher, #11668027). Crystal Violet powder was obtained from Sigma-Aldrich (St. Louis, MO; #C0775) and brought into solution with dH₂O at 0.1%. Antibodies used in immunocytochemistry are the following: 1) Anti-phospho-Histone H2A.x (Ser139) Antibody, mouse monoclonal, clone JBW301 (EMD Millipore; #05–636) 2) Rabbit anti-53BP1, polyclonal, (Bethyl Laboratories; A300–272A); anti-mouse (Alexa-Fluor 488) and anti-rabbit secondary antibody Cy3 (both from Jackson ImmunoResearch Laboratories; #715–165-150/#711–165-152). ProLong® Gold Antifade agent with DAPI (4', 6-diamidino-2-phenylindole) was used to mount and counterstain nuclei in immunocytochemistry (Cell Signaling Technology; #8961). Comet assay slides and lysis buffer were acquired from Trevigen (CometSlide™ #4250–200-03; CometAssay® Lysis Buffer #4250–050-01). Low melting agarose (NuSieve® GTG® Agarose; Lonza #50084). SYBR® Gold Nucleic Acid Gel Stain (10,000× concentrate in DMSO) ThermoFisher (Invitrogen™; #S11494) was used to stain comet assay slides.

2.2. Cell culture and treatments

A172 glioma cells were obtained from ATCC and the 10B1 glial cells were obtained from the laboratory of Dr. Eugene O. Majors from NINDS/NIH (Ferenczy et al., 2013). TET1 - deficient cell lines (shTet1) were established as previously reported (Coulter et al., 2017). In brief, transfections were done using Lipofectamine 2000 and scaled per the recommendations from the manufacturer. Following prior screening of pLKO plasmids containing shRNA against *TET1* for knockdown efficiency, clone TRCN0000075026 was used to generate shTet1 A172 and 10B1 cell lines. The control A172 and 10B1 cell lines (shEV) were transduced with empty vector pLKO. Cells were selected with 1 µg/mL puromycin and deficiency was validated by qRT-PCR. Cells were cultured in high-glucose (4.5 g/L) DMEM supplemented with 10% fetal bovine serum.

IR was delivered using a cesium-137 source gamma cell irradiator at a dose rate of 5.55 cGy/s. The topoisomerase inhibitors etoposide (ETO) and camptothecin (CPT) were used at 10 µM and 3 µM, respectively, for the lengths of time indicated.

2.3. Colony forming unit assay

Cells were irradiated in suspension at the indicated doses, plated in 6-well plates at 500 cells per well, and cultured for 12 days. Cells were then fixed with 3.7% formaldehyde for 15 min, permeabilized with ice-cold methanol, and stained with 0.1% Crystal Violet. Cells were de-stained by washing with PBS and plates were air dried. Colonies of 50 cells were included in calculating survival fraction.

2.4. Immunocytochemistry

5 × 10⁴ cells were plated on glass coverslips in 35 mm dishes and allowed to adhere overnight. Cells were irradiated at the indicated doses and lengths of time and fixed with 3.7% formaldehyde for 15 min. Cells were washed with PBS and permeabilized with 1%

Triton-X 100 in PBS. Following blocking with 2.5% BSA, cells were incubated with mouse anti- γ H2A.x (1:400) or with rabbit anti-53BP1 (1:400), at room temperature for 45 min. Coverslips were washed then incubated with 1:150 Alexa-Fluor 488 anti-mouse/anti-rabbit secondary antibody or 1:150 Cy3 for 30 min at room temperature. Coverslips were washed and mounted. 20 $\times$ and oil immersion 40 $\times$ and 63 $\times$ images were taken using a fluorescent microscope (Zeiss Apotome System). γ H2A.x and 53BP1 foci were quantified with hand counting. To compute percentage positive cells, the numbers of cells with 5 foci or more were divided by the total number of cells measured by DAPI staining.

2.5. Comet assays

Neutral and alkaline comet assays were performed accordingly to adapted protocol recommendations from Trevigen. In summary, 30 μ L of single cell suspension of 2×10^5 was mixed with 270 μ L of 0.1% low melting agarose. 50 μ L of the cell-agarose solution was pipetted onto each well per CometSlideTM and were allowed to set-up at 4 $^{\circ}$ C for 30 min. For maximum sensitivity, slides lysed overnight at 4 $^{\circ}$ C. Slides were rinsed in 1 $\times$ TBE buffer for 15 min prior to being electrophoresed at 21 V for 40 min at 4 $^{\circ}$ C. Slides were then washed in dH₂O and 70% ethanol for 5 min each and then transferred to 37 $^{\circ}$ C until the gel became level with the slide. Slides were stained with a 1:10,000 solution of SYBR[®] Gold Nucleic Acid Gel Stain in dH₂O for 30 min at room temperature in the dark and rinsed with dH₂O. After slides had dried at 37 $^{\circ}$ C, images were taken using a fluorescent microscope (Zeiss Axiovision System).

The alkaline comet assays varied from the neutral comet assay protocol described above with a 20 min alkaline solution unwinding step at room temperature, which replaced the 15 min 1 $\times$ TBE rinse step prior to electrophoresis. Comets were then electrophoresed in the same alkaline solution for 30 min at 21 V.

Comets were analyzed using CometScore software (TriTek) to acquire comet tail moment of individual cells.

2.6. RT-PCR

Total RNA was extracted following the manufacturer's guidelines using the Qiagen RNeasy Mini Kit and A260/A280 ratios < 1.8 were discarded. 1–3 μ g of total RNA was reverse transcribed into cDNA using MuLV Reverse Transcriptase and Oligo(dT) primers by Applied Biosystems (ThermoFisher). qRT-PCR was performed using an iQ5 detection system (Bio-Rad) and the expression of target genes was detected using Power SYBR green PCR kit (Applied Biosystems, ThermoFisher). Samples were analyzed in triplicate and relative gene expression was analyzed using the Bio-Rad iQ5 software and normalized to GAPDH.

2.7. Data analysis

Graphs and statistics were generated using GraphPad Prism v6. Bar graphs represent the average and standard deviation (SD) plotted. Scatter plots were also used to show distribution of data points. Unless otherwise noted, two-Way ANOVA and Tukey Post-Tests were performed and significant differences were considered at $*p < 0.05$.

3. Results

3.1. Responses of TET1-deficient cells (shTet1) in cell survival

We asked whether the survival advantage of TET1-deficient glial (shTet1) cells in response to IR would also be observed in response to cytotoxic agents that do not target DNA. A > 85% decrease was observed in levels of TET1 mRNA in both shTet1 A172 glioma (Fig. 1A) and shTet1 10B1 glial cell lines (Fig. 1D) compared to the control shEV cells. Differences were not observed in levels of TET2 mRNA levels. Differences were also not detected in TET3 mRNA in the 10B1 cells whereas TET3 mRNA was not detected in the A172 cells. The shTet1 A172 glioma (Fig. 1B) and shTet1 10B1 glial cell lines (Fig. 1E) displayed significantly higher colony forming efficiency after treatment with IR compared to the shEV cells. Ouabain at 10 nM drastically reduced cell survival of the A172 (Fig. 1C) and the 10B1 (Fig. 1 F) cell lines but difference were not observed between shTet1 cell and shEV for either one. We compared other Na⁺/K⁺-ATPase inhibitors on the A172 cell line but did not find differences between the shTet1 and shEV (Fig. S1). These results suggest TET1 deficiency provides a survival advantage relatively specific for DNA damaging agents.

3.2. TET1 involvement in the induction of γ H2A.x foci in glial cells

To further characterize the involvement of TET1 in the process of initiating DNA repair, we compared shTet1 and shEV glial cells for the formation of γ H2A.x foci formation in response to DNA damaging agents. The 10B1 cell line was examined to investigate cells that are not derived from tumors (Ferenczy et al., 2013). Foci were observed in the shTet1 and shEV 10B1 cells at 3 h after IR (Fig. 2A). The peak response in the shEV 10B1 cell line occurred at 8 h whereas 24 h were required to observe the peak response in the shTet1 10B1 cells. At all of the time points examined, the peak responses were greater in the shEV cells compared to the shTet1 cells.

To determine whether the effects of TET1 expression on γ H2A.x induction were restricted to IR, we studied γ H2A.x foci formation in 10B1 cells after treatment with topoisomerase 1 and 2 inhibitors CPT and ETO, respectively (Swift and Golsteyn, 2014). Similar to the responses observed after IR treatment, the maximum induction of γ H2A.x foci following CPT treatment was significantly less in the shTet1 10B1 glial cells compared to the shEV glial cells (Fig. 2B). The percentage of shEV cells with γ H2A.x foci at 3 h and 24 h after treatment with CPT was significantly higher compared to the shTet1. Also, differences were observed between the shTet1 and shEV glial cells in response to ETO treatment (Fig. 2C). These results show TET1 is required to sense double strand breaks and suggest TET1 is a critical determinant of the DDR.

3.3. 53BP1 response in shTet1 glial cells

Considering that activation of DNA-PK promotes NHEJ (Burma et al., 2006; Dobbs et al., 2010; Pawelczak et al., 2011) and that TET1-deficient glial cells lose DNA-PK expression (Coulter, Lopez-Bertoni et al. 2017), we investigated whether TET1 deficiency attenuates the recruitment of the NHEJ 53BP1 protein to DNA foci. Concurrent with the defects observed in γ H2A.x foci formation, shTet1 10B1 cells also displayed diminished 53BP1 foci formation compared to shEV l cells after exposure to IR (Fig. 3A). The response to

topoisomerase inhibitors was examined for the possibility that the effects of TET1 deficiency were specific to IR. At one hour after treatment with CPT, an increase was observed in the percentage of 53BP1 foci in shEV cells but not in shTet1 cells (Fig. 3B). At 3 h after CPT, the percentage of cells with 53BP1 foci declined in shEV cells although it remained greater than the basal levels. Similarly, the percentage of cells with 53BP1 foci increased in response to ETO in shEV cells but not in shTet1 cells (Fig. 3C). These results indicate the involvement of TET1 in NHEJ in response to DNA damaging agents.

3.4. DNA repair in shTet1 and shEV A172 cells

We turned our attention to glioma cell lines to understand the potential clinical relevance of our findings to cancer treatment. shTet1 and shEV A172 glioma cells were treated with IR to assess the γ H2A.x and 53BP1 foci responses. The peak γ H2A.x response was observed earlier and to a greater extent in the shEV cells than the shTet1 glioma cells lines (Fig. 4A). Moreover, the response was delayed in the shTet1 cells. In shEV cells the response declined after 3 h but continued to increase at 24 h in the shTet1 cells.

Interestingly, A172 control cells displayed a higher percentage of 53BP1 foci even in the absence of IR (Fig. 4 B). At one hour after treatment, an increase in foci was observed in both shTet1 and shEV A172 glioma cells. Although the maximum response at one hour was higher in the control cells, the fold-induction did not appear different. The shEV A172 glioma cells maintained the 53BP1 response at 4 h after IR but the number of foci greatly diminished in the shTet1 cells.

To measure the ability of A172 glioma cells to repair DNA strand breaks, comet tail moments were measured at different lengths of time after treatment in both neutral and alkaline comet assays, which detect double and single strand breaks respectively (Glei et al., 2016). In the neutral Comet Assay, shTet1 A172 cells exhibited a greater tail moment without IR treatment, which is indicative of more DNA double strand breaks, compared to the shEV A172 glioma cells (Fig. 4C and B). An increase was observed in tail moment after IR treatment in shEV A172 cells at 4 h and it decreased at 8 h (Fig. 4C). Similarly, an increase in tail moment was observed at 4 h, which decreased at 8 h in shTet1 A172 cells. The tail moment in shTet1 A172 glioma cells, however, was higher than it was in the control cells even at 24 h after treatment with IR. In the alkaline assay, it was necessary to measure tail moments at earlier time points because the highest responses were observed at one hour after treatment in shEV and shTet1 A172 glioma cells (Fig. 4D). Decreases were observed in tail moment at 4 h after IR in both the shEV and shTet1 A172 glioma cells. Again, higher tail moments were observed in the shTet1 compared shEV A172 glioma cells. Overall, shTet1 A172 glioma cells carry out DNA repair but contain a higher basal level of strand breaks than the shEV A172 glioma cells.

3.5. DNA-PK levels in glioma cells

DNA-PK activation is an early step in the NHEJ pathway for DNA repair. Western blot analysis confirmed our previous findings (Coulter, Lopez-Bertoni et al. 2017) that shTet1 cells displayed lower levels of DNA-PK than shEV cells (Fig. 5A). The involvement of DNA-PK in patient survival was examined using data from the GlioVis portal, which is a

web-based tool designed to access data relevant to brain tumor research (<http://gliovis.bioinfo.cnio.es>). Better survival was found in patients with higher levels of DNA-PK (Fig. 5B). Also, a positive Pearson correlation coefficient of 0.48 was determined when compared TET1 and DNA-PK expression, which indicates a positive correlation between the expression of TET1 and DNA-PK (Fig. 5 C).

4. Discussion

Chemotherapy resistance is often due to mutations and/or alterations in gene expression (Holohan et al., 2013). This report, which complements our earlier findings (Coulter, Lopez-Bertoni et al. 2017), shows that TET1 deficiency in glial cell lines display significantly better survival after treatment with IR compared to control glial cells (Coulter, Lopez-Bertoni et al. 2017). IR activates the ATM- and DNA-PK pathways resulting in DNA repair, but apoptosis ensues if the repair is insufficient. IR also induces cell death through pathways not involving DNA damage such as through endoplasmic stress (Kim et al., 2010; Zhang et al., 2010). The evidence presented here indicates that the effects of TET1 deficiency in the colony assay were due to an impaired DDR. Differences were observed in survival in response to IR but not to ouabain, which targets Na⁺ K⁺ ATPase and has been reported to induce death by disrupting calcium homeostasis and inducing hypoxia (Winnicka et al., 2007; Alonso et al., 2013; Xie et al., 2013). Interestingly, a recent studies found an inverse relation between incidence of glioma and use of cardiac glycosides (Sun et al., 2013; Chen et al., 2014; Garofalo et al., 2017). The study suggests that cardiac glycosides could be effective in preventing gliomas. Moreover, studies have shown the induction of apoptosis in glioma cell lines treated with cardiac glycosides (Nigim et al., 2015; Yan et al., 2015).

Evidence is also presented showing lower levels of DNA-PK in the TET1 deficient A172 glioma cell line. The inverse relation between DNA-PK and Tet1 was validated in human gliomas. DNA-PK is activated by DNA damage and evokes DNA repair, cell cycle inhibition, and apoptosis. More specifically, DNA repair evoked through DNA-PK activation is through the NHEJ pathway (Hill and Lee, 2010). The lower levels of DNA-PK would be expected to attenuate this pathway. Indeed, the formation of γ H2A.x foci is delayed and the numbers of foci were much lower in TET1 deficient cells after treatment with IR and topoisomerase inhibitors. Similarly, the formation of 53BP1 foci was delayed and the levels of foci were much lower in TET1-deficient cells than in controls after IR and treatment with topoisomerase inhibitors. 53BP1 acts as a scaffold to recruit several proteins to damaged chromatin (Panier and Boulton, 2014) to mediate NHEJ repair and its recruitment requires DNA-PK activity (Burma et al., 2006). In addition to maintaining DNA-PK expression, TET1 might also have a more direct role in DNA repair. 5-hydroxymethylcytosine was reported to accumulate at sites of DNA damage and co-localize with γ H2A.x and 53BP1 foci (Kafer et al., 2016). It is possible that active DNA de-methylation occurs at sites of DNA damage sites and is needed to promote DNA repair. Consequently, there might two or more distinct mechanisms involving TET1 in DNA repair: expression of DNA-PK and converting the nearby methyl cytosine to hydroxymethyl cytosine.

Comet assays revealed that TET1-deficient cells were capable of repairing DNA but the levels of DNA strand breaks remained elevated before and after treatment. DNA repair is

likely due to other pathways in TET1-deficient cells such as homologous repair. Our previous study did not find changes in levels of ATM in TET1 deficient cells (Coulter et al., 2017). Diminished DNA-PK could also compromise the apoptotic pathway and allow cells with genomic instability to survive. Genomic instability has been shown to result in an increased mutation rate and an increase the probability of the survival of a tumor cell subpopulation (Godek et al., 2016). Low TET1 expression could also result in the survival of cells with mutations and other types of DNA damage during the early stages of neoplastic progression (Laughney et al., 2015; Bakhoun and Landau, 2017). Lower levels of TET1 could come about from changes in the cellular microenvironment. Hypoxia and oxidative stress have been shown to affect TET1 regulation though the nature of the effect (increase or decrease) depends on the type of cells (Aguilera and Gomez-Gonzalez, 2008; Wu, 2016).

Overall, we suggest low levels of TET1 provide gliomas cells with a survival advantage by increasing genomic instability. Moreover, novel treatment paradigms for gliomas should be directed toward identifying and adding small molecular weight compounds that kill glioma cells without targeting DNA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

TET1-deficient cells display selective growth advantage following exposure to ionizing radiation. qRT-PCR was conducted to measure TET1 -deficient following transduction with lentivirus encoding empty vector (shEV) or shRNA constructs targeting TET1 (shTet1) in A172 glioma cells (A) and non-tumor-derived 10B1 glial cells (D). TET1 mRNA levels are relative to GAPDH and normalized to shEV control. Colony formation was measured in A172 glioma cells following treatment with 1 or 4 Gy IR (B) or ouabain at 1 and 10 nM (C). Colony formation was also measured in 10B1 glial cells following treatment with 1 and 4 Gy IR (E) or ouabain at 1 and 10 nM (F). Bar graphs represent mean and error bars show standard deviation. Two-Way ANOVA, Tukey Post-Test, *p < 0.05.

Fig. 2.

shTet1 10B1 cells display attenuated γ H2AX foci formation in response to DNA damaging agents. shTet1 and shEV 10B1 cells were treated for different lengths of time with IR, camptothecin, CPT (5 μ M), or etoposide, Eto, (5 μ M). To quantify foci, cells were stained with DAPI and antibody against γ H2AX. Percentages were computed by dividing the number of γ H2AX positive cells (minimum 5 foci per cell) by the total cell number (identified with DAPI) multiplied by 100. Bar graphs represent mean and error bars show standard deviation. Two-Way ANOVA, Tukey Post-Test, *p < 0.05.

Fig. 3.

shTet1 10B1 cells display attenuated 53BP1 foci formation in response to DNA damaging agents. shEV and shTet1 10B1 cells were treated for different lengths of time with IR, CPT (5 μ M), or Eto (5 μ M). To quantify foci, cells were stained with DAPI and antibody against 53BP1. Percentages were computed as described in Fig. 2. Bar graphs represent mean and error bars show standard deviation. Two-Way ANOVA, Tukey Post-Test, * $p < 0.05$.

Fig. 4. shTet1 A172 glioma cells display attenuated γ H2AX and 53BP1 foci formation and increased DNA breaks in response to IR. shTet1 and shEV A172 cells were treated with IR at 4 Gy for different lengths of time and percentages of γ H2AX (A) and 53BP1 (B) foci were computed as described in Fig. 2. Bar graphs represent mean and error bars show standard deviation. Two-Way ANOVA, Tukey Post-Test, * $p < 0.05$. Comet assay was performed under neutral (C) and alkaline (D) conditions to quantify strand breaks. Data are expressed as average comet tail moments in shEV and shTet1 A172 nucleoid. Significance determined by Student's t -test and error bars are standard deviation. * $p < 0.05$. 50 nucleoids were measured per group in each experiment.

Fig. 5.

Correlation between PRKDC and TET1 gene expression in GBM clinical specimens. (A) Western blot measuring DNA-pk (PRKDC is the gene name for DNA-pk) expression in shEV and shTet1 A172 glioma cells (B) Kaplan-Meier survival curves comparing GBM patients. Survival data was retrieved from the Gliovis portal (<http://gliovis.bioinfo.cnio.es/>). Optimal expression cutoff was set using statistical algorithm provided by the Gliovis portal. (C) Positive correlation between TET1 and PRKDC (DNA-PK) gene expression in GBM clinical specimens. Gene expression data was retrieved from the Gliovis portal <http://gliovis.bioinfo.cnio.es/>