The possible prognostic role of histone deacetylase and transforming growth factor β/Smad signaling in high grade gliomas treated by radio-chemotherapy: a preliminary immunohistochemical study

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Abstract

Glioblastoma (GBM) is the most common and aggressive tumor of the central nervous system. Unfortunately, patients affected by this disease have a very poor prognosis, due to high level of invasiveness and resistance to standard therapies. Although the molecular profile of GBM has been extensively investigated, the events responsible for its pathogenesis and progression remain largely unknown. Histone Deacetylases (HDAC) dependent epigenetic modifications and transforming growth factor (TGF)-β/Smad pathway seem to play an important role in GBM tumorigenesis, resistance to common therapies and poor clinical outcome. The aim of this study was to evaluate the involvement and the possible interaction between these two molecular cascades in the pathogenesis and prognosis of GBM. Immunohistochemistry (IHC) was performed on microdissected GBM samples, collected from 14 patients (6 men and 8 women) ranging in age from 43 to 74 years. The patients were previously divided, on the basis of their overall survival (OS), into two groups: short and long OS. Patients with poor prognosis showed hyperexpression of HDAC4 and HDAC6, an activation of the TGF-β/Smad pathway, with high levels of IL-13, SMAD2, PDGF and MMP3 expression, compared to the long survivors. The short OS group exhibits a decrease in SMAD7 expression and also low levels of p21 immunostaining, which represents a common target of the two pathways. The IHC data was confirmed by quantitative analysis and Immunoblotting. Our preliminary results suggest that both HDAC4 and HDAC6 together with the TGF-β/Smad pathway may be involved in progression of GBM and this cross talking could be a useful prognostic marker in this deadly disease.

Introduction

Gliomas are the most common group of primary brain tumors. Glioblastoma (GBM) defines grade IV of astrocytic tumors showing anaplasia and mitotic activity (anaplastic astrocytoma) and the appearance of microvascular proliferation and/or necrosis.[1]

GBM heterogeneity and genomic instability confers high capacity of invasion and refractoriness to several therapies and survival rates are poor with approximately only 34% of patients surviving at one year, 12% at two years, and less than 5% at five years following initial diagnosis. The median survival rarely exceeds 12 months from the diagnosis.[2]

Several genetic alterations have been supposed to be involved in the etiology of different grades of astrocytoma, including epigenetic alterations that result in changes in gene expression without altering the DNA sequence *per se*. Some of the most thoroughly studied mechanisms in the epigenetic regulation of expression are post-translational histone modification and DNA methylation (DNA-met) which are potentially reversible.[3]

Post-translational histone modification depends on the actions of two main enzymes, histone acetylases (HAT) and histone deacetylases (HDAC), on the lysines of histone tails. It is well understood that in normal cells histone proteins play a central role in controlling gene expression by modulating chromatin structure and function during cell growth and differentiation. Histone modifications are involved in tumorigenesis, tumor growth and resistance to chemotherapy and radiotherapy treatment and for these reasons they are a very attractive model to treat cancer.[4,5] HDACs are classified into four major classes with different structure, biological function and cellular localization and their involvement in oncogenesis.

Human class I HDACs includes HDAC1-2-3 and 8 which are proteins similar to the yeast Rpd3, ubiquitously expressed in many organs and generally localized to the nucleus.

Class II HDACs can be subdivided into two subclasses: IIa, that includes HDAC4,5,7,9a,9b, and IIb composed of HDAC6 and 10. Both classes are homologous to yeast Hda1 and their enzymes can be located in the nucleus and cytoplasm suggesting a versatile extranuclear functions.[6,7] HDAC class II proteins are abundant in many tissues characterized by low levels of mitotic activity such as skeletal muscle, heart and brain.[4,7-9] The third class of HDACs is represented by Sirtuins (SIRT1-7) which are homologous to the yeast Sir2 family of proteins and required NAD+ as enzymatic mechanism for deacetylase activity, in contrast to the mechanism used by class I and II HDACs. Sirtuins are mainly localized in the nucleus and mitochondrion.[10,11] HDAC class IV only contains HDAC11, a nuclear HDAC and it is an additional enzyme phylogenetically different from both class I and II.[12] The expression of HDAC is frequently altered in several malignancies and several evidences indicate that various agents are able to inhibit HDAC activities inducing growth

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arrest, intrinsic and extrinsic apoptotic mechanisms, inhibition of angiogenesis in cancer cells and improvement in NK cell-mediator tumor immunity.[4,13-15] HDAC inhibitors (HDACIs) as a class of agents that target the aberrant epigenetic characteristics of different cancer cells without acting on DNA sequence, have emerged as a promising new class of multifunctional anticancer drugs.[16,17]

Among the signal transduction pathways that play a critical role in GBM, the transforming growth factor-β (TGFβ) pathway is involved in the initiation and maintenance of malignant cells. Elevated levels of this inflammatory cytokine have been found in the blood of patients with GBM and a crucial correlation seems to be present between elevated TGFβ levels, high tumor grade and poor patients prognosis.[18] In normal conditions, TGFβ is considered a tumor suppressor as it is an inhibitor of proliferation in immune cells, epithelial cells and astrocytes whereas in certain malignant tumors, including GBM, TGFβ can switch from tumor suppressor to oncogenic activity, promoting proliferation, neoangiogenesis, immune suppression and metastasis and extracellular matrix deposition (ECM).[19-22]

TGFβ signal transduction pathway is mediated mainly by Smad proteins. Upon ligand binding and activation of TGFβ receptors (I, II and III) phosphorylated SMAD2 and SMAD3 bind with the common mediator SMAD4. The SMAD2/3-SMAD4 complex translocates into the nucleus where it regulates specific TGFβ target genes.

In glial cells, TGFβ changes its role from being a growth inhibitor of normal glial cells to promoting the proliferation of neoplastic cells and tumor progression through the induction of PDGFβ with an unmethylated PDGFβ gene.[23] It has been demonstrated that in gliomas with low levels of proliferation, the induction of PDGF by TGFβ/Smad pathway is impaired due to methylation of the PDGF promoter and therefore TGFβ/Smad pathway is poorly efficient. On the contrary, in aggressive gliomas the PDGFβ gene is not methylated and TGFβ pathway is hyperactive.[23]

In order to better understand the molecular mechanisms that govern oncogenesis in GBM, the aim of this study was to evaluate the correlation between different HDAC classes expression and TGFβ/Smad pathway activity in patients affected by highly aggressive gliomas with different term survival.

Materials and Methods

Patients and samples

Fourteen patients with tissue confirmed diagnosis of GBM (WHO grade IV) were selected from the Pathology Division archives of San Salvatore Hospital. Patients were eligible for the study if a diagnosis of GBM was established histologically by an experienced neuropathologist in accordance with the WHO classification.[1] This study protocol was approved by Institutional Ethic Committee, University of L'Aquila (n. 30622).

The patients (8 females, 6 males) were 43 to 73 years old at the time of diagnosis (mean age 59.8 years; 95% CI 56.22 to 66.82). In all cases, the tumor samples were obtained before treatment with radiation and TMZ. After surgery, the patients received radiotherapy to limited fields (2 Gy per fraction, once a day, 5 days a week, 60 Gy total dose) and concomitant TMZ (75 mg per square meter of body surface area, per day) for 7 days a week from the first to the last day of radiotherapy followed by six cycles of adjuvant TMZ (150-200 mg per square meter of body surface area on days 1 to 5) given at 4 week intervals. Survival was calculated from the date of surgery when a diagnosis of GBM had been established and on the basis of overall survival[5,24] the patients were divided into 2 groups: short survival (SS) and long survival (LS).[24]

Histology and immunohistochemistry

Samples collected during surgery were immediately immersed in 4% buffered formalin in phosphate buffer saline (PBS pH 7.4) for 12 h at room temperature, then dehydrated in graded ethanol and embedded in low-temperature-fusion paraffin for histological and immunohistochemistry studies. Serial 3 µm-thick sections were stained with Hematoxylin and Eosin (H&E) in order to check the extension of the neoplastic area as well GBM histological features (necrosis, microvascular proliferation and pseudopalisade arrangement). For immuno-

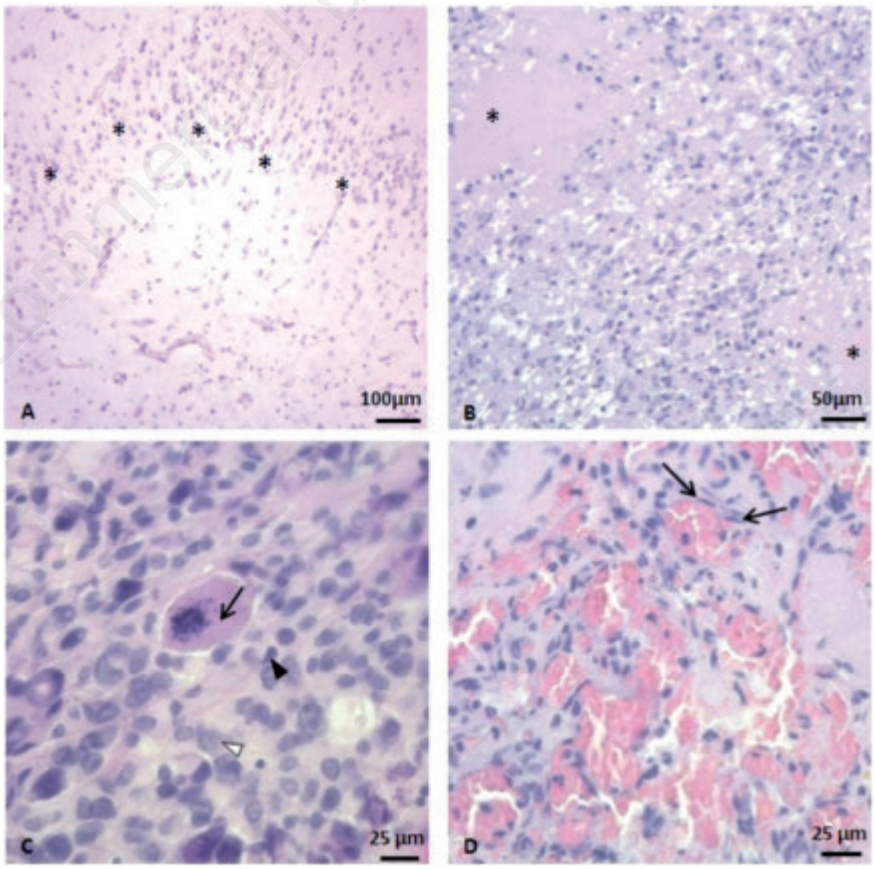

Figure 1. Typical histological features of GBM. H&E staining; original magnification: A) 10X; B) 20X; C) 40X; D) 40X. Microphotograph show 'pseudopalisading' cells (A, asterisks) and colliquative necrosis (B, asterisks), nuclei with phenomena of pyknosis (C, black head arrows), karyorrhexis (C, arrow) karyolysis (C, white head arrow), and glomeruloid vascular structures with hyperplastic endothelial cells (D, arrows).

histochemical (IHC) analyses 3 µm-thick-sections were immersed for 40 min in methanol and 3% hydrogen peroxide solution and then rinsed in PBS. Thereafter, sections were incubated overnight at 4°C with specific antibodies against HDAC1 (sc-7872), HDAC2 (sc-7899), HDAC3 (sc-81600), HDAC6 (sc-28386), HDAC8 (sc-365620) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) used at a dilution of 1:50, IL-13 (sc-1292), SMAD 2 (sc-6202), SMAD7 (sc-9183), PDGF (sc-128), MMP3 (sc-6839) and p21 (sc-397) (Santa Cruz Biotechnology Inc.) used at a dilution of 1:100 and HDAC4 (Ab 632-A00429) (GenScript, Piscataway, NJ, USA) used at dilution 1:50.

The samples were washed in PBS three times, for 2 min and then incubated with HRP conjugated antirabbit IgG secondary antibody (SV0002-1, Boster Biological Technology, Ltd., Pleasanton, CA, USA) for HDAC1,2,4 and PDGF (SV0001-1, Boster Biological Technology, Ltd.), for HDAC3,6,8 with HRP conjugated anti-mouse IgG secondary antibody (SV0001-1, Boster Biological Technology, Ltd) and with HRP conjugated antigoat IgG secondary antibody (SV0001-3, Boster Biological Technology, Ltd).

After three washes in PBS for 10 min, the sections were subject to incubation with 3,3'-Diaminobenzidine-tetrahydrochloride (DAB, Sigma Aldrich, Milan, Italy) for 1-3 min. The specificity of immune reaction was revealed by keeping off the primary antibodies. Finally, the samples were stained with Hematoxylin of Mayer and observed under the Olympus BX51 Light Microscope (Olympus, Optical Co. Ltd., Tokyo, Japan). Observations were processed with an image analysis system (IAS, Delta system, Rome, Italy) and were independently performed by two pathologists (RS, AV) in a blinded fashion.

Quantitative digital image analysis of immunohistochemical staining

The sections were observed at 20X and quantitative comparison of immunohistochemical staining was measured by the ImageJ digital image analysis public domain software.[25] The IHC profiler software plugin was required.[26] The immunopositivity was expressed as a percentage of the total software-classified areas and the data obtained were plotted as histograms.

Immunofluorescence

Serial 3-µm sections from SS and LS patients were immersed in 5% PBS Bovine Serum Albumine (BSA) for 1 h at room

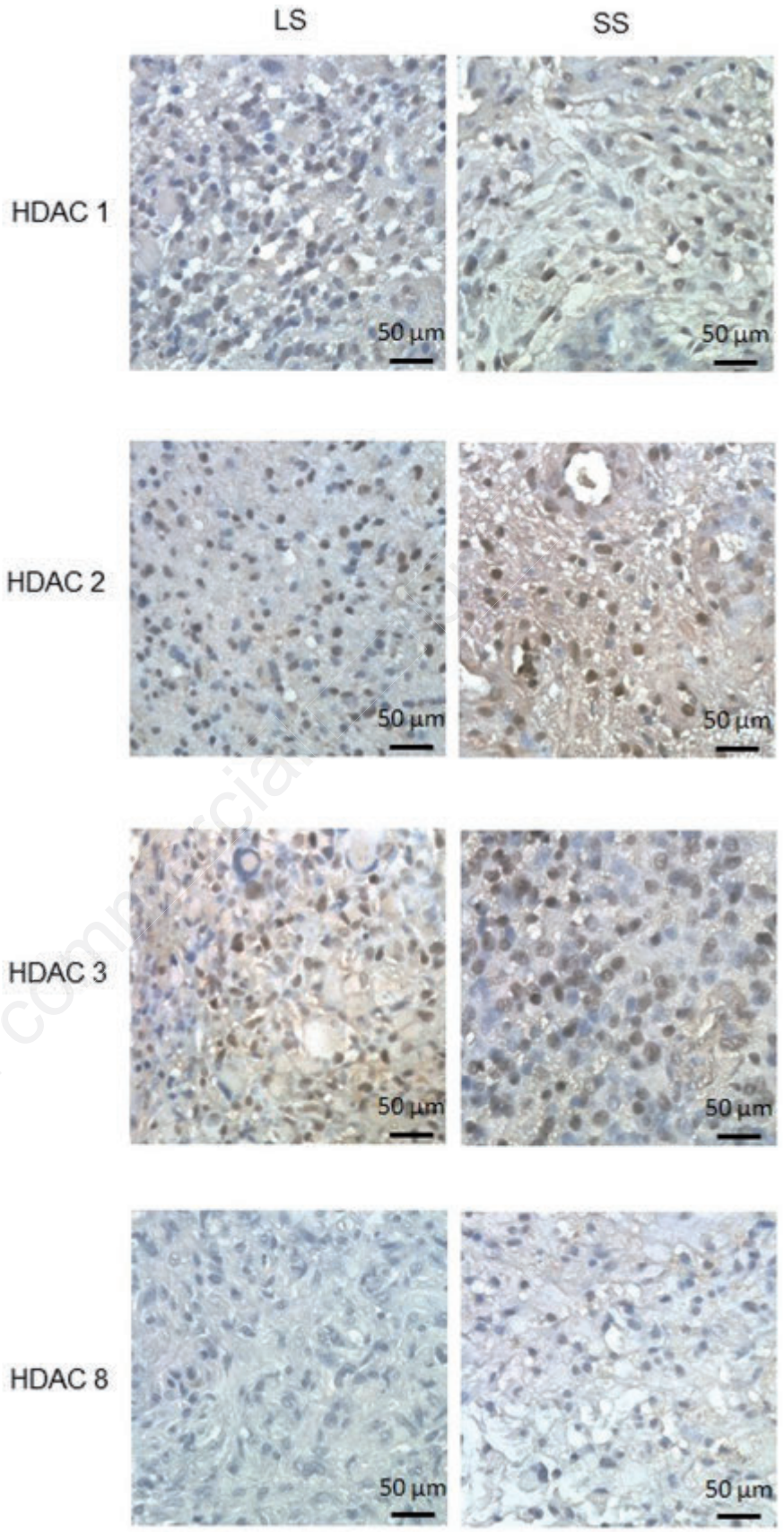

Figure 2. Class I HDAC expression in GBM. Immunohistochemistry; original magnification: 20X. Class I HDAC genes (HDAC1,2,3,8) shows lower levels of expression without significantly differences among LS and SS of patients.

temperature in order to prevent non-specific bindings. Samples were then incubated overnight at 4°C with HDAC6 antimouse and SMAD7 antigoat antibodies used at 1:100 dilution. After three washes in PBS for 10 min, the sections were incubated for 30 min at room temperature with secondary fluorescent antibodies donkey antigoat IgG-FITC and goat antimouse IgG-TR (Santa Cruz Biotechnology Inc.) used at dilution 1:200 and 1:100 respectively. Nuclei were treated with 4',6-diamidino-2-phenylindole (DAPI) at dilution 1:10, for 5 min at room temperature. Negative controls were obtained by keeping off the primary antibodies. Finally, the samples were observed under the Zeiss Axio Imager 2 with DFC 250 video camera (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA). Microphotographswere processed with Image J software and analyzed by two pathologists (AV, RS) in a blinded fashion.

Cell cultures, shRNA transfection, radiation exposure, tumor sphere and colony formation assays

The human GBM cell lines, U118MG, U138MG and U251MG, cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). NHA (normal human astrocytes) cell line was obtained from the LONZA (Rockville, MD, USA). Cells were maintained according to the manufacturer's instructions in Dulbecco's modified Eagle's medium with 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10% fetal bovine serum. Periodically, DNA profiling using the GenePrint 10 System (Promega Corporation, Madison, WI, USA) was carried out to authenticate cell cultures, by comparing the DNA profile of our cell cultures with those found in GenBank. A pool of 3 target-specific 19-25 small interfering RNAs (siRNAs) were used to knock-down HDAC6 (sc-35544, Santa Cruz Biotechnology, Ltd.) gene expression. Transfections were performed following Santa Cruz Biotechnology's instructions, by using siRNA Transfection Reagent (sc-29528), siRNA Transfection Medium (sc-36868) and siRNA Dilution Buffer (sc-29527).

Preparation of cell lysates and Western blot analysis

Tumor cells were plated (5×10^5 cells/dish) in 10-cm dishes, for 72 h. After this time, adherent cells were harvested and lysed in RIPA buffer; protein preparations (30-40 µg) were resuspended in reducing Sample buffer (Bio-Rad Laboratories, Segrate, Mi, Italy) and heated at 95°C for 5 min. Following electrophoretic separation by SDS-PAGE, proteins were transferred onto nitrocellulose membrane. After blocking, membranes were incubated with the primary antibodies against HDAC-6, SMAD7, α-actin and α-tubulin. Detection was done using horseradish peroxidase-conjugated secondary antibodies, and enhanced chemiluminescence ECL-Prime reagents. Total extracts were normalized by using an anti α-tubulin antibody.

In vitro radiation treatment and surviving fraction analysis

Radiation was delivered at room temperature using an x-6 MV photon linear accelerator, as previously described.[27,28] In brief, the total single dose of 4 Gy was delivered with a dose rate of 2 Gy/min using a source-to-surface distance (SSD) of 100 cm. A plate of Perspex thick 1.2 cm was positioned below the cell culture flasks in order to compensate for the build-up effect. Tumor cells were then irradiated placing the gantry angle at 180°. Non-irradiated controls were handled identically to the irradiated cells with the exception of the radiation exposure.

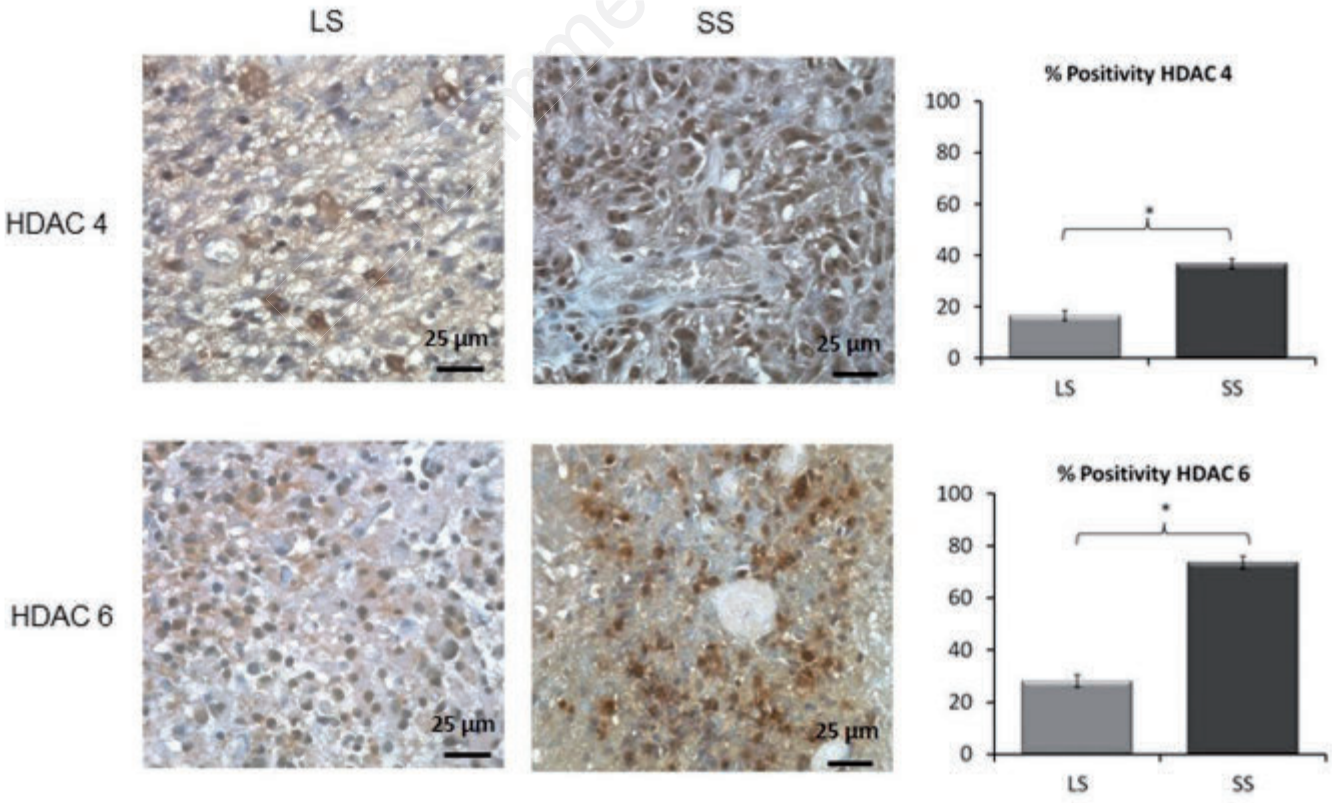

Figure 3. Class II HDAC expression in GBM. Immunohistochemistry; original magnification: 40X. HDAC4,6 are increased in patients with short overall survival. Data are confirmed by quantitative analyses.

Statistical analysis

Statistical analysis was performed using Students' *t*-test for comparison of two groups and one-way ANOVA test for more than two groups. Continuous variables were expressed as means ± SD. A P-value <0.05 was considered statistically significant.

Results

Microscopy, immunohistochemistry and immunofluorescence

Histopathologic examination showed necrotic and hemorrhagic foci surrounded by 'pseudopalisading' cells in a configuration that is relatively typical to glioblastomas. Different morphological features of cell death as apoptosis and colliquative necrosis with cellular nuclei that exhibit phenomena pyknosis, karyolysis and karyorrhexis. Glomeruloid vascular structures with hyperplastic endothelial cells were also present (Figure 1). Class I HDAC genes (HDAC1,2,3,8) showed lower levels of expression without significantly differences among the two groups of patients (Figure 2). The immunopositivity of HDAC4,6 was increased in patients with short survival compared to long. These data were confirmed by quantitative analyses (Figure 3). Quantitative evaluation of immunohistochemical staining showed a significantly increased expression of IL13, SMAD2 and PDGF in SS patients compared to LS patients in which the immunopositivity was mild and/or very low (Figure 4). On the other hand, a significant reduction of SMAD7 in SS patients was found (Figure 4). Finally, the MMP3 immunopositivity was significantly higher in SS patients respect to LS (Figure 4). These results were in accordance with those obtained by quantitative analyses with a statistically significant difference among two groups of patients (P≤0.05) (Figure 4). In SS patients p21 immunostaining showed a low positivity compared to LS and also these results were in accordance with those obtained by quantitative analyses with a statistically significant difference among two groups of patients (P≤0.01) (Figure 5). Double immunofluorescence analysis showed a localization of both SMAD7 and HDAC6 in neoplastic cells. In particular, in SS patients, HDAC6 signal was prevalent respect to SMAD7 whereas in LS we observed the contrary (Figure 6).

High HDAC6 and low SMAD7 expression predict GBM radiation sensitivity

Radiosensitivity was assessed by clonogenic assays, in a panel of glioblastoma cells (Figure 7A). Survival (S) data after a radiation dose (D) was fitted by a weighted, stratified, linear regression according to the linear-quadratic formula as described in materials and methods. U251MG, U118MG and U138MG respectively showed a high, intermediate and low radiosensitivity with a surviving fraction that decreased in a dose dependent manner (Figure 7A). HDAC6, SMAD7 expression levels and cell lines radioresensitivity were cross-matched. Decreasing levels of HDAC6 (Figure 7 B,C) or increasing of SMAD7 (Figure 7 B,C) were directly related to GBM radiosensitivity (Figure 7D). The relationship between HDAC6, SMAD7 expression and GBM radiosensitivity was confirmed by silencing HDAC6 expression in U138MG highly radioresistant cell line (Fig 8). Silencing HDAC6 increased SMAD7 protein expression (Figure 8A) inducing a significant (P<0.05) dose-dependent radiosensitization of U138MG cells (Figure 8B).

Discussion

GBM's genetic profiling has been thoroughly studied in order to identify the alterations responsible for its pathogenesis and progression. Among these alterations it was possible to observe epimutations that regulate gene expression without modifying the DNA sequence.[3] Epimutations affect *histones* through a variety of post-translational modifications. Variations in histone acetylation levels, caused by the opposing enzymatic activities of HATs and HDACs, involve histone tails rich in lysine, arginine and serine. Lysine deacetylation induced by HDACs causes an increase in positive charge density, leading to a stronger interaction with the DNA and, consequently, to a more compact chromatin structure associated to a transcriptional inactivity of some genes, among which many proto-oncogenes.[29,30] It has been demonstrated that mRNA expression of Class I HDACs does not reveal significant statistic differences between GBM, low-grade gliomas and normal brain tissue, so that these molecules may not directly influence the prognosis. On the contrary, class II and IV HDACs' levels are lower in GBM.[5] In our study the immunohistochemical analysis showed that HDAC1, 2, 3 and 8 were slightly expressed in the two groups of patients whereas immunopositivity for HDAC4,6 was significantly higher in patients with low overall survival. These data are strongly related to radiation therapy bad response and to the outcome of GBM patients. It is conceivable that GBM pathogenesis and aggressiveness are not only determined by epimutations but also by HDACs action on non-histone substrates.[31] Multiple pathways are associated with gliomas and the TGFβ/Smad can be considered a very crucial signaling not only in tissue remodeling and fibrosis in many organs,[21] but also in regulating tumor progression due to its oncogenic role.[32] Different proteins of the TGF-β/Smad pathway are known to be hyperexpressed in high-grade gliomas[32] resulting in overexpression of some target molecules such as PDGF and MMP3 involved in ECM rearrangement that leads to GBM invasiveness.[33,34] In our study, SS group of patients showed a marked immunopositivity of the upstream proteins (IL-13, SMAD2) and final effector proteins (PDGF, MMP3) of the cascade whereas in the same group immunohistochemistry analyses showed a very mild expression of SMAD7. On the contrary, the molecular pathway in the LS group was less active at all levels, both upstream and downstream, showing instead a higher immunopositivity of SMAD7. SMAD7 function is influenced by the localization in the various cell compartments and by the modulation of the protein stability, depending on the opposing action of HATs and HDAC different isoforms.[33] In addition, SMAD7 participates in the formation of the HDAC1/SMAD7/E2F-1 ternary complex that is involved in regulating gene transcription, and thus in controlling cell proliferation.[35] In our study, we noticed that in SS patients immunofluorescence analysis showed that SMAD7 signal was very low respect to HDAC6. Therefore, it seems plausible that SMAD7 deacetylation in GBM may depend on the action of HDAC6.

Although the meaning of our findings is still partly uncertain, we may hypotize the possible role of HDAC6 in a protein complex acting as transcriptional modulator besides the well-known interaction between deacetylates and SMAD7 lysine.[36] Considering the expression of the different tested proteins, we suggest that the modulation of the acetylation level may influence TGF-β/Smad signaling activity. These preliminary findings suggest a possible crosstalking between HDACs and the TGF-β/Smad pathway and it seems able to influence the prognosis of GBM, since TGF-β hyperactivity and HDACs expression promote proliferation and cell survival, partly responsible for the neoplasm's refractoriness to standard treatments. TGF-β acts as a proliferation inhibitor in many cells and its cytostatic activity is also due to induction of cyclin-dependent kinase inhibitors like p21.[37] In the light of these considerations regarding the activity of TGF-β/Smad path-

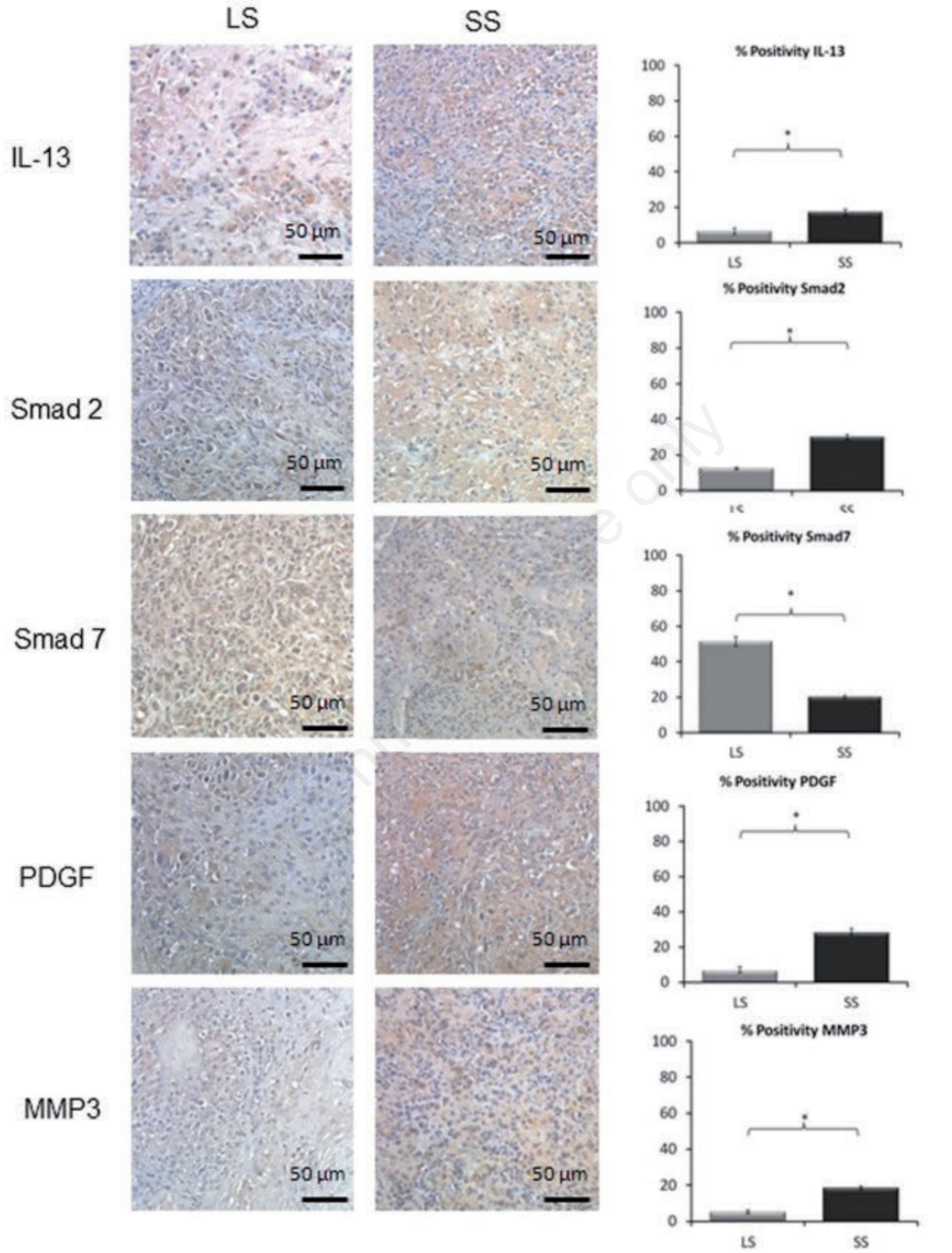

Figure 4. TGF-β/Smad pathway and MMP3 expression in GBM. Immunohistochemistry; original magnification: 20X. IL-13, SMAD2, PDGF and MMP3 immunostaining are significantly increased in SS patients compared to LS patients. SMAD7show a significant reduction of in SS patients.

way, p21 levels in group SS might be expected to be rather high. On the contrary, patients with most unfavorable prognosis showed lower levels in comparison to the other group. This result may be explained through the TGF-β paradox theory,[19,38] according to which the pathway functional switch from tumor suppressor to tumour promoter may at least partially depend on TGF-β inability to induce p21.[23,39]

Hypothesizing that all other functions of the cascade remain unaltered, the TGF-β paradox theory also explains the simultaneous expression of other target molecules such as MMP3 and PDGF. An alternative explanation is based on HDAC capacity to suppress p21 activity, as demonstrated in manyother tumors,[40,41] and in our results we hypnotize that p21 hypoexpression in SS group may be linked to p21 repression by HDAC4 or HDAC6, that are more expressed in the same group of patients. Taken together, our data may suggest that HDAC4, HDAC6 and TGF-β pathway's proteins may be considered among GBM prognostic markers. Limitation to this study includes the small number of patients and although our findings seem to indicate an association between HDAC4,6 and the TGF-β/Smad pathway further research is needed to confirm these preliminary data in order to improve disease control and current standard therapy.

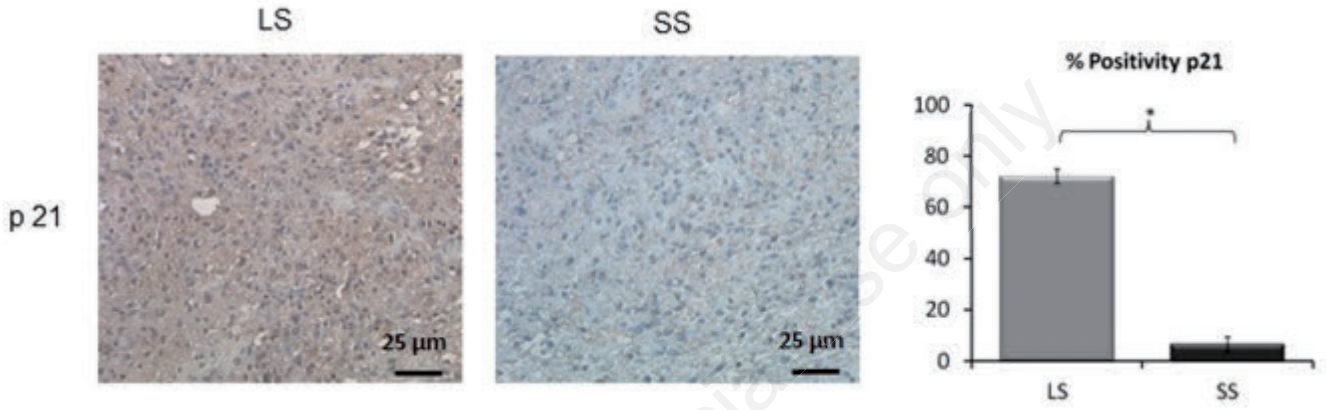

Figure 5. p21 expression in GBM. Immunohistochemistry; original magnification: 40X. In SS patients p21 immunostaining shows a low positivity compared to LS.

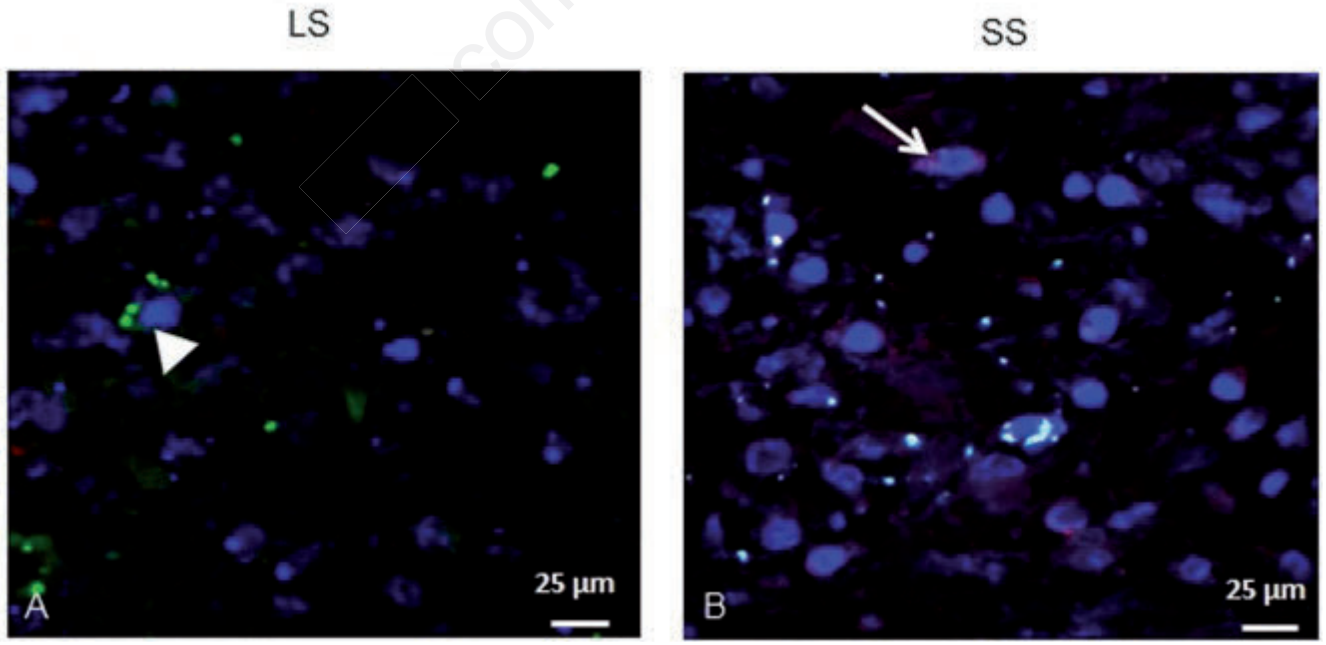

Figure 6. Double immunofluorescences analysis for SMAD7 and HDAC6 in LS and SS patients. Microphotographs show a red signal for HDAC 6 and a green signal for SMAD7. HDAC6 expression is prevalent respect to SMAD7 in SS patients (white arrow) whereas in LS patients SMAD7 immunopositivity is higher respect to HDAC6 (white head arrow); original magnification: 40X.

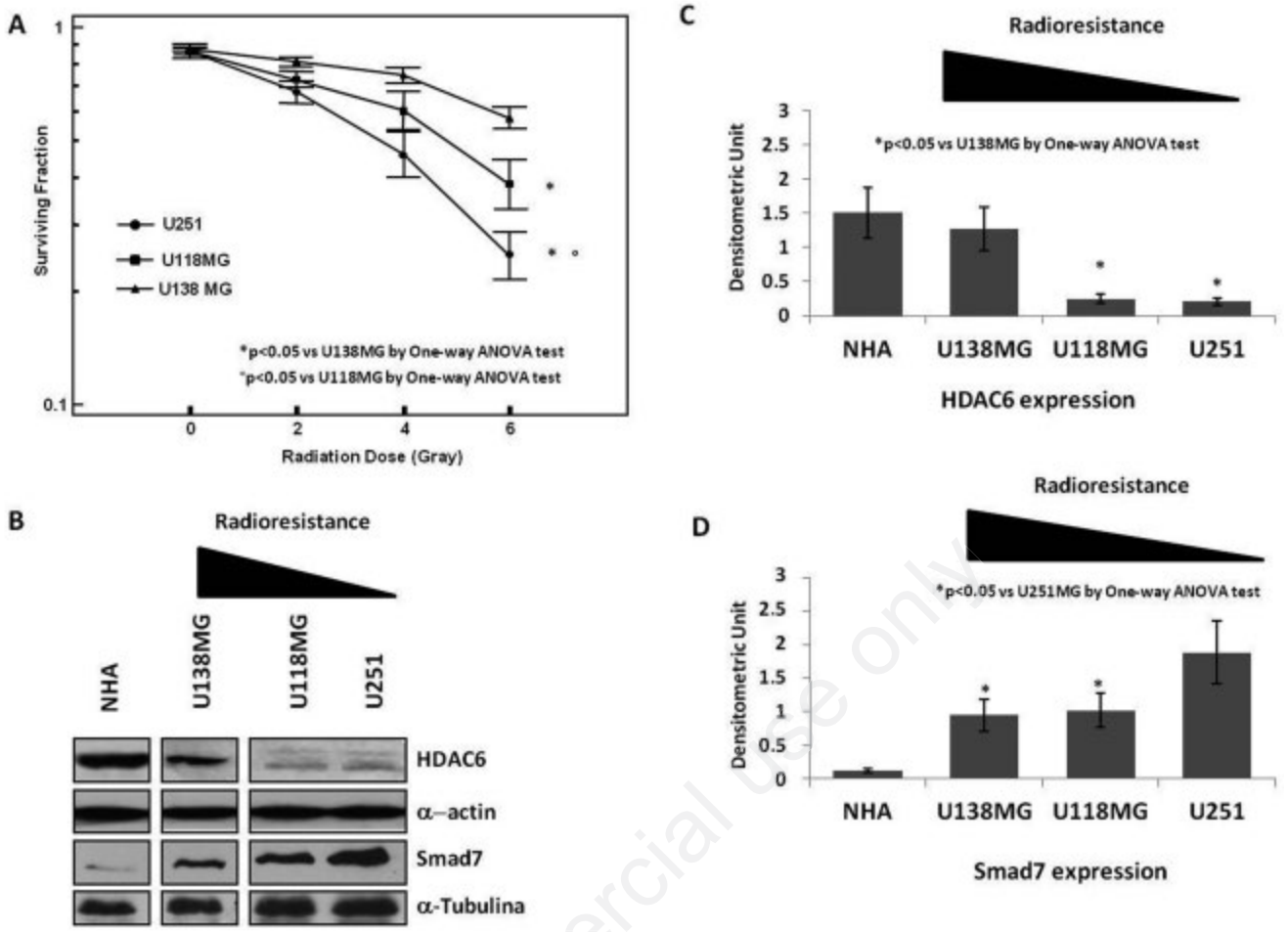

Figure 7. High HDAC6 and low SMAD7 expression correlate with GBM radiosensitivity. The glioblastoma cell lines were plated at clonal density for clonogenic assay and treated with ionizing radiation (0-6Gy). Cells were left for further 14 days when cultures were stained with 10% crystal violet in methanol and stained colonies were counted. Each point represented the mean surviving fraction calculated from three independent experiments done in triplicate for each treatment condition; error bars represent the standard deviation (SD) (A). Western blot analysis (B) was carried out with an anti-HDAC-6 and anti-SMAD7 antibodies according to manufacturer's instructions. C,D) Densitometric evaluation of HDAC-6 and SMAD7 expression levels. One representative of three different experiments is shown for each of the analyses performed.

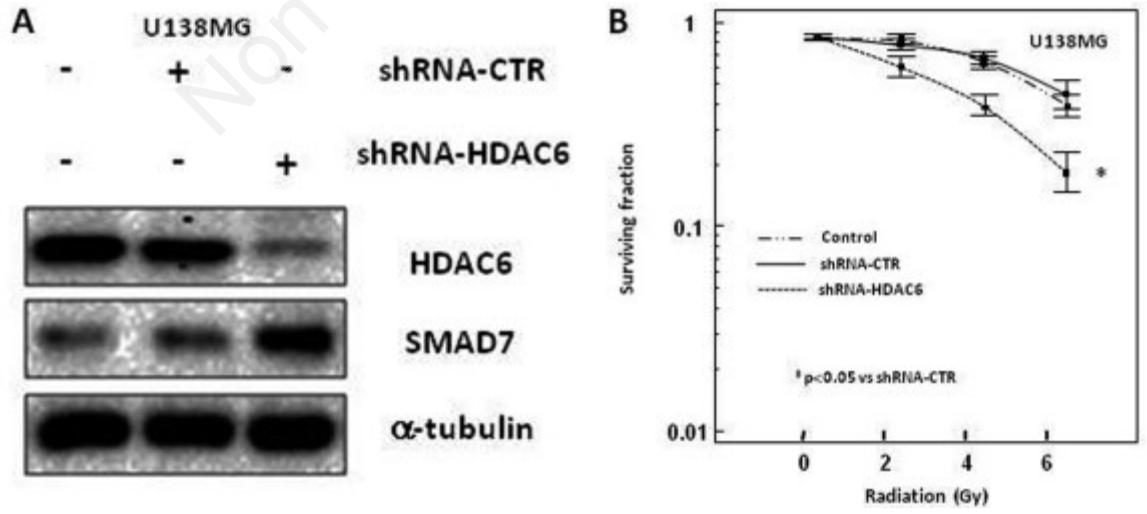

Figure 8. Silencing HDAC6 downregulates SMAD7 protein expression levels and radiosensitizes U138MG cells. U138MG cell lines were transiently transfected with siRNA for HDAC6: 72 h later, cells were assessed for HDAC6 and SMAD7 expression levels by Western blot (A) and treated or not with increasing doses of ionizing radiation (0-6 Gy) (B). Cells were left for further 14 days when cultures were stained with 10% crystal violet in methanol and stained colonies were counted. Each point represented the mean surviving fraction calculated from three independent experiments done in triplicate for each treatment condition; error bars represent the standard deviation (SD).

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