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Original Article

HDAC4 and HDAC6 sustain DNA double strand break repair and stem-like phenotype by promoting radioresistance in glioblastoma cells

Francesco Marampon [a, *, 1], Francesca Megiorni [b, 1], Simona Camero [b, c], Clara Crescioli [d], Heather P. McDowell [b, e], Roberta Sferra [a], Antonella Vetuschi [a], Simona Pompili [a], Luca Ventura [f], Francesca De Felice [g], Vincenzo Tombolini [g], Carlo Dominici [b], Roberto Maggio [a], Claudio Festuccia [a, 1], Giovanni Luca Gravina [a, 1]

[a] Department of Biotechnological and Applied Clinical Sciences, University of L'Aquila, Coppito, L'Aquila, Italy
[b] Department of Pediatrics and Infantile Neuropsychiatry, Sapienza University of Rome, Rome, Italy
[c] Department of Experimental Medicine, Sapienza University of Rome, Rome, Italy
[d] Department of Movement, Human and Health Sciences, University of Rome Foro Italico, Rome, Italy
[e] Department of Oncology, Alder Hey Children's NHS Foundation Trust, Liverpool, United Kingdom
[f] Department of Pathology, San Salvatore Hospital, Coppito, L'Aquila, Italy
[g] Department of Radiological, Oncological and Pathological Sciences, Sapienza University of Rome, Rome, Italy

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ABSTRACT

The role of histone deacetylase (HDAC) 4 and 6 in glioblastoma (GBM) radioresistance was investigated. We found that tumor samples from 31 GBM patients, who underwent temozolomide and radiotherapy combined treatment, showed HDAC4 and HDAC6 expression in 93.5% and 96.7% of cases, respectively. Retrospective clinical data analysis demonstrated that high-intensity HDAC4 and/or HDAC6 immunostaining was predictive of poor clinical outcome. *In vitro* experiments revealed that short hairpin RNA-mediated silencing of HDAC4 or HDAC6 radiosensitized U87MG and U251MG GBM cell lines by promoting DNA double-strand break (DSBs) accumulation and by affecting DSBs repair molecular machinery. We found that HDAC6 knock-down predisposes to radiation therapy-induced U251MG apoptosis- and U87MG autophagy-mediated cell death. HDAC4 silencing promoted radiation therapy-induced senescence, independently by the cellular context. Finally, we showed that $p53^{WT}$ expression contributed to the radiotherapy lethal effects and that HDAC4 or HDAC6 sustained GBM stem-like radioresistant phenotype. Altogether, these observations suggest that HDAC4 and HDAC6 are guardians of irradiation-induced DNA damages and stemness, thus promoting radioresistance, and may represent potential prognostic markers and therapeutic targets in GBM.

Introduction

Epigenetic mechanisms, including enzymatic modifications to histones and DNA methylation, contribute to tumor development, progression, chemoresistance, and radioresistance [1,2]. Because of their reversible nature and their role in gene expression, epigenetic alterations, such as histone acetylation, are a current focus for therapeutic targeting in clinical research. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) control a wide array of biological processes by transferring and removing acetyl groups to or from histones/proteins, respectively [3–5]. The aberrant expression and activity of HDACs, which result in the

Abbreviations: GBM, glioblastoma; TMZ, temozolomide; RT, radiotherapy; HDACs, Histone deacetylases; HATs, Histone acetyltransferases; DSBs, DNA double-strand breaks; OS, Overall survival; shRNA, short hairpin RNAs; NHEJ, non-homologous end-joining; HR, homologous recombination; HCN-2, human cortical neuronal; NHA, normal human astrocytes; siRNA, small interfering RNAs; p53, Tumor Protein p53; ATM, Ataxia-telangiectasia mutated; DNAP-PKcs, DNA-dependent protein kinase, catalytic subunit; H2AX, H2A histone family, member X.

* Corresponding author. Department of Biotechnological and Applied Clinical Sciences, University of L'Aquila, Via Vetoio 1, 67100 Coppito, L'Aquila, Italy. Fax: +39 0862 433523.
E-mail address: f.marampon@gmail.com (F. Marampon).
[1] Equal contribution.

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repression of antiproliferative genes, have been shown to promote tumor development and progression [1,2,5]. So, targeting HDACs is being increasingly appreciated as an effective anticancer therapy in preclinical and clinical studies [5–7]. Glioblastoma multiforme (GBM) is a highly malignant cancer of the central nervous system which still remains incurable due to its aggressiveness and resistance to conventional treatments, based on surgical resection, chemotherapy and radiotherapy (RT) [8]. Despite a clear survival advantage has been demonstrated with post-operative RT, the frequent tumor relapse proves the intrinsically high radioresistance of GBM cells, which is likely sustained by multiple, complex, and largely unknown molecular mechanisms. RT induces cell death by promoting the accumulation of DNA double-strand breaks (DSBs), but the aberrant activity of non-homologous end-joining (NHEJ) and/or homologous recombination (HR) repair pathways frequently increases the level of cancer radioresistance [9,10]. HDACs [11], such as HDAC4 [12,13] and HDAC6 [14,15], have been shown to be essential in GBM development, progression and resistance to treatment, but the HDAC-related molecular mechanisms which are responsible for the GBM radioresistance are still largely unknown. In this manuscript, HDAC4 and HDAC6 protein expression was analyzed in tumor samples obtained from GBM patients, and a close relationship of their expression levels with poor response to temozolomide (TMZ) and RT combined treatment, and with lower overall survival (OS) was found. Starting from this evidence, we decided to investigate the relationship between HDAC4 and HDAC6 expression levels and the response to radiations in the p53-wild type U87MG or p53-mutant U251MG GBM cells [16]. Herein, short hairpin RNAs (shRNAs)-mediated silencing of HDAC4 or HDAC6 affected GBM cell radioresistance by promoting RT-induced apoptosis, autophagy or senescence as well as by impairing GBM stemness potential. Furthermore, our results indicate that HDAC4 and HADC6 are strategic components of the complex molecular machinery responsible for GBM radioresistance, this suggesting HDAC4 and HDAC6 as possible prognostic markers and therapeutic targets in this malignancy.

Materials and methods

Cell cultures, shRNA and siRNA transfection, radiation exposure, tumorsphere and colony formation assays

The human GBM cell lines, U87MG and U251MG, as well as the HCN-2 (human cortical neuronal) cell line were obtained from the American Type Culture Collection (Rockville, MD). NHA (normal human astrocytes) cell line was obtained from the LONZA (Rockville, MD). 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) 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 or 5 target-specific plasmids, each encoding 19–25 nt (short hairpin) shRNAs designed to knock-down gene expression of HDAC4 (sc-35540-SH) or HDAC6 (sc-35544-SH), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Control-shRNA (sc-108060, Santa Cruz Biotechnology) was used as negative control. Stable Control-, HDAC4- or HDAC6-shRNA transfectants were selected by maintaining GBM cells in puromycin-containing media (500 ng/ml; Sigma–Aldrich, MI, Italy). A pool of 3 target-specific 19–25 small interfering RNAs (siRNAs) were used to knock-down Beclin-1 (sc-29797, Santa Cruz Biotechnology), $p21^{WAF1}$ (sc-29427, Santa Cruz Biotechnology), or p53 (sc-29435, Santa Cruz Biotechnology) gene expression. Transfections were performed following Santa Cruz Biotechnology's instructions, by using shRNA Plasmid Transfection Reagent (sc-108061) and shRNA Plasmid Transfection Medium (sc-108062), or siRNA Transfection Reagent (sc-29528), siRNA Transfection Medium (sc-36868) and siRNA Dilution Buffer (sc-29527). The autophagy inhibitor 3-methyladenine (3-MA) was obtained from Sigma–Aldrich. Neurospheres, radiation exposure and colony formation assays were performed as already described [17,18].

Western blot, subcellular fractions, immunoprecipitation, γ-galactosidase activity assay and flow cytometry analysis

Western blot, immunoprecipitation, and nuclear/cytoplasmic fractions were carried out as previously described [18,19]. Immunoblotting was performed with the following antibodies: anti-HDAC4 (A-4), -HDAC6 (H-300), -Tumor Protein p53 (p53) (DO-1), -Beclin1 (H-300), -Ataxia-telangiectasia mutated (ATM) (H-248), -DNA-depenent protein kinase, catalytic subunit (DNA-PKcs) (E-6), -H2A histone family, member X (H2AX) (M-20), -γ-H2AX (3C10), and α-tubulin (TU-02) from Santa Cruz Biotechnology at the concentration of 1:250; anti-Microtubule-associated protein light chain 3 (LC3) (2775) was from Cell Signaling Technology (Danvers, MA) and used at the concentration of 1:1000; anti-$pospho^{Ser1981}$-ATM (10H11.E12) and -$pospho^{Thr2609}$-DNA-PKcs (10B1) were obtained from Abcam (Cambridge, UK) and used at the concentration of 1:1000. Peroxidase-conjugate anti-mouse or anti-rabbit IgGs (Amersham GE Healthcare, UK) were used 1:2000 for enhanced chemiluminescence detection. γ-Galactosidase activity was measured by using mammalian-γ-galactosidase assay kit (75707) from ThermoFisher (Waltham, MA) according to manufacturer's instructions.

Patients and in vivo densitrometric quantification of HDAC4 and HDAC6 proteins

Patients with histologically confirmed diagnosis of GBM (WHO grade IV) diagnosed and treated at San Salvatore Hospital, University of L'Aquila, Italy, were selected. All patients provided written informed consent according to the research proposal, which was approved by the Ethical Committee of the San Salvatore Hospital. Patients were eligible for the study if a diagnosis of GBM was established histologically by the neuropathologists in accordance with the WHO classification [8]. Patients were 43 to 73-years-old at the time of diagnosis (mean age, 59.8 yrs; 95% CI 56.22 to 66.82). In all cases, tumor samples were obtained by surgical resection before treatment with radiation and TMZ. After surgery, patients received RT to limited fields (2 Gy per fraction, once a day, 5 days a week, 60 Gy total dose) and concomitantly TMZ (75 mg/square meter/day) for 7 days a week from the first to the last day of radiotherapy, followed by six courses of adjuvant TMZ (150–200 mg/square meter on days 1–5) given at 4-week intervals. Survival was calculated from the date of the diagnostic surgery. The disease was considered to have progressed if the sum of the products of perpendicular diameters of all enhancing lesions was increased by 25% of initial measurements, if a new lesion appeared on axial contrast-enhanced T1-weighted magnetic resonance imaging scan, or if the patient's neurologic condition worsened and required an increased dose of steroids [8]. Only those patients who completed RT and concomitant TMZ treatment were included. Immunohistochemical analysis was performed by using HDAC4 (A-4) and HDAC6 (D-11) antibodies from Santa Cruz Biotechnology. Protocols for the *in vivo* densitometric quantification of proteins have previously been described [20]. Observations were independently performed by two pathologists (AV, RS) in a blinded fashion.

Statistical methods

Overall survival was calculated from the date of diagnostic surgery to death from any cause. Survival curves were plotted using the Kaplan–Meier method and differences between groups were evaluated using the log-rank test. Multivariate analysis for survival was performed by using the Cox proportional hazards model by controlling for age, Karnofsky performance status (KPS), Ki67 measurement values, MGMT promoter methylation, expression of HDAC4 and HDAC6. Continuous variables, which were not normally distributed, were reported as median values, at a 95% confidence interval (CI), and their comparison was performed using the Mann–Whitney U test. Continuous variables, which were normally distributed, were reported as mean and standard deviation (SD), and their comparison was performed using the Student's t test. Comparison of categorical variables between two groups was performed by the Chi-square statistics or by using the Fisher exact test, when appropriate. All p-values are based on Student's t two-tailed test and differences were considered statistically significant when $p \leq 0.05$. Asterisks indicate the level of statistical significance: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

Results

HDAC4 and HDAC6 are expressed in primary GBMs and negatively correlate with overall survival rates after temozolomide combined with radiation therapy treatment

The relationship between HDAC4 and HDAC6 expression levels and RT response was assessed in 31 GBM patients by immunohistochemical analysis (Fig. 1). HDAC4 positivity was found in 29/31 (93.5%) cases, of these 22 (70.9%) showing high expression and 7 (22.6%) low expression. HDAC6 positivity was observed in 30/31 (96.7%) cases, of these 22 (70.9%) showing high expression and 8 (25.8%) low expression. Next, we examined the associations of the high expression of HDAC4 and HDAC6 proteins with the clinical outcome. Patients were followed for a median period of 13.8 months (range = 7–45). By analyzing retrospective data of these patients, we found that high expression levels of both HDAC4 (Low expression: 13.5 months versus high expression: 9 months;