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miRNA regulation of Sirtuin-1 expression in human astrocytoma

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Abstract. Sirtuins are a family of 7 histone deacetylases largely involved in the regulation of cell proliferation, survival and death. The role of sirtuins in tumorigenesis and cancer progression has been previously studied in certain cancer types. Few studies have investigated sirtuin expression in gliomas, with controversial results. The aim of the present study was to investigate the expression of sirtuin-1 (Sirt-1) in diffuse astrocytoma [low grade astrocytoma (LGA)], anaplastic astrocytoma (AA) and glioblastoma multiforme (GBM) and in primary glioma cell lines: PLGAC (primary LGA cells); PAAC (primary AA cells); and PGBMC (primary GBM cells). Tumor samples were obtained from patients who underwent craniotomy for microsurgical tumor resection at the Neurosurgery Unit of the University of Messina between 2011 and 2014. Sirt-1 expression was qualitatively analyzed in 30 human glial tumor samples and 5 non-neoplastic brain tissue (NBT) specimens using immunohistochemistry and western blotting techniques. Sirt-1 expression was quantitatively analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In addition, Sirt-1 expression in primary cell lines was investigated by immunoblotting and RT-qPCR. Sirt-1 expression was downregulated in gliomas compared to NBTs. Sirt-1 levels also varied among different tumor grades, with more evident downregulation in high-grade ($P<0.001$) than low-grade tumors ($P<0.01$). These data were confirmed in cell lines, with the exception of upregulation of protein level in the highest malignancy grade cell lines. The present results suggest a role for miRNA-34a, miRNA-132 and miRNA-217 in the epigenetic control of Sirt-1 during gliomagenesis and progression, and demonstrate the different implications of Sirt-1 in human tissues and cell lines. Furthermore, the present results reveal that Sirt-1 may be an intrinsic regulator of tumor progression and the regulation of Sirt-1 involves complex molecular pathways. However, the biological functions of Sirt-1 in gliomagenesis require additional investigation.

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Introduction

Sirtuins, homologues of the *Saccharomyces cerevisiae* silent information regulator 2 (Sir2) protein, are nicotinamide adenine dinucleotide (NAD^+)-dependent, class III histone deacetylases (HDACs), conserved from bacteria to humans (1). In total, 7 types of sirtuins (Sirt-1-7) have been identified in mammals, with each of them containing a conserved 275-amino acid catalytic domain and a NAD^+ binding domain (2). Sirtuins may be located in different subcellular compartments, including the nucleus (Sirt-6 and Sirt-7) and mitochondrion (Sirt-3, Sirt-4 and Sirt-5), while certain sirtuins (Sirt-1 and Sirt-2) shuttle between the nucleus and cytoplasm (3-5). Certain sirtuins may re-localize depending on the cell or tissue type, developmental stage, stress condition and metabolic status, suggesting that their localization is important for the regulation of their activity (6). Sirtuins are involved in various cellular functions, such as chromosomal stability and gene expression, cell proliferation and survival, cell cycle progression, cell division control, cell death, cell metabolism and calorie restriction (1). In detail, sirtuins may affect gene expression epigenetically by histone acetylation (7). Through acetylation, histones accumulate negative charges that decrease the interaction of their N-terminal regions with the negatively charged phosphate groups of DNA. As a consequence, chromatin is transformed into a more relaxed structure that permits gene transcription. De-acetylation, conversely, causes chromatin condensation that reduces the level of transcriptional activity.

Over the past decade, sirtuins have emerged as the principal factor in sensing and regulating cellular response to oxidative and metabolic stress (1,6,8,9). These proteins are involved in cell senescence, cell differentiation, stress-induced apoptosis cell growth, angiogenesis and blood supply (8). In addition, the epigenetic regulation of sirtuins has been characterized in numerous types of cancers, including lymphomas, leukemia, prostate, breast, intestine and colon cancers, and the effect of sirtuins on the expression of proteins involved in tumorigenesis, such as p53, p73, nuclear factor-κB (NF-κB) and forkhead box subgroup O (FOXO), has been well studied (9-15).

The 'double-face' of Sirt-1 in tumorigenesis was recently reported (16). It appears that Sirt-1 can inhibit inflammation and multistage carcinogenesis by acting as a tumor

suppressor (15,16). Paradoxically, it may also accelerate tumorigenesis via multiple mechanisms, such as inactivation of tumor suppressors, activation of oncoproteins and development of a microenvironment favorable to tumor survival (16). Accordingly, studies have shown that Sirt-1 may be either upregulated (17,18) or downregulated in human gliomas (19,20). Qu *et al* (21) demonstrated that Sirt-1 knockdown significantly delays mitotic entry of glioma cells, inhibits their growth and proliferation and promotes apoptosis via the phosphatase and tensin homolog (PTEN)/phosphoinositide 3-kinase (PI3K)/AKT signaling pathway, suggesting that Sirt-1 may be a promoter of tumorigenesis in glioma through the PTEN/PI3K/AKT signaling pathway (21). By contrast, it was recently shown that Sirt-1 expression levels are lower in glioblastoma, bladder, prostate and ovarian carcinomas compared to normal controls (20,22). In addition, previous studies strongly suggest critical roles for microRNAs (miRNAs) in regulating Sirt-1 (23-25). miRNAs are short non-coding endogenous RNAs that post-transcriptionally regulate the expression of a large number of target genes involved in physiological and pathological pathways. miRNAs are considered to play important roles in cancer by regulating the expression of various oncogenes and tumor suppressors (26), and alteration of miRNA profiles has been demonstrated in glioma tissues (26-28).

Although the pattern of Sirt-1 expression and its relevance has been reported in several types of cancer, to the best of our knowledge, there are no studies investigating the expression pattern of Sirt-1 in human glioma tissues and primary glioma cell lines, and the role of Sirt-1 in gliomagenesis remains largely unknown (11-15). In the present study, to better characterize the role of Sirt-1 in human gliomas, the miRNA expression of Sirt-1 and its associated regulators was investigated in astrocytic tumors with different grades of malignancy [World Health Organization (WHO) II-IV] (29), and also in primary human glioma cell lines for the first time.

Materials and methods

Tissue samples. The Institutional Review Board of the Department of Neurosciences of the University of Messina (Messina, Italy) discussed and approved the current study. Written informed consent was obtained from all subjects or their caregivers at the moment of diagnostic procedures, including for research purposes, according to the Declaration of Helsinki. In total, 30 tumor specimens of astrocytomas of different histological grades were obtained from adult patients who had undergone craniotomy for microsurgical brain tumor removal at the Neurosurgery Unit of the University of Messina between 2011 and 2014. Samples were immediately frozen in liquid nitrogen and stored at -80˚C for biomolecular analyses and formalin fixed for subsequent histological evaluation.

No patients received neoadjuvant therapy (radiotherapy and/or chemotherapy). According to the revised WHO classification (29), tumors were histologically classified as: Diffuse fibrillary astrocytoma [low grade astrocytoma (LGA)] (n=10); anaplastic astrocytomas (AA) (n=10); and glioblastoma multiforme (GBM) (n=10). Furthermore, samples of non-neoplastic brain tissue (NBT) (n=5) derived from the temporal lobes of patients who underwent brain surgery at the Neurosurgery Unit of the University of Messina between 2011 and 2014 for cerebral hemorrhage, including histologically verified normal cortex and white matter, were used as controls.

Cell lines. Primary glioma cell lines and normal human astrocytes (NHAs) were used as a control for the present study. The human glioma cell lines were obtained from samples of grade II, III and IV glioma, as follows: PLGAC, primary LGA cells (grade II); PAAC, primary AA cells (grade III); and PGBMC, primary GBM cells (grade IV). Briefly, fresh tumor samples were washed extensively with phosphate-buffered saline and were processed by mincing the tissue with operative scissors, followed by enzymatic digestion with 150 U/ml hyaluronidase and 250 U/ml collagenase type IV (Sigma-Aldrich, St. Louis, MO, USA). The single-cell suspension was filtered through a 100-μm cell strainer, washed and then plated in at a density of $5x10^4$ cells in DMEM medium supplemented with 10% FCS, penicillin at 100 U/ml, streptomycin at 100 μg/ml and 2 mM L-glutamine (Lonza, Verviers, Belgium) at 37˚C in 5% CO_2. The presence of glioma cells was assessed by staining proliferating cells with a monoclonal mouse anti-glial fibrillary acidic protein antibody (#M0761; Dako, Glostrup, Denmark; dilution, 1:100) and analyzing ploidy by fluorescence-activated cell sorting analysis.

Western blotting. Tumor specimens were homogenized on ice in lysis buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; 1 mM ethylenediaminetetraacetic acid (EDTA); 2 mM ethylene glycol tetraacetic acid (EGTA); 150 mm NaCl; 0.1% sodium dodecyl sulfate (SDS); 1% Nonidet P-40; 0.5% deoxycholate acid, pH 7.5; a mixture of protease inhibitors; 0.5 mm phenylmethylsulfonyl fluoride; 10 μg/ml aprotinin; 10 μg/ml leupeptin; and 10 μg/ml pepstatin), purchased from Sigma-Aldrich. Cell lines were lysed in radioimmunoprecipitation assay buffer (10 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 2% Triton), including proteinase inhibitors. The concentrations of protein were determined by the Lowry method. Protein samples (30 μg) were loaded onto 8% SDS-polyacrylamide gel electrophoresis and transferred onto methanol-activated polyvinylidene difluoride membrane. Membranes were incubated for 1 h in a solution containing 1% bovine serum albumin (Bio-Rad Laboratories, Inc., Milan, Italy) in Tris-buffered saline with Tween 20 (Sigma-Aldrich), then incubated overnight with mouse monoclonal antibodies against Sirt-1 (clone 1F3; #sc-74465; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; dilution, 1:500) and β-actin (#sc-8432; Santa Cruz Biotechnology, Inc.; dilution, 1:1,000). The membranes were incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G antibody (#P0260; Dako Italia Srl, Milan, Italy; dilution, 1:5,000) for 2 h. Immunoreactive bands were visualized using the ECL Chemiluminescence kit (Amersham; GE Healthcare Life Sciences, Chalfont, UK).

Semi-quantitative evaluation. Semi-quantitative evaluation of protein levels detected by immunoblotting was performed with computer-assisted densitometry (UN-SCAN-IT gel version 6.1; Silk Scientific, Inc., Orem, UT, USA). Different

exposure times were used for each blot. Protein amounts were normalized to the β-actin signal. Data were acquired and integrated density values were expressed as a percentage of densitometric levels using arbitrary densitometric units.

RNA, miRNA isolation and cDNA synthesis. Total RNA was extracted from specimens of glioma tissues and cell cultures using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA USA), according to the manufacturer's instructions. Total RNA concentration and integrity were checked using an Agilent Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Subsequently, 300 ng of total mRNA per sample was reverse transcribed (RT) into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.).

miRNAs were extracted from glioma tissue and cell lines using the miRVana Isolation kit (Ambion; Thermo Fisher Scientific, Inc.), following the manufacturer's protocol. The enriched miRNAs fraction was converted in cDNA using the TaqMan MicroRNA Reverse Transcriptase kit (Applied Biosystems; Thermo Fisher Scientific, Inc.).

In total, 2 μl of cDNA was used for specific miRNA TaqMan assay, which was amplified using Applied Biosystems TaqMan MicroRNA assay (hsa-miR-34a, hsa-miR-217 and hsa-miR-132; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. All RT reactions, including no-template controls and RT controls, were run in triplicate.

RT-quantitative polymerase chain reaction (qPCR) of Sirt-1 and miRNAs. RT-qPCR for Sirt-1 was performed using a standard TaqMan PCR kit procedure on an AB-7300 RT-PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.).

For miRNA quantification, 2 μl of cDNA was used for each specific miRNA TaqMan assay (hsa-miR-34a, hsa-miR-217 and hsa-miR-132) according to the manufacturer's instructions. All RT reactions, including no-template controls and RT controls, were performed in triplicate. RNU6 small nuclear RNA was used to normalize miRNA expression levels owing to its claimed expression stability and its wide use as loading control in several published miRNA expression studies (28,30,31). Relative fold expression and changes were calculated using $2^{-\Delta\Delta Ct}$ method. The expression levels of miRNAs are indicated as either fold expression (<0.3 downregulation and >3 upregulation) compared to NBT or NHA. Results were represented as $Log_{10}RQ$.

Target prediction tools. miRNAs that target Sirt-1 were identified by examining the Sirt-1 3'-untranslated region (UTR) with bioinformatics algorithms that predict miRNA target sites (32). Specifically, four online databases, miRDB (http://mirdb.org/miRDB/), TargetScan (www.targetscan.org), microRNA.org (www.microrna.org) and PicTar (http://pictar.mdc-berlin.de), were used for the analysis of the alignment between miRNAs and the 3'-UTR of Sirt-1.

Statistical data analysis. Statistical analysis was performed using INSTAT, version 3.0, and PRISM, version 4.0 (GraphPad, San Diego, CA, USA). Unpaired t-test with Welch correction was used to compare Sirt-1 expression levels. P<0.05 was considered to indicate a statistically significant difference.

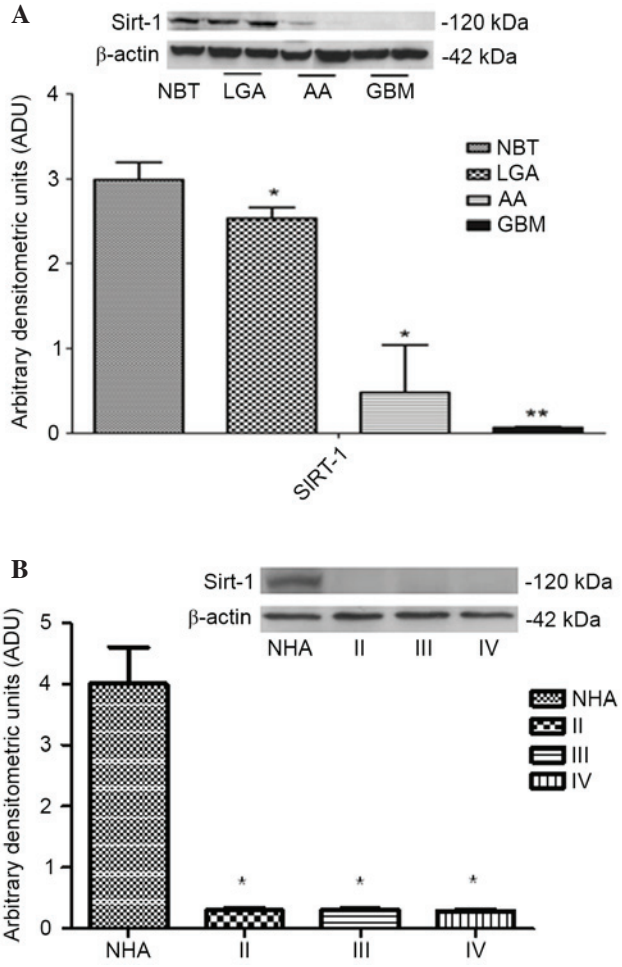

Figure 1. (A) Western blot analysis of Sirt-1 in NBT, LGA, AA and GBM tissues. Representative autoradiography shows Sirt-1 and β-actin expression. Quantitative data indicate the mean ± standard deviation (error bars). *P<0.01, LGA and AA vs. NBT; **P<0.001, GBM vs. NBT. (B) Western blot analysis of Sirt-1 on primary glioma cell cultures. Representative autoradiography shows Sirt-1 expression and β-actin in NHA, primary LGA cells (grade II), primary AA cells (grade III) and primary GBM cells (grade IV). Quantitative data indicate the mean ± standard deviation (error bars). *P<0.001 vs. NHA. Sirt-1, sirtuin-1; NBT, neoplastic brain tissue; LGA, low grade astrocytoma; AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; NHA, normal human astrocyte.

Results

Downregulation of Sirt-1 in glioma tissues and cell lines. Sirt-1 protein expression was downregulated in high-grade tumors (AAs and GBMs) compared to LGAs and controls (NBTs), as shown in Fig. 1 (LGA and AA vs. NBT, P<0.01; GBM vs. NBT, P<0.001). In addition, a similar pattern of Sirt-1 expression was observed in glioma cell lines compared to NHAs (P<0.0001; Fig. 1B).

To better understand the involvement of Sirt-1 in glioma development and progression, Sirt-1 mRNA levels were also analyzed by RT-qPCR in human NBTs, LGAs (WHO grade II), AAs (grade III) and GBMs (grade IV) and in primary glioma cell lines: PLGAC (primary LGA cells); PAAC (primary AA cells); and PGBMC (primary GBM cells). RT-qPCR analysis of glioma samples was consistent with the Sirt-1 protein levels revealed by western blotting. In particular, Sirt-1 mRNA levels in high-grade glioma (AA and GBM) were significantly lower

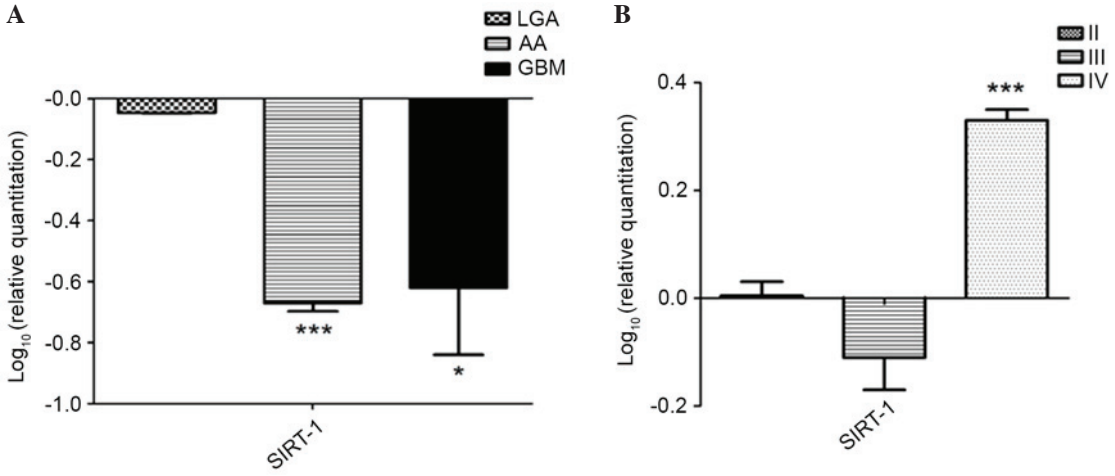

Figure 2. Expression of Sirt-1 mRNA in (A) tumor tissues and (B) primary glioma cell cultures. (A) Sirt-1 expression was quantified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis on tumor tissues and normalized vs. NBT. Data are represented as log10 (relative quantification). Error bars indicate the standard deviation. ***P<0.0001, AA vs. NBT; *P<0.01, GBM vs. NBT. (B) mRNA levels of Sirt-1 assessed by RT-qPCR in grade II (primary LGA cells), III (primary AA cells) and IV (primary GBM cells) glioma cell lines and normalized vs. NHA. Data are indicated as log10 (relative quantification). Error bars represent the standard deviation. ***P<0.0001 III cells vs. NHA. Sirt-1, sirtuin-1; LGA, low grade astrocytoma; AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; NBT, neoplastic brain tissue; NHA, normal human astrocyte.

than in low-grade tissues (LGA) as compared to NBT (LGA vs. NBT, P>0.05; AA vs. NBT, P<0.0001; GBM vs. NBT, P<0.01; Fig. 2A).

In primary LGA and AA cells, reduced expression of Sirt-1 was observed, according to the results obtained for protein expression in the same cells. Notably, in primary GBM cells a different expression pattern was observed, with an upregulation of Sirt-1 (GBM cells vs. NHA, P<0.0001; Fig. 2B).

Expression of miR-132, miR-34a and miR-217. Furthermore, the level of expression of miR-132, miR-34a and miR-217 was quantified, which were chosen by analyzing the alignment details of the 3'-UTR region of Sirt-1 with a computational miRNA target prediction tool (www.microRNA.org/) and comparing the results with validated pathways (Fig. 3). miR-217 (P<0.001 vs. LGA and II primary cells; Fig. 3A and B) and miR-34a (P<0.001 vs. LGA and PGLAC (grade II) cells; Fig. 3E and F) were consistently upregulated in high-grade glioma and tumorous cell lines, while a downregulation of miR-132 (P<0.001 vs. LGA and PGLAC (grade II) cells; Fig. 3C and D) was observed in high-grade glioma cell lines. These data suggest that the assessed miRNAs inversely modulate Sirt-1 expression, depending on the glioma grade. Analysis of Sirt-1 and miRNA expression may be a valuable marker of glioma progression.

Discussion

Previous studies have demonstrated that Sirt-1 is overexpressed in tumors such as lymphomas (11), leukemia (12), and prostate (13), breast (14) and colon cancers (15).

Usually, Sirt-1 is located in the nucleus, bound to histones H1, H3 and H4 (8,33). Similar to yeast Sir2, human Sirt-1 remodels chromatin through histone deacetylation and silences the transcription of numerous genes by heterochromatin formation (3-5,8,34). The genes repressed by this mechanism at their promoters include: E-cadherin; mismatch repair gene mutL homolog 1 ; transcription factors GATA-4 and GATA-5; p27; and cellular retinoid binding protein 1 (35). All these genes are actively involved in tumorigenesis, progression and metastasis (35,36).

Furthermore, Sirt-1 deacetylases non-histone substrates in cancer cells, such as the following transcription factors: p53; B-cell lymphoma-associated X protein, NF-κB, E2F1, FOXO1 (37,38), components of the core RNA polymerase I and the histone acetyltransferase p300/cAMP response element binding protein binding protein (39), thereby reducing gene expression (40). Other non-histone substrates include signaling factors, such as endothelial nitric oxide synthase, and DNA repair proteins, such as Ku-70 (36). Although the role of Sirt-1 it has been reported in a variety of cancers, to the best of our knowledge, there are no studies investigating its expression pattern in human glioma tissues and primary glioma cell lines of different grades.

The present results showed that Sirt-1 expression is reduced in human astrocytic tumors, with a more prominent downregulation in high-grade tumors (AAs and GBMs) compared with non-neoplastic brain tissue, thus suggesting that Sirt-1 expression is correlated with glioma tumor stages. In addition, the expression level of Sirt-1 mRNA in primary glioma cell lines is upregulated in high-grade PGBMCs as compared with normal human astrocytes (NHA). This different expression between high-grade tissues and and high-grade primary GBM cells suggests that Sirt-1 mRNA may undergo regulation at the transcriptional and translational levels, with more discordant expression patterns and protein levels, or the difference may be due to epigenetic effects on Sirt-1 mRNA or the variability of the microenvironment of brain cancer, leading to destabilization of the mRNA product or translational repression (41). The apparently controversial role of Sirt-1 may depend on the presence of other molecules regulating Sirt-1 during glioma progression or on the different cell-context specificity (immortalized cell cultures vs. human glioma cells). Among these molecules, microRNAs could play a key role. In fact, certain miRNAs

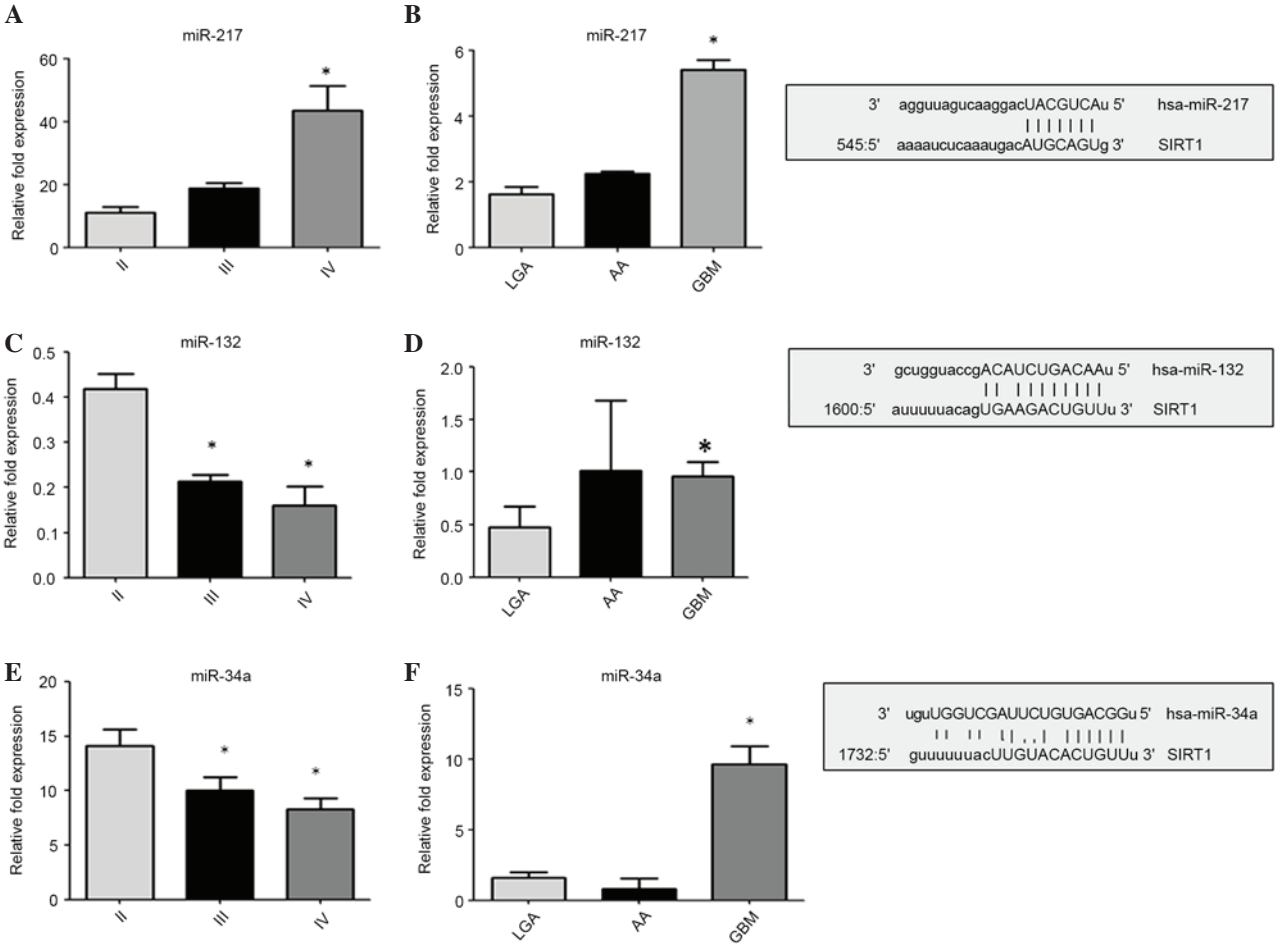

Figure 3. Expression of miR-132, miR-34a and miR-217. miRNAs expression were quantified by reverse transcription-quantitative polymerase chain reacion in primary glioma cell lines (panels A, C and E) and in tumor tissues (panels B, D and F). The data are expressed as the log10 RQ relative to U6. On the right: Aligment details with 3'-UTR region of SIRT-1. Sirt-1 is direct target of miR-34a, miR-217 and miR-132. The predicted highly conserved miRNAs targeting sequence located at the 3' UTR of Sirt-1 mRNA found on microRNA.org. Sirt-1, sirtuin-1; LGA, low grade astrocytoma; AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; miR, microRNA; 3'-UTR, 3'-untranslated region.

are overexpressed in tumors and act as oncogenes, promoting carcinogenesis by targeting tumor suppressors. Other miRNAs are downregulated in tumors and generally participate in oncogene overexpression. Additionally, under baseline conditions, miRNAs appear to act as moderate regulators of gene expression, but under conditions of stress or disease, miRNAs appear to exert more pronounced functions (23).

Overall, >16 miRNAs regulate Sirt-1 expression and activity. Among these miRNAs, miR-34a has been the most studied. miR-34a was identified as a tumor suppressor gene in neuroblastoma (42). One mechanism by which miR-34a expression decreases in cancers is aberrant CpG methylation of the promoter of miR-34a (43). Reduced expression of miR-34a alters cellular function in cancer cells. Ectopic expression of miR-34a induces cell cycle arrest in the G1 phase and apoptosis in several cancer cells. Numerous potential targets of miR-34a have been identified (44-46). However, the precise molecular targets of miR-34a remain unknown. Sirt-1 regulation by miR-34a has been reported in colorectal cancer, prostate cancer, hepatocarcinoma, glioma and pancreatic cancer (48-52).

miRNA-132 affects Sirt-1 regulation in adipocytes. Overexpression of miR-132 decreases Sirt-1 expression, which blocks deacetylation of p65 NF-κB (23). Proliferation and colony formation of pancreatic cancer cells was suppressed in cells transfected with miR-132 mimics and enhanced in cells transfected with miR-132 inhibitor by negatively regulating the Akt-signaling pathway (53).

Computational target prediction identified homology between miR-217 and 3'-UTR of human Sirt-1 mRNA. In a previous study comparing the expression of miR-217 during the aging process, from young to senescent endothelial cells, overexpression of miR-217 suppressed Sirt-1 expression in young endothelial cells as compared with senescent cells (54). By contrast, inhibition of miR-217 increases Sirt-1 expression and promotes cellular senescence in older human umbilical vein endothelial cells. This study confirms that miR-217 level is negatively associated with Sirt-1 expression (54).

One of the most notable aspects of miRNA biology is that one miRNA often regulates multiple genes and one gene is modulated by various miRNAs (55).

In the present study, data obtained suggest that miR-34a and miR-217 upregulation in high-grade tissue and cells is responsible for Sirt-1 downregulation, while miR-132 downregulation may be the cause of Sirt-1 overexpression in high grade primary cell lines. This intriguing profile of expression between gliomas tissues and cells may be due to a number of factors.

As aforementioned, regulation of Sirt-1 mRNA expression may be affected at the transcriptional and translational level or may be associated with various microenvironmental conditions. In addition, it is well known that miRNAs regulate gene expression by interacting with multiple networks and complex mechanisms, and this may account for the different profile of expression between human glioma tissues and cells. Indeed, every miRNA target contains miRNA response elements (MREs), to which miRNAs are attracted. At this level, the activity of the miRNAs may be modulated by the presence of competitive endogenous RNAs that, acting as sponges of miRNAs, vie for the miRNAs with shared MREs and affect the available levels of miRNAs in a cell, thus modulating the expression of target proteins (56-59). However, miRNAs may also activate gene expression, switching between repression and stimulation in response to different cell types, specific cellular and environmental conditions, and cofactors, which allows the cells to rapidly adapt to a variety of stimuli (60-62). The ambiguous effects of miRNAs make it challenging to study and fully understand the mechanisms by which miRNAs regulate gene expression.

The role of Sirt-1 in cancer is currently under debate due to numerous controversial findings suggesting that Sirt-1 acts as an oncosuppressor or as an oncogene (16,22,63). Few data specifically addressing the role of Sirt-1 in astrocytic tumors are currently available. Whereas certain studies reveal that overexpression of Sirt-1 is associated with gliomagenesis and its downregulation induces apoptosis and increases radiosensitivity in cancer cells, other studies show low levels of expression of Sirt-1 in gliomas (17,18,20). In particular, Liu *et al* (18) found that in GBM stem cells, Sirt-1 expression is 4.9-fold higher in poorly differentiated, CD133-positive tumor cells than in more differentiated CD133-negative tumor cells. Chang *et al* (17) reported that the knockdown of Sirt-1 expression in GBM stem cells enhances radiosensitivity and apoptotic activity *in vitro* and *in vivo*, suggesting that Sirt-1 could be a key regulator of GBM resistance to radiotherapy.

Wang *et al* (20) revealed a decreased expression level in GBM, although this result was not obtained by direct measurements, but rather by indirect analysis of public microarray datasets. The same decrement of Sirt-1 expression was observed by Lages *et al* (19) in GBM human samples and was associated with the different deregulation of Sirt-1 as an miRNA target. These data agree with the present findings of Sirt-1 downregulation in human astrocytic tumors.

To the best of our knowledge, the present study is the only study that has analyzed the expression of Sirt-1 in human samples of glioma with different grades of malignancy and Sirt-1 level expression in primary human glioma cell lines.

Whether Sirt-1 acts as an oncogene or tumor suppressor remains to be determined. However, the ability of Sirt-1 to protect the cell and expand the life span of the cell is evident. The unclear role of Sirt-1 may depend on the presence of other molecules, such as miRNAs, regulating Sirt-1 during glioma progression or on the different cell-context specificity (immortalized cell cultures vs. human glioma cells).

In conclusion, the present results support the important role for Sirt-1 as intrinsic regulator of tumor progression. However, the biological functions of Sirt-1 and its complex molecular pathways in gliomagenesis require additional investigation, with analysis of Sirt-1 gene silencing and functional analysis of involved miRNAs, for an in-depth characterization of the role of Sirt-1 in glioma development and progression.

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