Abnormal Methylation of Histone Deacetylase Genes: Implications on Etiology and Epigenetic Therapy of Astrocytomas

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Abstract. *Background: A growing body of evidence has revealed the involvement of epigenetic alterations in the etiology of astrocytomas. In the present study, we aimed to evaluate the association of DNA methylation of histone deacetylase genes (HDAC) with the etiology of astrocytoma, and the implications for epigenetic therapy. Materials and Methods: Methylation of the HDAC4, HDAC5 and HDAC6 genes was assessed in 29 tumor samples (astrocytomas grades I, III, and IV) and in the glioblastoma cell lines U87, U251, U343, SF188, and T98G by methylation-specific quantitative PCR (MSED-qPCR). Results: Significantly increased methylation of the HDAC5 gene was observed in astrocytomas when compared to non-neoplastic brain samples (p=0.0007) and to glioblastomas cell lines (p=0.001). A heterogenic methylation pattern was evidenced when compared to the glioblastoma cell lines. Distinct effects on methylation and gene expression were observed after in vitro treatment of the different cell lines with decitabine. Conclusion: Our results suggest that abnormal methylation of HDAC genes is involved in the etiology of astrocytomas and indicate that loci-specific epigenetic interindividualities might be associated to the differential responses to treatment with decitabine.*

Astrocytomas are the most common primary tumors of the central nervous system, accounting for 30% of adult primary

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brain tumors. These tumors are classified into four clinical grades according to the World Health Organization (WHO): grade I (pilocytic astrocytoma), grade II (low-grade difuse astrocytoma), grade III (anaplastic astrocytoma) and grade IV (glioblastoma and gliosarcoma) (1).

Glioblastomas are the most common and aggressive kinds of astrocytomas, characterized by high capacity of invasion and refractoriness to several therapeutic approaches. These tumors may develop from grade II and III astrocytomas (secondary) or more frequently without evidence of a precursor lesion (primary). Despite the efforts to develop novel therapies against glioblastomas, these tumors present an extremely malignant behavior and result in high mortality rate, with a median survival rarely exceeding 12 months (2, 3).

Several genetic alterations have been identified among different grades of astrocytoma, demonstrating the high heterogeneity and genomic instability of these tumors. Nevertheless, although the accumulation of some genetic alterations can be considered peculiar to this neoplasia, they are not specific markers of astrocytoma development (2, 4).

Genetic heterogeneity may contribute to the current limitations in predicting patient survival on the basis of histologic analysis of astrocytoma type and grade alone. A classification based on tumor genetic background could lead to a more accurate prediction of survival and response to therapy.

The understanding of the molecular genetic abnormalities involved in the pathogenesis and progression of astrocytomas has advanced significantly over the past decades. The epigenetic alterations at specific genomic loci have emerged as important factors of tumor ethiopathogeny and include DNA methylation and histone acetylation/deacetylation (5).

Aberrant methylation of CpG island-associated genes was associated with the inactivation of a large number of tumor suppressor genes involved in the cell cycle (*RB, p16INK4A,*

p73, RASSF1A, PTEN and *p53*), DNA repair (*O6MGMT*), apoptosis (*DAPK*), and invasion (*TIMP3*) (6-9). Abnormal histone deacetylation of lysine residues of histone tails is generally associated with a relaxed chromatin structure and a higher transcriptional activity. For this reason, several histone deacetylase inhibitors have been developed and tested in experimental and clinical trials of epigenetic therapy of cancer (10).

In a previous study, our group described a negative correlation between the expression of histone deacetylase genes (*HDAC*), especially class II genes, and astrocytomas when compared to non-neoplastic white matter. This reduced expression was found to be associated with tumor grade, suggesting that decreased expression of *HDAC* genes might play an important role in the malignancy of astrocytomas (11).

Based on this previous evidence of decreased expression, we aimed to test the hypothesis that DNA methylation in promoter regions could be involved in the loss of expression of *HDAC* class II genes in astrocytomas. Furthermore, we hypothesized that if DNA methylation controls *HDAC* gene expression, this might be a primary event that could precede histone modification in astrocytoma tumorigenesis, indicating the potential of demethylation-based therapies for the epigenetic treatment of astrocytomas.

Materials and Methods

Patients and samples. We analyzed 29 freshly frozen microdissected tumor samples obtained from gross total surgical resection including 17 grade IV, 2 grade III and 10 grade I primary astrocytomas from consecutive patients admitted to our institution for diagnosis and treatment. Diagnoses were made according to WHO criteria (1). Five samples of microdissected white matter obtained from patients with mesial temporal lobe epilepsy refractory to medical treatment were used as control. Five glioblastoma cell lines (U87, U343, U251, SF188 and T98G) and a peripheral blood sample (negative control) were also analyzed.

The study was approved by the Research Ethics Committee of the University Hospital, Faculty of Medicine of Ribeirão Preto, University of Sao Paulo (protocol number 9375/2003 and 7645/99) and was based on the Helsinki convention criteria. All patients and the persons responsible for the children gave written informed consent to participate.

Methylation analyses. We used the Methprimer software (12) for identification of CpG island within the promoter region of *HDAC* class II genes (*HDAC4, HDAC5* and *HDAC6*) considering the parameters of fragment length >100bp, % GC> 50%, Obs/Exp > 0,6 as criteria for the prediction of CpG island.

A DNA sample was obtained from tumors after microdissection or from cultivated cells by standard phenol-chloroform protocol. Methylation analysis at specific sites within the CpG island in promoter regions of *HDAC* genes was performed by the MSED-qPCR as previously described (13, 14). Real-time PCR reactions were performed in triplicates in an ABI 7500 Thermocycler (Applied Biosystems, Foster City, CA, USA) in a final volume of 12 ul containing Sybr Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and the

primers: *HDAC4*s: 5' CAGCAGCCAATGAGGTCCAG 3', *HDAC4*as: 5' CCGTCTGC TCCCACCCCTC 3', *HDAC5*s: 5' GTTGAGCCCC AGCA TCCATACT 3', *HDAC5*as 5' CACCACCAACCCCTCCCCTG 3', *HDAC6*s 5' GTCCCCACAGGTCGCCAGAAAC 3', *HDAC6*as 5' CTCCGCCCCCTTCCCGCCT 3'. The methylation sensitive enzyme *Hpa*II was used for methylation analysis of *HDAC4* and *HDAC5* genes and the *Hha*I for analysis of *HDAC6* gene.

Total cellular RNA was extracted using the Trizol® Reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to single-stranded cDNA using a High Capacity Kit (Applied Biosystems) according to the manufacturer's protocol. Messenger RNA expression level for each *HDAC* was evaluated using an ABI 7500 machine (Applied Biosystems). Amplifications were obtained using on demand TaqMan® probes (Applied Biosystems) for each *HDAC*. To normalize differences in the amount of total cDNA added to each reaction, *GAPDH* gene expression was used as an endogenous control. Non-tumor brain cells were used as a calibrator sample (reference sample for relative quantification). All reactions were carried out in triplicate at 4°C.

Cell lines culture and treatments. The human adult glioblastoma cell lines U87, U343, U251 and T98G were purchased from the American Type Culture Collection and the pediatric glioblastoma cell line SF188 was kindly provided by Dr. Nada Jabado and Dr. Damien Faury (McGill University, Canada). Cells were cultured in HAM F10 (Gibco BRL, Life Technologies®, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, penicillin (100U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified 5% CO_2 incubator.

Decitabine (5-aza 2-deoxycitidine) (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile phosphate buffer saline (PBS) solution, pH 7.2, and stored at –20°C to prevent decomposition.

For treatment, a total of 1×10^5 cells were seeded in 25 cm² tissue culture flasks containing 5 ml of culture medium. After 24 hours, the medium was replaced with fresh medium containing 1 μM and 10 μM decitabine every 24 hours for 4 days (4 pulses). At the end of treatment, the medium was replaced with fresh culture medium without Decitabine. Cells were cultured for an additional 24 hours, and used for molecular and functional assays. Control cultures were treated under similar experimental conditions in an equal volume of PBS.

Colony formation assay. The effects of decitabine on cell reproductive health were evaluated by the clonogenic assay (15). After trypsinization, a single-cell suspension of 300 cells was added to 6-well plates and treated as described above. After 7-10 days, the colonies were rinsed with PBS, fixed with methanol, stained with Giemsa and colonies of >50 cells were counted. Assays were performed in triplicate for each independent experiment.

Statistical analysis. Statistical analyses were performed by the non-parametric Mann-Whitney test and by one-way analysis of variance (ANOVA) using SPSS 15.0 (SPSS Inc, Chicago, IL, USA) and PRISM 4.0 (GraphPad Inc, San Diego, CA, USA) software. The level of significance was set at $p<0.05$ in all analyses.

Results

Methylation pattern of HDAC genes in astrocytomas. Quantitative analysis revealed a statistically significant difference ($p=0.007$) in methylation in the promoter region of *HDAC4* gene in astrocytoma when compared to the
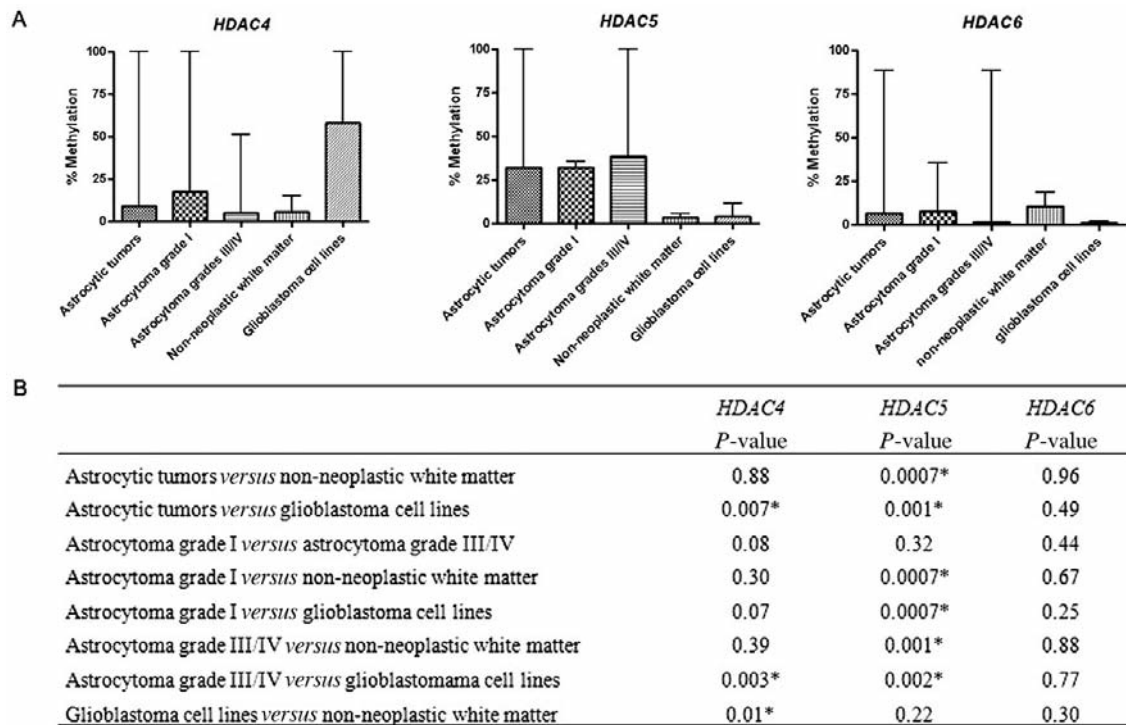
Figure 1. *A: Methylation pattern of the HDAC genes (median values and ranges) expressed in astrocytic tumors, grade I astrocytoma, grade III/IV astrocytoma, non-neoplastic brain tissue and cell lines. B: Statistical comparison of the HDAC methylation pattern between the groups analyzed. *Statistically significant difference (p<0.05, Mann-Whitney test).*

Table I. *Values of methylation patterns of HDAC4, HDAC5 and HDAC6 genes in the sample groups.*

	HDAC4			HDAC5			HDAC6		
	Median	Minimum	Maximum	Median	Minimum	Maximum	Median	Minimum	Maximum
Astrocytic tumors	9.11	0.78	100	32.	3.45	100	6.33	0.05	88.57
Astrocytoma grade I	17.61	0.93	100	32.04	18.36	35	7.60	0.28	35.60
Astrocytoma grade III/IV	5.01	0.78	50.9	38.7	3.45	100	1.74	0.05	88.57
Non-neoplastic white matter	5.59	4.41	15.21	3.44	1.56	5.44	10.37	0.20	18.49
Glioblastoma cell lines	57.94	15.16	100.00	4.13	1.94	11.53	0.93	0.88	1.89

methylation pattern observed in glioblastoma cell lines (Figure 1). Nevertheless, a statistically non-significant increase in methylation was observed when astrocytomas and non-neoplastic brain tissues were compared ($p>0.05$) (Figure 1).

Regarding *HDAC5*, a significant increase in methylation was observed in tumors when compared to non-neoplasic brain tissues ($p=0.0007$) and to glioblastoma cell lines ($p=0.001$) (Figure 1). No significant difference in methylation was observed for the *HDAC6* gene ($p<0.05$) (Figure 1). No significant difference was observed when the methylation pattern of *HDAC4, HDAC5* or *HDAC6* was compared to tumor grading ($p>0.05$) (Figure 1). The

median, maximum and median values of methylation in astrocytomas, non-neoplastic brain tumors and cell lines are shown in Table I.

Methylation pattern of HDAC in glioblastoma cell lines. A heterogeneous methylation pattern (mainly for *HDAC4* and *HDAC5* genes) was observed when the different glioblastoma cell lines were compared separately (Figure 2).

Effects of treatment with decitabine on methylation and expression of HDAC genes. Treatment with decitabine proved to be effective in reducing *HDAC4* methylation in a dose-
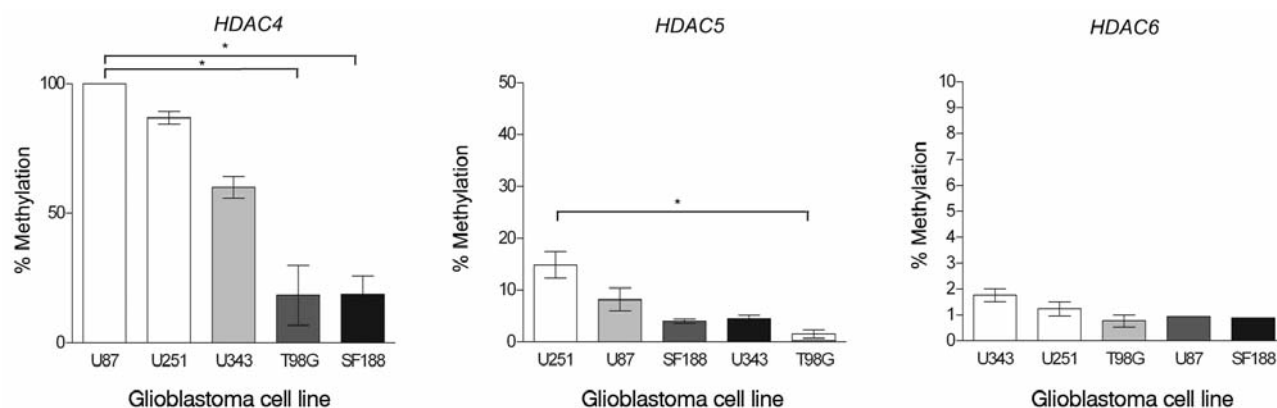
Figure 2. *Methylation pattern of the HDAC gene in different glioblastoma cell lines. *Statistically significant difference (p<0.05).*

dependent manner (1 μM or 10 μM) in U87 and U251 cell lines (Figure 3 A and C). A corresponding dose-dependent increased expression of *HDAC4* gene was observed in the U87 but not in the U251 cell line (Figure 3 B and D). An increased methylation not accompanied by lower expression of *HDAC4* gene was observed after treatment of the T98G cell line (Figure 3 E and F). No significant difference in methylation pattern or *HDAC4* expression was observed after treatment of U343 cell line (Figure 3 G and H). An increased methylation accompanied by reduced expression of *HDAC4* gene was observed in the SF188 cell line (Figure 3 I and J).

Regarding *HDAC5*, an increased gene expression independent of significant changes of methylation was observed in U87 cell line after treatment (Figure 3 A and B). A decreased methylation accompanied by an increased gene expression was observed after treatment with 1 μM or 10 μM of decitabine in the U251 cell line (Figure 3 C and D). No significant changes were observed in methylation pattern or gene expression for T98G and U343 cell lines (Figure 3 E to H). Increased methylation not accompanied by significant changes in gene expression was observed for the SF188 cell line after treatment (Figure 3 I and J)

No change in methylation of the *HDAC6* gene was observed after treatment with decitabine for all cell lines (Figure 3 A, C, E, G and I). However, a methylation-independent increased expression of *HDAC6* gene was observed in U343 and U87 cell lines (Figure 3B and H). In contrast, a dose-dependent decreased expression of *HDAC6* gene was observed in T98G and SF188 cell lines independent of significant changes of methylation pattern (Figure 3 F and J).

Effects of treatment with decitabine on the reproductive viability of glioblastoma cell lines. Treatment of the glioblastoma cell lines with both the 1 μM and 10 μM decitabine doses significantly affected (p<0.001) the *in vitro* reproductive ability of the cells (Figure 4).

Discussion

Elucidating the role of epigenetic alterations in the etiology of tumors has been the aim of many studies, mainly due to the potential of these alterations to be used as molecular markers for an early diagnosis and prognosis and even as targets of alternative cancer therapies.

Genome-wide studies have identified various cancer-associated candidate genes, with a direct association being observed between loss of expression and promoter hypermethylation (16, 17).

A significantly decreased expression of *HDAC* genes has been demonstrated to be associated with etiology and tumor grade progression in astrocytoma (11). However the mechanism that promotes this loss of expression in astrocytoma is unknown.

In the present study, we observed a statistically significant increased methylation pattern in the promoter region of *HDAC5* gene of astrocytomas when compared to non-neoplastic white matter brain tissue, but this pattern was not associated with tumor grade. These results may indicate, at least in part, that the lower *HDAC* gene expression described previously could be due to a higher methylation status of promoter regions of *HDAC* in astrocytoma.

A statistically significant difference in methylation of *HDAC4* was observed when comparing astrocytoma and glioblastoma cell lines. However, no significant difference in the methylation pattern of *HDAC4* or *HDAC6* genes was observed in astrocytoma according to tumor grade or when compared with white matter brain.

A heterogeneous methylation pattern was evidenced when comparing the methylation of the *HDAC* genes of tumors and glioblastoma cell lines, revealing that epigenetically, cell lines might not correspond to tumors. Supporting the evidence of incompatibility between cell lines and primary tumors, a series of genomic differences and differences in gene
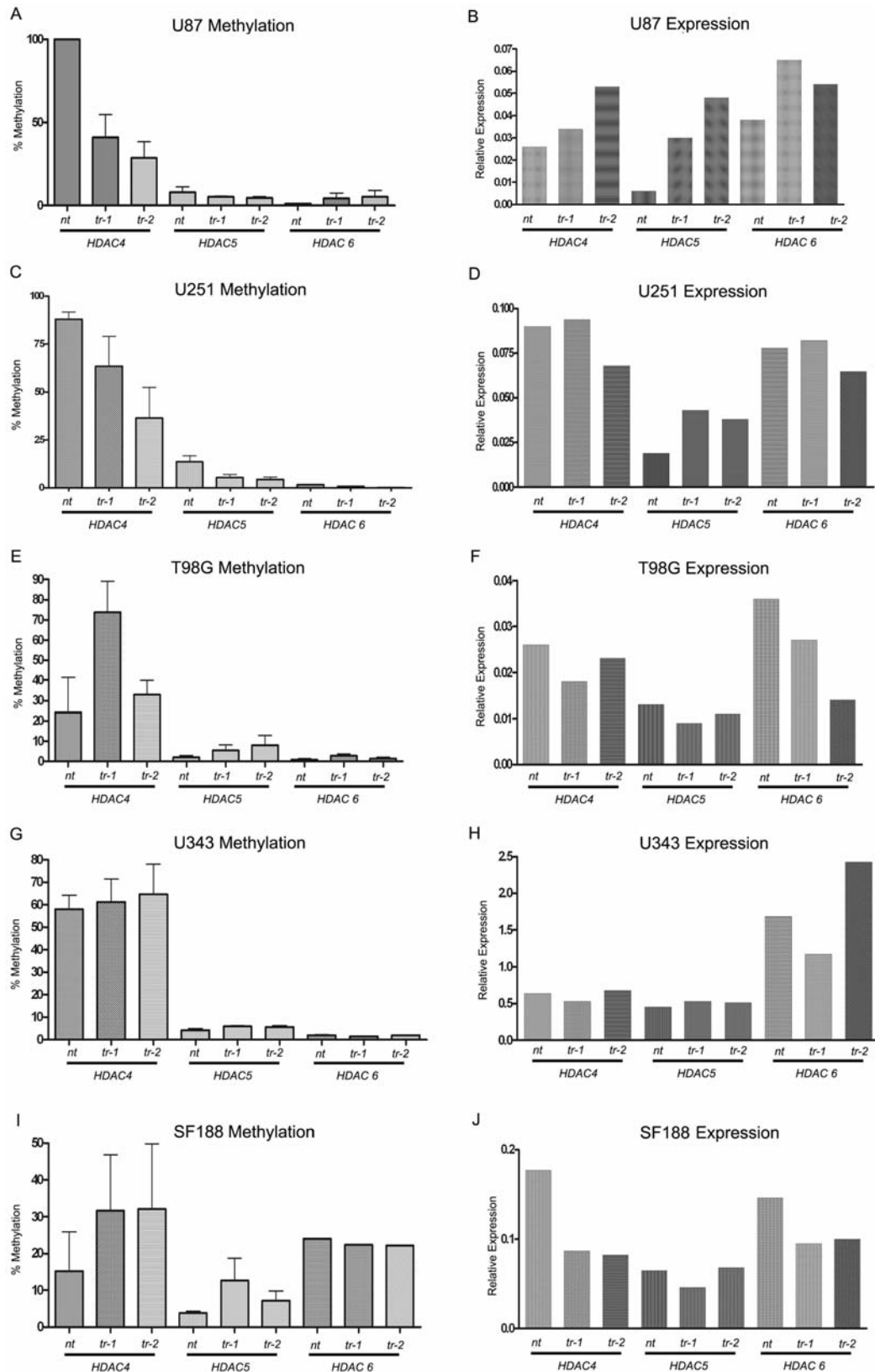
Figure 3. *Effects of treatment with decitabine on the methylation and expression of HDAC genes in different glioblastoma cell lines. nt, Non-treated; tr-1, treatment with 1 µM decitabine; tr-2, treatment with 10 µM decitabine.*
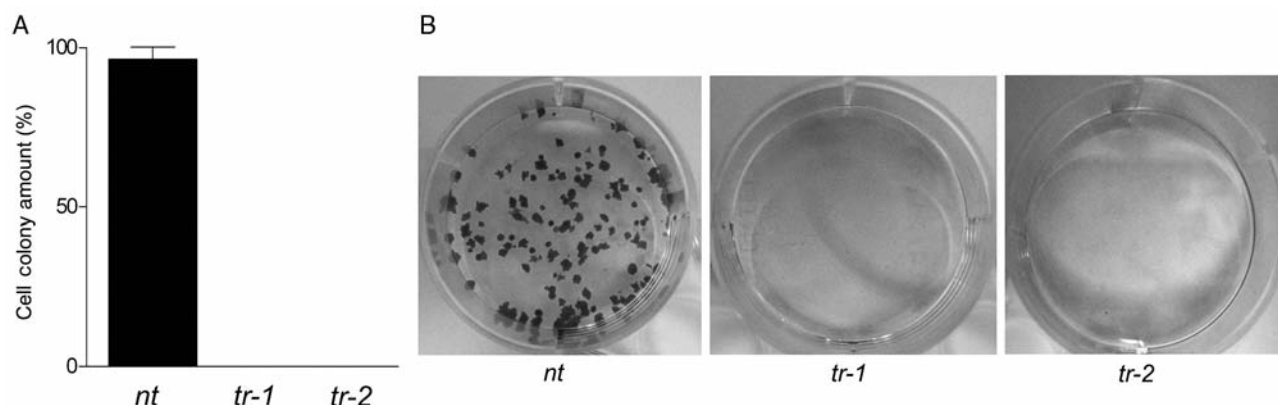
Figure 4. *Effects of treatment with decitabine on the reproductive viability of the U251 glioblastoma cell line. Graphic (A) and in vitro (B) demonstration of the results. nt, Non-treated; tr-1, treatment with 1 μM decitabine; tr-2, treatment with 10 μM decitabine.*

expression pattern were recently reported in astrocytoma (18). Although genomic alterations presents in primary tumors are also generally present in cell lines, various *de novo* chromosome alterations are detected exclusively in cell lines and a distinct gene expression profile is commonly observed when cell lines and tumors are compared (18). Considering the distinct methylation pattern observed in the present study in tumors and cell lines, we speculate that the genomic alterations described in cell lines compared to primary tumors could be consequent to epigenetic adaptations of tumor cells to the *in vitro* microenvironment. However, future studies are needed to test this hypothesis.

Due to the reversibility of epigenetic patterns inherent to transcriptionally active cells, treatment with specific drugs, such as demethylating or histone deacetylases, is becoming an interesting option for cancer treatment.

The fact that the previously reported decreased expression of *HDAC* genes of astrocytomas is associated with hypermethylation of promoter regions, leads us to hypothesize that the use of histone deacetylase inhibitors may not be the most effective epigenetic treatment for astrocytomas.

Decitabine is a nucleoside analogue that promotes demethylation by substituting cytosine residues during DNA replication (S phase of the cell cycle) and by inhibiting the activity of DNA methyl transferase enzymes (DNMT) through covalent binding (19). Many lines of evidence have revealed the important role of decitabine in inducing re-expression of tumor suppressor genes, reestablishing cell growth control or inducing apoptosis in tumors in *in vitro* or *in vivo* models (20-22).

Clinical trials have demonstrated that decitabine is more effective for the treatment of myelodysplastic syndrome than conventional chemotherapy (23). Pharmacokinetic tests have revealed the excellent distribution of decitabine throughout the body after intravenous injection and its ability to cross the blood–brain barrier, indicating its probable efficiency in reaching brain tumors (19, 24).

Different effects on methylation and expression of *HDAC* genes was observed in the present study, when comparing the *in vitro* treatment with decitabine of different glioblastoma cell lines. The expected hypomethylation associated with hyperexpression was observed in the *HDAC4* and *HDAC5* genes after treatment of the U87 and U251 cell lines, respectively. Increased expression of *HDAC5* and *HDAC6* independent of changes in methylation was observed after treatment of the U87 and U343 cell lines. A contradictory increase in the methylation of the *HDAC4* gene was observed in the SF188 and T98G cell lines and an increased methylation of the *HDAC5* was observed in SF188. These findings indicate that the effects of *in vitro* treatment with decitabine might depend on the type of cell line suggesting the influence of interindividual characteristics on the response to epigenetic treatments.

We speculate that in some cell lines decitabine might present a direct effect on the methylation and expression of *HDAC* genes, whereas for other cell lines, decitabine might indirectly affect *HDAC* genes. This indirect effect might be consequent to the activation and/or inactivation of an undetermined selective control pathway of *HDAC* expression. Furthermore, the effect of the genetic heterogeneity commonly observed in solid tumors on cellular response to epigenetic treatments is completely unknown. Future studies are needed to clarify these matters.

Decitabine had a devastating effect on cell viability and reproductive health after *in vitro* treatment of glioblastoma cell line at both doses (1 μM and 10 μM). This finding obtained by a clonogenic assay confirms that decitabine affects the reproductive capacity of glioblastoma. However, future studies in *in vitro* and *in vivo* models are needed to elucidate the molecular mechanism and the cellular pathway affected by treatment.

Taken together, our findings suggest that abnormal methylation in the promoter regions of *HDAC* genes is involved in the etiology of astrocytomas. Furthermore, the epigenetic interindividuality observed in the *HDAC locus* of different glioblastoma cell lines and the evidence of differential effects on methylation and *HDAC* gene expression after the treatment with decitabine indicates a possible implication of loci-specific epigenetic interindividuality to the effects of genome-wide demethylation therapies.

Acknowledgements

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