Folate Supplementation Limits the Aggressiveness of Glioma via the Remethylation of DNA Repeats Element and Genes Governing Apoptosis and Proliferation

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Abstract

Purpose: We have investigated whether the folate supplementation could be used to limit the aggressiveness of glioma through the DNA remethylation because (*a*) the cancer genome is characterized by a low level of DNA methylation (or 5-methylcytosine, 5 mC); and (*b*) folate is the main generator of S-adenosyl-methionine, the methyl donor molecule in the DNA methylation reaction catalyzed by the DNA methyltranferases.

Experimental Design: The effects of folate supplementations were analyzed on the global DNA methylation status, the methylation status of DNA repeat element, the sensitivity of temozolomide-induced apoptosis, and the proliferation index of glioma cells. Finally, we analyzed whether the DNA methylation level could be used as a prognostic factor and/or a biomarker in an antiglioma therapy using folate supplementation as an adjuvant.

Results: Our data show that gliomagenesis is accompanied by a reduction in 5 mC levels and that this low level of 5 mC is a poor prognostic factor in Glioblastoma Multiforme patients. We also show that folate supplementation enhanced the DNA remethylation through the Sp1/Sp3-mediated transcriptional up-regulation of genes coding for Dnmt3a and Dnmt3b proteins, two *de novo* methyltranferases. Finally, we show that the folate-induced DNA methylation limits proliferation and increases the sensitivity to temozolomide-induced apoptosis in glioma cells through methylation of the genes implicated in these processes (*PDGF-B, MGMT, survivin*, and *bcl-w*).

Conclusion: This study suggests that folate supplementation could be a promising adjuvant for the future design of antiglioma therapies in preclinical and/or clinical studies.

Despite the irrefutable role of genetic mechanisms in triggering tumorigenesis, epigenetic modifications, and particularly the DNA methylation modifications, are now recognized as frequent alterations playing a crucial role in the development and progression of human malignancies (1–3). Two distinct DNA methylation abnormalities are observed in cancer. The first is an overall genome-wide reduction in DNA methylation (global hypomethylation) and the second is regional hypomethylation or hypermethylation within the CpG islands of specific gene promoters. Both forms of hypomethylation are believed to induce proto-oncogene activation and chromosomal instability, whereas regional hypermethylation is strongly associated with transcriptional silencing of tumor suppressor genes (4). Thus, DNA methylation can function as a "switch" to activate or repress gene transcription, providing an important mechanism for overexpressed or silenced genes involved in the regulation of the cell cycle, DNA repair, growth signaling, angiogenesis, and apoptosis, and by ricochet in the initiation and the development of tumors (5).

To date, an increasing number of reports investigating epigenetic signatures in malignant gliomas and more particularly the hypermethylation of tumor suppressor genes have been published. For example, it has been reported that *PTEN* methylation occurs frequently in gliomas and may be associated with the focal loss of *PTEN* expression and this methylation of the *PTEN* promoter defines low-grade gliomas from secondary Glioblastoma Multiforme (6, 7). Thus, based on these observations and on the fact that DNA methylation is a reversible process, several preclinical and clinical studies have explored the

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Translational Relevance

Gliomas are the most common tumors of the brain and the most malignant form Glioblastoma Multiforme is virtually incurable. Several studies on the molecular mechanisms governing the development and the progression of gliomagenesis have been and are currently undertaken to design successful therapeutic protocols based on rational molecular targeting. The data presented here show that a decrease in DNA methylation levels occurring during gliomagenesis confers a poor prognosis in glioma patients. However, we show that this decrease can be limited by specific folate supplementations through the methylation of the (*a*) oncogene (*PDGF-B*), (*b*) DNA repeat elements, whose the low level of methylation is associated with the tumorigenesis, and (*c*) gene(s) implicated in the sensitivity to temozolomide-induced apoptosis (*MGMT*). Thus, this study suggests that specific folate supplementations are a promising alternative resource to epigenetic-based adjuvant treatment against glioma.

effects of DNA hypomethylating agents (DHA) in cancer because DHA have the capacity to reactivate the dormant tumor suppressor genes by demethylation of their hypermethylated promoter. Nevertheless and despite the successful and the promising results of some clinical studies, the use of these drugs can seem paradoxical in anticancer therapies because many tumors are characterized by a global DNA hypomethylation (8, 9). Moreover, the use of DHA to suppress the regional hypermethylation gene process in gliomas seems in contradiction with the fact that the *MGMT* or *bax* gene hypermethylations have been reported to be a marker of the response to temozolomide, an alkylating agent (10, 11), or associated with an increased survival of Glioblastoma Multiforme patients, respectively (12). Finally, the use of DHA in antiglioma therapy could have "oncogenic effect" because many reports have stated that global DNA hypomethylation can act as an oncogenic factor by enhancing the loss of imprinting, the reactivation of transposable elements, the chromosomal instability through the hypomethylation of DNA repeat element, or by promoting the gene-specific hypomethylation of oncogenes such as *platelet-derived growth factor (PDGF)-B* (13–15). Thus, it seems that the use of DHA could have possible unfavorable effects. Indeed, the induction of matrix metalloproteinase with 5-aza-2-deoxycytidine was observed in pancreatic cancer and lymphomas, associated with a matrix metalloproteinase–mediated increase in the invasive potential of cancer cells (16, 17). Increased invasiveness after the exposure to DHA was also observed in selected breast and prostate cancer cells where it was attributed to the induction of urokinase (urokinase-type plasminogen activator), which is a member of the serine protease family implicated in the degradation of various components of the extracellular matrix favoring tumor cell invasion (18–20).

Supported by these arguments and based on the fact that DNA methylating agents may result in a reduction in growth and invasive potential of cancer cells (21), we asked whether the DNA methylating agent treatment was a tool to limit the aggressiveness of glioma by enhancing the DNA remethylation. Among the DNA methylating agent, we chose to use folate because folate is the main generator of S-adenosyl-methionine, the methyl donor molecule in the DNA methylation reaction catalyzed by the DNA methyltranferases (Dnmts). Our results show that a tumor-grade specific treatment of folate devoid of an antioxidant property increased the level of DNA methylation. At the molecular level, we show that the folate supplementation devoid of pro-oxidant effects increased the DNA methylation levels in glioma cells through the Sp1/Sp3-mediated transcriptional up-regulation of genes encoding for the Dnmt3a and Dnmt3b proteins, two *de novo* methyltranferases. We also observed that the folate treatment limited the proliferation and increased the sensitivity of temozolomide-induced apoptosis of glioma cells through the methylation of genes implicated in these processes such as the *PDGF-B*, *MGMT*, *survivin*, and *bcl-w* genes. Thus, our work suggests that the folate supplementation could be used as an adjuvant in antiglioma therapy to limit the low DNA methylation level because this confers a poor prognosis in Glioblastoma Multiforme patients.

Materials and Methods

Cell culture and treatments. U373 were obtained from American Type Culture Collection and cultured in 1 g/l glucose DMEM supplemented with 10% FCS and 1% penicillin-streptomycin at 37°C with 5% CO_2. U251 and Ntv-a cells were obtained from the laboratory of Eric C. Holland (Memorial Sloan Kettering Cancer Center, New York, New York). The primary cultured tumor cells were obtained after mechanical dissociation according to the technique previously described (22). Briefly, tumor tissue was cut into pieces of 1 to 5 mm^3 and plated in a 60-mm^2 tissue culture dish with DMEM with 10% fetal bovine serum and antibiotics. In parallel, minced pieces of tumor were incubated with 200 U/mL collagenase I (Sigma) and 500 U/mL DNaseI (Sigma) in PBS for 1 h at 37°C with vigorous constant agitation as previously described (23). The single-cell suspension was filtered through a 70-μm cell strainer (BD Falcon), washed with PBS, and suspended in DMEM-10% fetal bovine serum. Cell cultures were subsequently split 1:2 when confluent and experiments were done before passage 3 to 8. All experiments were done after 7 d of treatment with folate (4 or 40 μg/mL) ± antioxidant cocktail (20 μmol/L β-carotene; 200 μmol/L ascorbic acid); medium was changed daily.

Immunostaining and flow cytometry. Briefly, fixed and denatured cells were treated with 2N HCl for 30 min. The preparation was then neutralized with 100 mmol/L Tris-HCl (pH 8) for 10 min. After washing in 0.05% Tween 20-PBS and blocking, the primary antibody against 5-methylcytosine (5 mC; Calbiochem) was detected with Alexa Fluor antibody (Molecular Probe). After mounting, the quantification of 5 mC signal was obtained after fluorescence microscopy and the analysis of 150 cells using the MetaVue program. Similar procedures were used for flow cytometry analysis.

Combined Bisulfite Restriction Assay of Alu and Line-1, two DNA repeat elements. DNA extraction was done using the DNA extraction kit (Qiagen). Next, bisulfite conversion was done using the EZ DNA methylation Gold kit (Zymo research) according to manufacturer's instructions. After PCR products amplified with specific primers of repeats, *Alu* and *line-1* (cf. Supplementary Table S2) were digested overnight with TaqI (Ozyme). After electrophoresis in 2% agarose gels and ethidium bromide staining, densitometric quantification of PCR bands was done using the ImageJ program.

Measure of reactive oxygen species production. Intracellular reactive oxygen species (ROS) levels were estimated with the fluorescent dye 2′, 7′-dichlorodihydrofluorescein diacetate (Molecular Probes), which is a nonpolar, nonfluorescent compound that is converted into a

polar nonfluorescent derivative (2′, 7′-dichlorodihydrofluorescein) by cellular esterases. 2′, 7′-Dichlorodihydrofluorescein is membrane permeable and is rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescein in the presence of intracellular ROS. Cells cultured in 24-well dishes until 70% to 80% confluence were incubated in saline buffer [135 mmol/L NaCl, 5 mmol/L KCl, 0.4 mmol/L KH2PO4, 1 mmol/L MgSO4, 20 mmol/L HEPES (pH 7.4), 5.55 mmol/L glucose, and 1 mmol/L CaCl2] with 1 mmol/L CM-2′, 7′-dichlorodihydrofluorescein-diacetate to estimate ROS contents. ROS production was estimated by comparing fluorescence immediately and 2 h later (fluorescence plate reader Victor; Perkin-Elmer) 2′,7′-dichlorofluorescein: ex, 485 nm; em, 535 nm. Cell number was then estimated in each well by crystal violet staining. The fluorescence intensity was normalized to cell number.

qPCR and reverse transcription-PCR. RNA was isolated from cells using trizol. Semiquantitative PCR reactions were done in triplicate by using the Multiplex Quantitative PCR system (Mx4000), qPCR Core Reagent kit (Stratagene) according to manufacturer's instructions, and by using primers specific of *DNMT3a*, *DNMT3b*, or *b-actin* as housekeeping gene (cf. Supplementary Table S2).

Chromatin Immunoprecipitation assay. Chromatin immunoprecipitation ChIP assays were done with the EZ ChIP kit (Millipore-Upstate) with specific antibodies directed against Sp1 (Santa Cruz; sc-59) and Sp3 (Santa Cruz; sc-644) and primers amplifying specific region of *DNMT3a* and *DNMT3b* genes (cf. Supplementary Table S2).

Clonogenicity assay. One hundred cells were seeded into 6-multiwell dishes and incubated at 37°C under 5% CO_2 and 95% humidity, and the medium added with folate or folate plus antioxidant were changed every 2 d over 1 wk. Colonies were fixed by 20% methanol, stained with crystal violet and photographed.

Methylation-specific PCR assays. The methylation status of *PDGF-B*, *bcl-w*, *survivin*, *bax*, and *PTEN* promoter was determined by methylation-specific PCR using specific primers (Supplementary Fig. S3), after sodium bisulfite conversion (EZ DNA methylation Gold kit-Zymo research). Methylation status of MGMT promoter was estimated according to Esteller et al. (10).

Statistical analysis. All experiments were done at least in triplicates. Significance of the differences in means were calculated using Student's *t* test, whereas correlations were determined using Pearson test. Survival curves were plotted according to Kaplan-Meier method and compared by means of log-rank test. All known prognostic parameters were entered into the proportional hazards Cox model for multivariate analysis. Proportional hazards assumption was verified for the final model by means of Schoenfeld residuals study and -ln(-ln(survival)) curves. All analyses were done with SAS 9.1.3 (SAS Institute, Inc.) and Stata 10.0 SE (Stata Corp.). All statistical tests were two-sided, and statistical significance was defined as *P* value of <0.05.

Patient characteristics. Overall survival was measured from the date of surgical resection to the death. All patients included in this study had similar management (Complete resection) and similar treatment [temozolomide 75 mg/m^2/d × 7 d/wk for 6 wk and fractionated radiotherapy (60 Gy total dose)]. Karnofsky performance score of patient are included in the [80-90] range and age is included into the [49-65] range.

Results

The amount of 5-methylcytosines decreases when glioma grade increases. To evaluate the global methylation status in human gliomas, we measured the amount of 5 mC presented in DNA extracted from gliomas included in a collection of 73 patient-derived biopsies of astrocytomas of different grades obtained from surgical resections. Five nonpathologic samples were used as a control. ELISA analysis and a Pearson's correlation test showed an inverse and significant correlation between the amount of 5 mC and the tumor grade ($P < 0.0001$; r = –0.497; Fig. 1). Consistently with some reports, our results indicated that the decrease in 5 mC (also referred as to global DNA hypomethylation) is a hallmark frequently observed in glioma (9, 14).

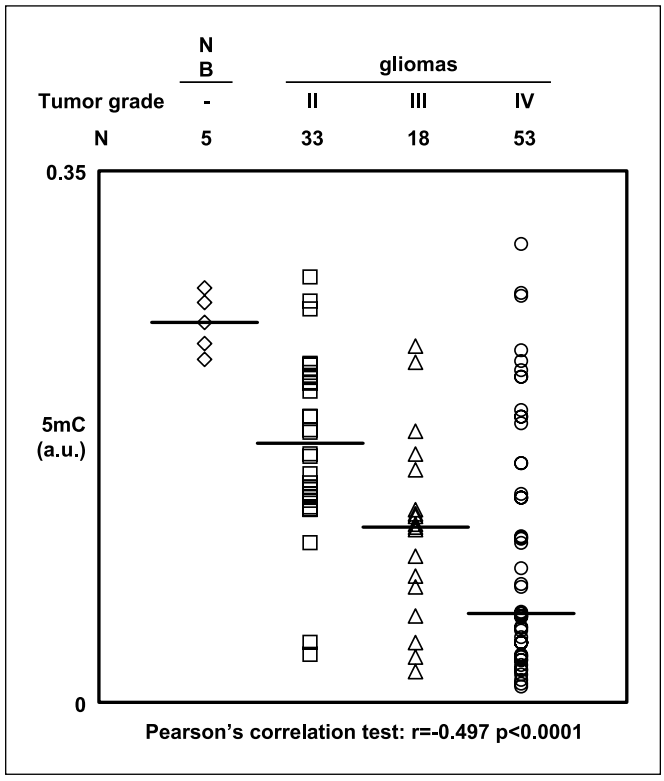

Fig. 1. Correlation between the 5 mC and the tumor grade in gliomas. The 5 mC number was assessed with the Methylamp Global DNA methylation Quantification kit (Euromedex-Epigentek) and was done on DNA from 5 normal brain samples (*NB*), 33 grade II astrocytomas, 18 grade III astrocytomas, and 53 grade IV astrocytomas or Glioblastome Multiforme patients.

Grade-specific abolition of Alu (a DNA repeat element) hypomethylation by folate supplementation. Some reports have already described that a folate supplementation has the capacity to inhibit DNA hypomethylation because folate is the main micronutrients required to maintain an adequate cellular pool of the methyl donor S-adenosylmethionine, which is the universal methyl group donor required for DNA methylation (24). Thus, we decided to investigate the effect of folate supplementation on the DNA methylation status of glioma cells. For this, we first choose to use the RCAS/Ntv-a cellular model of gliomagenesis (25, 26). This system allows gene transfer to specific cell types in culture or *in vivo*, if the transgenic mice or the primary brain cultures have been engineered to express the RCAS viral receptor tv-a. Thus, we obtained Ntv-a/PDGF and Ntv-a/Ras-Akt cells by RCAS-PDGF or RCAS-Ras-Akt infections in astrocyte progenitor cells expressing the tv-a from nestin promoter (Ntv-a cells). *In vivo*, this infection promotes the formation grade II and grade IV gliomas, respectively. The PDGF and the Ras-Akt dependence are supported by the use of a control infection with the RCAS-LacZ because the Ntv-a/LacZ cells are not malignant.

Flow cytometry, ELISA, and immunostaining analyses revealed that the mouse RCAS/Ntv-a cell model, such as the human gliomagenesis, is characterized by a decrease in the number of 5 mC as the tumor grade increases. Indeed,

the number of 5 mC in Ntv-a/PDGF cells decreased compared with Ntv-a/LacZ cells (flow cytometry, $P = 0.0074$; ELISA, $P = 0.0181$; and immunostaining, $P = 0.0133$), and the 5 mC number in Ntv-a/Ras-Akt cells decreased compared with Ntv-a/PDGF cells (flow cytometry, $P < 0.0001$; ELISA, $P < 0.0001$; and immunostaining, $P = 0.0003$; Fig. 2A). To illustrate the decrease in 5 mC observed in cells of RCAS system, we investigated the methylation status of two DNA repeat elements, *Alu* and

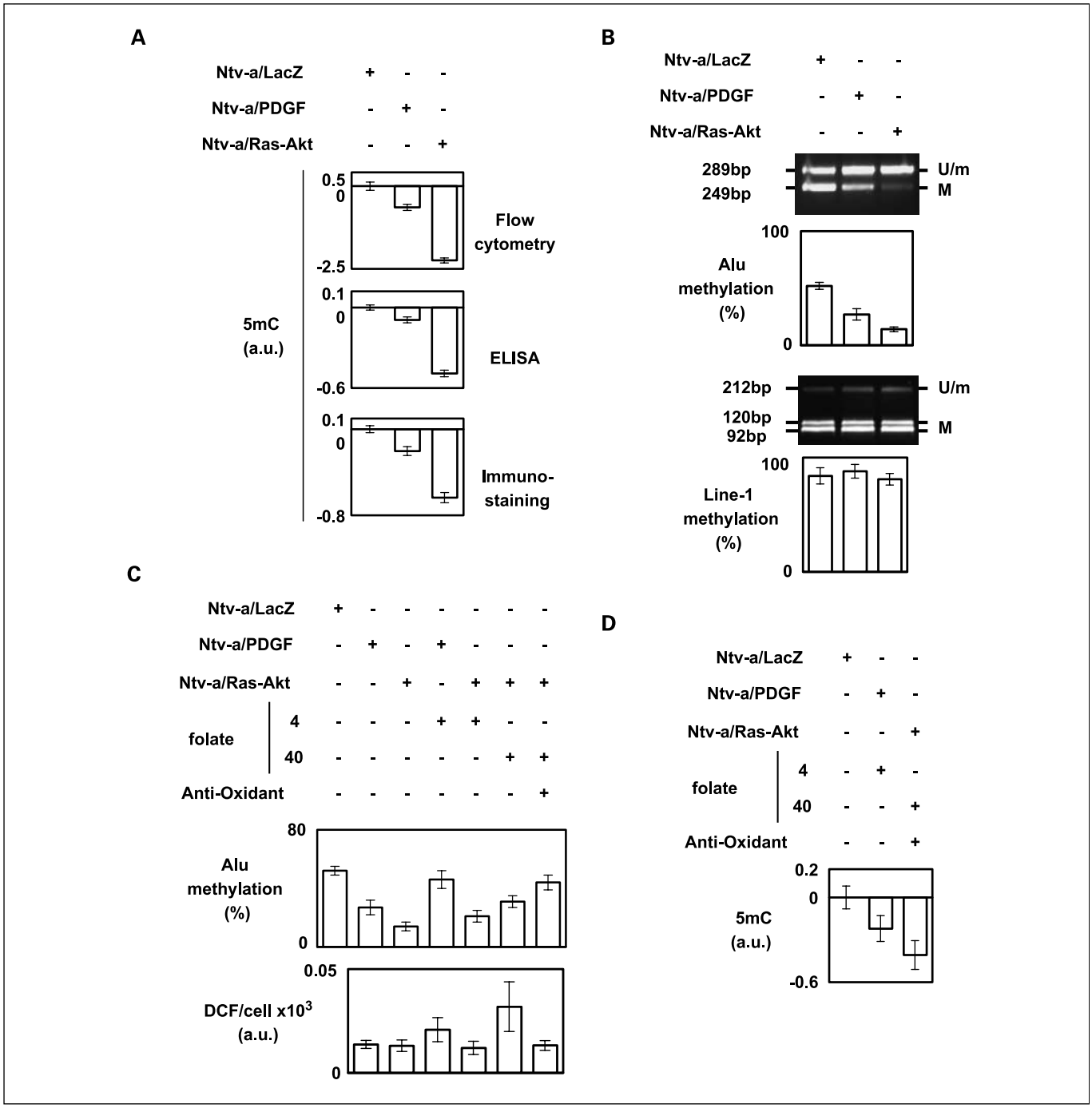

Fig. 2. Folate supplementations reverse the hypomethylation of Alu, a DNA repeat element. *A,* the 5 mC number was measured in Ntv-a cells by flow cytometry, by ELISA, and the use of the Methylamp Global DNA methylation Quantification kit (Euromedex-Epigentek) and by immunostaining assay. *B,* DNA methylation status of Alu and Line-1, two DNA repeat elements in Ntv-a cells by using Combined Bisulfite Restriction Analyses. After isolation of genomic DNA, bisulfite conversion was realized by using the EZ DNA methylation Gold kit (Zymo Research). PCR was done to amplify a pool of DNA repetitive elements. PCR products were then digested with TaqI for Alu and Line-1, which only cuts repetitive elements that were originally methylated. The Alu PCR products assays a single site digested by TaqI, generating 2 digestions products at 249/36 bp (36 bp bands are not observed in gels). The Line-1 PCR products assays one site digested by TaqI, generating two digestions products. After electrophoresis, the lower cut bands represent methylated repetitive elements (*M*), whereas the upper bands represent unmethylated or mutated repetitive elements (*U/m*). Densitometric quantification of PCR bands was done through the ImageJ program, and the methylation percentages (*m%*) are shown in graphs. *C,* effect of folate supplementations on the methylation status of Alu and the ROS production. *D,* effect of folate supplementations on global DNA methylation status. Global DNA methylation status is assessed by the use of the Methylamp Global DNA methylation Quantification kit (Euromedex-Epigentek).

LINE-1, by Combined Bisulfite Restriction analysis. This assay showed that *Alu* was hypomethylated in tumor cells compared with control cells (Ntv-a/LacZ; $P = 0.0018$, $P < 0.001$), and that the *Alu* hypomethylation increased in parallel to the tumor grade ($P = 0.0139$; Fig. 2B), whereas the methylation status of *LINE-1* remained unchanged in tumor RCAS/Ntv-a cells compared with Ntv-a/LacZ cells ($P = 0.4007$ and $P = 0.7078$). Thus, these data indicate that the DNA methylation status of each DNA repeat element did not reflect the cellular methylation status. Nevertheless, the change in the *Alu* methylation profile was similar to that of the number of 5 mC in the RCAS/Ntv-a cells. To this end, we decided to determine the minimal dose of folate needed to abrogate the DNA hypomethylation in RCAS/Ntv-a cells, in the hope that this concentration of folate could readjust *Alu* methylation status in the Ntv-a/PDGF and Ntv-a/Ras-Akt cells to that observed in Ntv-a/LacZ cells. Also, because folate has a pro-oxidative property at high dose, we combined the use of a high dose of folate with that of an antioxidative cocktail (Vitamin C + β-carotene) to abrogate the folate-induced ROS production (Fig. 2C). In Ntv-a/PDGF cells, 4 μg/mL folate (or F4 supplementation) was enough to abolish the *Alu* hypomethylation status ($P = 0.196$). Strikingly, we noted that a folate supplementation of 40 μg/mL (F40 supplementation) induced the partial remethylation of *Alu* in Ntv-a/Ras-Akt cells, whereas the *Alu* hypomethylation was abolished when these cells were treated with 40 μg/mL folate supplemented with a cocktail of antioxidant (40Ax supplementation; $P = 0.0763$). We spoke of partial remethylation for the F40 supplementation because under this condition, the remethylation of *Alu* was significantly superior to that seen in Ntv-a/RasAkt cells ($P = 0.0375$) and significantly inferior to that observed in Ntv-a/LacZ cells ($P = 0.0004$; Fig. 2C). Next, we measured the 5 mC level in Ntv-a/PDGF and Ntv-a/Ras-Akt cells treated with F4 and F40Ax supplementation to determine the effect on the cellular methylation status. ELISA results showed that both treatments partially abrogated the global DNA hypomethylation in Ntv-a/PDGF and Ntv-a/Ras-Akt cells because the 5 mC level remained inferior to that of the Ntv-a/LacZ cells ($P = 0.012$ and $P = 0.0021$) but were superior to that observed in the Ntv-a/PDGF and Ntv-a/Ras-Akt cells ($P = 0.042$ and $P < 0.0001$; Fig. 2D). These data show that two different doses of folate supplementation were able to abrogate the hypomethylation of Alu without abolishing the global DNA hypomethylation in the Ntv-a/PDGF and Ntv-a/Ras-Akt cells. This suggests that the gliomagenesis-associated aberrations of the DNA methylation machinery affect mechanisms other than those restored by the folate supplementation used in our experiments.

Folate supplementation regulates Sp1 and Sp3 recruitments on dnmt3a and dnmt3b promoters. The fact that the abrogation of Alu hypomethylation in Ntv-a/Ras-Akt cells was more efficient with the F40Ax supplementation than with the F40 supplementation suggested that folate could regulate the DNA methylation machinery through other mechanisms than that implicating SAM. To determine a putative role of folate on the DNA methylation machinery, we next hypothesized that the folate supplementation could promote a gain of methylation through the up-regulation of the *de novo* methyltransferases. Thus, we decided to analyze by semiquantitative PCR the expression levels of Dnmt3a and Dnmt3b, two major *de novo* methyltransferases. Interestingly, semiquantitative PCR analyses revealed that the treatments of Ntv-a/PDGF and Ntv-a/Ras-Akt cells with the F4 and the F40Ax supplementation, respectively, increased the expression levels of the Dnmt3a and Dnmt3b mRNA compared with that observed in Ntv-a/LacZ cells ($P = 0.0289$ and $P = 0.0356$ in Ntv-a/PDGF, and $P = 0.0101$ and $P = 0.0095$ in Ntv-a/Ras-Akt). The expression levels of the Dnmt3a and Dnmt3b mRNA remained unchanged when the Ntv-a/Ras-Akt cells were treated with F40 supplementation ($P = 0.1311$ and $P = 0.0853$; Fig. 3A).

In parallel to these experiments, we analyzed the expression levels of Dnmt3a and Dnmt3b mRNA in Ntv-a/PDGF and Ntv-a/Ras-Akt cells overexpressing Sp1 or Sp3 as Jinawath et al. (27) showed that Sp1 and Sp3 play a crucial role in the transcriptional regulation of the *dnmt3a* and *dnmt3b* genes.

After electroporation of pN3-Sp1 or pN3-Sp3 plasmids into cells, reverse transcription-PC analyses showed that the Dnmt3a and Dnmt3b mRNA expression were not significantly overexpressed in Sp1-overexpressing cells ($P = 0.452$ and $P = 0.406$), whereas both mRNA were overexpressed in Sp3-overexpressing cells ($P = 0.0084$ and $P = 0.0108$; Fig. 3B). Consistent with the fact that Sp3 can be a gene activator, our data indicated that Sp3 plays a more important role than Sp1 in the up-regulation of the *dnmt3a* and *dnmt3b* genes in Ntv-a/PDGF and Ntv-a/Ras-Akt cells (28). Thus, we hypothesized that a Sp3 predominant expression compared with the Sp1 expression induced by the folate supplementation devoid of ROS production (F4 and F40Ax) could be at the origin of the Dnmt3a and Dnmt3b mRNA overexpression. However, Western blot analyses showed no change in Sp1 and Sp3 expressions when cells were treated with the F4 and F40Ax supplementations. On the contrary, we noted that the treatment of Ntv-a/Ras-Akt cells with the F40 supplementation induced the overexpression of Sp1 without modifying the Sp3 expression (Fig. 3C). Because our results show no correlation between the Sp1 and Sp3 expression levels and the Dnmt3a and Dnmt3b mRNA expression levels, we hypothesized that the folate supplementation might modulate the Sp1 and Sp3 recruitment on *dnmt3a* and *dnmt3b* promoters. To test this hypothesis, we did ChIP asays. Products of PCR amplification revealed that the binding of Sp1 to the *dnmt3a* and *dnmt3b* promoters decreased in response to the F4 and F40Ax supplementations in the Ntv-a/PDGF and Ntv-a/Ras-Akt cells, respectively, whereas the recruitment of Sp3 to the *dnmt3a* and *dnmt3b* promoters increased under these conditions (Fig. 3D). We then observed that the F40 supplementation weakly increased the recruitment of Sp1 and reduced the Sp3 recruitment to the *dnmt3a* and *dnmt3b* promoters, compared with the quantity of Sp1 and Sp3 binding these promoters in Ntv-a/Ras-Akt cells (Fig. 3D). Consistent with what has been previously observed, our data show that the folate supplementations in absence of ROS production (F4 and F40Ax) promote the overexpression of Dnmt3a and Dnmt3b mRNA without changing the Sp1/Sp3 ratio but by promoting the Sp3 recruitment to the *dnmt3a* and *dnmt3b* promoters to the detriment of the Sp1 recruitment. Our results associated the F40 supplementation and its ROS production with the increase of the Sp1 recruitment on the *dnmt3a* and *dnmt3b* promoters to the detriment of the Sp3 recruitment, and with the relative abundance of Sp1 compared with Sp3. Thus, all these results suggest that the ROS-induced abundance of Sp1 compared with Sp3 can be at the origin of the Sp1 recruitment on the *dnmt3a* and *dnmt3b* promoters to the detriment of the Sp3 recruitment.

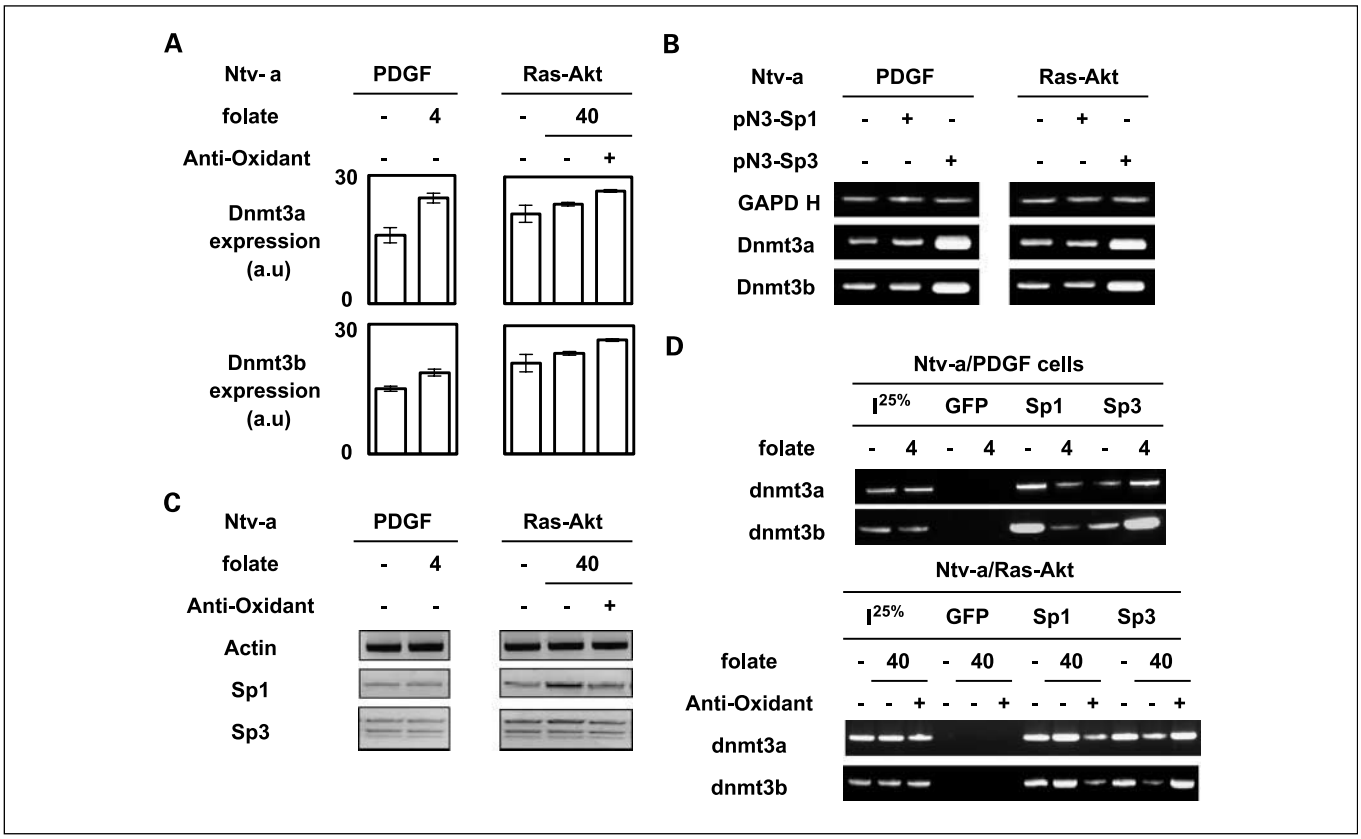

Fig. 3. Folate supplementation regulates the Dnmt3a and Dnmt3b mRNAs through the modulation of Sp1 and Sp3 recruitments on *dnmt3a* and *dnmt3b* promoters. *A,* RNA isolation is done by trizol method, and semiquantitative PCR were done according to material and methods section. *B,* expression level of Dnmt3a and Dnmt3b mRNA in Ntv-a cells overexpressing Sp1 or Sp3. Total RNA from cells transfected with pN3-Sp1 and pN3-Sp3 were amplified by semiquantitative PCR method using primers for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), Dnmt3a and Dnmt3b. *C,* effects of the F4, F40, and F40Ax supplementations on the Sp1 and Sp3 expression levels in Ntv-a/PDHG and Ntv-a/Ras-Akt cells. *D,* ChIP assays were done with the EZ ChIP kit (Millipore-Upstate) and specific antibodies directed against Sp1 (Santa Cruz sc-59) and Sp3 (Santa Cruz sc-644), and the indicated primers.

To validate this idea, the ROS production was induced in Ntv-a/Ras-Akt cells through a H_2O_2 treatment. As illustrated in Supplementary Fig. S1, Sp1, and not Sp3 expression, increased when Ntv-a/Ras-Akt cells were treated with H_2O_2. ChIP assays collaborated this observation, depicting an increase in Sp1 recruitment to the detriment of Sp3 on the *dnmt3a* and *dnmt3b* promoters. Thus, these results showed that ROS production increased the Sp1 expression and the binding of Sp1 on the *dnmt3a* and *dnmt3b* promoters, resulting in a decrease in Sp3 recruitment on both promoters. To summarize, it seems that the F40-induced ROS production is the cause of the partial remethylation of Alu shown in Fig. 2C because this dose of folate supplementation blocks the Dnmt3a and Dnmt3b augmentation by ROS-induced and Sp1-dependent mechanisms. Moreover, these data revealed that the folate supplementation devoid of ROS production (F4 and F40Ax) promoted the DNA remethylation by supplying methyl groups as well as inducing the up-expression of Dnmt3a and Dnmt3b mRNA.

Folate treatment decreases the clonogenicity and the proliferation but increases UV-induced apoptosis in Ntv-a/PDGF and Ntv-a/Ras-Akt cells. Because aberrant DNA methylation affects tumor cell biology, we focused on the effects of folate on the growth, the proliferation, and the sensitivity to apoptosis of the Ntv-a/PDGF and Ntv-a/Ras-Akt cells. The effect of folate supplementation on the proliferation of the Ntv-a/PDGF and Ntv-a/Ras-Akt cells was evaluated by using the Cell titer blue cell viability assay (Promega). The proliferation of Ntv-a/PDGF cells was significantly decreased in presence of F4 supplementation ($P < 0.0001$). A significant decrease in the proliferation was also observed when the Ntv-a/Ras-Akt cells were treated with the F40Ax supplementation ($P = 0.0024$; Fig. 4A). The effect of folate supplementation on the clonogenicity of the Ntv-a/PDGF and Ntv-a/Ras-Akt cells was also determined. The number of colonies was scored using the ImageJ program. F4 and F40Ax supplementation in the Ntv-a/PDGF and Ntv-a/Ras-Akt cells resulted in a significant reduction in the number of colonies ($P = 0.0023$ and $P = 0.0013$; Fig. 4B). Thus, all these results showed that the folate-induced methylation reduced the clonogenicity and the proliferation of glioma cells. In parallel, the assessment of apoptosis through a Trypan Blue exclusion and DEVDase assay (caspase activation assay) showed that the F4 and F40Ax supplementation increased UV-induced apoptosis in the Ntv-a/PDGF and Ntv-a/Ras-Akt cells (Fig. 4C). We then extended our studies to two human glioma cell lines (U373 and U251) and two primary cultured tumor cells obtained after mechanical dissociation of two different patient-derived glioma biopsy. As expected, this treatment diminished the proliferation and increased temozolomide-induced apoptosis in glioma cells (Fig. 5A and B). Thus, our results show that two hallmarks of tumor biology namely the high proliferation rate and the

apoptosis evasion phenotype are epigenetically regulated because both of these hallmarks were influenced by the folate-induced methylation of DNA.

We next analyzed whether the increase in the temozolomide-induced apoptosis correlated with the folate-induced methylation of *MGMT* because reports in the literature suggested that *MGMT* methylation was associated with temozolomide treatment (10, 11). Methylation-specific PCR analyses revealed that this was the case for U373, primary cultured tumor cell#1 and #2 but not U251 because these cells have a methylated *MGMT* gene in the absence of a folate supplementation (Fig. 5C). We also observed that the increase in the temozolomide-induced apoptosis in all of these cells was associated with a methylation of the *survivin* and *bcl-w* genes, both of which encoded for anti-apoptotic proteins. The choice of these genes was supported by the fact that survivin and Bcl-w overexpressions are associated with a poor prognosis in glioma patients and with highly invasive gliomas, respectively (29–31), and the *survivin* gene was frequently unmethylated in glioma (32). In parallel to this, we investigated whether the decrease in proliferation observed with a folate supplementation was associated with a methylation of the *PDGF-B* gene because the expression of this growth factor is epigenetically regulated in gliomas (15), and the PDGF-B acts as an oncogenic factor in gliomagenesis (25, 33). Methylation-specific PCR analyses showed that this was the case. Thus, our experiments indicated that the folate treatment enhanced the temozolomide-induced apoptosis and reduced the proliferation of glioma cells by increasing the DNA methylation and by promoting the methylation of specific gene among which were the *MGMT*, *survivin*, *bcl-w*, and *PDGF-B* genes.

Hypomethylation status of PDGF-B, survivin, and bcl-w genes is frequently observed in short survival time patients suffering from Glioblastoma Multiforme characterized by a high proliferation index and a low sensitivity to apoptosis. Consistent with the fact that folate-induced DNA methylation influences the proliferation and the sensitivity to apoptosis in glioma cells, we next asked whether the DNA methylation level correlated with the proliferation rate and/or the sensitivity to apoptosis in glioma cells. For this, DNA methylation was estimated by measuring the number of 5 mC using an ELISA assay, the proliferation rate was determined by immunohistochemical analyses of the Ki67 level, and apoptosis was estimated through the measure of caspase activity (as previously described ref. 12) in a collection of 53 Glioblastoma Multiforme. Ki67 and 5 mC levels were plotted against each other and a statistical analysis using the Pearson's correlation test showed a significant and inverse correlation between these parameters ($P = 0.0174$; Fig. 6A). Similar analyses also revealed a significant correlation between the caspase activity and the number of 5 mC ($P = 0.0037$; Fig. 6B). The DNA methylation level correlated with two markers of cancer capable of influencing the aggressiveness of tumors, we then determined whether the DNA methylation level was a prognostic marker of survival. Thus, 53 Glioblastoma Multiforme patients were divided into 2 groups: 27 patients with tumors having a low number of 5 mC (below the median value) were included in group A and 26 patients with tumors having a high number of 5 mC (above the median value) composed the group B. Survival curves of these two groups were established using the Kaplan-Meier method and compared using Log-rank test (Fig. 6C and Table 1). A significant difference was

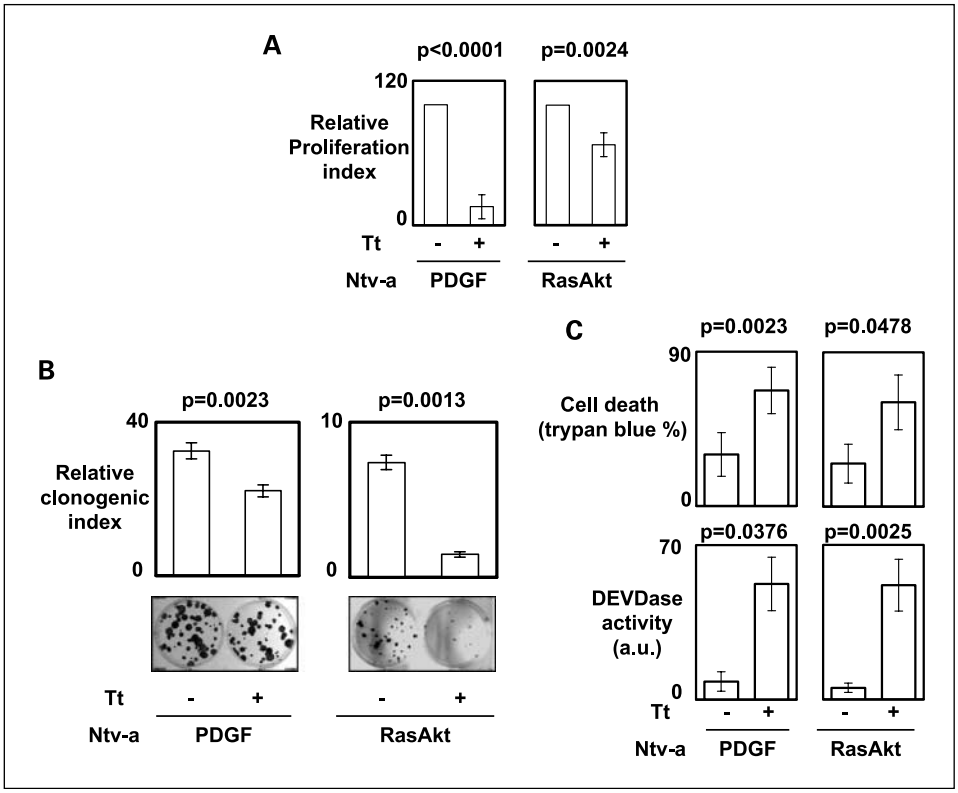

Fig. 4. Effect of folate supplementation on the invasion, the proliferation, the clonogenicity, and the sensitivity of UV- or temozolomide-induced apoptosis in mice glioma cells. *A,* proliferation index of Ntv-a cells is assessed by using the Cell Titer Blue Cell Viability Assay (Promega). *B,* effect of the F4 and F40Ax supplementations on the clonogenicity of the Ntv-a/PDGF and Ntv-a/Ras-Akt cells. *C,* apoptosis is assessed 24 h after UV induction (200 J/m^2) by measuring cell death through a Trypan Blue exclusion and by the measure of DEVDase activity according to previous protocol (40).

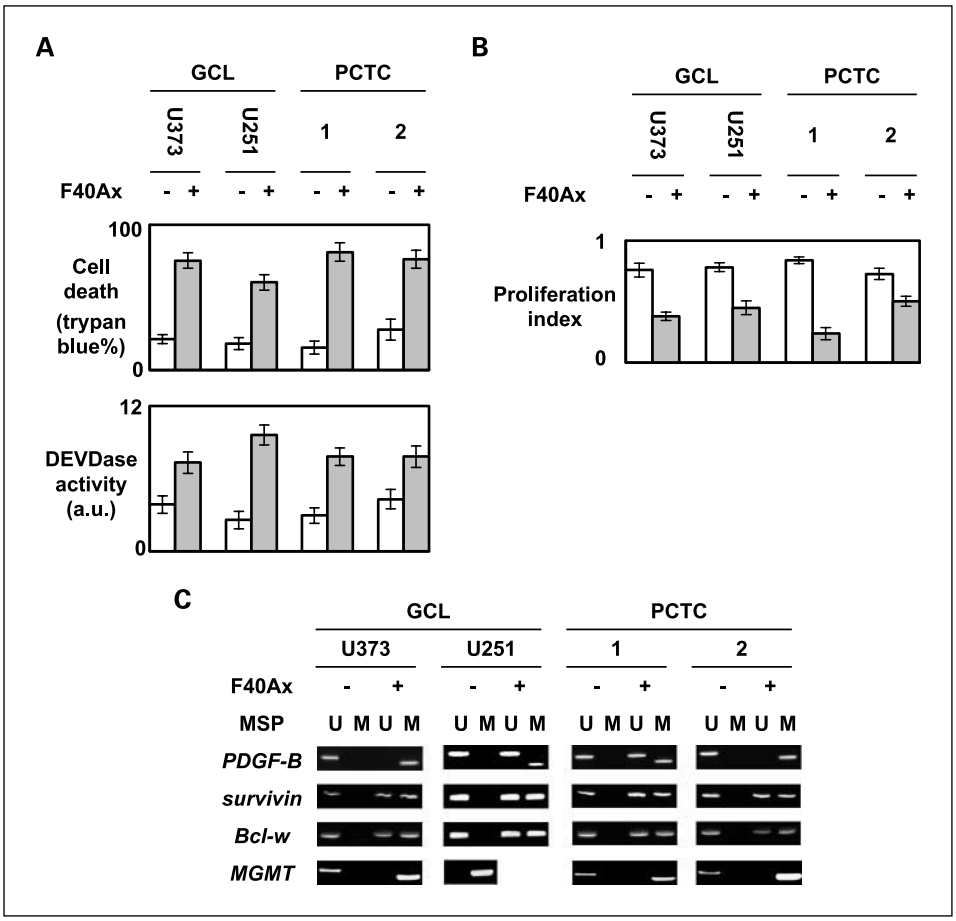

Fig. 5. Effect of folate supplementation on the invasion, the proliferation, the clonogenicity, and the sensitivity of UV- or temozolomide-induced apoptosis in human glioma cells. *A,* proliferation index of human glioma cells is assessed by using the Cell Titer Blue Cell viability Assay (Promega). *B,* apoptosis is assessed 24 h after temozilomide induction (0.2 mmol/L) by measuring cell death by Trypan Blue exclusion and by the measure of DEVDase activity according to previous protocol (40). *C,* effect of F40Ax supplementation on the methylation status of the *PDGF-B, survivin, bcl-w,* and *MGMT* genes in human glioma cells. Methylation status of these genes is evaluated by methylation-specific PCR method after bisulfite conversion of DNA.

observed between the two groups of patients ($P = 0.002$). All these results indicated that the low DNA methylation status correlated with a high proliferation, a low sensitivity to apoptosis in glioma cells, and was a poor prognostic factor in Glioblastoma Multiforme patients. Univariate and multivariate statistical analyses complemented and corroborated this point by showing that the shorter survival time associated with a low 5 mC level was not affected by established prognostic factors such as age, Karnofsky Performance Score, the *MGMT* methylation status (Supplementary Table S1; Fig. 6C). Thus, the 5 mC level could be used as an alternative prognostic factor in Glioblastoma Multiforme patients. Consistent with our study, this last point suggests that the DNA methylation level could be used as a molecular biomarker for patient stratification in antiglioma therapies conjugating temozolomide/radiotherapy treatment and folate supplementation.

Discussion

To improve therapeutic approaches in glioma patients and to better understand glioma biology, current studies have focused on epigenetic alternations associated with the development and progression of gliomas. Due to the two forms of DNA methylation alterations occurring in glioma (hypomethylation and hypermethylation), we here have chosen to investigate the effect on markers of glioma such as the rampant proliferation and the apoptotic evasion of DNA methylating agent rather than DHA because gliomas are characterized by a low DNA methylation state (8). Our data show that the folate-induced methylation involves *Alu*, a DNA repeat element weakly methylated in glioma cells (this hypomethylation has been described as a cause of chromosomal instability and tumorigenesis ref. 14), a recognized oncogenic factor in gliomagenesis: the *PDGF-B* gene and several genes associated with the increased sensitivity to temozolomide-induced apoptosis: *MGMT*, *survivin*, and *bcl-w* (Fig. 6D). In addition, we noted the folate-induced methylation promoted the methylation of *PTEN* gene but not that of *bax* (Supplementary Fig. S2). This folate-induced methylation of *PTEN* was associated with a PTEN overexpression. These findings corroborated the studies already published that there was no general correlation between *PTEN* promoter methylation and PTEN protein expression (6, 34). At the phenotypic level, the folate-induced methylation reduced the proliferation rate and increased the sensitivity of temozolimide-induced apoptosis, suggesting that the folate-induced methylation could be use as adjuvant in an antiglioma therapeutic protocol. This point is an ongoing subject in our group and the first results seem to indicate that the folate-supplemented diet in the mouse/rat model of gliomagenesis is associated with a decrease in tumor volume in tumors having a low level of 5 mC (data not shown).

At the molecular level, we have shown that a folate supplementation enhanced the DNA methylation through a Sp1/Sp3-mediated transcriptional up-regulation of genes coding for two DNA methyltransferases: the Dnmt3a and Dnmt3b. Moreover, our data indicate that Sp3 acted as an activator/enhancer of the

dnmt3a and *dnmt3b* gene expression. These results are similar to that reported by Jinawath et al. (27). Indeed, the Sp3 overexpression in HEK-293T cells significantly increased the Dnmt3a and Dnmt3b mRNA levels, whereas the Sp1 overexpression did not induce significant up-regulation of Dnmt3a and Dnmt3b mRNA. Although discussed in the literature, the transcriptional function of Sp3 has been already reported for the activation of the CD11a and CD11b promoters for example (35). Moreover, in our experiments, the enhancer role of Sp3 on the *dnmt3a* and *dnmt3b* gene expression is associated with F4 and F40Ax supplementations. To date, there are very few published observations associating folate and the modulation of DNA methylation machinery. Ghoshal et al. (36) have shown that folate and a methyl-deficit diet altered the expression of Dnmt, whereas Piyathilake et al. (37) reported that a mandatory fortification with folic acid was associated with an increased expression of Dnmt1 in the cervix. However, our data are the first to show that the folate-induced regulation of *dnmt3a* and *dnmt3b* results from the binding and the expression of Sp1 and Sp3.

Among the molecular determinants, which could explain the folate-induced decrease in proliferation is the methylation of the *PDGF-B* because the epigenetic regulation of PDBF-B gene dictates the oncogenic activity of TFGβ in proliferation in gliomas (15). Moreover, the fact that PDGF-B is clearly implicated in gliomagenesis and that folate induces its methylation reinforces the idea to use folate supplementation as adjuvant in antiglioma therapy (25, 38). We have identified that the folate-induced methylation of *survivin* is also a molecular determinant that could explain the decrease in proliferation and the gain in sensitivity in apoptosis in glioma cells because many literature reports have suggested that survivin promotes cell division through the control of the checkpoint in the G_2-M -phase of cell cycle and inhibits apoptosis through its interaction with caspase-3 (39). Moreover, our

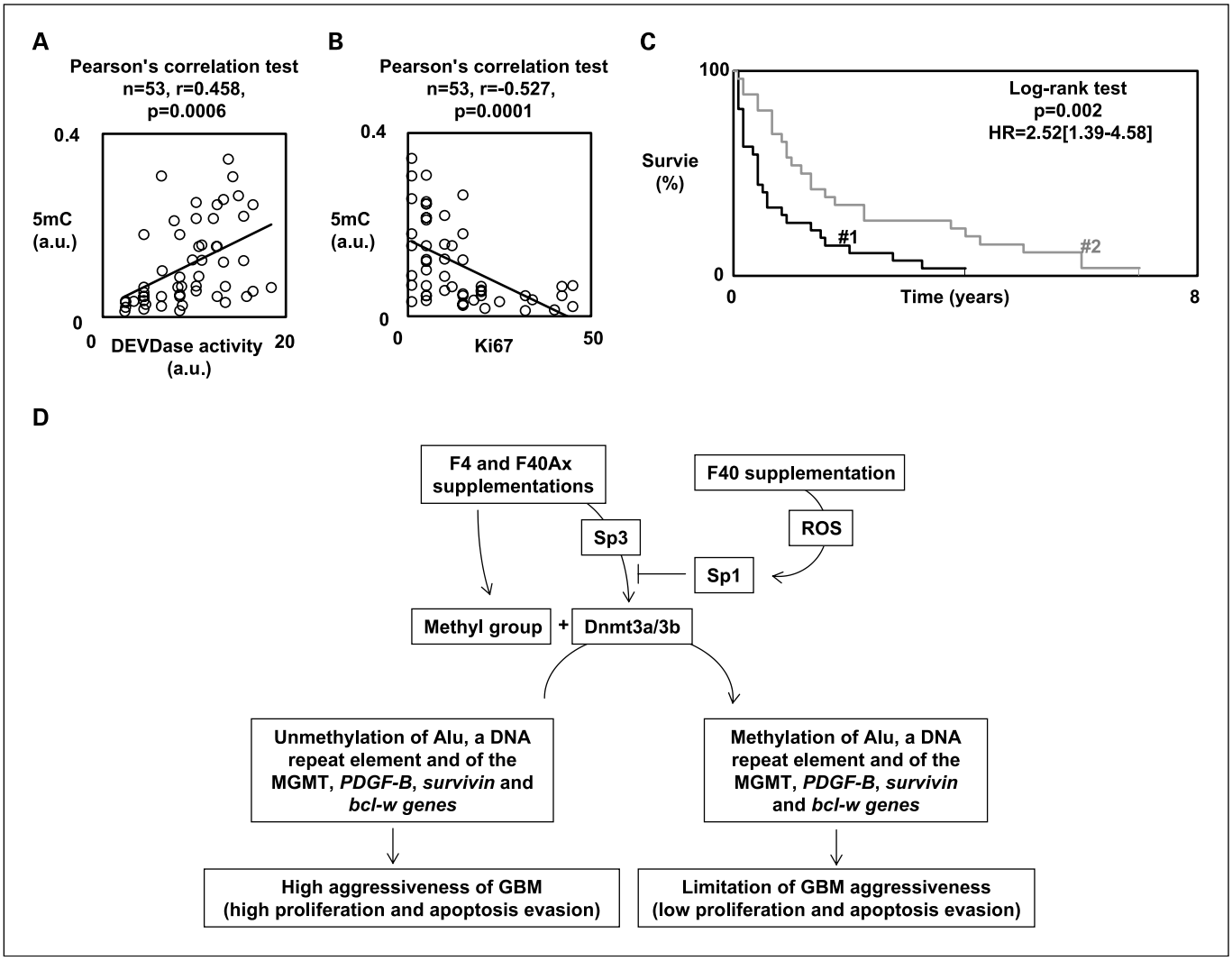

Fig. 6. Low level of 5 mC is a poor prognosis factor for survival time in GBM patients. *A,* correlation between the 5 mC number obtained by the use of the Methylamp Global DNA methylation Quantification kit (Euromedex-Epigentek) and the DEVDase activity obtained as previously described. *B,* correlation between the 5 mC number obtained by the use of the Methylamp Global DNA methylation Quantification kit (Euromedex-Epigentek) and the Ki67 rate obtained from immunohistochemical staining of human glioma sections. *C,* estimation of survival curves by Kaplan-Meier method and comparison by Log-rank test. *D,* the F4 and F40Ax supplementations induce the remethylation of Alu, a DNA repeat element, and of the *PDGF-B, survivin*, and *bcl-w* genes limiting the GBM aggressiveness. The remethylation is effective through the folate-induced contribution of methyl group and also through the Sp3-induced transcriptional up-regulation of Dnmt3a and Dnmt3b. On contrary, the F40 supplementation and/or the ROS production inhibit the transcriptional up-regulation of Dnmt3a and Dnmt3b through Sp1.

Table 1. Comparison of survival curves by log-rank test

	Low 5 mC (Group A, *n* = 27)	High (Group B, *n* = 26)	*P*
Age (y)	56 (49-65)*	57 (49-65)*	0.367
KPS	80 (80-90)*	80 (80-90)*	0.188
KSP (80/90)	15/12	19/7	0.254
Sexe (M/F)	17/10	13/13	0.412
MGMT (U/M)	18/9	12/14	0.170

*Median (range).

work corroborates the fact that the folate-induced methylation decreases the apoptosis evasion phenotype of glioma cells by demonstrating that folate treatment promotes the methylation of genes coding for the antiapoptotic protein Bcl-w. Although not being a gene coding for an antiapoptotic protein, similar observation also prevails for the folate-induced methylation of *MGMT* gene. Nevertheless, we have noted that the folate-induced methylation of *survivin* and *bcl-w* gene in cells presenting a methylated *MGMT* gene is also associated with a gain in temozolomide-induced apoptosis (case illustrated by the U251 cells), suggesting that the folate-induced methylation of genes other than *MGMT* play a crucial role in the gain of temozolomide-induced apoptosis in glioma cells treated with folate.

To conclude, our work identifies the use of folate as a promising alternative resource to the use of DHA as an anticancer treatment including an epigenetic-based adjuvant. Consistently with our results, this opens a new door to a better management of Glioblastoma Multiforme because the folate-induced methylation seems to be an efficient tool (*a*) to limit the low level of DNA methylation, a biomarker conferring poor prognosis in Glioblastoma Multiforme patient; and (*b*) to promote the *MGMT* methylation, a biomarker associates with a benefit from temozolomide treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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