Genome-wide Hypomethylation in Human Glioblastomas Associated with Specific Copy Number Alteration, Methylenetetrahydrofolate Reductase Allele Status, and Increased Proliferation

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Abstract

Genome-wide reduction in 5-methylcytosine is an epigenetic hallmark of human tumorigenesis. Experimentally induced hypomethylation in mice promotes genomic instability and is sufficient to initiate tumorigenesis. Here, we report that global hypomethylation is common in primary human glioblastomas [glioblastoma multiforme (GBM)] and can affect up to an estimated 10 million CpG dinucleotides per haploid tumor genome. Demethylation involves satellite 2 (Sat2) pericentromeric DNA at chromosomes 1 and 16, the subtelomeric repeat sequence D4Z4 at chromosomes 4q and 10q, and interspersed Alu elements. Severe hypomethylation of Sat2 sequences is associated with copy number alterations of the adjacent euchromatin, suggesting that hypomethylation may be one factor predisposing to specific genetic alterations commonly occurring in GBMs. An additional apparent consequence of global hypomethylation is reactivation of the cancer-testis antigen MAGEA1 via promoter demethylation, but only in GBMs and GBM cell lines exhibiting a 5-methylcytosine content below a threshold of ~50%. Primary GBMs with significant hypomethylation tended to be heterozygous or homozygous for the low-functioning Val allele of the rate-limiting methyl group metabolism gene *methylenetetrahydrofolate reductase* (*MTHFR*), or had a deletion encompassing this gene at 1p36. Tumors with severe genomic hypomethylation also had an elevated proliferation index and deletion of the *MTHFR* gene. These data suggest a model whereby either excessive cell proliferation in the context of inadequate methyl donor production from *MTHFR* deficiency promotes genomic hypomethylation and further genomic instability, or that *MTHFR* deficiency-associated demethylation leads to increased proliferative activity in GBM. (Cancer Res 2006; 66(17): 8469-76)

Introduction

Glioblastoma multiforme (GBM) is the most common primary brain tumor in adults and is characterized by a median survival of ~1 year for newly diagnosed cases (1). Although the role of genetic mechanisms in oncogene activation and tumor suppressor inactivation in GBM is well documented (2), very little is known about the epigenetic component of this disease. Localized hypermethylation of gene-associated CpG islands and a more extensive genome-wide reduction in 5-methylcytosine are epigenetic alterations that typify many cancers (3–5).

Methylation is the only known covalent modification of genomic DNA in humans and occurs at cytosines followed by guanines (CpG). CpG islands are ~1 kb on average and contain a 5-fold excess of CpGs relative to the rest of the genome. The estimated 30,000 CpG islands comprise ~7% of all CpGs (6), most of which are unmethylated in normal tissues. Normally methylated sequences include CpG islands associated with the inactive X chromosome and some imprinted and tissue-specific genes, as well as non-CpG island sequences such as juxtacentromeric DNA, intragenic regions, and transposon sequences. Aberrant methylation of some CpG islands in cancer has been associated with silencing of tumor-suppressor genes (7). In GBMs, aberrant methylation in the promoter of the *O6-methylguanine-DNA methyltransferase* gene has been associated with significantly longer survival in patients treated with radiation and the alkylating agent temozolomide (8). This and other studies underscore the importance of addressing the changes in the DNA methylation landscape in GBM and their effect on brain tumorigenesis.

Global genomic hypomethylation has been documented for multiple malignancies compared with matching normal tissues (9–11), and is associated with tumor progression in a mouse model (12). Three mechanisms by which hypomethylation contributes to malignancy have been proposed, including oncogene activation, loss of imprinting, and promoting genomic instability via unmasking of repetitive elements. Experimental support for a role of hypomethylation in driving tumorigenesis via genomic instability and an elevated rate of mitotic recombination is derived from studies of mice carrying hypomorphic alleles of DNA methyltransferase 1 (*DNMT1*; ref. 13). These mice develop aggressive lymphomas with high penetrance, and the tumors are commonly characterized by trisomy of chromosome 15. Similarly, global DNA hypomethylation achieved through reduced DNMT1 function accelerates the onset of tumor formation in a p53 and NF1 mutant mouse model of sarcoma (14). Also, the removal of DNMT1 in murine embryonic stem cells increases mutation rates and the incidence of aneuploidy at reporter genes (15), whereas double knockout of DNMT1 and DNMT3b in the near diploid HCT116 colorectal cancer cell line results in dramatic hypomethylation and genomic instability manifested by chromosomal translocations (16). Loss of imprinting generated through transient deficiency of DNMT1 activity during murine development leads to formation of a spectrum of solid tumors and hematologic cancer (17). These studies indicate that hypomethylation can cause genomic instability and initiate tumorigenesis.
The demethylation of repetitive elements, which comprise ~45% of the human genome, accounts for the majority of 5-methylcytosine loss in human cancers (9). Repetitive elements consist of tandem repeats of simple or complex sequences as well as interspersed repeats derived from transposable elements, such as the short interspersed nucleotide element Alu and the long interspersed nucleotide element LINE-1, which comprise ~10% and ~20% of the human genome, respectively (18). The tandem repeat satellite 2 (Sat2) DNA located at the juxta centromeric region of chromosomes 1 and 16 (Chr1 and Chr16), as well as the non satellite D4Z4 repeats located at the sub telomeric region of Chr4q35 and Chr10q26, are also targets for pronounced demethylation in several cancers (19–21), in addition to lymphoblast and fibroblast cell lines derived from patients with the immunodeficiency-centromeric instability-facial anomalies (ICF) syndrome, which usually involves germ-line mutations in DNMT3B (22). Breakage events at Chr1 also occur in the ICF cells, but only after stimulating the cells to divide. This suggests a possible model whereby the hypomethylated state followed by cell division lead to chromosome breakage in ICF syndrome. In GBM, however, the genomic locations affected and the potential contribution of hypomethylation to the genetic alterations characteristic of GBMs are unknown.

There is also correlative evidence from human tumors, suggesting that repeat-specific DNA demethylation may cause structural alterations of adjacent chromosomal regions. For instance, chromosome 1 pericentromeric rearrangements and Sat2 DNA hypomethylation are frequent in ovarian epithelial carcinomas (23). These studies suggest hypomethylation may lead to, or be caused by breakage events. Likewise, q-arm gain of Chr1 in hepatocellular carcinoma correlates with satellite DNA hypomethylation (24). Taken together with the data from ICF syndrome and DNMT-deficient cells, however, it seems most likely that hypomethylation precedes rearrangements.

Cancer-associated hypomethylation affecting single-copy genes was first reported in the gene body of Ras oncogenes (4). Recent examples of genes marked by aberrant promoter hypomethylation and up-regulated expression that might contribute to tumorigenesis have also been reported (25). MAGEA1, which belongs to a broad category of genes commonly called cancer-testis antigens, is also demethylated and its expression is activated in tumors. MAGEA1 activation correlates with genome-wide hypomethylation in tumors and tumor cell lines (26). Whether this mechanism also underlies MAGEA1 expression in GBM has not been reported.

The cause of hypomethylation in cancer is unknown. Because no mutation in DNMT genes has been reported in cancer, impaired DNMT function and altered DNA methylation levels may occur through perturbations in the production of the universal donor of methyl groups, S-adenosyl-1-(methyl-3H)methionine (15.0 Ci/mmol; GE Healthcare, Piscataway, NJ) using either M.SssI or dam methylases, respectively. M.SssI methylase catalyzes the transfer of a methyl group from SAM to cytosine residues within CpG dinucleotides; whereas dam methylates the adenine residue within GATC sequences. The percentage of methylated CpG was calculated from the ratio between M.SssI and dam methylase 3H incorporation (SssI/dam ratio) as follows: percentage of methylated CpG = [1 – (SssI/dam ratio / 3.2)] × 100, where 3.2 corresponds to the estimated frequency of the dam recognition site (1 of 256), compared with that of the M.Sss recognition site (1 of 80; ref. 26).

Immunohistochemistry. Five-micrometer paraffin sections of formalin-fixed tissues were mounted on poly-l-lysine-coated glass slides. Tissues were deparaffinized, fully hydrated through graded ethanol, and incubated in methanol with 0.5% H2O2 for 30 minutes at 22°C to block endogenous peroxidase activity. The sections were then processed by heating with a 750-W microwave for 25-minute intervals in 10 mmol/L citrate buffer (pH 6). Primary mouse monoclonal antibodies (Ki-67, MIB-1 clone; Dako A/S Carpinteria, CA) were applied (1:100) and allowed to react for 1 hour at 37°C. Visualization of the primary antibody used SuperPicture-Plus Polymer Mouse Detection horseradish peroxidase-3,3′-diaminobenzidine (Zymed, South San Francisco, CA) with a Mayer’s hematoxylin counterstain. To calculate the nuclear labeling index for Ki-67 ~900 to 1,700 total nuclei were examined for each tissue section in several regions with the highest labeling. The labeled nuclei were scored as a percentage (labeling index) of the total nuclei examined. Additional methods are available at Cancer Research online (Supplementary Data).

Results

Global DNA hypomethylation in human primary GBMs and glioma cell lines. The genomic 5-methylcytosine content of 10 primary GBMs (WHO grade 4 astrocytoma) and six glioma cell lines was measured by methyl acceptance and compared with values obtained for normal adult brain. The presence of slightly more elevated 5-methylcytosine levels in brain relative to PBLs (mean brain = 76.5%, mean PBLs = 72.8%) served as an indication of the reliability of this assay, because earlier 5-methylcytosine measurements using the sensitive high-performance liquid
chromatography technique revealed a similar relationship between the DNA methylation content of these tissues (34).

Genome-wide hypomethylation was observed in 8 of 10 primary tumors, two of which (GBM28 and GBM30) had a more dramatic reduction in 5-methylcytosine levels with values <50% (Fig. 1A). Assuming 30 million CpG per haploid genome (6), we estimate that 10.3 million CpGs are demethylated in GBM28 and 8.2 million CpGs were demethylated in GBM30. On average, the global 5-methylcytosine content of cultured glioma cell lines was more reduced than that of hypomethylated primary GBMs (mean GBMs = 59.8%, mean glioma cell lines = 48.4%; t test P = 0.01; Fig. 1B), a pattern that was previously noted for tumor cell lines of the breast, colon, and lung relative to their primary tumor counterpart (10).

Hypomethylation of juxtacentromeric, subtelomeric, and interspersed repetitive sequences in GBMs. More than one third of DNA methylation in normal tissues occurs in repetitive elements (6). The juxtacentromeric Sat2 DNA region of Chr1 and Chr16, the non-satellite D4Z4 subtelomeric repeats predominantly located at Chr4q35 and Chr10q26, and interspersed Alu elements have been shown to be demethylated in several cancers, but their methylation status in gliomas or normal brain is not known. Using bisulfite sequencing analyses, we found that DNA methylation content of Sat2 was higher than that of D4Z4 repeats in brain (range %5mC: 90-91% and 72-79%, respectively), whereas interspersed Alu element paralleled severe genomic hypomethylation in one of two GBMs (51% in GBM30; 10% in GBM28; Fig. 2C). It is of interest to consider the effect of Alu demethylation on the global levels of 5-methylcytosine in light of its considerable abundance in the human genome. On average, 36.7% of CpG dinucleotides have not undergone sequence drift or spontaneous deamination according to our bisulfite sequencing analyses. If we take into account that there are 22 CpG dinucleotides in the consensus Alu repeat (Genbank accession no. U14567) and there are ~1 to 1.4 million copies of Alu per haploid genome, then it can be estimated that Alu repeats contribute ~8 to 11 million CpGs to DNA methylation in the human genome. A reduction of 51% in GBM30 would be estimated to include loss of methylation at 4 to 5 million CpGs. Demethylation of other abundant interspersed repetitive elements, such as the long interspersed nucleotide elements LINE-1, may also contribute extensively to genomic hypomethylation in some GBMs.

Sat2 demethylation is associated with copy number alterations of adjacent euchromatin. We investigated whether satellite repeat DNA hypomethylation was associated with copy number alteration of nearby genomic sequences using array comparative genomic hybridization (aCGH) analysis. Concomitant with Sat2 hypomethylation, a gain in copy number spanning 1.6 Mb (RP11-29M22 to RP11-192J8; Ip11-12, UCSC May 2004 freeze), and a deletion of ≥35.3 Mb (RP11-29M22 to RP11-25E16; Ip11-22, UCSC May 2004 freeze) were noted in the pericentromeric region of chromosome 1p for severely hypomethylated GBM28 and GBM30, respectively (Fig. 2B). Additionally, a Chr1q copy gain with a 1q12 breakpoint was found in GBM28. For GBM30, copy number in the 1q12 region was not interpretable. Interestingly, no alteration was detected in the pericentromeric region of Chr1 for GBM19 and GBM27, which had only small decreases in Sat2 methylation (Fig. 2A). Cumulatively, these observations lend support to the proposal that classic satellite DNA hypomethylation predisposes to chromosomal breakage and specific copy number alteration at this region of chromosome 1 in GBMs.

MAGEA1 promoter demethylation and gene expression in extensively hypomethylated GBMs and glioma cell lines. We examined whether a relationship exists between MAGEA1 expression and the extent of genomic hypomethylation in primary
GBMs and cultured glioma cell lines. MAGEA1 expression was silenced in normal brain, but found to be reactivated in two tumors that exhibited the most dramatic genome-wide demethylation (Fig. 3A). Also, MAGEA1 was reactivated at a higher frequency in glioma cell lines relative to their primary tumor counterparts (Fig. 3B), coinciding with the more common occurrence of global hypomethylation in glioma cell lines (Fig. 1B). Interestingly, both primary GBMs and cultured glioma cells that were positive for MAGEA1 expression were segregated from nonexpressing tumors by exhibiting a global 5-methylcytosine threshold of $f_{50\%}$ or less (the mean of MAGEA1+ and MAGEA1/C0 samples were significantly different, $P = 0.003$; Fig. 4).

To determine whether DNA methylation exerted a negative influence on MAGEA1 expression, nonexpressing glioma cell lines were exposed to the demethylating agent 5-aza-2'-deoxycytidine. In each cell line examined, MAGEA1 expression was induced upon drug treatment, but remained silenced in corresponding mock-treated cells (Fig. 3C). Because promoter methylation of MAGEA1 is a primary mechanism silencing this gene in somatic tissues (35), we next evaluated whether MAGEA1 reactivation in GBM was coupled with demethylation of its promoter. Although MAGEA1 silencing in normal brain and GBMs was associated with significant methylation at critical CpG sites (35), the extent of demethylation at these CpG sites was paralleled by the degree of MAGEA1 expression in hypomethylated GBMs (Fig. 3D). For example, the more abundant expression of MAGEA1 detected in GBM30 relative to GBM28 correlated with a more extensive demethylation of the MAGEA1 promoter in GBM30 (Fig. 3A and D). Similarly, MAGEA1 expression was higher and promoter demethylation more pronounced in glioma cell lines LN229 and LN18 relative to LN319 (Fig. 3B and D).

Reduced global 5-methylcytosine content is associated with the presence of the low-functioning Val allele of MTHFR. We screened for the low-functioning C667T variant of MTHFR, a
rate-limiting enzyme in the production of the universal methyl donor, SAM, in primary GBMs and glioma cell lines using PCR followed by RFLP analysis (28). In primary GBMs, genotype frequencies were 30% for C/C homozygous (Ala/Ala), 30% for C/T heterozygous (Ala/Val), and 40% for T/T homozygous (Val/Val) MTHFR (Fig. 5A). A similar distribution of the MTHFR genotypes was observed among glioma cell lines (Fig. 5B). The MTHFR gene is located on chromosome 1p36, in a region previously shown to exhibit deletion in a proportion of GBMs (2). Among the 10 GBMs, GBM24, GBM28, GBM30, and GBM31 displayed a clear allelic loss of the region encompassing MTHFR (Supplementary Fig. S1). Normal tissue from these patients was not available, precluding the determination of the original MTHFR genotype. The frequency of the Val/Val genotype for MTHFR is ~10% in the general population (36). The higher than expected prevalence (40%) of apparent homozygosity for the low-functioning allele of MTHFR in primary GBM results in part from loss of heterozygosity (LOH) at Chr1p36 in GBM24 and GBM28.

Notably, MTHFR allelic loss occurred in GBMs displaying significant degrees of genomic hypomethylation, including the two most extensively hypomethylated tumors, highlighting a potentially important requirement of normal MTHFR activity in the maintenance of normal DNA methylation levels. This is reinforced by the observation that the only tumor with an Ala/Ala genotype (GBM19) showed no reduction in total 5-methylcytosine, whereas hypomethylated tumors tended to carry at least one copy of the lower functioning (Val) allele for MTHFR or LOH in the MTHFR region. Similarly, the presence of the Val allele was associated with a more profound reduction in global 5-methylcytosine content in three of four glioma cell lines (Figs. 1B and 5B). These results suggest that the constitutional MTHFR genotype, and modifications to it via distal Chr1p loss in the tumor may be one cause of hypomethylation in GBM.

GBMs with severe genomic hypomethylation and LOH of MTHFR exhibit a very high proliferation index. A distinguishing feature of GBMs is their high rate of proliferation relative to lower grade gliomas. Using MIB-1 immunoreactivity as an indicator of the proliferative index of GBM, all 10 GBM samples exhibited a significant fraction of mitotically active cells. Relative to the two GBMs without hypomethylation and the six with moderate hypomethylation, the two severely hypomethylated GBMs had a much higher mitotic index (33.9% for GBM28 and 69.1% for GBM30; Fig. 6). These two highly proliferative GBMs also had LOH for MTHFR. These data suggest a model whereby reduced MTHFR activity could cooperate with enhanced proliferation to exacerbate genomic hypomethylation and chromosome breakage events.

Discussion

Genome-wide hypomethylation is a hallmark of many cancers and is thought to contribute to tumorigenesis independently of CpG island hypermethylation (9, 33, 37). Expanding on early observations from a very limited number of gliomas (5, 38), we found that genomic hypomethylation occurs at a high frequency in GBM and GBM cell lines. Interestingly, global hypomethylation was not observed in two primary GBMs, indicating that a small proportion of GBMs may arise in the absence of extensive demethylation, although these two tumors exhibit clear differences in genetic and proliferative characteristics relative to severely hypomethylated tumors. However, the whole-genome approach to quantify DNA methylation is not sufficiently sensitive to detect potentially important regional reductions in 5-methylcytosine that can contribute to genomic instability. The high prevalence of genomic hypomethylation in GBM supports an important role for 5-methylcytosine reduction in GBM pathology.
Global decreases in methylation correlate with advancing age (39) as does overall cancer incidence. The extent of the age-related decrease seems to be significantly less than that observed in tumors. The extent of global DNA methylation changes in aging brain, if any, is not known. We did not observe a relationship between the extent of genomic hypomethylation and age of the patients with GBM, suggesting that hypomethylation is primarily a feature of the GBM rather than aging.

Several observations lend support to the proposal that regional chromosomal rearrangements may be attributable to repeat-specific hypomethylation in GBM. First, there was a trend between the methylation status of the juxtacentromeric Sat2 DNA repeat region and global DNA methylation levels in GBM, similar to that reported in cancers of the breast (20), liver (24), ovary (37), and in Wilms’ tumors (9). Second, severe demethylation of Sat2 in two GBMs was accompanied by copy number alterations defined by apparent breaks precisely next to the centromere-adjacent region of Chr1p, whereas this region was of normal copy number in tumors with marginal decreases in Sat2 methylation. Last, similar to that previously noted in human hepatocellular carcinoma (24), a Chr1q copy gain with a 1q12 breakpoint was also found in one GBM, which correlated with Sat2 hypomethylation in this tumor. In interpretation of the Sat2 methylation status and Chr1 copy number alterations, it should be noted that we cannot distinguish the Sat2 elements of Chr1 from those of Chr16 using the bisulfite assay. Other categories of chromosomal rearrangements might be relevant to hypomethylation-induced chromosome breakage, but are not detectable by aCGH, such as inversions and translocations. In contrast to Sat2 demethylation, alteration of the size and/or methylation status of D4Z4 repeats are thought to give rise to altered gene expression of nearby genes, rather than leading to chromosome breakage events (40). One must also consider the proposed model of hypomethylation-induced chromosome breakage in the larger context of know genetic alterations, such as defective p53 or RB pathways, that themselves drive additional chromosomal instability.

Unlike Sat2, demethylation of the D4Z4 repeat did not occur in GBMs with normal global DNA methylation levels, and was manifested to a lesser extent than Sat2 in severely hypomethylated GBMs. The distinct behavior of Sat2 and D4Z4 methylation patterns in GBM suggests that either regional or sequence-specific determinants influence the propensity for demethylation of repeat regions in cancer. It will be interesting to determine whether differential methylation between distinct classes of repetitive elements occurs in other tissues, and whether these are under the influence of sequence-specific determinants because of the important consequences these differences could bear on the manifestation of cell type-specific patterns of genomic instability resulting from genomic hypomethylation in cancer.

The level of MAGEA1 expression in GBM correlated with the level of demethylation at key CpG sites within its promoter, indicating that promoter methylation is a primary mechanism for silencing MAGEA1 in brain. It was recently shown that Brother of the Regulator of Imprinted Sites (BORIS) actively promotes demethylation/derepression of MAGEA1 (41) by competing with its paralog CTCF for binding in a methylation-insensitive manner in the promoter region of this gene. Like MAGEA1, BORIS expression is restricted to hypomethylated germ cells during spermatogenesis in conjunction with CTCF silencing, and is aberrantly reactivated in various cancers (42). It is, therefore, possible that MAGEA1 expression in GBM is not a random consequence of severe genome-wide hypomethylation, but may result from epigenetic modifications targeted by CTCF/BORIS-binding sequences. It will be interesting to test whether aberrant BORIS expression occurs in GBM in association with progressive genome-wide hypomethylation or itself leads to genome-wide hypomethylation in brain cancer. This proposed scenario might explain a threshold effect for the reactivation of the downstream target MAGEA1, although this remains to be determined.

The expression of cancer-testis antigens are being exploited for the development of immunologically based therapy in cancer.

**Figure 4.** Aberrant MAGEA1 expression in primary GBMs and glioma cell lines segregates with a global hypomethylation threshold below ~50%. Conventional RT-PCR analysis reveals that the normally silenced MAGEA1 gene is reactivated in primary GBMs and established glioma cell lines that contain an ~50% or less 5-methylcytosine content, suggesting a global hypomethylation threshold associated with the derepression of this cancer-testis antigen. Note that the percentage of 5-methylcytosine content of normal brain was not included in the calculation of the mean value (horizontal bar) in MAGEA1-negative samples.

**Figure 5.** Genotype of the MTHFR C677T polymorphism in primary GBMs and glioma cell lines. Restriction enzyme analysis of the 198 bp PCR product from genomic DNA amplification containing the C677T polymorphism that leads to the Ala→Val change in MTHFR. The C677T substitution creates a HinfI recognition sequence, which digests the 198 bp PCR product into 175 and 23 bp fragments; the latter fragment has been run off the gel.

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including MAGEA1 in GBM (43). However, despite its expression in ~40% of primary cultures of human GBM and glioma cell lines (43), MAGEA1 mRNA was not detected in 20 noncultured primary GBMs previously (44) nor in 8 of 10 primary GBMs in our study. Our study now shows that promoter hypomethylation is one likely mechanism of MAGEA1 reactivation in GBM. These findings reinforce the proposal that a DNA demethylating agent may allow for enhanced sensitivity to immunotherapy against MAGEA1 and be of benefit for the clinical management of GBM (43). In fact, a similar strategy was recently shown to confer responsiveness of GBM cells to tumor-necrosis factor-related apoptosis-inducing ligand-induced death through reactivation of caspase-8 (45).

There seems to be an important contribution of MTHFR allelic status to genomic hypomethylation in GBM. First, the low functioning (Val) allele of MTHFR tended to be present in primary GBMs and glioma cell lines that displayed varying degrees of genomic hypomethylation. Conversely, there was no global demethylation in a GBM that was homozygous for the normal (Ala) MTHFR allele. Second, aCGH indicated a reduction in copy number of Chr1p in a region that encompasses MTHFR in four hypomethylated GBMs, including two tumors with the most severe reduction in total 5-methylcytosine, supporting a protective role for normal MTHFR activity against genomic demethylation. It is important to note that the deletions involving MTHFR are not contiguous with the copy number alterations near Sat2 on the same chromosome, suggesting they arise from at least two different breakpoint events. Similarly, the presence of either the Val/Val and Ala/Val genotypes of MTHFR were found to be strongly associated with a low content of 5-methylcytosine in the DNA of both tumors and adjacent normal tissue of the patients with either colorectal, breast, or lung cancer (12). Taken together, these observations suggest an early influence of MTHFR status on susceptibility to progressive hypomethylation that may lead to or exacerbate chromosomal instability in GBM.

A manifestation of the Val allele type of MTHFR upon genomic hypomethylation is presumed to occur through reduced SAM production and is contingent upon limited nutritional folate and vitamin B6 bioavailability (27). Interestingly, all cultured glioma cell lines exhibited significant hypomethylation irrespective of MTHFR status despite their growth in supraphysiologic levels of all methyl group donors. Unlike normal fibroblasts, genome-wide demethylation in GBM may not be reversible by nutritional supplementation either due to the presence of genetic alterations that disrupt the normal activity of DNMTs or polymorphisms in other key enzymatic components of the methyl group metabolism pathway, such as methionine synthase and cystathionine β-synthase (12), which could synergize with reduced MTHFR activity. Alternatively, chronic exposure to exaggerated levels of micronutrients—for example, the >1,000-fold excess of folic acid in GBM culture medium relative to normal serum levels—may lead to a down-regulation of genes that enable the capacity of the cell to adequately use these micronutrients.

Interestingly, a more elevated mitotic index observed in the two severely hypomethylated GBMs was accompanied by Chr1p deletion spanning MTHFR in these tumors. A clear inverse relationship was observed between elevated proliferation and genomic hypomethylation in cultured PBL (33). Similarly, a high frequency of Sat2 hypomethylation and Chr1 pericentric rearrangements are also observed in mitogen-stimulated lymphocytes isolated from ICF patients with DNMT3B mutations (22), and increased proliferative activity and DNA hypomethylation co-occur in the rectal mucosa of patients with ulcerative colitis (46). Whether genomic hypomethylation leads to a deregulated cell cycle in cancer or tumor-associated proliferation drives hypomethylation is not known. We hypothesize that cells that are deficient for MTHFR activity are unable to sustain adequate levels of SAM necessary for maintaining normal genomic DNA methylation in instances of increased cellular proliferation, and that these cells gradually accrue genomic instability as a consequence of progressive hypomethylation. This model predicts that genomic hypomethylation will be less frequently encountered in lower grade gliomas, which are characterized by a lower proliferative index than GBMs despite the high incidence of Chr1p deletion encompassing MTHFR in oligodendrogliomas.

Experimental hypomethylation shifts the spectrum of tumor types induced by genetic events in mice. Pioneering studies showed that DNA hypomethylation strongly suppresses overall tumorigenesis in the intestine of ApcMin/+ mice (47). Similarly, induced hypomethylation has recently been shown to be protective against the onset of tumorigenesis in a mouse model of prostate cancer (48). In contrast, DNMT1 deficiency alone is sufficient to induce sarcomas in mice (13). Therefore, given the high frequency of genomic hypomethylation of GBM, it will be of considerable interest to determine whether genome-wide demethylation exerts a promoting or protective influence on the progression of this disease.

Due to the limited number of cases analyzed, it was not possible to determine whether genomic hypomethylation, or lack thereof, has prognostic value in GBM. In fact, the coordinate expression of a group of cancer-testis antigens has recently been shown to be indicative of advance disease and predict poor outcome in patients with non–small cell lung carcinoma (49). In this respect, it will be pertinent to address whether severe genomic hypomethylation or MAGEA1 expression is associated with expression patterns that define recently recognized subsets of GBM, in particular those characterized by a proliferative gene expression pattern and poor prognosis (50).

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