Differential Epigenetic Inactivation of Genes in Gliomas

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Abstract

Normal epigenetic modifications such as DNA methylation or histone modifications regulate normal cell differentiation while abnormal epigenetic changes could lead to oncogenic transformation of normal cells. Aberrant or altered DNA methylation cause genomic stability, which is often linked to various pathologies including cancer. Specifically, in glioma epigenetic dysfunctions are often shown to drive oncogenic transformation of low grade glioma to high-grade glioblastoma multiforme (GBM). While few epigenetic events are linked to glioma transformation, more transformation events remain to be identified and validated as viable targets for reversing the epigenetic gene silencing. Reversing the epigenetic silencing or resetting these genes hold a promise in preventing or arresting the progression of low grade glioma to GBM. We treated glioma cells with demethylating agent 5-aza (DNA methyltransferase inhibitor) and Trichostatin A (TSA, Histone deacetylase inhibitor) and whole genome expression array was performed to identify genes that were reactivated by treatment. Array results were confirmed by RT-PCR analysis using glioma cells treated with 5-aza and TSA. Genes were filtered by the following criteria: 2-fold reactivated by treatment as determined by microarray; CpG island in their promoter region; reactivated by treatment in majority of the glioma cell lines, as determined by RT-PCR. Methylation specific PCR (MSP) was carried out to determine methylation status of the promoter region using low grade and high-grade glioma cell lines.

Our results indicate that there is a significant difference in the expression of genes between low and high-grade glioma cells, when treated with 5-aza plus TSA. Induction of CLDN6, NDN, OSMR, ADFP, CDCP1, ANGPT2, ZFP42, BTG4 and MYEOV was seen in most of the glioma cells treated with 5-aza and TSA. MSP-DNA methylation analysis revealed that ADFP, CDCP1 and ZFP42 that were uniquely silenced in high-grade glioma cell lines were reactivated after 5-AZA treatment. This result suggests that studying the methylation status of ADFP, CDCP1 and ZFP42 in brain tumor biopsies may indicate the potential aggressive nature of glioma that helps doctors in accurate and diagnosis and clinical treatment decisions.

Keywords: Epigenome; Glioma Transformation; GBM; Epigenetic Silencing; CpG Island Hypermethylation

Abbreviations

5-aza: 5-aza-2’-deoxycytidine (DNA methyltransferase inhibitor) and Trichostatin A (TSA, Histone Deacetylase Inhibitor); MSP-PCR: Methylation Specific Polymerase Chain Reaction; HDAC: Histone Deacetylase; 5-AZA: 5-Azacytidine (Vidaza); CDCP1: CUB Domain Containing Protein 1; ZFP42: Zinc Finger Protein 42; ANGPT2: Tumor Angiogenic Switch; ADFP: Adipocyte Differentiation-Related Protein

Introduction

Gliomas are the most common primary tumors that arise within the central nervous system in adults accounting for 78% of malignant brain tumors. In recent years increasing use of genetic analysis in primary tumors resulted in identification of molecular events and pathways involved in the etiology of brain tumors. However, our understanding of molecular basis of most brain tumor cases remains...
poor. DNA methylation plays an important role in various cellular functions such as transcriptional silencing, X-chromosome inactivation, genomic imprinting, and genomic stability. Aberrant or altered DNA methylation is linked to various pathologies, including cancer [1]. Tumor cells exhibit global hypomethylation of the genome accompanied by region-specific hypermethylation events. Global hypomethylation occurs mainly in the repetitive sequences leading to genomic instability and tumor formation [2]. Aberrant hypermethylation occurs at CpG islands found in the promoter region of genes and is usually associated with the transcriptional silencing of that gene [3]. Another form of epigenetic gene silencing is the covalent modification of histone proteins. These are post-translational modifications that occur at the amino-terminal tail and include acetylation, methylation, phosphorylation, and ubiquitination. These forms of epigenetic modifications are closely linked to each other. Recent studies suggest that DNA methylation might be dependent on histone modifications and acts to preserve the silenced state rather than initiate silencing [3]. Increasing evidence suggests that epigenetic modifications, in addition to genetic changes, play an important role in carcinogenesis [4-6]. DNA methylation changes, particularly CpG island hypermethylation are frequent, early, and common events (as common as mutations) in many types of cancers leading to the inactivation of tumor suppressor genes [7] and potentially aiding the transformation of low grade tumors to higher grades.

Several genetic changes have been identified in astrocytic gliomas and glioblastomas involving heterozygous deletion of 19q13, inactivation/deletion of tumor suppressor genes namely p16INK4A [8], p14ARF [9], RB1 [10], PTEN and p53 gene [11] and amplification of EGF receptor gene (EGFR) [12]. Investigations of the role of epigenetics in glioma pathogenesis revealed several of these genes to be epigenetically silenced by promoter CpG island hypermethylation, e.g., cell cycle regulatory proteins RB1 [13], p16INK4A [14-17], myelin related gene EMP3 [1], DNA repair protein MGMT and matrix metalloproteinases inhibitor TIMP3 [2]. Comprehensive whole-genome microarray studies using inhibitors of epigenetic modification identified several genes like CST6 (putative metastatic suppressor), BIK (apoptosis inducer), TSPYL5 (unknown function), BEX1, and BEX2 (uncharacterized function) as putative tumor suppressors that are frequently methylated in primary gliomas [18,19]. Another genome-wide study using restriction landmark genomic scanning identified as many as 1500 CpG islands to be aberrantly methylated in low grade gliomas [16], highlighting a role for DNA methylation in gliogenesis.

DNA methylation status not only serves as a diagnostic or prognostic marker but is a potential therapeutic target because of the reversible nature of methylation. Since the primary DNA sequence of epigenetically modified genes remains intact, it is possible to reactivate genes using inhibitors of DNA methylation or histone modifications [20,21]. Clinical trials are being carried out using DNA methylation and histone deacetylase inhibitors to reactivate silenced genes in cancers. Some of the DNA methyltransferases inhibitors, 5-azacytidine (Vidaza), and 5-aza-2'-deoxycytidine (Decitabine), have been used with reasonable success in the treatment of hematologic malignancies [22]. Combination of HDAC inhibitors with DNA methyltransferases inhibitors seems to have a synergistic effect in inducing expression of silenced genes [6]. We set out to study whether there are unique hypomethylation events during the genotypic transformation of low to high-grade gliomas. A better understanding of the molecular basis of glioma progression, particularly identifying tumor suppressor genes that trigger glioma transformation to an aggressive high-grade will provide an opportunity to design novel therapeutic strategies to control an otherwise hard-to-treat brain tumor.

**Material and Methods**

**Cell culture**

Established glioblastoma cell lines Hs683, U-87 MG, U-118, LN18, LN229, SW1088, SW1783 and A172 were obtained from American Type Culture Collection (Manassa, VA). SW1088 and SW1783 cells were maintained in Libovitz's Media supplemented with 10% FBS and 2 mM L-Glutamine at 37°C with no CO₂. All other cells were maintained in DMEM supplemented with 10% FBS and 4 mM L-Glutamine at 37°C with 5% CO₂. Hs683 cell line represent low grade glioma and other cell lines (U-87 MG, U-118, LN18, LN229, SW1088, SW1783 and A172) represent high-grade gliomas.

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Drug treatment

5-aza-2'-deoxycytidine (5-aza) was dissolved in PBS and stored at -70°C. Trichostain A (TSA) was dissolved in DMSO and stored at -20°C. Glioma cells were treated with 5-aza (10 µM) for 4 days (fresh 5-aza added every day) followed by a 24 hour treatment with TSA (100 nM).

RNA extraction and RT-PCR

Total RNA was extracted from glioma cell lines using TRizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) using 1 µg of total RNA according to manufacturer's instructions. PCR primers for all genes were designed using Primer3 software as shown in Table 1, and the reaction carried out using AmpliTaq Gold polymerase. PCR products were separated on a 2% agarose gel. GAPDH expression was used as a loading control for treated and untreated samples.

<table>
<thead>
<tr>
<th>GENE</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLDN</td>
<td>ttcgtaacctggcattgtgg</td>
<td>ttc gta cct gcc att gac tgg</td>
</tr>
<tr>
<td>NDN</td>
<td>GGTCCCCGGACTGTGAGATGC</td>
<td>CGAGGTTAGGGCCAGAGAA</td>
</tr>
<tr>
<td>OSMR</td>
<td>TGGTACGACATCAAGCGACA</td>
<td>CAAAGGCTCCTCGCCTAGC</td>
</tr>
<tr>
<td>ADFP</td>
<td>GAAATGCCTATTTCTGAATCAGCC</td>
<td>TTTGCCACTTGGCAACACCT</td>
</tr>
<tr>
<td>CDCP1</td>
<td>TGGTTCCACCCAGAAATGT</td>
<td>GATGATGCACAGGCATTTATAGTA</td>
</tr>
<tr>
<td>ZFP42</td>
<td>ACT GGT ACC TCG GAT TCC AAA TGG AGA GGT CCT GC</td>
<td>AAT CTG GCT AGC AGT GGA AAC GTG GAC TGC CCT GGG</td>
</tr>
<tr>
<td>ANGPT2</td>
<td>GAGGAACGTGTCCTGAACACT</td>
<td>GTGGAAGAGGACACAGTG</td>
</tr>
<tr>
<td>MYEOV</td>
<td>CCT AAA TCC AGC CAC GTC AT</td>
<td>GAC ACA CCA CGG AGA CAA TG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>gaagatggtgatggatttc</td>
<td>gaagttgaaggtcggagt</td>
</tr>
</tbody>
</table>

Table 1: The primer sequences of all genes tested in this experiment by Real-Time PCR.

Bisulfite modification of DNA

Genomic DNA was isolated by lysing cells in Proteinase K containing buffer overnight at 37°C, followed by phenol-chloroform extraction and ethanol precipitation. Bisulfite modification of DNA was carried out using the EZ DNA Methylation kit (Zymo Research Co., Orange, CA). Briefly, 0.5 - 1 µg-denatured genomic DNA was treated with sodium bisulfite for 16 hrs at 50°C in dark. The samples were then applied to columns, washed, desulfonated and subsequently eluted with 20 µL of elution buffer. Modified DNA was used in subsequent PCR reactions.

Methylation specific PCR and Bisulfite genomic sequencing

Bisulfite modified DNA was used in a PCR reaction containing either methylation specific PCR (MSP) or bisulfite genomic sequencing (BGS) primers. Primers for BGS of ADFP, CDCP1 and ZFP42 were designed as shown in Figure 1, using MethPrimer software, to cover the predicted CpG island for the particular gene. PCR was carried out using AmpliTaq Gold polymerase using 1 µL of bisulfite modified genomic DNA. PCR products were extracted from the gel using Qiagen gel extraction kit (Qiagen, Valencia, CA) and cloned using TOPO TA cloning kit (Invitrogen, Carlsbad, CA). These clones were sequenced at Anderson Cancer Institute, Savannah, GA, USA Genomics Core Sequencing facility using either M13 forward or M13 reverse primers. BGS was done using genomic DNA from glioma cell line U118 or LN229, and at least five independent clones for each gene were sequenced.
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Figure 1: Primers for MSP and BGS were designed, using MethPrimer software, to cover the predicted CpG island for the ADFP, CDCP1 and ZFP42.

MSP was carried out using 1 µL of bisulfite modified DNA as template and two sets of primers, one to detect methylated sequence (M primer) and the other to detect unmethylated sequence (U). MSP was carried out in all glioma cell lines for each of the genes selected. Primers were designed using MethPrimer software. Placental genomic DNA, unmethylated or in vitro methylated using SssI methyltransferases (NEB, Ipswich, MA), was used to optimize the U and M primers respectively.

Gene expression array/Data analysis

Whole genome expression analysis, comparing treated and untreated glioma cell lines, representing low grade and high-grade gliomas (Hs 683 and LN18) was performed at our Genomics Core Facility. Cells were treated with 5-aza and TSA as indicated above. RNA was extracted from treated and untreated cells using TRIZOL reagent according to manufacturer’s protocol. RNA samples were further cleaned using RNeasy Minielute columns and RNA quality accessed using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Five to ten micrograms of RNA were processed and hybridized to Affymetrix GeneChip Exon array. Data obtained from the array was analyzed using Partek Genomics Suite software. Cell lines’ samples, (Hs683 and LN18) treated and untreated were run in triplicates. Genes that showed ≥ 5 fold induction in the treated vs. untreated sample and had a CpG island in their promoter region were selected for further analysis.

RT-PCR to validate microarray targets

As a first step towards validation of the array data, RT-PCR analysis was performed using RNA extracted from various glioma cell lines that were treated with 5-aza and TSA. One microgram RNA was used for cDNA synthesis using Superscript III reverse transcriptase (Invitrogen). PCR was performed using the cDNA and primers designed for genes that were significantly upregulated in 5-aza treated sample compared to untreated sample.

Results

Data obtained from the array heat map (Figure 2) was analyzed using Partek Genomics Suite software. Array results were confirmed by RT-PCR analysis using glioma cell lines treated with 5-aza and TSA. Genes were filtered by the following criteria: 2-fold upregulation by

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treatment as determined by microarray; CpG island in their promoter region; upregulated by treatment in majority of the glioma cell lines, as determined by RT-PCR. Methylation specific PCR (MSP), and bisulfite genomic sequencing (BGS) was carried out to determine methylation status of the promoter region using cell lines. Finally, out of eleven genes probed, we identified eight differentially methylated genes such as CLDN6, NDN, OSMR, ADFP, CDCP1, ANGPT2, ZFP42, and MYEOV in gliomas (low and high-grade) by RT-PCR following reactivation with 5-AZA. We believe that these genes may have functional roles in gliomas that can be potentially exploited for glioma treatment by reversing their methylation and possibly delaying the progression from low grade to high-grade gliomas.

**Figure 2:** Microarray data analysis showing Heat Map with differential expression of genes between low (Hs683) and high-grade glioma cells (LN18), untreated (UT) and treated (T) with 5-AZA and TSA.

Our results with cell lines representing low and high-grade gliomas (Hs683 and LN 18 respectively) indicate that there is a differential expression of genes between low and high-grade gliomas, when treated with 5-aza plus TSA. Induction of several genes was seen in most of the cell lines when treated with 5-aza and TSA as shown by their mRNA expression levels (Figure 3). Since CLDN6, NDN, OSMR, ADFP, CDCP1, ZFP42, ANGPT2, and MYEOV reactivation were noticeably altered, we selected them for further analysis.

**Figure 3:** mRNA of low grade (Hs 883) and high-grade glioma cell lines (U-87 MG, U-118, LN18, LN229, SW1088, SW1783 and A172 untreated and treated with 5-AZA and TSA. RT-PCR products (mRNA) of 11 genes identified by microarray gene expression analysis were analyzed. RNA was extracted from 7 glioma cell lines untreated or treated with 5-AZA and TSA and used in semi quantitative RT-PCR.

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Genes whose suppressed activity reversed following 5-aza/TSA treatment and having a CpG island in their promoter region were selected for Methylation-specific PCR (MSP) to confirm that 5-aza and TSA treatment effect was the direct result of demethylation and not a secondary effect. Primers for methylated and unmethylated sequence were designed in the regions as shown in Figure 1 and PCR carried out using TaqGold (ABI) DNA polymerase. PCR products (mRNA) were visualized on a 2% agarose gel for the selected genes as shown in Figure 4, which appears to correlate with the expression data from RT-PCR analysis.

**Figure 4:** MSP-PCR was carried out to discriminately amplify and detect a methylated region of interest using methylated-specific primers on bisulfite-converted genomic DNA. The mRNA expression of RT-PCR products of ADFP, CDCP1 and ZFP42 in three high-grade glioma cell lines are shown above. Bisulfite modified DNA was used in a PCR reaction containing either MSP or BGS primers. DNA methylation analysis by MSP-PCR was performed for ADFP, CDCP1 and ZFP42 that were reactivated after 5-AZA treatment. However, these genes were not activated by 5-AZA in low grade (Hs 883) glioma cell line. U: Unmethylated DNA Specific Primer; M: Methylated DNA Specific Primer.

**Discussion**

Increasing evidence suggests that disruption of DNA methylation plays an important role in cancer development. Tumor cells exhibit global hypomethylation of the genome accompanied by region-specific hypermethylation. Global hypomethylation occurs mainly in the repetitive sequences leading to genomic instability while aberrant hypermethylation occurs at CpG-rich regions known as CpG islands in the promoter regions of genes. Hypermethylation of CpG islands, usually associated with transcriptional silencing, has been observed in all types of cancers including brain tumors, with unique patterns of individual gene methylation exhibited by each cancer type. DNA methylation changes in gliomas, particularly CpG island hypermethylation are very frequent and early events of tumorigenesis. However, no study has attempted to correlate the extent of methylation with progression of glioma from low to a high-grade. We set out to study whether there is differential silencing of tumor suppressor genes between low and high-grade gliomas and that the extent of methylation correlates with the grade of gliomas.

DNA methylation servers as not only as a diagnostic and prognostic marker but is also a potential therapeutic target because methylation is a reversible process. Genes silenced by methylation can be reactivated using inhibitors of DNA methyltransferases like 5-aza-2'-deoxycytidine (5-aza). Our strategy was to treat low and high-grade glioma cells with DNA methyltransferases inhibitor and then identify genes that are reactivated in treated cells compared to untreated cells, using global gene expression array (Figure 2). These up-reactivated genes could be potential tumor suppressor genes that may be targeted to alleviate or slow glioma progression. Validation of the array data by RT-PCR was done using glioma cell lines representing low and high-grade gliomas.

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Many studies have reported that lower grade glioma may be transformed to high-grade glioma (GBM), due to underlying genetic as well as epigenetic modifications resulting in different biological behaviors with serious consequences for glioma management [28,29]. These genetic and epigenetic modifications have potential application for diagnosis, prognosis, and treatment of gliomas. They also pave the way for developing novel therapies to reverse epigenetic changes in gliomas. CT and MRI have been used to study the evolution of low-grade gliomas into malignant gliomas, but how and when these transformation processes occur or the underlying epigenetics is yet to be studied. We investigated if there are subtle differences in methylation of unique genes that may be extrapolated to possible involvement in the transformation processes. In low grade and secondary gliomas, certain genetic and epigenetic alterations including TP53, IDH1 and ATRX are frequently mutated leading to aberrant DNA methylation, while mutations in the important chromatin modifier ATRX affect chromatin structure [29]. The genome expression analysis data with low and high-grade glioma cell lines that were treated 5-aza plus TSA showed significant difference in the expression of genes between low and high-grade gliomas (Figure 2). In particular, CLDN6, NDN, OSMR, ADFP, CDCP1, ANGPT2, ZFP42 and MYEOV were significantly reactivated (Figure 3) in glioma cells. This observation is consistent with other comprehensive studies on colorectal cancers where high rates of aberrant hypermethylation in promoters were reported as CpG island methylator phenotype (CIMP) positive [30]. Latest studies involving genome-wide analyses have shown the presence of CIMP in breast cancers, lung cancers and gliomas [31-33]. Lai A., et al. [34] reported that glioma-CIMP tumors are frequently found in secondary GBM, which shows a step-wise progression from low-grade glioma to high-grade GBM. Furthermore, the presence of transformation epigenetic markers from low grade to high-grade tumors are shown in low grade pure and mixed astrocytomas, but is observed in less than half of those with low grade oligodendroglioma and has impacted the overall survival of patients. In addition, they showed that low grade astrocytomas were negative for p53 but were positive upon transformation into malignant gliomas in two patients [35].

RT-PCR validation of eight genes that were identified to be activated by 5-AZA in Array analysis (Figure 2), showed that CLDN6, NDN, OSMR, ADFP, CDCP1, ANGPT2, ZFP42 and MYEOV were suppressed in both low grade high-grade glioma cell lines (Figure 3). Interestingly, further MSP-DNA methylation analysis indicated that ADFP, CDCP1 and ZFP42 that were uniquely silenced in high-grade glioma cells lines, but not in low grade glioma cell line, were reactivated after 5-AZA treatment (Figure 4). This observation need to be validated, first with well-planned in vivo xenograft glioma tumor models and secondly with well-histologically and molecular pathologically classified brain tumors. Some of the known roles of ADFP, CDCP1 and ZFP42 in normal and cancers are listed in Table 2. Nevertheless, studying the methylation status of ADFP, CDCP1 and ZFP42 in brain tumor biopsies may prove to be useful in glioma classification and proper diagnosis of grades, which might eventually impact clinical decisions in glioma treatment and management.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Location</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDCP1</td>
<td>3p21.31</td>
<td>CUB domain containing protein 1. May be involved in cell adhesion and cell matrix association. May play a role in the regulation of anchorage versus migration or proliferation versus differentiation via its phosphorylation. This protein is found to be overexpressed in colon and lung cancers.</td>
<td>23</td>
</tr>
<tr>
<td>ZFP42</td>
<td>4q35.2</td>
<td>zinc finger protein 42 homolog. Potential human stem cell marker. May be involved in transcriptional regulation. Reduced expression in renal tumors.</td>
<td>25</td>
</tr>
<tr>
<td>ANGPT2</td>
<td>8p23.1</td>
<td>Tumor angiogenic switch</td>
<td>26</td>
</tr>
<tr>
<td>ADFP</td>
<td>9p22.1</td>
<td>ADFP in peripheral tissues may play a critical role in regulating the formation and turnover; and hence metabolic consequences, of ectopic fat.</td>
<td>27, 28</td>
</tr>
</tbody>
</table>

Table 2: The location and known functions of the genes that were found to be reactivated in high grade glioma cells following treatment with 5-AZA. The gene location and known cellular function with relevant references are shown above.

Next, we seek to study biological role of tumor suppressor genes (ADFP, CDCP1 and ZFP42) in mice with human high-grade glioma xenografts. We plan to treat mice bearing intracranial high-grade glioma xenografts with 5-aza and assess (using imaging and other methods) tumor regression compared to untreated mice with glioma xenografts. In addition, we will evaluate the effect of reintroducing the tumor suppressor gene in high-grade glioma cell line and its effect on brain tumor growth in intracranial xenograft model. Therapies for gliomas by targeting epigenetic molecules will be routinely in clinical trials and clinical use in the near future provided new line of HDAC and DNA methyltransferases inhibitors have low toxicity [37-39]. Alternatively, as one study showed aberrant epigenetic silencing of tumor suppressor genes may be reactivated by engineering of sequence-specific epigenome editing tools such as CRISPR/dCas9 VP64. This study outlined a novel sequence-specific, combinatorial epigenome editing approaches to reactivate highly methylated TSGs as a promising therapy for cancer and other diseases [40].

Conclusion

Presently, we have limited knowledge on the precise role of ADFP, CDCP1 and ZFP42 contributions in glioma progression from low to high-grade. Additionally, studies are required to establish how new line of HDAC and DNA methyltransferases inhibitors selectively target ADFP, CDCP1 and ZFP42 and the mechanisms by which such molecules work in vivo. Many more biomarker candidates identified [41] through this type of research must be investigated for development of a multi-gene panel of biomarkers. Such study will help in accurate diagnosis and management of low grade glioma that may aggressively develop to high-grade glioma in some patients. Our study is one step in that direction in understanding the role of epigenome in the dynamic regulation of glioma cells that contributes to the growth of devastating tumor. Therefore, complete understanding how the epigenome regulates certain genomic loci is critical for targeting the epigenome for developing new therapeutic approaches for GBM management.

Conflict of Interest

None of the authors have any conflict of interest in this research article.

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