The Contribution of Genetic and Epigenetic Mechanisms to Gene Silencing in Oligodendrogliomas

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Abstract

Very little is known of the genes and mechanisms contributing to the genesis of oligodendrogliomas, a subtype of primary brain tumors. Using an integrated genetic and epigenetic analysis of oligodendrogliomas, we show that aberrant CpG island methylation is the most prevalent alteration in these tumors, and the majority of methylated genes are independent of regions affected by deletion. In contrast, a subset of the gene-associated CpG islands are preferentially affected by converging methylation and deletion, including a putative zinc finger gene, ZNF342, located in a commonly deleted region at chromosome 19q13. ZNF342 expression is specifically decreased in primary oligodendrogliomas and up-regulated in glioma cell lines treated with a demethylating agent, whereas the expression level of the adjacent gene, Gemin7, is not consistently altered in these samples. This initial integrated approach identifies novel targets of gene silencing, and provides a more comprehensive view of the genes and mechanisms underlying oligodendrogliomas.

Introduction

Tumorigenesis is a multistep process that involves a series of genetic and epigenetic alterations, yet the current models of tumorigenesis and many cancer therapies are mainly based on the results of the genetic approach. Epigenetic modification, such as aberrant methylation of normally unmethylated cytosines within CpG islands, is also a prevalent gene inactivation mechanism in human sporadic cancer (1, 2). Typically, CpG island methylation is assessed on genes known to play roles in tumorigenesis, especially in tumor samples where those genes do not have genetic alterations. This candidate gene approach has identified many genes that are silenced by aberrant methylation including genes involved in differentiation, cell growth, apoptosis, and tumor progression (1, 2). These and other studies have demonstrated that aberrant methylation contributes to gene deregulation and tumorigenesis, but have focused on those genes first identified through genetic approaches and only on a few of the estimated 29,000 CpG islands in the genome (3).

RLGS4 (4) is a two-dimensional gel-based method that allows a reproducible and quantitative assessment of the methylation status of thousands of gene-associated CpG islands. We developed a custom computer program to identify the sequence and chromosomal position of each RLGS fragment using the human genome sequence (5), thus making RLGS results amenable to alignment with high-resolution copy number analyses, such as array CGH (6). We recently applied RLGS and array CGH to the analysis of astrocytic gliomas, a common subtype of glial brain tumors (5). This integrated approach identified novel targets of gene silencing and showed that most aberrant methylation is independent of deletions in astrocytic gliomas. This and other data suggest that an integrated genomic and epigenomic approach provides a more comprehensive understanding of the genes and mechanisms underlying tumorigenesis.

To determine whether our findings in astrocytomas are more universally applicable to brain tumors, here we studied the genetic and epigenetic alterations in oligodendrogliomas, a second glioma subtype. Oligodendrogliomas comprise a continuous spectrum ranging from well-differentiated (WHO, grade II) to anaplastic tumors (WHO, grade III). Deletions of chromosome 1p and 19q are the most common genetic abnormalities in oligodendrogliomas (7–9), and are associated with a favorable antitumor response to chemotherapy and longer patient survival (8–10). However, the putative tumor suppressor genes at these loci have not yet been identified. In contrast, there are only a few studies reporting aberrant methylation of candidate genes in oligodendrogliomas (11–15) and little or no knowledge about the extent of aberrant methylation across the genome of oligodendrogliomas. Therefore, we applied a large-scale integrated approach to gain insight into the relative contribution of these mechanisms in the formation of oligodendrogliomas. We identified genes not suspected previously to be involved in oligodendrogliomas, including one gene in the commonly deleted region of chromosome 19q13 that is inactivated by methylation of one allele and deletion of the other. Furthermore, the data indicate that aberrant CpG island methylation is the most frequently occurring alteration in these tumors.

Materials and Methods

Primary Brain Tumors and Human Glioma Cell Lines. We obtained tumor and nontumor control brain tissues from the Neurosurgery Tumor Bank at the University of California, San Francisco. The tumor samples included 9 WHO grade II oligodendrogliomas and 13 WHO grade III anaplastic oligodendrogliomas. We also obtained five nontumor brain samples from different individuals, including two samples of brain adjacent to tumor (BAT4 and BAT6), one autopsy sample (Aut1 and Aut2; white matter; Aut4; gray matter; Aut13: white matter + gray matter), and two surgical samples from individuals with epilepsy (NB3 and NB8). Samples were obtained with informed consent, and their usage was approved by the Committee on Human Research at the University of California, San Francisco.

Four human glioma cell lines, T98, U178, LN443, and U373 were plated at low density and incubated with 5-aza-2’-deoxycytidine (1 μM). Drug-containing medium was replaced every 24 h for 3 days. After day 3, total RNA was isolated for additional experiments.

RLGS. RLGS was performed as described previously for the NotI/EcoRV/Hinfl combination (5). We compared profiles from tumors and normal brain DNA by visual inspection of overlaid autoradiographs. The analysis was conducted independently by two individuals. The consistency of visual analysis was validated with two-dimensional gel analysis software (Nonlinear Dynamics) as described previously (5).

Array CGH. To determine the copy number of loci throughout the tumor genome, we performed comparative genomic hybridizations on arrays consist-
ing of 2400 mapped bacterial artificial chromosome clones distributed at \ (~1 Mb intervals across the genome (6)). Briefly, DNA from tumor and normal tissues was labeled with Cy-3 and Cy-5, respectively, and cohybridized to the arrayed clones. The bacterial artificial chromosome DNA was counterstained with 4',6'-diamidino-2-phenylindole hydrochloride. The image of the three fluorochromes was captured and processed using custom software (16), and the raw ratios and mean log2 ratios of triplicates of tumor to normal reference DNA hybridization were calculated and plotted for deletions and gains along each chromosome.

RNA Isolation, Reverse Transcription, and PCR. Glial cells and brain tissue were lysed with TRIzol (Life Technologies, Inc.) for RNA isolation. Total cellular RNAs were quantified using a spectrophotometer and then treated with amplification grade DNase I (Roche). After quantifying DNase-treated RNAs, we incubated 800 ng RNA with oligodeoxynucleotidyl acid and random primers in a 20 \( \mu \)l reverse transcription reaction, heated to 65°C for 5 min, and placed on ice. A mixture containing 1 × reaction buffer (Invitrogen), 10 mM DTT, 0.5 mM each deoxynucleoside triphosphates and 40 units of reverse-transcribed template in each sample. Water and genomic DNA were treated with amplification grade DNase I (Roche). After quantifying DNase-out RNase inhibitor (Invitrogen) was added to each sample. After samples were incubated at 42°C for 2 min, 200 units of Moloney murine leukemia virus reverse transcriptase was added and incubated at 25°C for 10 min, 42°C for 50 min, and 70°C for 15 min. We performed PCR amplification in a 25-\( \mu \)l reaction with 1 \( \mu \)l of each reverse transcription reaction product as template. We amplified ZNF342 and Gemin7 by incubating the reaction at 95°C for 5 min, followed by 32 cycles of 95°C 1 min, 68°C for 1 min, and 72°C for 1 min, and then incubated at 72°C for 10 min before cooling to 4°C. The primers used for ZNF342 and Gemin7 are as follows: ZNF342: forward primer: 5’-CCCGCAACCCAGACGAGCATGATG-3’, reverse primer: 5’-CAAGTCGGTCGCGTTGAGGT-3’ and Gemin7: forward primer: 5’-GGGGGTCGGCCAGAAGTTC-3’, reverse primer: 5’-CTCTCTGGTG-GCCCGGTGCT-3’. These amplicons span at least one intron. ACTB (encoding \( \beta \)-actin) was used as a control for comparison of the amount of reverse-transcribed template in each sample. Water and genomic DNA were used as negative controls for each gene. Sample duplicates and independent experiments yielded similar results.

Bisulfite Treatment, PCR, and Sequencing. We conducted bisulfite treatment of DNA as described (5). Nested PCR was carried out with 30 cycles for outer primers and 30 cycles for the nested primers with 1 \( \mu \)l of treated DNA and PCR products were gel purified (Qiagen) and cloned with the TOPO TA Cloning/PCR 2.1 TOPO kit (Invitrogen). Individual bacterial colonies were subject to PCR using vector-specific primers, or in some cases bacterial cultures were grown for plasmid isolation (Qiagen), and the products were sequenced. The primers used for nested PCR are as follows: 3D09 region I, outer forward: 5’-GAAAAGGGGAG-GATGGTGTGGT-3’, outer reverse: 5’-CCCAATACTACATTCATAAC-3’, inner forward: 5’-GGTGGTTGTAGTAGGTAGTGGG-3’, inner reverse: 5’-ACCAACTCTCTACACCATC-3’, 3D09 region II: outer forward: 5’-GAAGGGAGGTGGGTGTTAG-3’, outer reverse: 5’-CCTCCTCCTATATACCT-3’, inner forward: 5’-GTATGCGGTAGGGAGTGGT-3’, inner reverse: 5’-CCTCTCCTCATAACCTC-3’. Total: Ref; 5; see Supplementary Data Fig. A5 now allows the large-scale integration of methylation data from RLGS with copy number data.

Integrated Analysis of Genetic and Epigenetic Alterations in Oligodendrogliomas. To investigate the interaction of genetic and epigenetic mechanisms in tumors, we combined RLGS analysis with array CGH. We detected 1p and 19q deletion in all 22 of the oligodendrogliomas, consistent with the neuropathologic assessment of these tumors. To determine the contribution of methylation and genetic mechanisms to RLGS fragment changes, we attributed RLGS fragment loss or decrease to: (a) partial methylation, if the decrease in NolI fragment intensity in tumor DNA is at least 30% but no more than 70% relative to normal tissue, and no deletion is detected; (b) homozgyous methylation, if the decreased intensity is >70% relative to normal tissue and no deletion is observed; (c) deletion, if the intensity is between 30 and 70% of the control, and deletion is observed on the arrays; (d) methylation and deletion, if the decrease in NolI fragment intensity is >70% relative to control and hemizygous deletion is detected; (e) gain, if array CGH shows gain of the flanking loci; and (f) gain and methylation, if array CGH shows gain, whereas the RLGS fragment intensity decreases.

In Fig. 2A, among a total of 4642 possible events (22 oligodendrogliomas, up to 211 loci per tumor), most loci (67.1%) were not affected by either genetic or epigenetic mechanisms. For the remaining 32.9% with alterations, methylation was the most common mechanism acting on the loci. In contrast, the convergence of methylation...
and deletion was uncommon overall (2.4% of all possible events) similar to that observed in astrocytic gliomas (5). This suggests that the majority of genes affected by aberrant methylation are independent of the well-defined, recurrent genetic mechanisms. However, the biallelic alteration involving methylation of one allele and deletion of the other was observed at a very high frequency at particular loci (Fig. 2B). Convergence of methylation and deletion at RLGS fragment 3D09 occurred in 6 of 9 grade II and 10 of 13 grade III oligodendrogliomas. The corresponding CpG island is located on chromosome 19q13 between a pair of divergently oriented genes, ZNF342 and Gemin7 (Fig. 3A). ZNF342 encodes a putative zinc finger protein, and Gemin7 encodes a component of survival of motor neuron protein complex.

Decreased Expression of ZNF342 in Primary Oligodendrogliomas and Demethylation-Induced Reactivation in Glioma Cell Lines. To examine whether the aberrant methylation at the 3D09 CpG island is associated with changes in the expression of the two adjacent genes, we conducted RT-PCR analysis of RNA from oligodendrogliomas. Whereas ZNF342 was expressed in normal brain, the level was lower in both grade II and grade III tumor samples (Fig. 3B). We expected that all of the samples would exhibit some ZNF342 expression, due to the admixture of nontumor cells, including normal brain cells, as well as potential tumor cell heterogeneity in methylation. In contrast, the expression of Gemin7 was not significantly different between normal brain and the tumors. The quantitation of expression of both genes, shown in Fig. 3, C and D, clearly indicates that ZNF342 and Gemin7 are expressed differently in primary brain tumors, suggesting that deletion alone does not account for the decreased ZNF342 expression in the tumors. In glioma cell lines, expression of ZNF342 was observed in LN443 cells, whereas T98, U178, and U373 cells lines had little or no ZNF342 expression. In cells treated with the demethylating agent 5-aza-2′-deoxycytidine, ZNF342 expression was reactivated or increased in the nonexpressing glioma cells (Fig. 3E), consistent with the results from real-time quantitative RT-PCR (Supplementary Data Fig. D5). In contrast, Gemin7 was uniformly expressed among these cell lines and was not altered by the 5-aza-2′-deoxycytidine treatment (Fig. 3E; Supplementary Data Fig. D5), demonstrating that aberrant methylation contributed to silencing of ZNF342 but not Gemin7.

Extensive Methylation in the 3D09 CpG Island in Primary Tumors. To confirm and extend the methylation analysis of the 3D09 CpG island, bisulfite sequencing was applied to DNA from 3 normal brain tissues and 4 oligodendrogliomas. Fig. 3A shows the two contiguous regions of methylation analysis that are within the CpG island encompassing exon 1 of ZNF342. Region I contains the NotI CpG site (CpG #24), and region II encompasses the putative transcription start site of the ZNF342. For region I, the overall level of CpG methylation was similar between normal brain (21.3%) and tumors (38.9%), although the part of region I adjacent to region II (CpGs #1–10) showed a tendency toward increased methylation in the tumor samples (Fig. 4A). Consistent with this, CpGs toward the start site of the gene (region II) were heavily methylated in tumor samples, whereas very little methylation was observed in normal brain tissues (Fig. 4B). Taken together, these results indicate that ZNF342 is a frequent target of converging aberrant methylation and deletion, which leads to ZNF342 gene silencing in oligodendrogliomas.

Discussion

In this study we report the first large-scale integrated analysis of aberrant CpG island methylation and genomic deletion, two primary mechanisms of gene silencing, in oligodendrogliomas. We found that the majority of aberrant methylation occurred independently from deletion, and methylation was the predominant mechanism affecting the loci studied here. Therefore, aberrant methylation in particular may contribute significantly to gene silencing in these tumors, al-
though it is expected that a proportion of these events may not have a direct impact on gene expression. For particular genes of interest, a more detailed analysis of methylation and gene expression, such as that provided here for \textit{ZNF342}, is needed to determine their potential importance to tumorigenesis. These observations and others suggest that an integrated approach provides a more comprehensive understanding of the genes and mechanisms contributing to the genesis of these tumors. Although our analysis covers a relatively large number of genes and CpG islands, additional technical developments in genomic and epigenomic screening methods will be required for complete genome coverage.

This is also the first global analysis of aberrant methylation in oligodendrogliomas, and suggests that CpG island methylation is widespread, highly recurrent at particular loci, and occurs early in the genesis of these tumors. Other studies have shown that tumor-related genes such as \textit{MGMT}, \textit{GSTP1}, \textit{DAPK}, \textit{p14ARF}, \textit{THBS1}, \textit{TIMP}-3, \textit{p73}, \textit{HIC}, and \textit{CDKN2B} (11–15) are aberrantly methylated in oligodendrogliomas. In most cases, however, the extent of methylation and its potential impact on expression of the associated tumor-related gene has not been tested in oligodendrogliomas. In addition to methylation and deletion of \textit{ZNF342}, we also detected recurrent homozygous methylation of RLGS fragment 3D41 (Supplementary Data Fig. A5). The CpG island corresponding to 3D41 is located at chromosome 12q23.2 and was shown previously to be aberrantly methylated in human astrocytomas (5). The gene associated with this CpG island was identified recently as \textit{SLC5A8}, which encodes a putative sodium transporter and is aberrantly methylated in colon cancer (17). Expression of exogenous \textit{SLC5A8} inhibited the growth of nonexpressing human colon cancer cells, suggesting the silencing of \textit{SLC5A8} by aberrant methylation may have important functional consequences for tumorigenesis. In oligodendrogliomas, the \textit{SLC5A8} CpG island was homozygously methylated in 12 of 22 tumors. Additional analysis of the expression and function of \textit{SLC5A8} will also be of interest to understanding the functional contribution of aberrant methylation to the genesis of oligodendrogliomas.

Numerous studies have shown that deletion of chromosomes 1p and 19q are the most common genetic alterations in oligodendrogliomas, but the putative tumor suppressor genes residing in these regions have not yet been identified. Chromosome 1p and 19q deletion was observed in all 22 of the oligodendrogliomas studied here, likely due to our selection of cases with classic oligodendroglioma pathology. The minimally deleted regions appear to be 1p36 (18, 19), 1p34–p35 (20), and 19q13.2–19q13.4 (21, 22). Related to the chromosome 19q locus, Wolf et al. (23) identified p190RhoGAP as a potential tumor suppressor gene in a platelet-
derived growth factor-induced mouse model of oligodendrogliomas. The corresponding locus of the human p190RhoGAP gene is 19q13.3, suggesting that this gene may be a candidate tumor suppressor in human oligodendrogliomas. The deleted and extensively methylated ZNF342 gene identified in our study maps to 19q13.32, which is deleted in 100% of our samples but appears to be proximal to a minimal region defined by others (22). In future studies, it will be of interest to determine whether ZNF342 can suppress the growth of tumor cells. The current lack of human oligodendroglioma cell lines that retain 1p/19q loss in culture remains a substantial hurdle to obtaining functional evidence for candidate cancer genes in these tumors.

References

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