Brain Tumor Pathol (2000) 17:49–56
© The Japan Society of Brain Tumor Pathology 2000

ORIGINAL ARTICLE

Joseph F. Costello · Christoph Plass · Webster K. Cavenee

Aberrant methylation of genes in low-grade astrocytomas

Abstract The underlying basis of the malignant progression of astrocytomas is a specific and cumulative series of genetic alterations, most of which are confined to high-grade tumors. In contrast, a proportion of low-grade astrocytomas have a relatively normal-appearing genome when examined with standard genetic screening methods. These methods do not detect epigenetic events such as aberrant methylation of CpG island, which result in transcriptional silencing of important cancer genes. To determine if aberrant methylation is involved in the early stages of astrocytoma development, we assessed the methylation status of 1184 genes in each of 14 low-grade astrocytomas using restriction landmark genome scanning (RLGS). The results showed nonrandom and astrocytoma-specific patterns of aberrantly methylated genes. We estimate that an average of 1544 CpG island-associated genes (range, 38 to 3731) of the approximately 45,000 in the genome are aberrantly methylated in each tumor. Expression of a significant proportion of the genes could be reactivated by 5-aza-2-deoxycytidine-induced demethylation in cultured glioma cell lines. The data suggest that aberrant methylation of genes is more prevalent than genetic alterations and may have consequences for the development of low-grade astrocytomas.

Key words Low-grade astrocytoma · Methylation · RLGS · CpG island

J.F. Costello (✉)
University of California-San Francisco, The Brain Tumor Research Center, 2340 Sutter, Room N261, San Francisco, CA, USA
Tel. +1-415-514-1183; Fax +1-415-502-6779
e-mail: jcostello@cc.ucsf.edu

C. Plass
Division of Human Cancer Genetics, The Ohio State university, Columbus, Ohio, USA

W.K. Cavenee
Ludwig Institute for Cancer Research, Department of Medicine and Center for Molecular Genetics, University of California-San Diego, La Jolla, CA, USA

Introduction

The underlying basis of astrocytoma progression is a series of genetic alterations that activate oncogenes and inactivate tumor suppressor genes. The genetic alterations are cumulative and often specific to the histology grade, although the majority are confined to high-grade gliomas.[1,2] A few genetic mutations such as those in the p53 tumor suppressor gene however, are found in approximately 30% of both low-grade and high-grade astrocytomas. This genetic definition of brain tumors may be biased by the exclusively genetic screening methods used over the past two decades. Epigenetic events such as aberrant methylation may also play a role in glioma genesis and malignant progression, particularly in low-grade tumors in which genetic alterations are rarer.

An imbalance in methylation is prevalent in human sporadic cancers.[3,4] Methylation defects include genome-wide demethylation and localized aberrant methylation of CpG islands.[3–5] Genome-wide demethylation may lead to chromosome breakage and is usually more pronounced in high-grade tumors.[5,6] Aberrant methylation of CpG island-containing genes leads to an inappropriate silencing of genes involved in growth regulation,[7,8] DNA repair,[9,10] apoptosis, angiogenesis,[11] and tumor cell invasion.[12] Thus, there is considerable evidence to suggest that methylation imbalance contributes to malignancy.

Usually, CpG island methylation is assessed in genes known to play roles in the development of a particular tumor type, particularly in tumor samples that do not harbor genetic alterations of the gene. To date, these studies have focused on fewer than 15 of the estimated 45,000 CpG islands in the genome, and very few studies have assessed methylation in low-grade gliomas.[3,4,13]

In contrast to the candidate gene approach, genome-wide screens of CpG island methylation could provide a more systematic and unbiased analysis for detection of novel gene targets and patterns of aberrant methylation related to tumor development. Restriction landmark genome scanning (RLGS) is an approach that is uniquely

suited for simultaneously assessing the methylation status of thousands of CpG islands.[14] The methylation sensitivity of the endonuclease activity of *Not*I provides the basis for differential methylation analysis, and *Not*I sites also have a predicted bias for occurrence in CpG islands and genes.[15] RLGS has been used to identify two novel imprinted genes,[16,17] novel targets of DNA amplification[18,19] and methylation in human cancer,[20,21] and to identify genetic and methylation abnormalities in a mouse model of tumorigenesis.[22] The RLGS system is well suited for tumor and normal tissue comparisons due to its reproducibility, ability to isolate identified fragments, and published profiles of chromosomal assignments for each *Not*I fragment on the profile.[23]

Here we have used RLGS to examine the methylation status of 1184 CpG island-associated genes in each of 14 low-grade astrocytomas. This analysis, combined with our analysis of six additional tumor types,[20] strongly suggests that the aberrant methylation in brain tumors is nonrandom and that the methylation patterns are specific to astrocytomas. We estimate that in many low-grade astrocytomas, hundreds to thousands of CpG islands may be aberrantly methylated. These data have important implications for understanding the molecular pathways by which human brain tumors arise.

Materials and methods

Tumors and cell lines

Fourteen gliomas (GLI), including 12 World Heath Organization (WHO) grade II astrocytomas and two WHO grade III anaplastic astrocytomas, were obtained from Saitama Medical School, the University of Tokyo, Teikyo University School of Medicine Komagome Metropolitan Hospital, and the University of Washington, Seattle. The patients were 10 females and 4 males with an age range of 7 to 57 years (average, 34 years). Brain tissue adjacent to the tumor was also obtained for one WHO grade II and one WHO Grade III tumor. Twelve cases were compared to three unmatched normal brain samples and to the two brain samples from the matched sets.

Restriction landmark genome scanning (RLGS)

RLGS was performed according to published protocols.[14] Briefly, nonspecific sheared ends of 2–5 μg of genomic DNA were blocked in a 10-μl reaction by the addition of nucleotide analogues (αS-dGTP, αS-dCTP, ddATP, and ddTTP) with 2U DNA polymerase I (37°C, 20min) followed by enzyme inactivation (65°C, 30min). The buffer was then adjusted and the DNA was digested (37°C, 2h) with 20U *Not*I (Promega, Madison, WI). Sequenase (version 2.O, U.S.B.) was then used to fill in the *Not*I ends with [α-^{32}P]-dGTP (New England Nuclear) and α-^{32}P]-dCTP (Amersham) for 30min at 37°C. The labeled DNA was digested (37°C, 1h) with 20U *Eco*RV (Promega), and a portion was electrophoresed through a 60-cm-long, 0.8% agarose tube gel (first-dimension separation). The agarose gel was then equilibrated in *Hin*fI digestion buffer and the DNA was digested in the gel with 700U *Hin*fI (Promega) at 37°C for 2h. The agarose gel was then placed horizontally (rotated 90° relative to the first electrophoresis) across the top of a nondenaturing 5% polyacrylamide gel, the two gels were connected with molten agarose, and the DNA was electrophoresed in the second dimension. The gels were dried and exposed to X-ray film in the presence of intensifying screens (Quanta III, DuPont) for 2–10 days.

Whole-genome estimates of aberrant CpG island methylation

A simplistic estimate was obtained by multiplying the total number of methylation sites detected by the quotient of 45,000 total CpG islands and the 1184 CpG islands analyzed.[20] Several important factors with potential to influence the whole-genome estimates were considered. First, methylation at *Not*I sites may reflect either methylation throughout a CpG island or a more localized event in a mostly unmethylated island. Most published reports, however, indicate that aberrant methylation can spread across the CpG island.[3,4,24,25] Second, 6% of the *Not*I fragments may represent non-CpG island DNA. Third, digestion at each *Not*I site generates two distinct fragments, although there is a less than 1% chance that both will be included in the analyzed fragments. Other events, such as multiple *Not*I sites in a single CpG island, unmethylated *Not*I sites within mostly methylated CpG islands (false negatives), and deletion and point mutation, are expected to occur, although at rates that would not have a significant impact on the estimates. Perhaps the most influential criterion affecting any such genome-wide estimate is the minimum level of methylation required to score a positive methylation event. Lowering the minimum value could significantly increase the estimate, and vice versa. Our minimum value for inclusion of a CpG island as methylated was set at 30% methylation of a diploid CpG island, a value reliably detected by our analytic system.

Drug treatment, RNA isolation, and reverse transcription-PCR

Two human glioma cell lines (T98, U251) were plated at low density and incubated with 1μM 5-aza-2′-dexycytidine. Drug-containing media were replaced every 24h for 3 days. After the third day, total cellular RNAs were isolated from each of two treated and one control untreated sample using TRIZOL (Gibco BRL). Total cellular RNA from normal adult and fetal brain was obtained from Clontech. RNAs were quantitated using a spectrophotometer and then treated with amplification grade DNAse I (Gibco). Two micrograms of DNAse-treated RNA was then incubated with oligo-dT and random primers in a 20-μl reaction, heated to 70°C for 10min, and placed on ice. A mix containing 1× reaction buffer (Gibco), 10mM DTT,

0.5 mM of each dNTP, and 80 U RNAsin (Promega) was added to each sample. The samples were divided into two tubes, each containing 19 µl, and incubated at 37°C for 2 min. M-MLV reverse transcriptase (RT, 200 U) was added to one of the two tubes, and each was incubated at 37°C for 1 h. Thirty microliters of DEPC-treated H_2O was added to each sample and heated in boiling H_2O for 5 min.

Polymerase chain reaction (PCR) amplification was performed in 10-µl reactions for each gene except for 2D20, 3C1, and 4D50, which were performed in 25-µl reactions.[20] All 10-µl amplification reactions contained 1.5 µl of the reverse transcription reaction, 1× reaction buffer, 0.5 U Taq polymerase (Boehringer Mannheim), 10 pmol of each gene-specific primer, and 250 µM of each dNTP. The 25-µl amplification reactions were similar, except that 1.25 U Taq polymerase and 20 pmol of each gene-specific primer were used. For each gene, separate amplification reactions were performed using RT-positive and RT-negative reactions as template. Amplification was not detected from the RT-negative reactions. Duplicate and independent drug-treated samples yielded similar RT-PCR results. β-Actin was used as a control for comparison of the amount of reverse-transcribed template in each sample.

Results

RLGS is designed to display the methylation status and copy number of *Not*I sites.[14] Differences in digestion are assessed by radiolabeling the DNA at cleaved *Not*I sites. Following further enzyme digestion, two-dimensional electrophoretic separation, and autoradiography, the intensity of a DNA fragment on the resultant RLGS profile quantitatively reflects the copy number and methylation status of the *Not*I fragment. A typical RLGS profile displays nearly 2000 single copy, unmethylated *Not*I fragments (Fig. 1A). A total of 210 *Not*I fragments have been cloned and the nucleotide sequences have been determined. Analysis of the nucleotide sequences indicates that RLGS primarily detects 5′ CpG islands associated with genes.[20]

A central region of the RLGS profiles having the best resolution of fragments was chosen for all analyses presented in this report (Fig. 1). In total, the same set of 1184 CpG islands was examined in each tumor and normal DNA profile. Methylation and/or deletion could contribute to decreased intensity of CpG islands on the RLGS profiles from tumors. To determine the relative contribution of each mechanism, we located in an arrayed genomic library of *Not*I/*Eco*RV fragments[26,27] the clones corresponding to 26 RLGS fragments exhibiting frequent loss or decreased intensity in tumors.[20] Each clone was used separately as a probe for Southern blotting of *Not*I/*Eco*RV-digested DNA. Figure 2 shows one example comparing fragment 3C1 on the RLGS profile, and the Southern blot of the same tumor DNAs hybridized with the cloned fragment (Fig. 2B). The clone exhibits perfect homology with a gene encoding an alpha subunit of a heterotrimeric G-protein (*GNAL*). Although deletions were not detected for any of the loss events, both complete and partial methylation was observed for the same CpG island in different tumors (Fig. 2B). This could reflect one or a combination of factors, including normal tissue contamination, cellular heterogeneity, or allele-specific methylation. The completely methylated loci indicate that homozygous methylation of *Not*I sites of particular CpG island loci occurs frequently in low-grade tumors. A complete concordance was observed between the detection of loss or decreased intensity on the RLGS profiles and methylation of the *Not*I site assessed by Southern blotting (Fig. 2A,B). This observation and our previous studies[20,21,27] strongly suggest that methylation is the predominant mechanism underlying loss of fragments from RLGS profiles.

We tested two human glioma cell lines for *GNAL* expression by reverse-transcription (RT)-PCR. Both cell lines were methylated at the *Not*I site and other CpG dinucleotides in the 5′ end of *GNAL* (data not shown). *GNAL* is expressed at a low level in T98 cells but was undetectable in U251 cells. Treatment of these cell lines with the demethylating agent 5-aza-2-deoxycytidien resulted in a reproducible increase in *GNAL* expression in both glioma cell lines, suggesting that the methylation may affect transcription of the associated gene.

Many of the low-grade astrocytomas displayed high inherent levels of methylation, whereas others displayed low levels (Table 1). Based on the number of CpG islands that were lost from individual tumor profiles, we estimated the average number of CpG islands affected per tumor genome. Assuming 45,000 CpG islands per genome,[13] we predict that aberrant methylation was initiated at an average of 1544 CpG islands (range, 38 to 3731) in the tumors (see materials and methods). The data suggest that aberrant methylation occurs throughout the genome

Table 1. Number of aberrantly methylated CpG islands in the genome of low-grade astrocytomas

Tumor	Number of methylated CpG islands of 1184 tested	Estimated number of methylated CpG islands in the tumor genome[a]
J2	68	2616
J4	5	192
J6	65	2500
J7	1	38
J9	77	2962
J12	6	231
J14	8	308
J16	83	3193
J26	47	1808
J30	97	3731
J48	35	1346
J49	6	231
MB5	27	1039
kMB8	37	1423
Avg.	40	1544

[a] A simplistic estimate was obtained by multiplying the total number of methylation sites detected by the quotient of 45,000 total CpG islands and the 1184 CpG islands analyzed

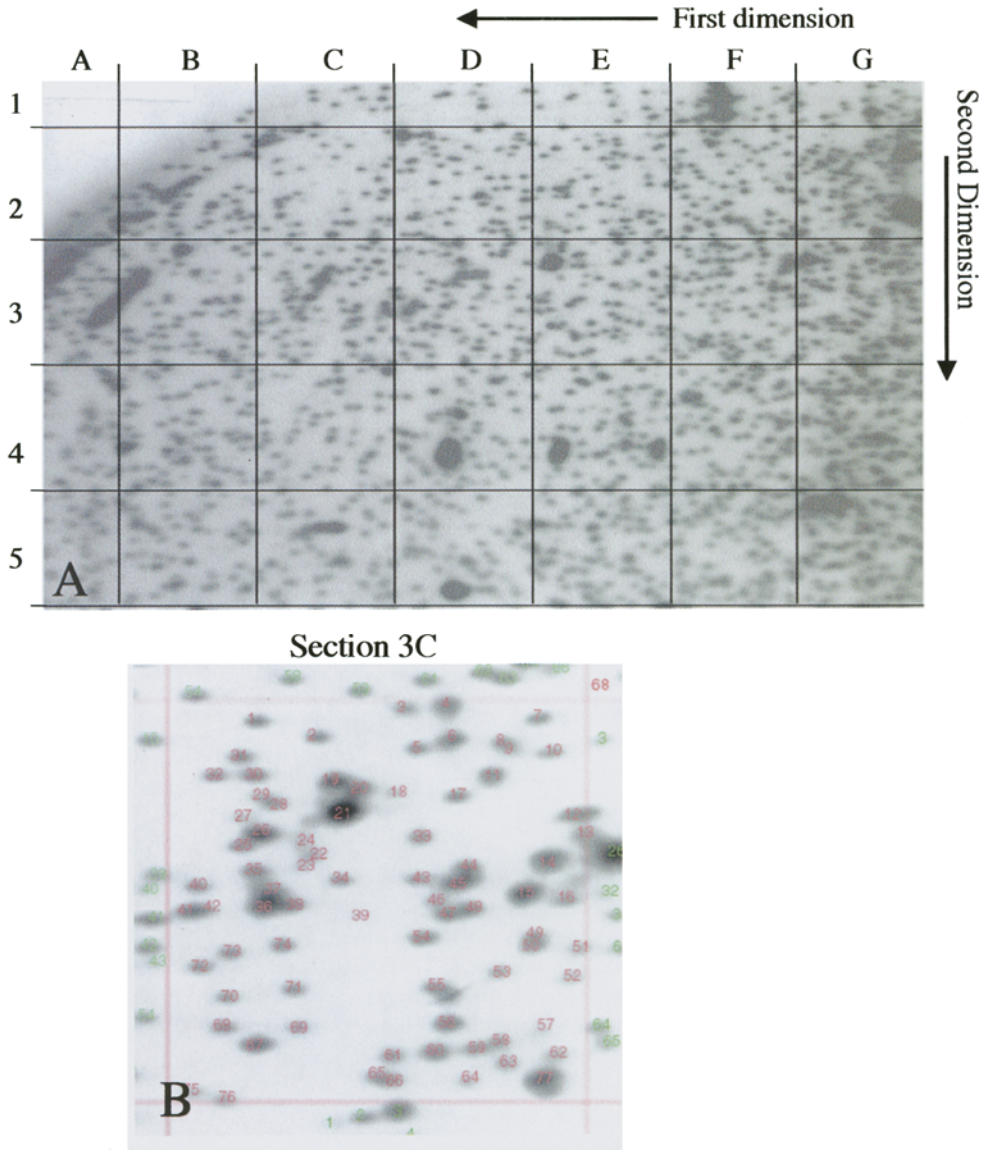

Fig. 1. Coordinate system for uniform analysis of RLGS profiles. **A** Typical RLGS profile of a WHO Grade II astrocytoma displaying more than 2000 single-copy *Not*I fragments and 15 to 20 high-copy-number fragments. Methylation detection in RLGS profiles depends on the methylation sensitivity of the endonuclease activity of *Not*I. Radiolabeling the DNA at cleaved *Not*I sites assesses differences in digestion. First-dimension separation of labeled *Not*I/*Eco*RV fragments extends from right to left horizontally, and following gel digestion with *Hin*fI, the fragments are separated vertically downward into a polyacrylamide gel and autoradiographed. To allow uniform comparisons of RLGS profiles from different samples and different laboratories, a coordinate system was established (http://pandora.med.ohiostate.edu/masterRLGS), which is superimposed on the profile. Each fragment is given a three-variable designation (Y coordinate, X coordinate, fragment number). The central region of the RLGS profile used for all comparisons described in this report has 28 sections (1 to 5 vertically and B to G horizontally; the 4G and 5G sections were excluded due to high density and lower resolution of fragments). The defined region contains 1576 fragments, of which 392 fragments were excluded from the analysis due to significant positional overlap with neighboring fragments or due to intensities reflecting greater than or less than diploid copy number on normal profiles. The less than diploid copy number intensities can result from polymorphism, partial methylation, or sex chromosome-derived fragments. Tumor and normal DNA profiles were compared by visual inspection of overlaid autoradiographs. Each CpG island was defined as unmethylated or methylated (a visually apparent decrease in intensity on the RLGS profile, which through corroboration with Southern blot data for 26 CpG island loci and more than 100 loss events, corresponded to a 30% or greater level of methylation). **B** Enlarged view of profile section 3C, showing the numbers assigned to each *Not*I fragment. Of the 210 clones, 184 clones were randomly chosen, and 26 corresponded to fragments that were frequently lost from tumor profiles. CpG islands have a GC content greater than 50% and a CpG value of 0.6 or greater, relative to bulk DNA (average GC content of 40% and CpG ratio of 0.2).[39] An average of 377 nucleotides per clone were analyzed (not indicative of actual CpG island size). The average *Not*I/*Eco*RV clone size was approximately 2 kb. [39]

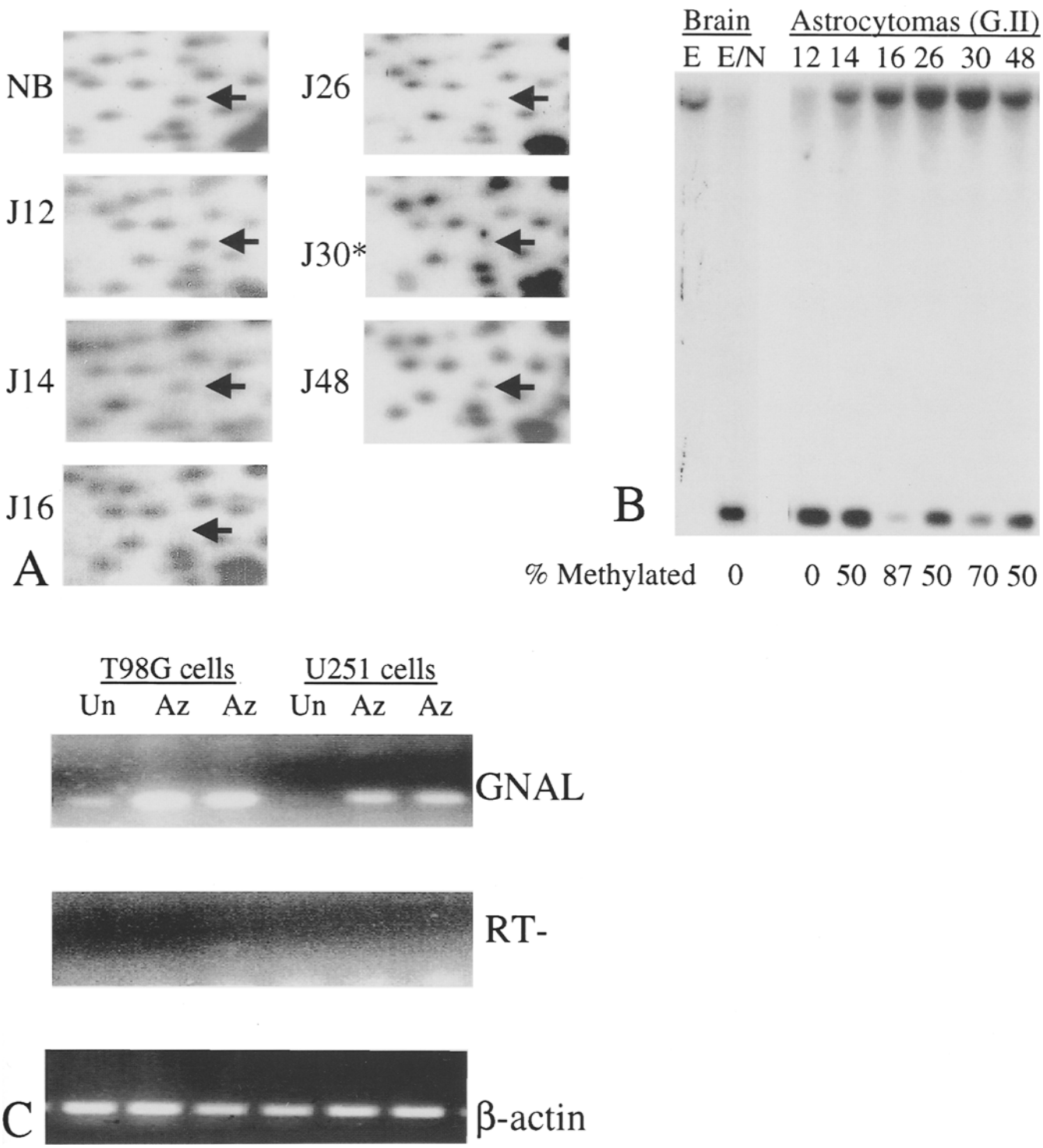

Fig. 2. Loss of *Not*I fragments from RLGS profiles of low-grade astrocytomas is due to methylation. **A** Enlarged view of RLGS profile within section 3C from normal brain (*NB*) and low-grade gliomas (*J12,J14,J16,J26,J30, and J48*). The *arrows* point to fragment 3C1, which is frequently diminished in intensity in the tumors. The *asterisk* in J30 indicates that an artifact spot is located just above the 3C1 fragment. **B** The fragment corresponding to 3C1 was cloned and shown to correspond to *GNAL*, encoding an alpha subunit of a G-protein. *GNAL* was used to probe a Southern blot of normal brain DNA digested with *Eco*RV only (*E*) or *Not*I/*Eco*RV (*E/N*) along with *Not*I/*Eco*RV digests of the tumor DNA from those samples presented in **A**. The probe detects a larger fragment (methylated), a smaller fragment (unmethylated), or both (partial methylation) upon double digestion with *Not*I and *Eco*RV. The ratio of the larger fragment intensity to total hybridization is a measure of the percentage of methylation in the sample. **C** Reverse transcription (RT)-PCR analysis of *GNAL* expression in two glioma cell lines (*T98*, *U251*). *Un*, untreated parental cells; *Az*, cells treated in duplicate with 1 μM 5-aza-2-deoxycytidine for 3 days. Control PCR reactions were also performed using the RT reaction lacking the reverse transcriptase and *GNAL*-specific primers (*middle panel*), and RT reaction with β-actin-specific primers (*lower panel*)

of most low-grade astrocytomas and may be involved in the early stages of brain tumor development.

There were certain CpG island targets that were methylated in a single tumor and other islands that were methylated in multiple tumors (Table 2). For example, 56 of the CpG islands were methylated in only 1 of the 14 tumors, the same 4 CpG islands were methylated in 10 of the 14 tumors, and none of the CpG islands were methylated in 14 of 14 tumors. Statistical analysis of these data indicated that these patterns are nonrandom.[20] This preferential methylation suggests that mechanisms such as growth selection pressures and/or differential susceptibilities of individual CpG islands may influence the patterns in the tumors.

Table 2. Frequency distribution of methylated CpG islands in astrocytomas

Number of methylated CpG islands (1184 tested)	Number of tumors showing methylation at the particular CpG islands
56	1 of 14
42	2 of 14
17	3 of 14
19	4 of 14
13	5 of 14
15	6 of 14
7	7 of 14
6	8 of 14
3	9 of 14
4	10 of 14
1	11 of 14
1	12 of 14
0	13 of 14
0	14 of 14

We compared the methylation patterns seen in low-grade astrocytomas to those of 84 additional tumors from 6 tumor types.[20] Some targets were affected at a high frequency in astrocytomas but not any of the other tumor types (astrocytoma-specific targets), whereas other methylation targets were shared by multiple tumor types. Table 3 displays examples of both shared and astrocytoma-specific CpG islands, which have been isolated and further analyzed for the presence of genes.

RT-PCR analysis showed that 14 of the 15 known genes or ESTs are normally expressed in adult brain (Table 3). Aberrant methylation of CpG islands containing the promoters of cancer-related genes is often associated with transcriptional inactivation. To determine whether the methylation events reported here affect transcription, RT-PCR analysis was performed. Expression of these genes was either variable or uniform across five randomly selected human glioma cell lines, and for several genes, expression was not detectable in certain cell lines.[20] To determine if the lack of transcription of these genes could be influenced by aberrant methylation, we treated the glioma cell lines with the demethylating agent 5-aza-2′-deoxycytidine. Expression of 6 of 16 genes (38%) was fully or partially reactivated following drug treatment, indicating that methylation regulated expression of these genes. Interestingly, *POU3F1* and *SIM2* are important for normal differentiation of glial cells. Silencing of methylation-regulated genes was associated with partial or complete methylation of the *Not*I site. Expression of 10 of 16 genes (63%) was unaffected by the demethylation treatment in all five cell lines, regardless of methylation status, constituting methylation-insensitive expression. These data suggest that a sizable proportion of the aberrant methylation events affect transcription.

Table 3. Shared and astrocytoma-specific methylation targets

CpG island	Gene	Methylation pattern in tumor types	Methylation frequency in tumors (%)	Expression in normal tissue	
				Fetal brain	Adult brain
2F50	*POU3F1*	Shared	13	+	+
3C1	*GNAL*	Shared	67	+	+
4D47	ESTs	Shared	53	−	+
4D50	EST	Shared	60	+	+
5E33	*SIM2*	Astrocytoma-specific[a]	33	+	+
5E34	*WIT1*	Shared	13	−	−
2C24	ESTs	Shared	47	+	+
2D20	ESTs[b]	Shared	47	+	+
3C32	ESTs	Astrocytoma-specific	40	+	+
3D41	ESTs[c]	Shared	33	+	+
3D48	ESTs	Astrocytoma-specific	40	+	+
3E3	ESTs	Astrocytoma-specific	13	+	+
3G49	*RPA2*	Astrocytoma-specific	33	+	+
4D12	ESTs	Shared	53	+	+
5C8	ESTs	Shared	33	+	+

[a] Astrocytoma-specific indicates methylation observed in astrocytomas but not in a total of 84 tumors of 6 other tumor types, including leukemias, primitive neuroectodermal tumors, and tumors of the breast, head and neck, colon, and testis
[b] EST has region of homology to *OTX1*
[c] EST has region of homology to *Na/I Symporter*
− Absence of RT-PCR product
+ Presence of RT-PCR product

Discussion

We have surveyed 1184 normally unmethylated *Not*I sites in 14 primary low-grade astrocytomas for a total of 16,576 possible aberrant methylation events. These data provide implications for understanding the mechanism(s) underlying CpG island methylation patterns observed in human tumors and the molecular pathways by which human astrocytomas arise. We propose that the descriptions of molecular defects in astrocytomas have been biased by the exclusively genetic screening methods used, and that their quality and number may be substantially underestimated.

Our gene cloning and expression analysis of some of the targets of shared and tumor type-specific CpG island methylation suggests that a proportion of the genes are silenced by methylation, consistent with numerous reports showing that methylation of 5′ CpG islands can lead to gene silencing.[3,8,28–32] The precise location and density of methylation can determine whether gene expression is silenced or unaffected. Since the methylation-regulated genes identified from primary astrocytomas include genes that are normally expressed in adult brain and that are important for normal glial cell differentiation (POU3F1[33] and SIM2[34]) and for signal transduction (GNAL[35]) in other organisms, it seems reasonable to propose that their methylation would have consequences for the tumor cell.

The genome-wide CpG island methylation may reflect a breakdown in the mechanisms that rigorously preserve CpG islands in an unmethylated state, a deregulation of methylating enzymes, or a cellular response to endogenous or exogenous signals. The potential genome-wide impact of CpG island methylation on chromatin structure and gene expression may play a significant role in modulating the tumorigenic potential of a cell. Thus, these alterations may be an integral part of the molecular pathways by which astrocytomas arise.

In contrast to the methylation-regulated genes, expression of a significant proportion of genes in our study was unaffected by demethylation treatment. It is possible that these methylation events have longer-range effects on chromatin or indirect effects on transcription of distant genes. The high frequency of aberrant methylation of these sites in human tumors could also result from an increased susceptibility or exposure of these sites to the methylation machinery in a background of methylation instability, rather than from a selection pressure. It is also possible that the consequence of the methylation within some of these genes is unrelated to inhibition of transcription.

Methylation appears to be the predominant mechanism acting on the gene-associated CpG islands studied here and in our previous reports,[20,21,27] and can be compared to genes and chromosome regions identified by traditional genetic screening methods such as comparative genome hybridization,[36] LOH studies,[37] or cytogenetics.[38] The distribution of imbalances detected by both genetic screens[38] and our CpG island methylation screens is nonrandom; there is considerable overlap in the imbalance profiles of different cancer types and frequencies of alteration at particular loci that display tumor type specificity. Virtually all chromosomal bands have been implicated in genetic loss within individual tumor types,[38] whereas in the limited number of tumors studied here, we detected methylation at 36% of the CpG islands tested.[20] Clearly, both genetic and epigenetic alterations have "background" alterations that may reflect an unstable genetic and epigenetic state of the tumor cell. The recurrent sites of genetic alteration often identify regions that harbor tumor suppressor genes and/or represent chromosomal fragile sites. Many, but not all, of the frequently methylated CpG islands for which chromosomal localizations have been determined do not map near regions of recurrent genetic loss in the same tumor type, suggesting that many of these targets are independent of recurrent genetic alterations. This is underscored by the fact that genetic analyses of low-grade astrocytomas show that a significant proportion of these tumors have relatively normal-appearing genomes,[2] whereas our epigenetic studies indicate that methylation is frequent and widespread, involving hundreds to thousands of genes. This raises important questions regarding the relative contribution of genetic and epigenetic mechanisms in the genesis of human astrocytomas.

Acknowledgments We would like to thank M.S. Berger, A. Asai, A. Tamura, and N. Shitara for glioma samples, and Y. Hayashizaki and the late V. Chapman for advice and encouragement. J.F.C. was supported sequentially by a Basic Science Fellowship from the American Association for Cancer Research and by a Frances Goodrich and Albert Hackett Postdoctoral Fellowship from the American Brain Tumor Association.

References

1. Kleihues P, Ohgaki H (1999) Primary and secondary glioblastoma: from concept to clinical diagnosis. Neuro-Oncology 1:44–51
2. Cavenee WK, Furnari FB, Nagane M, et al. (2000) Diffusely infiltrating astrocytomas. In: Cavenee WK, Kleihues P (eds) Tumours of the nervous system. IARC Press, Lyon, pp 9–52
3. Baylin SB, Herman JG, Graff JR, et al. (1998) Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res 72:141–196
4. Jones PA, Laird PW (1999) Cancer epigenetics comes of age. Nature Gene 21:163–167
5. Gama-Sosa MA, Slagel VA, Trewyn RW, et al. (1983) The 5-methylcytosine content of DNA from human tumors. Nucl Acids Res 11:6883–6894
6. Chen RZ, Pettersson U, Beard C, et al. (1998) DNA hypomethylation leads to elevated mutation rates. Nature 395:89–93
7. Stirzaker C, Millar DS, Paul CL, et al. (1997) Extensive DNA methylation spanning the Rb promoter in retinoblastoma tumors. Cancer Res 57:2229–2237
8. Herman JG, Latif F, Weng Y, et al. (1994) Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. Proc Nat Acad Sci USA 91:9700–9704
9. Kane MF, Loda M, Gaida GM, et al. (1997) Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. Cancer Res 57:808–811
10. Costello JF, Futscher BW, Kroes RA, et al. (1994) Methylation-related chromatin structure is associated with exclusion of transcription factors from and suppressed expression of the O-6-methylguanine DNA methyltransferase gene in human glioma cell lines. Mol Cell Bio 14:6515–6521

11. Li Q, Ahuja N, Burger PC, et al. (1999) Methylation and silencing of the thrombospondin-1 promoter in human cancer. Oncogene 18:3284–3289
12. Graff JR, Herman JG, Lapidus RG, et al. (1995) E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. Cancer Res 55:5195–5199
13. Antequera F, Bird A (1993) Number of CpG islands and genes in human and mouse. Proc Nat Acad USA 90:11995–11999
14. Hatada I, Hayashizaki Y, Hirotsune S, et al. (1991) A genomic scanning method for higher organisms using restriction sites as landmarks. Proc Nat Acad Sci USA 88:9523–9527
15. Lindsay S, Bird AP (1987) Use of restriction enzymes to detect potential gene sequences in mammalian DNA. Nature 327:336–338
16. Plass C, Shibata H, Kalcheva I, et al. (1996) Identification of Grf1 on mouse chromosome 9 as an imprinted gene by RLGS-M. Nature Genetics 14:106–109
17. Hayashizaki Y, Shibata H, Hirotsune S, et al. (1994) Identification of an imprinted U2af binding protein related sequence on mouse chromosome 11 using the RLGS method. Nature Genet 6:33–40
18. Costello JF, Plass C, Arap W, et al. (1997) Cyclin-dependent kinase 6 (CDK6) amplification in human gliomas identified using two-dimensional separation of genomic DNA. Cancer Res 57:1250–1254
19. Frühwald MC, O'Dorisio MS, Rush L, et al. (2000) Gene amplification in PNET/medulloblastoma: mapping of a novel amplified gene within the MYCN amplicon. J Med Genet 37:501–509
20. Costello JF, Fruhwald MC, Smiraglia DJ, et al. (2000) Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. Nature Genet 24:132–138
21. Plass C, Yu F, Yu L, et al. (1999) Restriction landmark genome scanning for aberrant methylation in primary refractory and relapsed acute myeloid leukemia; involvement of the WIT-1 gene. Oncogene 18:3159–3165
22. Akama TO, Okazaki Y, Ito M, et al. (1997) Restriction landmark genomic scanning (RLGS-M)-based genome-wide scanning of mouse liver tumors for alterations in DNA methylation status. Cancer Res 57:3294–3299
23. Yoshikawa H, de la Monte S, Nagai H, et al. (1996) Chromosomal assignment of human genomic *Not*I restriction fragments in a two-dimensional electrophoresis profile. Genomics 31:28–35
24. Graff JR, Herman JG, Myöhänen S, et al. (1997) Mapping patterns of CpG island methylation in normal and neoplastic cells implicates both upstream and downstream regions in de novo methylation. J Biol Chem 272:22322–22329
25. Graff JR, Greenberg VE, Herman JG, et al. (1998) Distinct patterns of E-cadherin CpG island methylation in papillary, follicular, Hurthle's cell, and poorly differentiated human thyroid carcinoma. Cancer Res 58:2063–2066
26. Plass C, Weichenhan D, Catanese J, et al. (1997) An arrayed human *Not*I-*Eco*RV boundary library as a tool for RLGS spot analysis. DNA Res 4:253–255
27. Smiraglia DJ, Frühwald MC, Costello JF, et al. (1999) A new tool for the rapid cloning of amplified and hypermethylated human DNA sequences from restriction landmark genomic scanning gels. Genomics 58:254–262
28. Bachman KE, Herman JG, Corn PG, et al. (1999) Methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene suggests a suppressor role in kidney, brain, and other human cancers. Cancer Res 59:798–802
29. Merlo A, Herman JG, Mao L, et al. (1995) 5′ CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nature Med 1:686–692
30. Myöhânen SK, Baylin SB, Herman JG (1998) Hypermethylation can selectively silence individual p16ink4A alleles in neoplasia. Cancer Res 58:591–593
31. Nan X, Tate P, Li E, et al. (1996) DNA methylation specifies chromosomal localization of MeCP2. Mol Cell Biol 16:414–421
32. Cameron EE, Bachman KE, Myöhänen S, et al. (1999) Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nature Genet 21:103–107
33. Bermingham JR Jr, Scherer SS, O'Connell S, et al. (1996) Tst-1/Oct-6/SCIP regulates a unique step in peripheral myelination and is required for normal respiration. Genes Dev 10:1751–1762
34. Nambu JR, Franks RG, Hu S, et al. (1990) The single-minded gene of Drosophila is required for the expression of genes important for the development of CNS midline cells. Cell 63:63–75
35. Offermanns S, Simon MI (1996) Organization of transmembrane signalling by heterotrimeric G proteins. Cancer Surv 27:177–198
36. Kallioniemi A, Kallioniemi OP, Sudar D, et al. (1992) comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science 258:818–821
37. Cavenee WK, Dryja TP, Phillips RA, et al. (1983) Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. Nature 305:779–784
38. Mertens F, Johansson B, Höglund M, et al. (1997) Chromosomal imbalance maps of malignant solid tumors: a cytogenetic survey of 3185 neoplasms. Cancer Res 57:2765–2780
39. Gardiner-Garden M, Frommer M (1987) CpG islands in vertebrate genomes. J Mol Biol 196:261–282