Biallelic Epigenetic Inactivation of the RASSF1A Tumor Suppressor Gene in Medulloblastoma Development

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ABSTRACT

Epigenetic inactivation of the RASSF1A tumor suppressor gene (TSG) at chromosome 3p21.3 was examined in medulloblastoma, the most common malignant brain tumor of childhood. Seventy-nine % (27 of 34) of primary tumors and 100% (8 of 8) of medulloblastoma cell lines displayed extensive tumor-specific DNA hypermethylation across the RASSF1A promoter-associated CpG island. Hypermethylation was associated with epigenetic silencing of RASSF1A transcription in medulloblastoma cell lines, and RASSF1A expression in these lines was restored after treatment with the DNA-methyltransferase inhibitor 5-aza-2′-deoxycytidine. No evidence was found of RASSF1A inactivation by genetic mechanisms (gene mutation or deletion) in either cases with no evidence of RASSF1A hypermethylation or paired normal/tumor cases and cell lines with evidence of total RASSF1A CpG island hypermethylation. Epigenetic inactivation by biallelic hypermethylation therefore represents the primary mechanism of RASSF1A gene inactivation in medulloblastoma. Furthermore, RASSF1A hypermethylation is a frequent event in medulloblastoma tumorigenesis detectable in adult (5 of 7) and pediatric patients (22 of 27) and in all histological variants and age and sex groupings. Importantly, these data demonstrate that comprehensive analysis of the genome and epigenome will be required for identification of the key tumor suppressor genes involved in medulloblastoma development.

INTRODUCTION

Medulloblastoma is an invasive embryonal tumor of the cerebellum that represents the most common malignant brain tumor of childhood. Despite recent improvements in treatment, 5-year survival rates stand at 50–60% with significant rates of long-term intellectual and neuroendocrine damage associated with current therapies. Current therapeutic classification systems are of little value in clinical management, and the accurate assessment of disease risk among children with medulloblastoma remains a major aim in the field of pediatric neurooncology (1).

An improvement in understanding of the molecular basis of medulloblastoma development may lead to improved therapies through the identification of novel prognostic indicators and therapeutic targets. Cyogenetic and molecular genetic studies have described a number of consistently observed, nonrandom chromosomal aberrations including isochromosome 17q, 17p loss, 11p loss, and 10q loss (1). Despite this, few consistent observed, nonrandom chromosomal aberrations including isochromosome 17q, 17p loss, 11p loss, and 10q loss (1). Despite this, few consistently observed, nonrandom chromosomal aberrations including isochromosome 17q, 17p loss, 11p loss, and 10q loss (1). Despite this, few consistently observed, nonrandom chromosomal aberrations including isochromosome 17q, 17p loss, 11p loss, and 10q loss (1).

A cohort of 34 primary medulloblastomas were analyzed (23 male and 11 female). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: TSG, tumor suppressor gene; CNS, central nervous system; CpG, 5′-CpG-3′ dinucleotide; COBRA, combined bisulfite and restriction analysis; 5-AzaC, 5-aza-2′-deoxycytidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
female), comprising 27 pediatric (mean age, 6.1 years; range, 1–11 years) and 7 adult cases. Six cases had nodular/desmoplastic, 3 anaplastic/large cell, and 25 classic histology. Surgical biopsies from nonneoplastic conditions of the cerebrum and cerebellum including examples of gliosis and vascular malformation comprised a series of control tissue samples \( n = 9 \). A panel of constitutional DNA samples from 50 normal individuals was obtained from the North Cumbria Community Genetics Project. DNA was extracted from formalin-fixed, paraffin-embedded sections using a Nucleon hard tissue kit (Nucleon Biosciences). Peripheral blood samples were available for 8 of the medulloblastoma patients, and DNA from these patients was prepared using a Nucleon II kit (Nucleon Biosciences).

**Analysis of RASSF1A Promoter Methylation Status.** Promoter methylation status was assessed after bisulfite DNA treatment, which converts unmethylated but not methylated cytosine to uracil. Methylated and unmethylated cytosines at a given position can then be distinguished either by sequencing or digestion using a restriction enzyme containing a CpG in its recognition sequence. Bisulfite DNA treatment was carried out using a CpG genome DNA modification kit (Intergen Co.) according to the manufacturer’s instructions. Amplification of bisulfite-treated DNA was carried out using the primers ML561, MU379, and ML730, as described by Dammann et al. (9). Control unmethylated and fully methylated DNA consisted of normal peripheral blood DNA, and this DNA was methylated in vitro by SssI methylase (New England Biolabs), respectively. PCR products were purified using a QIAquick PCR purification kit (Qiagen Ltd.). Initial COBRA was performed as described by Lo et al. (11) and Agathanggelou et al. (13) by overnight digestion at 65°C with TaqI (MBI Fermentas). TaqI cuts twice within the 204-bp PCR product if methyl-cytosine is contained within its restriction sites, generating products of 92, 81, and 31 bp on complete digestion or partially digested bands of 173 and 112 bp. Digested PCR products were separated on a 4% NuSieve 3:1 agarose gel in 1 X TBE (0.09 M Tris-Borate, 0.002 M EDTA (pH 9))). Products were directly sequenced with a CEQ DTC5 kit (Beckman Coulter) using the primer ML561 to obtain the reverse sequence and the primer 5’-TGGATTAATGATGTTAGT-3’ to obtain the forward sequence. Sequenced products were analyzed on a CEQ 2000XL DNA analysis system (Beckman Coulter). The methylation status at each Cpg residue was determined by assessment of the relative intensities of methylated (C/G) and unmethylated (T/A) peak heights on forward and reverse sequences.

**Loss of Heterozygosity Analysis.** Loss of heterozygosity at 3p21.3 was assessed using polymorphic microsatellite markers contained within the cosmids LUCA8.1 and LUCA11.1 (Accession ID: GDB9865466 and GDB9865469 respectively). The forward primer from each set was end-labeled with a fluorescent Beckman Dye (Invitrogen). PCR conditions were 94°C for 1 min, 50°C (LUCA 8.1) or 52°C (LUCA11.1) for 1 min, and 72°C for 1 min for 35 cycles. Size markers (Beckman Coulter) were added to a 1:10 dilution of the PCR products, which were then run and analyzed on a CEQ 2000XL DNA analysis system.

**Mutation Screening.** Exons were amplified using primer sets and amplification conditions described previously by Lo et al. (11) with the exception of exon 1, which was amplified as a single product (478 bp) using primers 5’-CTGGGAGAGGGCTGGAATG-3’ (forward) and 5’-CTCTCTGCGC- GACCTGACC-3’ (reverse). Amplification conditions were 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, for 35 cycles. PCR products were analyzed for heteroduplexes using high performance liquid chromatography (Transgenomic Wave DNA Fragment Analysis System), according to the manufacturer’s instructions. PCR products detected as containing a heteroduplex were sequenced directly on a CEQ 2000XL DNA analysis system.

**Reexpression of RASSF1A by 5-Aza CdR Treatment.** Three cell lines (MED-8A, Daoy, and D283 Med) were grown in the presence or absence of 5-Aza CdR (5 μM) for 4 days. Medium was renewed daily. HeLa cells were also grown as a positive control for RASSF1A expression (9). RNA was extracted from 107 cells using an RNeasy kit (Qiagen Ltd.). RNA concentration was determined by spectrophotometry, and agarose gel electrophoresis was used to confirm consistent RNA integrity and quantity from each cell line. One μg of total RNA was used to synthesize cDNA using a reverse transcription system (Promega). cDNA was synthesized using random primers and oligo-d(T) primers in separate reactions, followed by pooling. cDNA was used for PCR amplification using the following primer sets and conditions:

(a) Amplification of RASSF1A and RASSF1C transcripts: primers used were described previously by Dammann et al. (9) with the lower primer for each being in the common exon 4, the upper primer for RASSF1A in exon 2 αβ, and the upper primer for RASSF1C in exon 2 γ. PCR conditions were 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, for 35 cycles for RASSF1A and 30 cycles for RASSF1C. Product sizes were 242 and 272 bp, respectively.

(b) Amplification of control transcripts: β-actin PCR was carried out using primers BA67 and BA68 to generate a 232-bp fragment (19). PCR conditions were 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, for 25 cycles. GAPDH primers (20) generated a 369-bp fragment. PCR conditions were 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min for 25 cycles. PCR products were separated on 2.5% agarose gels in 1 X TBE. PCR results shown were reproducible over multiple replicates.

**RESULTS**

**Epigenetic Inactivation of RASSF1A in Medulloblastoma Cell Lines.** We first determined the hypermethylation status of the RASSF1A promoter-associated CpG island in a panel of eight medulloblastoma cell lines. Initial analyses using a COBRA method (TaqI digestion, which cuts twice within the CpG island if methylcytosine is contained within its restriction sites) detected the presence of hypermethylated CpG residues within the RASSF1A CpG island of all eight lines (Fig. 1A). Further detailed analysis of CpG methylation status by direct sequencing revealed intensive hypermethylation across the entire CpG island in all lines (Fig. 1B). All 16 CpG sites were either partially or fully methylated in all eight cell lines, indicating that RASSF1A promoter hypermethylation is a common event in medulloblastoma cell lines.

To establish the functional significance of the promoter hypermethylation observed, we investigated the expression of RASSF1A and RASSF1C transcripts by reverse transcription-PCR in three of our cell lines (Daoy, D283 Med, and MED-8A). RASSF1C is an alternatively spliced transcript of the RASSF1 gene, which arises from an alternative promoter distal to the RASSF1A CpG island (9). Expression of RASSF1A was absent (D283 Med and MED-8A) or reduced (Daoy) in these cell lines, correlating with promoter hypermethylation status. Expression of the RASSF1A transcript was restored in each line after treatment with the demethylating agent 5-Aza CdR, confirming that promoter hypermethylation is responsible for the reduced expression levels observed (Fig. 1C). We found no evidence of transcriptional silencing of RASSF1C. Equivalent expression levels were observed for RASSF1C, β-actin, and GAPDH control transcripts in all cell lines and experimental conditions tested, demonstrating that hypermethylation at the RASSF1 locus is specific to the RASSF1A CpG island in medulloblastoma and consistent with reports from other adult tumor types (9, 10, 14, 16).

**RASSF1A Promoter Hypermethylation in Medulloblastoma Tumor Samples.** We next sought to determine the incidence and nature of RASSF1A promoter hypermethylation in a series of 34 primary tumor samples (see “Materials and Methods” for cohort details). After bisulfite treatment, samples were analyzed initially using the COBRA/TaqI digestion method. Twenty-seven of 34 cases (79%) showed digestion with TaqI, suggesting promoter hypermethylation. A more detailed examination of hypermethylation patterns across the entire CpG island by direct sequencing was successful for 31 of the samples. For COBRA-positive cases \( n = 27 \), a striking pattern of dense hypermethylation across the entire CpG island was revealed (Fig. 2). All of these cases showed partial or total methylation of \( ≥15 \) of 16 CpG sites, with 8 tumors demonstrating 100% methylation at all CpG sites. COBRA-negative cases \( n = 7 \) were uniformly demonstrated to be unmethylated across the entire island.

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\(^{1}\) Internet address: http://www.gdb.org.
showing no evidence of methylation at 15 of the 16 CpG sites. This concordance between results from COBRA and direct sequencing assays of hypermethylation status (see Fig. 2) demonstrates that, although COBRA examines only 2 of 16 CpG sites, it is a rapid and reliable test for RASSF1A promoter methylation status in medulloblastoma cases.

To determine whether RASSF1A promoter hypermethylation is a tumor-specific event in medulloblastoma, 9 control nonneoplastic brain samples were examined. Eight of 9 cases showed no evidence of RASSF1A methylation by either COBRA or direct sequencing analysis (Fig. 2), suggesting that RASSF1A promoter hypermethylation is tumor specific. A single sample (N9, Fig. 2) showed methylation of 11 of 16 CpG sites, and the significance of this observation remains unclear. The pattern of methylation in this sample was distinct from that seen in tumors with promoter hypermethylation, where at least 15 of 16 sites show methylation, and raises the possibility that methylation at certain CpG sites could be compatible with a functional level of expression.

A preliminary assessment of clinical and pathological data showed that hypermethylation of the RASSF1A promoter is observed in both adult (5 of 7) and pediatric patients (22 of 27) and in all histological variants; nodular/desmoplastic (4 of 6), anaplastic-large cell (3 of 3), and classic (20 of 25). No further relationships were apparent between methylation status and patient age or sex (data not shown).

Mechanisms of RASSF1A Gene Inactivation in Medulloblastoma. To investigate the relative contributions of genetic and epigenetic mechanisms to RASSF1A gene inactivation in medulloblastoma, we next looked for evidence of RASSF1A gene mutation and deletion alongside hypermethylation analysis in 8 tumor samples for which paired tumor and constitutional (blood) DNA samples were available and in our panel of eight cell lines.

Gene deletion was assessed by PCR-based loss of heterozygosity analysis using two polymorphic microsatellite markers, LUCA8.1 and LUCA11.1, which lie approximately 140 and 60 kb upstream of the RASSF1A locus, respectively. No evidence of loss of heterozygosity was seen for any of the paired tumor samples (Fig. 3). Where markers were informative, allelic ratios were equivalent (<15%) in both tumor and normal DNA samples. Likewise, all eight cell lines were heterozygous for at least one marker, suggesting that deletion of this region is uncommon in medulloblastoma. These data are consistent with our recent comparative genomic hybridization study, which demonstrated that chromosome 3p loss is not a common feature of medulloblastoma (21).

No genetic mutations in the RASSF1A coding region were found.
in any of the paired tumor or cell line samples analyzed, or additionally, in the six further tumors that displayed no evidence of RASSF1A promoter hypermethylation. The previously reported exon 3 variation G435T (ALA133SER, see Refs. 11, 13, 17; Fig. 3) was found in one methylated paired tumor sample (T42) and two unmethylated tumor samples (T7 and T27). This change was also detected in a matched blood DNA sample (for T42) and in 9 of 100 chromosomes analyzed from our normal panel of constitutional DNA, indicating that this variation represents a reasonably frequent polymorphism. Equivalent frequencies of this polymorphism were observed in medulloblastoma patients (3 of 42 chromosomes) and normal controls (9 of 100 chromosomes), suggesting that it is unlikely to be associated with medulloblastoma predisposition. RASSF1A hypermethylation, gene deletion, and mutation analyses for our paired samples and cell lines are summarized in Fig. 3. For tumors where methylation was detected, 100% hypermethylation was frequently observed at multiple CpG residues across the RASSF1A CpG island (see Figs. 2 and Fig. 3, samples T28, T39, T41, and T42). Although we cannot exclude the presence of smaller or intragenic deletions, these levels of hypermethylation coupled with the heterozygosity detected at the RASSF1 region and the lack of evidence for tumor-specific mutations observed in these cases are consistent with biallelic hypermethylation of the RASSF1A promoter. Epigenetic inactivation of RASSF1A by biallelic promoter hypermethylation (in the absence of gene deletion or mutation), therefore, appears to represent the primary mechanism of RASSF1A gene inactivation during medulloblastoma development.

**DISCUSSION**

In this study, we have demonstrated that hypermethylation of the RASSF1A promoter occurs at high frequency in medulloblastomas (~80% of cases) and causes epigenetic silencing of RASSF1A gene transcription in medulloblastoma cell lines. RASSF1A hypermethylation defines medulloblastomas of all histopathological subtypes and age and sex groupings. Moreover, RASSF1A inactivation represents the most common genetic or epigenetic defect identified to date in medulloblastoma development.

Epigenetic inactivation of RASSF1A has been shown recently to play a role in adult malignancies including lung, breast, nasopharyngeal, bladder, prostatic, and renal cell carcinomas (9–17). A recent study in neuroblastoma (a childhood tumor of the peripheral nervous system) provided an initial precedent for a role for RASSF1A in pediatric malignancies (22). Our results extend these findings to define a role in CNS tumors and, in view of the frequency of inactivation observed in medulloblastomas, highlight the need to determine any wider involvement in pediatric tumors, in particular those arising from the CNS (e.g., ependymoma and astrocytoma).

Medulloblastomas are genetically distinct from most tumor types with evidence of RASSF1A inactivation, which display frequent allelic losses at the RASSF1A locus at chromosome 3p21.3 alongside RASSF1A promoter hypermethylation (e.g., lung, breast, nasopharyngeal, bladder, prostatic, and renal cell carcinomas and neuroblastoma; Refs. 9–17, 22). However, in medulloblastomas, we found no evidence of specific allelic losses involving the RASSF1 region and only rare general losses involving chromosome 3p (by comparative
genomic hybridization analysis; Ref. 21). In common with other investigators, we found no inactivating mutations in the RASSF1A coding region, suggesting that mutational inactivation is also infrequent. In contrast, multiple cases showed complete methylation of all CpG sites in the absence of gene mutation or deletion, indicating that biallelic epigenetic inactivation represents the primary mechanism of RASSF1A inactivation during medulloblastoma development. This provides an important precedent for RASSF1A inactivation by biallelic hypermethylation in tumor types that do not display chromosome 3p abnormalities. Our data further support the revision of Knudson’s two-hit hypothesis to take into account epigenetic mechanisms of gene inactivation alongside genetic events and show that both copies of a TSG may be inactivated epigenetically (23, 24). More broadly, these findings show that the identification of the critical TSG events in medulloblastoma tumorigenesis will require comprehensive analysis of the epigenome alongside genetic investigations.

Our studies in cell lines demonstrate that hypermethylation at the RASSF1 locus in medulloblastoma is specific to RASSF1A and does not affect expression of the RASSF1C transcript. Epigenetic gene inactivation has not been studied widely in medulloblastoma. However, a recent report demonstrated hypermethylation of the caspase-8 promoter region occurs in 55% of primary primitive neuroectodermal tumor/medulloblastomas (25), and Fruhwald et al. (26) analyzed the promoter methylation status in medulloblastoma of five candidate genes, which are methylated in other tumor types (p16INK4a, von Hippel-Lindau, TP53, E-Cadherin, and p16INK4A/536) but demonstrated only low level methylation (10–15%) of E-Cadherin and p16INK4A/536. Taken together with our data, these findings suggest that RASSF1A methylation is likely to be a specific event in medulloblastoma and do not support the existence of a common “CpG hypermethylator” phenotype affecting multiple genes in this tumor type.

It will now be important to evaluate the utility of RASSF1A promoter hypermethylation as a marker of clinicopathological disease features and its potential for use in the assessment of disease risk/prognosis and patient stratification in a larger cohort of primary medulloblastoma samples taken from uniformly treated cases. In addition, the parallel analysis of the methylation status of further candidate genes in medulloblastoma should give insights into patterns of promoter hypermethylation in medulloblastoma, as well as potentially allowing the assessment of multitgene methylation status predictors for similar purposes.

Although a role for the RASSF1A gene product in the suppression of tumorigenicity has been clearly demonstrated in different tumor cell types (9, 16, 17), the normal intracellular role(s) of RASSF1A and the functional consequences of its inactivation during tumorigenesis remain unclear. RASSF1A encodes a 340-amino acid protein that is predicted to contain a distal Ras-association domain (9). Initial evidence has suggested that the RASSF1C protein may function as a Ras effector and play a role in Ras-dependent apoptosis (27); however, any role for RASSF1A in Ras-dependent pathways requires investigation and clarification. RASSF1A also contains a predicted NH2-terminal diacylglycerol binding domain and a PEST domain, which are commonly found in proteins regulated by ubiquitin-mediated proteolysis (9, 17). This PEST domain contains a phospho-serine residue phosphorylated in vitro by ataxia telangiectasia mutated and related protein kinases (28). Further studies are clearly necessary to elucidate RASSF1A function, and studies are under way to determine its role in medulloblastoma tumorigenesis. Notably, many tumors analyzed in our study showed variable levels of methylation at individual CpG sites, and greater insights into RASSF1A function should allow a better understanding of the level and pattern of promoter hypermethylation consistent with gene inactivation and downstream consequences.

The widespread involvement of RASSF1A inactivation in the development of medulloblastoma (~80% of cases), coupled with its role in multiple other tumor types, highlights its putative functional consequences as potential targets for the development of novel therapeutic strategies once they are better understood. Moreover, unlike genetic alterations which lead to TSG inactivation, TSG inactivation by promoter hypermethylation is a reversible epigenetic event. RASSF1A inactivation in medulloblastoma therefore represents a target for therapeutic strategies aimed at restoration of gene function, potentially involving treatment with either demethylating agents such as 5-Aza CdR or novel less mutagenic inhibitors of DNA methyltransferase (29).

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