Analysis of DNA Mismatch Repair Proteins in Human Medulloblastoma

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ABSTRACT

During replication, the primary function of the eukaryotic DNA mismatch repair (MMR) system is to recognize and correct mismatched base pairs within the DNA helix. Deficiencies in MMR have been reported previously in cases of hereditary nonpolyposis colorectal cancer and sporadic tumors occurring in a variety of tissues including gliomas. Furthermore, recent evidence indicates that the MMR system may be involved in mediating therapeutic sensitivity to alkylating agents. In this study, 22 neoplastic tissue samples from 22 patients who underwent surgical resection for medulloblastoma, a common cerebellar tumor of childhood, were assayed for the presence or absence of MMR polypeptides using Western blot and immunohistochemical techniques. Results from these experiments indicate that the MMR system is not commonly deficient in medulloblastoma.

INTRODUCTION

The primary role of the DNA MMR system is to correct DNA polymerase errors produced during replication by recognizing and repairing mismatches in the DNA double helix. In Escherichia coli, four proteins have been identified as crucial to the MMR system: MutS, MutL, MutH, and MutU. MutS recognizes and binds to the mispaired base, MutL binds MutS, and MutH serves as an endonuclease by nicking at a GATC site of hemimethylated DNA on the unmethylated strand near a mismatch. That portion of the incised strand spanning the nick and the mismatch is excised in a reaction dependent on the MutU helicase, and the ensuing gap is repaired and ligated (1).

Proteins homologous to bacterial MutL and MutS have been identified in eukaryotes, and a strand-specific MMR system has been demonstrated in human cells (2). Both systems function similarly by protecting DNA from mutation and direct repair to the nascent strand of DNA. A major difference between the bacterial and eukaryotic MMR systems, however, is that the eukaryotic system contains multiple homologues for each bacterial component. For example, hMSH2, hMSH6, and hMSH3 are human homologues of the bacterial MutS protein. The hMSH2 and hMSH6 polypeptides form a heterodimer called MutSα, which binds to and participates in repair of base-base and insertion/deletion mismatches (3, 4). In addition, hMSH2 also forms another heterodimer with hMSH3, another MutS homologue. This complex known as MutSβ binds to insertion/deletion mispairs (4, 5).

Tumor-specific hypermutability caused by deficiency in MMR has been observed in HNPCC. According to genetic analysis, four key MMR genes are mutated in many HNPCC cells: hhMSH2, hMLH1, hhMSH6, and hPM2 (6–13). In addition to containing mutations in genes encoding MMR proteins, a subset of sporadic colon tumors and most tumors occurring in HNPCC patients exhibit instability in microsatellite repeats (14, 15).

Several hypotheses have been proposed to describe the mechanism by which deficiency in the human MMR system contributes to tumorigenesis. One interesting possibility is that cells deficient in MMR exhibit a mutator phenotype (16, 17) and potentially accumulate somatic mutations in the proto-oncogenes and tumor suppressor genes implicated in neoplastic formation. This hypothesis implies that mutation in these crucial genes need not result from gross chromosomal alterations such as inversions, deletions, or translocations. Another possibility is that cells deficient in MMR fail to arrest in the G2 phase of the cell cycle and proliferate uncontrollably when exposed to alkylating agents that induce DNA mismatches (18).

Colon cancers have been subdivided into subsets of tumors deficient in MMR. These neoplasms not only include those historically grouped as HNPCC tumors but also comprise a group of tumors from patients diagnosed with Muir-Torre syndrome and Turcot’s syndrome (19, 20). The Turcot’s syndrome patients can be divided into two subsets; those patients with loss of heterozygosity within the APC gene have a high incidence of medulloblastoma, whereas the patients with MMR deficiency have a relatively high incidence of glioma (21, 22).

Microsatellite instability, a phenotype that generally indicates a deficiency in MMR, has been observed in a variety of extracolonic neoplasms including brain tumors (23–25). Among the primary human brain tumors surveyed thus far, microsatellite alterations have been found in minor subsets of astrocytomas, gangliogliomas, meningiomas, schwannomas, pituitary adenomas, benign oligodendromas, and anaplastic...
Fig. 1 Western blots (A, B, and C) of medulloblastoma tissue nuclear extracts. Blots were consecutively incubated with hMSH2, followed by hPMS2 and hMSH2, followed by hMLH1. The polypeptides detected are as follows: row 1, hPMS2 and hMSH2; row 2, hMSH2 and hMLH1; row 3, α-tubulin.

oligodendromas, and in a small subset of medulloblastomas (23, 24). Blaeker et al. (24) also assayed medulloblastomas for microsatellite instability and found that zero of six tumors, three of which (TB 285 1D, TB 558 1F, and TB 637 1A) were assayed for MMR deficiency in the present study, exhibited microsatellite instability. In contrast, Friedman et al. (26) recently reported microsatellite alterations and MMR deficiency in a human glioblastoma multiforme xenograft with laboratory-generated resistance to procarbazine.

MMR deficiency has not been examined in medulloblastoma, a tumor characterized by malignant neuronal progenitor cells of the cerebellar vermis. Medulloblastoma has the capacity to spread to the cerebellar hemispheres and to the brain stem (27) and accounts for about 25% of all pediatric CNS tumors (28, 29). In this study, the presence or absence of full-length hMLH1, hMSH2, and hPMS2 polypeptides in the cells of 22 human medulloblastomas was determined using Western blot and immunohistochemical techniques to ascertain whether this type of neoplasm commonly shows a deficiency in MMR.

MATERIALS AND METHODS

Tissue Samples. We studied a series of 22 patients with medulloblastoma (19 newly diagnosed and 3 with recurrent tumors) surgically treated at five hospitals (Duke, Johns Hopkins, Charlotte, University of Alabama at Birmingham, and Roanoke) between 1983 and 1996. The mean age of these patients was 9.8 years, and 16 were younger than age 17 at the time of surgical resection. In addition, the three patients with recurrent tumors had received chemotherapy treatment prior to resection. Information pertaining to the stage, clinical course, and variant status of each tumor was unavailable. All tumors occurred sporadically. Tumor samples were cut in 1-cm³ sections, frozen in liquid nitrogen, and maintained at −170°C in the Duke Brain Tumor Tissue Bank until analyzed.

Western Blot Analysis. Crude nuclear fractions from medulloblastoma tumor sections were obtained by lysis in hypotonic buffer [20 mM HEPES (pH 7.5), 5 mM KCl, 0.5 mM MgCl₂, 0.1% phenylmethylsulfonyl fluoride saturated solution in isopropanol, 0.5 mM DTT, and 10 μg/ml leupeptin], and nuclear proteins were extracted with SDS buffer [60 mM Tris-Cl (pH 6.8), 100 mM DTT, and 2% SDS]. The amount of protein present in the samples was quantified using the Bradford protocol (30). Protein extracts (10 μg) were separated on reducing 7.5% SDS-polyacrylamide gels and then electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Polyvinylidene difluoride membranes were then incubated with primary mouse monoclonal IgG α-hMLH1 (Ab-1), α-hMSH2 (Ab-1), α-hPMS2 (Ab-1), or α-tubulin (Ab-1) (all from Oncogene) in TBS-T [0.8% NaCl, 20 mM Tris-Cl (pH 7.6), and 0.1% Tween 20] containing BLOTTO (5% nonfat milk in TBS-T), followed by horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham). The enhanced chemiluminescence (ECL) system (Amersham) was used to develop the protein signals on the membrane.

Immunohistochemistry. Immunoperoxidase staining was performed on formalin-fixed, paraffin-embedded tissue sections. Briefly, 5 μm tissue sections were mounted on Superfrost/Plus slides (Fisher), deparaffinized, rehydrated, and incubated with 0.6% H₂O₂ in absolute methanol to block endogenous peroxidase activity. Antigen retrieval was performed using the microwave citrate method (Citra, 600 W for 5 min × 2; Vector Laboratories) as described (31). Nonspecific protein binding was blocked by incubation with normal horse serum diluted 1:20 in 0.01 M PBS (pH 7.2–7.4; 10.4 mM Na₂HPO₄, 3.16 mM...
**RESULTS**

Western blots of medulloblastoma nuclear extracts indicate that all 22 tumor samples contained full-length hMLH1 ($M_1$, $85,000-90,000$), hMSH2 ($M_2$, $105,000$), and hPMS2 ($M_3$, $95,000-105,000$) polypeptides (Fig. 1). These proteins were also detected in normal human cerebellum found in Lane 1 of blots A, B, and C, respectively (Fig. 1). α-Tubulin antibody was used to determine that approximately equivalent amounts of protein were loaded in each lane.

Paraffin sections were available from 11 of the 22 tumors examined by Western blot analysis. Immunohistochemistry was performed on medulloblastoma tumor sections and human tonsil control tissue, using α-hMLH1 or α-hMSH2 mouse monoclonal antibodies, as well as the anti-nuclear antibody, α-Ki-67/MIB-1. Anti-hPMS2 was observed to be unreactive with any of the formalin-fixed, paraffin-embedded tissues. Fig. 2 shows the staining observed in human tonsil tissue and in three of the medulloblastomas using these antibodies. The staining pattern observed with α-Ki-67/MIB-1 indicated that 10-60% of the cells in the medulloblastoma sections were not in G$_0$. Immunohistochemistry confirmed the nuclear location of both MMR enzymes in a significant number of tumor cells in 10 of 11 cases. In one of the medulloblastoma sections (TB410 1D), only minor areas of tumor exhibited immunoreactivity to either hMLH1 or hMSH2, which was believed to be related to extensive surgical electrocautery artifact in the tissue.

**DISCUSSION**

Western and immunohistochemical studies were performed on 22 human medulloblastomas, 3 of which were obtained at recurrence. These studies were conducted to determine the presence or absence of MMR polypeptides in the tumor samples.
Our results indicate that the present immunohistochemical method is a less sensitive, although specific assay, because in one of the tumor samples, only a minor fraction of the cells was stained after reaction with MMR-specific antibodies, whereas the Western blot did not show an equivalent low level of the MMR polypeptides.

Treatment of children with medulloblastoma invariably includes chemotherapy with alkylating agents such as cyclophosphamide, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, and cisplatin. Medulloblastoma is considered to be a chemosensitive tumor; however, acquired drug resistance is commonly seen, particularly in patients with high-risk disease, and newer agents are needed for the treatment of this neoplasm (27).

Methylating agents have been reported in laboratory and clinical studies to be active against medulloblastoma. Procarbazine alone, or in combination with the Vinca alkaloid vincristine and nitrosourea 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, has demonstrated modest activity against medulloblastoma in Phase II clinical trials (33). Laboratory studies have also demonstrated the activity of the DNA-methylating agent temozolomide against a broad spectrum of human CNS tumors, including medulloblastoma, growing as s.c. xenografts in athymic nude mice (34). In addition, the Children’s Cancer Group and Pediatric Oncology Group have just opened a joint Phase II trial for children with recurrent CNS tumors, including medulloblastoma.

Resistance of neoplastic cells to methylators has been observed in a spectrum of neoplasms with only two mechanisms identified to date: (a) methylator resistance may arise from overexpression of AGT, an enzyme that dealkylates guanine nucleotides, thereby reducing the number of potentially cytotoxic lesions in the tumor cell DNA (35–38); and (b) methylator resistance may arise via MMR deficiency (18, 39). Methylator resistance mediated by MMR deficiency has been found in brain tumors in addition to colorectal and lymphoblastoid tumors. Friedman et al. (26) reported observing microsatellite alterations and MMR deficiency in a procarbazine-resistant glioblastoma multicentric xenograft showing an absence of AGT activity. All methylating agents tested, including temozolomide, were ineffective against this procarbazine-resistant tumor. Our present studies with medulloblastoma, which show little evidence of a deficiency of DNA mismatch repair capacity, are in contrast to our results with glioblastoma multiforme, in which a high proportion of tumors with MMR-deficient cells have been observed (40). These results suggest that such methylating agents may be effective in the treatment of medulloblastoma. Furthermore, if MMR deficiency does not significantly contribute to methylator resistance, reduction of tumor AGT levels with O6-benzylguanine should prevent any AGT-mediated resistance to methylating agents. This approach would not be rational if there were a high frequency of MMR deficiency (39).

In summary, MMR deficiency does not appear common in newly diagnosed medulloblastoma. Further studies in tumor samples obtained after failure of therapy, particularly with methylators, will need to be conducted to determine more extensively the incidence and mechanisms of clinically acquired MMR deficiency.

REFERENCES


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