Promoter Hypermethylation of Mismatch Repair Gene hMLH1 Predicts the Clinical Response of Malignant Astrocytomas to Nitrosourea

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ABSTRACT

Purpose: In certain types of human cancers, transcriptional inactivation of hMLH1 by promoter hypermethylation plays a causal role in the loss of mismatch repair functions that modulate cytotoxic pathways in response to DNA-damaging agents. The aim of the present study was to investigate the role of promoter methylation of the hMLH1 gene in malignant astrocytomas.

Experimental Design: We examined the hMLH1 promoter methylation in a homogeneous cohort of patients with 41 malignant astrocytomas treated by 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-2(2-chloroethyl)-3-nitrosourea chemotherapy in combination with radiation and interferon therapy, and assessed the correlation of such methylation with clinical outcome.

Results: hMLH1 promoter methylation was found in 6 (15%) of the 41 newly diagnosed malignant astrocytomas. Hypermethylation of the hMLH1 promoter corresponded closely with a loss of immunohistochemical staining for hMLH1 protein (P = 0.0013). Patients with hMLH1-methylated tumors displayed a greater chance of responding to adjuvant therapy as compared with those with hMLH1-unmethylated tumors (P = 0.0150). The presence of hMLH1 hypermethylation was significantly associated with a longer progression-free survival on both univariate analysis (P = 0.0340) and multivariate analysis (P = 0.0161).

Conclusions: The present study identified hMLH1 methylation status as a predictor of the clinical response of malignant astrocytomas to chloroethyl nitrosourea-based adjuvant therapy. The findings obtained suggest that determination of the methylation status of hMLH1 could provide a potential basis for designing rational chemotherapeutic strategies, as well as for predicting prognosis.

INTRODUCTION

Alkylating chloroethylnitrosoureas such as 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-2(2-chloroethyl)-3-nitrosourea, 1,3-bis(2-chloroethyl)-1-nitrosourea, and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea have been employed as the main chemotherapeutic agents in the treatment of malignant astrocytomas (anaplastic astrocytoma and glioblastoma multiforme) and have been shown to modestly improve survival (1). These types of drugs attack the O6 position of guanine and then form potent cytotoxic DNA adducts, which cross-link with the opposite cytosine residues, leading to DNA strand breaks (2). The major determinant of chloroethyl nitrosourea resistance is the activity of O6-methylguanine DNA methyltransferase (MGMT) which directly and specifically removes cytotoxic alkyl adducts from the O6 position of guanine (2). Recent clinical studies, including work at our institution, have shown that epigenetic inactivation of MGMT by promoter hypermethylation can increase the sensitivity of malignant astrocytomas to chloroethyl nitrosourea chemotherapy (3–5).

The DNA mismatch repair (MMR) system plays a prominent role in maintaining genomic integrity by mediating the activation of cell cycle checkpoints and apoptosis. It is well documented that MMR-deficient cell lines or xenografts are resistant to killing by platinum compounds such as cisplatin and carboplatin (6). This has been presumed to result from a process impairing the ability of the neoplastic cells to detect platinum-induced DNA adducts and to activate signaling pathways that contribute to the triggering of apoptotic activity (6). The MMR system also recognizes the O6-methylguanine DNA adducts formed by monofunctional alkylating agents such as procarbazine and temozolomide, which cause mispairing with thymine during DNA replication and potentially trigger the apoptotic pathway by invoking MMR (2, 6). However, it remains unclear whether or not the MMR system mediates the cytotoxicity of bifunctional chloroethyl nitrosoureas that form the O6-chloroethylguanine DNA adducts generating interstrand cross-links (6–9).

Among the MMR genes, human Mut L homologue 1 (hMLH1) gene seems to be particularly susceptible to aberrant promoter methylation. In colorectal and gastric cancers exhibiting microsatellite instability, a hallmark of the genomic instability due to loss of MMR, hypermethylation of the hMLH1 gene promoter was found to be extremely frequent and often accompanied by down-regulation of hMLH1 expression (10, 11). Reversal of methylation with 5-aza-2′-deoxycytidine resulted in restoration of MMR function in MMR-deficient cell lines (10). These findings suggest that hypermethylation of hMLH1 may be the most...
prevalent mechanism of MMR defects. In diffuse gliomas, loss of hMLH1 expression was frequently observed (12), and hMLH1 hypermethylation was detected in a subset of glioblastoma multiforme with microsatellite instability (13). However, no study has yet systematically analyzed such hypermethylation in a large series of malignant astrocytomas and assessed its clinical implications.

The aim of the present study was to investigate the role of promoter methylation of the hMLH1 gene in malignant astrocytomas. We examined the hMLH1 promoter methylation in a homogeneous cohort of patients with malignant astrocytomas treated by 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea chemotherapy in combination with radiation and interferon (IFN) therapy, and evaluated its clinical usefulness.

**MATERIALS AND METHODS**

**Patient Population.** Between April 1991 and December 2000, a total of 58 patients with a new histologic diagnosis of supratentorial anaplastic astrocytoma (grade 3) and glioblastoma multiforme (grade 4) classified according to the WHO criteria (14) were included in a prospective study designed to evaluate the efficacy of human fibroblast IFN-β, 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea, and radiation therapy (termed IAR therapy) as reported previously (15). Patients who were < 18 years of age at the time of operation were excluded from the present study. All cases showed contrast enhancement upon T1-weighted magnetic resonance imaging (MRI) at preoperative examination. Among the 58 malignant astrocytomas, 41 tumor samples were available for analysis of their hMLH1 methylation, and these constituted the study population. The study protocol was approved by the Clinical Research Ethics Committee, and all patients gave informed consent for the genetic analyses to be undertaken.

Postoperative MRI scans with contrast were done within 72 hours of the operation. The radiographic responses were assessed by comparing the immediate postoperative MRI scans with follow-up scans obtained on the same MRI scanner, employing standard cross-sectional diameter measurements of the enhancing tumor. A complete response was defined as total disappearance of all enhancing tumor on consecutive MRI scans at least 1 month apart. Partial response was defined as a 50% or more reduction in the area of the enhancing tumor on consecutive MRI scans at least 1 month apart. Stable disease was defined as no change in the area of the tumor or a < 50% reduction or a < 25% increase in the tumor size. Progressive disease was defined as a ≥ 25% increase in the area of the enhancing tumor or the appearance of new lesions.

**Methylation-Specific PCR.** Genomic DNA was extracted from paraffin sections as described previously (16). Promoter hypermethylation of the hMLH1 gene was determined by the methylation-specific PCR (17). Sodium bisulfite modification was done with a CpGenome DNA Modification Kit (Intergen, Oxford, United Kingdom) as described previously (18, 19). The primer sequences of hMLH1 for the methylated and unmethylated reactions were as reported previously (10, 11). The PCR was carried out using a Robocycler (Stratagene) in a total volume of 10 μL, consisting of PCR buffer [10 mmol Tris (pH 8.3), 50 mmol KCl, 2 mmol MgCl2, deoxynucleotide triphosphates (250 μmol/L each), sense and antisense primers (0.3 μmol/L each), 0.5 units of Platinum Taq DNA polymerase (Life Technologies, Grand Island, NY) and ~ 40 ng bisulfite-modified DNA. Initial denaturing at 95°C for 10 minutes was followed by 35 cycles consisting of denaturing at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 50 seconds. A final extension step at 72°C for 10 minutes was added. The amplified products were electrophoresed on 3% agarose gels, and were visualized with ethidium bromide. CpGenome Universal Methylated DNA (Intergen) and normal blood DNA were included in each PCR set as methylated and unmethylated controls, respectively.

**Immunohistochemistry.** Staining for hMLH1 protein was done using anti-mouse hMLH1 monoclonal antibody (clone G168-15; PharMingen, San Diego, CA). Sections were deparaffinized in xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature. After deparaffinization and rehydration, the sections were incubated overnight at 4°C with an anti-hMLH1 antibody (1:100 dilution). The reaction was visualized using a Vectastain avidin-biotin complex method kit and diaminobenzidine (Vector Laboratories, Burlingame, CA). For the hMLH1 immunostaining, only nuclear staining was considered to ensure retention of hMLH1 protein, and the cytoplasmic reactivity may be nonspecific. Tumors were scored as exhibiting loss of hMLH1 expression if the neoplastic cells had nuclear staining, whereas the adjacent normal cells showed adequate nuclear staining as an internal positive control.

**Statistical Analysis.** The follow-up period of the present study was terminated on April 1, 2002. The Kaplan and Meier method was employed to calculate the progression-free survival (PFS) and overall survival (OS). We designated PFS as the time period from the start of IAR therapy to the point when radiographic evidence of progressive disease was noted. When progressive disease was evident at the time of the first evaluation, the PFS was set to zero. OS was defined as the interval between the start of IAR therapy and the date of death or the most recent evaluation. The log-rank test was used to assess the degree of significance of the differences in different subgroups. The Cox proportional hazards model was employed to identify the multivariate predictors of survival. When a potential prognostic factor was judged to be independent and appropriate for the model, the relative risk and 95% confidence interval were calculated. The relationships between the various parameters were analyzed statistically using the χ² test, Fisher’s exact test, or the Mann-Whitney U test as appropriate. The significance level chosen was P < 0.05, and all tests were two-sided. The statistics were analyzed with a personal computer running StatView J-5.0 software (Abacus Concepts, Berkeley, CA).

**RESULTS**

**Methylation-Specific PCR and Immunohistochemistry for hMLH1.** Among all of the tumors investigated, hMLH1 promoter methylation was present in 6 of the 41 samples (15%). All of the methylated tumors displayed evidence of unmethylated hMLH1, probably indicating the presence of normal
containing tissue (Fig. 1). Thirty-one samples were available for immunohistochemical examination of their hMLH1 protein expression. Among these, 19 tumors (61%) expressed hMLH1 in a relatively heterogeneous pattern, whereas 12 (41%) were judged to have loss of hMLH1 expression. When the methylation data were matched to the study of the hMLH1 expression, all 6 methylated tumors lacked hMLH1 expression, whereas 19 of the 25 unmethylated tumors retained hMLH1 expression (P = 0.0013 by Fisher’s exact test). In all of the 6 methylated samples, the neoplastic cells displayed a homogeneous absence of nuclear staining for hMLH1 protein (Fig. 2).

**Correlation Between hMLH1 Methylation and Clinicopathologic Parameters.** The characteristics of the patients according to their hMLH1 methylation status are summarized in Table 1. The MGMT methylation, TP53 mutation, and MIB-I staining had been examined previously (5). hMLH1 methylation was more frequent in tumors with MGMT methylation (4 of 15, 27%) than in those without MGMT methylation (2 of 26, 8%), although this trend did not reach statistical significance (P = 0.1683 by Fisher’s exact test). An association also existed between age and hMLH1 methylation. Patients with hMLH1-methylated tumors had a median age of 48 years, as opposed to a median age 56 years in the patients with hMLH1-unmethylated tumors (P = 0.1210 by Mann-Whitney U test). Regarding the histologic grade, hMLH1 methylation was present in 3 of 14 (21%) anaplastic astrocytomas, as compared with 3 of 27 (11%) glioblastoma multiformes (P = 0.3935 by Fisher’s exact test). None of the other factors including sex, Karnofsky performance scale, MIB-I labeling index, extent of surgery, and TP53 mutation revealed a correlation with hMLH1 methylation (P > 0.5 for each comparison).

**Responses.** Of the 41 patients, 21 had radiographically measurable disease on immediate MRI scans. Overall, six patients (29%) responded to treatment, with a complete response in two patients and a partial response in four patients. There were 15 nonresponders (9 with stable and 6 with progressive disease).

hMLH1 methylation was strikingly associated with responsiveness to IAR therapy. All 3 methylated tumors responded to therapy, as compared with only 3 of the 18 (17%) unmethylated tumors (P = 0.0150 by Fisher’s exact test). Responses were also observed more frequently in patients with MGMT-methylated tumors than in those with MGMT-unmethylated tumors, as reported previously (5).

**Survival Analysis.** The presence of hMLH1 hypermethylation was significantly associated with a longer PFS (Fig. 3). The median PFS for the patients with methylated versus unmethylated tumors was estimated to be 22 and 7 months, respectively (P = 0.0340). As we have shown in a previous study (5), age, histologic grade, Karnofsky performance scale, and extent of surgery also influenced the PFS significantly. To assess the predictive power of hMLH1 methylation independently of the other factors, we next examined these variables simultaneously in a Cox proportional hazards model. The hMLH1 methylation status was of independent prognostic value for the PFS in this model (relative risk, 5.109; 95% confidence interval, 1.354-19.274; P = 0.0161). The OS of the patients with hMLH1-methylated tumors was also longer than that of those with hMLH1-unmethylated tumors (the estimated median OS values being 38 and 15 months, respectively; Fig. 3), although this difference failed to achieve statistical significance (P = 0.0712).
We next analyzed the anaplastic astrocytoma and glioblastoma multiforme patients separately. The hMLH1 methylation status did not correlate with either the PFS or OS in each subgroup; however, the sizes of the subsets were too small to draw any firm conclusions (data not shown).

DISCUSSION

The present study is the first to show unequivocally that aberrant methylation of the promoter region of the hMLH1 gene accompanied by subsequent loss of hMLH1 protein expression is implicated in a significant fraction of malignant astrocytomas. We further observed that the presence of hMLH1 hypermethylation was significantly associated with an increased sensitivity to IAR therapy and prolonged PFS. Although our relatively small patient number and selected patient population could have affected the results of the statistical analysis, the above findings are novel in suggesting a predictive role for hMLH1 hypermethylation in the clinical response of malignant astrocytomas to adjuvant therapy.

A similar correlation has been reported among ovarian cancers treated by platinum drug-based chemotherapy, in which loss of hMLH1 expression was significantly associated with responsiveness to treatment or improved survival (20, 21). On the contrary, reduced hMLH1 expression after chemotherapy was found to be associated with poor survival in breast cancers (22) and esophageal cancers (23). Such conflicting findings could imply that different roles of MMR defects are operative in the treatment sensitivity of each cancer type. In colorectal cancers, inactivation of the MMR system enhanced the susceptibility to mutations of several critical growth regulatory genes, and microsatellite instability was predictive of a favorable outcome and a reduced likelihood of developing metastases (24). These observations suggest that a deficiency in MMR could invoke certain kinds of unique mutations, which may contribute to the less aggressive clinical behaviors seen in certain types of cancers (24).

Although the therapeutic contribution of concomitant IFN and radiation therapy remains to be elucidated, another possibility for achieving a favorable outcome in hMLH1-methylated malignant astrocytomas is that loss of MMR could confer an increased sensitivity to 1-(4-amino-2-methyl-5-pyrimidiny)methyl-3-2(2-chloroethyl)-3-nitrosourea chemotherapy. This view is, however, contradictory to the data provided by experimental studies which indicated that MMR-deficient cancer cells are resistant to the tumoricidal effects of chemotherapeutic agents such as platinum compounds and methylating agents (6). Unlike the DNA adducts produced by platinum compounds and methylating agents, the O6-chloroethylguanine DNA adducts formed by chloroethylnitrosoureas do not seem to be recognized as mismatches by the MMR system (6). Such adducts are cytotoxic rather than mutagenic, and thereby form a cross-link with the opposite strand cytosine yielding interstrand DNA cross-link, which is repaired by MGMT, base, and nucleotide excision repairs (2). There has been speculation that MMR might aid nucleotide excision repair in excising

Table 1 Characteristics of 41 patients with malignant astrocytomas according to methylation status of the hMLH1 gene

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Unmethylated (n = 35)</th>
<th>Methylated (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (y)</td>
<td>56</td>
<td>48</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>20 (57%)</td>
<td>3 (50%)</td>
</tr>
<tr>
<td>Female</td>
<td>15 (43%)</td>
<td>3 (50%)</td>
</tr>
<tr>
<td>Karnofsky performance scale</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>80</td>
<td>85</td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anaplastic astrocytoma</td>
<td>11 (31%)</td>
<td>3 (50%)</td>
</tr>
<tr>
<td>Glioblastoma multiforme</td>
<td>24 (69%)</td>
<td>3 (50%)</td>
</tr>
<tr>
<td>Radical surgery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>17 (49%)</td>
<td>3 (50%)</td>
</tr>
<tr>
<td>No</td>
<td>18 (51%)</td>
<td>3 (50%)</td>
</tr>
<tr>
<td>Response to IAR therapy*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>3 (17%)</td>
<td>3 (100%)</td>
</tr>
<tr>
<td>Nonresponders</td>
<td>15 (83%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>MIB-1 labeling index†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (%)</td>
<td>8.2</td>
<td>10</td>
</tr>
<tr>
<td>MGMT methylation‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>24 (69%)</td>
<td>2 (33%)</td>
</tr>
<tr>
<td>Positive</td>
<td>11 (31%)</td>
<td>4 (67%)</td>
</tr>
<tr>
<td>TP53 mutation‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>27 (77%)</td>
<td>4 (67%)</td>
</tr>
<tr>
<td>Positive</td>
<td>8 (23%)</td>
<td>2 (33%)</td>
</tr>
</tbody>
</table>

*Twenty patients were not assessable for their response to adjuvant therapy.
†The MIB-1 staining, MGMT methylation, and TP53 mutation were examined previously (5).

Fig. 3 PFS and OS of 41 patients with malignant astrocytomas, according to methylation status of the hMLH1 promoter.
interstrand DNA cross-links and could thereby protect against chloroethylnitrosourea-induced cytotoxicity (8). Lack of MMR capacity could thus contribute to an increased sensitivity to chloroethylnitrosoureas. In support of this, Aquilina et al. (8) provided proof of a hypersensitivity to 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea in MMR-deficient cancer cell lines. However, this mechanism has yet to be clarified in detail in vitro, and the link between MMR defects and sensitivity to chloroethylnitrosoureas has never been consistently identified in other experimental studies (7, 9). Further in vitro and in vivo investigations are required to define the precise contribution of MMR functions to the cellular responses to chloroethylnitrosoureas.

The tumor suppressor P53 protein also plays a crucial role in regulating cell cycle checkpoints and apoptosis upon genotoxic stress. Several experimental studies have provided evidence to suggest that P53 and the MMR system interact to control sensitivity to the cytotoxic effects of DNA-damaging agents (25, 26). Unfortunately, we failed to observe a significant correlation between hMLH1 methylation and TP53 mutation in our limited number of samples. An interesting interplay also exists between MGMT and the MMR system. In MGMT-deficient cell lines, an acquired resistance to alkylating agents is associated with a loss of capacity for MMR (27). In gastric and colorectal carcinomas, MGMT promoter hypermethylation was found to occur predominantly in tumors without hMLH1 methylation or microsatellite instability (28, 29). In contrast, we observed a relatively high frequency of hMLH1 methylation in tumors with MGMT methylation, suggesting that hMLH1 may determine the sensitivity of gliomas to treatment partially dependently on MGMT.

Microsatellite instability has been employed as a molecular marker for defective MMR genes in a wide range of human cancers including those of the stomach and colon (10, 11). In diffuse gliomas, several lines of evidence have supported the involvement of microsatellite instability in pediatric patients (30, 31). However, microsatellite instability has been found to be absent or extremely rare in adult gliomas (13, 30, 31), which is inconsistent with our data demonstrating a relatively high frequency of hMLH1 hypermethylation in adult high-grade astrocytomas. In the study of Wei et al. (12) using multiplex reverse transcriptional-PCR assay, low expression of hMLH1 was detected in 7 of 33 gliomas (21%), a frequency similar to the hMLH1 hypermethylation observed in the present study. They also showed that microsatellite instability was found in only one of five tumors with a reduced expression of MMR genes. The relationship between hMLH1 hypermethylation and microsatellite instability in human gliomas needs to be confirmed in a larger number of samples.

In conclusion, the present results indicated that epigenetic inactivation of hMLH1 by promoter methylation was implicated in a significant proportion of malignant astrocytomas. The presence of hMLH1 hypermethylation was predictive of an increased sensitivity to adjuvant therapy and a prolonged PFS. Our findings suggest that methylation of the hMLH1 promoter may represent a potential molecular marker that could assist in assessing the prognosis and guiding chemotherapeutic decisions.

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