

Analysis of MLH1 and MSH2 Expression in Ovarian Cancer before and after Platinum Drug-based Chemotherapy¹

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ABSTRACT

Preclinical studies have demonstrated a relationship between DNA mismatch repair (MMR) status and sensitivity to cisplatin and carboplatin. MMR-deficient cells are resistant to both drugs, and selection for cisplatin resistance *in vitro* is sometimes accompanied by loss of MMR protein expression. We used immunohistochemical staining techniques to investigate hMLH1 and hMSH2 expression in paired ovarian tumor sections from 54 ovarian cancer patients before and after platinum-based therapy. We sought associations between hMLH1 and hMSH2 protein expression and clinical parameters known to be of prognostic significance as well as response to treatment and overall survival. hMLH1 and hMSH2 staining decreased significantly after platinum-based therapy. The percent of malignant cells that stained positive correlated with the intensity of nuclear staining for both proteins; staining for hMLH1 correlated well with staining for hMSH2. Unexpectedly, expression of nuclear hMLH1 correlated negatively with response to treatment. Expression of nuclear hMLH1 and hMSH2 was positively correlated with pretreatment CA125 level, and expression of nuclear hMSH2 was positively correlated with change in CA125 level after treatment. Tumor stage was associated with expression of nuclear hMSH2, and tumor histological subtype was associated with both hMLH1 and hMSH2 staining. No association was found between

expression of either protein and overall survival. These results indicate that the tumor is biologically altered after chemotherapy consistent with treatment-induced selection for cells expressing lower hMLH1 and hMSH2 levels. However, immunohistochemical staining for either hMLH1 or hMSH2 was not highly predictive of drug sensitivity as measured by response or survival.

INTRODUCTION

The MMR³ system detects DNA nucleotide mismatches, small insertions and deletions, and several types of drug-induced adducts in damaged DNA, and recruits factors to repair the damage (reviewed in Ref. 1). Recognition and possibly processing of drug-induced adducts by the MMR system appear to generate a proapoptotic signal (2). Cells that have lost MMR activity are less able to detect certain types of adducts and have an impaired ability to react to damaged DNA with an appropriate apoptotic response.

Several studies have suggested a relationship between MMR status and sensitivity to certain classes of chemotherapeutic agents (3–6). MMR-deficient cells are resistant to the platinum-containing drugs cisplatin and carboplatin, both of which produce similar adducts in DNA and are particularly important because of their widespread clinical use. The MMR-deficient HCT116 colon cancer and HEC59 endometrial cancer cell lines both exhibit low-level resistance to cisplatin compared to their MMR-proficient counterparts (4). In addition, an MSH2-knockout murine embryonic stem cell line was found to be 2.1-fold more resistant to cisplatin than its isogenic MMR-proficient parental counterpart (3).

Further evidence linking loss of MMR function to resistance to cisplatin is provided by the observation that selection for cisplatin resistance *in vitro* is sometimes accompanied by loss of expression of some MMR proteins. A cisplatin-resistant derivative of the human ovarian adenocarcinoma cell line 2008 was found to have lost hMLH1 protein expression (4). In another study, 9 of 10 sublines of the drug-sensitive A2780 ovarian cancer cell line that were independently selected for acquired cisplatin resistance were found to be deficient in hMLH1. Western blot analysis of human ovarian carcinoma samples obtained directly from patients before initial chemotherapy demonstrated that 10% lacked MLH1 expression, whereas in tumor samples obtained from other patients after platinum-based chemotherapy, 36% lacked MLH1 expression (5). Finally, a limited immunohistochemical study of paired ovarian cancer samples obtained from the same patient before and after at least three cycles of

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³ The abbreviations used are: MMR, DNA mismatch repair; FIGO, Fédération Internationale de Gynécologie et d'obstétrique.

Table 1 Patient characteristics

Characteristic	No. of patients	Percent
Histologic subtype		
Clear cell	5	9
Endometrioid	8	15
Mucinous	2	4
Serous	21	39
Not otherwise specified	18	33
FIGO grade		
2	12	26
3	34	74
FIGO stage		
3	41	82
4	9	18
Response		
Progression of disease	11	21
Stable/persistent	11	21
Partial response	23	43
Complete response	8	15

platinum-based primary chemotherapy demonstrated that MLH1 expression was reduced in 66% of cases (6).

The increased mutation rate that occurs throughout the genome in cells that lack MMR is often detected as microsatellite instability (7). An estimated 15–20% of sporadic ovarian tumors exhibit microsatellite instability (5), and there is a high probability that an even larger fraction contain at least some cells that are MMR-deficient. Presently available evidence suggests that resistance should emerge more rapidly in tumors that contain a larger number of MMR-deficient cells (1), and this may be the basis for the acquired resistance to primary chemotherapy that emerges so frequently in patients with ovarian cancer.

In this study, we used immunohistochemical staining techniques to quantify hMLH1 and hMSH2 expression in paired ovarian tumor sections from patients before and after platinum-based treatment. All clinical data on these patients had been collected prospectively as they had all been treated as part of either a Memorial Sloan-Kettering Cancer Center or Southwest Oncology Group trials (8). We sought associations between expression of these two MMR proteins and parameters known to be of prognostic significance as well as response to treatment and overall survival.

MATERIALS AND METHODS

Patients. The study population consisted of patients with ovarian cancer who had been entered on one of several different Memorial Sloan-Kettering Cancer Center studies (patients 1–41) or the Southwest Oncology Group study 8835 (patients 42–54). All cases from these two trials in which paired fixed and paraffin-embedded tumor tissues were available from both before and after at least two cycles of chemotherapy were included. Each patient had received cisplatin- or carboplatin-based chemotherapy as part of her first line treatment, followed by various second and third line treatments. Each patient's age, stage, histological grade, histological subtype, type of chemotherapy, and clinical response were eventually attained, and survival status was available for analysis. Data on CA125 level

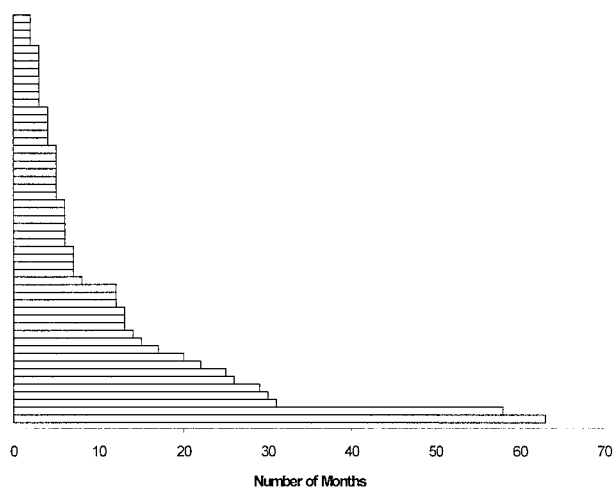


Fig. 1 Distribution of time elapsed between pre- and posttreatment tumor biopsies.

(pre- and post-first line treatment) and number of cycles of treatment received were available for patients 1–41 only. Patient characteristics are summarized in Table 1.

Immunohistochemical Staining. Sections of 3–4 μm were cut from paraffin blocks, deparaffinized with two 10-min soaks in xylene, and hydrated through two 5-min soaks in graded alcohol (100% ethanol followed by 95% ethanol), followed by a 10-min soak in distilled water. Antigen retrieval was performed by boiling the slides at 100°C in citrate buffer for two 5-min periods. The slides were then allowed to cool for 20 min and were rinsed in PBS. Immunohistochemical staining was performed according to the guidelines of the Catalyzed Signal Amplification System (DAKO, Carpinteria, CA), with some alterations. Endogenous peroxidase activity was blocked with a 30-min incubation in 3% hydrogen peroxide, followed by a rinse in Tris-buffered saline. Nonspecific protein binding was blocked with a 20-min incubation with serum-free protein in PBS and 0.015 M sodium azide. The slides were incubated with a 1:500 dilution of either anti-hMLH1 antibody (PharMingen, San Diego, CA, clone G168–728) or anti-hMSH2 antibody (Calbiochem, La Jolla, CA) for 1 h at a concentration of 1 $\mu\text{g}/\text{ml}$ in PBS/1% BSA. As a negative control, parallel sections were incubated with nonimmune mouse IgG1 sera (DAKO); as a technical positive control, slides were incubated with antivimentin antibody (DAKO). Staining was completed as described by the manufacturer using Tris-buffered saline with 0.1% Tween 20 for all rinses. After a rinse in distilled water, the slides were counterstained using Mayer's hematoxylin, mounted, and covered.

Immunohistochemical Analysis. Each slide was scored blindly for the percentage of malignant cells that stained positively (0–100% in 10% increments) and intensity of staining (0–4) for hMLH1 or hMSH2 by an independent pathologist who was unaware of the treatment status of the patient from whom the tissue sample was obtained.

Statistical Analysis. Based on available clinical and staining data, the following associations were examined for patients before treatment, after primary treatment, and for the difference between pre- and posttreatment: percent staining *ver-*

Fig. 2 Distribution of percent of malignant cells that stained positive for hMLH1 (top) or hMSH2 (bottom) before and after platinum-based treatment. The difference in percent staining following platinum-based treatment was found to be statistically significant using sign-rank analysis ($P = 0.0020$ and 0.0179 , respectively).

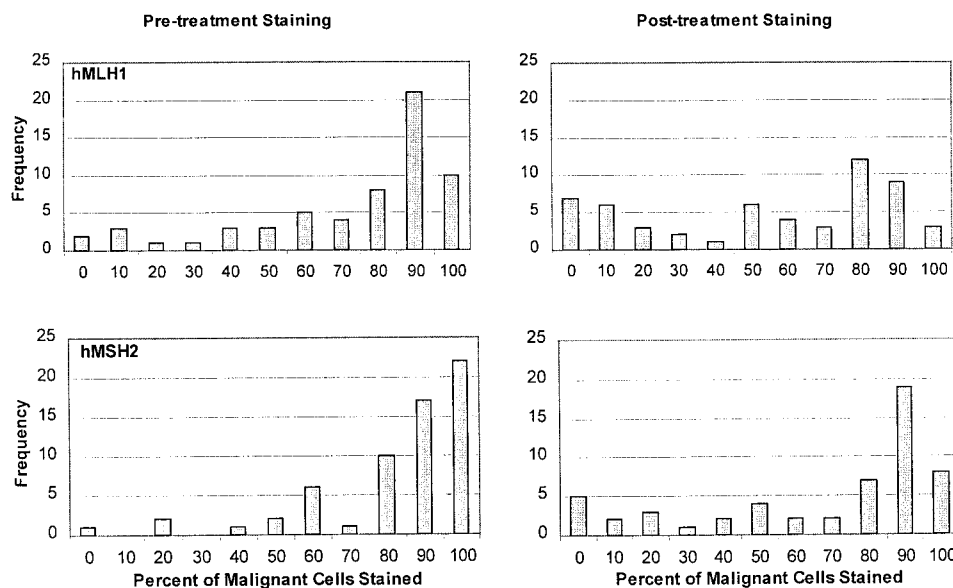


Table 2 Correlation between the percent of malignant cells that stained positive and intensity of staining in the same tissue sample

	Spearman correlation coefficient	<i>P</i>
MLH1		
Pretreatment samples	0.48859	0.0001
Posttreatment samples	0.43257	0.0011
Pre- versus posttreatment difference	0.53695	0.0001
MSH2		
Pretreatment samples	0.75180	0.0001
Posttreatment samples	0.56819	0.0001
Pre- versus posttreatment difference	0.48935	0.0002

Table 3 Correlations between the staining characteristics for hMLH1 and hMSH2

	Spearman correlation coefficient	<i>P</i>
Percent of cells staining		
Pretreatment samples	0.52033	0.0001
Posttreatment samples	0.55394	0.0001
Pre- versus posttreatment difference	0.60475	0.0001
Intensity of staining		
Pretreatment samples	0.39845	0.0015
Posttreatment samples	0.50984	0.0001
Pre- versus posttreatment difference	0.46537	0.0004

versus intensity of staining of hMLH1 and hMSH2 (Spearman correlation coefficients); percent and intensity of staining for hMLH1 *versus* hMSH2 (Spearman correlation coefficients); and percent and intensity of staining of hMLH1 and hMSH2 *versus* grade, stage, histological subtype (Kruskal-Wallis ANOVA), CA125 levels (for patients 1–41 only), age, response to treatment (Spearman correlation coefficients), and overall survival (comparison of Kaplan-Meier curves by log-rank statistic). In addition, patients were grouped based on their change in percent staining for hMLH1 and hMSH2 after primary platinum-based therapy and analyzed for association with overall survival (comparison of Kaplan-Meier curves by log-rank statistic).

RESULTS

The clinical parameters of the patients included in this study are summarized in Table 1. Fig. 1 shows the distribution of the time elapsed between the pretreatment and subsequent biopsy for all 54 patients. The median time between the pretreatment and posttreatment tumor samples for all patients was 6 months (range, 2–63 months). The median number of cycles

was 4 (range, 2–10). Fig. 2 shows the distribution of the percent of malignant cells stained in the samples obtained before and after treatment. The mean change in percent staining of cells for hMLH1 (pre- minus posttreatment) was $18.7 \pm 5.7\%$ (SE). In the case of staining for hMSH2, mean change in percent staining of cells was $15.0 \pm 5.1\%$ (SE). For both hMLH1 and hMSH2, the difference in percent staining after platinum-based treatment was found to be statistically significant using sign-rank analysis ($P = 0.0020$ for hMLH1 and 0.0179 for hMSH2). Thus, the expression of both proteins tended to be lower in the posttreatment samples. Subset analyses were undertaken to determine whether the magnitude of the decrease in staining varied as a function of the time elapsed between the first and second biopsies. Even in patients whose second biopsy was obtained ≤ 4 months after the first, a trend toward decreased staining was observed for both hMLH1 and hMSH2, although due to the small number of patients in this subset, the changes did not reach statistical significance. The magnitude of the decrease was not significantly greater than the mean for all patients in the subset whose second biopsy was ≥ 10 months after the first.

Table 2 shows that there were highly significant correla-

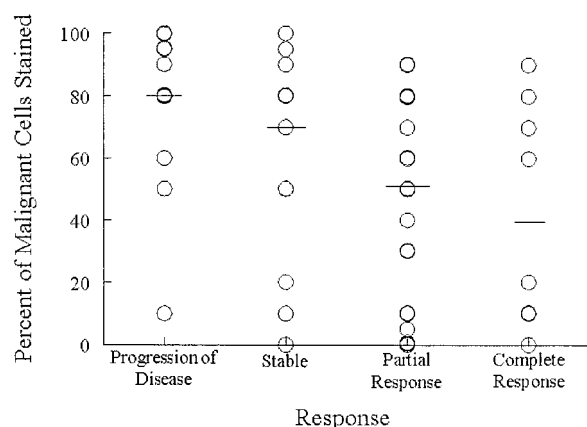


Fig. 3 Association between percent of malignant cells staining positive for hMLH1 after treatment and response to treatment ($r = -0.35995$; $P = 0.0081$). Horizontal lines, median values.

tions between the fraction of the malignant cells in a tumor sample that stained positive for hMLH1 and the intensity of the staining for hMLH1 in the same tissue sample. This was also observed for hMSH2. Strong correlations were observed in both the pretreatment and posttreatment tumor samples. In addition, the difference in percent of cells staining positively between the pre- and posttreatment samples within the same patient was correlated with the difference in intensity of staining between the pre- and posttreatment samples. Thus, tumors in which a large fraction of cells stained positively also tended to stain with greater intensity, suggesting that these two staining characteristics are not independent of each other.

Analysis also revealed that the percent of malignant cells staining positively for hMLH1 was strongly correlated with the percent of cells staining positively for hMSH2 in both the pretreatment and posttreatment samples (Table 3). Likewise, the difference in percent of cells staining positively for hMLH1 between the pre- and posttreatment samples within the same patient was correlated with the difference in the percent of cells staining positively for hMSH2. Similar correlations were found between the intensity of staining for hMLH1 and the intensity of staining for hMSH2 in the pre- and posttreatment samples, and the differences in intensity of staining for the two proteins in the pre- and posttreatment samples. Thus, tumors that expressed one of the proteins at a high level tended to express the other at a high level and *vice versa*. When there was a change in expression of one protein between the pre- and posttreatment samples from the same patient, there tended to be a change in the other protein in the same direction. These results provide evidence for coordinate regulation of the level of hMLH1 and hMSH2 in ovarian cancer.

Associations were sought between the hMLH1 and hMSH2 staining characteristics and clinical parameters including FIGO stage, histological subtype of ovarian carcinoma, response, and overall survival. With regard to response, there were significant differences in hMLH1 staining (both percent of cells stained and intensity) among the four response categories (progressive disease, stable disease, partial response, and complete response). This association was observed in both the pre- and posttreatment samples, although it reached statistical significance only in the

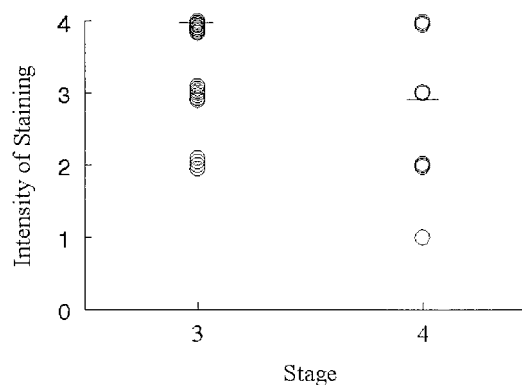


Fig. 4 Association between intensity of staining for hMSH2 before treatment and stage ($P = 0.0236$). Horizontal lines, median values.

posttreatment tumor samples. As shown in Fig. 3, posttreatment staining for MLH1 was greatest in patients in the progressive disease category and least for patients who eventually attained a complete response ($r = -0.35995$; $P = 0.0081$). As expected, intensity of staining of hMLH1 in the same group of patients also correlated with response to treatment ($r = -0.32447$; $P = 0.0178$). Interestingly, no association was found between the hMLH1 staining characteristics of the pretreatment samples and response, nor between the difference in staining characteristics between the pre- and posttreatment samples and response. These results suggest that, contrary to previous findings, lower expression of hMLH1 does not in itself indicate a lack of *in vivo* sensitivity to platinum-based therapy. No association was found between hMSH2 staining (percent of cells positive or intensity) and response to treatment for either the pre- or posttreatment samples.

An association between staining characteristics and either pretreatment CA125 or the change in CA125 that occurred in association with treatment could only be assessed in the subset of 41 patients for whom CA125 measurements were made. In this subset, the only association between pretreatment CA125 and a staining characteristic was for the pre- versus posttreatment difference in staining intensity. Interestingly, this association was statistically significant for both hMLH1 and hMSH2 ($r = 0.40679$, $P = 0.0113$ and $r = 0.39809$, $P = 0.0133$, respectively). Patients who had higher CA125 levels before treatment had more malignant cells staining positive. Of the 41 patients with pretreatment CA125 level measurements, 38 patients had additional measurements made after the primary platinum-based treatment. Significant associations were found between the change in CA125 levels and pretreatment intensity ($r = 0.34042$; $P = 0.0365$), as well as the pre- versus posttreatment difference in staining intensity ($r = 0.36278$; $P = 0.0252$), of hMSH2. Patients whose CA125 decreased after treatment had a greater intensity of staining of hMSH2 before treatment and also lost hMSH2 expression after treatment based on immunohistochemical analysis.

With respect to FIGO stage, no associations were found for hMLH1, but there were statistically significant differences in pretreatment intensity ($P = 0.0236$), and in the change in intensity ($P = 0.0270$), in the case of hMSH2. As shown in Fig.

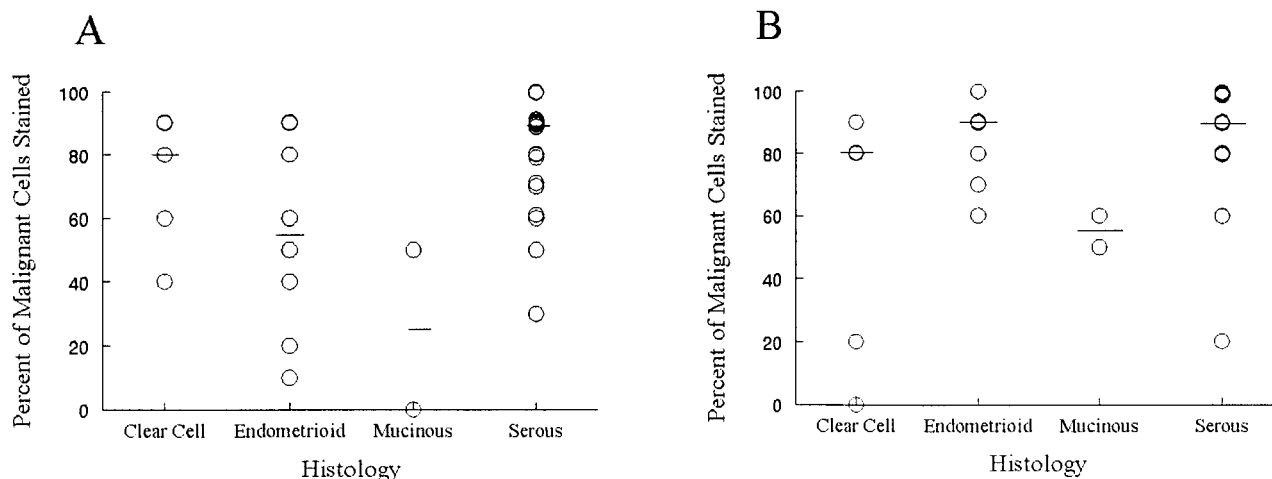


Fig. 5 Association between histological subtype and pretreatment percent of malignant cells staining positive for hMLH1 (A; $P = 0.0156$) and hMSH2 (B; $P = 0.0218$). Horizontal lines, median values.

4, patients with higher stage had a significantly lower median staining intensity.

Fig. 5 shows that the percent of malignant cells staining positive before treatment was associated with histological subtype for both hMLH1 ($P = 0.0156$) and hMSH2 ($P = 0.0218$). As expected from the finding that percent staining correlated well with intensity of staining, the intensity of staining of hMLH1 and hMSH2 in patients before treatment was also associated with histological subtype ($P = 0.0253$ and 0.0096 , respectively), with a majority of patients with serous histology staining for hMLH1 and hMSH2 at an intensity of 3 and 4 before treatment.

No association was found between hMLH1 or hMSH2 staining characteristics and FIGO grade, age, posttreatment CA125 levels, or overall survival. Furthermore, when patients were categorized based on their change in percent staining for hMLH1 and hMSH2 after treatment, no association was found with overall survival.

DISCUSSION

This exploratory study sought to identify possible associations between either the fraction of malignant cells staining positive for hMLH1 or hMSH2, or intensity of immunohistochemical staining, and a variety of histological and clinical parameters in paired tumor samples obtained before and after primary chemotherapy with platinum drug-based combination regimens. It is important to point out that because we tested for possible associations between a large number of different variables, there is a chance that even those that showed up as being statistically significant are nevertheless spurious. It is also uncertain whether those correlations that turned out to be numerically significant are biologically important.

The first observation of interest is that staining for both hMLH1 and hMSH2 was demonstrable in a large fraction of the malignant cells in most human ovarian carcinomas before the start of treatment. Second, there was a statistically significant difference in the expression of both proteins and in the percent-

age of malignant cells expressing these proteins after platinum-based therapy. Subset analyses did not detect any clear difference in the magnitude of the decrease as a function of the timing of the second biopsy, although the small size of the subsets limited the power of this analysis. Thus, after drug exposure, the persistent tumor mass was biologically altered with respect to the expression of these two proteins. This observation is consistent with a previous smaller study, which disclosed that hMLH1 expression was decreased in 66% of 38 patients with ovarian carcinoma after at least three cycles of primary chemotherapy (6). These results are in contrast to the initial report of another study by Mackean *et al.* (9), which examined the prognostic significance of MMR deficiency. Immunohistochemical staining of paraffin-embedded samples in the study by Mackean *et al.* (9) did not identify any significant change in hMLH1 or hMSH2 expression in 26 paired ovarian carcinoma samples after cisplatin-based chemotherapy. There has been speculation that loss of hMLH1 expression *in vitro* may be due to hypermethylation of its promoter (10), although this mechanism has yet to be studied in detail *in vivo*. There has been no investigation, to our knowledge, into the mechanism of loss of hMSH2 expression in these tumors.

The strongest and most consistent association identified in the present study was that between the fraction of the malignant cells that stained positive for either hMLH1 or hMSH2 and the intensity of staining for that protein in the same sample. Highly significant correlations were found for both hMLH1 and hMSH2 in both pre- and posttreatment samples. *A priori* it is difficult to know which of these two parameters might be more important. There is a reasonable biological basis for anticipating that they might be independent variables. For example, a few cells that have lost all hMLH1 or hMSH2 expression may be more important to the development of drug resistance than a large number of cells that have low intensity staining. However, the results of this study indicate that, in the case of human ovarian carcinoma, these two variables are clearly associated. The larger the fraction of tumor cells that stained positively, the

higher the intensity of staining for that sample. This suggests that it is reasonable to search for associations between either one of these and clinical parameters separately rather than trying to develop a scoring system based on contributions from each as has been reported previously (9).

One interesting but unexpected association identified was that between the staining characteristics of hMLH1 and those of hMSH2. Both the percent of malignant cells that stained positive and the intensity of staining for hMLH1 were well correlated with the same parameters for hMSH2. This association was demonstrated for the pretreatment and post-treatment samples and for the difference following treatment. Previous studies have reported that loss of hMLH1 protein expression is coupled with loss of PMS2 protein expression (5). The loss of PMS2 was ascribed to the lack stabilization by its heterodimeric partner hMLH1. Our results are consistent with a similar coordinate regulation of hMLH1 and hMSH2 levels in ovarian cancer.

Based on the observation that loss of MSH2 is associated with cisplatin and carboplatin resistance *in vitro* (3) and that loss of hMLH1 expression frequently accompanies acquired cisplatin resistance *in vitro* (5), one would expect that low hMLH1 or hMSH2 expression would correlate with a poor response to these agents *in vivo*. However, our results indicate only a relatively weak association between staining characteristics and therapeutic outcome, and this association was in the opposite direction than that predicted by the *in vitro* data. In the case of hMLH1, response did differ as a function of the fraction of cells staining positively for hMLH1, and this association was corroborated by the fact that the same association was observed for hMLH1 staining intensity. However, patients attaining a complete response had a lower mean intensity of staining of hMLH1 posttreatment, compared to other patients ($P = 0.0178$), and patients who had disease progression had a higher mean percent staining of hMLH1 posttreatment, compared with other patients ($P = 0.0081$). In addition, there was no association of hMSH2 staining with response, and no association of staining for either protein with overall survival. Thus, these results do not support the hypothesis that lack of hMLH1 or hMSH2 staining predicts *in vivo* resistance to the platinum drugs.

Associations were identified between pretreatment CA125 levels and pre- versus posttreatment difference in staining intensity for hMLH1 and hMSH2. Even more interesting, an association was identified between the change in CA125 level after treatment and pretreatment intensity as well as the change in intensity of staining of hMSH2. Higher CA125 levels before treatment correlated with a greater fraction of malignant cells staining positive for both proteins. A decrease in CA125 level after treatment correlated with a higher pretreatment intensity of staining as well as a decrease in intensity of staining of hMSH2. This is an important observation in that changes in CA125 levels have been used as an indirect measure of tumor responsiveness, with a decrease in CA125 levels indicating a better response to treatment (11).

A relation was found between tumor stage and hMSH2 staining. With respect to both the fraction of positively staining cells and intensity of staining, the data permit rejection of the null hypothesis that all stages expressed hMSH2 at the same level. Patients with stage 4 tumors had significantly lower

hMSH2 staining intensity in pretreatment samples compared with stage 3 patients ($P = 0.0236$). Therefore, patients with a worse prognosis based on FIGO stage had decreased hMSH2 expression before treatment. This is in contrast to a previous study in which MMR deficiency, as assessed by microsatellite instability, was demonstrated in 75% of stage 1 patients with ovarian neoplasms compared to 11% of stage 2, 3, or 4 patients (12).

The next most interesting observation was that tumors of different histological subtypes had different levels of expression of both hMLH1 and hMSH2. Serous adenocarcinomas stood out as having greater staining (both in terms of the fraction of cells that stained positively and the intensity of staining) than the other histological subtypes. It is of interest that serous carcinoma are also generally the most responsive to platinum drug-based chemotherapy, but the data do not permit the conclusion that there is a causal link. The analysis demonstrated that there was no discernible association between MMR protein expression and FIGO grade. This was not surprising because there was only a limited distribution of grades represented, with almost all patients having grade 2 or 3 tumors.

Our results also indicate that there was no association between MMR protein expression and overall survival. Based on previous findings that MMR deficiency is associated with acquired resistance to cisplatin treatment *in vitro*, it was expected that expression of MMR proteins be predictive of survival. However, our analysis did not discern a correlation between either hMLH1 or hMSH2 expression and overall survival. It would have been of interest to examine for an association with progression-free survival, but information on the time to progression of disease was not available for the patients in the present study.

The association between loss of MMR activity and resistance to cisplatin and carboplatin has now been very well established using molecularly engineered tumor cell lines. However, it is not clear that immunohistochemical staining for hMLH1 or hMSH2 measures MMR activity even indirectly. Unfortunately, tools for making direct measurements of MMR activity in human tumor samples are not available, and thus it was important to explore the question of whether immunohistochemical staining for hMLH1 or hMSH2 could be used as a surrogate. This study contributes importantly to the field by suggesting a negative answer to this question.

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