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Immunohistochemical staining of hMLH1 and hMSH2 reflects microsatellite instability status in ovarian carcinoma

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Microsatellite instability is due to defects in the family of DNA repair genes, primarily *hMLH1* and *hMSH2*, which can be detected by immunohistochemical staining. However, it is unclear whether immunohistochemical staining can accurately predict microsatellite instability status. We sought here to evaluate the sensitivity, specificity, and predictive values of immunostaining for the expression of the DNA mismatch-repair genes *hMLH1* or *hMSH2* in predicting microsatellite instability in ovarian carcinoma. Tissue microarrays with specimens from 322 women with primary ovarian carcinoma were stained with antibodies to hMLH1 and hMSH2; cases in which either hMLH1 or hMSH2 were negative were analyzed for microsatellite instability with the five-marker panel recommended by the National Cancer Institute (*BAT26*, *BAT25*, *D5S346*, *D2S123*, and *D17S250*). Microsatellite instability was also analyzed in another 19 cases selected at random in which both hMLH1 and hMSH2 were positive. Tumors with instability at two or more of the five NCI markers were considered to have a high level of microsatellite instability; tumors showing instability at only one marker were considered microsatellite instability-low; and tumors in which no markers exhibited microsatellite instability were considered microsatellite stable. We found that negative staining for hMLH1 protein (five cases) or hMSH2 protein (two cases) was associated with high level of microsatellite instability. The sensitivity and specificity of immunohistochemical staining for hMLH1 were 62 and 100% and those of hMSH2 alone were 25 and 100%. Combining loss of expression of both hMLH1 and hMSH2 led to sensitivity, specificity, and positive and negative predictive values of 87, 100, 100, and 95%. These results suggest that use of a two-molecule panel (hMLH1 and hMSH2) can accurately determine the microsatellite instability status of patients with ovarian cancer.
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Microsatellite instability is due to defects in a family of DNA mismatch-repair genes. Microsatellite instability refers to changes in the patterns of polymorphic, two- to three-nucleotide repeat segments, called microsatellites that are distributed throughout the genome.[1] Both microsatellite instability and germline mutations have been found in the colorectal, endometrial, and gastric carcinomas that develop in patients with the hereditary nonpolyposis colon carcinoma (HNPCC) syndrome or Lynch syndrome II, a hereditary syndrome in which female family members are at increased risk of ovarian and endometrial cancer in addition to colon cancer and other malignancies of the gastrointestinal and genitourinary system.[2] These genes include *hMSH2*, *hMLH1*, *PMS1*, *PMS2*, *hMSH6*, and *hMSH3*,[3–9] although *MLH1* and *MSH2* are the most frequent germline mutations found in Lynch syndrome cases.[10,11] Ovarian carcinoma occurs in almost 10% of women with HNPCC who manifest germline mutations in either *hMLH1* or *hMSH2*.[12,13] Microsatellite instability has been noted in 10–17% of sporadic ovarian cancers, but the prevalence rises to 30–50% if the analysis is limited to the endometrioid histotype.[14–17] The prevalence of microsatellite instability in sporadic ovarian carcinoma in general remains largely unknown.

The assessment of mismatch-repair defects has also become an important tool in clinical practice.

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As individuals who show evidence of high levels of microsatellite instability are at increased risk of metachronous cancers (ovarian, endometrial or gastric carcinomas), they should be observed closely during long-term follow-up periods. Some authors have proposed that any individual with colorectal cancer that also shows evidence of microsatellite instability should be evaluated to rule out inherited conditions.[18] A 1997 Consensus Development Conference report from the National Cancer Institute[13] indicated that colorectal carcinomas with high microsatellite instability were associated with better prognosis than those with low microsatellite instability or stable microsatellites but that such tumors could also be resistant to some chemotherapy agents.[13,18] In light of these findings, rapid and sensitive screening methods for detecting microsatellite instability would be of great value.

Immunohistochemical analysis with specific antibodies directed against mismatch-repair proteins has proven useful for investigating mismatch-repair defects in both familial and sporadic forms of cancer in humans.[19] Recent studies have shown that use of commercial antibodies against hMSH2 and hMLH1 can reliably detect microsatellite instability.[18–24] The prevalence of loss or mutation of these mismatch-repair genes in sporadic human ovarian carcinoma has not been clear. We sought here to investigate the prevalence of mismatch-repair defects in ovarian cancer and to determine the sensitivity, and specificity of hMLH1 or hMSH2 immunostaining for predicting microsatellite instability.

Materials and methods

Patients and Tissue Samples

Subjects who provided tissue samples were 322 consecutive women with primary epithelial ovarian cancer who had undergone initial surgery at The University of Texas MD Anderson Cancer Center between 1990 and 2005. Histopathologic diagnoses were based on Gynecologic Oncology Group criteria, and disease was staged according to the International Federation of Gynecology and Obstetrics (FIGO) system. Epithelial tumors of low malignant potential, non-surface epithelial ovarian tumors, or benign epithelial lesions were excluded. All tissue samples were linked to a Microsoft Access database containing demographic, treatment-related, and survival information for the women who donated the samples. Follow-up was updated through June 2005 by review of medical records and the US Social Security Index. None of the patients had a personal or family history of HNPCC syndrome. The use of tissue blocks and chart review was approved by MD Anderson's institutional review board.

Construction of the Tissue Microarrays

The tissue microarrays were created from tissue blocks that had been stored at approximately 24°C. Hematoxylin-and-eosin-stained sections were reviewed by a pathologist to select representative areas of tumor from which to acquire cores for the microarray. The tissue microarray blocks were constructed with a precision instrument (Beecher Instruments, Silver Spring, MD, USA) as previously described.[25] For each case, two replicate 1-mm core-diameter samples were collected and each was placed on a separate recipient block. The final tissue microarray consisted of four blocks, the first two (1a and b) containing duplicates of 164 spots and the second two (2a and b) containing duplicates of 158 spots, with samples spaced 0.5 mm apart. Sections (5-μm) were obtained and stained with hematoxylin-and-eosin to confirm the presence of tumor and to assess tumor histology. Of the 322 cases, 168 were serous carcinoma, 73 were mixed-type carcinoma, 34 were endometrioid adenocarcinoma, 16 were clear cell carcinoma, 14 were malignant mixed müllerian tumors, eight were undifferentiated carcinoma, seven were mucinous adenocarcinoma, and two were transitional cell carcinoma. Serous carcinoma was the major histotype component found in mixed carcinoma histotypes ($>60\%$) combined with endometrioid carcinoma (47 cases), clear cell carcinoma (five cases), poorly differentiated carcinoma (17 cases), or transitional cell carcinoma (four cases).

Tumor samples were randomly arranged on the blocks. Sample tracking was based on coordinate positions for each tissue spot in the tissue microarray block; the spots were transferred onto tissue microarray slides for staining. Each core was scored individually, and the results were presented as the mean of the two replicate core samples.

Immunohistochemical Staining for hMSH2 and hMLH1

Immunohistochemical staining was carried out with 5-μm sections from selected formalin-fixed, paraffin-embedded normal and tumor tissue samples placed on coated slides. The antibodies used for the immunohistochemical analyses were mouse monoclonal anti-hMLH1 (PharMingen International, San Diego, CA, USA), used at a 1:30 dilution, and mouse monoclonal anti-hMSH2 (Ab-2; Oncogene, La Jolla, CA, USA), used at a 1:100 dilution. Immunostaining was done with an EnVision Plus System, Peroxidase kit (DAKO, Carpinteria, CA, USA) for hMLH1 and with an LSAB horseradish peroxidase kit (DAKO) for hMSH2 with diaminobenzidine used as a chromogen. Sections were lightly counterstained with hematoxylin. Loss of hMSH2 or hMLH1 expression in cancer tissues was defined as the total absence of detectable nuclear staining of neoplastic cells. Infiltrating lymphocytes as well as stromal

cells served as internal positive controls. Two pathologists assessed all of the cases with no knowledge of the microsatellite instability results or clinicopathologic information; scoring discrepancies were resolved by a third pathologist (JL).

Microsatellite Instability Analysis

Microsatellite instability was analyzed in DNA from cases that showed loss of *hMLH1* or *hMSH2* in the immunohistochemical staining and from an additional 19 cases selected at random that showed positive staining for both either hMLH1 and hMSH2. DNA was extracted from two 10- to 11-μm sections cut from selected tissue blocks. Tumor tissues were manually microdissected from adjacent normal tissue to ensure that each tumor sample contained at least 70% neoplastic cells. Specimens from a nonmetastatic lymph node, the appendix, or segments of normal fallopian tube from the same patients were used as normal tissue controls. DNA was extracted as previously described[26] and analyzed for microsatellite instability in five microsatellite markers: two mononucleotide repeats (BAT26 and BAT25) and three dinucleotide repeats (D5S346, D2S123, and D17S250). Oligonucleotide forward primers were labeled with fluorescence at the 5′ end. Multiple polymerase chain reactions (PCR) were carried out in a 12-μl mixture containing approximately 40 ng DNA, 1 × PCR buffer, and 0.5 μM of each PCR primer (Life Technologies, Gaithersburg, MD, USA), plus 2.5 mM $MgCl_2$, 200 μmol deoxynucleotide triphosphate, and 2 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, USA). PCR amplification was carried out in a Perkins-Elmer Gene Amp Thermo Cycler 9600. Samples were denatured at 95°C for 7 min and then subjected to three cycles consisting of denaturation (94°C, 1 min), annealing (58°C, 30 s), and extension (72°C, 45 s) followed by 42 cycles consisting of 45 s at 94°C, 30 s at 54°C, 40 s at 72°C, and a final extension step at 72°C for 30 min. Then, 1 μl of the labeled PCR products of paired normal and tumor tissues were mixed with 12 μl of deionized formamide and 1 μl GeneScan TAMRA 500 Size Standard (Applied Biosystems). The mixtures were denatured at 94°C for 5 min, cooled on ice, and then loaded on an ABI PRISM 310 Genetic Analyzer (Applied Bioystems), where data on the size of PCR products and the amount of fluorescent signal were collected and analyzed with the GeneScan 3.1 and Genotyper Analysis programs (Applied Biosystems). Microsatellite instability was defined as the presence of novel peaks in the tumor tissue that were not present in normal control tissue from the same patient or as a difference in microsatellite lengths in two samples. Tumors showing instability at two or more markers were considered to have a high level of microsatellite instability. Tumors showing instability at only one marker were defined as having a low level of microsatellite instability. Tumors in which no markers exhibited microsatellite instability were defined as microsatellite-stable. All positive samples were assayed at least twice to confirm the results.

Statistical Analysis

Sensitivity, specificity, positive predictive value, and negative predictive value of the immunohistochemical findings were calculated by using microsatellite instability-high or microsatellite instability-low/microsatellite-stable as the standard. Sensitivity was defined as the ratio of negative immunohistochemical results found to the total number of microsatellite instability-high tumors. Specificity was the ratio of positive immunohistochemical results to the total number of microsatellite instability-low/microsatellite-stable tumors. Positive predictive value was calculated as the ratio of microsatellite instability-high to the total of negative immunohistochemical results. Negative predictive value was calculated as the ratio of microsatellite-stable/L to the total number of positive immunohistochemical results. SAS software was used for all statistical analyses (SAS Institute, SAS Language Reference, Version 8, SAS Institute Inc., Cary, NC, USA, 1999).

Results

Sample anti-hMLH1 and anti-hMSH2 immunostains are shown in Figure 1. The normal staining pattern of both hMLH1 and hMSH2 was nuclear. Of the 322 cases subjected to immunohistochemical staining, only seven showed negative hMLH1/hMSH2 staining: five did not stain for hMLH1 and two did not stain for hMSH2 (Table 1). The remaining 315 samples stained for both hMLH1 and hMSH2. Thus, a total of 26 samples were subjected to microsatellite instability analysis by PCR (these seven cases plus 19 others selected at random) (Table 1). The mean age of the seven subjects negative for hMLH1 and hMSH2 staining was 57 years (range, 39–83 years). One case was diagnosed as malignant mixed müllerian tumor, three as mixed type carcinomas, two clear cell carcinomas, and one endometrioid carcinoma. Four cases presented at advanced stage disease and three at early stage disease. In only one cases there was a family history of a first relative with cancer. Two other cases presented a synchronic endometrial endometrioid cancer. The main components of the mixed-type carcinoma diagnosed were transitional, clear cell, and endometrioid carcinoma, respectively. All seven samples were found to be microsatellite instability-high. From the remaining 19 tested cases only one showed microsatellite instability-high and positive expression of both hMLH1 and hMSH2 (case 8). Hence, the calculated sensitivity and specificity of negative

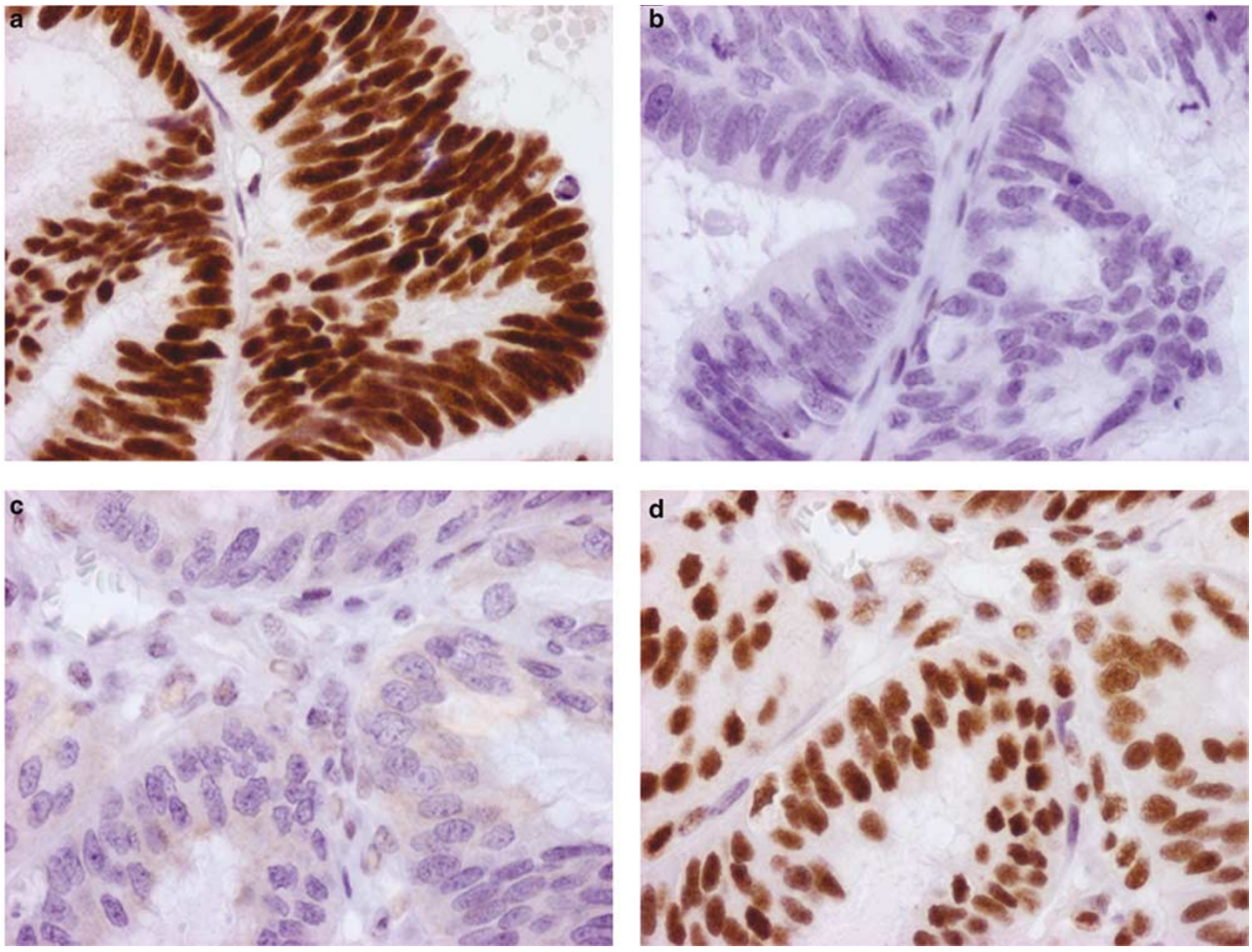

Figure 1 (**a**) Positive staining for hMLH1 and (**b**) negative expression of hMSH2 in case 3 (endometrioid component of mixed type carcinoma). (**c**) No staining for hMLH1 and (**d**) positive staining for hMSH2 in case 7 (endometrioid carcinoma).

immunohistochemical staining of hMLH1 for detecting microsatellite instability were 62 and 100%, respectively, and sensitivity and specificity of hMSH2 were 25 and 100%, respectively. When lack of staining for both hMLH1 and hMSH2 was considered together, the sensitivity, specificity, positive predictive value, and negative predictive value were 87, 100, 100, and 95% (Table 2).

Discussion

In this study, we examined the protein expression of hMLH1 and hMSH2 by immunoperoxidase technique in tissue microarrays of 322 cases of primary ovarian carcinoma. We then assessed the microsatellite instability status by PCR in the seven cases in which the protein expression of either hMLH1 or hMSH2 was absent and in 19 other cases in which both protein products were expressed. We found that the lack of expression of hMLH1 protein (five cases) or hMSH2 protein (two cases) was associated with microsatellite instability-high. In other words, immunohistochemical staining for hMLH1 and hMSH2 was highly specific (100%) and sensitive (87%) in predicting microsatellite instability status.

The reported prevalence of microsatellite instability in ovarian carcinoma ranged from 0 to 53%.[20–24,26] This wide discrepancy in the various reports reflects differences between studies in sample size, tumor histotype, microsatellite markers used, and criteria used to define microsatellite instability positivity. Many of these studies were done before the advent in 1998 of a standard panel of microsatellite markers or standardized criteria for determining microsatellite instability. These standard markers have since been proven suitable for examining microsatellite instability in ovarian carcinoma,[27] Unfortunately, in most of the published studies done to date, not only were different microsatellite markers used but instability at only one locus was considered to indicate microsatellite instability, which is no longer the case which is no longer. Therefore, the choice of markers for detecting microsatellite instability is crucial.

Table 1 Microsatellite marker patterns in seven hMSH2- or hMLH1-negative cases and in 19 other randomly selected cases

Case no.	*Histotype*	*Age*	*Stage*	*Grade*	*Family history of cancer*	*2nd primary*	*Microsatellite instability markers*					*Micro-satellite instability status*	*IHC*	
							BAT25	*BAT26*	*D2S123*	*D5S346*	*D17S250*		*hMSH2*	*hMLH1*
1	MMMT	83	IIIC	3	No	No	+	+	+	–	+	High (4/5)	+	–
2	Mixed type ca.	42	IIIC	3	No	No	–	–	NA	+	+	High (2/4)	+	–
3	Mixed type ca.	48	IV	3	No	Endometrial	+	+	NA	+	+	High (4/4)	–	+
4	Mixed type ca.	39	IIIC	3	No	Endometrial	+	+	NA	+	+	High (4/4)	+	–
5	CCC	46	IC	3	Breast	No	+	+	+	+	+	High (5/5)	–	+
6	CCC	60	IC	3	No	No	+	+	NA	+	+	High (4/4)	+	–
7	Endometrioid ca	83	IC	1	No	Endometrial	–	+	NA	+	–	High (2/4)	+	–
8	Mixed type ca.	54	IIIC	3	No	No	–	+	NA	+	–	High (2/4)	+	+
9	Transitional cell ca.	71	IIIC	3	No	No	–	–	–	–	–	Stable	+	+
10	CCC	59	IV	3	No	No	–	–	–	–	–	Stable	+	+
11	Mixed type ca.	44	IIC	3	No	No	–	–	–	–	–	Stable	+	+
12	Endometroid ca.	64	IC	2	Colorectal	No	–	–	–	–	–	Stable	+	+
13	Serous ca.	56	IIIC	3	No	No	–	–	–	–	–	Stable	+	+
14	Serous ca.	59	IIIC	3	Colorectal	No	–	–	–	–	–	Stable	+	+
15	Serous ca.	73	IIIC	3	No	Endometrial	–	–	–	–	–	Stable	+	+
16	Mucinous ca.	63	IA	1	No	No	–	–	–	–	–	Stable	+	+
17	Serous ca.	67	IV	3	No	Breast	–	–	–	–	–	Stable	+	+
18	Serous ca.	47	IIIC	3	Breast	No	–	–	–	–	–	Stable	+	+
19	Serous ca.	59	IIIC	3	No	Breast	–	–	–	+	–	Low	+	+
20	Serous ca.	27	IIIB	3	No	No	–	–	–	–	–	Stable	+	+
21	Mixed type ca.	54	IIIC	3	No	No	–	–	–	–	–	Stable	+	+
22	Mixed type ca.	47	IIC	3	No	No	–	–	–	–	–	Stable	+	+
23	Mixed type ca.	47	IIIC	3	No	No	–	–	–	–	–	Stable	+	+
24	Mixed type ca.	68	IV	3	No	No	–	–	–	–	–	Stable	+	+
25	CCC	57	IIIC	3	No	No	–	–	–	–	–	Stable	+	+
26	CCC	59	IIIC	3	Breast	No	–	–	–	–	–	Stable	+	+

MSI, microsatellite instability; IHC, immunohistochemical analysis; MMMT, malignant mixed müllerian tumor; NA, not available; CCC, clear cell carcinoma; ca, carcinoma.

Table 2 Sensitivity, specificity, positive predictive value and negative predictive value of immunohistochemical staining for determining microsatellite instability status

Marker	*Sensitivity (%)*	*Specificity (%)*	*Positive predictive value (%)*	*Negative predictive value (%)*
hMLH1	62	100	100	86
hMSH2	25	100	100	75
hMLH1+hMSH2	87	100	100	95

Molecular analysis is normally used to identify microsatellite instability in sporadic and familial tumors. The techniques involved in genotyping are difficult, time-consuming, expensive, and are often constrained by the availability of fresh tumor samples that have been appropriately collected and stored. Molecular analysis of microsatellite instability also does not identify the genes involved, a question that would require further molecular-level tests. Immunohistochemical analysis, on the other hand, has become routine in many pathology laboratories. The techniques are relatively inexpensive, fast, and simple. Several reports have demonstrated that immunohistochemical staining of hMLH1 and hMSH2 is a highly sensitive and specific screening method for the detection of microsatellite instability-high in tumor samples,[28] unlike staining for hMSH3 or hMSH6, mutations in which are less common (at least in ovarian endometrioid adenocarcinomas) and thus unsuitable for mutation screening.[29] Use of widely validated tissue microarray technology allows high-throughput immunohistochemical staining.[25] Therefore, the combination of immunoperoxidase staining and tissue microarray analysis, besides reducing cost and time, can be a sensitive screening method to identify genetic events that are relatively rare in large populations.

Testing microsatellite instability in ovarian carcinoma represents an unusual challenge because of the large number of tumor histotypes. Indeed, increasing evidence suggests that microsatellite instability is more likely to be associated with certain histologic types of ovarian carcinoma than with others, being more common in endometrioid, mucinous, and clear cell carcinoma than in serous carcinoma.[16,17,30,31] However, one study has shown that microsatellite instability in ovarian cancer is not definitely associated with a specific histopathologic subtype.[15] We previously demonstrated that high microsatellite instability was present in 20% of ovarian endometrioid carcinomas[32] and in 14% of clear cell carcinomas.[26] Our current findings are consistent with our previous ones; none of the 168 serous carcinomas in the microarray showed loss of

hMLH1 or hMSH2 expression. Further, of the seven microsatellite instability-high cases, two were clear cell carcinoma and one was an endometrioid carcinoma; endometrioid, transitional, and clear cell carcinoma were the main components in the three mixed-type carcinomas included in this study. These findings suggest that the presence of high microsatellite instability may be associated with some aspect of tumor development or histogenesis in at least some endometrioid or clear cell carcinomas of the ovary.

Two studies describe a high correlation (100%) between microsatellite instability status and immunohistochemical staining patterns;[18,20] in other studies, the correlations were not quite as high but ranged mostly from 75 to 95%.[19,21–23,33] In a large multicenter effort, Lindor *et al*[24] studied microsatellite instability with immunoperoxidase staining in colorectal tumors from 1144 patients. Immunohistochemical detection of hMLH1 and hMSH2 had a sensitivity of 92% and a specificity of 100%. In the present study, either hMLH1 or hMSH2 was not expressed in only seven cases (2%). This small number of negative cases reflects the low incidence of microsatellite instability in sporadic ovarian carcinoma. Our study also demonstrated that use of an hMLH1 and hMSH2 antibody panel was highly sensitive and specific for detecting microsatellite instability; in other words, the likelihood of predicting microsatellite instability-high when either hMLH1 or hMSH2 is negative would be 100%. A positive screen such as this, with a positive predictive value of 100%, would not require any additional molecular testing. We had only one false-negative finding (positive for hMLH1 and hMSH2 with microsatellite instability-high; case 8), which makes the likelihood of the immunohistochemical test detecting a true negative case 95%. One possible explanation for this false-negative finding is that the specimen may have harbored mutations in other of the nontested mismatch-repair genes.

In summary, our results suggest that the use of a dual molecular panel (hMLH1 and hMSH2) can quite accurately determine the microsatellite instability status of patients with ovarian cancer. As these two immunohistochemical stainings can be routinely used in pathology laboratory, they can be useful to predict the status of mismatch-repair gene for individuals with suspected hereditary conditions.

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