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Defective DNA Mismatch Repair Influences Expression of Endometrial Carcinoma Biomarkers

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Introduction

Endometrial carcinoma (EC) is the most common gynecologic malignancy and is the fourth most common cancer in women in the United States. The American Cancer Society estimated that there will be 52,630 new diagnoses and 8,590 deaths from endometrial cancer in 2014 [1]. High levels of microsatellite instability (MSI-high) result from mismatch repair (MMR) defects, which are seen in approximately 20% of endometrial cancers including sporadic endometrial cancer and endometrial carcinomas associated with Lynch syndrome (Hereditary Nonpolyposis Colorectal Cancer/HNPCC) [2]. Sporadic endometrial cancers with DNA MMR defects demonstrate immunohistochemical loss of MLH1 secondary to methylation of the *MLH1* gene promoter. In Lynch Syndrome, germline mutations occur in a family of mismatch repair genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*) [3,4,5]. Both sporadic and Lynch Syndrome associated endometrial carcinomas with mismatch repair defects are typically MSI-high. Detection of patients with MSI-high endometrial cancers due to germline mutations is important, as these patients are predisposed to develop a variety of other malignancies, especially colorectal cancer.

Numerous studies have shown that endometrial carcinomas of endometrioid type are related to estrogen excess, while non-endometrioid carcinomas are not related to hormone exposure [6,7,8]. Therefore, endometrioid-type endometrial carcinomas typically express the hormone receptors estrogen receptor (ER) and progesterone receptor (PR), but the actual percentage of cells expressing these hormone receptors can be extremely variable from tumor to tumor, and this variability is not always explained by differences in tumor grade [9,10]. Such variability is clinically relevant, as grade 1 endometrioid carcinomas can be conservatively

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treated with progestin therapy, with variable efficacy [11,12,13]. In this study we sought to investigate if DNA mismatch repair status may potentially play a role in influencing expression of hormone receptors and other biomarkers relevant to endometrial carcinoma. Past studies have shown that colorectal carcinomas with high levels of microsatellite instability have altered immunophenotypic characteristics, namely reduced expression of CK 20, which is typically present in 70–100% of colorectal carcinomas [14]. Other studies have shown a significant correlation between microsatellite instability in breast cancer and decreased expression of ER and PR [15]. In addition to examining expression of ER and PR, we also examined Pax-8, CK7, and CK20. CK 7 and CK 20 are the most commonly used cytokeratin stains used to aid in the classification of tumors according to their site of origin [16,17]. Both of these markers are often used by pathologists to distinguish mullerian primary tumors from tumors derived from the lower gastrointestinal tract. Endometrial carcinomas are typically CK 7 positive and CK 20 negative [18,19,20]. Pax-8, although not specific, is a useful marker for indicating mullerian differentiation. Previous reports have shown that Pax-8 expression in EC is high, ranging from 84% to 98% [21–23]. Strong and diffuse Pax-8 expression is especially associated with higher EC tumor grade [24].

Materials and Methods

Unselected cases of endometrial carcinoma involving women who underwent hysterectomy at MD Anderson Cancer Center through 2011 were identified. Endometrial cancers were included if the patient was 18 years of age or greater and sufficient tissue from the surgery was available for molecular analysis. Relevant clinical data were extracted from the electronic medical record, and all hysterectomies were pathologically reviewed by a gynecologic pathologist (RRB). The carcinomas were graded according to the International Federation of Gynecology and Obstetrics (FIGO) grading system. Four-hundred-eleven patients with endometrial carcinoma met inclusion criteria, and their tumors were subjected to immunohistochemistry testing for MLH1, MSH2, MSH6, and PMS2. *MLH1* methylation was also performed in patients with immunohistochemical loss of MLH1. One hundred and fifteen endometrial cancers with mismatch repair defects were identified. The majority of these tumors were endometrioid tumors, and a considerable amount of these endometrioid tumors were grade 2 tumors. To eliminate any possible variation in biomarker expression that could be attributed to the histotype or grade of tumor, we sought to select and study grade 2 endometrioid tumors, since a large proportion of the tumors were grade 2 endometrioid tumors. Specific mismatch repair deficient cases were then selected based on the presence of adequate tumor and age of patient for matching to a mismatch repair intact control. Eighty grade 2 endometrial endometrioid adenocarcinoma cases were selected for further study, 40 mismatch repair deficient and 40 controls with intact mismatch repair. These controls were not only matched for histotype and grade, but were also age matched to the 40 selected grade 2 cases with MMR defects. Immunohistochemical analysis for ER, PR, CK 7, CK 20 and PAX-8 were performed on all 80 cases. The methods are outlined in Figure 1.

Immunohistochemistry and molecular analysis

All tissue-based analyses were performed using formalin-fixed, paraffin-embedded endometrial carcinoma sections. All of the cases were fixed in the same pathology laboratory, for approximately the same length of time, and under the same protocols. After review of all the H&E stained slides for each case, one representative block that had an adequate amount of viable tumor for immunohistochemistry studies and was best representative of grade 2 endometrioid histology was selected for further study. Immunohistochemistry of MMR proteins was performed using standard techniques for MLH1 (G168-15 1:25; BD Biosciences Pharmingen), MSH2 (FE11, 1:100; Calbiochem), MSH6 (44, 1:300; BD Biosciences Pharmingen), and PMS2 (Alb-4, 1:125; BD Biosciences Pharmingen) [25]. MLH1, MSH2, MSH6, and PMS2 immunohistochemistry was scored as protein intact or deficient using light microscopic examination. Complete absence of mismatch repair protein expression was required in order for a case to be designated as mismatch repair deficient. Stromal cells served as an internal positive control. For tumors with loss of MLH1 protein expression, PCR-based *MLH1* promoter methylation analysis was performed. DNA was isolated from mapped formalin-fixed, paraffin-embedded tissue sections that were dissected with a scalpel blade to provide relatively pure tumor samples for analysis. Isolated DNA was treated with bisulfite to convert unmethylated cytosine nucleotides to uracil using the Zymo EZ DNA Methylation-Gold Kit according to the manufacturer's instructions (Zymo Research, Orange CA). Methylation of *MLH1* was assessed using a modified version of methylation-specific PCR followed by capillary electrophoresis using FAM labeled reverse primer and unlabeled forward primers (Integrated DNA Technology). The primer sequences were the following: methylated forward, 5'-GAT AGC GAT TTT TAA CGC-3', unmethylated forward, 5'-AGA GTG GAT AGT GAT TTT TAA TGT-3' and labeled reverse primer, 5'-FAM-TCT ATA AAT TAC TAA ATC TCT TC-3' and labeled reverse primer, 5'-FAM-TCT- ATA AAT TAC TAA ATC TCT TC-3'. The forward primers were designed to distinguish the methylated amplicon from the unmethylated by difference in size. The bisulfite treated DNA was then amplified by PCR using primers specific for methylated and unmethylated DNA. The methylated PCR product of 85 bp was separated from unmethylated PCR product of 91 bp by capillary electrophoresis using an ABI Prism 3130 Genetic Analyzer. Chromatograms for tumor were compared to those generated for the RKO colon carcinoma cell line (positive control known to have loss of MLH1 protein due to *MLH1* promoter methylation).

For the 80 endometrial carcinomas, immunohistochemistry for cytokeratins 7 and 20, PAX-8, ER, and PR was performed. The CK 7 antibody was clone OV-TL 12/30 (Dako) at 1:100 dilution, and the CK 20 antibody was clone KS20.8 (Dako) at 1:4000 dilution. The PAX-8 polyclonal antibody (10336-1-AP; Proteintech) was at 1:100 dilution. The progesterone receptor antibody clone was PgR 1294 (Dako) at 1:200 dilution, and the estrogen receptor antibody clone was 6F11 (Novacastra) at 1:35 dilution. Immunoprofiling for CK7, CK 20 and Pax-8 were done on deparaffinized sections with avidin-biotin-peroxidase complex method in a Dako Autostainer after elimination of endogenous peroxidase and antigen retrieval. Immunoprofiling for ER and PR was performed on deparaffinized sections in a Dako Autostainer post pretreatment and subsequent citrate buffer in the steamer for 30 minutes (ER) and 20 minutes for (PR).

For immunohistochemical expression of CK 7, CK 20, Pax-8, ER and PR, the percentage of carcinoma cells with positive staining was semi-quantitated and tabulated in quintiles while blinded for tumor mismatch repair status. Cases with less than 1% staining were classified as negative. High vs. low cut points for each biomarker were then established based on the distribution of the per cent tumor cells with positive expression of a biomarker, while attempting to create high vs. low groups with at least 10–15 individual cases each for the purposes of rational comparison. For both CK 7 and Pax-8, the cut point for high vs. low expression was set at 50%. For ER and PR, the cut points for high vs. low expression was 60% and 70%, respectively. For ER and PR, in addition to quantifying the percentage of positive tumor cells, we also evaluated the intensity of staining.

A Wilcoxon rank sum test was conducted to compare the expression percentage for CK7 between MMR deficient and MMR intact groups. A Fischer exact test was used to compare prevalences of immunoprofiles by MMR status.

Results

Of the 40 selected grade 2 EC's that had an MMR deficiency, 30 were due to MLH1 methylation, and 10 cases had either loss of expression of MSH2, MSH6, PMS2 or MLH1 without associated *MLH1* methylation. CK 7 expression was significantly decreased in the MMR deficient group compared to the MMR intact group. The median percentage of CK 7 positive tumor cells in the MMR deficient group was 45%, compared to 78% in the MMR intact group ($p=0.008$, Figures 2 and 3). Fifteen percent of MMR deficient EC's had 5% or less of tumor cells expressing CK 7, while none of the MMR intact EC's had 5% or less CK 7 immunohistochemical expression. There were 35 cases with CK7 expression in 50% or less of the tumor cells; 22/35 (63%) had a MMR deficiency. CK 20 was not expressed in any of the tumor cells in either the MMR deficient group or the MMR intact group (data not shown). Pax-8 expression was not significantly different between the MMR deficient and MMR intact groups. The median percentage of tumor cells exhibiting ER expression was similar in both the MMR intact (78%) and MMR deficient (80%) groups. In addition, no difference was noted in the degree of intensity of staining between the two groups. However, there was greater variability in biomarker expression in the MMR deficient group compared to the MMR intact group. Only 5 cases of MMR intact EC's had a percentage of ER positive tumor cells that was less than 55%, but 11 cases of MMR deficient EC's had an ER positive percentage less than 55%. There were 16 cases with ER expression in 50% or less of the tumor cells; 11/16 (69%) had a MMR deficiency. Thus, if a case had low ER expression, it was more likely to be MMR deficient (Figure 4). The percent of PR + tumor cells in both the MMR intact and the MMR deficient groups were both similar, 78% and 80%, respectively. There was no difference noted in the degree of intensity of staining between the two groups.

Each of the cases were stratified to either high vs. low expression of CK 7, Pax-8, ER, and PR as outlined in the Materials and Methods section. Tumors with the immunophenotype of low CK 7 low and Pax-8 low were more commonly MMR deficient than MMR intact, 42.5% vs. 20% respectively (Table 1). Additionally, the combined immunophenotype of CK 7 high, ER high and PR high was more common in the MMR intact group (42.5%), as compared to the MMR deficient group (20%) (Table 2).

Among the EC's with an MMR defect, we evaluated if the type of MMR defect resulted in differences in immunohistochemical expression. We found that 70% of EC's with *MLH1* methylation had less than 70% CK7 positive tumor cells, while only 40% of carcinomas with a MMR defect not due to *MLH1* methylation had less than 70% CK7 positive tumor cells. Therefore, there was a trend for the endometrial carcinoma cases with *MLH1* methylation to have a lower percentage of CK 7 staining than endometrial carcinomas with other types of MMR defects. With regards to ER, no difference was seen in the percentage of tumor cells that were positive for ER between the carcinomas with *MLH1* methylation vs. the carcinomas with other MMR defects. There was, however, a tendency for the carcinomas with *MLH1* methylation to have weaker ER staining as compared to MMR deficient carcinomas that were non-methylated. 23.3% of the *MLH1* methylation EC cases had weak ER staining, while none of the carcinomas with non-methylation MMR defects had weak staining.

Discussion

Mismatch repair defects resulting in microsatellite instability have been detected in up to 20% of EC's, making this one of the most common molecular alterations in this tumor type [26,27]. Some studies have suggested distinctive histologies in EC's with mismatch repair defects, such as the increased presence of tumor infiltrating lymphocytes and the presence of a dedifferentiated carcinoma component [28]. EC's centered on the lower uterine segment have an increased likelihood of mismatch repair defect [29]. It has been well documented that some colorectal carcinomas with MMR defects can have a histologically distinctive phenotype, which enables distinction from colorectal carcinomas with intact MMR machinery [30–33]. Although specific histologic features have been associated with MMR deficient colorectal carcinomas, such histologic features are not always reliable in predicting MMR deficiency, as many MMR deficient carcinomas may not have these distinctive features. Alexander et al. evaluated the relationship between histopathologic features and MSI status in a cohort of colon cancers and found that histopathology alone did not reliably discriminate between MSI-H cancers and microsatellite-stable (MSS) cancers [34]. They found that despite the fact that certain morphologic features are associated with MMR deficient carcinomas, the morphological predication of MSI-H had a low sensitivity [34].

Several studies have noted a difference in the immunohistochemical expression of tissue biomarkers in MMR deficient colorectal tumors, namely decreased CK 20 expression [14,35]. McGregor et. al., evaluated immunohistochemical expression of CK 7 and CK 20 in 22 MSI-H and 22 MSS colorectal carcinomas that were matched for clinical-pathologic characteristics and found that the mean percentage of CK 20 + tumor cells was lower in the MSI-H group (37% $\pm$ 8%) in comparison to the MSS group (84% $\pm$ 6%) [14]. Positive expression of CK 7 was infrequent and pancytokeratin staining was universal. In a subsequent study, Lugli, et al., evaluated the phenotypic expression of CDX2/CK 20/CK 7 by immunohistochemistry in a large series of colorectal carcinoma, stratified by MMR status [35]. They found that CK20 and CDX2 negativity was more frequently found in MMR deficient colorectal carcinomas than in MMR intact carcinomas (19.3 vs. 7.5% and 21.6 vs 6.7%) respectively [35].

Such studies examining the expression of relevant biomarkers in endometrial carcinomas with mismatch repair defects, to our knowledge, has not been previously reported in EC. We have shown that CK 7 expression was significantly lower in MMR deficient EC. Chu et al, found that 100% of the endometrial carcinomas studied were positive for CK 7 [18], but these cases were not stratified according to MMR status. In addition, despite controlling for tumor histotype (endometrioid), grade, and patient age, we observed that the MMR deficient cases had more heterogeneity with regards to immunohistochemical expression of relevant biomarkers. 16.7 % of MMR deficient EC's had less than 5% staining for CK 7, while none of the MMR intact EC's had less than 5% staining. The mechanism of decreased CK 7 expression in MMR deficient endometrial carcinoma is unknown. The transcriptional regulation of keratins is complex. Nuclear receptors for retinoic acid and thyroid transcription factor are known to regulate the majority of keratins, but the regulation of cytokeratin 7 has not been well elucidated [36]. Interestingly, we saw a tendency for CK 7 expression to be further decreased in MMR deficient cases with *MLH1* methylation vs MMR deficient cases with an unmethylated *MLH1* promoter. This may be related to more general methylation of other promoters in addition to the promoter for *MLH1* [37].

Our finding of decreased cytokeratin 7 expression in MMR deficient endometrial carcinomas has important clinical implications. In routine pathology practice, cytokeratin 7 and cytokeratin 20 expression is used in the diagnostic work-up of tumors of unknown origin. Endometrial carcinomas typically have a CK 7 +/-CK 20 – phenotype. Thus, our findings of decreased cytokeratin 7 in MMR deficient EC's is clinically relevant, as lack of CK 7 or minimal CK 7 immunostaining would not automatically rule out an EC as the primary site of origin of the tumor in question. This is especially important when evaluating tumors taken from small needle biopsies, where the tissue sampled may be limited and not representative of the tumor as a whole. 16.7% of our MMR deficient endometrial carcinoma cases had less than 5% CK 7 immunohistochemical staining. Thus, when evaluating small tissue samples, the very minimal and focal CK 7 staining may lead to an errant pathological diagnosis.

In MMR deficient colorectal carcinomas, *BRAF* mutation is rarely seen in the setting of Lynch syndrome [38,39]. Therefore, in MSI-H tumors with *MLH1* loss by immunohistochemistry, *BRAF* mutation analysis is clinically useful to help exclude Lynch syndrome. *BRAF* mutations in colorectal carcinoma have been found to be closely linked to high-level CpG island methylator phenotype, which can include methylation of *MLH1* [40]. Nevertheless, the exact mechanisms as to why *BRAF* mutation is typically not seen in Lynch syndrome related carcinoma is not clearly known. In sporadic EC *BRAF* mutations are relatively infrequent, so *BRAF* molecular analysis to help exclude Lynch syndrome in such cases is not of utility [41–44]. From our current study, decreased cytokeratin 7 expression in an EC may be a potential indicator of underlying MMR deficiency. This is important, because a subset of these patients may subsequently have Lynch syndrome. In the appropriate clinical setting, decreased cytokeratin 7 expression in an endometrial carcinoma may be one of several factors that could prompt additional screening for Lynch syndrome.

ER and PR are usually positive in endometrioid carcinomas. Our data showed no statistically significant differences in the degree of ER and PR staining between the MMR deficient and

MMR intact groups. However, greater variability was noted in ER expression in the MMR deficient group compared to the MMR intact group. Thus, a tumor with a lower expression of ER was more likely to be in the MMR deficient group. Furthermore, when evaluating a combination of immunophenotypes, the CK 7 high/ER high/PR high immunophenotype, which would traditionally be expected in the majority of endometrioid-type endometrial carcinomas, was seen more commonly in the MMR intact group versus the MMR deficient group (42.5% vs. 20%). Although the underlying mechanism for these changes is not entirely clear, given the decreased number of CK 7 high/ER high/PR high tumors in the MMR deficient group, MMR defects may contribute to the variability of responsiveness of low grade endometrioid adenocarcinomas to hormonal manipulation [11,12,13].

As previously noted, decreased expression of CK7 or ER was associated with a tendency for an endometrial carcinoma to be mismatch repair deficient. This effect was especially strong for decreased CK7 expression. At this point, we have insufficient germline sequencing data to determine if expression of CK7 or ER is associated with germline mutation of a mismatch repair gene. In many centers, universal screening of endometrial cancer patients for mismatch repair defects is not practiced. Decreased expression of CK7 or ER could be a microscopic tumor feature that might trigger a pathologist to initiate more definitive testing. Our data may also be viewed from a different perspective. Mismatch repair deficiency may help to explain, at least in part, the variable biomarker expression pathologists observe in endometrioid-type endometrial carcinomas.

The results of our study show that mismatch repair deficiency in endometrial carcinoma affects biomarker expression, especially decreased CK 7 expression. Knowledge of this is particularly important when considering and evaluating carcinomas of unknown primary origin, as absent or decreased CK 7 expression would not necessarily rule out an endometrial primary site. Additionally, in the appropriate clinical setting, decreased CK 7 expression in an endometrial carcinoma may be another pathologic factor to consider when assessing which patients may need further screening for Lynch syndrome.

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List of Abbreviations

EC	Endometrial carcinoma
EEC	Endometrioid endometrial carcinoma
MMR defect	Mismatch repair defect
MMR intact	Mismatch repair intact

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Figure 1.

Schematic overview of methodology. 411 endometrial carcinomas were screened for mismatch repair deficiency using immunohistochemistry and *MLH1* methylation analysis when a tumor had loss of MLH1 protein expression. 293 carcinomas had intact DNA mismatch repair protein expression, while 118 were mismatch repair deficient.

Figure 2.
Immunohistochemical expression of CK 7 in representative MMR intact (A) and MMR deficient (B) endometrial carcinomas.

Figure 3.
Distribution of CK 7 immunohistochemical expression amongst the MMR intact and deficient endometrioid carcinomas.

Figure 4.

Percentage of ER positive tumor cells in the MMR deficient and MMR intact groups. While mean ER+ tumor cells was comparable between the 2 groups, the MMR deficient group shows greater variability, with more cases showing less than 55% ER positive tumor cells.

Table 1

Combined expression of CK 7 and Pax-8. For both CK7 and Pax-8, the cut point for a high/low designation was set at 50%.

Immunoprofile	MMR intact % (number of cases)	MMR deficient % (number of cases)
CK 7 low/Pax-8 low	20% (8)	42.5% (17)
CK 7 low/Pax-8 high	5% (2)	10% (4)
CK 7 high/Pax-8 low	50% (20)	22.5% (9)
CK 7 high/Pax-8 high	25% (10)	25% (10)

Table 2

Combined expression of CK 7, ER and PR. For ER, the cut point for a high/low designation was set at 60%. For PR, the cut point for high/low was set at 70%.

Immunoprofile	MMR intact % (number of cases ^I)	MMR deficient % (number of cases ^I)
CK 7 high/ER&PR high	42.5% (17)	20% (8)
CK 7 low/ER&PR low	0% (0)	7.5% (3)
CK 7 low/ER&PR high	25% (10)	37.5% (15)
CK 7 high/ER&PR low	12.5% (5)	7.5% (3)

^I Note that total does not add to 40 because of smaller numbers of cases with other immunoprofiles, such as CK7high/ER high/PR low, CK7 high/ER low/PR high, etc.