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Variable Expression of MSH6 in Endometrial Carcinomas with Intact Mismatch Repair and with MLH1 Loss due to *MLH1* Methylation

Nidhi Tandon, M.D[1], **Courtney Hudgens, BS**[1,2], **Bryan Fellman**[3], **Michael T. Tetzlaff, M.D., Ph.D.**[1,2], **Russell R. Broaddus, M.D.,Ph.D.**[1]

[1]Department of Pathology, The University of Texas M.D. Anderson Cancer Center Houston, Texas

[2]Department of Translational and Molecular Pathology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas

[3]Department of Biostatistics, The University of Texas M.D. Anderson Cancer Center, Houston, Texas

Summary

Immunohistochemistry for mismatch repair proteins MLH1, MSH2, MSH6, and PMS2 is an effective screen to detect individuals at risk for Lynch syndrome. College of American Pathologists guidelines stipulate that protein expression should be reported as present vs. absent, as most patients with germline mutations in a mismatch repair gene have complete loss of protein expression in tumor cells. A similar approach is employed to screen for cancer patients eligible for immune checkpoint blockade. This “all or none” interpretive approach ignores substantial evidence that mismatch repair may be more finely regulated by other mechanisms. We have observed clinically that MSH6 expression is variable, even in carcinomas that are overall considered positive for MSH6 expression. A proof-of-principle study was therefore designed to more rigorously quantify the protein expression of MSH6 and its binding partner, MSH2, using image analysis applied to age-matched endometrioid grade 2 subsets that were either mismatch repair intact or MLH1-deficient due to *MLH1* gene methylation. In both endometrioid groups, MSH6 expression was significantly lower than MSH2 expression. MSH6 expression increased in higher grade, mismatch repair intact serous carcinomas, but it was still significantly lower than that for MSH2. MSH2 expression was consistently high across the three different tumor groups. These results suggest that MSH6 expression is subject to wide fluctuations in expression, even when overall its expression is considered intact. While such fluctuations are likely not relevant for Lynch syndrome screening, they may be more impactful when considering patients eligible for immune checkpoint blockade.

Corresponding author: Russell R. Broaddus, M.D., Ph.D., Department of Pathology, Unit 85, University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, rbroaddus@mdanderson.org, Office phone: (713) 745-2794, Office fax: (713) 792-5532, After September 16, 2019: rbroaddus@med.unc.edu.

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Keywords

endometrial carcinoma; mismatch repair; MSH6; digital pathology

Introduction

It has become standard of care to evaluate all endometrial cancer patients for the hereditary cancer syndrome, Lynch syndrome. As an initial part of this evaluation, immunohistochemistry for the four major mismatch repair proteins, MLH1, MSH2, MSH6, and PMS2, is a commonly employed first screening step. Germline mutation of a mismatch repair gene is typically associated with complete immunohistochemical loss of the corresponding mismatch repair protein by immunohistochemistry. Thus, guidelines have typically recommended that the immunohistochemistry results be reported in a binary fashion, either positive (intact nuclear expression in tumor cells) or negative (nuclear expression lost in tumor cells) (1–3).

When it was discovered that mismatch repair deficiency was associated with improved survival following treatment with PD-L inhibitors (4–6), the same immunohistochemistry reporting algorithms for identifying patients with Lynch syndrome were then applied to screening cancer patients with advanced solid tumor malignancies for eligibility for immune checkpoint blockade therapeutic trials. This “expressed or not expressed” binary approach to reporting the immunohistochemical expression of mismatch repair proteins ignores substantial published evidence that these proteins may be more finely regulated, with important biological consequences. For example, in cell lines in which the expression of individual mismatch repair genes has been knocked down with shRNA, total loss of mismatch repair function was detected with 25% expression of *MLH1* and *MSH2* (7). Mismatch repair was decreased significantly when *MSH6* and *MSH2* were at 75% expression (7). *PMS2* partial knockdown was also associated with significantly decreased mismatch repair (8). Partial downregulation of *MSH2* resulted in microsatellite instability in the BAT26 mononucleotide repeat and telomere shortening (9). Given these compelling *in vitro* data on the impact of fine regulation of DNA mismatch repair, we designed a proof-of-principle study to determine if MSH6, compared to its binding partner MSH2, exhibits variability in protein expression in different groups of endometrial carcinoma previously characterized by immunohistochemistry for mismatch repair status.

Materials and Methods

Patient selection

The study was approved by the University of Texas MD Anderson Cancer Center’s institutional review board (Protocol LAB01–718). Retrospective review from 2013–2017 revealed 449 endometrial carcinomas previously tested for DNA mismatch repair protein immunohistochemistry for evaluation of Lynch Syndrome. After excluding small biopsies, cases with immunohistochemical loss of PMS2 in isolation, MSH2, or MSH6, and cases with unavailable slides, 384 endometrial carcinomas (317 endometrioid, 67 non-endometrioid) remained for potential study. To minimize possible impact of tumor grade

on expression of mismatch repair proteins in the endometrioid group, we chose to only examine FIGO grade 2 tumors, which was the most common histology (231/317 endometrioid, 73%). In the grade 2 group, 175/231 (76%) were mismatch repair intact, while 56/231 (24%) had immunohistochemical loss of MLH1 and PMS2 secondary to *MLH1* methylation. All of the grade 2 tumors with *MLH1* methylation were included for study. To minimize the possible impact of age on mismatch repair protein expression, age matching was employed to generate a group of mismatch repair intact grade 2 endometrioid carcinomas for examination. The non-endometrioid group was composed of a variety of histologies, including serous carcinoma, clear cell carcinoma, undifferentiated carcinoma, and carcinosarcoma. We focused for further examination on the 37 tumors that were pure serous carcinoma or predominantly serous carcinoma with intact expression of mismatch repair proteins. Identification of the patient groups included in this study is summarized in the flow diagram in Figure 1.

Assessment of DNA mismatch repair

Mismatch repair protein testing were performed according to methodology previously described (10). Immunohistochemistry of mismatch repair 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). Immunohistochemistry was scored as mismatch repair 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, polymerase chain reaction (PCR)-based *MLH1* promoter methylation analysis was performed. DNA was isolated from mapped formalin fixed, paraffin-embedded tissue sections from the same block in which immunohistochemistry was performed. Tissue sections 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 unalabeled forward primers (Intergrated 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 with those generated for the RKO colon carcinoma cell line (positive control known to have loss of MLH1 protein due to *MLH1* promoter methylation).

Quantification of MSH2 and MSH6 protein expression using automated image analysis

MSH2 and MSH6 immunohistochemistry slides were analyzed using image analysis software (Aperio ImageScope) in order to quantify the number of positive cells within designated areas. Slides were scanned at 20X (Aperio Scanscope AT Turbo; Leica Biosystems) utilizing methods previously described (11). Tumor cell expression of MSH2 and MSH6 was designated according to image analysis software (Aperio ImageScope) within designated areas using a modified Nuclear v9 algorithm, with the intensity thresholds adjusted manually to remove background artifacts and to account for variable differences in cell size. The image analysis system scored nuclei as 0 (no expression), 1+, 2+, and 3+ and quantified the relative numbers of these in five 1 mm^2 boxes within the tumor. The boxes were placed by visual inspection to include areas that were enriched with tumor cells. One of the boxes was the hotspot, defined as the focus with highest expression of mismatch repair protein as determined by light microscopic examination. The other four 1 mm^2 boxes were designated randomly throughout the tumor. These four boxes were placed in the same locations in the MSH2 and MSH6 slides to minimize any possible impact of regional variability on mismatch repair protein expression. With five 1 mm^2 boxes quantified per tumor, a total of 760 boxes were characterized for MSH2 and MSH6 analysis. For hotspot and random regions, the number of total MSH2- or MSH6-positive cells (1+, 2+, and 3+) was summed and divided by the total area (mm^2) in which the cells were counted. The results for MSH2 and MSH6 were reported as per cent positive cells. In addition, the number of tumor cells with the strongest (3+) MSH2 and MSH6 expression was determined.

Statistical Analysis

Summary statistics of means, standard deviations, and ranges for MSH2 and MSH6 immunohistochemical expression were calculated by tumor groups and compared using a Kruskal-Wallis test. Tumor group categories were mismatch repair intact endometrioid grade 2, endometrioid grade 2 with MLH1 protein loss secondary to *MLH1* gene methylation, and mismatch repair intact non-endometrioid. Comparisons of MSH2 vs. MSH6 within each tumor group were performed using a signed-rank test. All statistical analyses were performed using Stata/MP v15.0 (College Station, TX). This was considered an exploratory/hypothesis generating analysis, and statistical significance was defined at the 0.05 level.

Results

The mean age for the 59 mismatch repair intact endometrioid patients was 60 years, while the mean age for the 56 patients with endometrioid tumors with MLH1 loss due to *MLH1* methylation was 66 years. The mean age for the mismatch repair intact non-endometrioid group was 69 years. There were some differences between the groups in the FIGO stage distribution at the time of diagnosis. The majority (26/37, 70%) of the non-endometrioid tumors were advanced stage (stages III and IV) at the time of diagnosis. In contrast, the vast majority of the endometrioid tumors in both groups were early stage (stages I and II) at the time of diagnosis (mismatch repair intact 55/59, 93%; MLH1 loss 44/56, 79%). For all tumor groups, the primary tumor was examined for MSH2 and MSH6 immunohistochemical expression.

In the mismatch repair intact endometrioid group, expression of MSH6 was significantly lower than expression of MSH2, despite the fact that both were overall considered positive (Table 1, first column). This lower MSH6 expression was seen as both lower per cent tumor cells expressing MSH6 and lower per cent tumor cells with strong 3+ MSH6 expression. As was suspected by routine light microscopic inspection of immunohistochemistry slides, MSH2 expression was diffuse and strong, present in more than 90% of tumor cells in the hotspot and nearly 90% when four random areas were assessed. This lower MSH6 expression was also observed in endometrioid tumors with mismatch repair deficiency due to *MLH1* gene methylation and subsequent MLH1 and PMS2 protein loss (Table 1, second column), suggesting that status of MLH1 or PMS2 does not impact protein expression of MSH6. Similarly, MSH6 expression was also significantly lower than that for MSH2 in mismatch repair intact non-endometrioid carcinomas (Table 1, third column). Note that for all three measures of immunohistochemical expression, MSH2 levels are fairly consistent across the 3 different tumor groups (Table 1, MSH2 row). However, the standard deviation is much greater for MSH6 than for MSH2, demonstrating the more dynamic range and variability for MSH6 expression.

There are data to suggest that differentiated cells have lower levels of mismatch repair proteins (12) and that mismatch repair proteins, especially MSH6, can be upregulated during the S phase of the cell cycle (13). Non-endometrioid endometrial carcinomas, such as endometrial serous carcinoma, are poorly differentiated with lower expression of hormone receptors such as estrogen and progesterone receptors, are more clinically aggressive, and associated with dysregulated cell cycle and a mitotic gene expression pattern (14). We therefore speculated that these non-endometrioid carcinomas would have increased MSH6 expression compared to expression in either of the more differentiated endometrioid groups. The hotspot for MSH6 density was slightly increased in the non-endometrioid group, but this was not statistically significant (Table 1, MSH6 row). However, the mean of the four random MSH6 foci and the mean of the 3+ tumor cells in the MSH6 four random foci showed significantly higher expression in the non-endometrioid group compared to both of the endometrioid groups (Table 1, MSH6 row). In aggregate, these data suggest that MSH6 expression may be more susceptible to dynamic regulation, compared to the regulation of expression of MSH2. One clinically relevant regulator of MSH6 may be dysfunction of the cell cycle and/or tumor grade, given the higher expression in the non-endometrioid carcinomas.

The observation that higher tumor grade, as seen in the non-endometrioid tumors, may be associated with higher MSH6 expression, was confirmed in three endometrioid cases from this cohort in which a small, more poorly-differentiated focus was immediately adjacent to a well-differentiated component (Figure 2). Note that for each of these cases, MSH2 expression was diffusely strong in both the poorly-differentiated solid and well-differentiated glandular components (Figure 2, B-D-F). However, differences in MSH6 expression between the two adjacent components were readily seen by light microscopic examination. In each of the 3 cases, the MSH6 expression was weaker and present in fewer tumor cells in the well-differentiated glandular component compared to the stronger and more diffuse expression in the immediately adjacent poorly-differentiated, solid component (Figure 2, A-C-E).

Discussion

We used automated image analysis to carefully characterize and quantify MSH6 and MSH2 protein expression in three different groups of endometrial adenocarcinoma. In these clinical samples, MSH6 protein expression was significantly lower than expression for MSH2, even when both were overall considered positive by immunohistochemistry. MSH6 expression increased in tumors that were more poorly differentiated and higher grade, but even in these non-endometrioid carcinomas, its expression was significantly lower than that of MSH2. The differences in MSH2 and MSH6 expression were also identified in a set of endometrial carcinomas that had well differentiated and poorly differentiated areas adjacent to each other on the same slide. As mismatch repair protein expression has not been previously characterized in depth, this study was designed as proof-of-principle to document that mismatch repair protein expression can be quantified in clinical samples and that such expression can be variable even when overall it is considered positive. While non-endometrioid histology was associated with significantly higher MSH6 expression, future studies with a different experimental design will be needed to determine if other pathological variables such as stage, depth of myometrial invasion, presence of lymphatic/vascular space invasion, endometrioid grade, or specific mutations within the tumor impact MSH6 expression.

There is pre-clinical and clinical evidence that such variable expression in MSH6 is biologically important. As previously mentioned, partial knockdown of mismatch repair gene expression results in decreased DNA repair *vitro* (7,8). In normal human fibroblasts, partial down-regulation of mismatch repair gene expression is associated with an increased rate of telomere shortening (9). Temozolomide is a cytotoxic chemotherapy agent used for treatment of glioblastoma multiforme. In part, its mechanism of action is dependent upon partial repression of DNA repair pathways, including repression of MSH2 and MSH6 (15). Interestingly, recurrence following temozolomide treatment is associated with complete loss of MSH6 protein expression, even when the tumor prior to treatment had intact MSH6 expression (16). In colorectal adenocarcinomas with intact immunohistochemical expression of mismatch repair proteins at baseline, chemoradiation therapy can result in weaker MSH6 expression in the residual tumor cells if the immunohistochemistry is performed post-treatment (17). This weaker expression is likely due, at least in part, to chemoradiation blocking cell cycle progression and promoting differentiation.

The current binary positive vs. negative reporting system for mismatch repair immunohistochemistry fails to take into account the cases in which heterogeneous expression is observed clinically. We previously identified that up to 2% of endometrial carcinomas have heterogeneous immunohistochemical expression of MLH1 and PMS2, characterized by tumor foci with strong nuclear expression of both proteins admixed with foci showing no nuclear expression in tumor cells (but retained nuclear expression in adjacent stromal cells) (18). Most of these endometrial carcinomas were associated with *MLH1* gene methylation (18). In such tumors, high levels of microsatellite instability and somatic mismatch repair gene mutations can be detected in the foci with retained expression of mismatch repair proteins (19). Thus, this observed heterogeneous expression is not artefactual and is associated with molecular defects.

Based on current knowledge, variability of mismatch repair protein expression likely has no utility in the screening for Lynch syndrome-associated endometrial or colorectal carcinomas. However, such variable expression could potentially be more important in the identification of patients who would benefit most from immune checkpoint blockade. As previously noted, tumor mismatch repair deficiency is associated with better survival after treatment with immune checkpoint blockade (4–6). Endometrial carcinomas with mismatch repair deficiency are associated with increased numbers of tumor-associated lymphocytes (20,21) and higher burden of somatically acquired mutations (14). Interestingly, the numbers of tumor-associated lymphocytes in mismatch repair-intact and –deficient cancers are highly variable so that lower levels in the mismatch repair deficient tumors may overlap with the higher numbers in the mismatch repair deficient tumors. Accordingly, we previously identified a subset of mismatch repair intact endometrioid carcinomas with increased PD-L1 expression and higher numbers of CD3+ and CD8+ lymphocytes within the tumor (22). Increased tumor-associated lymphocyte numbers may be important, as they have been associated with better outcomes in colorectal adenocarcinomas (23,24), but this possible impact on survival has not yet been examined in endometrial cancer. Similarly, it was recently shown that mismatch repair intact tumors with higher tumor mutation burden were also responsive to immune checkpoint blockade (25).

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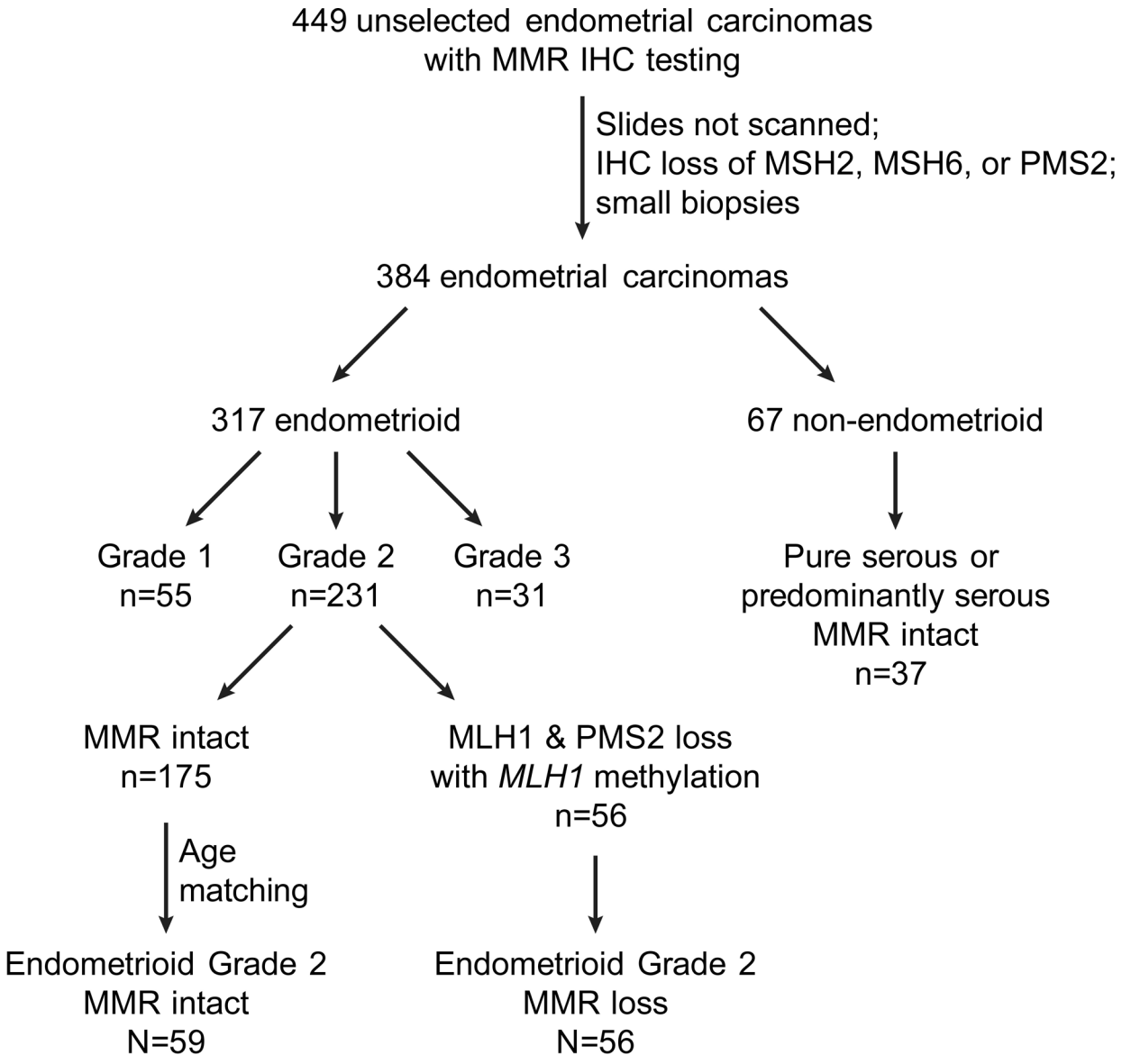

Figure 1.

Schematic overview of methodology. In the course of screening for Lynch Syndrome, 449 endometrial carcinomas were tested for mismatch repair deficiency using immunohistochemistry and *MLH1* gene methylation analysis when a tumor had loss of MLH1 protein expression. For the endometrioid tumors, to control for possible impact of grade on mismatch repair protein expression, this study was restricted to FIGO grade 2. For the non-endometrioid tumors, the study was restricted to cases in which serous carcinoma was the only histology or predominant histology.

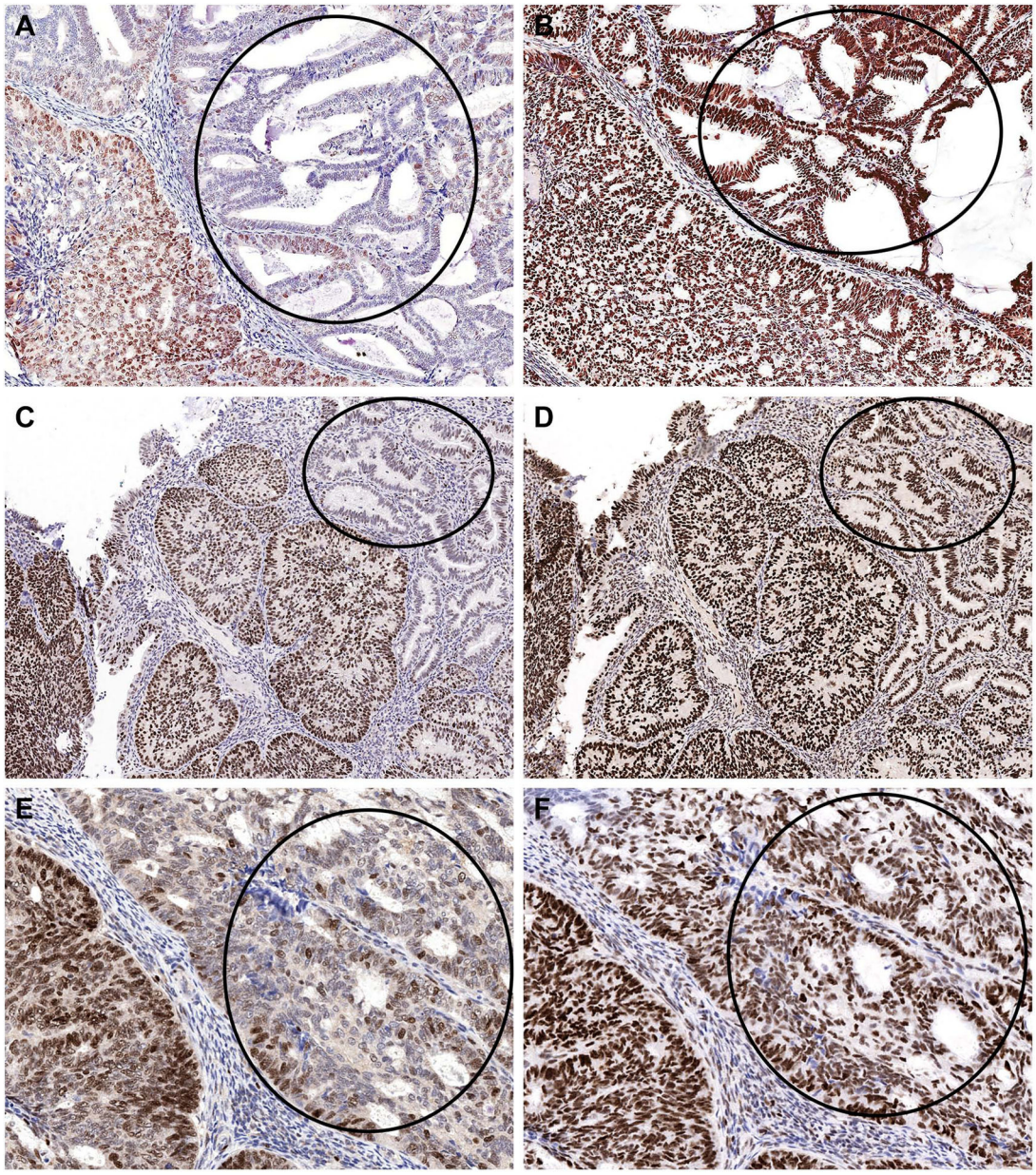

Figure 2.

Variable expression in MSH6 depending on differentiation status within an endometrioid carcinoma. MSH6 (A-C-E) and MSH2 (B-D-F) immunohistochemical expression are shown in three different endometrioid carcinomas with poorly differentiated solid foci immediately adjacent to well-differentiated glandular foci (well-differentiated foci delineated by a circle). In each case, the MSH2 protein expression was diffusely and strongly positive in both the poorly differentiated and well-differentiated foci. However, MSH6 expression was more variable, with weaker and blunted expression in the glandular foci compared to the adjacent solid components.

Table 1.

Quantification of MSH2 and MSH6 immunohistochemical expression in three different endometrial carcinoma groups.

	Endometrioid MMR Intact (n=59)	**Endometrioid MLH1 Lost (n=56)**	**Non-Endometrioid MMR Intact (n=37)**	***p*-value between groups**
MSH2 (mean % cells positive, SD)				
Hotspot	91 (10)	93 (9)	94 (7)	0.314
Mean Random 4	87 (13)	92 (7)	92 (7)	0.069
Mean 3+ Random 4	60 (22)	66 (21)	68 (21)	0.165
MSH6 (mean % cells positive, SD)				
Hotspot	66 (21)	69 (20)	71 (22)	0.245
Mean Random 4	39 (23)	48 (21)	59 (23)	<0.001
Mean 3+ Random 4	19 (18)	23 (20)	38 (24)	<0.001
MSH2 vs MSH6, *p*-value intra-group				
Hotspot	<0.001	<0.001	<0.001	
Mean Random 4	<0.001	<0.001	<0.001	
Mean 3+ Random 4	<0.001	<0.001	<0.001	