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Immunohistochemistry staining for DNA mismatch repair proteins in endoscopic biopsies and the corresponding surgical specimen in colorectal cancer

Carmen Martínez Lapiedra 1, Alfonso García-Fadrique 2, Zaida García Casado 3, Samuel Navarro Fos 4, Isidro Machado Puerto 5

1 Gastroenterology Department. Instituto Valenciano de Oncología. Valencia. Spain.

2 Gastrointestinal Surgery Department. Instituto Valenciano de Oncología. Valencia. Spain.

3 Laboratory of Molecular Biology. Instituto Valenciano de Oncología. Valencia. Spain.

4 Pathology Department. University of Valencia. Valencia. Spain.

5 Pathology Department. Instituto Valenciano de Oncología. Valencia. Spain.

AUTOR DE CORRESPONDENCIA:

Carmen Martínez Lapiedra

Instituto Valenciano de Oncología

Gastroenterology Department

C/Beltrán Báguena 8

46009, Valencia, Spain

mmartinezl@fivo.org

ABSTRACT:

Microsatellite instability is found in 15% of sporadic colorectal cancers (CRC) and 95% of hereditary CRC cases. Lynch syndrome (LS) diagnosis begins with the analysis of the surgical specimen using methods such as immunohistochemistry (IHC), which identifies changes in the nuclear expression of DNA mismatch repair (MMR) proteins. However, IHC analysis on endoscopic biopsies could provide substantial benefits. Our goal was to assess the accuracy of MMR IHC status on endoscopic biopsies in comparison to corresponding surgical specimen in a series of CRC. We retrospectively selected patients who had undergone CRC surgery between February 2011 and January 2020 and had IHC testing for MMR proteins on the surgical specimen. The study was then performed on the corresponding endoscopic biopsies and results were compared. MMR IHC staining on surgical specimens were available for 361 CRC patients and only in 154 cases for preoperative endoscopic biopsies. The concordance between MMR IHC status of the endoscopic biopsy and the surgical specimen analysis was 98.6% for the MLH1/PMS2 proteins and 100% for MSH2/MSH6. In conclusion, endoscopic biopsies of colorectal tumors serve as a suitable tissue source for the immunohistochemical analysis of DNA repair proteins. The correlation with results from the surgical specimen was notably high and discrepancies were primarily as a result of intratumoral heterogeneity within the same sample. The features of MMR protein loss in endoscopic biopsies can provide clinicians with valuable information for specific therapeutic approaches and genetic counseling.

KEYWORDS: colorectal cancer, DNA mismatch repair proteins, immunohistochemistry, microsatellite instability, biopsies.

Abbreviations

Colorectal cancers/colorectal carcinoma: CRC
Lynch syndrome: LS
Immunohistochemistry: IHC
Mismatch repair: MMR
Microsatellite instability: MSI
Microsatellite stability: MSS

INTRODUCTION

Colorectal carcinoma (CRC) is one of the most common neoplasms in developed countries (1,2). A mechanism involved in its carcinogenesis is microsatellite instability (MSI), characterized by alterations in the error-repair system during DNA replication, controlled by the mismatch repair (MMR) genes, primarily MLH1, MSH2, MSH6, and PMS2. Tumors developed by this pathway present hundreds of mutations in highly repetitive sequences called microsatellites (3). This phenomenon is observed in 15% of sporadic CRCs due to the epigenetic silencing of MLH1 (4) and in 95% of hereditary CRCs, notably Lynch syndrome (LS) (5–7) .

The definitive diagnosis of LS requires the identification of a specific germline mutation in one of the DNA repair genes, although prior study of microsatellite instability (MSI) by molecular biology and/or immunohistochemistry (IHC) analysis of MMR proteins is recommended. IHC has the advantage of direct analysis of the gene that encodes the unexpressed protein (8–10).

Universal LS screening is recommended in all diagnosed CRCs, using IHC or molecular methods to detect MSI, as a cost-effective method for increasing the rate of early diagnoses (for CRC and extracolonic tumors) through proper monitoring of carrier relatives (11–15). Moreover, MSI is highly relevant in adjuvant treatment decision making for patients with stage II CRC, as its presence suggests a better prognosis (16–18). It has also been observed that metastatic colorectal tumors with MSI have a better response and longer progression-free survival when treated with immunotherapy than with conventional chemotherapy (17,19). Finally, in recent studies, neoadjuvant immunotherapy has shown a high pathological response rate in patients with locally advanced MMR-deficient colon cancer, so it could potentially become the standard treatment for these patients (20,21). Despite the above, screening programs for LS have been inconsistently implemented in different hospitals (22,23), and when performed, the studies for MSI are usually carried out on the surgical specimen after surgery.

Performing IHC for MMR on CRC endoscopic biopsies offers significant advantages. Preoperative detection of LS cases would allow decisions regarding the extent of surgery (colectomy vs. segmental resection, prophylactic hysterectomy or not). Moreover, in cases of rectal cancer with a complete pathological response after neoadjuvant therapy, there will be no tumor material in the surgical specimen on which to conduct the IHC

study. Additionally, some studies suggest that neoadjuvant treatment might alter the MMR possibly due to the hypoxia and oxidative stress produced by treatments (24–26). Given the importance of identifying tumors with MSI, especially at the time of CRC diagnosis, this study assesses the efficacy of IHC analysis of DNA MMR proteins in endoscopic biopsies, by comparing the results with those of the corresponding surgical specimen.

MATERIALS AND METHODS

This is a retrospective, cross-sectional, single-center study based on a prospective surgical database, which includes all patients undergoing surgery for CRC at our center. Cases that underwent surgery between February 2011 and January 2020 in which the IHC study of MMR proteins had been conducted on the surgical specimen were selected. Subsequently, IHC was determined on the corresponding endoscopic biopsies carried out prior to surgery for those patients for whom sufficient material was available for diagnosis.

The study was approved by the Research Ethics Committee of our center (CEIM file number: 2020-04). The development of the study did not alter the diagnosis, therapy and follow-up of the patient, since it was carried out through normal clinical practice. As this was a non-drug, observational study, safety parameters were not collected. The provisions of title 1 of Article 5 of Law 14/2017 on biomedical research regarding the protection of personal data and guarantees of confidentiality were guaranteed at all times.

Immunohistochemical Staining

Endoscopic biopsy samples in which there were fixation and processing defects that did not allow IHC techniques to be performed or that did not have sufficient tumor material were excluded.

The formalin-fixed, paraffin-embedded tissue block was recovered and cut into 3-micron sections. The samples were deparaffinized for 12 hours in an oven and mounted on microscope slides.

IHC staining to detect 4 MMR proteins (MLH1, MSH6, MSH2, PMS2) was performed automatically using the EnVision FLEX visualization system (DAKO Omnis) with prediluted DAKO primary antibodies. Each staining session included an external positive quality control sample. The antibodies used are shown in Table 1.

Interpretation of the endoscopic biopsy IHC staining was carried out blindly by two experienced pathologists (IM and SN). Adjacent non-neoplastic tissues (vascular endothelial and inflammatory cells and normal colonic tissue) were used as internal controls. “Loss of expression” was considered when nuclear staining was absent in the tumor in the presence of staining of adjacent non-neoplastic tissues. "Preserved expression" was considered when nuclear staining was observed both in the tumor tissue and in the surrounding tumor-free tissues. The result was deemed "inconclusive" when there was no staining of the internal control or staining could not be interpreted due to an unusual pattern.

Statistical Analysis

All collected study variables were analyzed using R software V.3.4.0. To evaluate the correlation between the IHC results in the biopsy and the surgical specimen, we calculated sensitivity, specificity, negative predictive value, positive predictive value, and the Kappa correlation index. Frequency comparisons were made using the chi-square test for categorical variables. For continuous variables, differences in means were assessed with the Student's t-test. When the data did not meet the criteria for normality, the Mann-Whitney U test was employed. All tests were two-sided and used a 5% significance level.

RESULTS

Demographic and Clinical Data of the Patients

A total of 732 patients underwent surgery for CRC during the inclusion period. In 361 cases the IHC study of MMR proteins was carried out on the surgical specimen, detecting loss of nuclear protein expression in 41 of these (11.35%).

The corresponding endoscopic biopsy was also available for 154 of the 361 patients with IHC on the surgical specimen. Of these cases, 70 were female (45.5%) and 84 were male (54.50%), with a median age at diagnosis of 67.75 years. By histological type, adenocarcinoma was the most common, accounting for 93.5% of patients (70% well-differentiated, 26% moderately differentiated, and 2.6% poorly differentiated), followed by mucinous tumors (6.5%). Regarding tumor location, the distribution was in the rectum for 22%, left colon for 40%, and right colon for 38%.

Immunohistochemistry of DNA Repair Proteins

The overall IHC results for the present series are summarized in Figure 1. All cases of non-informative staining in the specimen were due to a failure of the internal control (absence of nuclear staining in stroma cells adjacent to the tumor), while the non-evaluable endoscopic biopsies were due to the absence of tumor tissue in the samples. All these cases were later removed from the comparative analysis.

Table 2 compares the results between the IHC analysis in biopsies and the surgical specimens. We found a complete agreement in the nuclear expression of both heterodimers: MLH1 with PMS2 and MSH2 with MSH6. There was a match between endoscopic biopsy and surgical specimen in 144 of the 146 patients studied for the MLH1 protein (98.63%), showing preserved expression in 130 patients and loss of expression in 14 patients. For the 145 patients with PMS2, there was a biopsy-specimen match in 143 cases (98.62%), with preserved expression in 129 patients and loss of expression in 14 patients. The remaining two cases did not match for either MLH1 or PMS2. One of these had preserved expression in the endoscopic biopsy with loss of expression in the respective surgical piece (Figure 2), while the other had preserved expression in the surgical piece and loss of expression in the biopsy (Figure 3).

Endoscopic biopsies showed a sensitivity of 93.33% (95% CI 68.05-99.83) and specificity of 99.24% (95% CI 95.82-99.98) in detecting loss of MLH1 expression, and a sensitivity of 93.33% (95% CI 68.05-99.83) and specificity of 99.23% (95% CI 95.79-99.98) in detecting loss of PMS2 expression. Regarding the MSH2 and MSH6 proteins, the biopsy and surgical specimen matched in 100% of the cases.

Neoadjuvant Treatment:

In our series, 38 (24.68%) of the 154 included patients had received treatment with chemotherapy or chemo-radiotherapy before surgery. Expression of MMR proteins was preserved in the endoscopic biopsy for all these 38 patients, and after neoadjuvant treatment the MMR status changed in only one patient for the proteins MLH1, PMS2, and MSH2. In this case, the expression was preserved in the endoscopic biopsy, while in the surgical piece it was not informative.

Discussion:

In our series, the concordance between the MMR IHC results of endoscopic samples and the corresponding surgical specimen of 154 patients who underwent CRC surgery was 99.3%. These results are in line with previous studies (27–31) and indicate that endoscopic biopsies are a suitable tissue source for the immunohistochemical analysis of DNA repair proteins.

Individual examination revealed a biopsy-specimen match of 98.63% in the case of MLH1 and 98.62% for the PMS2 protein with a discrepancy in the results for only two patients. In one case, expression was preserved in the endoscopic biopsy and lost in the respective surgical specimen, and viceversa in the other, where expression was preserved in the specimen and lost in the biopsy. We attribute this discrepancy to intratumoral heterogeneity which is estimated to appear in about 5% of colorectal cancers (8,32,33), and is higher in endoscopic biopsies as they represent only a small part of the tumor. MMR status depends on the sample source area and inadequate sampling can lead to false positives and false negatives.

Regarding the MSH2 and MSH6 proteins, there were no discrepancies in the results.

Cases with non-informative IHC results were excluded from the comparative study. In the present series, there was a higher percentage of non-evaluable MMR IHC samples in the endoscopic biopsy than in the surgical specimen. There are two possible justifications for these results: the samples had scant tumor material, or what material there was had been used up for earlier molecular studies.

Very few studies (27–31), all with lower cases than ours, assess the reliability of MMR IHC in the tumor tissue of endoscopic biopsies. Nevertheless, they all demonstrate an

excellent correlation of IHC performed on biopsy and the corresponding surgical specimen. Table 3 compares the present study with others of similar characteristics published in the literature. O'Brien et al. (27) compared the expression of the MLH1, MSH2, MSH6, and PMS2 proteins in the tumor tissue of endoscopic biopsies and the corresponding surgical specimen in 53 patients diagnosed with CRC, finding some loss of protein expression in 10 patients (18.87%) and a biopsy-surgical specimen match rate of 100%. As in our study, the interpretation of staining results was qualitative, meaning the presence of nuclear staining was considered as positive expression, and its absence as negative expression. Kumarashinge et al. (28) studied the IHC-MMR of the same 4 proteins in the biopsy and the corresponding surgical specimen of 112 patients with CRC surgery. IHC was previously available in the surgical specimen of 21 patients, hence the study was conducted in the specimen of the remaining 91 patients and in the 112 corresponding biopsies. They found 10 immunostainings with non-informative results in the surgical specimen of 9 patients, while all the stains of the endoscopic biopsies were informative. In cases where the specimen stains were adequate, the biopsy-specimen match rate was 100%. Similarly, Warrier et al. (30) showed a match of 100% in a cohort of 33 patients with LS and a control group matched by age meeting Bethesda criteria. Shia et al. (29) performed IHC-MMR on both the endoscopic biopsy and the surgical specimen of 70 patients with gastrointestinal cancers (67 CRC), observing a discrepancy in 4% of cases for MLH1 and 3% for MSH6. Preserved expression in the biopsy and loss of expression in the surgical specimen was associated with a small biopsy volume. Loss of expression in the biopsy and preserved expression in the surgical specimen was attributed to possible sampling errors due to intratumoral heterogeneity and, in one sample, possibly related to neoadjuvant treatment in the patient. Vilkin et al. (31) included 96 patients with colon cancer and without neoadjuvant treatment, demonstrating a match of 93.2%. In another study, Vilkin et al. (25) compared the IHC staining for all four MMR proteins in 32 patients with rectal cancer before and after neoadjuvant treatment, compared to a control group of 39 patients with sigma cancer who underwent direct surgery. They identified a greater discrepancy between the IHC of endoscopic biopsies and the surgical piece of those patients who had received neoadjuvant treatment (18.5%) compared to those who had not (7.7%). On the other hand, loss of MSH6 expression, regardless of MSH2 expression status in CRC with

microsatellite stability (MSS) after neoadjuvant therapy has also been described (24). In our study, 38 of the 154 included patients (24.68%) had received prior treatment. And unlike other studies, there was no change in the MMR status in any patient.

One of the challenges posed by the interpretation of IHC staining is the small volume of biopsies, which can limit the performance of IHC if insufficient tumor material is available. No clinical guidelines exist that indicate the optimal number of biopsies required to diagnose CRC, nor are there recommendations regarding the amount of tumor tissue needed to conduct an adequate molecular study (34). One way to sidestep this issue is to obtain as many samples as possible in the presence of a colorectal tumor; in our center, we obtain a minimum of 10 endoscopic biopsies from different areas, aiming to select the areas with highest infiltration.

The methodology used for IHC analysis can also pose another challenge. Some studies similar to ours employed a quantitative method to determine the degree of loss of MMR protein expression. We, like O'Brien et al. and Warrier et al., used only presence or absence of staining, which implies less subjectivity in the interpretation of results and greater agreement between the IHC-MMR of the endoscopic biopsy and the surgical specimen.

The quality of IHC staining is determined by several factors, such as antigen preservation, which relies on proper tissue fixation. One of the advantages of endoscopic biopsy samples is that they have a higher surface/volume ratio, so they fix easily and quickly, producing higher-quality staining (25). Although there are no clinical guidelines for specimen handling and fixation, having standardized protocols in each institution reduces the risk of variability in IHC stains (34). In our Pathological Anatomy laboratory, CRC surgical specimens are fixed with formalin for 24 hours before processing. We have found that interpreting results by two pathologists offers no significant advantages.

Our study population came from a surgical database, meaning the surgical specimen was available for all cases. Nevertheless, the endoscopic biopsies were not available for all these cases as many were referrals from other centers.

A limitation of this study is that the data on sensitivity and specificity were obtained using IHC analysis of the surgical specimen as the "gold standard" and not from the germline mutation of the MMR genes, which were not analysed in most cases, due to the fact our study population came from screening for LS in patients with CRC and not from individuals with high clinical suspicion of LS. Another weak point of our study is that it was retrospective, which influenced patient selection and data collection. This is also a single-center study, although it is worth noting that the number of cases exceeds those published in the literature so far.

In conclusion, endoscopic biopsies of colorectal tumors have an excellent correlation with those of the surgical specimen and provide a suitable tissue source for the immunohistochemical analysis of MMR proteins. The few cases in which there is a discrepancy between the two are attributable to the intratumoral heterogeneity present in a single tumor sample. The search for MMR gene status in endoscopic biopsies can guide clinicians in specific therapeutic approaches and genetic counseling. One of the benefits is the preoperative detection of LS since it helps in making decisions about the therapeutic management of these patients. When an individual with LS is diagnosed with CRC the surgical options are a segmental colectomy or a total colectomy that takes into account the increased risk of metachronous cancer and the circumstances of the individual patient. Likewise, during surgery, a prophylactic hysterectomy should be performed after finishing family planning. All of the above could reduce the number of surgeries and therefore the costs.

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Table 1. Antibodies, sources and dilutions

Antibody	Clone	Source	Dilution
MLH1	ES05	DAKO Agilent	Prediluted
MSH2	FE11	DAKO Agilent	Prediluted
MSH6	EP49	DAKO Agilent	Prediluted
PMS2	EP51	DAKO Agilent	Prediluted

Table 2. Comparison between immunohistochemistry mismatch protein expression in colorectal carcinoma endoscopic biopsy and surgical specimen

	MLH1 surgical specimen			
MLH1 biopsy	**PRESERVED**	**LOSS**	**Total**	**p-value**
PRESERVED	130 (99.24%)	1 (0.76%)	131 (89.73%)	<0.001
LOSS	1 (6.67%)	14 (93.33%)	15 (10.27%)	
Total	**131 (89.73%)**	**15 (10.27%)**	**146 (100.00%)**	
	PMS2 surgical specimen			
PMS2 biopsy	**PRESERVED**	**LOSS**	**Total**	**p-value**
PRESERVED	129 (99.23%)	1 (0.77%)	130 (89.66%)	<0.001
LOSS	1 (6.67%)	14 (93.33%)	15 (10.34%)	
Total	**130 (89.66%)**	**15 (10.34%)**	**145 (100.00%)**	
	MSH2 surgical specimen			
MSH2 biopsy	**PRESERVED**	**LOSS**	**Total**	**p-value**
PRESERVED	144 (100.00%)	0 (0.00%)	144 (98.63%)	<0.001
LOSS	0 (0.00%)	2 (100.00%)	2 (1.37%)	
Total	**144 (98.63%)**	**2 (1.37%)**	**146 (100.00%)**	
	MSH6 surgical specimen			
MSH6 biopsy	**PRESERVED**	**LOSS**	**Total**	**p-value**
PRESERVED	146 (100.00%)	0 (0.00%)	146 (98.65%)	<0.001
LOSS	0 (0.00%)	2 (100.00%)	2 (1.35%)	
Total	**146 (98.65%)**	**2 (1.35%)**	**148 (100.00%)**	

Table 3. Comparative analysis between our study and previous publications

	N	IHC Sequence	Agreement Biopsy-surgical specimen (SE)	IHC study	dMMR	Total number of pathologists
Present study	145	SE ⇄ Biopsy	98% MSH1/PMS2 100% MSH2/MSH6	qualitative (4 proteins)	11.35%	2 pathologists
O'Brien 2018	53	SE ⇕ Biopsy	100%	qualitative (4 proteins)	-	2 pathologists
Vilkin 2015	96	SE ⇄ Biopsy	93.2%	qualitative (4 proteins)	20.8%	2 pathologists
Warrier 2011	66	SE ⇕ Biopsy	100%	Qualitative (4 proteins)	-	1 pathologist
Shia 2011	70	SE ⇕ Biopsy	94% MLH1 96% MSH6 100% PMS2/MSH2	Qualitative (4 proteins)	41.4%	1 pathologist
Kumarashinge 2010	112	SE ⇕ Biopsy	100%	Qualitative (4 proteins)	13%	1 pathologist

⇕ Available IHC results in biopsy or surgical specimen or done in both samples.

⇄ Available IHC results in the surgical specimen and done later in the endoscopic biopsy.

SE: surgical specimen

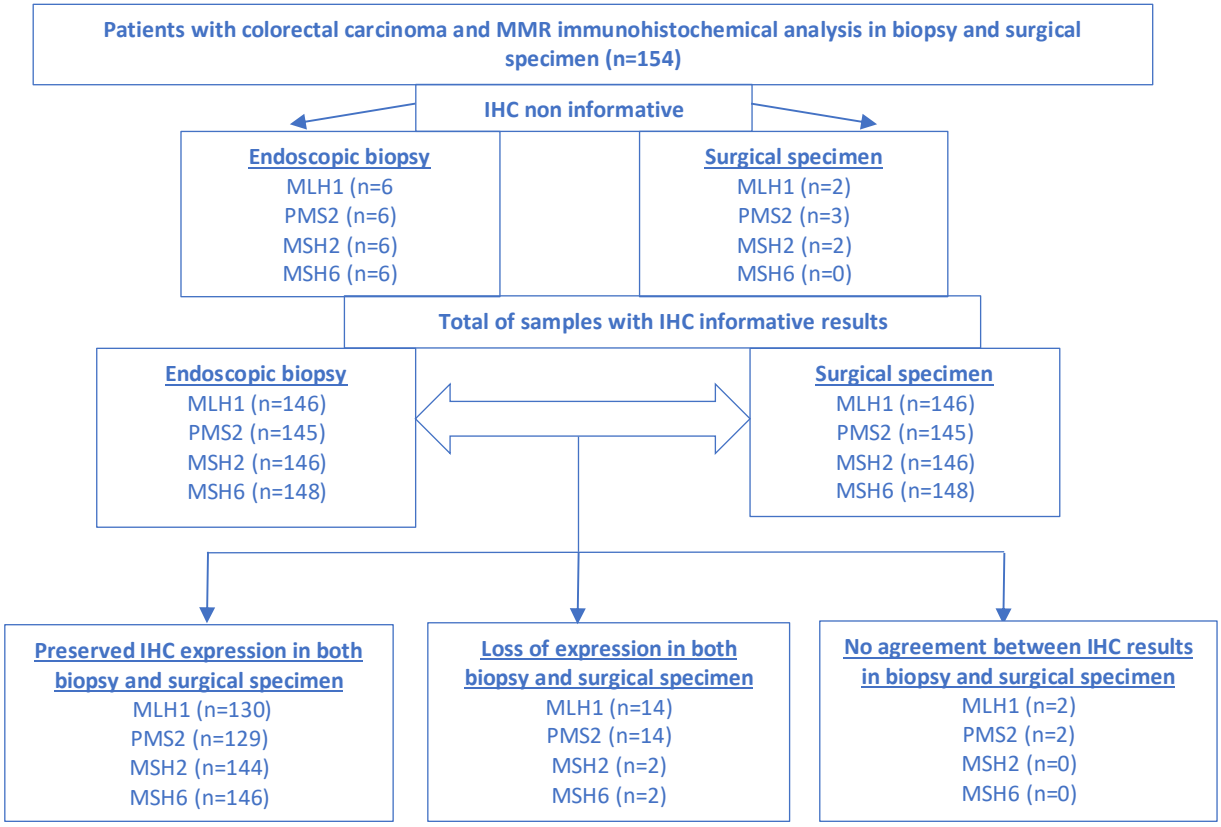


Figure 1. Comparative analysis of immunohistochemistry results (MMR proteins) in biopsies and surgical specimens.

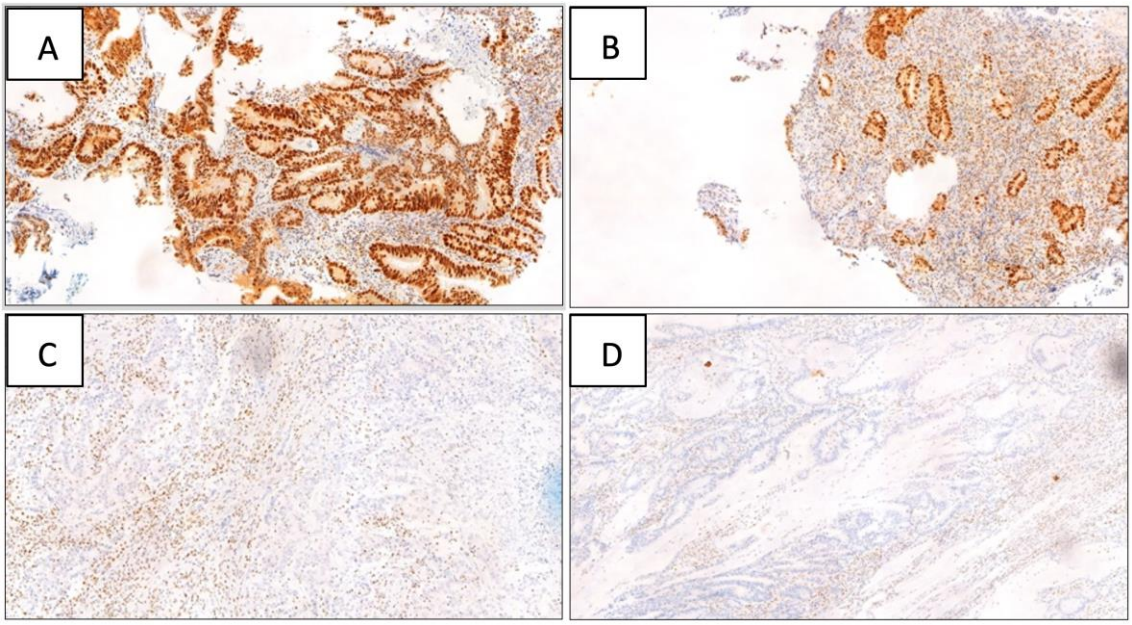
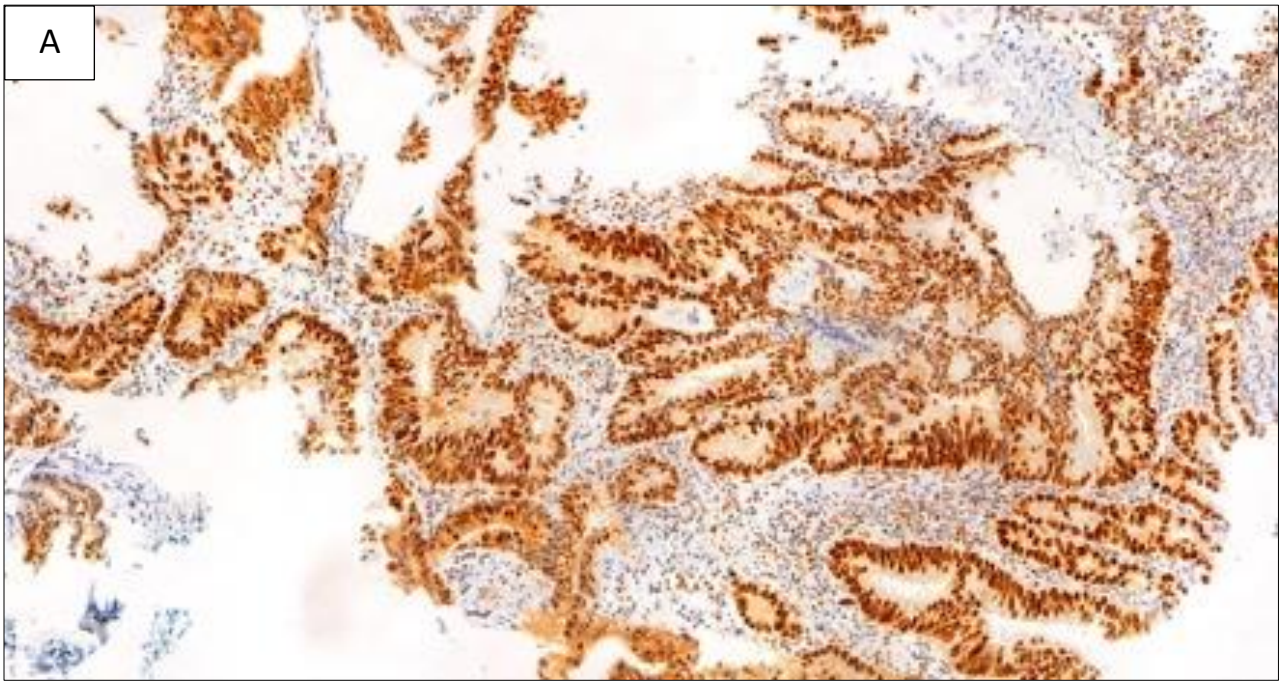


Figure 2. Immunohistochemical staining for MLH1, PMS2 in the endoscopic biopsy (preserved expression) and in the respective surgical piece (loss of expression)
A, B: *biopsy*; C, D: surgical piece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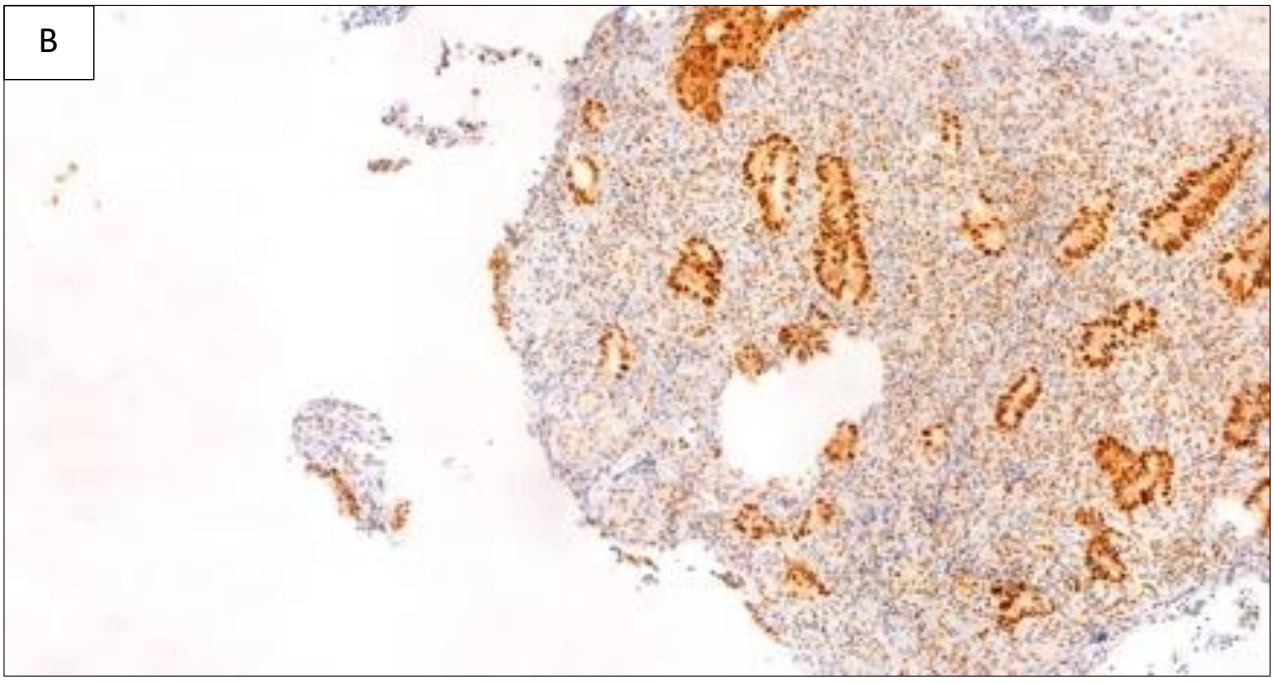
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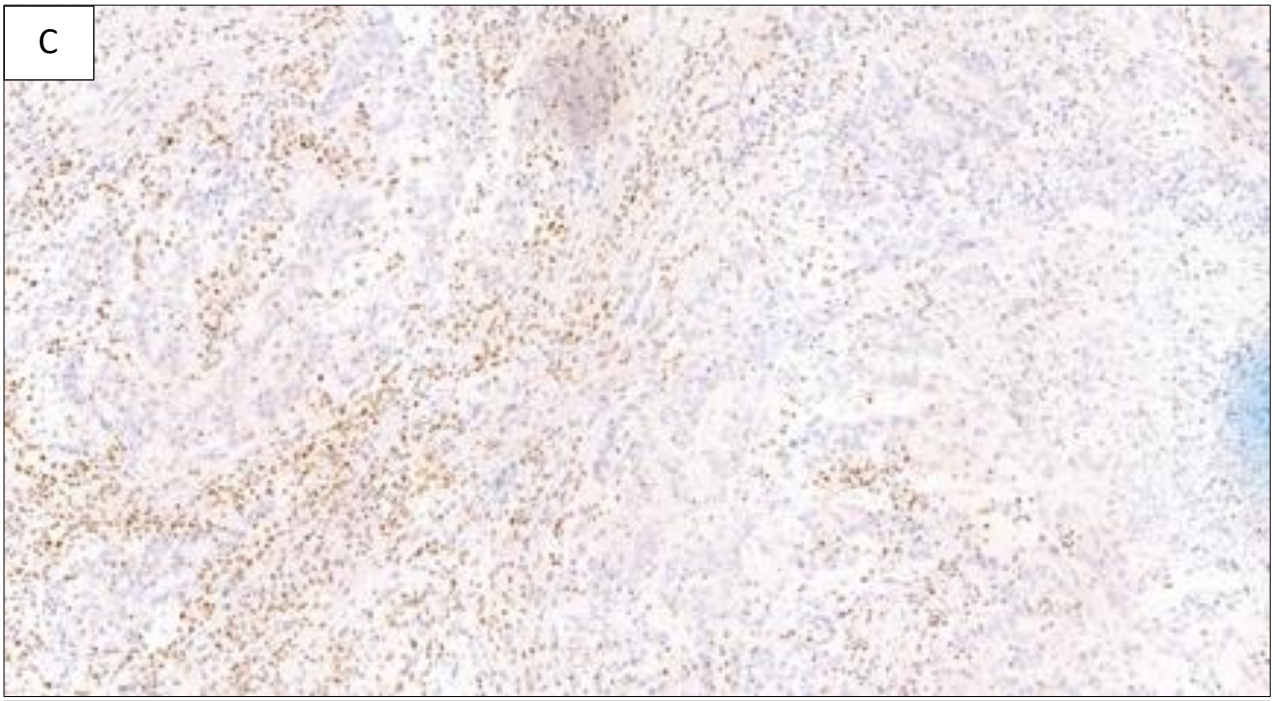
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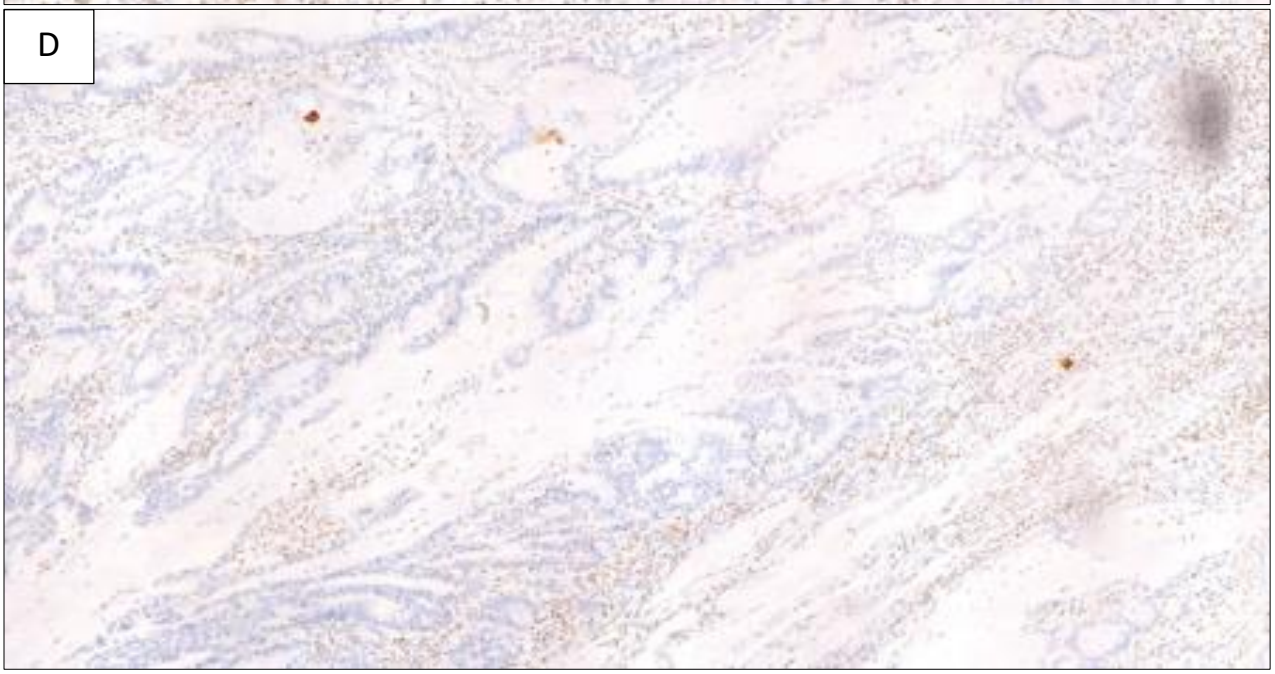
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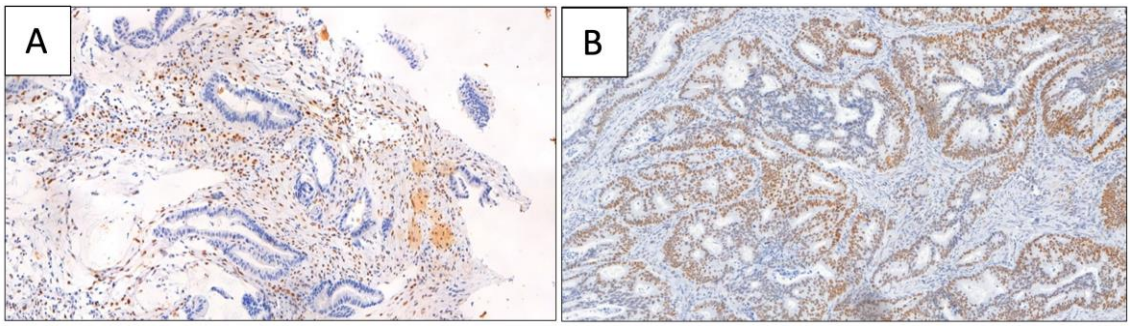

Figure 3. Immunohistochemical staining for MLH1 in the endoscopic biopsy (A: loss of expression) and in the respective surgical piece (B: preserved expression)