Mismatch Repair Deficiency in Colorectal Adenocarcinoma: Clinical, Pathological and Prognostic Features, a Single Center's Experience of 1002 Cases

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Abstract

Background and Study Aims: Microsatellite instability pathway caused by loss of DNA "Mismatch Repair genes" (MMR) is responsible of Lynch Syndrome-related tumors and 10-15% of sporadical colorectal cancers. Although MSI-test is regarded as the golden standard for detection of "Lynch Syndrome-related tumors", there are increasing evidence on similar analytic sensitivity of immunohistochemical evaluations. Patinets and Metods: We retrospectively evaluated 1002 colorectal tumors for loss of DNA MMR protein (MLH1, PMS2, MSH2, MSH6) immunohistochemically. The results were correlated with clinicopathological features and high level-microsatellite instability (MSI-H) related histological parameters. Results: MMR protein expression loss was observed in 9.8% of the cases. MLH1-PMS2 loss (53.2%) was the most common loss followed by MSH2-MSH6 (31.6%), isolated PMS2 loss (12%), and isolated MSH6 loss (2%). MMR deficiency was more frequent under 50 years-old ($p<0.0001$), in right colon tumors ($p<0.0001$), poorly differentiated tumors ($p<0.0001$), tumors with tumor infiltrating lymphocytes ($p<0.0001$), mucinous component ($p=0.001$), and medullary component ($p<0.0001$). Also MMR deficiency was less frequent in tumor with tumor budding ($p<0.0001$) and dirty necrosis ($p<0.0001$). The 5 years-survival rate was 55.7%. No significant correlation was found with MMR deficiency and survival. Conclusions: MMR deficiency was observed in 9.8% of the cases with distinct clinicopathological features. The results were consistent with previous studies. Unlike the literature, we did not find any statistically significant difference between MMR deficiency and prognosis.

INTRODUCTION

Microsatellite instability (MSI) pathway, which is one of the colorectal carcinogenesis pathways, accounts for 15-20% of sporadic colorectal cancers (CRC) and tumors related to the hereditary nonpolyposis colorectal cancers (HNPCC-Lynch Syndrome).[1,2] Lynch syndrome is an autosomal dominant syndrome characterized by a mutation in DNA Mismatch Repair (MMR) genes accounting for 2-5% of all colorectal cancers.[2,3] There is also an increased incidence of endometrial, renal pelvic, small bowel, and ureter tumors and an increased incidence of CRC. Since the syndrome is characterized by the development of synchronous and metachronous tumors at an early age affecting multiple generations of a family, the recognition of an individual with Lynch Syndrome enables screening of other family members before the cancer development and allows more efficient patient management.[4]

Many guidelines have recommended using a PCR-based MSI testing as the gold standard method for selecting patients who undergo further genetic testing concerning Lynch Syndrome.[2,5] However, MSI testing is not an appropriate method for screening purposes as the test is costly and time-consuming. For this reason, recent studies have compared the analytical sensitivity of the loss of expression in MMR proteins identified by immunohistochemical (IHC) analysis with that of PCR-based MSI testing and reported similar sensitivity

rates.[6–8] In light of these data, identifying the loss of expression of MMR proteins using IHC methods has taken the first step in the diagnostic algorithm for Lynch Syndrome. The fact that IHC directly identifies the MMR protein expression or the loss of expression and thus focuses mutation analysis directly on the gene resulting in protein loss is designated as an additional advantage of IHC analysis.[1] There are also studies suggesting the use of reflex testing with MMR immunohistochemistry due to this finding's possible role in predicting response to therapy and prognosis and the fact that the loss of MMR protein expression is also observed in sporadic cases of CRC.[2,9]

The identification of MSI is also crucial as the presence of MSI is a prognostic marker in terms of survival and is of predictive value in the selection of chemotherapy. Many studies to date have found that sporadic MSI tumors are associated with better prognosis and improved overall survival than microsatellite stable (MSS) tumors.[10,11]On the other hand, it is reported that high-level microsatellite instability (MSI-H) tumors often poorly respond to 5-fluorouracil (5-FU)-based chemotherapy used in the classical treatment of CRC.[5,12] Also, it was established in recent years that individuals with tumors displaying loss of MMR protein expression benefit from the blockage of the programmed cell death protein 1 (PD-1).[13,14] For this reason, the identification of MSI status has an important place in patient-centered selection of the treatment modality before proceeding with chemotherapy.

The present study evaluates histological or clinical (age, gender, localization) parameters that might be of value in predicting MMR protein deficiency and investigates whether the results of immunohistochemical studies define a patient profile that is a candidate for further MSI testing.

In the scope of the present study, clinicopathological data (age, gender, tumor localization, tumor diameter), histological data (tumor type, tumor grade, pT, pN, number of metastatic lymph nodes, LVI, PNI, presence of satellite nodule), and histological data related to the MSI-H phenotype (TIL, Crohn's-like lymphoid reaction, mucinous tumor component, signet ring cell tumor component, medullary tumor component, micropapillary tumor component, cribriform comedo-type necrosis tumor component, presence of dirty necrosis, presence of tumor budding, infiltrative tumor margins) were evaluated in 1,002 patients. Overall survival data as of March 2017 was retrieved, and the results were interpreted concerning the loss of MMR protein expression identified by IHC.

MATERIALS AND METHODS

Study Group: The study included 1,002 patients who underwent surgery due to colon and rectum adenocarcinoma between 2002 and 2011, the resection material of whom were examined in the Department of Pathology at Ege University Faculty of Medicine. Patients with a rectal tumor who received neoadjuvant chemotherapy were excluded from the study. The study was granted approval by the Clinical Trials Ethics Committee of Ege University Faculty of Medicine (Decision No: 12-5.1/4; Date: 03.07.2012).

The patients' clinicopathological and macroscopic data were retrieved from the hospital's information system, and survival data were obtained from the database of the Cancer Registry Center of the Department of Cancer (KIDEM) in Izmir Public Health Directorate. Histopathological analysis consisted of retrospective examination of hematoxylin-eosin (HE)-stained sections of formalin-fixed and paraffin-embedded tissue block.

Histological Characteristics: The depth of invasion (pT) and the number of metastatic lymph nodes (pN) were recorded according to the eighth edition of the AJCC-UICC TNM classification.[15]Histological findings were re-assessed according to the 2019 recommendations of the World Health Organization.[16]Histological tumor type, differentiation, satellite tumor focus, lymphovascular invasion (LVI), and perineural invasion (PNI) were evaluated.

Phenotypic Characteristics of MSI-H:

The characteristics to be evaluated for MSI-H tumor risk were determined according to the Bethesda criteria and the data collected from the literature.[17]

Tumor-infiltrating lymphocytes (TIL): Tumor-infiltrating lymphocytes were evaluated by observing

lymphocytes with a halo and a small, blue nucleus in H&E-stained tissue sections. The mean value was calculated by evaluating the number of TIL in 10 microscopic high power fields (HPF). The presence of two or more TIL in an individual HPF was considered to indicate a positive result for MSI-H phenotype.[18,19]

Crohn 's-like lymphoid reaction: The presence of two or more lymphoid aggregates in tumor-infiltrated borders was considered to indicate a positive reaction.[19]

Mucinous differentiation: The tumor was classified as "mucinous adenocarcinoma" if the extracellular mucin rate was more than 50% and as a tumor exhibiting "focal mucinous differentiation" if the rate was less than 50% (Figure 1A).[19]

Signet ring cell differentiation: The tumors containing more than 50% signet ring cells were classified as "signet ring cell carcinoma", and the tumors containing less than 50% signet ring cells were classified as "focal signet ring cell differentiation" (Figure 1B).[19]

Poorly Differentiated Histological Subtypes Associated with MSI-H: The tumor was considered to exhibit MSI-H phenotype if histological subtypes containing medullary component, and poorly differentiated component exhibiting less than 5% gland formation comprised more than 10% of the tumor (Figure 1C).[16]

Infiltrative Tumor Margins: Infiltrative tumor margins were examined in a low power field (x4 magnification) and evaluated in two categories as expansive and infiltrative growth pattern.

Tumor Budding: The presence of single tumor cells or small cell clusters of less than five cells with an anaplastic character at the infiltrative tumor margin that is independent from the primary tumor mass was evaluated (Figure 1D).[20]

Dirty Necrosis: Dirty necrosis was considered to be positive in the presence of more than 10% cell debris and "dirty" necrosis containing inflammatory cells. The presence of geographic necrosis related to the rapid growth of the tumor was not taken into consideration.[19]

Immunohistochemistry: Immunohistochemical analysis involved the examination of tissue microarray 5 mm in diameter prepared from H&E-stained tissue sections exhibiting an average tumor area. All IHC staining were performed using a fully automated IHC staining device (Ventana BenchMark XT, Ventana Medical Systems, Tucson, AZ). MLH1 (Clone: ES05 Novacastra; Dilution:1/150), MSH2 (Clone: 25D12 Novacastra; Dilution:1/75), MSH6 (Clone: PU29 Novacastra; Dilution:1/50), and PMS2 (Clone: M0R4G Novacastra; Dilution:1/75) primer antibodies were used in IHC examination. A nuclear staining for each antibody was considered a positive reaction during examination, and the heterogeneity of staining was separately evaluated. A lack of staining in all tumor cells or less than 1% positive nuclear staining was considered to indicate a loss of "Mismatch Repair Gene" protein.

Evaluation of Survival Data: Survival analysis involved the assessment of "overall survival" from the date of operational diagnosis to the last control visit on March 2017 for survivors, and from the date of diagnosis to death in "months" for nonsurvivors. Early postoperative deaths in the first three months were excluded.

Statistical Analysis: SPSS 18.0 software package was used in statistical analysis. The patients' clinicopathological data and MMR immunohistochemistry results were evaluated using the frequency analysis, and survival was evaluated using Kaplan-Meier survival analysis. Nonparametric tests were used to compare the loss of MMR protein expression with clinicopathological data and survival (Pearson's chi-square and Fisher's exact test, where appropriate), and a Mann-Whitney U test was used to compare age, tumor diameter, number of metastatic LN, and TIL count with other data. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Clinicopathological data

Age: Included in the study were 1,002 patients with a mean age of 63±12.7 years in the range of 22-94 years. At the time of diagnosis, 18% (n=180) of the patients were under 50 years.

Gender: Of the patients, 603 (60.2%) were male, and 399 (39.8%) were female. The mean age at the time of diagnosis was lower in females than in males (61±13.7 versus 65±11.9).

Tumor Localization: Of the tumors in these patients, 445 (45.4%) were located in the rectum, 324 (32.3%) were in the left colon, and 223 (22.3%) were in the right colon. The rate of tumors localized in the left colon, including the rectum, was 77.7% (n=779).

Multiple Tumor: There were multiple tumors in 64 patients (6.4%). Of these tumors, 36 (56.3%) were diagnosed at the time of initial diagnosis, and 28 (43.8%) were diagnosed in an area distant from the primary tumor at least six months after the operation.

Tumor Stage (pT): The examination of the tumor stages of the patients revealed that the majority of the patients had an advanced-stage tumor. The rate of pT3 tumors was 79.7% (n=798), and the rate of pT1 tumors was only 1.5% (n=15).

Number of Lymph Nodes and Stage (pN): The majority of 1,002 patients (47.9%) included in the study had pN0 disease. Lymph node metastasis (LNM) was identified in 472 patients (47.2%), and the mean number of metastatic lymph nodes was 2.05±3.8. Thirty-eight patients (3.8%) did not have an LNM but have a satellite nodule; these patients were analyzed in the pN1c category.

Tumor Diameter: The mean tumor diameter in the resection materials was 5±2.3 cm (range: 1-26 cm). The diameter of the second tumor was not separately evaluated in patients with multiple tumors. It was found that the diameter of the primary tumors localized in the right colon tended to be larger than the diameter of tumors localized in the left colon (6.13±2.7 vs. 5.1±2.2, $p<0.05$).

Histological Findings:

Tumor Type: The examination of the histological subtype revealed that 89.6% (n=898) were adenocarcinoma, 9.8% (n=98) were mucinous adenocarcinoma, and 0.6% (n=6) were signet ring cell carcinoma.

Tumor Differentiation and Grade: Of cases with adenocarcinoma, 80.8% (n=727) had moderately differentiated tumors. When the tumors were divided into two groups as low-grade and high-grade tumors, the rate of adenocarcinomas among the low-grade tumors was 91.2% (n=821).

Lymphovascular, Perineural Invasion, and Satellite Nodule: The rates of LVI and PNI were close to each other (25% and 23.5%, respectively). A satellite nodule was observed in 17.3% of the patients.

Histological Findings Related to MSI-H: Lymphocytic Reaction around the Tumor: In microscopic examination based on the presence of two lymphocytes in one high power field that is considered to be related to MSI-H phenotype, the TIL count was considered to be "positive" in 104 patients (10.4%). Crohn's-like lymphoid reaction was detected in 385 patients (38.4%).

Mucinous Tumor Component: The presence of a focal or diffuse mucinous component was observed in 343 patients (34.4%). The majority of these patients exhibited focal mucinous differentiation (n=238, 69.3%). Thirty-nine patients (11.3%) with mucinous component had signet ring cell tumor component.

Other Tumor Components: Medullary tumor component was observed in 5% of the cases (n=50). Aside from these, 64 cases had cribriform-comedo-type necrosis pattern, and 30 cases had micropapillary tumor pattern.

Dirty Necrosis and the Assessment of Infiltrative Tumor Margins: Dirty necrosis was observed in approximately half of the patients (n=544, 54.3%). Expansive tumor growth pattern was detected in only 71 cases (7.1%). There was tumor budding in 238 patients (25.5%) with infiltrative tumor growth patterns.

Findings Related to the Loss of MMR Protein Expression: A loss of expression in one or more MMR proteins was observed in 98 (9.8%) out of 1,002 patients included in the study. Of these patients, 52

(5.2%) had a deficiency in MLH1-PMS2 expression, 31 (3.1%) had a deficiency in MSH2-MSH6 expression, 12 (1.2%) had isolated PMS2 deficiency, and 2 (0.2%) had isolated MSH6 deficiency. Only one patient had a deficiency in MLH1-PMS2 and MSH6 expression.

Heterogeneity: The percentage and intensity of staining was heterogeneous in 40.9% (n=410) of the tumors. The extent of heterogeneity was the highest in the expression of MSH-6 (19%) and PMS2 (15%).

The Relationship between the Loss of MMR Protein Expression and Clinicopathological, Histological and MSI-H-Related Findings

Although the rate of the loss of MMR protein expression was higher in males, the difference relative to females was of no statistical significance. The rate of the loss of MMR protein expression was significantly higher in patients under 50 years (18.3% vs. 8.1%; $p<0.0001$). MMR-deficient tumors were more often localized in the right colon (63.3% vs. 36.7%; $p<0.0001$) and had a larger diameter (6 ± 3.2 vs. 4.7 ± 2; $p<0.0001$).

MMR-deficient tumors were mostly composed of poorly differentiated high-grade tumors ($p<0.0001$). The rate of MMR protein deficiency was 31.6% among high-grade tumors and 7.4% among low-grade tumors ($p<0.0001$). Furthermore, MMR protein deficiency was significantly more common in pT4 and pN0 tumors (16.9%, $p=0.05$; 8.1%, $p<0.0001$; respectively).

The presence of a satellite nodule was uncommon in MMR-deficient tumors (10.6% vs. 5.8%; $p=0.05$). The presence of TIL was more common in MMR-deficient tumors ($p<0.0001$). The rate of Crohn's-like lymphoid reaction was significantly higher in MMR-deficient tumors (13% vs. 7.8%; $p=0.005$).

The rates of focal mucinous differentiation and mucinous adenocarcinoma were significantly higher in patients with MMR-deficient tumors (15.5% and 12.4% vs. 7.3%, $p=0.001$). Similarly, the rate of medullary tumor component was significantly higher in patients with MMR-deficient tumors (44% vs. 8%, $p<0.0001$).

The rates of tumor budding (4.2% vs. 11.5%, $p=0.001$) and dirty necrosis (6.4% vs. 13.8, $p<0.0001$) were significantly lower in MMR-deficient tumors.

No significant relationship was found between the loss of MMR protein expression and gender, tumor type, LVI, PNI, signet ring cell component, and the configuration of infiltrative tumor margins. The relationship between the loss of MMR protein expression and clinicopathological data is presented in Table 1.

Histopathological Characteristics of MLH1-PMS2-deficient Tumors

MLH1-PMS2-deficient tumors were more commonly localized in the right colon ($p<0.0001$). The loss of MLH1-PMS2 protein expression was significantly more common in poorly differentiated tumors and tumors with mucinous and medullary components ($p<0.0001$ for each). The rate of TIL was also higher ($p<0.0001$). The rates of tumor budding and dirty necrosis were lower ($p=0.007$ and $p<0.0001$, respectively). No significant relationship was found with other parameters (Figure 2).

Histopathological Characteristics of MSH2-MSH6-deficient Tumors

MSH2-MSH6-deficient tumors were often localized in the right colon ($p<0.0001$). The rate of MSH2-MSH6 deficiency was significantly higher in tumors with a medullary component and lower in the presence of tumor budding ($p<0.0001$ and $p=0.008$, respectively) (Figure 3).

No significant relationship was found with histopathological parameters in tumors with isolated PMS2 and isolated MSH6 deficiency.

Survival: The examination of 942 patients for whom overall survival data were available revealed that 48% of these patients survived. The overall survival rate was 47.9% among patients with a loss of MMR protein expression versus 43.9% among patients without a loss of MMR protein expression. However, no statistically significant relationship was found between the loss of MMR protein expression and overall survival. Also, the relationship between overall survival and individual protein deficiencies revealed no statistically significant

relationship. The relationship between the loss of MMR protein expression and overall survival is presented in Table 2.

DISCUSSION

The Loss of MMR Protein Expression

IHC revealed a loss of expression in one or more MMR proteins in 98 patients (9.8%). Various studies have reported a rate of MMR protein deficiency as high as 20% in sporadic colorectal cancers.[21,22] Consistent with the literature data, the majority of patients with DNA MMR deficiency was composed of patients with MLH1-PMS2 deficiency.[1] The loss of MSH2 expression is often the result of germline mutations, while the loss of MLH1 expression can result from either germline mutations or somatic hypermethylation; thus, MLH1 is one of the most frequent deficiencies.[23]

One of the patients was evaluated with repeat IHC analysis, which revealed combined MLH1-PMS2 and MSH6 deficiency. This patient was female older than 50 years and had a moderately differentiated tumor with expansive growth pattern; the patient exhibited only Crohn's-like lymphoid reaction among other phenotypic characteristics of MSI-H. According to a recent theory, a secondary mutation occurs in some individuals with Lynch syndrome, resulting in an extraordinary immunohistochemical staining pattern.[24] For example, a somatic mutation can occur in the MSH6 gene over time in a carrier of hereditary mutation in the MLH1-PMS2 gene. These abnormal combinations are generally identified in MSH2-MSH6-PMS2 proteins on rare occasions, and it was asserted that these combinations are independent from the carcinogenesis process.[7] It was considered that a similar pathway is involved in this particular case, while there is a need for further molecular evidence.

Relationship between the Loss of MMR Protein Expression and Clinicopathological Data

Age: The age at tumor diagnosis was significantly lower in patients with a loss in MMR protein expression than in patients without MMR deficiency. This finding is in agreement with the previous studies reporting a younger age in patients with tumors related to Lynch syndrome than in patients with sporadic colorectal tumors.[21,25] The age is often in the range of 50 to 74 years in patients with sporadic tumors exhibiting a loss of MMR protein expression.[3,10,21] In the examination of patients younger than 50 years in our series, MMR protein deficiency was detected in 18.3% of the patients, and this rate was significantly higher than that in patients older than 50 years and exhibiting MMR protein deficiency.

Gender: There are studies in the literature reporting a high rate of females with loss of MMR protein expression, while there are also studies reporting no significant gender difference.[11,26] In a series of patients reported by Belizzi et al., no significant difference was found between males and females with tumors related to Lynch Syndrome; interestingly, they reported females' predominance among patients with sporadic MSI tumors.[27] The present study reports no significant relationship with gender.

Diameter: There are numerous studies reporting larger diameter in MMR-deficient tumors.[11,28] The present study also found a larger tumor diameter in association with the loss of MMR protein expression. It seems reasonable that the finding of MSI tumors with MMR protein deficiency being more often localized in the right colon can be related to larger tumor diameter.[9,21,25]

Localization: The localization of the tumor in the right colon was remarkable in MMR-deficient tumors. The rate of right colon tumors is lower than that of left-sided tumors, including rectum; however, the localization of the tumor in the right colon seems predominant compared to patients without MMR deficiency. Chapusot et al. reported a rate of 35% for right colon predominance in MMR-deficient tumors and emphasized that this tumor localization has a highly sensitive predictive value for poor tumor differentiation and MMR deficiency.[29]

Stage: The tumors with MSI-H phenotype tend to have high tumor invasion depth (pT) and low overall TNM stage. It has been widely accepted that this phenotype is associated with a low rate of lymph node metastasis.[11] Similar to the literature, the present study found a significant relationship between MMR

deficiency and pT and pN. Raut et al. found a lower rate of LNM in MMR-deficient tumors than in tumors without MMR deficiency.[11]

Relationship between MMR Protein Expression and Histological Data

Tumor Type and Differentiation: In the present study, the rate of poorly differentiated tumors was significantly higher among MMR-deficient tumors than in tumors without MMR deficiency. This difference is particularly more remarkable in MLH1 and PMS2-deficient tumors. It has been reported that patients with a loss of MMR protein expression more often have poorly differentiated and high-grade tumors,[10,28] despite low predictive value in predicting the loss of MMR protein expression.[10,29]The fact that such tumors are still described by the Bethesda criteria mandates immunohistochemical screening of these tumors.

The diagnosis of these tumors is not always straightforward, and the evaluation of these tumors in different categories in different studies complicates the interpretation of the results. For example, medullary tumors have an extremely low rate among all colorectal cancers. For this reason, medullary tumors are often included in the poorly differentiated tumor category in the studies.[19] In our series, it has been problematic to differentiate undifferentiated or poorly differentiated tumors with medullary tumor-like areas from medullary tumors. It is a remarkable finding to note that one of the patients with medullary carcinoma in the present study had multiple tumors, and synchronous secondary tumor had mucinous adenocarcinoma histology, and two tumors have MLH1-PMS2 deficiency.

Furthermore, the presence of a medullary tumor component was analyzed in a separate category isolated from patients with medullary carcinoma. Among patients with a medullary tumor component, the rate of patients with loss of MMR protein expression was significantly higher than the rate of patients without MMR protein deficiency. In a study by Alexander et al., the presence of medullary tumor component was reported in approximately 25% of patients with loss of MMR protein expression.[22] It was emphasized that the presence of medullary tumor component offers a specificity of 97% in predicting the loss of MMR protein expression.[22] When MLH1-PMS2 and MSH2-MSH6-deficient tumors were evaluated separately, the rate of medullary tumor component was significantly higher in tumors without MMR protein deficiency.

Mucinous tumor component is another phenotypic characteristics for MSI tumors. Many other studies have established that the loss of MMR protein expression is the most important predictive characteristic for the mucinous component.[28,30] Also, in the present study, the rate of focal mucinous differentiation and mucinous adenocarcinoma was significantly higher among MMR-deficient tumors. In a study by Greenson et al. involving 528 patients, 29.1% of MMR-deficient tumors showed focal mucinous differentiation, and 28.6% showed more than 50% mucinous component.[19] The same study also reported a sensitivity of 67.3% and a specificity of 81.9% for the presence of mucinous component in predicting MSI-H phenotype.

TIL: The most remarkable histological characteristic of tumors with an MSI-H phenotype is the lymphocytic reaction to tumor. TIL count has an important place among these lymphocytic reactions.[18] There is no consensus in the literature regarding the method of determining the TIL count. The studies on the TIL count based on immunohistochemical studies using T-cell markers (CD3 or CD8) have found higher values than using HE-stained preparations.[19,22] Also, the cut-off value for TIL count that might be associated with MSI-H phenotype varies according to the method used. There are studies considering a cut-off value of 4-7 lymphocytes in immunohistochemical analysis,[22,31]while the investigators relying on the H&E-stained slides have considered a cut-off value of 2-3 lymphocytes.[19,32]Greenson et al. performed TIL counts using H&E staining and found that the most significant cut-off value is 2 lymphocytes associated with an MSI-H phenotype.[19] They suggested that the presence of 2 or more lymphocytes in one high power field offers a sensitivity of 90.4% and a specificity of 76.7% in predicting MSI-H phenotype.[19] In the present study, the mean TIL count was 0.87 ± 2 in patients with loss of MMR protein expression, which is significantly higher than that in patients without MMR deficiency. A cut-off value of 2 lymphocytes was used as recommended by Greenson et al.[19] According to this analysis, TIL count was higher than 2 in 20% of patients with MMR protein deficiency and only in 8.3% of patients without MMR protein deficiency, and the difference was statistically significant.

Crohn 's-like Lymphoid Reaction: Crohn's-like lymphoid reaction is another lymphocytic reaction pattern.[11,18]The rate of Crohn's-like lymphoid reaction was significantly higher in patients with MMR deficiency. In a study by Alexander et al. Involving 204 patients, the rate of Crohn's-like lymphoid reaction was reported to be as high as 49%.[22] They, however, emphasized that this feature is of low value in predicting MSI-H phenotype.[29] It has been proposed that this reaction reflects the response of the host immune system to the tumor. It was also suggested that this reaction reduces the rate of metastasis and offers survival benefit to a certain extent.[29]

Growth Pattern: The growth pattern, along with lymphoid reaction surrounding the tumor, may give an idea about the MSI-H phenotype.[21,30] In a study by Joost et al., expansive growth pattern has appeared as the most significant parameter indicating the loss of MMR protein expression.[21] The present study did not find such a relationship. It was considered that this finding might have been caused by a small number of patients having tumors with an expansive growth pattern.

Tumor budding: There were studies reporting that the presence of dedifferentiated cells (tumor budding) observed in some of the tumors with an infiltrative growth pattern might be an unfavorable prognostic parameter.[20,33] However, tumor budding is more often observed in tumors without MMR protein deficiency.[34]Also, in the present study, the rate of tumor budding was higher in patients without MMR protein deficiency. In a study by Kevans et al. involving 258 patients with stage II colorectal cancer, 48% of tumors with tumor budding were MSS tumors.[34]

Absence of Dirty Necrosis: The absence of dirty necrosis is another histological parameter linked to MSI-H phenotype in recent studies.[19] In a series of patients reported by Greenson et al., the absence of dirty necrosis offered a sensitivity of 82.7% and a specificity of 76.6% in predicting MSI-H phenotype.[19] In the present study, the rate of dirty necrosis was 35.7% in MMR-deficient tumors and 64.3% in tumors without MMR protein deficiency, and the difference was statistically significant ($p<0.0001$).

The Effect of the Loss of MMR Protein Expression on Overall Survival

It is known that MSI is a prognostic marker for survival in patients with colorectal cancer.[10,11] Many studies have emphasized that the loss of MMR protein expression favorably affects disease-specific survival.[10,28] A study by Gafa et al. investigated the effect of histological parameters (tumor diameter, localization, differentiation, mucin component, medullar characteristic, lymphocytic reaction, expansive growth pattern) and MSI status on disease-specific survival, and MSI status was found to be the most important predictive parameter increasing disease-specific survival.[35] Only overall survival data was available in the present study. Although the overall survival was longer in patients with MMR protein deficiency, no significant relationship was found between the loss of MMR protein expression and survival. The inconsistency with the literature data can be explained by the low rate of these patients in the entire study group and the fact that disease-specific survival data was not available in our study.

In conclusion, the characteristics associated with the loss of MMR protein expression are age under 50 years, advanced tumor stage, low number of metastatic lymph nodes, right colon localization, poor differentiation and high tumor grade, mucinous and medullary tumor component, increased TIL and Crohn's-like lymphoid reaction, and low rates of tumor budding and dirty necrosis. Clinicopathological findings of the present study are consistent with those reported in the literature. However, no significant relationship was found between MMR protein deficiency and survival, although survival was longer in patients with MMR-deficient tumors. Unlike many other studies, the present study evaluated medullary components in a separate category independently of poorly differentiated tumors. This distinction has shown that the presence of medullary tumor component is an important parameter in predicting the loss of MMR expression.

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Figure Legends

Figure 1: The morphologic features of tumor that most determined MMR status in colon adenocarcinoma. MMR deficient mucinous carcinoma with large areas of extracellular mucin and cellular lymphocytic nodules in the surrounding fibrosis (A), MMR deficient signet ring cell carcinoma that was containing more than 50% signet ring cells (B), Tumor-infiltrating lymphocytes in medullary carcinoma that was a rare subtype of poor differentiation tumors (C), Tumor budding is presence of single tumor cells or small cell clusters of less than five cells with an anaplastic character at the infiltrative tumor margin (D). (H&E.)

Figure 2. Mismatch repair immunohistochemistry: Loss of MLH1-PMS2 protein expression in moderately differentiated adenocarcinoma cells while stromal lymphocytes show nuclear positivity. Immunohistochemical staining indicates loss of expression MLH1 (A) with PMS2 (B) and expression of MSH2 (C) with MSH6 (D).

Figure 3. Mismatch repair immunohistochemistry**:** Loss of MSH2-MSH6 protein expression in poorly differentiated adenocarcinoma cells while normal colon crypts and stromal lymphocytes show nuclear positivity. Immunohistochemical staining indicates expression of MLH1 (A) with PMS2 (B) and loss of expression MSH2 (C) with MSH6 (D).

Table 1: Clinicopathological and IHC characteristics of MMR deficient CRCs and MMR intact CRCs

	MMR deficient CRC n=98 (9,8 %)	MMR intact CRC n=904 (90,2 %)	p value
Gender			0.08
Female	46 (46,9 %)	353 (39 %)	
Male	52 (53,1 %)	551 (61 %)	
Localisation of Tumor			<0,0001
Left side	36 (36,4 %)	744 (82,3 %)	
Right side	62 (63,3 %)	160 (17,7 %)	
Tumor Differentiation			<0,0001
Well	7 (7,1 %)	87(9,6%)	
Moderate	54 (55,1 %)	673 (74,4 %)	
Poor	25 (25,5 %)	54(6%)	
Mucinous	12 (12,2 %)	90 (10 %)	
pT Stage			0,005
T1	2 (2,0 %)	13(1,4%)	
T2	5(5,1 %)	94 (10,4 %)	
T3	76 (77,6 %)	723 (80,0 %)	
T4	15 (15,3 %)	74(8,2%)	
pN Stage			<0,0001
N0	59 (60,2 %)	421 (46,6 %)	
N1	24 (24,5 %)	273(30,2 %)	
N1c	2(2%)	36(4%)	
N2	9 (9,2%)	170(18,8)	
Nx	4(4%)	4(0,4%)	
LVI			0,502
Present	24 (27,8 %)	227(25,1%)	
Absent	74(75,5%)	677(74,9%)	
PNI			0,593
Present	17 (17,3 %)	218(24,1%)	
Absent	81(82,7%)	686(75,9%)	
Satellite Nodule			0,03
Present	10 (10,2 %)	163(18%)	
Absent	88(89,6%)	741(82%)	
TILs			<0,0001
Present	25 (25,5 %)	79(8,7%)	
Absent	73(74,5%)	825(91,3%)	
Crohn-like Reaction			0,005
Present	50 (51 %)	335(37,1%)	
Absent	48(49%)	569(62,9%)	
Tumor Budding			<0,0001
Present	10 (10,2 %)	228(25,2%)	
Absent	88(89,8%)	676(74,8%)	
Dirty Necrosis			<0,0001
Present	35(35,7 %)	509(56,3%)	
Absent	63(64,3%)	395(43,7%)	
Tumor Border			0,391
Expansive	8 (8,2 %)	63(7%)	
Infiltrative	90(91,8%)	841(93%)	

Table 2. Evaluation of MMR deficient CRC and MMR intact CRCs in terms of overall survival.

	Estimate time (month)	Std Error (month)	95% Confidence Interval		p value
	Mean		Lower Bound	Upper Bound	
					0.264
Overall Survival (5 years)	**101.077**	**2.488**	**96.201**	**105.952**	
MMR deficient CRC	102.270	6.936	88.676	115.864	
MMR intact CRC	100.219	2.609	95.201	105.331	

Evaluation was made using Kaplan-Meier analysis