Analysis of the MLH1, MLH2, MLH6, PMS2 genes and their correlations with clinical data in rectal mucinous adenocarcinoma

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BACKGROUND: *Microsatellites are short repeated DNA sequences normally found in the human genome. Following specific mutations, microsatellites can vary in the number of repeats thus making the DNA unstable. Microsatellite instability (MSI) is responsible for approximately 20% of rectal cancers, while the remaining 80% are caused by chromosomal instability. One of the following genes, MLH1, MLH2, MLH 6, and PMS2, is inactivated, leading to MSI colorectal cancers.*
AIM: *This study aimed to analyze the expression of some MMR system genes presenting mutations in mucinous rectal cancer and their correlations with clinical data.*
METHODS: *A retrospective study was performed on patients with rectal mucinous adenocarcinoma who underwent surgery between January 2000 and January 2017. We collected a total of 42 patients and analyzed the demographic data, histopathological results and MMR system genes mentioned above.*
RESULTS: *Almost 93% of the cases analyzed had MSI-H and only 7% were MSI-L. For MLH1, 50% of stage T2 and 50% of stage T4 had weak expression, while in stage T3, 42.50% had moderate expression. Regarding the N stage, we found that 66.67% of the patients with moderate gene expression (2+) were N2, while 42% of the patients with weak expression were N0. For MSH2, the majority of patients with strong gene expression were in stage T3 (27%). Weak expression was found in 50% of the patients in stage T2, 35% of the patients in stage T3, and 33.3% in T4. In 44.44% of the weak expression was N2, while for strong expression, there was an equivalent percentage of 33.33% in stages N1 and N2. Describing the MSH6 gene, we found that the most heterogeneous results were in stage T3. Weak expression was observed in 38.46% of the patients, while moderate and strong expression was observed in 30.77% and 11.54% respectively. Analysis of PMS2 revealed that 66.67% of the patients in stage T4 had a weak expression of the gene, while the same expression was found in 38.46% of the patients in stage T3. A total of 23.08% of patients in stage T3 had strong gene expression. We also analyzed the overall gene expression. Thus, we found that three patients (7.14%) had only 1, three genes were expressed, nine (21.42%) had two genes and the remaining 27 patients had all 4. The 1-year survival rate in the analyzed lot was 75%, decreasing to 60% in the second year and 35% in the 3rd. There were no statistically significant differences in survival data between the stages or gene expression.*
CONCLUSIONS: *Our study showed no statistical difference regarding the survival on different gene expression or staging, consistent with studies that found that mucin expression does not have a significant impact on local recurrence, nor does it affect nodal down staging.*

KEY WORDS: Mucinous adenocarcinoma, Microsatelites instability

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Introduction

Colorectal cancer (CRC) represents the 3rd most common cancer in men and the 2nd in women worldwide [1,2]. The most common histological subtype is classical adenocarcinoma, followed by mucinous adenocarcinoma (MAC). MAC is one of the less common histological subtypes of colorectal cancers accounting for 10-15% of all colorectal tumors. Due to its rarity, few studies and data are available in the medical literature. Other rare subtypes include signet cell carcinoma, neuroendocrine carcinoma and adenosquamous carcinoma [3-5].

The WHO defines MAC as a tumor characterized by glandular formation and pools of extracellular mucin that comprise >50% of the tumor volume, while a mucin content of <50% designates "adenocarcinoma with mucinous component" compared to classical adenocarcinoma; MAC appears to develop more frequently in younger age groups; in female patients, MAC is more frequently observed in the right hemicolon, often present at higher stages and are less likely to show vascular, lymphatic or perineural invasion [5-7].

Multiple studies have shown that mucinous adenocarcinomas are found at higher rates in patients with inflammatory bowel diseases, Lynch syndrome (HNPCC) and radiotherapy-induced CRCs [5,8-10].

There are three types of genetic instability in colorectal cancer: chromosomal instability pathway (CIN), microsatellite instability pathway (MIS), and CPG island methylator or CIMP. The microsatellite pathway is found in approximately 15% of sporadic colorectal cancers. Microsatellites are prone to mutations that appear during DNA replication when there is a defect in the microsatellite mismatch repair (MMR) system. When this error occurs, this phenomenon is called microsatellite instability to tumor development. MSI concerns gene repair processes, such as hMLH1 (human mut-L hologue). In hereditary nonpolyposis colorectal cancer – HNPCC), the mutation is also encountered in other genes, such as hMSH2, hMSH6, and pPMS2. Still these genes inactivation – called mismatch repair – MMR can be inherited (HNPCC) or can be achieved by the methylation of one or more genes involved [11-13].

MSI-H sporadic colorectal cancers are more often (about 70-95 %) caused by alteration of MLH1 gene via somatic promotor hypermethylation [11].

Colorectal adenocarcinoma tumor grading was established based on microsatellite stability or instability. The aim of this study was to determine whether there is a correlation between gene expression, tumor stage and differential degree.

Material and method

We performed a retrospective study to analyze the histopathological results of rectal mucinous adenocarcinoma operated between January 2000 and January 2017 at the Regional Institute of Gastroenterology and Hepatology "O. Fodor" Cluj-Napoca, Romania. In 42 patients, we performed genetic testing of the MLH1, MLH2, MLH6 and PMS2 genes. Other parameters included in the analysis were: demographic data and histopathological results. Immunohistochemistry was interpreted as absent (0+), weak (1+), moderate (2+), or strong (3+). We considered cases that displayed two or more genes as MSI-H and cases with only one gene expression as MSI-L. We followed the TNM staging system according to the American Joint Committee on Cancer Staging Manual. Statistical analyses were performed using Microsoft Excel software. We excluded patients with missing data or those with no consent forms.

For each case, a paraffin-embedded block containing the tumor area adjacent to the normal mucosa was selected. Three-micron-thick sections were obtained for the immunohistochemical analysis. The primary antibodies used were as follows: anti-hMSH2 (clone 79H11, RTU, catalog number: PA0048; Leica), anti-hMSH6 (clone PU29, catalog number: MSH6-L-CE; Leica), anti-hMLH1 (clone ES05, catalog number MLH1-L-CE; Leica) and anti-PMS2 (clone M0R4G, catalog number PMS2-L-CE; Leica). The BOND-III staining instrument (Leica Biosystems) and Bond Polymer Refine Detection Kit (Leica Biosystems) were used for all antibodies.

Normal colorectal mucosa and lymphoid tissues were used as the positive controls. Normal expression was defined as nuclear staining in tumor cells, while negative expression of protein was defined as the complete absence of nuclear staining in tumor cells with concurrent positive internal controls.

A mean percentage of stained tumor cells has been identified and classified into four categories: 0 (complete absence of nuclear staining of tumor cells); 1+ (<30% positive cells); 2+ (30-60% positive cells); 3 + (>60% positive cells).

Results

Of the 42 patients, 22 (52%) were women and 20 (48%) were men (W:M = 1.1:1), with a median age of 60 years (range, 41-82 years).

Considering the TNM classification, the majority of the patients, 20, were in stage III (47.6%), and in stage II, 18 (42.85%). There were only three patients in stage I (7.14%) and one in stage IV (2.38%).

The expression of each gene was analyzed using the TNM score. Concerning MLH1, 5 patients (11.9%) had no expression of the gene, while the most patients had a weak expression, 50% of them in stage T2 and T4 and 42.5% had a moderate expression, in stage T3 (Fig. 1). Regarding the N stage, we found that 66.67% of the patients with moderate gene expression (2+) were N2,

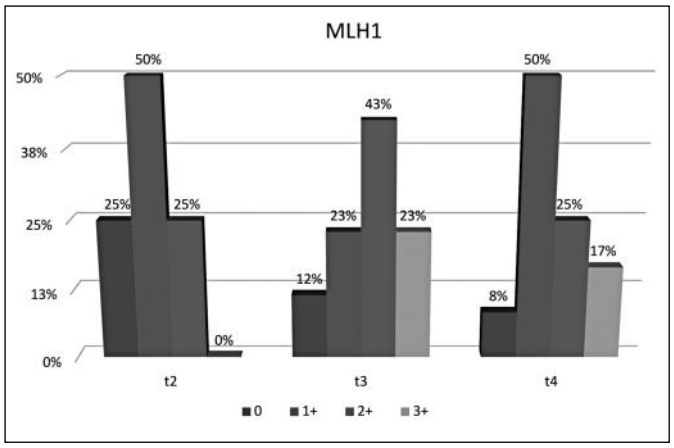

Fig. 1: MLH1 - T score.

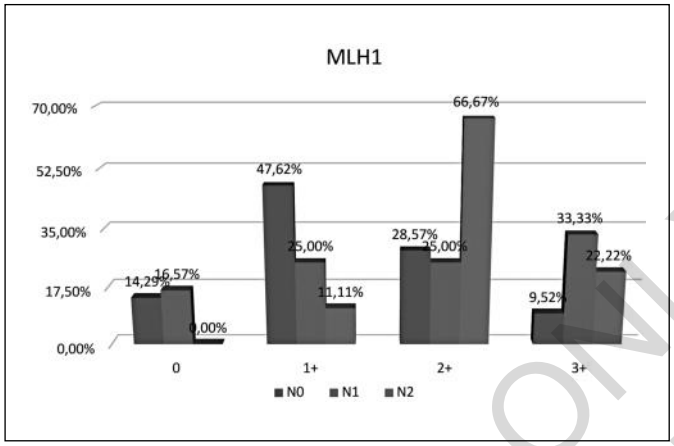

Fig. 2: MLH1 - N score.

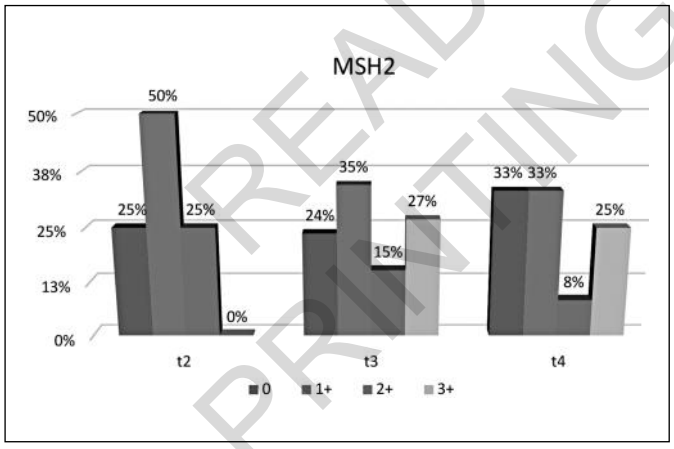

Fig. 3: MSH2 - T score.

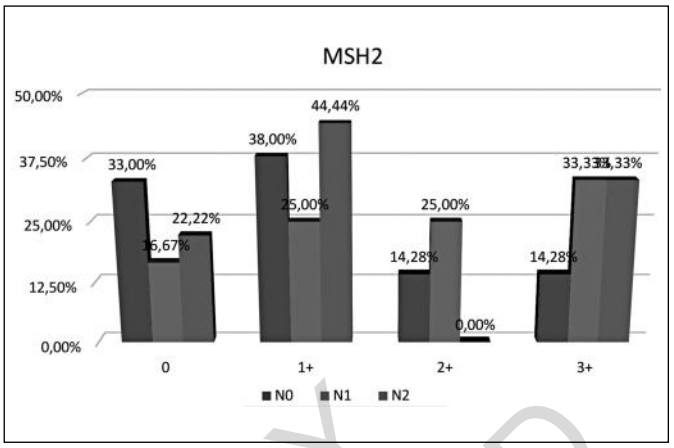

Fig. 4: MSH2 - N score.

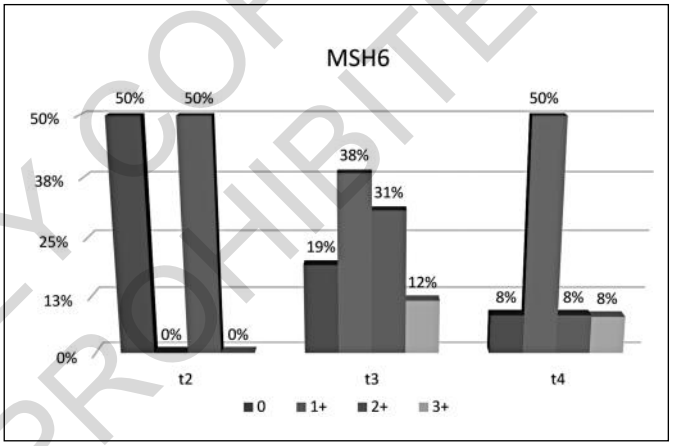

Fig. 5: MSH6 - T stage.

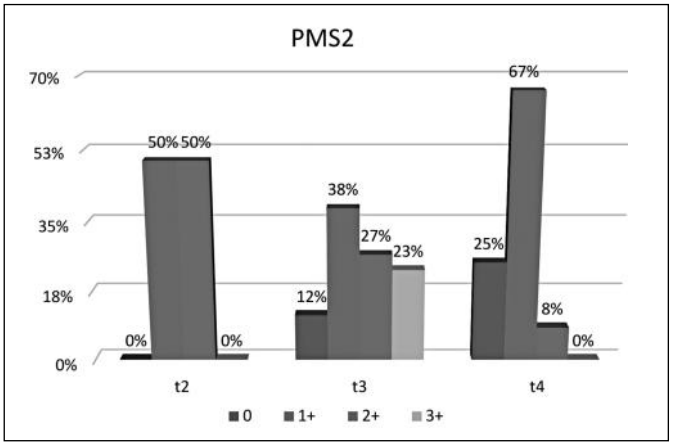

Fig. 6: PMS2 - T stage.

while 42% of the patients with weak expression were N0 (Fig. 2).

For MSH2, the majority of patients with strong gene expression were in stage T3 (27%). Weak expression was found in 50% of the patients in stage T2, 35% of the patients in stage T3, and 33.3% in T4 (Fig. 3).

Analysis of the same gene revealed that 44.44% of weak expression was N2, while for strong expression, there was an equivalent percentage of 33.33% in stages N1 and N2 (Fig. 4).

Describing the MSH6 gene, we found that the most heterogeneous results were in stage T3. Weak expression was observed in 38.46% of the patients, while moderate and strong expression was observed in 30.77% and 11.54% respectively 19.23% of the patients, respectively (Fig. 5).

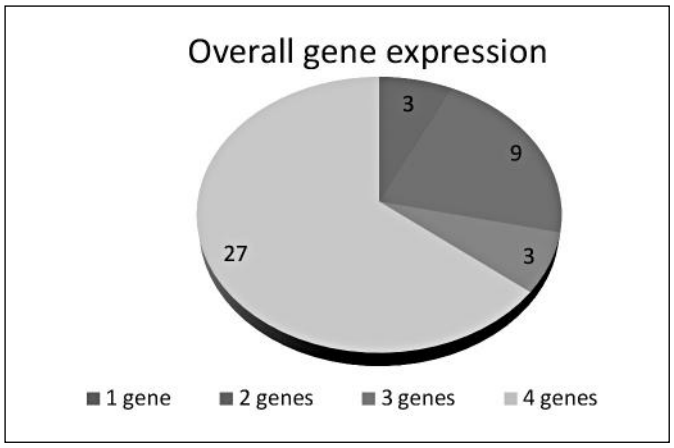

Fig. 7: Overall gene expression.

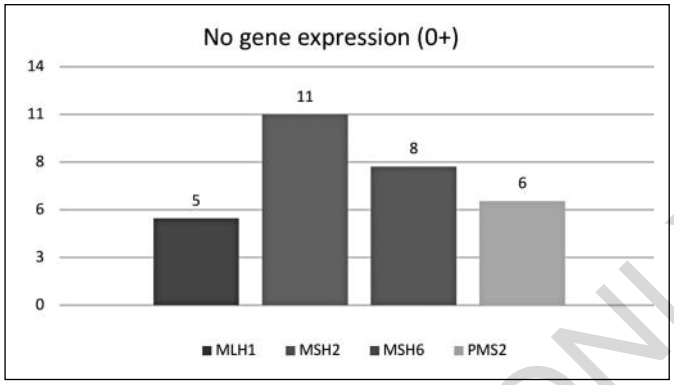

Fig. 8: No gene expression.

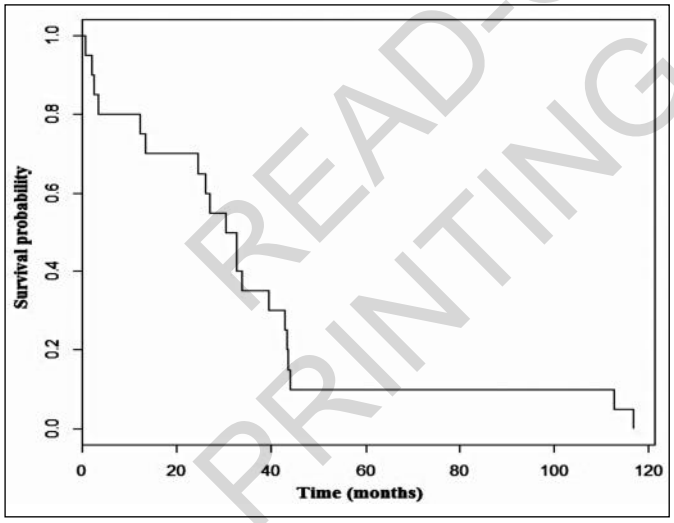

Fig. 9: Kaplan-Meier survival.

Analysis of PMS2 revealed that 66.67% of the patients in stage T4 had a weak expression of the gene, while the same expression was found in 38.46% of the patients in stage T3. A total of 23.08% of patients in stage T3 had strong gene expression (Fig. 6).

We also analyzed the overall gene expression. Thus, we found that three patients (7.14%) had only 1, three genes were expressed, nine (21.42%) had two genes and the remaining 27 patients had all 4 (Fig. 7). Therefore, almost 93% of the cases analyzed had MSI-H and only 7% were MSI-L.

In the absence of gene expression, we found that MSH2 was the least expressed gene, where 11 out of the 42 patients had no expression, followed by MSH6 in 8 patients (Fig. 8).

The 1-year survival rate in the analyzed group was 75%, which decreased to 60% in the second year and 35% in the 3rd. There were no statistically significant differences in survival data between the stages or gene expression.

Discussions

As we find ourselves in an era of personalized medicine where genetic testing is no longer considered only an academic pursuit, identifying gene mutations has become necessary in providing appropriate care[14]. MSI defects are found in sporadic colon, gastric, endometrial, ovarian, non-small cell lung cancer and most other cancers; therefore, the identification of MSI status in CRC has both prognostic and therapeutic effects[11,15]. Therefore, microsatellite instability analysis is becoming increasingly significant for patients with CRC neoplasm[11,14]. Mutation rates in single nucleotides were higher in MSI-positive CRCs than in CIN-positive CRCs.

Microsatellites are short tandem repeats (STRs), which are small repeating stretches of DNA dispersed across the genome. Because of their structure, microsatellites are prone to a high mutation rate[11]. This alteration is a unique molecular defect and hypermutable phenotype and is in fact the result of an incomplete DNA mismatch repair (MMR) system. The MMR system is responsible for the recognition and repair of nucleotide polymerase incorporation defects that occur during replication, thereby preserving the integrity of the genome[11]. The central players in the Escherichia coli MMR are MutS, MutL and MutH, while the main proteins involved in eukaryotes are MSH2, MSH3 and MSH6, which are homologs of MutS, MLH1, MLH2 and MLH3, which are MutL homologs [11,16,17]. There are also other homologs of MutL, more precisely, post-meiotic segregation proteins (PMS1 and PMS2). The mismatch repair system works through a series of phases: MSH2 interacts with MSH6 or MSH3, causing the formation of MutSα and MutSβ heterodimers. They recognize single-base mismatches as well as insertion or deletion loops. Subsequently, they can recruit MutLα, MutLβ or MutLγ heterodimers (if MLH1 couples with PMS2, PMS1 or MLH3,) and form a complex that creates a sliding clamp around the DNA and moves along the new chain. Proteins found in the sliding clamp interact with exonuclease-1 and proliferating cell nuclear antigen (PCNA). The error was excised and a new strand was synthesized.

Finally, the DNA polymerase and ligase are correcting the synthesis and the ligation [11,14,17].

A certain number of genes have been found to include microsatellite sequences within their coding region and MSI manifests as altered signaling, transduction, apoptosis, DNA repair, transcriptional regulation and protein translocation. Mutation rates in single nucleotides were higher in MSI-positive CRCs than in CIN-positive CRCs.

The hereditary type of CRC is divided into five subtypes: familial adenomatous polyposis, Lynch syndrome, MUTYH-associated polyposis, Peutz-Jeghers syndrome and serrated polyposis syndrome [11,14,17]. Familial adenomatous polyposis is the most common hereditary polyposis syndrome and is caused by a mutation in the APC gene located on chromosome 5q21. The APC gene is a tumor suppressor gene that produces the APC protein, which controls cell growth and helps prevent tumor development [18-20]. Besides APC mutations, other mutations, such as K-RAS, DCC, P53, COX-2, and BCL-2 have been identified in malignant tumor development. In Lynch syndrome, also known as hereditary non-polyposis colorectal cancer, it has been established that the following mutations in DNA mismatch repair genes account for its appearance: MSH2 on chromosome 2p16, MLH1 on chromosome 3p12, MSH6 on chromosome 2p16, and PSM2 on chromosome 7p22. MSH2 and MLH1 mutations are responsible for most Lynch syndrome cases. MMR genes are responsible for coding proteins that are necessary for the appropriate repair of DNA mismatches and corrections over base mismatches or small deletions and insertions. If these genes are inactive, the DNA repair process is interrupted, which causes and alters the short-tandem DNA repetitive sequence, generating a microsatellite instability phenotype. This phenotype is blameworthy for approximately 2-3 % of the total CRC cases [11]. The demographic data analyzed were consistent with those of other studies [21–23]. Most of our patients were women, with a median age of 60. It is known that mucinous adenocarcinoma is associated with a poor prognosis and a low overall survival rate; however, in our study, the 2-year survival rate was 60%, decreasing to 35% in the 3rd year. These data are consistent with other studies, which also have low survival rates (77% in the first year, 33% in the 3rd). In addition, our study showed no statistical difference regarding the survival on different gene expression or staging, consistent with studies that found that mucin expression does not have a significant impact on local recurrence, nor does it affect nodal down staging. While 38 (93%) of the patients were MSI-H and only 3 patients were considered MSI-L, we cannot state that there were significant differences between the two groups considering the analyzed parameters. Nevertheless, all the patients considered for this study had a rectal mucinous adenocarcinoma histopathological diagnosis, which made the selection criteria very strict.

A study performed on 681 patients with CRC[24] showed that the MLH1/MSH2 phenotype represents a distinct subtype of sporadic CRC and is an independent predictive and prognostic factor for the outcome of stage II and III CRC. There were 395 patients in stage II, 286 in stage III and by gender division, there were 387 men and 294 women. Regarding MLH1/MSH2, 131 (19.24%) patients were negative, which indicated the presence of the tumor, while the remaining 550 (80.76%) were positive. The present study included patients in all four tumor stages, but most of them were stage II (42.85%) and stage III (47.6%). MLH1/MSH2-negative appeared more often in the right colon than in the left colon, as revealed by our study and, more frequently, in the colon than in the rectum. Furthermore, MLH1/MSH2-negative results were observed in poorly differentiated CRC compared to well-moderately differentiated CRC. Moreover, MLH1/MSH2-negative CRC is characterized by LN metastasis and mucinous tumors [11,24]. In this study, they concluded that MLH1/MSH2 expression was not associated with any of the following: age, sex, tumor stage and size, lymphocytic infiltration, or circumscribed margin. The median follow-up period was 56 months a range, 8-72 months. Patients with MLH1/MSH2-negative stage II or III CRC showed a favorable trend for OS (68.62 and 62.11%, respectively). Patients with MLH1/MSH2-negative stage II CRC had longer OS than those with MLH1/MSH2-positive CRC, and patients with stage III CRC and MLH1/MSH2-negative CRC had a longer OS. The 5-year survival rate for patients with MLH1/MSH2-negative CRC was 86.9%, compared with 59.1% for patients with MLH1/MSH2-positive CRC[24].

Markovic et al.[25] published a study on 125 patients with colorectal cancer, 21 of which had high MSI representing 20% and 101 of which had low MSI or MSS representing 80%. The median follow-up period for these patients was 31 months. The mean age was 63 years in the MSI-H group and 62 in the MSI-l/MSS group, similar to the present study. Even though most patients in the MSI-H group were male, there was no significant correlation between sex and MSI status. The prevalence of proximal colon lesions was higher in the MSI-H group [11,25]. Tumors with MSS/MSI-L status had a higher recurrence rate than those with MSI-H status. The study revealed no difference in survival between MSI-H and MSI-L/MSS tumors. There are two important differences compared to our study because this study did not include only patients with MAC and excluded patients who underwent preoperative radiotherapy and chemotherapy, patients with IBD and those with HNPCC.

In another study of 186 patients with colorectal cancer who underwent surgery for adenocarcinoma between 2008 and 2012, the authors analyzed the association between colorectal cancer and MLH-1, MSH-2, PMS-2, and MSH-6 expression [26]. The cases were retrospectively analyzed in terms of demographic data, tumor

characteristics, including mucinous differentiation, staging, lymphovascular and perineural invasion, surgical border and lymph node metastasis. Comparing this article to ours, it can be observed that there were 124 men and 62 women in this study, with a mean age of 66 years. The loss of expression of the following immunohistochemical markers: MLH-1, MSH2, PMS-2, and MSH was correlated with poorly differentiated and mucinous adenocarcinoma histology. Furthermore, there was a correlation between the localization of the tumor in the right colon and the loss of PMS-2 and MSH-6 [11,26]. Approximately half of the cases with loss of MLH-1 expression were located in the cecum and ascending colon. In addition, a lack of MLH-1 expression was observed in tumors with mucinous components.

Moreover, it was recorded that tumors that exhibited loss of MLH-1 and PMS-2 expression presented with lymphovascular invasion and intense intratumoral lymphocytic infiltration more often.

The purpose of this study was to show that there are specific clinicopathological features of CRC with loss of gene expression[26].

To assess the survival of patients with stage II colorectal cancer, their data were correlated with the presence of MSI in a review of 39 studies comprising 12.110 patients. Many studies have investigated the relationship between the survival of CRC patients and MSI status, often regarding individuals in both the early and advanced stages [27]. In this review, the instability of two or more microsatellite loci was defined as high frequency, while the instability of one or no marker was outlined as a low-frequency or stable microsatellite status [27]. However, there is another method to evaluate the MMR status, more precisely, the analysis of MMR proteins (MLH1, MSH2, MSH6 and PMS2)[11,27] using immunohistochemical staining. The main interests of this review are the overall survival of patients with stage II CRC and the prognostic significance of MMR status. As for the results of the meta-analysis, the median follow-up of the patients was 68.5 months, and the median age was 65.5 years which is comparable to our study where the median age was 60 years. In the overall survival analysis, the review showed that in 27 studies, patients with MSI-associated CRC had a reduced risk of death and also regarding disease-free or relapse-free survival, the patients from 27 studies had a reduced risk of relapse [27].

Conclusions

Our study shows heterogeneity regarding the expression of microsatellite instability genes in both T and N stages. Analysis of the survival rate between the T and N stages and overall TNM stages showed no significant differences.

Mucinous adenocarcinoma has a worse prognosis and lower survival rates, and a more aggressive therapy and closer follow-up should be considered when managing these patients.

Early diagnosis of colon cancer will lead to more efficient treatments with better results and better genomic characterization of these tumors will select groups of patients for genetic testing screening and customized oncological and surgical treatments.

Riassunto

I microsatelliti sono brevi sequenze ripetute del DNA presenti normalmente nel genoma umano. A seguito di specifiche mutazioni, i microsatelliti possono variare nel numero di ripetizioni rendendo in tal modo il DNA instabile.

L'instabilità dei microsatelliti (MSI) è responsabile di circa il 20% dei tumori del retto, mentre il restante 80% è causato da instabilità cromosomica. Uno dei seguenti geni, MLH1, MLH2, MLH 6 e PMS2, è inattivato, portando a tumori del colon-retto MSI.

Questo studio retrospettivo mirava ad analizzare l'espressione di alcuni geni del sistema MMR che presentano mutazioni nel cancro del retto mucinoso e le loro correlazioni con i dati clinici. Lo studio è stato condotto su pazienti con adenocarcinoma mucinoso rettale sottoposti a intervento chirurgico tra gennaio 2000 e gennaio 2017. Abbiamo raccolto un totale di 42 pazienti e analizzato i dati demografici, i risultati istopatologici ei geni del sistema MMR sopra menzionati.

Risultati: Quasi il 93% dei casi analizzati aveva MSI-H e solo il 7% era MSI-L. Per MLH1, il 50% dello stadio T2 e il 50% dello stadio T4 avevano un'espressione debole, mentre nello stadio T3, il 42,50% aveva un'espressione moderata. Per quanto riguarda lo stadio N, abbiamo riscontrato che il 66,67% dei pazienti con espressione genica moderata (2+) era N2, mentre il 42% dei pazienti con espressione debole era N0. Per MSH2, la maggior parte dei pazienti con una forte espressione genica era allo stadio T3 (27%). Una debole espressione è stata riscontrata nel 50% dei pazienti allo stadio T2, nel 35% dei pazienti nello stadio T3 e nel 33,3% nello stadio T4. Nel 44,44% l'espressione debole era N2, mentre per l'espressione forte c'era una percentuale equivalente del 33,33% negli stadi N1 e N2.

Descrivendo il gene MSH6, abbiamo scoperto che i risultati più eterogenei erano nello stadio T3. Un'espressione debole è stata osservata nel 38,46% dei pazienti, mentre un'espressione moderata e forte è stata osservata rispettivamente nel 30,77% e nell'11,54%. L'analisi della PMS2 ha rivelato che il 66,67% dei pazienti allo stadio T4 aveva una debole espressione del gene, mentre la stessa espressione è stata trovata nel 38,46% dei pazienti allo stadio T3. Un totale del 23,08% dei pazienti allo stadio T3 aveva una forte espressione genica. Abbiamo anche analizzato l'espressione genica complessiva. Pertanto, abbiamo scoperto che tre pazienti

(7,14%) avevano solo 1, tre geni erano espressi, nove (21,42%) avevano due geni e i restanti 27 pazienti avevano tutti 4. Il tasso di sopravvivenza a 1 anno nel lotto analizzato era del 75%, scendendo al 60% nel secondo anno e al 35% nel 3°. Non c'erano differenze statisticamente significative nei dati di sopravvivenza tra gli stadi o l'espressione genica.

CONCLUSIONE: Il nostro studio non ha mostrato differenze statistiche riguardo alla sopravvivenza su differenti espressioni geniche o stadiazione, coerentemente con gli studi che hanno scoperto che l'espressione della mucina non ha un impatto significativo sulla recidiva locale, né influisce sulla stadiazione nodale verso il basso.

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