Microsatellite instability and B-type Raf proto-oncogene mutation in colorectal cancer: Clinicopathological characteristics and effects on survival

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ABSTRACT

Prognostic significance of microsatellite instability (MSI) status and B-type Raf proto-oncogene (*BRAF*) mutation in colorectal cancer is controversial. The aim of this study was to examine the clinical and pathological characteristics associated with microsatellite stability and the effect of MSI and *BRAF* mutation on the survival of patients with colorectal cancer. The study included 145 colorectal cancer cases. All the patients were examined for DNA mismatch repair (MMR) proteins with an immunohistochemical method. Molecular assessment of MSI was available in a subset of 41 patients. In addition, *BRAF* mutation analysis was performed in 30 cases. Immunohistochemically, MMR deficiency was present in 28 (19.3%) patients. Female gender (p = 0.001), lesion size ≥5 cm (p = 0.013), Crohn-like response (p = 0.035), and right-sided localization (p < 0.001) were significantly more frequent among MMR-deficient patients. The overall survival was 44.1 ± 5.1 months (95% confidence interval [CI], 33.7-54.4). Multivariate analyses identified only high tumor grade as an independent predictor of poor overall survival: odd ratio, 6.7 (95% CI 2.1-21.7), p = 0.002. In the subset of patients with available *BRAF* assessment (n = 30), a negative *BRAF* status was associated with better survival when compared to a positive *BRAF* status (36.7 ± 2.1 vs. 34.1 ± 7.2 months, p = 0.048). The sensitivity and specificity of the immunohistochemical method in predicting positive MSI status, with the molecular method as a reference, were 85.7% (95% CI: 56.2%-97.5%) and 88.9% (95% CI: 69.7%-97.1%), respectively. *BRAF* appears to be a significant predictor of a worse outcome in patients with colorectal cancer. Further studies with a large spectrum of clinical and biological variables are warranted.

KEY WORDS: Microsatellite instability; DNA mismatch repair genes; B-type Raf proto-oncogene mutation; survival; polymerase chain reaction; immunohistochemistry

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies in the developed world, and it is on the rise in developing countries; thus, representing a global public health challenge [1-3]. Most CRCs develop sporadically without evidence of family history or genetic predisposition [4].

The microsatellite instability (MSI) pathway is among the several proposed mechanisms for the development of colon cancer. Microsatellites are repeats of short DNA motifs, 1-5 base pairs in length, distributed throughout the genome, particularly in noncoding regions. MSI is a change in the composition of these microsatellites within tumor tissue [5]. Such instabilities result from deficient function of DNA mismatch repair (MMR) genes, thus indicating a tendency toward increased genetic mutations [6-8]. Actually, DNA MSI represents a molecular manifestation of DNA MMR deficiency. Deficiency of MMR can be detected by immunohistochemical methods targeting MMR proteins in the tumor or using molecular methods utilizing polymerase chain reaction (PCR). Several studies examined the association between MSI and prognosis of CRC, most demonstrating better prognosis in MSI patients [9-16].

The B-type Raf proto-oncogene (*BRAF*) encodes an enzyme that takes part in intracellular signaling and cell growth, and *BRAF* mutation has been frequently observed in CRC, supporting its role in tumorigenesis [4,17]. *BRAF* mutation is also associated with worse outcomes in colorectal cancer, although conflicting results have been reported [18-21].

This study aimed to examine the clinicopathological characteristics associated with microsatellite stability and the

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effect of MSI and *BRAF* mutations on the survival of patients with colorectal cancer. In addition, we examined the reliability of immunohistochemical evaluation of microsatellite stability status in comparison with PCR-based assessment.

MATERIALS AND METHODS

Patients

This retrospective study included 145 colorectal cancer cases diagnosed between 2012 and 2013, with available MSI findings. The medical records were reviewed for demographical and clinical characteristics. In addition, hematoxylin and eosin stained preparations were re-examined. Cases with subserosa (T3) and peritoneal invasion (T4) were selected. All patients were examined for DNA MMR proteins with an immunohistochemical method, and molecular assessment of MSI was available in a subset of 41 patients. In addition, *BRAF* mutation analysis was performed in 30 cases. During follow-up, all patients or relatives were assessed by phone to get information on survival status.

Immunohistochemical assessment of DNA mismatch repair proteins

All immunohistochemical staining procedures were performed using an automated device (BenchMark XT IHK/ISH Staining Module, Ventana Medical Systems Inc., Medical Systems, Tucson, AZ, US). About 4 μm thick sections of 10% paraffin-fixed blocks were obtained from a site best representing the tumor and left overnight at 40°C on positively charged slides. Deparaffinization was attained using solutions compatible with the staining system, and the preparations were rehydrated using decreasing concentrations of alcohol solutions and distilled water. To retrieve antigens, they were left in a 10 mmol/l buffered citrate solution at 36°C for 30 minutes. Then commercial primary monoclonal antibodies were applied to the sections: MLH1 (Clone GM011, Genemed Biotechnologies, South San Francisco, USA), MSH2 (Clone G219-1129, Cell Marque Corporation. Rocklin, California, USA), MSH6 (Clone GM024, Genemed Biotechnologies, South San Francisco, USA), and PMS2 (Clone A16-4, Biocare Medical, Pike Lane, Concord, USA 1:50). After that, the preparations were counterstained with 0.01% hematoxylin and rinsed. Negative protein expression was defined as the absence of nuclear staining in tumor cells with concurrent internal positive controls, which were non-neoplastic colonic mucosa, stromal cells, infiltrating lymphocytes, or the centers of lymphoid follicles. If at least one marker showed negative expression, then the tumor was considered DNA MMR protein deficient. Figure 1 shows example images for the immunohistochemical evaluation of the MMR protein status.

Analysis of microsatellite instability with PCR

MSI was examined using molecular methods in 41 patients. For each patient, a pair of tissue samples (one from the tumor and one from a tumor-free site) was selected from formalin fixed paraffin embedded (FFPE) tissue. Using microtome, 5-6 sections, 5-10 μm thick, were obtained from each tissue and placed in Eppendorf tubes separately. The Qiagen FFPE Tissue Kit was used for DNA extraction. Following deparaffinization and lysis, released DNA was passed through nitrocellulose filter colons, purified with rinsing solutions, and prepared for use or storage with elusion solution. The purity and concentration of DNA were confirmed with spectrophotometry. Next, samples from normal and tumor tissues were amplified using a Techne 3200 PCR device and appropriate software. For the amplification reaction, forward primers were labeled with a WellRED Dye while reverse primers were unlabeled; thus, both strands of the target DNA segments were amplified and became visible on the device. The following MSI

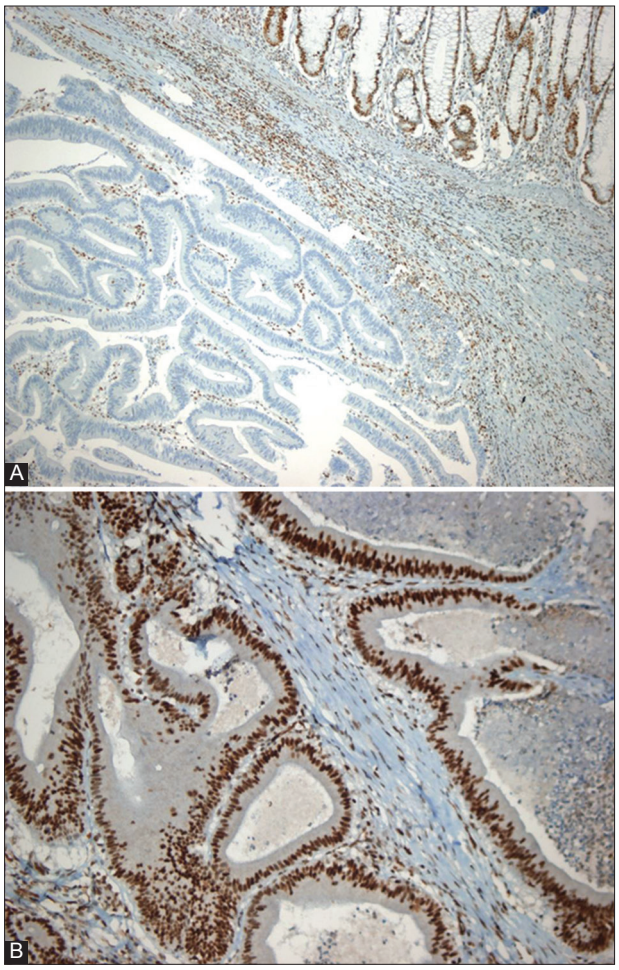

FIGURE 1. Immunohistochemical evaluation of mismatch repair protein status in colorectal carcinoma. (A) Loss of MLH-1 expression is evident in carcinoma tissue (lower part of the figure) while it is preserved in normal tissue (upper part of the figure) (100× magnification). (B) Normal expression of MSH2 in carcinoma tissue. (200× magnification)

markers were used: *BAT26, BAT25, NR24, NR21,* and *NR27*. DNA fragment analysis was carried out on a Beckman-Coulter DNA sequence analysis device using the capillary electrophoresis method. The samples were loaded on a 96-well plate, and the analysis was initiated with the sequence analysis device. If allelic profiles of the microsatellite markers of normal DNA and tumor DNA are similar, then there is no MSI. But if the allelic profiles do not match each other, this indicates MSI.

Results were interpreted as follows: High-frequency MSI (MSI-H) was considered when two or more of the 5 markers showed instability; low-frequency MSI (MSI-L) was considered when instability was observed at a single locus; microsatellite stable (MSS) was determined when none of the markers showed instability.

BRAF analysis with real-time PCR

Appropriately prepared DNA from the tumor region of the samples (as described above) from 30 patients was used for *BRAF* analysis. Conditions for the analysis were optimized for a Light Cycler480 Real Time PCR device (Roche Diagnostics GmbH). Identification of mutations was performed using the Entrogen BRAF Codon600 Mutation Analysis Kit (V600E) (Entrogen Inc.). Master mixture for the reaction was prepared as instructed by the kit manufacturer with following constituents: ×2 PCR reaction solution 15 µl, primer solution 6 µl, DNA sample 20 ng, completed to 30 µl with water. A positive control (PC) was used for the construction of reference curve, and contamination was checked with a negative control (NTC). After loading the device, PCR program was initiated: denaturation, at 95°C for 10 minutes; quantification, during denaturation at 95°C for 15 seconds through 40 cycles; annealing, at 60°C for 30 seconds. The amplification and quantification of target DNA were carried out simultaneously throughout the cycles. Complete conformity of target DNA and fluorescent-labeled primer pairs, specifically designed to detect the mutation site, results in a fluorescent emission which is recorded and analyzed using a specific software and based on cycle threshold (Ct) values. If a mutation is present, the fluorescence emission occurs before the 37th cycle. In the case of a wild-type sequence (no mutation), only basal fluorescent emission is detected.

Statistical analysis

SPSS version 21 was used for the analyses of data. Continuous variables were compared using the Student *t*-test for independent samples, and categorical variables were compared using the Pearson's Chi-square test or Fisher's exact test, where appropriate. Overall survival estimates were calculated using the Kaplan-Meier test and univariate comparisons were performed using the log-rank test. Overall survival was defined as the time elapsed between the date of diagnosis and death from any cause, and patients alive at the last follow-up were censored. For multivariate analysis, potential univariate predictors of overall survival were entered into a Cox proportional hazards model to identify independent predictors. In addition, diagnostic performance of the immunohistochemical method in predicting MSI status was calculated in reference to the molecular method for the patients with both assessments available. Two-sided $p < 0.05$ was considered statistically significant.

TABLE 1. Distribution of clinical parameters by MMR status assessed by the immunohistochemical method

Parameters	MMR competent (*n*=117)	MMR deficient* (*n*=28)	*p* for difference
Age, year (mean±SD)	63.8±11.0	64.7±16.3	0.777
Female gender (%)	43 (36.8)	20 (71.4)	0.001
Lesion size≥5 cm (%)	47 (41.6)	19 (67.9)	0.013
Lesion location (%)			
Right colon	42 (35.9)	23 (82.1)	
Left colon	43 (36.8)	4 (14.3)	<0.001
Rectum	32 (27.4)	1 (3.6)	
Histological type (%)			
Adeno NOS	53 (45.3)	10 (38.5)	
Mixed	46 (39.3)	8 (30.8)	
Mucinous	18 (15.4)	8 (30.8)	0.182
High tumor grade	11 (11.3)	4 (22.2)	0.250
Crohn-like response	44 (40.4)	17 (63.0)	0.035
Lymph node metastasis (%)			
Negative	53 (45.3)	16 (57.1)	0.260
Positive	64 (54.7)	12 (42.9)	

Unless otherwise stated, data presented as n (%). *If there was loss of expression of at least one of the MMR proteins, then the tumor was considered MMR protein deficient. MMR: Mismatch repair, SD: Standard deviation, NOS: Not otherwise specified

TABLE 2. Distribution of clinical parameters by MSI status

Parameters	MSI-H (*n*=14)	MSS (*n*=27)	*p* value
Age, year (mean±SD)	59.9±14.8	58.3±13	0.729
Female gender (*n*=41) (%)	10 (71.4)	11 (40.7)	0.062
Lesion size≥5 cm (%)	9 (64.3)	10 (37)	0.219
Lesion location (%)			
Right colon	10 (71.4)	11 (40.7)	
Left colon	4 (28.6)	11 (40.7)	0.097
Rectum	0 (0)	5 (18.6)	
Histological type (%)			
Adeno NOS	3 (25)	17 (63)	0.086
Mixed	6 (50)	6 (22.2)	
Mucinous	3 (25)	4 (14.8)	
High tumor grade	3 (33.3)	2 (9.1)	0.131
Crohn-like response	9 (69.2)	8 (47.1)	0.131
Lymph node metastasis (%)			
Negative	11 (78.6)	16 (59.3)	0.305
Positive	3 (21.4)	11 (40.7)	

SD: Standard deviation, MSI-H: High-frequency microsatellite instability, MSS: Microsatellite stable, NOS: Not otherwise specified

RESULTS

Patients

The mean age was 64.0 ± 12.1 years. Immunohistochemically, there was MMR protein deficiency in 28 (19.3%) patients. The distribution of the clinical parameters by the MMR status is given in Table 1. The MMR status did not differ with regard to the age, histological type, tumor grade, or lymph node involvement ($p > 0.05$ for all, respectively). However, the female gender ($p = 0.001$), lesion size ≥5 cm ($p = 0.013$), and Crohn-like response ($p = 0.035$) were significantly more frequent among the MMR protein deficient patients; in addition, the tumor was located more commonly at the right colon in these patients ($p < 0.001$). The distribution of clinical parameters by the MSI status, summarized in Table 2, was similar to the distribution of the MMR status.

Survival

During the mean 23.8 ± 10.1 months of the follow-up, 33 deaths occurred (22.8%). The overall survival was 44.1 ± 5.1 months (95% confidence interval [CI], 33.7-54.4). Figure 2A shows the Kaplan-Meier curve of the overall survival for the entire study population. Table 3 shows the univariate analysis results for potential predictors including the MMR status for the overall survival. A high tumor grade was associated with shorter overall survival (26.0 ± 3.6 vs. 51.8 ± 6.1 months, $p = 0.002$). The multivariate analyses identified only high tumor grade as an independent predictor of poor overall survival: Odd ratio, 6.7 (95% CI 2.1-21.7), $p = 0.002$. Figure 2B shows Kaplan-Meier curves by the tumor grade status. In the subset of the patients with available *BRAF* assessment ($n = 30$), the percentage of *BRAF* mutation was 33.3% (10/30) and a negative *BRAF* status was associated with better survival when compared to positive *BRAF* status (36.7 ± 2.1 vs. 34.1 ± 7.2 months, $p = 0.048$) (Figure 2C). About 4 of 10 *BRAF* positive cases and 7 of 20 *BRAF* negative cases were MSI, all of which were MSI-H. When combinations with microsatellite stability status were further analyzed, *BRAF* positive plus MSI tumors had poorer overall survival (14.4 ± 7.6 months) when compared to *BRAF* negative plus MSI tumors (36.8 ± 1.2, $p = 0.001$) and *BRAF* negative plus MSS tumors (35.8 ± 3.7, $p = 0.011$).

Molecular versus immunohistochemical analysis of MSI status

The molecular analysis of the MSI status was also available in a subset of patients ($n = 41$). Twenty-seven cases were

TABLE 3. Univariate analysis for overall survival with the log-rank test

Overall survival (months)	Mean (SE)	95% CI	*p*
Gender			
Female (*n*=63)	45.0 (5.7)	33.8-56.2	0.513
Male (*n*=82)	31.7 (1.4)	29.1-34.4	
Age (years)			
<60 (*n*=48)	50.5 (6.9)	36.9-64.1	0.265
≥60 (*n*=97)	39.1 (1.9)	35.4-42.9	
Lesion size (cm)			
<5 (*n*=75)	33.0 (1.3)	30.4-35.6	0.493
≥5 (*n*=66)	54.5 (3.3)	48.0-61.0	
Lesion location			
Right colon (*n*=65)	31.8 (1.8)	28.3-35.2	
Left colon (*n*=47)	34.8 (1.8)	31.3-38.3	0.573
Rectum (*n*=33)	48.1 (6.8)	34.9-61.4	
Histological type			
Adeno NOS (*n*=63)	48.5 (6.0)	36.8-60.1	
Mixed (*n*=54)	38.9 (1.6)	31.7-38.0	0.197
Mucinous (*n*=26)	28.3 (3.2)	22.1-34.6	
Tumor grade			
Low (*n*=100)	51.8 (6.1)	39.8-63.7	0.002
High (*n*=15)	26.0 (3.6)	18.9-33.1	
MMR status*			
Competent (*n*=117)	44.7 (5.4)	34.1-55.4	0.637
Deficient (*n*=28)	30.7 (2.5)	25.9-35.6	
MMR-deficient*			
Low (*n*=9)	28.6 (4.8)	19.2-38.0	0.533
High (*n*=19)	32.0 (2.7)	26.7-37.4	

*Based on immunohistochemistry findings; Low: Cases with loss of expression of only one of the MMR proteins. High: Cases with loss of expression of more than one MMR proteins. MMR: Mismatch repair

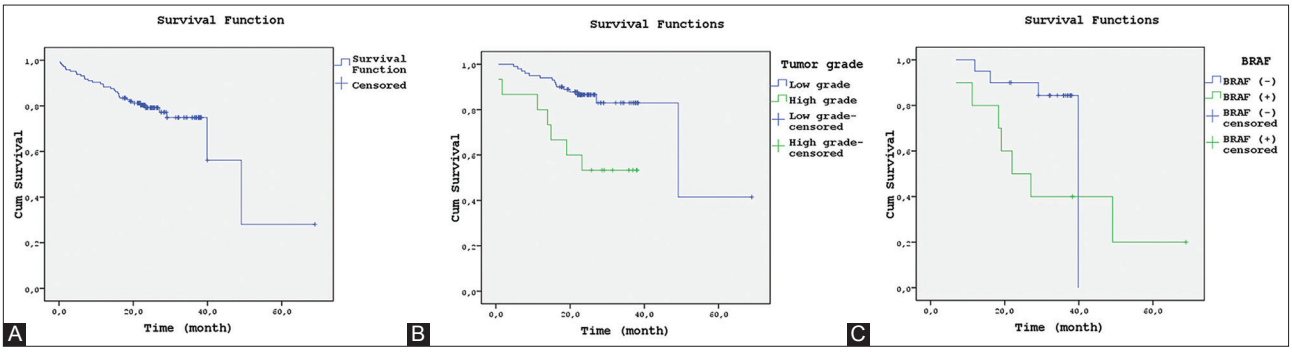

FIGURE 2. (A) Kaplan-Meier curve of the overall survival for the entire study population, (B) Kaplan-Meier curves by the tumor grade status. The blue curve indicates low grade and green curve indicates high-grade tumor, (C) Kaplan-Meier curves by B-type Raf proto-oncogene (*BRAF*) status. The blue curve indicates *BRAF* negative status and green curve indicates *BRAF* positive status

MSS, 14 cases were MSI-H and there were no MSI-L cases. The sensitivity and specificity of the immunohistochemical method in predicting positive MSI status, with the molecular method as a reference, were 85.7% (95% CI: 56.2%-97.5%) and 88.9% (95% CI: 69.7%-97.1%), respectively. False positivity and false negativity rates were 7.3% and 4.9%, respectively.

DISCUSSION

This study examined the microsatellite stability status of colorectal cancer patients along with its relation to clinicopathological variables and effects on survival. Although no effect of MSI on survival was detected, *BRAF* mutation was significantly associated with worse survival. In addition, the immunohistochemical and molecular methods were in good agreement in identifying the MSI status of the patients.

The overall prevalence of the MSI status evaluated by the MMR immunohistochemistry was 19.3% in this study, showing significant association with the female gender, large lesion size at diagnosis, Crohn-like response, and right-sided localization. These findings are largely in agreement with previous studies although different methods have been utilized for the assessment of MSI. Merok et al. found an overall MSI prevalence of 14% among colorectal cancer patients who underwent resection, with highest prevalence in females (19%) and in proximal colon cancer (29%) [9]. Another study by Vogelaar et al. found an overall prevalence of 23%, where MSI positivity was significantly associated with female sex, right-sided location, and poor differentiation [22]. Another study examining specifically the clinicopathological features of MSI-H colon cancers showed significant association with poor differentiation, proximal location, high mucin content, and female predominance [23]. However, in the study by Kaur et al. where the overall prevalence of abnormal DNA MMR protein expression was 18.7%, this condition was associated with proximal lesion, right-sided location, mucinous type, and poor differentiation, but not with the age, gender, or ethnic status of the patient [24]. In our study, we could not find a statistically meaningful correlation between the MSI status and histological grade; this is most probably due to low/high-grade case ratio which is high in this study. Regarding peritumoral Crohn's-like lymphoid reaction, it emerged as an intensive marker for MSI-H tumors in the study by Alexander et al. [25].

To date, several studies with diverse methodologies have examined the role of MSI status in predicting survival and treatment outcomes in colorectal cancer, mostly supporting favorable outcomes in association with MSI. The survival advantage of MSI tumors has been demonstrated in a number of studies [15,23,26], and two large meta-analyses confirmed these findings. One of these meta-analyses examined data from a large pool of colorectal cancer patients (n = 12782) and showed survival advantage of MSI both in terms of overall survival and disease-free survival (DFS) [12]. In another meta-analysis with pooled data from 7642 cases, MSI had advantage in terms of overall survival and survival advantage persisted for the subgroups of patients with locally advanced disease and patients treated with adjuvant fluorouracil (FU) [11].

Several studies have relatively distinct findings with regard to different subgroups and different outcomes. For example, a study from Italy examined the prognostic significance of MSI particularly in patients with pT3NoMo tumors and found an advantage for MSI-H tumors in terms of relapse-free survival [27]. Benatti et al. found survival advantage of MSI-H particularly for stage II and III cancers [13]. In another study, MSI was strongly associated with a decreased likelihood of lymph node and distant organ metastases at diagnosis, regardless of the pathologic features of the tumors [14]. A Norwegian study found survival advantage in favor of MSI tumors, but this finding was only confined to stage II tumors [9].

On the other hand, several studies did not find advantage for MSI tumors. In a Belgian study, MSI status did not emerge as an independent predictor of survival, instead tumor stage and differentiation significantly affected survival outcome [28], similar to the findings of our study. In a study by Shin et al., overall survival and DFS did not differ with regard to MSI status. However, in the subgroup of patients with stage II disease, DFS was worse for patients with MSI-H tumors [29]. In a recent study, with stage II colon cancer patients, a trend toward worse overall survival was seen in patients with an MSI tumor when compared to patients with an MSS tumor; however, the difference did not reach statistical significance [22].

Another issue to be addressed regarding the outcomes for MSI-H tumors is their response to therapy relative to microsatellite stable tumors. Some evidence suggests that MSI patients may not benefit or may get reduced benefit from FU-based adjuvant therapy, despite overall better survival of these patients [30-32]. Thus, some of the discrepancies for survival advantage of MSI status across different studies may well arise from the differences in the treatment protocols (i.e., with or without adjuvant treatment).

In contrast to the findings for colorectal cancer, Samowitz et al. found more than two times risk of dying of rectal cancer for patients with MSI-H tumors, and the authors attributed this finding to the relatively high frequency of Lynch-associated cancers among rectal MSI-H tumors [33].

Similar to several abovementioned studies, the MSI status did not emerge as an independent predictor of survival in this study. Previous studies suggest that the effect of microsatellite stability status on survival seem to be substantially affected by several other parameters; thus, an accurate stratification of MSI patients for other parameters such as tumor stage,

treatment type, tumor site, and other molecular markers seems to be necessary to better interpret MSI status and to utilize it for decision-making.

Several studies tested the predictive value of *BRAF* mutation in diverse settings of colorectal cancer. Farina-Sarasqueta et al. examined the prognostic significance of *BRAF* mutations in stage II and stage III disease, where *BRAF* mutation was an independent prognostic factor indicating worse outcomes in terms of overall survival and cancer-specific survival [34]. Another study found an association with *BRAF* mutation and worse survival in stage III colon cancer [20]. Yokota et al. identified *BRAF* mutation as a powerful prognostic factor in advanced and recurrent colorectal cancer treated with anti-epidermal growth factor receptor therapies [21]. However, studies stratifying cases according to both *BRAF* mutation and MSI status obtained diverse results. In the study by French et al., *BRAF* V600E mutation status did not affect disease-free or overall survival. However, a subgroup analysis of patients without *BRAF* mutation but with deficient MMR genes had significantly improved overall survival compared to other subgroups with regard to combined BRAF and MMR status [19]. In another study, *BRAF* mutation was not predictive for recurrence-free survival, but associated with worse overall survival, particularly for MSI-low and MSI-stable tumors [35]. Taieb et al. examined the prognostic importance of *BRAF* mutation in stage III colon cancer patients that received adjuvant chemotherapy and found that in patients with microsatellite-stable tumors *BRAF* V600E mutation was independently associated with worse clinical outcomes. In contrast, in patients with MSI tumors, it was associated with significantly longer DFS [36]. The present study found an association between better overall survival and the MSI plus *BRAF* negative status and MSS plus *BRAF* negative status. However, it is important to emphasize that the number of patients in each subgroup was relatively small.

This study found relatively high diagnostic value of the immunohistochemical method when the PCR method is used as a reference, with almost 90% sensitivity and specificity and less than 5% false negativity rate. Although the use of PCR method is recommended in most settings, in situations where PCR is not available, the immunohistochemical method may be an alternative.

The main limitation of this study is the retrospective design and a low number of patients, which did not allow testing the effects of too many parameters on the study outcomes.

CONCLUSION

In our study, *BRAF* mutation seems to be a significant predictor of worse outcome in colorectal cancer patients with MSI. However, further studies with better design, taking a large spectrum of clinical and biological variables into consideration, are warranted to draw firm conclusions.

DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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