Medicine®

OPEN

Molecular profiling of the colon cancer in South-Eastern Romania
Results from the MERCUR study

Razvan Catalin Popescu, MD, PhD[a,b], Cristina Tocia, MD, PhD[a,c,*] [iD], Costel Brînzan, PhD[d],
Georgeta Camelia Cozaru, MD, PhD[d,e], Mariana Deacu, MD, PhD[a,e], Andrei Dumitru, MD[c], Nicoleta Leopa, MD[a,b],
Anca Florentina Mitroi, MD, PhD[d,e], Anca Nicolau, MD, PhD[d,e], Eugen Dumitru, MD, PhD[a,c,d]

Abstract

Colorectal cancer is a heterogeneous disease with multiple epigenetic alterations and different molecular features. The molecular classification is based on 2 major distinct pathways: microsatellite stable pathway and the microsatellite instability pathway. Molecular profiling of colorectal cancer provides important information regarding treatment and prognosis. Aim of the study was to assess the frequency of microsatellite instability in colon cancer and the clinicopathological characteristics of the tumors with high level of microsatellite instability (MSI-H) in our region. The secondary outcome was to assess the frequency of v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) mutations in colon cancer.

The study included 129 patients with colon cancer fit for surgery. Demographic data, clinical and pathological data, immunohistochemistry staining pattern (4 mismatch repair proteins were investigated), and *BRAF* gene mutations were assessed. According to microsatellite instability status by polymerase chain reaction, patients were divided into 3 groups: microsatellite stable (MSS) = 108 patients, high level of microsatellite instability (MSI-H) = 15 patients and low level of microsatellite instability (MSI-L) = 6 patients. Different clinicopathological comparisons between MSS and MSI-H patients, and between MSS and MSI-L patients were performed.

Microsatellite instability was found in 16.3% patients: 11.6% had MSI-H and 4.7% had MSI-L. Significantly more patients in the MSI-H group than in the MSS group were female (*P* = .01) and had a family history of colon cancer (*P* < .001). MSI-H and MSI-L groups were associated with the ascending colon location of the tumors, were mostly type G3, T2, and stage I whereas MSS tumors were mostly G2, pT3, and stage III. Overall, *BRAF* mutations were identified in 18/129 patients (13.9%). BRAF mutant tumors were predominantly associated with MSI-H and MSI-L tumors. Immunohistochemistry had a sensitivity of 76% and a specificity of 89% in detecting MSI tumors and an accuracy of 87.6%.

The frequency of microsatellite instability in our study was 16.3%. MSI-H is a distinct molecular phenotype of colon cancer with particular features: female gender, family history of colorectal cancer, a predilection for the ascending colon, poorly differentiated, predominantly T2, and stage I. The frequency of *BRAF* mutations was 13.9% and mutations were more often present in the MSI tumors.

Abbreviations: BMI = body mass index, *BRAF* = v-raf murine sarcoma viral oncogene homolog B1, CRC = colorectal cancer, dMMR = deficient mismatch repair, FFPE tissue = formalin-fixed paraffin-embedded tissue, IHC = immunohistochemistry, MMRP = mismatch repair proteins, MSI = microsatellite instability, MSI-H = high level of microsatellite instability, MSI-L = low level of microsatellite instability, MSS = microsatellite stable, PCR = polymerase chain reaction, PCR = polymerase chain reaction.

Keywords: *BRAF* gene, colon cancer, microsatellite instability, mismatch repair proteins

1. Introduction

Colorectal cancer (CRC) is the third most common cancer and the second cause of cancer deaths worldwide.[1] CRC is a heterogeneous disease with multiple genetic and epigenetic alterations and different molecular features. Molecular profiling of CRC has the advantage of providing essential information in the pathogenesis of cancer and, also, information about prognosis and therapy.

The molecular classification of CRC is based on 2 major distinct pathways: chromosomal instability or microsatellite stable (MSS) pathway and the microsatellite instability (MSI) pathway; chromosomal instability/MSS accounts for 80% of all CRC and MSI for about 20%.[2] MSI occurs by 2 different mechanisms in sporadic and hereditary CRC: in sporadic cancer, the cause is a hypermethylation of the MLH1 promoter and sometimes sporadic mutations, and in Lynch Syndrome, the cause is a mutation in 1 of the 4 DNA mismatch repair proteins (MMRP).[3]

The DNA mismatch repair system has the role to identify and correct DNA defects; errors in the mechanisms of this system lead to MSI status which is defined by deficient mismatch repair (dMMR) during DNA replication.[3,4] The proteins involved in the mismatch repair system are MLH1, MSH2, MSH6, PMS2; usually, these proteins form a complex represented by a tetramer composed of 2 heterodimers: MLH1/PMS2 and MSH2/MSH6.[5] Their expression is interdependent: if 1 protein is absent, the partner protein is degraded, and the consequence is a dMMR, finally resulting in MSI.[6]

At present, there are 2 methods to detect the status of MSI: by fluorescent based polymerase chain reaction (PCR) assay followed by capillary electrophoresis fragment size analysis and by immunohistochemistry (IHC); IHC detects dMMR and PCR detects MSI. Based on guidelines,[7] a standard set of 5 microsatellites sequences are tested and, according to the number of markers which show instability, tumors are classified as MSI-H (when 2 or more microsatellites are instable), MSI-L (when only 1 microsatellite is instable) and MSS if markers show no expression.[6] The IHC method is based on the detection of expression of MMRP (MLH1, MSH2, MSH6, PMS2) in the tumor cells. In tumors with dMMR, MMRP show a loss of nuclear expression.[8] The majority of the dMMR tumors are characterized by loss of expression of both MMR proteins in a heterodimer (MLH1/PMS2 or MSH2/MSH6), but unusual IHC patterns can also occur, especially in the setting of Lynch syndrome.[9] Fluorescent PCR based assay is considered the gold standard for the detection of MSI in CRC.[10] Several studies showed that the detection of MSI by IHC is similar to the fluorescent PCR based method.[11,12] Although these 2 methods are complementary, they provide different information. There is a trend that all CRC, regardless of age, should be tested for MSI using either IHC, PCR, or both for better results.[6]

Molecular classification provides important information regarding treatment and prognosis. Potential roles of testing MSI in CRC could be: a screening tool for hereditary nonpolyposis colorectal cancer, prediction for chemotherapy response, and, also, a prognostic biomarker.[13] Genetics has an important role in individual risk of developing CRC, but modifiable risk factors (such as diet, lifestyle, obesity, alcohol) also contribute significantly by increasing the individual risk. Advanced research showed that the broad spectrum of genetic, epigenetic, and molecular alterations in CRC is likely to be more extensive than previously reported, thus investigating the underlying genetic phenotype would provide quality data for basic research in the etiopathogeny, prognosis, personalized treatment or even response to treatment, given the opportunity to health workers to apply the concept of "personalized medicine."

Although the tumor stage has the most important role in the prognosis of CRC, the molecular phenotype is also associated with different outcomes. Beside MSI status, BRAF (v-raf murine sarcoma viral oncogene homolog B1) gene mutations mainly through mutations at codon 600 (V600E) showed different associations with survival.[14,15] MSS tumors with BRAF mutations have a negative prognostic with poor survival rates,[16,17] but little is known about MSI-H tumors and associated BRAF mutations.

The present study aimed to assess the frequency of MSI by the fluorescent PCR based assay in colon cancer and the clinico-pathological characteristics of the MSI-H tumors in our region. The secondary outcome was to assess the frequency of BRAF mutations in colon cancer according to MSI status.

2. Methods

The present study included all patients diagnosed with CRC and prospectively admitted for elective surgery to the Surgery Department of Constanta County Clinical Emergency Hospital between January 01, 2019 and December 31, 2019. Inclusion criteria: patients with previously histopathological diagnosed colon cancer at colonoscopy with biopsies and scheduled for surgery (stage 0–III), colon location of the tumor (from sigmoid to the cecum), more than 16 years old and fit for surgery, informed written consent. Exclusion criteria: rectal cancers, stage IV tumors, nonelective surgery for colon cancer, unfit for surgery (severe comorbidities which contraindicate surgery), absence of the consent for surgery or for the inclusion in the study, age less than 16 years old.

Out of 163 patients prospectively admitted to the Surgery Department, only 129 patients met the inclusion and exclusion criteria and were enrolled in the study.

Demographic data, clinical data, pathological data, IHC staining pattern, MSI status by fluorescent PCR based assay, and BRAF mutations status were assessed in all patients.

Demographic and clinical data included age, gender, body mass index (BMI) (<18.5 = underweight, $18.5–24.9$ = normal weight, $25–29.9$ = overweight, ≥ 30 = obese),[18] alcohol consumption status (according to National Institute on Alcohol Abuse and Alcoholism,[19] moderate alcohol consumption is up to 1 drink per day for women and up to 2 drinks per day for men, binge drinking is defined as ≥ 5 drinks (male) and ≥ 4 drinks (female) in about 2 hours, and heavy alcohol use is defined as ≥ 4 drinks on any day for men and ≥ 3 drinks for women), smoking status (according to Center for Disease Control and Prevention,[20] patients were divided into current smoker, former smoker, or never smoker), and family history of CRC (we included patients with available data about 1st-degree relatives with a positive diagnosis of CRC). Patients who met the definitions for moderate and heavy alcohol use were categorized as drinkers and patients who were current and former smokers were categorized as smokers. (We included patients with available data about 1st degree relatives with a positive diagnosis of CRC; other details about relatives were not requested).

Pathological data included details about tumor location, histologic grading (G1 = well-differentiated, G2 = moderate dif-

ferentiated, G3 = poor differentiated), lymphovascular and perineural invasion (present/absent), pTNM classification and tumor stage, IHC staining pattern for MMPR (4 MMPR were investigated: MLH1, MSH2, MSH6, PMS2), MSI status by PCR and BRAF mutations status.

2.1. Mismatch repair proteins immunohistochemistry

Immunohistochemistry for the 4 most common MMRP was performed in all cases using the standard procedure recommended by Vitro, Master Diagnostica. Tumor representative blocks were selected for analysis with normal junction to assess staining results properly. Primary monoclonal antibodies against MLH1 (clone BS29, ready to use, Vitro SA, Master Diagnostica, Spain), MSH2 (clone FE11, ready to use, Vitro SA, Master Diagnostica), MSH6 (clone EP49, ready to use, Vitro SA, Master Diagnostica) and PMS2 (clone BS29, ready to use, Vitro SA, Master Diagnostica) were applied on 4 μm deparaffinized, rehydrate and heat-induced epitope retrieval sections. The reaction was visualized with Master Polymer Plus Detection System (DAB included) and slides were counterstained with hematoxylin.

Non-neoplastic colonic mucosa and appendix were used as internal positive controls. The known MMRP deficient colorectal carcinomas served as external negative controls. Two experienced pathologists evaluated the staining results independently and blindly to the MSI status. Positive expression was defined as nuclear staining within tumor cells, while negative protein expression was defined as a complete absence of nuclear staining within tumor cells with concurrent internal positive controls. If internal non-neoplastic tissues showed invalid negative staining, the procedure was routinely repeated.

2.2. DNA Isolation

Genomic DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. For DNA isolation, tissue areas up to 250 mm², and up to 8 sections with a maximum thickness of 10 μm were used for each case. Hematoxylin and eosin stained sections were used as a reference and the largest tumor area (at least 50% tumor cells) was scraped off with a scalpel under a dissecting microscope.

2.3. BRAF mutation analysis

For identification of *BRAF* mutations we used a method based on PCR and reverse hybridization (Strip Assay, Vienna Lab Diagnostics GmbH, Austria) following the manufacturer's instructions. The assay covers 9 mutations in the BRAF gene (codon 600 and 601: V600A, V600D, V600E, V600G, V600K, V600M, V600R, K601E). Procedure includes a PCR amplification using biotinylated primers hybridization of amplification products to a test strip containing allele-specific oligonucleotide probes immobilized as an array of parallel lines. Bound biotinylated sequences are detected using streptavidin-alkaline phosphatase and color substrates.

2.4. MSI status by fluorescent PCR based analysis

The MSI status was evaluated using fluorescently-labeled microsatellite PCR primers, followed by separation of the amplicons by capillary electrophoresis using a 3500 Genetic Analyzer (Applied Biosystems, Fitchburg, WI), and analysis of

data was performed using GeneMapper software, version 5 (Applied Biosystems). Typically, MSI analysis involves comparing allelic profiles of microsatellite markers generated by amplification of DNA from matching normal and test sample, which may be mismatch-repair (MMR) deficient. Alleles that are present in the test sample but not in corresponding normal samples indicate MSI. The MSI assay (MSI Analysis System version 1.2 kit, Promega Corporation) determined 5 quasimonomorphic mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24, and MONO-27) and 2 pentanucleotide markers (Penta C and Penta D). Each PCR amplification assay consists of 2 ng of genomic DNA and 8 μL master mix (5.85 μL Nuclease-Free Water, 1 μL Gold STAR 10X Buffer, 1 μL MSI 10X Primer Pair Mix and 0.15 μl AmpliTaq Gold DNA polymerase). Samples were incubated in a thermocycler with the following parameters: 95°C for 11 minutes, 96°C for 1 minute, then 10 cycles (94°C for 30 seconds, 58°C for 30 seconds, 70°C for 1 minute), followed with 20 cycles (90°C for 30 seconds, 50°C for 30 seconds, 70°C for 1 minute) and final extension for 30 minutes at 60°C. In the capillary electrophoresis analysis, 1 μL of the amplified product was combined and mixed with a loading cocktail (0.5 μL × samples + 9.5 Hi-Di Formamide × sample) and loaded in a PCR thermal cycler (3 minutes at 95°C and cooled at 4°C) before load in the ABI 3500 sequencer.

MSI was defined as a marked alteration in repeat length or as a discrete band below or above the expected allele. Analysis was performed by 2 experienced geneticists who evaluated the results independently and blindly to the IHC results. Following National Cancer Institute guidelines,[10] MSI at more than 2 loci was defined as a high level of microsatellite instability (MSI-H), MSI at a single locus was defined as low level of microsatellite instability (MSI-L), and absence of instability at any of the loci was defined as microsatellite stable group (MSS).

According to MSI status by PCR, patients were divided into 3 groups: MSS = 108 patients, MSI-H = 15 patients and MSI-L = 6 patients. Different comparisons (demographic, clinical, pathological, IHC staining patterns, BRAF mutations status) between MSS and MSI-H patients, and between MSS and MSI-L patients were performed. All analyses were performed with the MSS group as the reference.

2.5. Statistical tests

Statistical analysis was performed using the JASP 0.11.1 statistic software package. Descriptive statistics were used for demographic, clinical, and pathological data: mean+/− standard deviation for continuous variables in the MSI-H group, and median+/− interquartile range in the MSI-H and MSI-L group; frequency was used for categorical variables. Comparisons were performed with the MSS group as the reference. For comparison between variables, the Mann–Whitney U-test was used for continuous variables, and the chi-square test or Fisher exact test (when cell count was 0) were used for categorical variables. Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of IHC for detecting MSI tumors (having PCR method as gold standard for MSI status) were calculated. Results were considered statistically significant if P-value < .05.

2.6. Ethical approval

The study was conducted according to good laboratory practice and in accordance with the Declaration of Helsinki and national

and institutional standards. Informed consent was obtained from all patients, and the study was approved by the Local Ethics Commission for the Approval of Clinical and Research Developmental Studies (approval no. 16/2018).

3. Results

Microsatellite instability was found in 21 (16.3%) patients: 15 (11.6%) had MSI-H and 6 (4.7%) had MSI-L. Most of the patients, 108 (83.7%) were MSS.

Demographic and clinical data of the patients according to the MSI status are illustrated in Table 1. There were no significant differences between groups regarding age, alcohol consumption status, and smoking status. Significantly more patients in the MSI-H group than in the MSS group were female. Patients in the MSS group had significantly higher mean BMI and subanalysis between categories of BMI showed that most of the patients in the MSI-H group and MSI-L were normal weighted in contrast with patients in the MSS group which were mostly overweighted. Diabetes and hypertension were more often encountered among patients with MSS tumors. Significant more patients from the MSI-H group than from the MSS group had a family history of colon cancer: 53.3% versus 12%, $P < .001$.

The pathological data of the tumors according to the MSI status is illustrated in Table 2.

Table 1

Demographic and clinical data of the patients according to the MSI status.

Variables	MSS (n = 108)	MSI-H (n = 15)	MSI-L (n = 6)
Age (yr)	66.1 +/−12.2	67 (57–61)	65.5 (58–68)
Mean +/− SD or median (IQR)	Ref	.81	.75
Gender, n (%)			
Female	50 (46.3%)	12 (80%)	3 (50%)
Male	58 (53.7%)	3 (20%)	3 (50%)
P-value	Ref	.01	.85
BMI			
Mean +/− SD or median (IQR)	26.4 +/− 4.29	19.7 (18.5–22.7)	20.1 (18.7–22.5)
	Ref	.02	.03
Underweight, n (%)	4 (3.7%)	1 (6.6%)	0 (0.0%)
P-value	Ref	.58	>.99
Normal weight, n (%)	20 (18.5%)	7 (46.7%)	4 (66.6%)
P-value	Ref	.01	<.001
Overweight, n (%)	58 (53.8%)	4 (26.6%)	2 (33.3%)
P-value	Ref	.04	.33
Obese, n (%)	26 (24%)	3 (20.1%)	0 (0.0%)
P-value	Ref	.71	.33
Diabetes, n (%)	65 (60.1%)	4 (26.6%)	2 (33.3%)
P-value	Ref	.01	.19
Hypertension, n (%)	60 (55%)	3 (20%)	3 (50%)
P-value	Ref	<.001	.78
Alcohol, n (%)	49 (45.3%)	6 (40%)	5 (83.3%)
P-value	Ref	.69	.06
Smoking, n (%)	72 (66.6%)	8 (53.3%)	4 (66.6%)
P-value	Ref	.31	>.99
Family history of colorectal cancer, n (%)	13 (12%)	8 (53.3%)	0 (0.0%)
P-value	Ref	<.001	>.99

Median and interquartile range (IQR) was used to assess age and BMI in the MSI-H and MSI-L groups. BMI = body mass index, MSI-H = high level of microsatellite instability, MSI-L = low level of microsatellite instability, MSS = microsatellite stable.

Table 2

Pathological data of the tumors according to the MSI status.

Variables	MSS (n = 108)	MSI-H (n = 15)	MSI-L (n = 6)
Location, n (%)			
P-value			
Sigmoid colon	34 (31.4%)	2 (13.3%)	0
	Ref	.14	.17
Descending colon	24 (22.2%)	2 (13.3%)	1 (16.6%)
	Ref	.42	.74
Transverse colon	8 (7.4%)	1 (6.7%)	0
	Ref	.91	>.99
Ascending colon	28 (26%)	8 (53.4%)	4 (66.6%)
	Ref	.02	.03
Cecum	14 (13%)	2 (13.3%)	1 (16.6%)
	Ref	.96	.79
Histologic grading, n (%)			
p-value			
G1	12 (11.1%)	1 (6.7%)	2 (33.3%)
	Ref	.59	.79
G2	74 (68.5%)	5 (33.3%)	2 (33.3%)
	Ref	<.001	.07
G3	22 (20.4%)	9 (60%)	2 (33.3%)
	Ref	<.001	.44
Lymphovascular invasion, n (%)	95 (87.9%)	5 (33.3%)	5 (83.4%)
P-value	Ref	<.001	.73
Perineural invasion, n (%)	77 (71.3%)	5 (33.3%)	4 (66.6%)
P-value	Ref	<.001	.80
pT, n (%)			
P-value			
T1	2 (1.8%)	0 (0.0%)	1 (16.6%)
	Ref	>.99	.1
T2	35 (32.4%)	11 (73.3%)	5 (83.4%)
	Ref	<.001	.01
T3	58 (53.7%)	3 (20.0%)	0 (0.0%)
	Ref	.01	.01
T4	13 (12.1%)	1 (6.7%)	0 (0.0%)
	Ref	.1	>.99
pN, n (%)			
P-value			
N0	67 (62.0%)	8 (53.3%)	4 (66.6%)
	Ref	.51	.81
N1	30 (27.7%)	4 (26.7%)	1 (16.6%)
	Ref	.92	.55
N2	11 (10.3%)	3 (20.0%)	0
	Ref	.26	>.99
Stage, n (%)			
P-value			
I	16 (14.8%)	10 (66.6%)	4 (66.6%)
	Ref	<.001	<.001
II	41 (38%)	4 (26.7%)	2 (33.3%)
	Ref	.39	.81
III	51 (47.2%)	1 (6.7%)	0
	Ref	<.001	.03

MSI-H = high level of microsatellite instability, MSI-L = low level of microsatellite instability, MSS = microsatellite stable, pN = lymph node metastasis, pT = depth of tumor invasion.

Location: MSI-H and MSI-L groups were associated with the ascending colon location of the tumors. There were no significant differences between groups regarding other locations of the tumors.

Histologic grading: MSI-H tumors were mostly type G3 whereas MSS tumors were mostly G2. Regarding MSI-L tumors, there were no significant differences.

Lymphovascular invasion and perineural invasion: Lympho-vascular invasion was more common in MSS tumors than in MSI-

H tumors. Similarly, MSS tumors were associated with perineural invasion. MSI-L tumors analysis did not show significant differences in contrast MSI-H tumors regarding lymphovascular and perineural invasion.

TNM classification: MSI-H and MSI-L tumors were predominantly T2 whereas MSS tumors were predominantly T3. There were no tumors classified as Tis. Analysis of lymph nodes involvement (pN) showed no significant differences between groups. All patients included in the study were without distant metastases (M0).

Stage: MSI-H tumors and MSI-L tumors were predominantly stage I and MSS tumors were predominantly stage III. There were no patients with stage 0 tumors.

Of all the 15 patients with MSI-H tumors, only 2 patients had a medical history of cancer: 1 patient had endometrial cancer, and 1 had urinary bladder cancer.

3.1. Frequency of BRAF mutations according to the MSI status

Overall, *BRAF* mutations were identified in 18/129 patients (13.9%). Out of the 9 mutations investigated (codon 600 and 601: V600A, V600D, V600E, V600G, V600K, V600M, V600R, K601E), only V600E was present. BRAF status of the tumors according to the MSI status is illustrated in Table 3. BRAF mutant tumors were significantly associated with MSI-H and MSI-L tumors in contrast with MSS tumors.

Demographic and clinical subanalysis of the MSI – *BRAF* mutant tumors (including both MSI-H and MSI-L) in contrast with MSI *BRAF* non-mutant tumors (illustrated in Table 4) showed an association of *BRAF* mutant status with female gender ($P=.01$) and family history of colon cancer ($P<.001$).

IHC staining data pattern is illustrated in Table 5. According to the IHC staining, we observed 11 (10.2%) patients in the MSS group that had 1 MMPR negative (false MSI tumors). Also, we observed 1 patient from MSI-H who had all MMPR positive (false MSS tumor). Most of the patients from the MSS group had all MMPR positive, and most of the patients from MSI-H had 2 MMPR negative. Regarding MSI-L tumors, 66.7% had all MMPR positive and 33.3% had 1 MMPR negative. The pattern with 3 or all 4 MMPR negative was not encountered in any of the 3 groups. Statistical analysis showed that IHC had a sensitivity of 0.76 (95% confidence interval [CI] 0.52–0.91) and a specificity of 0.89 (95% CI 0.82–0.94) in detecting MSI tumors with a positive predictive value of 0.59 (95% CI 0.44–0.72), a negative predictive value of 0.95 (95% CI 0.90–0.97), a positive likelihood ratio of 7.48 (95% CI 4.07–13.75), a negative likelihood ratio of 0.27 (95% CI 0.12–0.57), and an overall accuracy of 0.87 (95% CI 0.80–0.92).

Table 4

Demographic and clinical data of the MSI patients according to the *BRAF* mutations status.

Variables	MSI *BRAF* mutant (n = 15)	MSI *BRAF* non-mutant (n = 6)	P-value
Age (yr)			
Median (IQR)	60 (56–69)	62 (60–67)	.67
Gender, n			
Female	13	2	.01
Male	2	4	
BMI			
Median (IQR)	19 (18.3–22.3)	20.3 (19.5–21.8)	.56
Diabetes, n	1	1	.17
Hypertension, n	2	1	.39
Alcohol, n	4	2	.17
Smoking, n	6	1	.82
Family history of colorectal cancer, n	8	0	<.001

Percentages in the MSI *BRAF* non-mutant group were not calculated due to the small number of patients.
MSI-H = high level of microsatellite instability, MSI-L = low level of microsatellite instability, MSS = microsatellite stable.

4. Discussion

The molecular classification of CRC is closely associated with clinicopathological features of the tumors, prognosis, treatment strategy and response to treatment, both in hereditary CRC and sporadic CRC, and detecting MSI is of paramount importance.[21–23]

In our study, microsatellite instability was found in 16.3% patients: 11.6% had MSI-H and 4.7% had MSI-L. Most of the patients (108) were MSS. Our results are in concordance with previous studies: Salovaara et al[24] found MSI in 12% of the 535 investigated CRCs, similar results being reported also by other authors.[25,26] One of the largest pooled analyses of more than 7600 CRC reported MSI in 16.7% of the cases.[21]

As reported previously, MSI-H tumors are known for having some distinctive features: early age of onset, proximal location, higher frequency of family history of CRC, mucinous type and poorly differentiated phenotype, and lymphocytic infiltration.[27,28] In the present study, we identified significant differences between the clinical characteristics of MSS and MSI-H tumors. MSI-H tumors were predominantly associated with female gender ($P=.14$), 53.3% had a family history of CRC ($P<.01$) and had a median BMI of 19.7 ($P=.02$), while MSS tumors were associated with male gender, 12% had a family history of CRC and the mean BMI was higher (26.4). Obesity is an established risk factor for CRC.[29] Regarding categories of

Table 3

BRAF status of the tumors according to the MSI status.

BRAF STATUS	MSS (n = 108)	MSI-H (n = 15)	MSI-L (n = 6)
Mutant, n (%)	3 (2.8%)	11 (73.3%)	4 (66.6%)
Non-mutant, n (%)	105 (97.2%)	4 (26.6%)	2 (33.3%)
p-value	Ref	<.001	<.001

BRAF = v-raf murine sarcoma viral oncogene homolog B1, MSI-H = high level of microsatellite instability, MSI-L = low level of microsatellite instability, MSS = microsatellite stable.

Table 5

IHC staining data pattern according to the MSI status.

IHC staining pattern, n (%)	MSS (n = 108)	MSI-H (n = 15)	MSI-L (n = 6)
One MMRP —	11 (10.2%)	2 (13.4%)	2 (33.3%)
Two MMRP —	0	12 (80%)	0
Three MMRP —	0	0	0
All 4 MMRP —	0	0	0
All MMRP +	97 (89.8%)	1 (6.6%)	4 (66.7%)

"—" = negative, "+" = positive, IHC = immunohistochemistry, MMRP = mismatch repair protein, MSI-H = high level of microsatellite instability, MSI-L = low level of microsatellite instability, MSS = microsatellite stable.

BMI, there were differences between normal weighted and overweighted patients within the 2 groups: most of the patients in the MSI-H group (46.7%) were normal weighted and only 26.6% were overweighted, in contrast with patients from the MSS group where only 18.5% were normal weighted and the majority (53.8%) were overweighted; obesity was present in both groups, irrespective of the MSI status: 24% of the patients with MSS tumors and 20.1% of the patients with MSI-H were obese. Similar to our results, a large case-control study on obesity,[30] CRC risk, and MSI status, concluded that higher BMI was associated with MSS phenotype. Nakayama et al[31] found also that diabetes is more common in MSS tumors than in MSI-H tumors. Unfortunately, there are no other studies on this topic and MSI status in Romania to compare the results.

Alcohol consumption and smoking are other well-established risk factors for CRC.[29] In our study, these risk factors were present in more than half of the patients in each group irrespective of the MSI status. We also investigated the association between MSI status with diabetes and hypertension and we found that patients with MSS tumors are more likely associated with these 2 diseases than MSI-H patients.

In contrast with other studies[27,28] that showed that MSI-H tumors are associated with early age of CRC onset, the median age of the patients from the MSI-H group in our study was 67 years and was similar to the mean age of the patients from MSS group or MSI-L group. This could be a particularity of the patients with MSI-H phenotype in our country, but because of the small number of patients in this group, larger studies are needed to confirm this characteristic.

As reported previously,[27,28] MSI-H cancers are usually located in the proximal colon and are poorly differentiated. In our study, 53.4% of the patients from the MSI-H group were located in the ascending colon in contrast with 26% of the patients from the MSS group (P=.28). Our results also indicated that tumors with MSI-H phenotype are characterized by poorer differentiation as 60% of them were classified as G3 type. Lymphovascular and perineural invasion were mostly reported in tumors from MSS group (87.9%, 71.3%, respectively) in contrast with tumors from MSI-H (33%, 33%, respectively), suggesting a more extensive disease in patients from MSS group. Regarding the depth of tumor invasion (pT), the majority of the tumors from MSI-H group (73.3%) were limited to muscularis propria (T2), suggesting a less invasive pattern of the tumors in contrast with the MSS tumors which were predominantly invading the subserosa (53.7%). Similar results were reported by Jung et al.[32] In our study, patients from the MSI-H group were predominantly classified as having stage I tumors (66.6%) in contrast with patients from the MSS group who were predominantly stage III (47.2%). This result suggests that patients with MSI-H phenotype have a less advanced disease in contrast with patients with MSS phenotype and can possibly explain the better prognosis of MSI cancers compared with MSS ones. Other studies who investigated the prognostic role of MSI status showed that MSI-H phenotype was an independent prognostic factor, along with stage, tumor grade differentiation, and histology type of the tumor.[32–34]

The distinction between MSI-H phenotype and MSS phenotype was proved in several studies and now it is universally accepted in the literature that MSI-H tumors have a distinct clinicopathologic phenotype and different prognostic and our results are in concordance with literature.[27,28,32–34] In contrast, the MSI-L phenotype was not associated with distinctive features

in literature, and usually this type of tumors have similar features to MSS tumors.[33–35] Despite this evidence, some authors tried to demonstrate that MSI-L is a distinct sub-group of sporadic CRC with specific molecular features.[36–38] Interestingly, in our study, some characteristics of the patients with MSI-L phenotype were different from MSS phenotype: 66.6% had tumors located in the ascending colon (P=.03), the majority of the patients (83.4%) had a depth of tumor invasion T2 while the majority of the MSS patients had T3. Also, most of the patients with MSI-L phenotype were stage I (66.6%), followed by stage II (33.3%), findings in contrast with features of MSS patients who were predominantly stage III (47.2%). Unfortunately, due to the small number of patients in the MSI-L group, these findings could not be assessed properly and larger studies are needed to confirm these characteristics.

About 10% of the CRC have *BRAF* mutations.[39] The prognostic role of *BRAF* mutations in CRC has been largely investigated and it was demonstrated that mutant tumors are associated with significantly poorer prognosis[15,40] Numerous studies[16,17,41] have shown that patients with MSS phenotype and *BRAF* mutations have a worse prognosis than MSI-H tumors with mutations. In line with the results of previous studies,[39,41] in our study, the frequency of *BRAF* mutations was 13.9%, and our analysis by MSI status showed that *BRAF* mutations were more frequent in the MSI-H group than in the MSS group (73.3% vs 4.6%, P<.01). Results from an analysis of a Romanian CRC cohort[42] showed that *BRAF* mutations were detected in 16% of the cases. Contrary to the similarities in age, BMI, presence of diabetes or hypertension in the subanalysis of the MSI patients with *BRAF* mutations vs. without mutations, female gender and family history of CRC were found to differ significantly between the 2 groups, *BRAF* mutations being predominantly associated with female gender (P=.04) and family history of CRC (<.001). Studies showed that *BRAF*-mutated tumors are often right-sided, more prevalent in women and associated with MSI.[43] Strikingly, among patients with MSS phenotype, we found 5 (4.6%) patients who had *BRAF* mutations, and 4 patients with MSI-L phenotype who had also mutations. According to literature, *BRAF* mutations in MSI-H phenotype has no prognostic effect, but *BRAF*-mutated CRC with MSS phenotype is a distinct molecular phenotype which place patient at risk for poor treatment response and worse prognosis.[43,44]

Regarding the 2 methods for MSI analysis, it has been reported that the IHC method for detection of MSI status has similar results with the PCR method.[11,12] The results obtained from IHC or PCR studies are complementary, but provide different information. The PCR method cannot detect which mismatch repair protein is deficient while IHC provides specific data regarding which protein in the mismatch repair tetramer is deficient.[6] Taking this into account, the PCR method cannot distinguish between sporadic or Lynch syndrome-associated MSI cancer. It is now generally recommended that all CRC patients should be tested for MSI either by PCR, IHC, or both.[8] The decision on which test to use is institution-dependent, but MSI analysis based on PCR remains the gold standard for detecting microsatellite status.[7,10] Using IHC method, tumors displaying loss of 1 or more MMRPs can be classified as deficient MMR and are considered to be MSI-H, whereas those with intact MMRPs can be classified as proficient MMR and are considered to be MSS or MSI-L.[45] Usually, MSI cancers are characterized by 2 negative MMPRs in a heterodimer: in sporadic MSI cancers, loss of MLH1-PMS2 is characteristic, whereas in Lynch syndrome

either heterodimer may be lost, but unusual IHC patterns are reported such as isolated loss of 1 of the 4 MMRPs.[7,8] When all 4 MMPRs are intact, tumors are assumed to be MSS.[7] Lynch syndrome is present in 1% to 2% of the MSI tumors, it is characterized by loss of expression of 1 MMPR, and, in contrast with the sporadic cancers, it is not characterized by BRAF mutations (Lynch tumors have a wild-type BRAF gene).[7,9] In our study, according to the IHC staining pattern only, 97 (89.8%) patients were MSS and 11 (10.2%) patients showed loss of expression of 1 MMPR; taking into account only the IHC data, these 11 patients could be classified as MSI, but PCR showed that these patients are MSS. It is also worth noting that there was 1 patient classified as MSI-H by PCR while all the 4 MMPRs were positively expressed, meaning that this case could be classified as MSS or MSI-L by IHC. This study identified 11 patients that were MSS yet had negative MMPRS by IHC; furthermore, the current study identified 1 patient that was MSI-H despite IHC staining. False-negative rates for IHC were previously reported in the literature.[34,46,47] Despite the close correlation between the 2 methods of detection, our findings among other studies suggest that IHC cannot substitute PCR. Therefore, we believe that cases with all 4 MMPR positive by IHC may be classified as MSS or MSI-L (given the data supporting that MSI-L tumors are similar to MSS tumors and that these 2 phenotypes can be grouped), but when any of the MMPR is deficient, further examination by PCR is necessary to clearly determine the MSI status.

To our knowledge, the current study is one of the few in our country which assessed the MSI status of the CRC by PCR method (additionally to IHC method) and investigated the BRAF mutations in all patients.[48] Molecular analyses on CRC were also performed in our region, but they were based on the investigation of microRNAs.[49]

A possible limitation of the study could be the small number of patients in the MSI-H group and MSI-L group (data in the literature are few and limited on these categories of patients) and this topic should be studied in larger analyses to assess more precisely the particular features of these patients and obtain more conclusive results.

MSI tests may be used for diagnosis of suspected Lynch syndrome and also to identify clinical and therapeutical implications of MSI-H phenotype in sporadic CRC. There is extensive data which indicates that molecular testing and analysis should be incorporated into our practice for better management of CRC.

In conclusion, the overall frequency of MSI in our study was 16.3%: 11.6% for MSI-H and 4.7% for MSI-L. MSI-H is a distinct molecular phenotype of colon cancer with particular features: female gender, normal BMI, family history of CRC, a predilection for the ascending colon, poorly differentiated, predominantly T2 and stage I. The frequency of BRAF mutations was 13.9%, mutations were more often present in MSI-H tumors and were associated with female gender and family history of CRC. PCR remains the gold standard for the detection of MSI status in contrast with the IHC method.

Author contributions

Conceptualization: Razvan Catalin Popescu, Eugen Dumitru.
Data curation: Razvan Catalin Popescu, Cristina Tocia, Andrei Dumitru, Nicoleta Leopa.
Formal analysis: Cristina Tocia, Costel Brînzan, Georgeta Camelia Cozaru, Mariana Deacu, Andrei Dumitru, Nicoleta Leopa, Anca Florentina Mitroi, Anca Nicolau.

Investigation: Razvan Catalin Popescu, Costel Brînzan, Georgeta Camelia Cozaru, Mariana Deacu, Andrei Dumitru, Nicoleta Leopa, Anca Florentina Mitroi, Anca Nicolau.
Methodology: Razvan Catalin Popescu, Cristina Tocia, Costel Brînzan, Georgeta Camelia Cozaru, Mariana Deacu, Anca Florentina Mitroi, Anca Nicolau, Eugen Dumitru.
Project administration: Eugen Dumitru.
Supervision: Eugen Dumitru.
Validation: Cristina Tocia.
Writing – original draft: Razvan Catalin Popescu, Cristina Tocia.
Writing – review & editing: Razvan Catalin Popescu, Eugen Dumitru.

References

[1] Available at: https://gco.iarc.fr/today/data/factsheets/cancers/10_8_9-Colorectum-fact-sheet.pdf [access date March 3, 2020].
[2] Al-Sohaily S, Biankin A, Leong R, et al. Molecular pathways in colorectal cancer. J Gastroenterol Hepatol 2012;27:1423–31.
[3] Vilar E, Gruber SB. Microsatellite instability in colorectal cancer-the stable evidence. Nat Rev Clin Oncol 2010;7:153–62.
[4] Markowitz SD, Bertagnolli MM. Molecular origins of cancer: molecular basis of colorectal cancer. N Engl J Med 2009;361:2449–60.
[5] Zaanan A, Meunier K, Sangar F, et al. Microsatellite instability in colorectal cancer: from molecular oncogenic mechanisms to clinical implications. Cellular Oncology (Dordrecht) 2011;34:155–76.
[6] Gibson J, Lacy J, Matloff E, et al. Microsatellite instability testing in colorectal carcinoma: a practical guide. Clin Gastroenterol Hepatol 2014;12:171–6.e1.
[7] Umar A, Boland CR, Terdiman JP, et al. Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst 2004;96:261–8.
[8] Zhang X, Li J. Era of universal testing of microsatellite instability in colorectal cancer. World J Gastrointest Oncol 2013;5:12–9.
[9] Ward RL, Hicks S, Hawkins NJ. Population-based molecular screening for Lynch syndrome: implications for personalized medicine. J Clin Oncol 2013;31:2554–3256.
[10] Boland CR, Thibodeau SN, Hamilton SR, et al. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res 1998;58:5248–57.
[11] Amira AT, Mouna T, Ahlem B, et al. Immunohistochemical expression pattern of MMR protein can specifically identify patients with colorectal cancer microsatellite instability. Tumour Biol 2014;35:6283–91.
[12] Shia J. Immunohistochemistry versus microsatellite instability testing for screening colorectal cancer patients at risk for hereditary nonpolyposis colorectal cancer syndrome. Part I. The utility of immunohistochemistry. J Mol Diagn 2008;10:293–300.
[13] Chapusot C, Martin L, Puig PL, et al. What is the best way to assess microsatellite instability status in colorectal cancer? Study on a population base of 462 colorectal cancers. Am J Surg Pathol 2004;28:1553–9.
[14] Ogino S, Shima K, Meyerhardt JA, et al. Predictive and prognostic roles of BRAF mutation in stage III colon cancer: results from intergroup trial CALGB 89803. Clin Cancer Res 2012;18:890–900.
[15] Roth AD, Tejpar S, Delorenzi M, et al. Prognostic role of KRAS and BRAF in stage II and III resected colon cancer: results of the translational study on the PETACC-3, EORTC 40993, SAKK 60-00 trial. J Clin Oncol 2010;28:466–74.
[16] Samowitz WS, Sweeney C, Herrick J, et al. Poor survival associated with the BRAF V600E mutation in microsatellite-stable colon cancers. Cancer Res 2005;65:6063–9.
[17] Ogino S, Nosho K, Kirkner GJ, et al. CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. Gut 2009;58:90–6.
[18] Available at: https://www.cdc.gov/healthyweight/assessing/bmi/adult_bmi/index.html [access date March 5, 2020].
[19] Available at: https://www.niaaa.nih.gov/alcohol-health/overview-alcohol-consumption/moderate-binge-drinking [access date March 5, 2020].
[20] Available at: https://www.cdc.gov/nchs/nhis/tobacco/tobacco_glossary.htm [access date March 5, 2020].

[21] Popat S, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. J Clin Oncol 2005;23:609–18.

[22] Ionov Y, Peinado MA, Malkhosyan S, et al. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. Nature 1993;363:558–61.

[23] Fallik D, Borrini F, Boige V, et al. Microsatellite instability is a predictive factor of the tumor response to irinotecan in patients with advanced colorectal cancer. Cancer Res 2003;63:5738–44.

[24] Salovaara R, Loukola A, Kristo P, et al. Population-based molecular detection of hereditary nonpolyposis colorectal cancer [published correction appears in J Clin Oncol 2000 Oct 1;18(19):3456]. J Clin Oncol 2000;18:2193–200.

[25] Lim SB, Jeong SY, Lee MR, et al. Prognostic significance of microsatellite instability in sporadic colorectal cancer. Int J Colorectal Dis 2004;19:533–7.

[26] Benatti P, Gafà R, Barana D, et al. Microsatellite instability and colorectal cancer prognosis [published correction appears in Clin Cancer Res. 2006 Jun 15;12(12):3868-9]. Clin Cancer Res 2005;11:8332–40.

[27] Alexander J, Watanabe T, Wu TT, et al. Histopathological identification of colon cancer with microsatellite instability. Am J Pathol 2001;158:527–35.

[28] Raut CP, Pawlik TM, Rodriguez-Bigas MA. Clinicopathologic features in colorectal cancer patients with microsatellite instability. Mutat Res 2004;568:275–82.

[29] Keum N, Giovannucci E. Global burden of colorectal cancer: emerging trends, risk factors and prevention strategies. Nat Rev Gastroenterol Hepatol 2019;16:713–32.

[30] Campbell PT, Jacobs ET, Ulrich CM, et al. Case-control study of overweight, obesity, and colorectal cancer risk, overall and by tumor microsatellite instability status. J Natl Cancer Inst 2010;102:391–400.

[31] Nakayama Y, Iijima T, Wakaume R, et al. Microsatellite instability is inversely associated with type 2 diabetes mellitus in colorectal cancer. PLoS One 2019;14:e0215513.

[32] Jung SB, Lee HI, Oh HK, et al. Clinico-pathologic parameters for prediction of microsatellite instability in colorectal cancer. Cancer Res Treat 2012;44:179–86.

[33] Gryfe R, Kim H, Hsieh ET, et al. Tumor microsatellite instability and clinical outcome in young patients with colorectal cancer. N Engl J Med 2000;342:69–77.

[34] Ward R, Meagher A, Tomlinson I, et al. Microsatellite instability and the clinicopathological features of sporadic colorectal cancer. Gut 2001;48:821–9.

[35] González-García I, Moreno V, Navarro M, et al. Standardized approach for microsatellite instability detection in colorectal carcinomas. J Natl Cancer Inst 2000;92:544–9.

[36] Laiho P, Launonen V, Lahermo P, et al. Low-level microsatellite instability in most colorectal carcinomas. Cancer Res 2002;62:1166–70.

[37] Kambara T, Matsubara N, Nakagawa H, et al. High frequency of low-level microsatellite instability in early colorectal cancer. Cancer Res 2001;61:7743–6.

[38] Whitehall VL, Walsh MD, Young J, et al. Methylation of O-6-methylguanine DNA methyltransferase characterizes a subset of colorectal cancer with low-level DNA microsatellite instability. Cancer Res 2001;61:827–30.

[39] Tejpar S, Bertagnolli M, Bosman F, et al. Prognostic and predictive biomarkers in resected colon cancer: current status and future perspectives for integrating genomics into biomarker discovery. Oncologist 2010;15:390–404.

[40] Yuan ZX, Wang XY, Qin QY, et al. The prognostic role of BRAF mutation in metastatic colorectal cancer receiving anti-EGFR monoclonal antibodies: a meta-analysis. PLoS One 2013;8:e65995.

[41] Jensen LH, Lindebjerg J, Byriel L, et al. Strategy in clinical practice for classification of unselected colorectal tumours based on mismatch repair deficiency. Colorectal Dis 2008;10:490–7.

[42] Brînzan C, Aşchie M, Grasa CN, et al. The mutation profiles of KRAS and BRAF genes in a Romanian colorectal cancer cohort. Rev Chim 2019;70:1346–50.

[43] Lochhead P, Kuchiba A, Imamura Y, et al. Microsatellite instability and BRAF mutation testing in colorectal cancer prognostication. J Natl Cancer Inst 2013;105:1151–6.

[44] Phipps AI, Limburg PJ, Baron JA, et al. Association between molecular subtypes of colorectal cancer and patient survival. Gastroenterology 2015;148:77–87.e2.

[45] Kawakami H, Zaanan A, Sinicrope FA. Microsatellite instability testing and its role in the management of colorectal cancer. Curr Treat Options Oncol 2015;16:30.

[46] Thibodeau SN, French AJ, Roche PC, et al. Altered expression of hMSH2 and hMLH1 in tumors with microsatellite instability and genetic alterations in mismatch repair genes. Cancer Res 1996;56:4836–40.

[47] Chen ML, Chen JY, Hu J, et al. Comparison of microsatellite status detection methods in colorectal carcinoma. Int J Clin Exp Pathol 2018;11:1431–8.

[48] Banias L, Jung I, Bara T, et al. Immunohistochemical-based molecular subtyping of colorectal carcinoma using maspin and markers of epithelial-mesenchymal transition. Oncol Lett 2020;19:1487–95.

[49] Brînzan C, Aşchie M, Matei E, et al. Molecular expression profiles of selected microRNAs in colorectal adenocarcinoma in patients from south-eastern part of Romania. Medicine 2019;98:e18122.