

Mutational status of *KRAS* and *MMR* genes in a series of colorectal carcinoma cases

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Abstract

Background: The *KRAS* gene mutation is the most common somatic change in colorectal carcinoma (CRC) and is predictive of resistance to anti-epidermal growth factor receptor (EGFR) therapy in the metastatic forms. Microsatellite instability (MSI), a mismatch repair (MMR) system defect, accounts for 15–20% of all CRCs, more frequent in early stages. CRCs with MSI present better prognosis, a distinct histopathological aspect and a different response to chemotherapy. Patients with both *KRAS* wild type and MSI have a reduced risk of dissemination and recurrence. **Materials and Methods:** Our study included formalin-fixed paraffin-embedded tissue samples from 40 patients with metastatic CRCs, aged between 40 and 71 years old, gender (males/females) ratio 2.33:1. The MMR proteins were analyzed using an indirect bistadial immunohistochemical (IHC) technique with monoclonal antibodies. *KRAS* mutations were detected by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis. **Results:** Of the 40 tumors analyzed, 40% presented *KRAS* mutations located in codon 12 or codon 13. IHC expression of MMR proteins revealed a microsatellite stable status in 35 cases, including 15 cases with mutated *KRAS*. MSI status was identified in five cases (four with *KRAS* wild type). All MSI tumors had a poorer histological differentiation and four cases revealed a mucinous phenotype. Eighty percent of the patients with MSI status were older women. **Conclusions:** Our study demonstrates a 20% frequency of mutated *KRAS* in MSI CRCs, the incidence of *KRAS* mutations being inversely correlated with MSI status in these tumors. MMR protein deficient CRCs tend to occur in older females, have a poorer differentiation and are frequently associated with *KRAS* wild type.

Keywords: colorectal carcinoma, *KRAS* gene, mismatch repair genes, microsatellite instability.

Introduction

Colorectal carcinoma (CRC) is a complex disease, a cluster of heterogeneous subtypes resulted from various combinations of genetic and epigenetic events [1]. It represents the third most common type of cancer worldwide and a leading cause of cancer death [2]. The incidence rates are higher in more developed countries, 737 000 cases per year, *versus* 624 000 cases per year in less developed ones, but mortality is higher in the latter, with 52% of total deaths, which indicates a poor survival [3].

In Romania, CRC registered an incidence of 17.74 cases/100 000 inhabitants in 2000, and was responsible for 19.05 deaths/100 000 inhabitants in 2002, while 8240 new cases have been diagnosed in 2006 [4].

CRC pathogenesis is generated by three major molecular pathways: the chromosomal instability (CIN) pathway, the CpG island methylator phenotype (CIMP) pathway and the microsatellite instability (MSI) pathway. They alter factors that regulate proliferation, differentiation, angiogenesis, apoptosis or DNA repair processes, generating activation of oncogenes and down regulation of tumor suppressor genes or mismatch repair genes [5–7].

The majority of CRCs arise from the CIN pathway (65–70%) or the classic adenoma–carcinoma sequence,

usually mediated by activating mutations of the *KRAS* proto-oncogene [8]. It is mostly associated with sporadic cancers and with a low percentage of hereditary forms (1%) [9].

CIMP phenotype is present in 20–30% of CRCs and is characterized by abnormal global promoter hypermethylation due to epigenetic causes. Is frequently associated with mutations of the *BRAF* proto-oncogene [10].

Microsatellite instability represents a hypermutable phenotype generated by the mismatch repair (MMR) system defects because of DNA mismatch repair genes mutations (especially *MLH1*, *MSH2*, *MSH6* and *PMS2*) and loss of MMR nuclear proteins expression [11].

High-level MSI (MSI-H) is found in about 15–20% of all CRCs, more frequent in early stages and especially in sporadic forms (12–15%), the rest being associated as a hallmark feature with the inherited autosomal dominant Lynch syndrome, the usual heritable cause of CRCs [12, 13]. The most common mechanism of sporadic CRCs associating MSI is the hypermethylation of *MLH1* gene promoter, often in the context of CIMP phenotype (80%) [14–16].

The MSI phenotype can cause accumulation of mutations in tumor suppressor genes, oncogenes and coding microsatellite sequences, but with a distinct gene

mutation profile, including frequent alterations of *BRAF* proto-oncogene; *KRAS* mutations are significantly less frequent [17–19].

CRCs with MSI status are characterized by better prognosis, a distinct histopathological aspect and a different response to chemotherapy, with lack of benefit from 5-fluorouracil (5-FU) adjuvant therapy demonstrated at least for patients in early stages [16, 20].

The Kirsten rat sarcoma viral oncogene homolog [*KRAS* – *Online Mendelian Inheritance in Man* (OMIM) #190070) mutation is the most common somatic mutation in CRC and is predictive of resistance to anti-epidermal growth factor receptor (EGFR) antibodies personalized therapy in the metastatic forms. *KRAS* is a proto-oncogene with cytogenetic location on the short arm of chromosome 12 (12p12.1), that encodes a guanosine triphosphate (GTP-ase) involved in cell division, differentiation and apoptosis [21, 22]. This guanosine diphosphate/guanosine triphosphate (GDP/GTP)-binding protein is part of the RAS/RAF/MEK/ERK pathway, which is activated by the EGFR [23]. Metastatic CRC patients with tumors presenting a mutant *KRAS* with constitutive activation (30–50%) are resistant to anti-EGFR therapy, with decreased progression free survival and overall survival, compared with patients with *KRAS* wild-type tumors [22, 24].

The aim of our study was to identify possible correlations between the mutational status of *KRAS* and *MMR* genes and other clinical and histopathological findings in a series of metastatic CRC cases, in order to develop a predictor using a combination of biomarkers and to identify distinct molecular tumor subtypes. Furthermore, our purpose was to detect potential particularities regarding our population, the investigation of MSI in Romanian patients with CRC being in early stages.

Materials and Methods

Our retrospective analysis included formalin-fixed paraffin-embedded tissue samples from 40 patients with metastatic CRC, aged between 40 and 71 years old, gender (males/females) ratio 2.33:1, with different metastatic localizations. The study material consisted specimens from the Archive of the “Victor Babeș” National Institute for Research and Development in Pathology and Biomedical Sciences, Bucharest, Romania, from the 2010–2013 period. All the analyzed cases were stage IV, metastatic, colorectal adenocarcinomas, in order to reflect the routinely *KRAS* mutation test for EGFR inhibitor therapy. In addition, we selected only the patients with investigated protein expression for all four principal components of the MMR system and with available clinical information. The exclusion criteria were the history of familial CRC and the presence of other synchronous or metachronous tumors specifics for the inherited syndromes with CRC.

Surgical specimens were analyzed regarding tumor location. Histological type and differentiation, mucinous character and the budding and deepness of parietal invasion were established by microscopic investigations. Paraffin-embedded surgical samples were used to determine *KRAS* gene status by molecular biology methods.

Tissue samples

Serial 5- μ m sections from all formalin-fixed, paraffin-embedded (FFPE) tissues were cut from each paraffin block and the histopathological diagnosis was performed on the first section stained with Hematoxylin–Eosin (HE). All cases were classified and graded according to *World Health Organization* (WHO) criteria [25].

In order to select the tumor area for DNA extraction, the sections were manually dissected, >80% of the test area being represented by tumoral tissue [26].

DNA extraction

Genomic DNA was isolated according to the manufacturer's protocols with QIAamp DNA Mini Kit (Qiagen, Biotrade, Austria). For deparaffinization, tumor sections were placed in three baths of xylene and three baths of ethanol; after that, the sections were put in lysis buffer with proteinase K (20 mg/mL), at 56°C. DNA was precipitated using 200- μ L ethanol 100% and was fixated on the QIAamp silica membrane by centrifugation. Two washes were performed and the purified DNA was eluted in 200- μ L buffer AE, preheated at 70°C. The DNA quality and quantity were determined spectrophotometrically, at 260 nm and 280 nm, using UV–VIS spectrophotometer ASP3700 (ACTGene, USA).

Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP)

KRAS gene mutations were detected by PCR–RFLP for codons 12 and 13. The primers were synthesized by Invitrogen, with sequences according to the published data [27].

Codon 12

After amplification, the PCR products of 135 base pairs were incubated overnight at 37°C and digested with the MvaI restriction enzyme (Fermentas). The wild-type fragment was cleaved in two fragments, with 106 and 29 base pairs. The mutations in codon 12 cancelled the restriction situs for MvaI and the mutant fragment remained uncut. The cases with mutation presented both alleles (wild type and mutated).

Codon 13

After amplification, the PCR products of 159 base pairs were incubated and digested overnight with the HaeIII restriction enzyme (Fermentas). The wild-type allele was cleaved by the restriction enzyme in three fragments of 85, 48 and 26 base pairs, while the mutant allele was cleaved only in two fragments of 85 and 74 base pairs.

The quality control of the method included two DNA samples with known *KRAS* gene mutational status for codons 12 and 13, a wild-type DNA as negative control (K-) and a mutant DNA as a positive control (K+); a no template control “nuclease-free” water (NFW) was used as PCR settings and amplification control, for monitoring potential contamination during the reaction.

Immunohistochemical reaction for MMR proteins

For immunohistochemistry (IHC), paraffin sections were deparaffinized, rehydrated and rinsed in phosphate-buffered saline (PBS), pH 7.4. Retrieval with cooking in specific buffer was raised in microwave oven (Whirlpool), at 800 W. The MMR proteins were analyzed using an indirect bistadial immunohistochemical technique performed with a polymer based automated detection system (Leica Biosystems, UK), according to the manufacturer's instructions. All specimens were counterstained with Mayer's Hematoxylin, examined, and photographed with a Leica ICC50HD microscope.

The cases were tested by IHC using primary mouse monoclonal antibodies against: MLH1 (clone ES05, product code MLH1-L-CE/NCL-L-MLH1), MSH2 (clone 25D12, product code MSH2-L-CE/NCL-L-MSH2), MSH6 (clone PU29, product code MSH6-L-CE/NCL-L-MSH6), and PMS2 (clone MOR46, product code PMS2-L-CE/NCL-L-PMS2), purchased from Novocastra Reagents, Leica Biosystems Newcastle Ltd., Newcastle upon Tyne, UK.

For internal positive control, the identical procedure was performed on adjuvant normal tissue from each sample, represented by epithelial cells at the base of normal colonic crypts or lymphocytes. MMR protein loss was defined as an absence of nuclear staining in tumor cells but positive nuclear staining in normal colonic epithelial cells/lymphocytes. The tumor was defined as MSI-H (or deficient MMR) when anyone of the MMR proteins was negatively expressed.

Statistical analysis

Statistical calculations were performed using Statistical Package for the Social Sciences (SPSS) software version 21.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical associations between the presence of CRC mutations and clinicopathological characteristics was assessed by Pearson's χ^2 (chi square)-test for categorical variables (gender, location, mucin production, differentiation, MMR status) and analysis of variance (ANOVA) test for continuous variables (age). A *p*-value lower than 0.05 was considered significant.

Results

All the 40 colorectal tumors analyzed had a histological diagnosis of adenocarcinoma. The location of the primary tumor was in nine cases in the right colon, in 21 cases in the left colon and in 10 cases in the rectum. The tumors presented different metastatic locations, including liver, pulmonary, ovarian, uterine, lymph nodes, mandibular, muscle, peritoneal and cutaneous metastases.

We identified conventional adenocarcinomas in 29 patients and mucus-secreting adenocarcinoma forms in 11 patients. Regarding the tumor grading, the majority of metastatic CRCs were moderately differentiated adenocarcinomas (G2) – 24 cases, 11 patients presented poorly differentiated forms (G3), and five cases were well differentiated (G1).

KRAS modifications were present in 40% of the cases, 16 patients having *KRAS* mutations located in exon 2, codons 12 and 13. Thirteen (32.5%) cases harbored an

activating mutation in codon 12 of the gene (Figure 1), while in three (7.5%) cases we identified a codon 13 mutation (Figure 2).

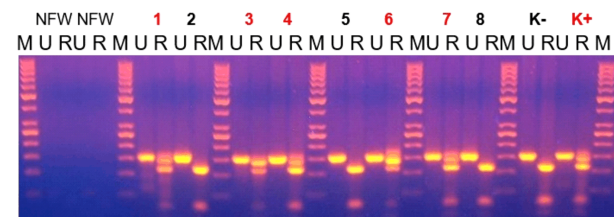


Figure 1 – Mutations in exon 2, codon 12 of the *KRAS* gene, detected by PCR–RFLP analysis (4% agarose gel electrophoresis of PCR products for *KRAS* codon 12, unrestricted and restricted fragments). NFW: “Nuclease-free” water (negative control of PCR); Samples 1, 3, 4, 6, 7: Heterozygous for codon 12 mutation (with both mutant and wild type alleles); Samples 2, 5, 8: Homozygous normal samples (with wild-type allele only); K-: Negative control; K+: Positive control; U: Unrestricted PCR amplicon; R: PCR products digested with *MvaI* restriction enzyme; M: GeneRuler 50 bp DNA Ladder (Fermentas); *KRAS*: Kirsten rat sarcoma viral oncogene homolog; PCR–RFLP: Polymerase chain reaction–restriction fragment length polymorphism.

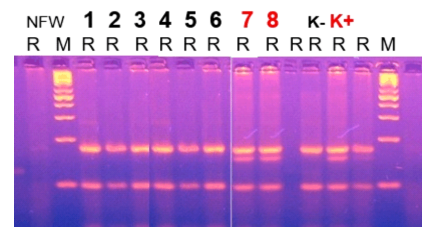


Figure 2 – Mutations in exon 2, codon 13 of the *KRAS* gene, detected by PCR–RFLP analysis (4% agarose gel electrophoresis of PCR products for *KRAS* codon 13, unrestricted and restricted fragments). NFW: “Nuclease-free” water (negative control of PCR); Samples 7, 8: Heterozygous for codon 13 mutation; Samples 1, 2, 3, 4, 5, 6: Homozygous samples without mutation; K-: Negative control; K+: Positive control; R: PCR products digested with *HaeIII* restriction enzyme; M: GeneRuler 50 bp DNA Ladder (Fermentas); *KRAS*: Kirsten rat sarcoma viral oncogene homolog; PCR–RFLP: Polymerase chain reaction–restriction fragment length polymorphism.

No correlations were found between *KRAS* mutant phenotype and the patients' gender and age, location of primary tumor, histological tumor grading or mucinous character. The presence of *KRAS* mutation was inversely associated with the presence of deficient MMR (Table 1).

Immunohistochemical expression of MMR proteins was positive for all four [microsatellite stable (MSS) status) in 35 cases, including 15 cases with mutated *KRAS* (Figure 3).

MSI status was identified in five (12.5%) patients. In all five cases, IHC reaction was negative for both MLH1 and PMS2 proteins and was positive only for MSH2 and MSH6 proteins (Figure 4).

In terms of gender and age, 80% of the patients with MSI status (four cases) were older women and only one case was a younger man of 40 years old. Regarding of primary tumor position, three cases with MSI were located in the right colon, signifying 1/3 of all tumors with this location considered in our study.

Of the five cases, four revealed a mucinous phenotype, representing 80% of MSI cases and more than 1/3 of all mucus-secreting tumors from our study group. All MSI tumors had a poorer histological differentiation, with two cases of moderately differentiated adenocarcinoma (G2) and three cases of poorly differentiated adenocarcinoma (G3).

Four (80%) cases with MSI associated a *KRAS* wild-type adenocarcinoma and only one case (20%) presented a mutation in *KRAS* gene located in codon 12 (Table 2).

The male patient with MSI had a *KRAS* wild type, right-sided, moderately differentiated colorectal adenocarcinoma with mucinous component.

Table 1 – Correlations between *KRAS* mutations and clinicopathological features of metastatic colorectal carcinoma, including MMR proteins status

Clinicopathological characteristics		Total (%) N=40 cases (100%)	<i>KRAS</i> WT (%) N=24 cases (60%)	<i>KRAS</i> MT (%) N=16 cases (40%)	P-value
Gender	Male	28 (70)	17 (42.5)	11 (27.5)	0.679
	Female	12 (30)	7 (17.5)	5 (12.5)	
Age [years]	≥65	26 (65)	15 (37.5)	11 (27.5)	0.783
	<65	14 (35)	9 (22.5)	5 (12.5)	
Primary tumor site	Right colon	9 (22.5)	6 (15)	3 (7.5)	0.924
	Left colon	21 (52.5)	12 (30)	9 (22.5)	
	Rectum	10 (25)	6 (15)	4 (10)	
Histology	Conventional adenocarcinoma	29 (72.5)	17 (42.5)	12 (30)	0.218
	Mucinous adenocarcinoma	11 (27.5)	7 (17.5)	4 (10)	
Tumor grade	G1	5 (12.5)	3 (7.5)	2 (5)	0.348
	G2	24 (60)	14 (35)	10 (25)	
	G3	11 (27.5)	7 (17.5)	4 (10)	
MMR status	pMMR	35 (87.5)	20 (50)	15 (37.5)	0.018
	dMMR (MSI-H)	5 (12.5)	4 (10)	1 (2.5) (codon 12)	

KRAS: Kirsten rat sarcoma viral oncogene homolog; MMR: Mismatch repair; p: proficient; d: deficient; MSI-H: High-level microsatellite instability; WT: wild type; MT: mutation.

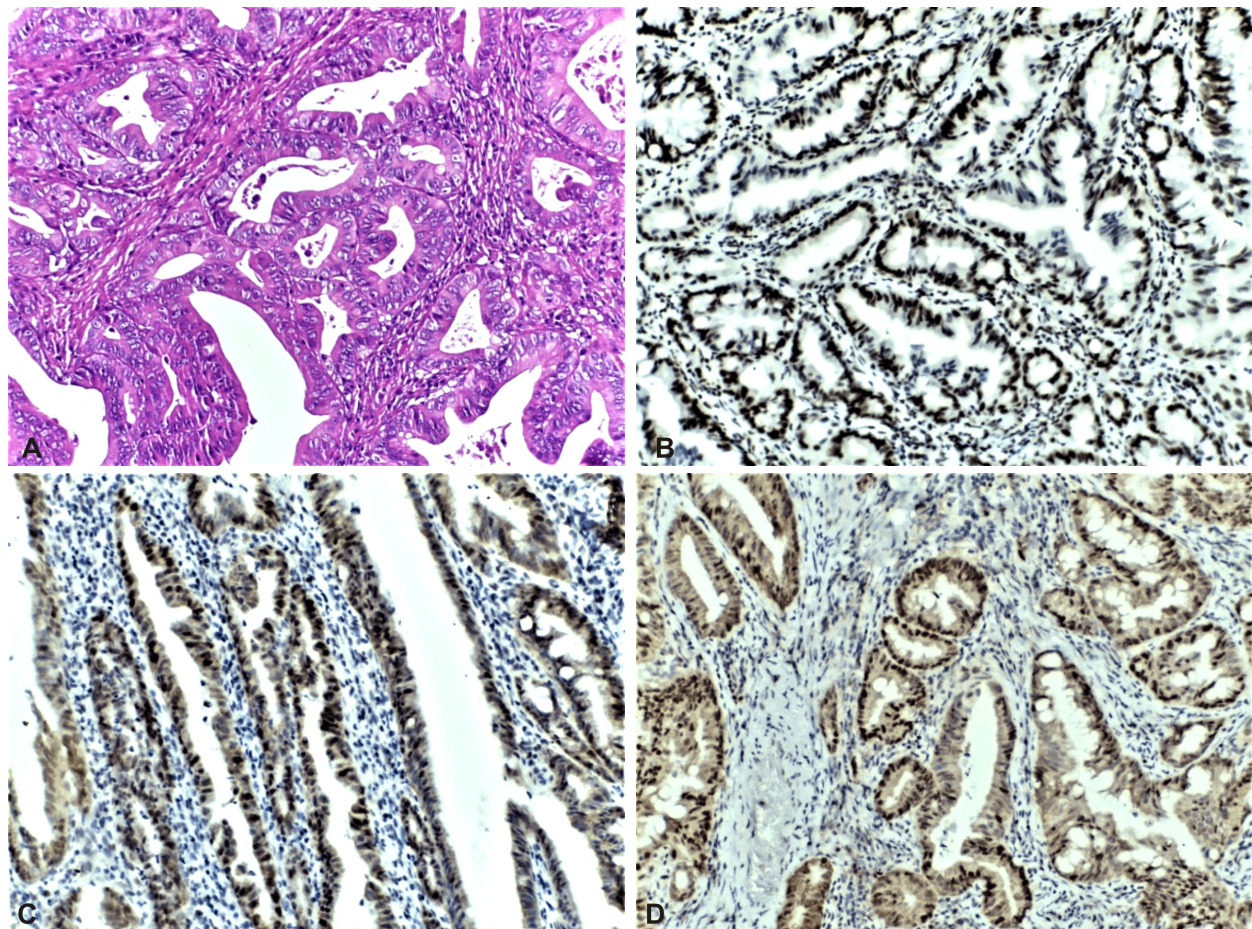


Figure 3 – Moderately differentiated MSS colorectal adenocarcinoma with well-differentiated areas, mucinous component <25%, a moderate anti-tumoral immune response and intact staining of MMR proteins: (A) HE staining, ×200; (B) Anti-MLH1 immunostaining, ×200; (C) Anti-MSH2 immunostaining, ×200; (D) Anti-MSH6 immunostaining, ×200. MSS: Microsatellite stable; MMR: Mismatch repair; HE: Hematoxylin–Eosin; MLH1: MutL homolog 1, colon cancer, non-polypoid type 2 (Escherichia coli) protein; MSH2: MutS protein homolog 2; MSH6: MutS protein homolog 6.

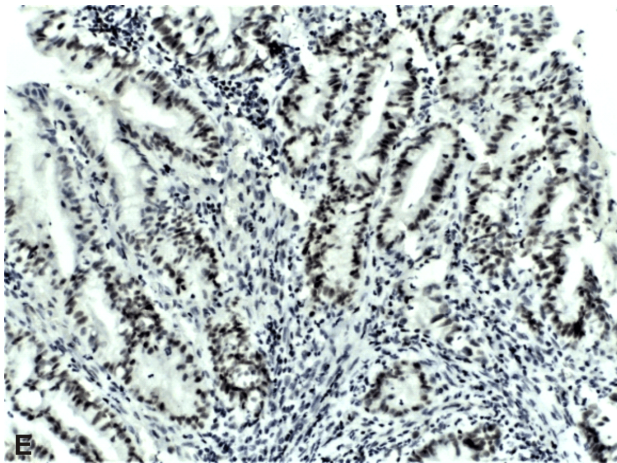


Figure 3 (continued) – Moderately differentiated MSS colorectal adenocarcinoma with well-differentiated areas, mucinous component <25%, a moderate anti-tumoral immune response and intact staining of MMR proteins: (E) Anti-PMS2 immunostaining, $\times 200$. MSS: Microsatellite stable; MMR: Mismatch repair; PMS2: Mismatch repair endonuclease.

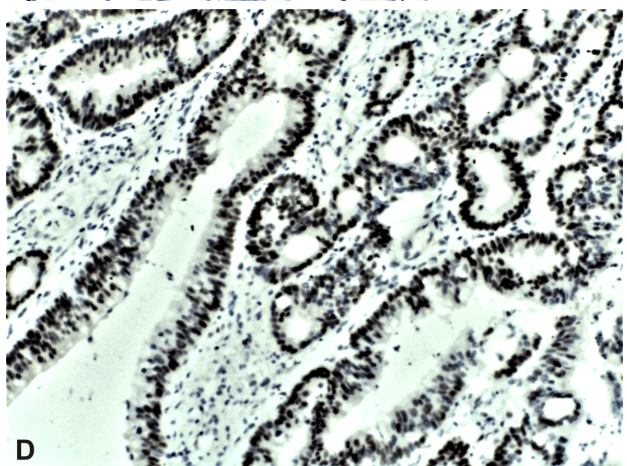
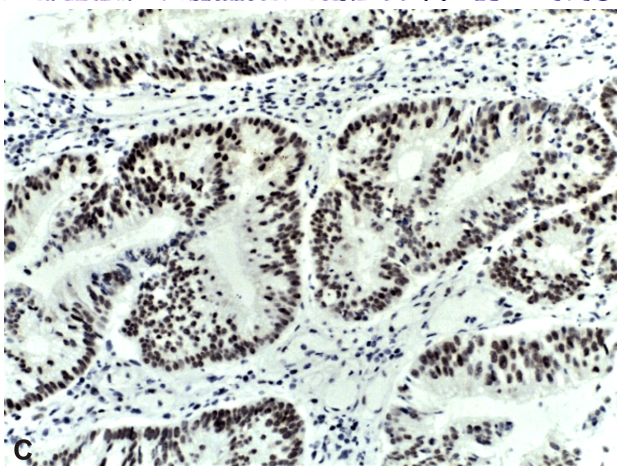
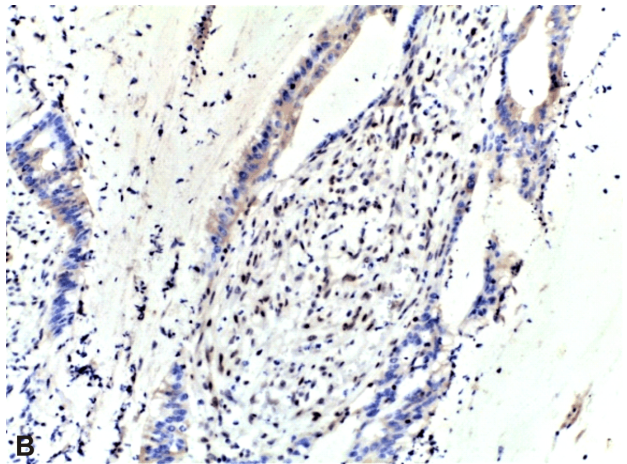
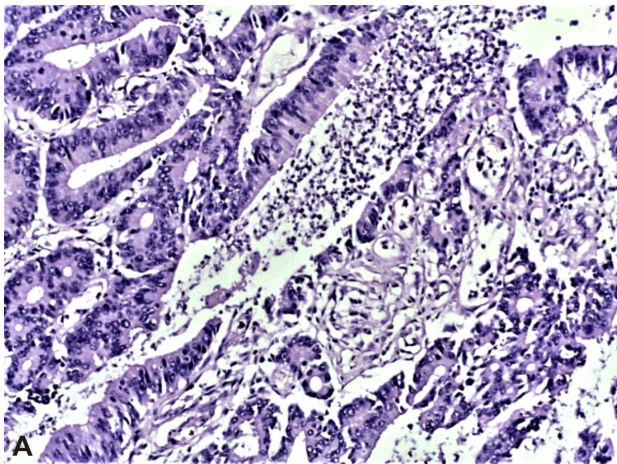


Figure 4 – Poorly differentiated MSI-H colorectal adenocarcinoma with moderately differentiated areas, mucinous component >55%, a marked anti-tumoral immune response, negative staining of MLH1 and PMS2 proteins and intact staining of MSH2 and MSH6 proteins: (A) HE staining, $\times 200$; (B) Anti-MLH1 immunostaining, $\times 200$; (C) Anti-MSH2 immunostaining, $\times 200$; (D) Anti-MSH6 immunostaining, $\times 200$; (E) Anti-PMS2 immunostaining, $\times 200$. MSI-H: High-level microsatellite instability; MLH1: MutL homolog 1, colon cancer, non-polyposis type 2 (*Escherichia coli*) protein; PMS2: Mismatch repair endonuclease; MSH2: MutS protein homolog 2; MSH6: MutS protein homolog 6; HE: Hematoxylin–Eosin.

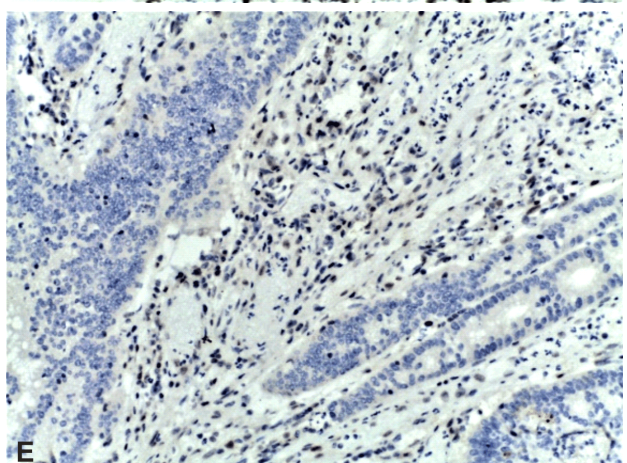


Table 2 – Clinicopathological characteristics of metastatic colorectal carcinoma with dMMR status

Clinicopathological characteristics		dMMR (%)
Gender	Male	1 (20)
	Female	4 (80)
Age [years]	≥65	4 (80)
	<65	1 (20)
Primary tumor site	Right-sided	3 (60)
	Left-sided	1 (20)
	Rectum	1 (20)
Histology	Conventional adenocarcinoma	1 (20)
	Mucinous adenocarcinoma	4 (80)
Tumor grade	G1	–
	G2	2 (40)
	G3	3 (60)
KRAS status	KRAS WT	4 (80)
	KRAS MT	codon 12 1 (20)
		codon 13 –

dMMR: Deficient mismatch repair; KRAS: Kirsten rat sarcoma viral oncogene homolog; WT: wild type; MT: mutation.

Discussions

Along with other genes, *KRAS* plays a key role in the prognosis of metastatic CRCs, its mutational status being the principal factor that influences the personalized therapy with monoclonal antibodies.

Our study revealed *KRAS* mutations in 16 cases, 40% of the patients having *KRAS* modifications located in exon 2, codons 12 and 13. The highest percent of mutations was detected in codon 12, 32.5% of all cases harboring an activating mutation in this location. A codon 13 mutation was detected in 7.5% of all cases.

Our results were similar with other literature data that identified the presence of *KRAS* gene mutations in ~40% of the CRCs, concerning in principal the codons 12 and 13 (in 90–95% of cases). Mutations in other codons, including 61, 146 or 154, are seldom described, in about 5–10% of cases [28, 29].

Neither of our cases presented double mutations in both codons, these positions being affected simultaneously in only 27% of CRCs [30].

We found no correlations between the position of *KRAS* mutations and the mucinous profile, even if other studies mention an association of the codon 12 mutations with the mucinous tumors and of the codon 13 mutations with non-mucinous, more aggressive neoplasms [31].

The present analysis also revealed the absence of statistically significant correlations between *KRAS* mutations and the patients' gender, age, location of primary tumor or histological tumors grading (resumed in Table 1), which is confirmed by other works as well [32].

Regarding MSI testing, the *MMR* genes status of our patients was identified using IHC techniques. IHC analysis detect MMR proteins expression in a much simple manner compared to molecular tests and allow a straightforward identification of the affected gene. When include all the four most frequent mutated MMR proteins, they have a predictive value virtually equivalent to MSI molecular testing, with almost similar clinical sensitivity (>90%) and an excellent specificity (100%) [16, 33]. Tumors

exhibiting loss of a MMR protein are considered to be deficient MMR (dMMR), with MSI-H profile, whereas tumors with intact MMR proteins are expected to be proficient MMR (pMMR), with MSI-L or MSS profile [16].

Our study group presented pMMR metastatic CRCs in most cases, MSI-H status being identified in only five patients. High-level MSI are generally found in about 15–20% of all CRCs but are less frequent in advanced stages of the disease [13]. This explains the lower percent of dMMR metastatic CRCs detected in our study, representing 12.5% of all cases.

We observed a strong nuclear staining for all four MMR proteins in every pMMR tumor; associating the patients' family history and their other pathological antecedents, we could exclude most probably the risk of a false-positive MMR reaction. Rarely tumors may present positive MMR protein staining despite MSI-H status. Missense mutations in *MMR* genes, frequently associated with inherited Lynch syndrome, can generate proteins that have lost their function but conserve their antigenicity, with false normal IHC staining, most frequently observed for MLH1 [33–35]. More than one third of *MLH1* alterations are missense mutations, generating mutant proteins that are catalytically inactivate but antigenically intact. Because of this, in cases with equivocal staining or in cases with high clinical suspicion of inherited forms, additional molecular analysis is recommended [5].

All our MSI cases revealed negative IHC reactions for MLH1 and PMS2 and positive IHC reactions for MSH2 and MSH6. The MMR active proteins form two functional heterodimeric complexes, MLH1–PMS2 and MSH2–MSH6, MLH1 and MSH2 proteins being principal, mandatory partners of their heterodimers [36]. Mutations in the *MLH1* and *MSH2* genes generate proteolytic degradation of the whole corresponding dimer and loss not only of the codified protein but also of the secondary partner [37]. In change, *PMS2* or *MSH6* genes mutations often cause loss of the codified proteins only, because their function can be compensated by others MMR proteins, such as MSH3, MLH3 or PMS1 [33].

In these conditions, our patients with MSI submitted most probably a *MLH1* mutation with degradation of the whole corresponding dimer and a consecutive false negative PMS2 reaction.

In terms of primary tumor position, tumor phenotype or histological differentiation, our MSI-H patients presented typical aspects for this status, resumed in Table 2: predominant right-sided tumors, with mucinous histology and a poorer differentiation, in accordance with other published data. As many studies described before, MSI status exhibits distinct features at the histological level, especially in the somatic form: mucinous and medullary histology, signet-ring cell differentiation, poor differentiation, a marked anti-tumoral immune response characterized by intra- and peri-tumoral lymphocytes and “Crohn-like” reaction, lack of “dirty” necrosis, “pushing” tumor margins with no or low-level of tumor budding and tumor heterogeneity [38–44]. Many studies also demonstrate that any mucinous differentiation should raise the suspicion of MSI [40].

Analyzing the gender and age of the MSI-H patients from our cohort, the majority were older women and only one case was a younger man of 40 years old. Many other studies prove that MSI-H lesions, especially those associated with CIMP phenotype, are generally diagnosed at an advanced age and with female preponderance [45, 46].

Our study identified four (80%) cases with MSI associated with *KRAS* wild-type adenocarcinoma and only one case (20%) presenting a mutation in codon 12 of the *KRAS* gene. Instead, 15 (42.8%) cases from 35 patients with MSS status associated mutations of the *KRAS* gene in both codons 12 or 13 (Table 1). The results corresponded to the published data, with a prevalence of MSI status inversely correlated with the presence of *KRAS* mutations in CRC [47]. MSI phenotype is more frequent in early stages of CRCs and the association with *KRAS* proto-oncogene alterations is significantly less common. As mentioned before in the literature, patients presenting both MSI status and *KRAS* wild type have generally a reduced risk of dissemination and recurrence and patients with *KRAS* mutated MSS tumors appear more likely to have disseminated disease, demonstrating that mutated *KRAS* and MSS may represent negative predictors for disease-specific survival [47, 48].

The case of the male patient with MSI required a differential diagnosis between a somatic form of CRC and the debut of a Lynch syndrome with germline mutation *de novo*, because of an early start of the disease, of the patient's gender and of a negative family history for inherited syndromic cancers. The differential diagnosis is very important considering the distinct evolution of the two forms and the impact on other members of the patient's family.

Personal antecedents of our patient did not include other synchronous or metachronous tumors specifics for the inherited syndromes with CRC, but the tumor location and histopathology were common for both sporadic and inherited forms of CRC. *MLH1* gene mutation is the most common alteration in the two forms; however, the molecular pathways are different. The differential diagnosis necessitates further molecular analysis for *BRAF* oncogene, highly associated with *MLH1* promoter methylation in sporadic MSI colorectal cancers and virtually absent in inherited Lynch syndrome [34, 49].

In summary, our data indicate the absence of significant correlations between *KRAS* mutations and most of the clinicopathological characteristics in Romanian patients with CRC, with a prevalence of MSI status inversely correlated with the presence of *KRAS* mutant type and certain particularities for MSI-H colorectal tumors, being similar to those reported previously in other Caucasian populations.

To our best knowledge, this is one of the few studies that have investigated combinational status of *KRAS* and MSI in Romanian CRC patients. The main limitations of the present work are the retrospective design, with lack of follow-up, and the reduced number of cases, so further investigations are needed to validate these findings and to identify the predictive and prognostic value of their association in CRC.

Conclusions

Our study demonstrates a 20% frequency of mutated *KRAS* in MSI-H CRCs, the presence of *KRAS* mutations being inversely correlated with MSI status in these tumors. *KRAS* mutational phenotype is not correlated with other clinicopathological findings. MSI-H status is less common in the advanced stages of the disease. In our cases, MMR protein deficient CRCs tend to occur in older females, have a poorer differentiation and are frequently associated with *KRAS* wild type. Identification of CRCs with MSI-H has a great clinical importance because represents the fundamental molecular feature of the Lynch syndrome and also a marker of favorable prognosis and a predictive factor of resistance to standard adjuvant chemotherapy. Testing for MMR deficiency using IHC or molecular analysis should be performed in all CRCs, if appropriate infrastructure is available, since the development of a predictor using a combination of biomarkers can represent a significant solution in CRC management, regarding the screening, the prognostic tests and the potential markers of response to chemotherapy and/or targeted therapies.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

Study conducted with the support of the Project PERSOTHER-SMIS-CSNR: 549/12.024.

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Received: December 18, 2017

Accepted: May 30, 2018