

ARTICLE

Oncogenic mutations and microsatellite instability phenotype predict specific anatomical subsite in colorectal cancer patients

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In colorectal cancer (CRC) oncogenic mutations such as *KRAS* alterations, are considered standard molecular biomarkers that predict the clinical benefit for targeted intervention with epidermal growth factor receptor (*EGFR*) inhibitors. In addition, these mutations are associated with specific anatomical area in colon tumor development, as *BRAF* mutations with the microsatellite instability (MSI). In this translational study, we aimed to assess the mutation frequencies of the *EGFR* (hotspot area and polyadenine deletions A13_del), *KRAS*, *BRAF*^{V600E}, and *PIK3CA* oncogenes in a series of 280 CRC patients. MSI phenotypes are also considered in this series. All patients' clinicopathological data were assessed for statistical analysis and its associations were validated. We verified multiple associations between oncogenic mutations and determined clinicopathological tumor features (1) *EGFR* A13_del mutations are associated with right colon carcinoma ($P < 0.005$), mucinous histotype ($P = 0.042$), G3 grading ($P = 0.024$), and MSI status ($P < 0.005$); (2) *PIK3CA* mutations are related mucinous histotype ($P = 0.021$); (3) *KRAS*^{G12} and *KRAS*^{G13} mutations are correlated, respectively, with the left and right colon cancer development ($P < 0.005$), and finally (4) MSI is associated with right colon tumors ($P < 0.005$). Mostly, we verified a higher frequency rate of the *KRAS*^{G13} and *EGFR* A13_del oncogene mutations in right colon cancer; whereas *KRAS*^{G12} codon mutation occurs more frequently in left colon cancers. In particular, we assessed that right vs left colon cancer are associated with specific molecular characteristics. These evidences, in association with clinicopathological data, can delineate novel approaches for the CRC classification and targeted intervention.

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INTRODUCTION

The mitogen-activated protein kinase (*MAPK*) pathway regulates important cellular activities, including the cell proliferation, differentiation, migration, and the apoptosis.¹ The *MAPK* activation is regulated through targeted tyrosine kinase receptor (TKR), as the *EGFR*. The active *EGFR* stimulates the *MAPK* cascade and the cell survival pathway.² The receptor dimerization causes activation of the intrinsic cytoplasmic kinase domain, resulting in the phosphorylation of several tyrosine residues.³

In colorectal cancer (CRC), oncogenic mutations damaging a TKR domain are considered a valid predictive biomarker for tumor targeted treatments, such as the *EGFR* inhibitors.⁴ Clinical studies demonstrated that patients with metastatic (m)CRC harboring mutations in the *EGFR* downstream molecules, named *KRAS* and/or *BRAF*^{V600E} genes, are resistant to the *EGFR* inhibitors, specifically to the anti-*EGFR* monoclonal antibody named 'cetuximab'.^{5–8} However, conventional hotspot *EGFR* mutations are rare in CRC;^{9,10} recently a new activating mechanism has been identified. This genetic disorder occurs *in vitro* at the A13 repeat of the *EGFR*

3'-UTR¹¹ in a subset of cancer samples with a microsatellite instability (MSI) phenotype.¹² Never it has been tested in human colon samples.

In CRC, other oncogenic mutations, such as the *PIK3CA* gene, can attack the *MAPK* cascade's function;¹³ in clinical setup, the presence of *PIK3CA* mutations is associated with an invasive cancer phenotype.¹⁴ In CRC treatment, the role of *PIK3CA* mutation as predictive therapeutic biomarker is not well defined.¹⁵

Colon tumors expressing MSI phenotypes correlate with specific clinicopathological features, as proximal location (right colon), poor differentiation, frequently mucinous histotype, lower tumor stage, and rare lymph nodal metastasis. The prognosis is generally good and long-term survival is higher.¹³

In this clinicomolecular study, we aimed to assess the mutation frequencies at the *EGFR*, *KRAS*, *BRAF*^{V600E}, *PIK3CA* oncogenes in a series of 280 CRC patients. For this analysis, we considered also the MSI status. Mutations profiles and MSI pattern were investigated in all cases and the associations between molecular data and patients' clinicopathological features were also considered.

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MATERIALS AND METHODS

Patient characteristics and genomic DNA extraction

Patients with primary CRC, histologically proven, were eligible for this translational study; we admitted 280 consecutive patients with written informed consent. These patients underwent a radical surgical procedure (R0 tumor classification). In this study for statistical analysis, we considered surgical procedures and clinicopathological data and molecular results (Table 1). Tumor and constitutional DNA were extracted from snap-frozen tissues; tumor concentration in tissues was assessed around 80%. About 30 mg of sample tissue was used for DNA extraction, using Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA) and the manufacturer's manual was followed for genomic DNA isolation.

This material was used to characterize the molecular alterations in genomic tumor DNA and in matched constitutional DNA.

Somatic mutation analysis of *EGFR*, *KRAS*, *BRAF*^{V600E} and *PIK3CA* oncogenes

For oncogenic mutation screening we adopted the method reported in detail by Corso *et al.*¹⁶ Briefly, we analyzed for *EGFR* mutation the hotspot kinase domain (exons 18, 19, 20, and 21) and the polyadenine (A13) repeat at the 3'-UTR. *KRAS* mutation analysis comprise codons 12 and 13 and *BRAF*^{V600E} point mutation.

To search for somatic alterations of *PIK3CA* gene, exons 9 and 20 were sequenced. All amplifications were performed in tumor and matched constitutional genomic DNA; all PCR products were directly sequenced and suspected alterations were validated with a second independent PCR.

MSI analysis

Microsatellite analysis was evaluated using five quasimonomorphic mononucleotide repeats BAT-26, BAT-25, NR-24, NR-21, and NR27. Tumor cases were considered as MSI whenever two or more markers showed instability on five loci considered. Method and data interpretation were already described.¹⁷

Statistical analysis

Analyses were performed using the Statistical Product and Service Solutions, SPSS 14.0 for Windows, 2006, SPSS Inc., Chicago, IL, USA. Statistical associations between the presence of CRC oncogenic mutations and clinicopathologic characteristics was assessed by χ^2 -test for categorical variables and Student's *t*-test or ANOVA test for continuous variables. A *P*-value lower than 0.05 was considered significant.

RESULTS

Overall mutation frequencies

A total of 120 (42.9%) mutations were identified in this genetic screening of 280 individual affected by CRC. All identified mutations showed a significant correlation with MSI phenotype (32/120; $P < 0.005$), and a statistical trend was assessed for mucinous carcinomas (42/120; $P = 0.007$).

EGFR screening

No hotspot *EGFR* mutations were identified at TK domain (0/280). Instead *EGFR* A13_del mutations (Figure 1) occurred with a frequency of 10% (28/280); these novel alterations were significantly associated with the following characteristics: (a) MSI pattern (28/28; $P < 0.005$), (b) right tumor site (22/28; $P < 0.005$), (c) mucinous carcinoma (12/28; $P = 0.042$), and (d) aggressive grading (G3) (12/28; $P = 0.024$) (Table 1).

KRAS, *PIK3CA*, and *BRAF*^{V600E} mutation status

We identified a total of 85 *KRAS* mutations (30.4%). Among this oncogene, we verified that 69 alterations (81.2%) flanked the codon 12, and 16 mutations (18.8%) the codon 13; respectively, we

observed: G12V 32.9%, G12D 24.8%, G13D 18.8%, G12C 12.9%, G12S 5.9%, and G12A 4.7%.

Table 1 resumed the correlations between patients with *KRAS* mutations *vs* wild-type and their clinicopathological features, no specific significant associations have been verified. Conversely, *KRAS* codons' stratification, named *KRAS*^{G12} *vs* *KRAS*^{G13}, showed interesting and novel results. We verified that *KRAS*^{G12} mutations occurred in proximal and distal CC with the frequencies of 59.3% (16/27) and 91.4% (53/58), respectively. Conversely, *KRAS*^{G13} mutations are identified in right and left colon cancer with the frequencies of 40.7% (11/27) and 8.6% (5/58), respectively ($P < 0.005$) (Table 1 and Figure 2). Comparing mutation G12 *vs* G13, we tested a higher frequency of *KRAS*^{G13} in a MSI subset 7.2% *vs* 18.7%, respectively.

PIK3CA mutations occurred with a frequency of 6.4% (18/280). In particular we identified the following hotspot alterations: (a) E542K (5/18), (b) E545K (5/18), (c) H1047R (5/18), (d) E545G (2/18), and (e) E545A (1/18). Statistical analysis revealed only a correlation between oncogenic alterations and mucinous carcinomas ($P = 0.021$). Although not significant, we verified a correlation between *PIK3CA* mutations (72.2%) and distal CC.

Among *BRAF* oncogene, we diagnosed a total of nine (9/280; 3.2%) mutate V600E phenotype; 6/9 (66.7%) were associate with a MSI pattern and 8/9 with right tumor location. However, the *BRAF* mutation number was rather small to perform a complete statistical analysis.

Concomitant oncogenic mutations

Eighteen patients (6.4%) of the mutant cases showed concomitant oncogenic mutations. From those cases with more than one mutation, eight had both *KRAS* and *PIK3CA* mutations (8/18; 44.5), four *KRAS* and *EGFR* A13_del (4/18; 22.2%), three *BRAF* and *EGFR* A13_del (3/18; 16.7%), and at least two *BRAF*^{V600E}, *PIK3CA* and *EGFR* A13_del (2/18; 11.1%). Our study confirmed that *KRAS* and *BRAF* mutations are mutually exclusive.

MSI phenotype and clinicopathology

MSI was identified in 38 patients (13.6%). Main clinicopathologic characteristics sharing with the MSI pattern were resumed in Table 1; in particular, MSI associated significantly with right colon tumor location ($P < 0.005$). A statistical trend was verified between stable pattern and mucinous carcinomas ($P = 0.057$).

DISCUSSION

Clinical studies have been demonstrated clearly that proximal CRC presents different pathological features and long-term survival impact.^{18–20} It has been suggested to consider CRC as three distinct tumor entities: right, left, and rectal cancer,²¹ in which specific genetic mechanisms and biological causes underlie these topographic differences. It was described that the UICC stage, metastatic, and lymphatic spread, T-stage, associates with specific colonic subsites, suggesting that the conventional cancer classification may be insufficient.²²

Recent studies emphasize that colorectal carcinogenesis relates with multigenetic causes. Specific oncogenes belonging to the *MAPK* cascade present different molecular pathways inside the same genetic structure.^{23,24} *KRAS* oncogene mutations occur with different frequencies in codons G12 and G13, and the most frequent amino acid changes observed are G12D, G12V, and G13D.²³ *KRAS* mutations affecting the codon G12 were more common in sporadic cases, whereas mutations at the hotspot G13 were predominant in a MSI hereditary setting.²³ Although, the reasons underlying this

Table 1 Variables and their associations with the identified mutations

Variable	EGFR _{del} mutations			PIK3CA mutations			Overall KRAS mutations			KRAS codon mutations ^b			Microsatellite status		
	Total	EGFR_MUT	EGFR_WT	PIK3CA_MUT	PIK3CA_WT	P-value	KRAS_MUT	KRAS_WT	P-value	KRAS ¹²	KRAS ¹³	P-value	MSI	MSS	P-value
	280	28 (%)	252 (%)	18 (%)	262 (%)		85 (%)	195 (%)		69 (%)	16 (%)		38 (%)	242 (%)	
Gender															
Male	157	13 (8.3)	144 (91.7)	9 (5.7)	148 (94.3)	0.278	45 (28.7)	112 (71.3)	0.591	36 (80)	9 (20)	0.768	21 (13.4)	136 (86.6)	0.914
Female	123	15 (12.2)	108 (87.8)	9 (7.3)	114 (92.7)		40 (32.5)	83 (67.5)		33 (82.5)	7 (17.5)		17 (13.8)	106 (86.2)	
Tumor location															
Proximal	99	22 (22.2)	77 (77.8)	5 (5)	94 (95)	<0.005	27 (27.3)	72 (72.7)	0.486	16 (59.3)	11 (40.7)	<0.005	28 (28.3)	71 (71.7)	<0.005
Distal	181	6 (3.3)	175 (96.7)	13 (7.2)	168 (92.8)		58 (32)	123 (68)		53 (91.4)	5 (8.6)		10 (5.5)	171 (94.5)	
Histotype															
Adenocarcinoma	205	16 (7.8)	189 (92.2)	9 (4.4)	196 (95.6)	0.042	60 (29.3)	145 (70.7)	0.021	49 (81.7)	11 (18.3)	0.857	23 (11.2)	182 (88.8)	0.057
Mucinous carcinoma	75	12 (16)	63 (84)	9 (12)	66 (88)		25 (33.3)	50 (66.7)		20 (80)	5 (20)		15 (20)	60 (80)	
Tumor staging															
pT1	6	1 (16.7)	5 (83.3)	0	6 (100)	0.539	0	6 (100)	0.799	0	0	0.281	1 (16.7)	5 (83.3)	0.925
pT2	80	5 (6.2)	75 (93.8)	6 (7.5)	74 (92.5)		27 (33.7)	53 (66.3)		23 (85.2)	4 (14.8)		10 (12.5)	70 (87.5)	
pT3	165	18 (10.9)	147 (89.1)	11 (6.7)	154 (93.3)		49 (29.7)	116 (70.3)		40 (81.6)	9 (18.4)		22 (13.3)	143 (86.7)	
pT4	29	4 (13.8)	25 (86.2)	1 (3.4)	28 (96.6)		9 (31)	20 (69)		6 (66.7)	3 (33.3)		5 (17.2)	24 (82.8)	
Lymphnode metastasis															
Absent (pN0)	152	14 (9.2)	138 (90.8)	7 (4.6)	145 (95.4)	0.631	52 (34.2)	100 (65.8)	0.175	43 (86.7)	9 (17.3)	0.653	20 (13.2)	132 (86.8)	0.808
Present (pN+)	128	14 (10.9)	114 (89.1)	11 (8.6)	117 (91.4)		33 (25.8)	95 (74.2)		26 (78.8)	7 (21.2)		18 (14.1)	110 (85.9)	
Grading															
G1	11	1 (9.1)	10 (90.9)	1 (9.1)	10 (90.9)	0.0243	2 (18.2)	9 (81.2)	0.791	2 (100)	0	0.489	1 (9.1)	10 (90.9)	0.339
G2	206	15 (7.3)	191 (92.7)	14 (6.8)	192 (93.2)		69 (33.5)	137 (66.5)		57 (82.6)	12 (17.4)		25 (12.1)	181 (87.9)	
G3	63	12 (19.1)	51 (80.9)	3 (4.8)	60 (95.2)		14 (22.2)	49 (77.8)		10 (71.4)	4 (28.6)		12 (19.1)	51 (80.9)	
Stage grouping															
I	60	3 (5)	57 (95)	2 (3.3)	58 (96.7)	0.440	20 (33.3)	40 (66.7)	0.532	17 (85)	3 (15)	0.824	7 (11.7)	53 (88.3)	0.304
II	82	10 (12.2)	72 (87.8)	5 (6.1)	77 (93.9)		27 (32.9)	55 (67.1)		22 (81.5)	5 (18.5)		12 (14.6)	70 (85.4)	
III	111	13 (11.7)	98 (88.3)	9 (8.1)	102 (91.9)		30 (27)	81 (73)		24 (80)	6 (20)		16 (14.4)	95 (85.6)	
IV	22	1 (4.5)	21 (95.5)	1 (4.5)	21 (95.5)		6 (27.3)	16 (72.7)		4 (66.7)	2 (33.3)		1 (4.5)	21 (95.5)	
NC	5	1 (20)	4 (80)	1 (20)	4 (80)		2 (40)	3 (60)		2 (100)	0		2 (40)	3 (60)	
Dukes															
A	60	3 (5)	57 (95)	2 (3.3)	58 (96.7)	0.455	20 (33.3)	40 (66.7)	0.674	17 (85)	3 (15)	0.936	7 (11.7)	53 (88.3)	0.925
B	82	10 (12.2)	72 (87.8)	5 (6.1)	77 (93.9)		27 (32.9)	55 (67.1)		22 (81.5)	5 (18.5)		12 (14.6)	70 (85.4)	
C	111	13 (11.7)	98 (88.3)	9 (8.1)	102 (91.9)		30 (27)	81 (73)		24 (80)	6 (20)		16 (14.4)	95 (85.6)	
NC	27	2 (7.4)	25 (92.5)	2 (7.4)	25 (92.6)		8 (29.6)	19 (70.4)		6 (75)	2 (25)		3 (11.1)	24 (88.9)	

Table 1 (Continued)

Variable	EGFR del mutations		PIK3CA mutations		Overall KRAS mutations		KRAS codon mutations ^b		Microsatellite status	
	EGFR_MUT	EGFR_WT	PIK3CA_MUT	PIK3CA_WT	KRAS_MUT	KRAS_WT	KRAS ^{G12}	KRAS ^{G13}	MSI	MSS
Total	280	252 (%)	18 (%)	262 (%)	85 (%)	195 (%)	69 (%)	16 (%)	38 (%)	242 (%)
	28	116.7)	6	100)	0	6	100)	0	1	5
MAC	6	116.7)	0	100)	0	6	100)	0	1	5
A	1	83.3)	0	94.9)	0	89	65.4)	0	18	118
B	12	91.2)	7	91.9)	47	81	81)	8	16	95
C	13	88.3)	9	92.6)	30	19	70.4)	6	3	24
NC	2	92.6)	2	93.8)	8	30	78.9)	2	38	—
MSI status	38	26.3)	3	92.1)	8	165	68.2)	3	0.155	—
MSI	242	100)	15	93.8)	77	165	68.2)	13	—	242

The different clinicopathological factors are classified in columns; in rows the correlated oncogenic mutations and microsatellite instability's results. Statistical analysis is reported in a dedicated column; significant results are indicated in emphasis (P-value).
^aThe numbers and percentages of KRAS codons' stratification (G12 vs G13) are referred to the overall KRAS mutation's number (left column).

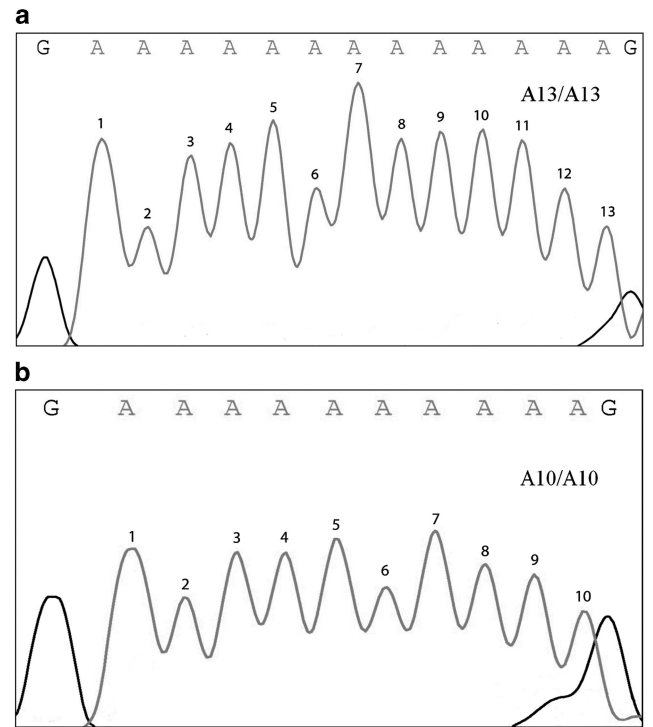


Figure 1 A 10/10 tumor deletion localized into the 3'-UTR of EGFR. In upper, chromatogram indicated a constitutional sequence of the EGFR polyadenine tract (A13/13). Below, the matched colon tumor sequence with a triple A deletion.

observation are not elucidated yet; it is supposed that different KRAS codon mutations are differentially expressed during the tumor development/progression. This variability probably depends on the specific tumor onset, as proximal vs distal colon. The clinical studies about KRAS oncogene are focused on two different areas: (a) KRAS as predictive biomarker of chemotherapeutic response; (b) KRAS as prognostic biomarker. The KRAS predictive role is unquestionable, it is well assessed that carries a mutation status present defect in response after the treatment with EGFR inhibitors. Conversely, the clinical application of KRAS as prognostic marker is strongly debating. A recent study analyzed the chemotherapeutic response in stage II and III resected CRC, demonstrating that KRAS did not exercise a major prognostic value.²⁵ In agreement with our study, we validated that KRAS mutation carriers did not correlate with specific clinicopathological features. However, we noted that KRAS codon's stratification presents other interesting results. It has been suggested that KRAS hotspot mutations may exercise a different impact in the colonic carcinogenesis, as the developing of proximal colon tumors. This factor is not well elucidated yet. Mostly, we verified that patients KRAS^{G13} mutation carriers presented a correlation with specific anatomical subsites, in particular with the proximal colon carcinoma. Exploring our results, we argued that KRAS^{G13} mutator pattern is a targeted hotspot for right colon tumor development; probably this data could define a novel KRAS role in colonic carcinogenesis. Conversely, we verified that KRAS^{G12} mutations occurred more frequently in left CC. Taking into this information, we could argue that KRAS^{G12} and KRAS^{G13} codon mutations participate delineate a different pathway during the colon carcinogenesis.
EGFR mutations represent a valid biomarker for the treatment with specific inhibitors in CRC patients.²⁶ However, hotspot mutations are

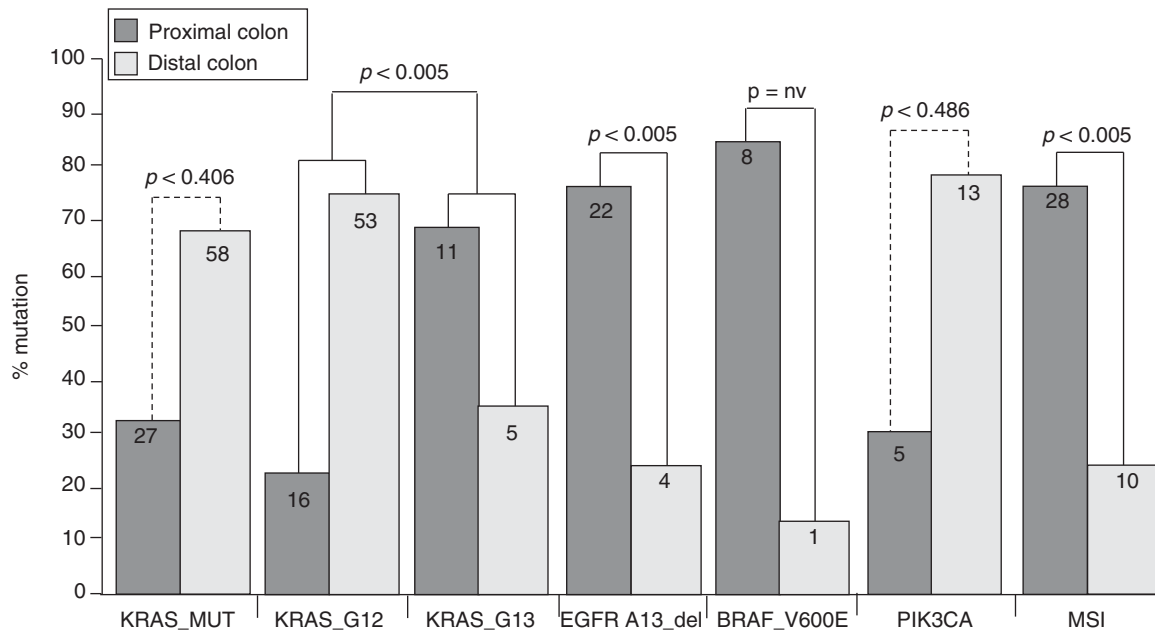


Figure 2 Mutation frequencies founded in proximal and distal CRC. In this panel the correlation between tumor sites and oncogenic mutation are depicted. Dotted and continuous lines indicate, respectively, not significant and significant statistical results, respectively. The *BRAF* mutation's number was rather low to perform a complete statistical analysis ($P = nv$).

very rarely identified in colon tumors expressing aberrant *EGFR*; in these cases, without mutations it is difficult to candidate patients at *EGFR* inhibitors. Interestingly enough, a novel activating mechanism deleting the *EGFR* polyadenine tract (A13) has been described. This genetic factor shows *in vitro* an oncogene overexpression in MSI colon carcinoma cell lines.¹¹ For the first time, we perform the *EGFR* A13 genetic screening in a human CRC samples. Previously, we verified that these deletions occur with a frequency of 47% in MSI gastric cancer. In CRC, we identified that these deletions were expressed in 10% of the overall cases; instead in MSI group, *EGFR* A13 deletions were diagnosed in 73% of samples. Comparing results from gastric vs colon cancers, we can elicit that *EGFR* A13 deletions are one of major genetic mechanisms in the CRC carcinogenesis with a MSI setting. Although not well clarified, this genetic mechanism is an emerging interest in clinical practice; it correlate with MSI status, right colonic subsite, mucinous carcinomas, and G3 grading.

Microsatellite disorders are a common event in CRC,²³ as it occurs with a frequency of about 22%. Owing to the presence of the mutator phenotype, MSI CRCs are associated with specific clinicopathological features, as proximal location, poor differentiation, and the presence of mucinous component.^{13,27} Moreover, MSI CRCs are associated with lower tumor stage at diagnosis and rare lymph nodes metastasis and/or distant organs.^{28,29} The prognosis is generally good with a long relapse-free survival time.^{28,30} Several clinical studies have been demonstrated that patients with MSI CRCs do not benefit from the treatment with 5-fluorouracil-based adjuvant chemotherapies.^{31–38}

In our study, we identified that tumors with MSI status showed a significant correlation with right CC, and a statistical trend with mucinous carcinomas. MSI results are similar to the cases with *EGFR* A13 deletions and *BRAF*^{V600E} mutations. Here, *BRAF*^{V600E} mutation occurs with a frequency of 3.2% and in MSI cases 23.7%, the majority diagnosed in right CC. These data are in accord with the literature report.³⁹

PIK3CA oncogenic mutations were described to occur in ~16% of the cases, whereas they occur preferentially in association with the

presence of *KRAS* or *BRAF*^{V600E} oncogenic mutations^{40–42} and with an invasive phenotype.¹⁴ The *PIK3CA* prognostic clinical impact is still debating and data about its prognosis in CRC patients are contrasting.⁴³ We identified only that *PIK3CA* mutations are associated with mucinous carcinomas; however, we don't have sufficient data to affirm that *PIK3CA* has a major role in CRC prognosis.

From this study, other important factor is to consider that concomitant oncogenic mutations occurred with a frequency of 6.4% in CRC patients. In particular, we noted an excess of associations between *KRAS* with *PIK3CA* mutations, since both alterations exercise a cumulative and/or synergic effect in a subset of colon carcinogenesis.¹³

Finally, we would consider that this study presents some limitations: (a) information about hereditary vs sporadic status, neo- and/or adjuvant treatments (chemotherapy, radiotherapy, etc) are not available in our series; (b) the mutation frequency affecting some oncogenes (such as *BRAF*) is rather low to perform a good statistical analysis. However, these results should encourage further studies to quantify the chemotherapeutic response rate, and long-term survival in association with the presence or absence of oncogenic mutations.

CONCLUSION

From this study we observed that colon carcinoma is a multigenetic disease, as several oncogenes are involved frequently in this process. Oncogenic mutations spread differently, based on specific anatomical regions. In particular, we can delineate a novel panel of molecular factors specific for right and left colon tumors. Our results demonstrated that (1) *EGFR* A13_del are associated with right colon carcinoma, mucinous histotype, G3 grading, and MSI status; (2) *PIK3CA* mutations are related mucinous histotype; (3) *KRAS*^{G12} and *KRAS*^{G13} mutations are correlated, respectively, with the left and right colon cancer development, and finally (4) MSI is associated with right colon tumors.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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