Original article

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Prevalence and prognostic role of microsatellite instability in patients with rectal carcinoma

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Background: Association between microsatellite instability (MSI) and favorable postoperative survival in patients with colorectal cancer receiving adjuvant chemotherapy has been indicated. To evaluate whether an analogous positive prognostic role of MSI could be present in rectal carcinoma (RC; most RC patients receive adjuvant radiotherapy), PCR-based microsatellite analysis of archival RCs and statistical correlation with clinico-pathological parameters were performed.

Patients and methods: DNA from paraffin-embedded paired samples of tumors and corresponding normal tissue from 91 RC patients was analyzed for MSI using five microsatellite markers (tumors were classified as MSI^+ when two or more markers were unstable).

Results: Seventeen (19%) RC patients exhibited a MSI^+ phenotype. Prevalence of instability was found in patients with earlier RC onset (28% in cases with diagnosis age ≤55 years versus 15% in cases >55 years), whereas similar MSI frequencies were observed in patients with different disease stage or receiving different adjuvant therapies. While MSI was detected in seven (64%) of 11 familial patients, a remarkably lower MSI incidence was observed in sporadic cases (10/80; 12.5%). A significant association with better disease-free survival (DFS) and overall survival (OS) was found for MSI^+ patients (median DFS/OS, 30/32 months) in comparison to MSI^- ones (median DFS/OS, 18/21 months) ($P < 0.001$).

Conclusions: MSI was demonstrated to be a strong molecular prognostic marker in rectal carcinoma, independent of the administered treatment (radiotherapy, chemotherapy or both).

Key words: microsatellite instability, polymerase chain reaction, prognosis, rectal cancer

Introduction

Rectal carcinoma (RC) accounts for ~10% of new cancer cases each year. It strikes men and women at nearly the same rate, generally in the age range from 50 to 80 years, with rising incidence with age [1]. Despite simple screening procedures rectal cancer is often advanced when discovered. Current trends in the management of cancer have focused on organ preservation and improved quality of life without compromising overall survival [2].

Rectal tumorigenesis is thought to occur by sequential accumulation of genetic alterations. To date, a multistep genetic model of tumorigenesis, based on specific genetic alterations in benign and primary malignant lesions, has been ascertained for colorectal carcinoma [3, 4]. For RC, several studies have implicated mutational inactivation of tumor suppressor genes (*p53*, $p21^{waf1/cip1}$, $p27^{kip1}$) [5–7] as well as activation of oncogenes (K-*ras*) [8] in its pathogenesis.

A molecular mechanism involved in the development and progression of some human malignancies, including colorectal cancer, is represented by a defective replication fidelity. Tumors with a non-functional DNA mismatch repair display a genomic instability as inferred by detection of ubiquitous somatic variation in length of microsatellite sequences [9, 10]. Microsatellite instability (MSI) is characterized by small insertions or deletions within short tandem repeats in tumor DNA when compared with the corresponding normal DNA. Microsatellite instability has been demonstrated in patients with hereditary nonpolyposis colorectal carcinoma (HNPCC) [11]. Frequency of MSI has been reported ranging from 10 to

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20% in sporadic colon carcinomas [12–14] and from 75% to nearly all tumors in HNPCC patients [15, 16].

For rectal carcinomas, MSI has been observed at a very low rate. The incidence of MSI seems to decrease moving from carcinomas originating in the proximal/transverse colon to those located in the distal colon and rectum, where frequency of MSI has been found to be <10% [17] (in some reports, frequencies were 1 [18] or 2% [19]). Despite its low prevalence in RC, MSI has been associated with the presence of mutations in the two principal mismatch DNA repair genes, *hMLH1* and *hMSH2* [12]. In fact, patients with RC tumors displaying MSI have clinical histories, including a family history positive for colorectal cancer and/or synchronous colorectal carcinomas, strongly suggesting hereditary cancer [19, 20]. This is in agreement with widely-reported data indicating that MSI is a molecular feature particularly frequent in familial colorectal cancers [12, 15, 21].

With regard to the prognostic value of MSI, most of data from different studies in colorectal and gastric cancer are consistent in defining a better outcome for patients with a MSI^+ phenotype [16, 22–24]. Results on patients with MSI^+ colorectal carcinoma (taking into consideration all tumor sites within the large bowel) have demonstrated a better survival only for cases with stage disease (Dukes' B and Dukes' C) receiving adjuvant chemotherapy (survival rates among untreated patients were found to be similar in the two groups, with and without MSI) [18, 25, 26].

The aim of this study was to investigate further both the prevalence and the prognostic role of MSI in rectal carcinoma, as well as the existence of some differences in the clinicopathological characteristics of RC patients with and without MSI that might be representative of distinctive tumor behavior. Considering that most RC patients undergo radiotherapy as the principal adjuvant treatment after surgical removal of the tumor, we indeed intended to test whether the MSI^+ phenotype could really predict the clinical outcome in RC, as well as to investigate the effects of the adjuvant treatments in MSI^+ RC patients.

Microsatellite analysis was performed on archival tissues, from a subset of 91 consecutive patients with RC at various stages of disease, using a PCR-based approach. Statistical correlation between the presence of MSI and histopathological or clinical parameters were thus inferred.

Materials and methods

Patient characteristics

Patients with a histologically-proven diagnosis of rectal carcinoma (RC) were included in the study. Eleven of 102 enrolled patients were excluded from the series because of DNA degradation in their samples. The remaining 91 RC patients presented with primary resectable disease and were classified according to the extension of the tumor following the Dukes' criteria.

The standard surgical treatment for colorectal cancers was Miles' procedure (30 cases), anterior resection of rectum (49 cases), Hartmann's procedure (seven cases) and palliative surgery (five cases). Median distance of the primary lesion from the anal verge was 4 cm (range 1–8 cm). The tumor volume included the primary with 3–5 cm margins.

The adjuvant radiation therapy was given 3 months after surgery; when chemoradiation was performed, adjuvant chemotherapy (5-fluorouracil and leucovorin for a total of four to six courses) was prescribed and radiotherapy began at 4–6 months following surgery. With the exception of four patients who were treated with a ^{60}Cobalto unit, the remaining 59 patients received radiotherapy using a linear accelerator (LINAC Saturno 15 CGR-GE; 12 MV photons). In 28 patients, one postero-anterior and one antero-posterior fields were used. In one patient the irradiation technique was of two fields (ant-post) and a cinetic field. In the remaining 34 patients the planning treatment was obtained by a computer-controlled intensity simulation and two computer software packages (convolution multigrid superposition and radiotherapy treatment planning), with a four-field 'box' technique. Median tumor dose was 54 Gy (range 44–63 Gy) and patients received total dose in 2–2.5 Gy daily fractions, five fractions per week.

Clinicopathological factors, such as family history, stage of disease at diagnosis, type of surgical resection, radio- and chemotherapy, metastatic or locally recurrent tumor during the follow-up (with diagnosis modality and therapy), disease-free survival, quality of life issues, time of last control (for overall survival), were abstracted from the hospital records for each patient. No therapeutic approach or clinical decision was made based on the results of the PCR assays.

Disease status at the time of diagnosis was defined depending on clinical staging as assessed by medical history, physical examination and instrumental tests. Clinical follow-up was performed over a median period of 45 months (range 12–69). All patients were informed about the aims of this study.

Anatomic sites of recurrence after initial treatment were as follows: local recurrences, nine; recurrence in the cervix uteri, one; recurrence in the bladder, one; distant metastases, 18 (six lung, 10 liver, one bone and one sovraclavicular lymph nodes). Diagnostic procedures for the evaluation of failure included proctosigmoidoscopy with biopsy, barium enema, computer tomography (TC), chest radiography and bone scans. Metastatic or locally recurrent tumors were treated with chemotherapy, radiotherapy and surgery—alone or in combination.

Tissue samples and DNA extraction

Tissue samples from RC patients were collected consecutively at the Departments/Institutes of Pathology participating in the study. Pathological review was performed on each case to confirm the diagnosis of rectal carcinoma. Patients originated from both the island of Sardinia (where the incidence of RC is similar to that of Western countries [27]) and other geographical areas in southern Italy; they were initially included in the study regardless of familial recurrence of the disease. However, family history of cancer was then evaluated by questionnaire interviews in RC patients after initial surgical treatment. The majority of interviewed patients were classified as sporadic due to the fact that no significant evidence of tumors in first- and second-degree relatives was registered. A case was classified as familial when at least three family members were affected by colorectal carcinoma.

Genomic DNA was isolated from tumor samples and corresponding normal tissue, as previously described [28, 29]. Percentage of neoplastic and normal cells in each tissue specimen was estimated by light microscopy. Tumor samples were estimated to contain ≥80% intact neoplastic cells.

Microsatellite analysis

Primers used to amplify simple sequence repeat markers were obtained from Life Technologies (Gaithersburg, MD, USA). Microsatellite instability was studied at five loci containing single- or dinucleotide repeat sequences and mapping to different chromosomal locations: BAT-25 (at locus 4q12), BAT-26 (2p16), D2S123 (2p16-p21), D5S346 (5q21-q22) and D17S250 (17q11.2-q12). All primer sequences were as reported in Genome DataBase (GDB; http://www.gdb.org).

Presence of MSI (referred to as MSI^+) was defined in each patient by the detection of at least two unstable (due to deletions or insertions) microsatellite markers in tumor DNA when compared with normal DNA. For each marker analysis, PCR was carried out as previously described [28, 29] using fluorescence-labeled primers. Electrophoresis of PCR products was carried out on a 377 Automated DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Using Pearson's chi-square test, presence of MSI was assessed for association with different clinical and pathological parameters: stage of disease, tumor grading, disease-free survival and overall survival.

The exact coefficient for sample proportion analysis was performed to determine all significant parameters (below 0.05 level). All analyses were performed with the statistical package SPSS for Windows, version 7.5 (SPSS, Chicago, IL, USA).

Results

Microsatellite analysis on archival tissues

Paired normal and tumor paraffin-embedded tissues from 91 patients with rectal carcinoma were analyzed for genetic instability by PCR-based microsatellite analysis. Table 1 shows the characteristics of all analyzed patients. About the same proportion of males and females were included in the study; median age was 63 years (range 39–84). No patient with distant metastases was included in the study. Following surgical resection, the majority of patients (63; 69%) received radiation therapy with or without fluorouracil (5-FU)-based chemotherapy. Using standardized selection criteria for family history (presence of at least three affected members), we identified 11 (12%) RC patients with recurrence of colorectal carcinomas who were classified as familial cases (in all families, two generations were represented; in seven of them, an onset age <45 years was also observed in at least one affected member, as indicated by the 'Amsterdam criteria' for identification of HNPCC syndrome) [30].

Comparison of amplified DNA from tumor and corresponding normal tissue allowed the identification of genetic instability as an electrophoretic mobility shift due to contraction or expansion of microsatellite repeats, with consequent changes in microsatellite length. For each marker, typical examples of MSI, as detected on an automated sequencer, are shown in Figure 1. Electrophoretic patterns in MSI^+ tumors ranged from 'smears' of abnormal peaks (like those reported for markers BAT-25 and BAT26 in Figure 1) to evident shifts in the PCR fragment sizes (like those reported for the remaining three markers in Figure 1), due to insertion/deletion of microsatellite repeats in tumor DNAs [in some cases, affecting both alleles and/or associated to loss of heterozygosity (like the example reported for marker D2S123 in Figure 1)]. In any case, all unclear or ambiguous results were confirmed in replicated experiments. Tumors were classified as MSI^+ when at least two out of the five markers used displayed evidence of mutant alleles in tumor DNA compared with the corresponding normal tissue DNA.

Among the 91 RC patients analyzed, 17 (19%) exhibited a MSI^+ phenotype; the remaining 74 (81%) cases (including nine tumors showing only one unstable marker) were classified as exhibiting a microsatellite stable (MSI^-) phenotype (Table 2). A similar MSI frequency was detected in the different disease stages (from 17% in Dukes' A to 20% in Dukes' C), whereas a prevalence of unstable tumors was found in patients with earlier RC onset (28% in cases with age at diagnosis ≤55 years versus 15% in cases >55 years) (Table 2). Although clinico-pathologically different, the same propor-

Table 1. Patient characteristics[a]

Characteristics	No. of patients	%
Total entered	102	
Total analyzed	91	
Males/females	49/42	54/46
Age (years)		
≤55	29	32
>55	62	68
Median	63	
Range	39–84	
Dukes'		
A	12	13
B	38	42
C	41	45
Primary site		
Rectum	66	73
Rectum-sigma	25	27
Therapy		
Follow-up only	5	6
CHT alone	23	25
RT/RT plus CHT	63	69
Total Gy (median)	54	
(range)	44–63	
Familial cases	11	12
Sporadic cases	80	88

[a]Staging was according to the Dukes' classification.
RT, radiotherapy; CHT, chemotherapy.

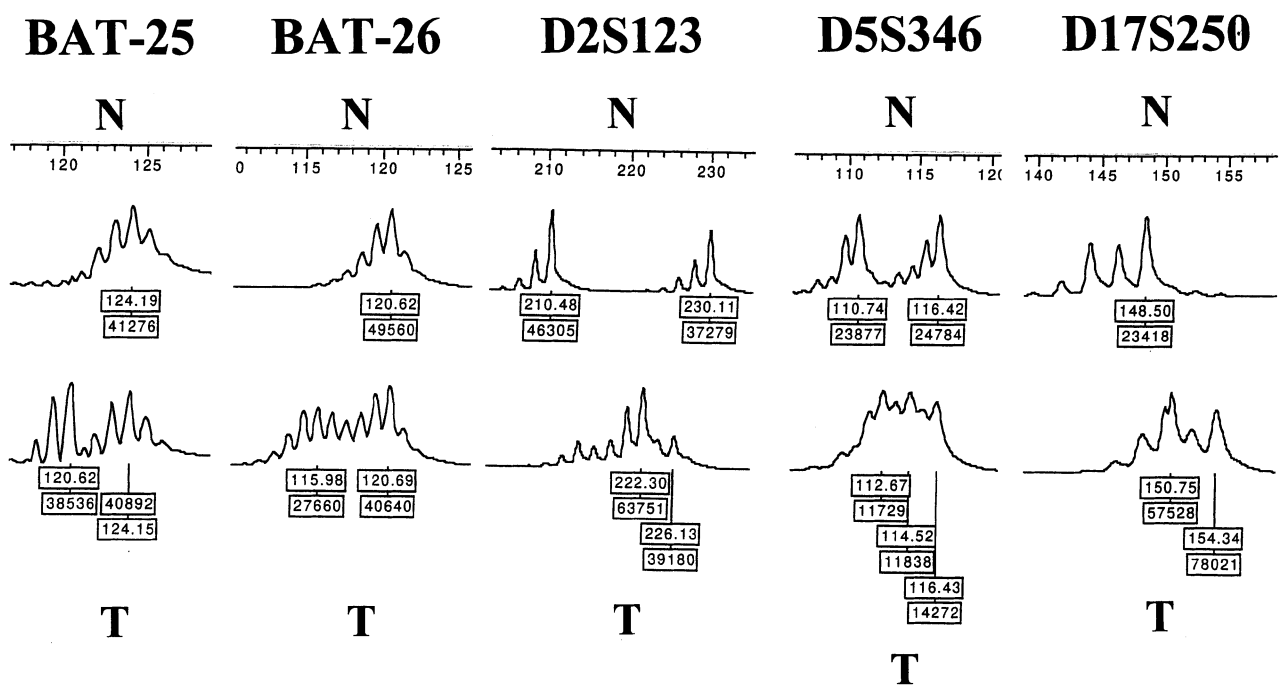

Figure 1. Representative examples of microsatellite instability at the five locus markers. Peaks visualized on the automated sequencer ABI 377 and corresponding to normal (top) and tumor (bottom) DNA are labeled N and T, respectively. The corresponding size bar is shown. Sizes are indicated at the bottom of each peak as base pairs (number below the size is the computer-calculated area under the peak).

tion of cases with rectal and recto-sigmoid cancer presented microsatellite instability (Table 2). Considering the therapy, no difference in MSI incidence was observed in the two groups of patients receiving radiotherapy (with or without association to chemotherapy; 12/63 patients, 19%) or chemotherapy alone (4/23 patients; 17%).

When we compared RC patients originating from Sardinia, whose population is genetically homogeneous (its historical isolation inducing a slow population growth rate and a high level of endogamy), to those from other geographical areas in southern Italy (where genetic heterogeneity, like that observed in general population, is present), some differences were observed in the frequency of MSI [altogether, 13 (21%) MSI^+ cases out of 61 Sardinian patients versus four (13%) MSI^+ cases out of 30 non-Sardinian patients] (Table 3). While the same high incidence (about two thirds of cases) was detected in patients with a positive family history, a quite remarkable difference in instability rates among the two groups was found in sporadic patients [8/53 (15%) versus 2/27 (7%) MSI^+ cases in Sardinian and non-Sardinian cases, respectively] and in patients with diagnosis age >55 years [7/38 (18%) versus 2/24 (8%) MSI^+ cases in Sardinian and non-Sardinian cases, respectively] (Table 3).

Clinical correlation

After a clinical follow-up over a median period of 45 months (range 12–69), a statistically significant correlation with a favorable outcome was demonstrated for patients with MSI^+ tumors. As reported in Table 4, univariate analysis showed a significant association with better survival [including both disease-free survival (DFS) and overall survival (OS)] in the MSI^+ group (17 patients: median DFS, 30 months; median OS, 32 months) in comparison to the group without MSI (74 patients: median DFS, 18 months; median OS, 21 months) (P <0.001). This positive significance was still observed when patients were classified according to disease stage (P <0.001) or therapeutic approach (P <0.05, regardless of the type of treatment administered: radiotherapy alone, chemotherapy alone or chemoradiation) (Table 4).

Discussion

In this study we performed a microsatellite analysis of unselected rectal carcinomas in order to investigate further the prevalence and the role of genetic instability in such a disease. Nevertheless, we evaluated the presence of a positive or negative correlation between microsatellite instability and clinical outcome.

Using a reference panel of five polymorphic markers (two mononucleotide repeats and three dinucleotide repeats), DNA from paraffin-embedded tissues of 91 RC patients was collected and investigated for genome-wide microsatellite instability. As recently recommended by a panel of experts [31], alterations in two or more locus markers were needed to identify tumors with high MSI (also referred to as MSI^+

Table 2. Results of microsatellite analysis. Correlation between number of rectal carcinoma cases with (MSI^+) or without (MSI^-) instability and several clinicopathological features

Characteristics (patients)	MSI^+	MSI^-
	n (%)	*n* (%)
Total patients (*n* = 91)	17	74
Dukes'		
A (12)	2 (17)	10 (83)
B (38)	7 (18)	31 (82)
C (41)	8 (20)	33 (80)
Age, years		
≤55 (29)	8 (28)	21 (72)
>55 (62)	9 (15)	53 (85)
Primary site		
Rectum (66)	12 (18)	54 (82)
Rectum-sigma (25)	5 (20)	20 (80)
Therapy		
RT/RT + CHT (63)	12 (19)	51 (81)
CHT alone (23)	4 (17)	19 (83)
Follow-up only (5)	1 (20)	4 (80)
Disease status		
Relapsed (29)	2 (7)	27 (93)
Disease-free (55)	15 (27)	40 (73)
Lost to follow-up (7)	0	7 (100)

MSI, microsatellite instability; RT, radiotherapy; CHT, chemotherapy.

Table 3. Distribution of MSI^+ rectal carcinoma cases according to patient origin

Characteristics (No. of patients)	MSI^+ patients	%
Sardinian patients		
Sporadic cases (53)	8	15
Familial cases (8)	5	62.5
Age, years		
≤55 (23)	6	26
>55 (38)	7	18
All cases (61)	13	21
Non-Sardinian patients		
Sporadic cases (27)	2	7
Familial cases (3)	2	67
Age, years		
≤55 (6)	2	33
>55 (24)	2	8
All cases (30)	4	13

MSI, microsatellite instability.

phenotype). This instability can be revealed through the shift in the electrophoretic mobility of analyzed fragments from tumor DNA, which is due to a change in the number of repeat units.

PCR-based screening with these markers revealed the presence of MSI in about one fifth of RC cases (17/91; 19%). The frequency of this genetic alteration in our series is higher than that reported by other authors for carcinomas of the rectum (typically, percentages have been <10%) [17–19]. Surprisingly, incidence of instability was detected at a higher rate in patients from Sardinia in comparison with those originating from other geographical areas in southern Italy (21 versus 13%, respectively). Such differences were even more evident for patients with an age of onset >55 years (18 versus 8%) or for sporadic patients (15 versus 7%) among Sardinian and non-Sardinian cases, respectively (as also shown in Table 3, the MSI incidence observed in the these two latter subgroups of non-Sardinian patients are similar to that previously reported in the general population [17–19]). Although there is no clear explanation for this phenomenon, one could speculate that the presence of a larger subset of unstable rectal carcinomas in patients from Sardinia could somehow be due to the high inbreeding rate and genetic homogeneity of the Sardinian population.

In a more comprehensive study including Sardinian and non-Sardinian patients with carcinomas from all colorectal tracts, the probability of detecting MSI seems to be steadily increased by the presence of both earlier age of onset and familial recurrence of colorectal cancer (M. Colombino, A. Cossu and G. Palmieri, unpublished data). Moreover, comparison between Sardinian and non-Sardinian cases among this larger series of patients should allow better statistical analyses to be performed and provide clues on the real role of these two different genetic backgrounds in determining different MSI frequencies. Finally, colorectal carcinomas are being screened for the presence of inactivation in all known mismatch repair genes (mainly *MLH1* and *MSH2*) at both somatic and germline levels (work in progress). Interestingly, preliminary data from immunohistochemistry using the anti-MLH1 antibody among nine MSI^+ RC tumors from our series revealed that a proportion of cases (four negative-staining cases; 44%) presented a loss of expression for this mismatch repair gene.

Large numbers of studies have documented microsatellite instability at various rates in many sporadic tumor types [10, 14, 32, 33], suggesting that the presence of MSI might be a marker of a tendency for replication errors in human cancers [32, 33]. This phenomenon was first documented in colorectal tumors of patients affected by hereditary nonpolyposis colorectal carcinoma (HNPCC) syndrome, where existence of a non-functional mechanism of DNA repair has been widely reported [12–14]. Therefore, deficit in mismatch repair with a subsequent increase of the replication error rate and a sequential accumulation of genetic mutations (affecting other target genes in the form of a 'cascade') may be the causative

Table 4. Correlation of clinical outcome to disease stage and therapy in MSI^+ and MSI^- rectal carcinoma patients[a]

Characteristics (No. of patients)	DFS median	OS median	DFS/OS median		*P*
			MSI+	MSI^-	
Dukes' A+B (50)	24	27	31/34	20/23	<0.001
Dukes' C (41)	18	20	27/29	15/18	
RT alone (25)	31	33	36/37	25/28	<0.01
RT + CHT (38)	30	33	34/37	24/26	<0.01
CHT alone (23)	14	16	18/20	12/15	0.03
MSI^+ (17)	30	32	–	–	<0.001
MSI^- (74)	18	21	–	–	

[a]Median values (in months) of both disease-free survival (DFS) and overall survival (OS) are indicated.
CHT, chemotherapy; MSI, microsatellite instability; RT, radiotherapy.

mechanism in tumors (including rectal carcinoma) with microsatellite instability [10, 34].

Considering the correlation with clinical parameters, a predictive value as prognostic factor was found for both the stage of disease (with a better outcome in Dukes' A + B patients in comparison to Dukes' C patients, as expected) and the MSI^+ phenotype (Table 4). Although presence of MSI was associated with neither the disease stage (Table 2) nor with tumor grading (data not shown), significantly more favorable survivals (including both disease-free and overall survivals) were observed in all subgroups of RC patients with MSI (regardless of the age of onset and the rectal or recto-sigmoid localization also). In addition, the strongly positive prognostic value of MSI that we have observed in our series of RC patients has been demonstrated to be independent of the administered treatment (radiotherapy, chemotherapy or both; Table 4). These findings differ slightly from the data in patients with unstable colorectal carcinomas (including all tumor sites, from the right colon to the rectum; it is important to keep in mind that rectal carcinomas represent the smallest fraction of MSI^+ tumors of the large bowel), who showed a better survival only when treated by adjuvant chemotherapy (thus, indicating that MSI might improve the sensitivity to this treatment) [18, 25, 26].

Our results demonstrate that MSI is a strong molecular predictive marker in rectal carcinoma and its presence should always be evaluated in paraffin-embedded tumor tissues from RC patients. However, our findings should be investigated further in prospective, randomized clinical trials based on microsatellite analysis. Therefore, a larger collection of rectal carcinomas from unselected and consecutively enrolled patients undergoing different adjuvant treatments should be helpful in better defining the role of MSI as a clinically useful predictive factor for the management of such a disease.

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