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ORIGINAL ARTICLE

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Distinct molecular patterns based on proximal and distal sporadic colorectal cancer: arguments for different mechanisms in the tumorigenesis

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Abstract *Background and aims:* Colorectal cancer (CRC) ranks as the fourth most frequently diagnosed cancer worldwide. CRCs that arise proximally or distally to the splenic flexure show differences in epidemiologic incidence, morphology, and molecular alterations, suggesting the existence of two categories of CRC based on the site of origin. The aim of the present work is to investigate the histological and molecular differences between CRCs located proximally and distally to the splenic flexure, and their potential involvement in tumor prognosis and therapeutic strategies. *Methods:* We evaluated 120 patients affected by sporadic CRC for clinicopathologic features, microsatellite instability (MSI), loss of heterozygosity (LOH) of chromosomes 18q, 8p, and 4p; they were also investigated for hMlh1, hMsh2, Fhit, p27, and Cox-2 immunostaining. *Results:* The mucinous histotype was more frequent in the proximal than in the distal CRCs ($p<0.004$). The frequency of MSI phenotype was higher in proximal than in distal tumors ($p<0.001$); moreover, reduced or absent hMlh1, Fhit, p27 immunohistochemical expressions were more frequent in proximal than in distal tumors ($p<0.001$ and 0.01 for p27). In contrast, the frequency of LOH in 18q was higher in distal than in proximal tumors ($p=0.002$). No significant differences were observed between proximal and distal tumors in the frequency of LOH in 8p and altered expression of hMsh2 and p53 protein. *Conclusion:* These different features may reflect different genetic pathways of carcinogenesis and support the hypothesis of a different mechanism of cancer development between the proximal and the distal colon, with potential implications in the therapeutic approach.

Keywords Colon cancer · Proximal colon · Distal colon · Molecular profile · Therapeutic approach

Introduction

Worldwide, colorectal cancer (CRC) is the fourth most common malignancy, after carcinoma of the lung, breast, and prostate, accounting in 2002 for nearly 148,000 new cases and 56,000 deaths in the United States and approximately 125,000 mortalities in Europe [1, 2]. More than a decade ago, the existence was proposed of two broadly different categories of CRCs according to whether the tumor is located proximally (right) or distally (left) to the splenic flexure [3–9]. This hypothesis has been supported by recent epidemiologic studies showing that the incidence of proximal tumors in Western countries has steadily increased, while that of distal tumors has shown a corresponding decrease [7, 10]. Moreover, cancers with a proximal location were found more frequently in elderly patients [11–13] and in the female gender [11]. It is well known that two different genetic pathways are involved in the carcinogenesis of CRC, one involving chromosomal instability and the other involving microsatellite instability

(MSI) [14–16]. The first pathway includes mutation in p53 and K-ras and loss of heterozygosity (LOH) of chromosome regions such as 4p, 5q, 8p, 17p, and 18q, 22q [17–20]. The second pathway is characterized by disruption of the DNA mismatch repair system (MMR) that normally maintains sequence fidelity during DNA replication [21].

The *FHIT* (fragile histidine triad) gene mapped at 3p14.2 functioning as tumor suppressor [22] encodes for a protein which low expression is associated with MMR deficiency in human advanced colorectal carcinoma [23].

More recently, an epigenetic model that is not accompanied by changes in DNA sequence has been characterized as an alternative mechanism for CRC initiation and progression [16, 24]. Epidemiologic studies have also shown a pathogenetic role in colonic tumorigenesis for $p27^{kip-1}$, a cyclin-dependent kinase inhibitor [25], with putative tumor suppressor function and for cyclooxygenase-2 (Cox-2), an enzyme catalyzing the biosynthesis of prostaglandins and thromboxanes [26]. The loss of p27 protein expression may result in tumor development and/or progression; however, this loss of expression does not appear to result from gene mutations [27, 28]. A large number of studies have characterized p27 as a prognostic factor in various human cancers, including colorectal adenocarcinoma [25, 29–31]. Multiple studies indicate that Cox-2 is overexpressed in many human malignant tumors [32–34] and is linked to the process of carcinogenesis [35], tumor survival [36], invasion [37], and metastasis [38].

Whether the pathogenetic role of Cox-2 and p27 differs with respect to proximal and distal CRCs is unknown. The aim of the present work is to investigate the possible differences between CRCs that arise proximally (right) and distally (left) to the splenic flexure from a histological and molecular point of view, and their potential involvement in the prognosis and therapeutic strategies.

Materials and methods

Patient population

The study population consisted of 133 unselected consecutive patients who had undergone curative colorectal resection for sporadic CRC between July 1996 and April 1999 at the our surgical unit. All cases were deemed sporadic, based on the absence of relevant family history as recorded prospectively at initial patient interview. A curative operation was defined as one in which no macroscopic tumor remained at the end of surgery and in which histopathologic examination of the operative specimen showed no tumor at the margins of resection. Distant metastases at the time of resection were excluded by preoperative liver ultrasonography, chest X-ray, and intraoperative exploration. Six patients were excluded on the basis of insufficient tissue for analysis, five patients because they were lost at follow-up, and two because of a double location of metachronous tumors (one located in the right colon and the other in the left colon), leaving 120 patients for study (63 males and 57 females).

During the study period, a uniform surgical management protocol was adopted. Proximal colon was defined as the large bowel proximal to the splenic flexure, and distal colon was defined as the large bowel distal to the splenic flexure.

All specimens underwent histopathological analysis by the same gastrointestinal pathologist (Bordi C.), who was unaware of the interim results of molecular genetic and immunohistochemical analysis. In accordance with the classification of tumors by the World Health Organisation [39], tumors were defined as mucinous when 50% or more of the tumor mass consisted of accumulated mucin, mostly extracellular; the other tumors were classified as "adenocarcinoma, not otherwise specified". Tumors were staged in accordance with the Tumour-Node-Metastasis (TNM) system [40].

Patients with stage III colon cancer under 75 years received adjuvant chemotherapy [41]. Patients with rectal cancer received irradiation therapy administered before surgery [42].

Patients were observed at 3-month interval for 24 months after the completion of therapy, then every 6 months for 3 years, and then yearly. History and physical examination, complete blood cell and platelet count, liver chemistries, ultrasound, and carcinoembryonic antigen measurement were performed at each visit, and chest X-ray, colonoscopy, and computed tomography (CT) were performed once a year.

Local recurrence was defined as the regrowth of the tumor in and around the tumor bed—including the pericolic fat, the adjoining mesentery, and lymph nodes—or in the suture or staple line of the bowel anastomosis, occurring either alone or in conjunction with generalized recurrence. We adopted the methodology to be followed in the reporting of studies of recurrences after resection of colorectal tumors recently suggested by Dent et al. [43].

Ethical approval for the study was obtained from the Human Ethics Committee of the University of Parma.

DNA preparation, LOH, and MSI testing

Specimens of freshly resected colorectal carcinomas were snap-frozen in liquid nitrogen and subsequently stored at −80°C. In all cases, fresh specimens of normal colon mucosa were also collected and used as matching controls. Only tumor samples containing at least 80% of neoplastic cells were included in the study. To verify this condition, one 10-μm-thick cryostat section from each tumor sample was stained with hematoxylin and microscopically examined by a pathologist. Fifteen to 25 cryostat sections (20-μm-thick) from the tumors included in the

study and from matching normal samples were submitted to DNA extraction by QIAGEN DNeasy tissue kit (QIAGEN, Hilden, Germany).

Polymerase chain reaction (PCR)

The following panel of six polymorphic microsatellite markers located on chromosomal regions potentially involved in CRC development and progression was used: D18S58 (18q22-23), D18S61 (18q22) [44], BAT26 (2p16), BAT40 (1p13) [45], D8S254 (8p22) [20], and D4S2397 (4p14-16) [17]. The markers were selected from the Genome database (http://www.gdb.org) on the basis of chromosomal location and heterozygosity. The PCR conditions and fragment analysis have been described in more detail previously [46].

Definition of allelic loss (LOH)

An imbalance factor was calculated as the ratio of relative allelic peak area in the tumor DNA to relative allelic peak area in the corresponding normal DNA on the basis of the following formula:

$$\left(lowerallele/higherallele\right)_{tum} \Big/ \left(lowerallele/higherallele\right)_{norm}$$

For informative markers LOH was scored when signal reduction for one allele was 40% or more [47].

Microsatellite instability (MSI)

The novel appearance in the tumor DNA of one or more alleles, i.e., new peaks in the electropherogram, not present in its paired normal DNA, was the indicator of MSI [48].

Tumors were classified as high frequency MSI (MSI-H) when instability was detected in at least 30% of the interpretable microsatellite markers investigated, or as low-frequency MSI (MSI-L) when instability was found in less than 30% of the markers, in accordance with international criteria [49]. Tumors without MSI were defined as microsatellite-stable (MSS) [49]. For the purposes of this study, MSS and MSI-L cases were considered together [50].

Immunohistochemical staining

For immunohistochemical analysis the specimens containing tumor and normal glands of the same snap frozen tumors were routinely fixed in buffered 10% formalin and embedded in paraffin. Sections of 5 μm were stained with hematoxylin and eosin for histological diagnosis and with the following primary antibodies: anti-hMsh2 (Clone FE11, Oncogene Research Products, Cambridge, Massachusetts, USA; working dilution:1/20); anti-hMlh1 (clone G168-728, Pharmingen, San Diego, California, USA working dilution:1/75), anti-Fhit clone (Polyclonal-ZR44, Zymed Laboratories, San Francisco, California, USA, working dilution: 1/50), with anti-p27 (clone SX53G8, Dako, Glostrup, Denmark; working dilution:1/50), anti-p53 (clone DO7, Dako, Glostrup, Denmark; working dilution: 1/50), and with anti-Cox-2 (Clone 4H12, Novocastra Laboratories, Benton Lane, Newcastle upon Tyne, UK, working dilution: 1/100).

The antibodies (Ab), clones, pretreatments, working dilutions, incubation time, and localisation of the immunostaining are listed in Table 1.

For antigen retrieval, sections were treated with 10 mM citrate at pH 6.0, in a 750-W microwave oven for three 5-min cycles. The sections were immunostained with the streptavidin-biotin kit (LSAB2, Dako) in accordance with the manufacturer's specifications and counterstained with haematoxylin. Positive controls were the normal glands of the intestinal crypts for anti-hMsh2, hMlh1, Fhit; peritumoral lymphocytes for anti-p27, and Cox-2; colorectal carcinomas strongly positive for anti p53. Negative controls consisted of substituting the primary antibodies with the normal serum.

Semiquantitative analysis

The immunostaining for each antibody except for p53 and Cox-2 was estimated on a semiquantitative score according

Table 1 Type and characteristics of antibodies used in the study

Antibodies	Clone	Treatment	Dilution	Incubation	Type of positivity
hMlh1	G168-728, Pharmingen	Citrate, MW	1:75	o/n	Nuclear
hMsh2	FE11, Oncogene	Citrate, MW	1:20	o/n	Nuclear
p27	SX53G8, Dako	Citrate, MW	1:50	o/n	Nuclear
Cox-2	4H12, Novocastra	Citrate, MW	1:100	o/n	Cytoplasmic
p53	DO7, Dako	Citrate, MW	1:50	o/n	Nuclear
Fhit	Pab- ZR744, Zymed	Citrate, MW	1:50	o/n	Cytoplasmic

MW microwave oven, *o/n* overnight

to the number of positive tumor cells as follows: 0% (0), <10%(1), 10 to 50% (2), 51 to 80% (3), or >80% (4). The intensity of staining was also evaluated as weak (1+), moderate (2+), or strong (3+). For each tumor case, the values of the two parameters were multiplied, resulting in scores ranging from 0 to 12 [51]. For the purposes of the study, staining of tumor nuclei for hMlh1 and hMsh2 and cytoplasms for Fhit was evaluated as absent (no protein) or present (any evidence). The 0–6 scores were considered as altered expression, 7–12 as preserved expression.

In addition, among tumors with preserved gene expression, two groups were distinguished, with a low (<50% of positive cells, or scores of 1 to 6) and high (>50%, or scores of 8 to 12) expression, respectively [52]. Moreover, for the expression of p27 scores <6 (cut off 50%) were considered as low protein expression [53]. The expression of Cox-2 and p53 was analyzed on the basis of the frequency of positive cells as + low expression; ++ and +++ high expression [54].

Real time FHIT analysis

RNA extraction and cDNA synthesis

RNA was extracted from paraffin-embedded specimens using TriZol Reagent as described by the manufacturer (Life Technologies, Gaithersburg, MD).

Reverse transcription of RNA was performed in a final volume of 20 μl containing 5× buffer (Tris, KCl, $MgCl_2$) 0.1 mM DTT, 1.5 mM total deoxynucleotide triphosphate, 5 U of RNase inhibitor (Roche, Penzberg, Germany), 10× Random Hexamer primers (Boehringer, Mannheim), 20 U of M-MuLV reverse transcriptase (Sigma, San Louis, Missouri, USA), and 5 μl (1 μg) of extracted RNA. The samples were incubated at 37°C for 1 h.

PCR conditions

PCR amplification was performed in the presence of specific target, fluorogenic probes (TaqMan probe) that allowed an automated quantification of the amplified products in real-time with the MJ Opticon. Primers and probes were chosen with the assistance of the Primer Amplify computer program to confirm the gene specificity of the chosen nucleotidic sequences for *FHIT*. The Taqman probe carrying a 5′ FAM reporter label and a 3′ MGB nonflourogenic quencher group was synthesized by PE Applied Biosystems.

For each PCR, 10 ng of cDNA template was used. The polymerase amplification was performed in a total volume of 20 μl containing 2× TaqMan Universal Master Mix, 2× Target Assay Mix (FHITprobe) or Endogenous Control Assay Mix (β-Actin probe).

The thermal cycling conditions were: 2 min at 50°C, 10 min at 95°C and then 45 cycles at 95°C for 15 s and 60°C for 1 min.

Quality control assessment was performed in standardized PCR conditions, including in each experiment a negative control (with no template) and the housekeeping gene (β-Actin) used for data normalization.

Data analysis

Calculation of the amounts of DNA is based on the cycle number, where fluorescence of each reaction passes the cycle threshold, which is set to the geometric phase of the amplification above the background. The amount of gene expression was determined using the comparative method of the ΔΔCT, always using a normal sample as control and a housekeeping gene (β-actin) as reference. Data analysis was based on the following formula:

$$\text{calculation of } \Delta CT : \left[\text{CTFHIT}\left(\text{sample}/\text{control}\right) - \text{CTACTINA}\left(\text{sample}/\text{control}\right)\right]$$

$$\text{calculation of } \Delta\Delta CT : [\Delta\text{CTsample} - \Delta\text{CTcontrol}]$$

$$\text{calculation of } \textit{Log ratio} = [-\Delta\Delta\text{CT}]2^{-\Delta\Delta\text{CT}} \begin{bmatrix} \text{this value shows how many times the gene} \\ \text{is overexpressed or underexpressed compared} \\ \text{to the housekeeping gene and the normal control} \end{bmatrix}.$$

Statistical analysis

Molecular data, immunohistochemical results, recurrence frequency, and patient survival were analyzed statistically in relation to the proximal or distal subsite location of the tumors.

Contingency tables and the χ^2 test were used to evaluate differences between percentages. The association of overall, local and distant recurrences with prognostic factors was evaluated by means of multivariable logistic regression.

The statistical analyses for the recurrences and for survival were performed excluding patients with palliative surgical treatment.

Disease-free interval in patients who had recurrence was measured as the interval between the date of resection and the date of diagnosis of recurrence.

Duration of survival was measured from the date of resection until the date of death from any cause or until the censoring date of April 30, 2004. In the survival analysis, deaths due to postoperative complications within 30 days were excluded. Survival curves were drawn according to the method of Kaplan and Meier, and differences in survival and disease-free interval were evaluated by means of the log-rank test. The simultaneous effect of more than one prognostic factor was estimated by the Cox proportional hazards model. Mortality rate ratios were used to assess the difference in deaths due to colorectal cancer.

Cohen's Kappa test was used to evaluate the concordance between the status of chromosomes 18q, 8p, and 4p.

All reported *P* values are two tailed. Statistical significance was set at an alpha level of 0.05.

Results

The clinical and histological data of 120 CRCs distributed in the proximal and distal colon are described in Table 2. From a histological point of view the carcinomas of mucinous type were more frequent in the proximal (57%) than in the distal (30%) colon (p=0.004) (Table 2). No significant correlations were found between tumor location and sex, mean age, distribution of TNM stage, or tumor grading, respectively.

Table 2 Clinico-pathological features of 51 proximal and 69 distal CRCs analyzed in the present study

Characteristics	Anatomic site		*P*
	Proximal[a]	Distal[b]	
N (%)	51 (43)	69 (57)	
Mean age (years±SE)	715±113	68.5±11	ns
Female (%)	49	46	ns
TNM stage (%)			ns
I	10	9	
II	49	48	
III	25	26	
IV	16	17	
Mucinous ADK (%)	57	30	0.004
Differentiation (%)			ns
Good–Moderate	59	68	
Poor	41	32	
Curative surgery (%)	82	84	ns

[a]Proximal colon was defined as the large bowel proximal to splenic flexure

[b]Distal colon was defined as the large bowel distal to splenic flexure

Several molecular features evaluated in this study resulted as being different from proximal to distal tumors. The incidence of the microsatellite instability (MSI) phenotype showed marked regional differences with a frequency of up to sixfold higher in proximal than in distal tumors (39 vs 6%, p<0.001) (Fig. 1). Moreover, reduced or absent hMlh1 immunohistochemical expression was more frequent in proximal than in distal tumors (46 vs 9%, p<0.001) (Fig. 2). The frequency of reduced or absent Fhit expression was fourfold higher in proximal than in distal tumors (28 vs 6%, p=0.001) (Fig. 3). This result was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR), which showed significantly lower gene Fhit expression levels in proximal tumors (p=0.019) (Fig. 4). Results of immunohistochemical analysis of Fhit expression significantly agree with those of RT-PCR (p=0.002) (Fig. 5).

Tumor loss of expression of cyclin-dependent kinase inhibitor, protein p27, was twice as frequent in proximal as in distal localizations (44 vs 20%; p=0.01) (Fig. 6). In contrast, the frequency of allelic losses at chromosomal regions in 18q (18qLOH) was twice as high in distal as in proximal tumors (57 vs 27%, p=0.002) (Fig. 7). The immunohistochemical expression of Cox-2 was more frequent in proximal (88%) than in distal tumors (72%), although the difference was not statistically significant (p=0.188).

No significant differences were observed between proximal and distal tumors in the frequency of allelic losses at chromosomal regions in 8p (17 vs 29%, p=0.209) and 4p (15 vs 18%, p=0.711), in the frequency of altered expression of hMsh2 protein (14 vs 8%, p=0.109). The immunohistochemical expression of p53 were found in 63% of proximal colon tumors and in 48% of the distal colorectal tumors without any statistical difference (p=0.29).

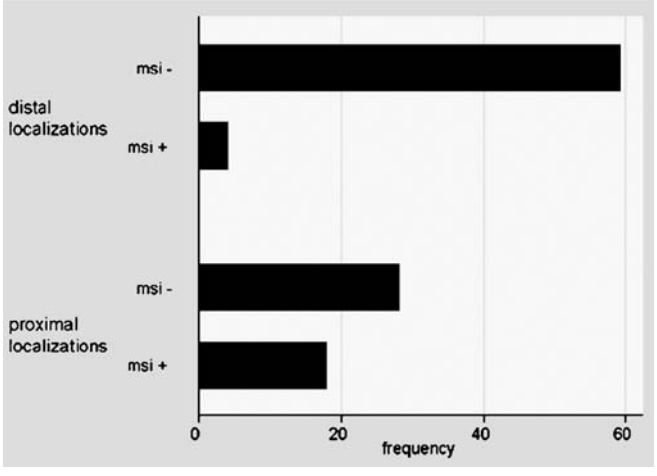

Fig. 1 Incidence of the microsatellite instability (MSI+) phenotype according to tumor site in 120 patients affected by colorectal cancer (p<0.001)

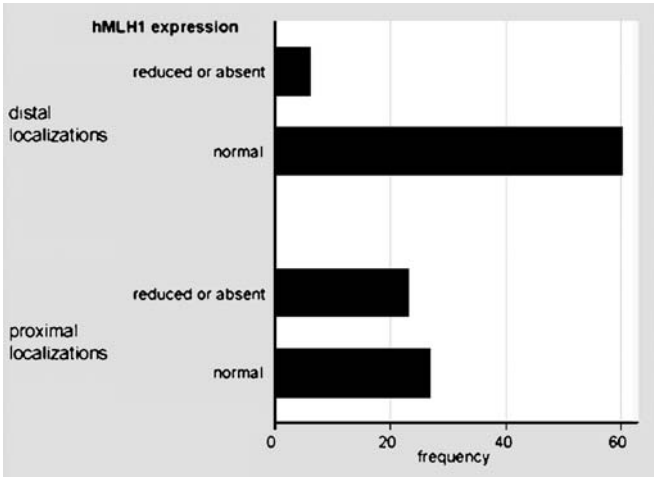

Fig. 2 Reduced or absent expression of hMlh1 according to tumour site in 120 patients affected by colorectal cancer ($p<0.001$)

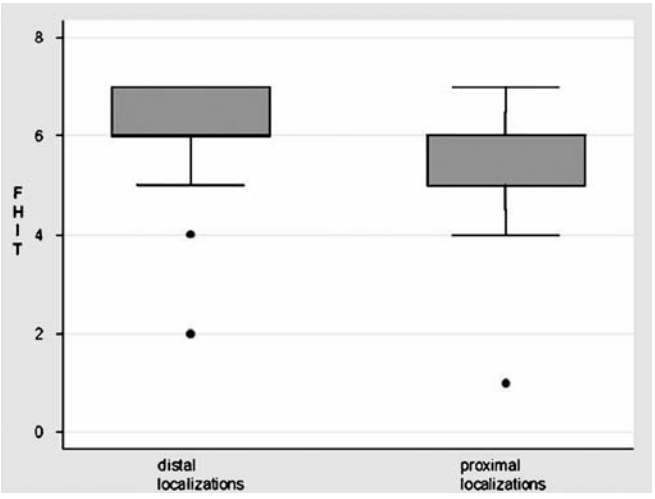

Fig. 4 Frequency of reduced or absent FHIT expression, by real time PCR amplification, according to tumour site in 120 patients affected by colorectal cancer ($p<0.019$)

In proximal tumors the MSI phenotype was significantly associated with low levels of hMlh1 ($p<0.001$) and Fhit expression ($p<0.001$), the absence of 18qLOH ($p=0.020$), and with mucinous histotype ($p=0.030$). Reduced or absent Fhit expression was significantly associated also with reduced or absent hMlh1 expression ($p=0.004$). Reduced or absent hMlh1 expression was correlated with altered hMsh2 expression ($p=0.003$). Loss of p27 expression was more frequently associated with MSI phenotype ($p=0.002$) and mucinous histology ($p=0.001$).

In distal colorectal tumors 18qLOH was more frequently associated with normal hMlh1 expression ($p=0.023$). MSI+ phenotype was more frequently associated with reduced or absent hMlh1 expression ($p<0.001$), and with mucinous histotype ($p=0.034$); reduced or absent Fhit expression was more frequently associated with altered hMsh2 expression ($p=0.003$) and with mucinous histotype ($p=0.052$); reduced or absent hMlh1 expression was more frequently associated with altered hMsh2 expression ($p<0.001$); loss of p27 expression was more frequently associated with mucinous histotype ($p=0.002$).

Frequency of recurrence and survival curves

The analyses relating to frequency of recurrence of the disease and to survival were carried out using data relating

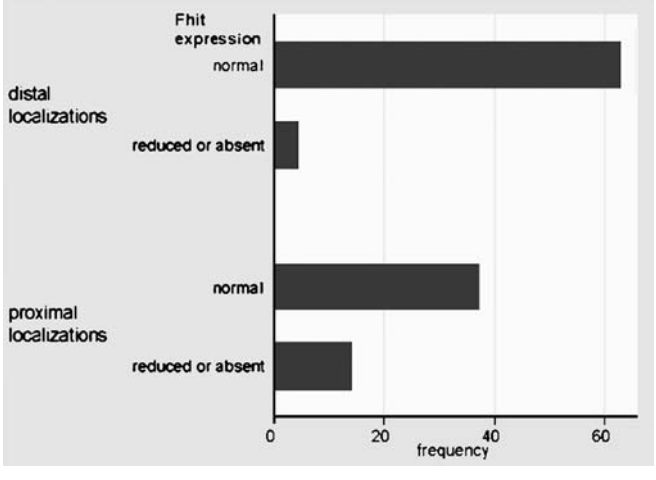

Fig. 3 Frequency of reduced or absent Fhit expression, by immunohistochemical staining, according to tumor site in 120 patients affected by colorectal cancer ($p<0.001$)

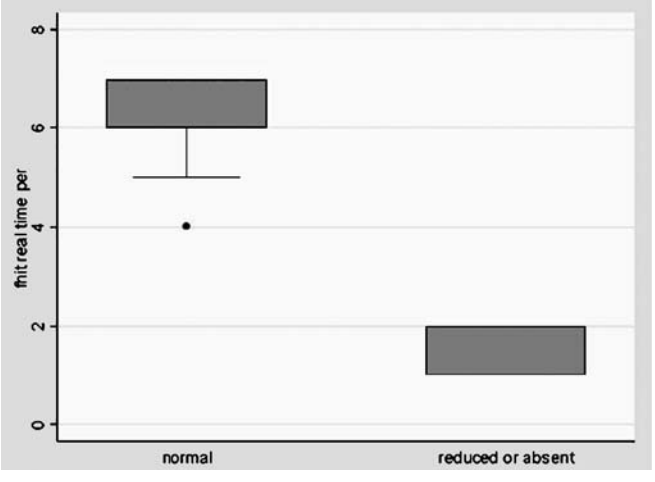

Fig. 5 Results of immunohistochemical analysis of Fhit expression significantly agree with gene FHIT expression by real time PCR ($p=0.002$).The *thicker horizontal line* identifies the median sample value and the *ends of the box* are the 25th and 75th quantiles. The whiskers extend from the ends of the box to the outer-most data point and are calculated according to: upper or lower quartile + 1.5* (interquartile range). *Points* represent outliers

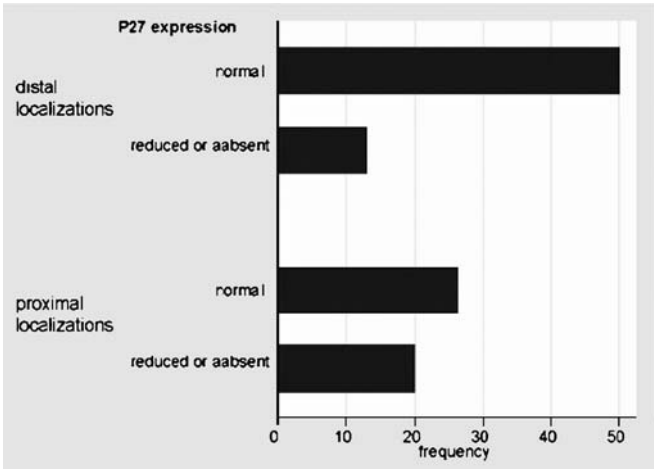

Fig. 6 Loss of the cyclin-dependent kinase inhibitor, protein p27, according to tumor site in 120 patients affected by colorectal cancer (p=0.01)

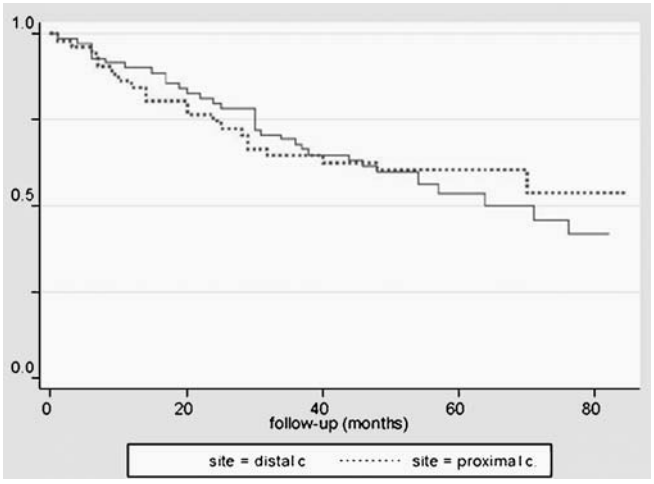

Fig. 8 Overall survival according to tumor localization in 99 patients who had undergone curative colorectal resection for sporadic colorectal cancer. The differences between the curves are not significant (p=0.712)

to 99 patients (82.5%) who had undergone curative surgery. Mean follow-up was 65 months (range 2–85). The frequency of neoplastic recurrence was 17% for proximal localizations and 29.8% for distal ones, although the difference is not statistically significant (p=0.128). Cumulative survival curves (p=0.712) (Fig. 8) and those for disease-free survival (p=0.143) (Fig. 9) do not differ significantly in the two groups. However, the difference between the survival curve of patients who had undergone curative colorectal resection of MSI + CRC and that of patients who had undergone curative colorectal resection of CRC with 18qLOH is close to statistical significance (p=0.0521), independently of the localization of the tumor (Fig. 10).

Discussion

Our study demonstrates several significant differences between proximal and distal CRCs from a histological and molecular point of view. The incidence of mucinous carcinomas resulted as being greater in the proximal colon, and approximately twice as low in frequency from the proximal to the distal colon. This result is in agreement with several studies [55–58], whereas other authors report a higher frequency of mucinous carcinomas in the rectum and/or distal localizations [59, 60], or do not find significant differences [56]. The frequency of mucinous adenocarcinomas in the sample examined in the present study accounts for 40% of all colorectal cases and is higher than that reported in other studies [55–58]. The well-

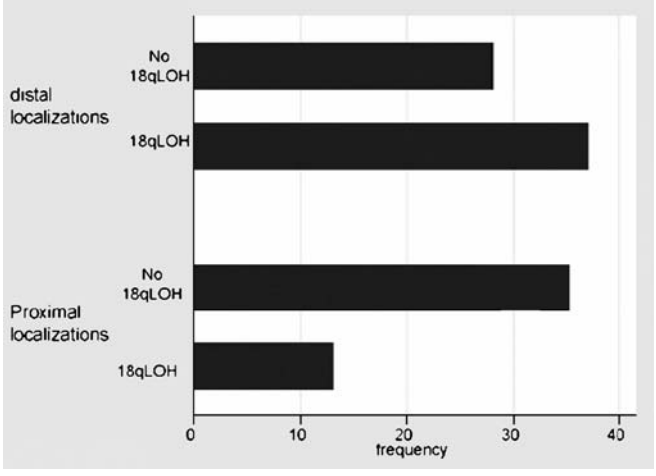

Fig. 7 Frequency of allelic losses at chromosomal regions in 18q (18qLOH) according to tumor site in 120 patients affected by colorectal cancer (p=0.002)

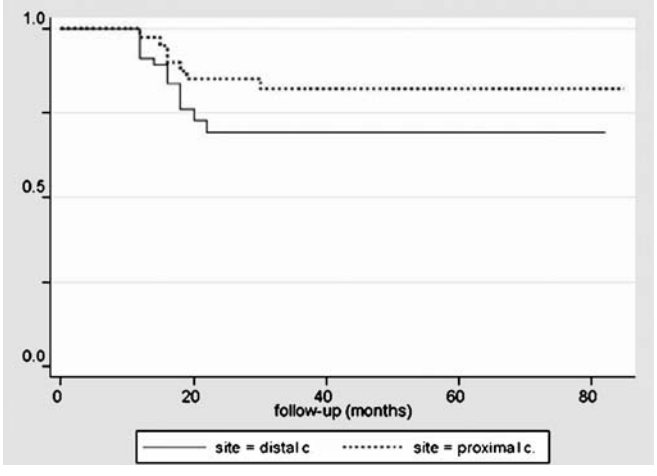

Fig. 9 Disease-free survival according to tumor localization in 99 patients who had undergone curative colorectal resection for sporadic colorectal cancer. The differences between the curves are not significant (p=0.143)

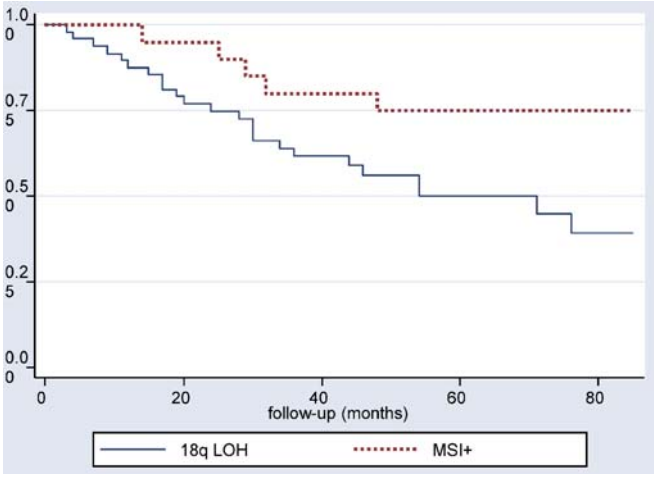

Fig. 10 Overall survival of 41 patients who had undergone curative colorectal resection for sporadic colorectal cancer with 18qLOH is significantly worse than overall survival of 19 patients who had undergone curative colorectal resection for sporadic colonic cancer with MSI (p=0.05)

known marked geographical variations in the epidemiological characteristics of colorectal cancer [11] probably account for these differences as well as for the discrepancy between our data and that previously reported. Indeed, the association of increasing age or female gender with proximal colorectal cancer shown in some other studies [11, 61, 62] was not observed in the present investigation, which was in keeping with another recent study that does not reveal differences in sex and age between patients with proximal and distal CRCs [63, 64].

In the sample of patients examined in this study, the pattern of genetic alterations is different in proximally and distally located tumors. In particular, MSI+ phenotype, reduced or absent hMlh1 expression, reduced or absent Fhit expression, and altered espression of p27 were significantly more frequent in proximal than in distal colonic tumors. The observation that MSI+ tumors are located predominantly in the proximal colon is in agreement with most previous investigations [45, 65–67]. The majority of sporadic MSI+ tumors arise following hypermethylation of the *hMLH1* gene [68]; it is not surprising, therefore, that MSI+ phenotype resulted as being correlated with reduced or absent hMlh1 expression. Even reduced or absent Fhit protein expression and *FHIT* gene expression evaluated by real time PCR resulted as being correlated with MSI+ phenotype and with reduced or absent hMlh1 expression. Moreover, the close correlation between Fhit protein expression evaluated by immunohistochemistry and *FHIT* gene expression evaluated by real time PCR has confirmed the reliability of the immunohistochemical analysis in the assessment of the *FHIT* gene alterations. In this regard, we previously suggested that a deficiency of an *MMR* gene could be responsible for the high frequency of altered tumor expression of Fhit, and *FHIT* gene alteration may be part of the MSI-associated genetic pathway of colonic carcinogenesis [52].

The correlation between proximal colorectal cancer with MSI and adenocarcinoma with mucinous phenotype, revealed in the present study, has already been reported in previous studies [50, 67, 69, 70].

In our CRCs the immunohistochemical expression of p27 is altered more frequently in proximal than in distal localization, in agreement with the results of Zhang [30] but in contrast with those of Manne et al. [31]. Several lines of evidence suggest that decreased p27 expression in tumors is associated with a more aggressive tumor phenotype such as poor histologic grade, the presence of lymphovascular invasion and higher growth fraction [71]. The absence of p27 protein expression is a predictor of poor prognosis in proximal CRCs [30] and a powerful negative prognostic marker in colorectal carcinomas, particularly in stage II tumors, thus helping in the selection of patients who will benefit from adjuvant therapy [25]. Our results also showed a significant correlation of the reduced expression of p27 with the mucinous type of colorectal adenocarcinoma and with the MSI+ phenotype. The latter result may suggest that also altered expression of p27 may be part of the MSI-associated genetic pathway of colonic carcinogenesis.

Distal colorectal tumors were characterized by significantly more frequent 18qLOH, normal hMlh1 expression, normal p27 expression, and normal Fhit expression. The higher frequency of allelic losses on 18q in distal CRC is in agreement with previous results [46, 62].

No significant differences in the Cox-2 expression were found between proximal and distal colorectal tumors. Nor were any correlations found between Cox-2 and the other parameters analyzed. Cox-2 expression is commonly involved in colorectal tumorigenesis [26], although its interrelationship and clinicopathological effects remain inconclusive. In a recent work, Nasir [72] found frequent expression of Cox-2 in left-sided CRCs, although this report is only based on 36 patients. The alterated immunohistochemical expression of the protein p53 was more frequent in proximal tumors but it did not reach the statistical significance in accordance with other works in which the mutations of the p53 gene were detected at similar frequencies in proximal and distal tumors [73, 74].

It has been suggested that MSI in CRC is a predictor of improved survival [45, 46, 75], whereas 18qLOH has been associated with an adverse clinical outcome [46, 62, 76–80]. It would be expected, therefore, that the outcome of proximal colonic cancers, which are more frequently MSI+, is better than that of distal CRCs, which are more often characterized by 18qLOH. Indeed, several studies conclude that patients with distal tumors have a poorer survival rate than those with tumors on the right side [44, 76, 81, 82]. Our results on the frequency of recurrent disease and on disease-free survival were in agreement with these observations, although the differences were not

statistically significant, possibly because of the small size of the sample examined.

In conclusion, the present study identifies the existence of at least two major groups of colorectal cancer based on histologic and molecular features. One group occurs predominantly in the proximal colon and is characterized by histologic mucinous type, MSI, altered expression of Mlh1, Fhit, and p27. The other group occurs predominantly in the left colon and is characterized by 18qLOH. These differing features may reflect two different genetic pathways of carcinogenesis in the proximal and the distal colon, as suggested by Ikeda et al. [83].

In this regard, we agree with Iacopetta [9] in considering that the anatomic site of origin of CRC tumors may provide a convenient discriminator for two subgroups of tumors having important biological and clinical differences with potential repercussions on therapeutic choices. They could, for example, lead to the identification of a subset of patients who benefit most from 5-fluorouracil-based chemotherapy. Recent literature reports differences in the response to 5-fluorouracil on the part of colorectal tumors with differing genetic profiles [84]. Results are not univocal [84–87], and further studies on wide sample sizes will be needed to identify more clearly the role of the differing genetic profiles in defining different responses to adjuvant chemotherapy treatment. On the basis of our results, we maintain that for this purpose, it would be useful to compare the response to chemotherapy of patients with proximal tumors and those with distal ones; these groups of patients, in fact, could constitute two large populations with important differences in distribution of tumor genetic profiles, one with a higher frequency of tumors with MSI and the others with a higher frequency of tumors with 18qLOH.

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