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ORIGINAL ARTICLE

Site impact on colorectal cancer biological behavior in terms of clinicopathological and molecular features

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Summary

Purpose: *We investigated the biological behavior of proximal and distal colorectal adenocarcinomas (CRC), intending to determine specific segmental differences, possibly arising from the distinct genetic pathways involved in their development.*

Methods: *Thirty-six proximal and 83 distal cancers were comparatively and retrospectively analyzed, regarding tumor stage, grade and Ki-67, p53 and Bcl-2 immunohistochemical expression.*

Results: *Proximal tumors were more likely to be poorly differentiated (p=0.005) and to exhibit low Ki-67 and p53 expression (<20% and ≤ 30% stained nuclei respectively; p=0.026 and 0.0014, respectively). Distal lesions were more likely to be moderately differentiated (p=0.001), to display moderate Ki-67 expression (20-50% stained nuclei, p= 0.013) and p53 staining higher than 30% stained nuclei (p= 0.0014). Such segmental variations regarding mainly p53 and to a lesser extent Ki-67 were seen within most of the specific sub-groups of patients (stratified by stage, grade, gender and age). An association between Bcl-2 expression and distal site was also observed among females (p=0.008).*

Conclusion: *Proximal and distal cancers displayed different clinicopathological and molecular patterns, reinforcing the proposal that they are genetically and biologically different entities. Potential clinical applications of these findings should be investigated.*

Key words: Bcl-2, colorectal tumors, distal, Ki-67, p53, proximal

Introduction

It has been well established that certain differences exist between proximal and distal CRC, concerning clinical manifestation and tumor's macroscopic picture. Epidemiological studies added further differences, according to age, gender, geographic area and race [1-3]. Proximal tumors were more common among elderly [1,2], females[1,2], areas displaying lower overall CRC incidence [3] and African – Americans [1,2]. Distal tumors were predominant in middle age [1,2], males [1-3], and areas with higher cancer incidence [3]. Differential trends by site are also reflected in the proximal shift in the distribution of cancer within the large bowel in Western countries [3].

Genetic alterations during colorectal tumorigenesis were described and linked with phenotypic changes in a genetic model presented by Fearon and Volgestein [4] in 1990. Since then, accumulated findings from the molecular biology field [5-11] indicated two distinct genetic categories based on tumor location, as was firstly proposed by Bufil [12]. In his opinion, the different embryologic origins and other developmental and biologic differences of the right and left colon, may result in differential susceptibilities to neoplastic transformation. The prementioned epidemiologic findings support this aspect. Moreover, treatment trials revealed a site-related difference in the response to chemotherapy [13].

Specific markers such as p53 oncosuppressor gene [7-9,11,14-18], Ki-67 proliferative antigen [10,17-

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23], and Bcl-2 antiapoptotic oncogene [11,17,24-26], extensively studied about their role in the malignant process and their impact on the disease outcome (which, however, remains rather unclear [27]), have been also found showing varying distribution [7-11,14,16,18] and prognostic significance [11,17,23] of their immunohistochemical expression or mutation according to tumor site. Other segmental differences of CRC regarding disease stage [1,2,28] and grade of differentiation [9,28] have been reported as well, although the former could be attributed to the tendency of proximal tumors to be less symptomatic at an early stage and/or inadequate screening strategies, leading to delayed diagnosis [1,28].

In this study we compared proximal and distal CRC in terms of both molecular (Ki-67, p53 and Bcl-2 expression) and clinicopathological features (stage and grade) looking for arguments supporting the hypothesis that they are biologically different entities.

Methods

The hospital records of 147 unselected, consecutive patients operated on for CRC in the 2nd Surgical Department of Tzaneion Hospital, Piraeus, from 2000 to 2003 were retrospectively analyzed. Cases with disease recurrence, synchronous tumors of double location, hereditary cancer, insufficient tissue for analysis and unclear pathology reports, were excluded, finally leaving 119 eligible patients (69 males and 50 females, with a mean age of 69.3 years). At the chosen period, none of them had received pre-operative chemoradiotherapy, being a homogeneous sample of sporadic, firstly diagnosed, untreated cases.

Immunohistochemistry

Five micrometer thick sections were taken from paraffin embedded tissue blocks of primary tumor specimens. Immunoperoxidase method was then performed in 3 steps, using Envision DAKO Kit (Glostrup, Denmark). Monoclonal antibodies MIB-1 (dilution 1:100, DAKO), DO-7 (dilution 1:100, DAKO), and Bcl-2 clone 124 (dilution 1:80, DAKO) were used for the assessment of Ki-67, p53 and Bcl-2, respectively. The enzymatic activity was revealed with the use of diaminobenzidine (DAB 0.6%) as chromogen and tissues were counter-stained with hematoxylin. Tumors with known Ki-67 and p53 status were used as positive controls whereas a normal lymph node served the same purpose for Bcl-2. Pre-immune rabbit serum was used as negative control to test for non-specific staining.

Analysis of expression status

Immunoreactivity was independently evaluated by two pathologists, blinded to clinical information, by assessing the percentage of positively immunostained tumor cells. Discrepancies were resolved by consensus. Areas displaying the less intratumoral heterogeneity in their staining pattern where chosen for scoring, avoiding section margins and areas of poor morphology [14,19]. Any lesion with ≥ 5% cells showing distinctly visible staining (nuclear for Ki-67 and p53, cytoplasmic for Bcl-2), even of relatively weak staining intensity, was considered positive.

In more detail, Ki-67 expression was scored as low (<20% stained cells), moderate (20-50%) and high (>50%), while p53 expression was classified as negative (<5%), low (5-30%), moderate (31-60%) and high (>60%). Finally, Bcl-2 expression was determined as negative (<5%) and positive (≥5%). Satisfactory reproducibility of staining and assessment methods was ascertained in 30 cases, stained and evaluated a second time.

The selection of the cut – off values for each particular marker was based on corresponding categorizations from other studies [15,18,21-26], despite the lack of uniformity in criteria used for the assessment of immunohistochemical findings [27]. The chosen thresholds were previously found as best showing prognostic [15,22,23,25], clinicopathological [26], and molecular [18,23,25] correlations of the examined markers. Classification in more than two staining groups for Ki-67 and p53 was considered as a proper approach for more accurate detection of site-specific differences of CRC exhibiting particular expression levels of these markers. A similar application for Bcl-2 was hampered by the relatively lower proportion of positive stains.

Clinicopathological classification

Patients were classified according to the results of their pathology reports as stage I, II, III, IV (TNM classification), and grade I (well differentiated), II (intermediately differentiated) and III (poorly differentiated). They were also classified by gender (males/females) and age (<70/≥70 years).

Statistical analysis

Cases were divided into proximal or distal in relation to the splenic flexure [2,9,11]. Clinicopathological and immunohistochemical data were then analyzed according to this division. Additional site-specific comparisons concerning Ki-67, p53 and Bcl-2 expressions

were performed within each particular stage, grade, gender and age group. Grade was also separately compared between proximal and distal cancers with early or advanced stage. Since all comparing variables were (or turned into) categorical, x^2 test (with Yates correction when necessary) and Fisher's exact test were used as appropriate for this analysis. Wilcoxon test was selectively used to analyze abnormal continuous distributions within certain categories. All p-values were two-sided. Statistical significance was set at the level of 0.05.

Results

The clinicopathological, demographic and immunohistochemical characteristics of the patients are presented in Table 1. In summary, there were 62 cases of early cancer (stages I and II) and 57 cases of advanced disease (III and IV). Most tumors (103/119; 86.5%) displayed intermediate differentiation (G_2). Finally, the observed frequencies of positive staining for Ki-67, p53 and Bcl-2 were 100%, 70.6% and 46.2%, respectively. Typical immunostainings are shown in Figure 1. Overall, 36 (30%) proximal cancers were compared to 83 (70%) distal lesions, for each one of the examining parameters and the results are listed in Table 2.

Poorly differentiated (G_3) carcinomas were more frequent in proximal than in distal site (19.5 vs. 2.5%; p= 0.005). Conversely, tumors with moderate (G_2) differentiation were predominantly distal (94 vs. 70%; p=0.001; Table 2). After stratification by stage, these segmental variations were seen only in advanced disease (stages III and IV pooled together; Figure 2). On the other hand, there were no significant site–related differences regarding stage (Table 2) or gender and age distribution. Proximal vs. distal male percents and mean age values were 55 vs. 59% and 70.2 vs. 69 years, respectively. Also, there was no segmental variation by site for any particular age subset (<50, 50-59, 60-69, 70-79, ≥80).

Table 1. Demographics, clinical, histological and immunohistochemical characteristics

Characteristics	*Number of patients (n=119)*	*%*	*Comments*
Tumor location			
Right	36	30	Cecum 13 (11%) Ascending 15 (12.5%) Transverse 8 (6.5%)
Left	83	70	Descending 15 (12.5%) Sigmoid 35 (29.5%) Rectum 33 (28%)
Gender			
Male	69	58	
Female	50	42	
Age (years)			
<70	56	47	Mean: 69.3
≥70	63	53	Median: 71 Range: 32-90
TNM stage			
I	12	10	
II	50	42	
III	44	37	
IV	13	11	
Grade			
I	7	6	
II	103	86.5	
III	9	7.5	
Positive immunohistochemical expression			
Ki-67	119*	100	Low expression 7 (6%) Moderate expression 50 (42%) High expression 62 (52%)
p53	84	70.6	Low expression 18 (15%) Moderate expression 36 (30%) High expression 30 (25%)
Bcl-2	55	46.2	

*Detected in all patients

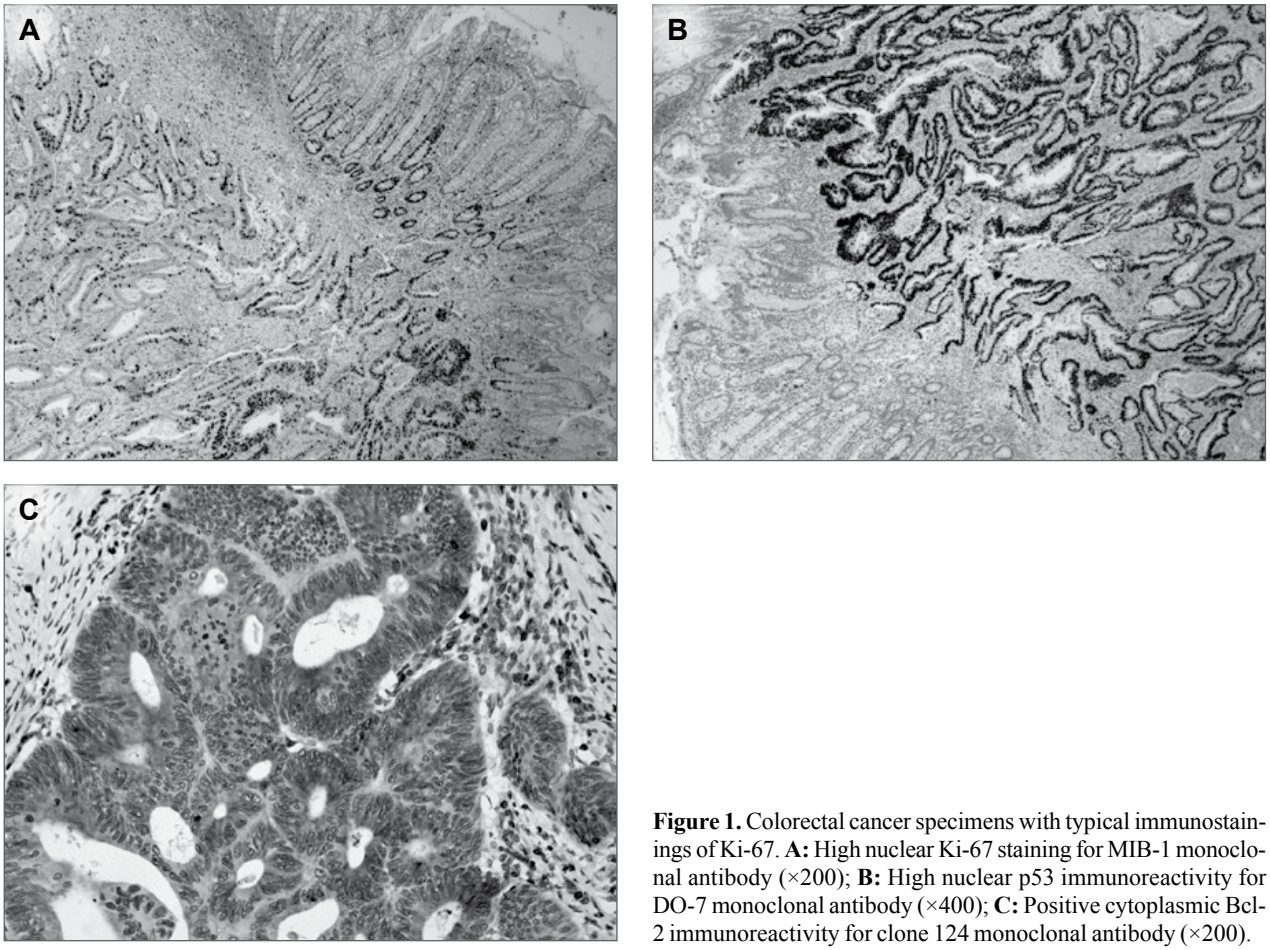

Figure 1. Colorectal cancer specimens with typical immunostainings of Ki-67. **A:** High nuclear Ki-67 staining for MIB-1 monoclonal antibody (×200); **B:** High nuclear p53 immunoreactivity for DO-7 monoclonal antibody (×400); **C:** Positive cytoplasmic Bcl-2 immunoreactivity for clone 124 monoclonal antibody (×200).

Table 2. Comparison between proximal and distal adenocarcinomas for stage, grade, p53, Ki-67, and Bcl-2

Parameter	*Right side*		*Left side*		*Total*	*p-value*
	n=36	*%*	*n=83*	*%*	*n=119*	
Stage						
I	3	8.5	9	11	12	NS
II	13	36	37	44.5	50	NS
III	15	41.5	29	35	44	NS
IV	5	14	8	9.5	13	NS
Grade						
I	4	11	3	3.5	7	NS*
II	25	69.5	78	94	103	0.001*
III	7	19.5	2	2.5	9	0.005*
Ki-67						
Low expression (<20%)	5	14	2	2.5	7	0.026**
Moderate expression (20-50%)	9	25	41	49.5	50	0.013
High expression (≥50%)	22	61	40	48	62	NS
p53						
Negative expression (<5%)	15	41.5	20	24	35	NS
Low expression (5-30%)	9	25	9	11	18	0.048
Moderate expression (31-60%)	5	14	31	37.5	36	0.011***
High expression (>60%)	7	19.5	23	27.5	30	NS
Bcl-2						
Negative expression (<5%)	20	55.5	44	53	64	NS
Positive expression (≥5%)	16	44.5	39	47	55	

*x^2 Yates, **Fisher's exact test, ***P-value from the comparison of overall proximal and distal distributions for p53 expression. Corresponding p-values from overall comparisons for the other parameters are not shown, as they were not significant (Stage) or because the use of x^2 was inappropriate, owing to number limitations (Grade, Ki-67). NS: non significant

Ki-67 expression was found to be significantly different between right and left sided CRC in both groups of low and moderate immunoreactivity. There was a superiority of proximal lesions in the former (14 vs. 2.5%; p=0.026) contrasting the preponderance of distal lesions in the latter (49.5 vs. 25%; p=0.013). Tumors with high immunoreactivity showed an insignificant trend for proximal site (62 vs. 48%; Table 2). However, this was reversed regarding lesions with very high expression values (>80% stained nuclei) displaying an almost significant predilection for distal site. The reversion of this trend appeared for lesions with staining level >80% and was progressively increased along with the staining level. When comparison was restricted to the higher expression group (62 cases) proximal vs. distal frequencies for tumors with Ki-67 expression ≥90% were 9 vs. 30%, respectively (p=0.06).

At subgroup analysis (Table 3), prevalence of proximal tumors with low Ki-67 staining was recorded for cases with advanced stage, intermediate differentiation (G_2) and younger age (p=0.046, 0.043 and 0.02, respectively). Predominance of distal tumors in the moderate expression group was ascertained in G_2 tumors (p=0.012), older patients (p=0.02), males (p=0.016) and was marginally suggested in advanced stage (p=0.054). Lack of any significant segmental difference in all particular subgroups was noted for tumors with high Ki-67 expression. Also, there was no association between site and staining of any level in females, or within particular stages (data not shown).

As for p53, overall proximal staining distribution was significantly different from the corresponding distal (p= 0.011; Table 2). In particular, there was a preponderance of right sided lesions in the group of lower expression, while left-sided predominated in the group of

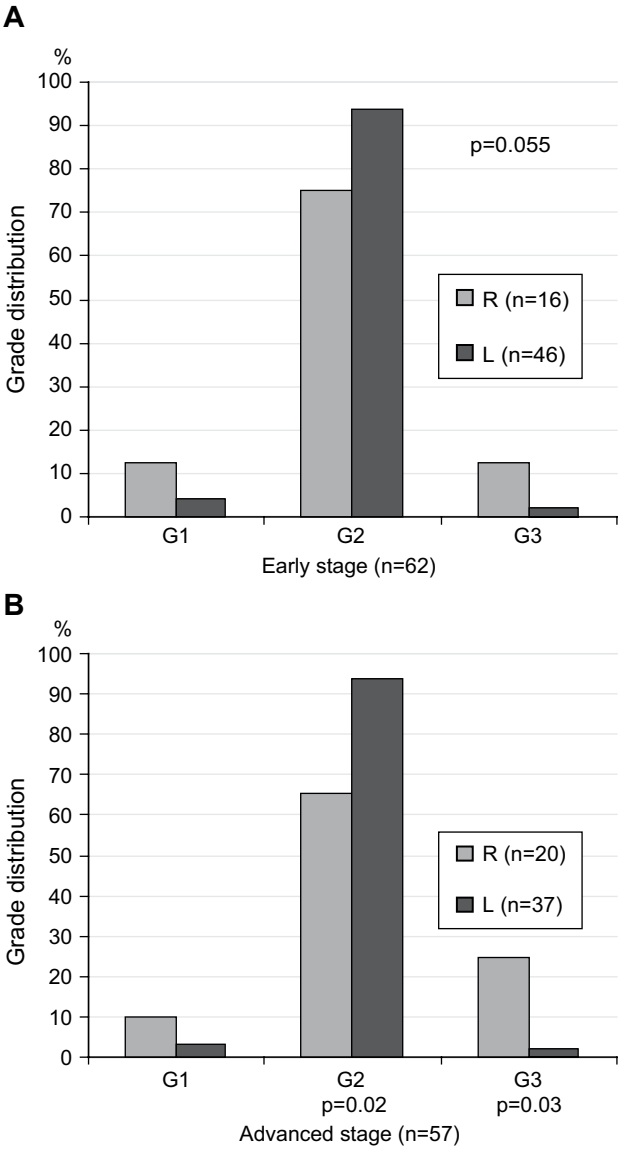

Figure 2. Grade distribution by site in early **(A)** and advanced stage **(B)**. The vertical columns represent the frequencies of particular grades (G_1, G_2, G_3) in right (R) and left (L) - sided tumors. The observed differences, though existing in both stage groups, were significant only in advanced stage (p=0.02 and 0.03 for G_2 and G_3 respectively - x^2 Yates).

Table 3. Ki-67 comparison within stage, grade, gender and age subgroups

Parameter	*Staining groups*	*Right*	*Left*	*p-value*
Stage				
Early (I+II)	Low	1	1	NS*
(n=62)	Moderate	4	22	NS
	High	11	23	NS
Advanced (III+IV)	Low	4	1	0.046*
(n=57)	Moderate	5	19	0.054
	High	11	17	NS
Grade				
G1 (n=7)	Low	2	–	NS*
	Moderate	1	2	NS*
	High	1	1	NS*
G2 (n=103)	Low	3	1	0.043*
	Moderate	5	38	0.012
	High	17	39	NS
G3 (n=9)	Low	–	1	NS*
	Moderate	3	1	NS*
	High	4	–	NS*
Gender				
Males (n=69)	Low	3	1	NS (0.07)*
	Moderate	5	28	0.016
	High	12	20	NS
Females (n=50)	Low	2	1	NS*
	Moderate	4	13	NS
	High	10	20	NS
Age (years)				
<70 (n=56)	Low	3	0	0.02*
	Moderate	5	19	NS
	High	8	21	NS
≥70 (n=63)	Low	2	2	NS*
	Moderate	4	22	0.02
	High	14	19	NS (0.06)

*Fisher's exact test, NS: non significant

moderate expression (p=0.048 and 0.011, respectively). Using only the level of 30% stained nuclei as threshold, we found exactly two thirds (24/36, 66%) of proximal tumors with lower staining values and a similar proportion (54/83, 65%) of distal tumors displaying higher expression levels (p= 0.0014). Specific analysis (with the same threshold) confirmed the presence of this trend within stages II, III and IV (p=0.03, 0.04 and 0.035, respectively), advanced disease (p=0.008), G_2 tumors (p=0.002), all gender and age groups (Table 4), and also within tumors Bcl-2 (+), Bcl-2(-) and those with Ki-67 staining <50% (p=0.02,0.011 and 0.0008). For these 3 molecular subsets comparative (proximal vs. distal) frequencies of tumors with p53 expression >30% were 19 vs. 52%, 45 vs. 77% and 7 vs. 60%, respectively.

On the other hand, Bcl-2 was almost uniformly expressed in both proximal and distal adenocarcinomas in the entire series (Table 2). However, a dual expression pattern was observed in relation to gender; among males, tumors with positive expression of Bcl-2 were prevalently proximal, although the difference was marginally non-significant (p=0.053), while among females the majority of Bcl-2 (+) stains were distal lesions (p= 0.008; Figure 3).

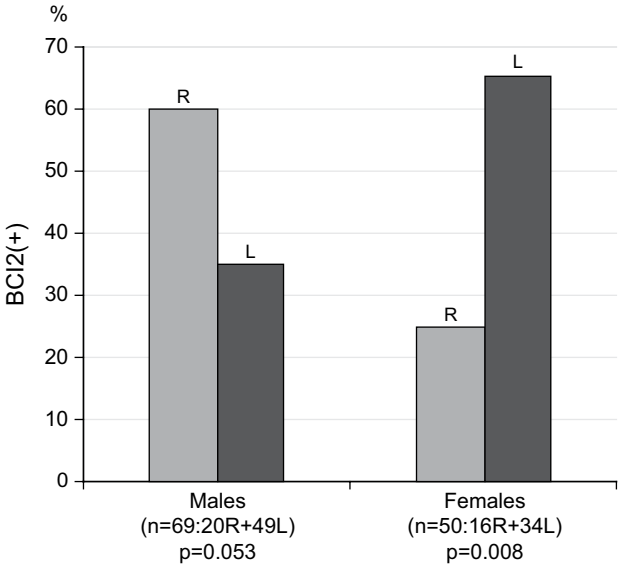

Figure 3. Bcl-2 expression by site in males and females. The vertical columns represent the frequencies of Bcl-2 positivity in right (R) and left (L) - sided tumors. A dual pattern of differences was observed according to gender, but the significance of these findings was restricted in females (x^2, p= 0.008).

Discussion

Molecular biology findings [5-11], strongly suggest that distinct site-related tumorigenic pathways are probably responsible for the differences observed between proximal and distal CRC [12]. A significant part of right-sided lesions is considered to follow the DNA instability mechanism (microsatellite instability – MSI), almost exclusively observed in proximal colon [8,13,29-31]. CIMP (CpG island methylator phe-

Table 4. p53 comparison within stage, grade, gender and age subgroups (with 30% stained nuclei as cut-off point)

Parameter	*Staining groups (% stained nuclei)*				*p-value*
	Right (n=36)		*Left (n=83)*		
	≤30%	*>30%*	*≤30%*	*>30%*	
Stage					
I (n=12)	1	2	4	5	NS*
II (n=50)	9	4	13	24	0.03
Early stage (I+II)	10	6	17	29	NS (0.07)
III (n=44)	11	4	2	17	0.04
IV (n=13)	3	2	–	8	0.035*
Advanced stage (III+IV)	14	6	12	25	0.008
Grade					
G1 (n=7)	3	1	1	2	NS*
G2 (n=103)	17	8	26	52	0.002
G3 (n=9)	4	3	2	–	NS*
Gender					
Males (n=69)	13	7	19	30	0.047
Females (n=50)	11	5	10	24	0.009
Age (years)					
<70 (n=56)	11	5	13	27	0.013
≥70 (n=63)	13	7	16	27	0.03
Overall (n=119)	24	12	29	54	0.0014

*Fisher's exact test, NS: non significant

notype) epigenetic pathway –frequently related but not restricted to MSI - also shows clear proximal predilection [30,31]. On the other hand, for left-sided tumors, chromosomal instability mechanism (CIN), predominant throughout the large bowel, has been incriminated for the great majority of them [6,9,31].

Alterations of p53 tumor suppressor gene have been proved to participate in the latter mechanism and consequently occur more frequently in distal than in proximal carcinomas [7-9,11,14,16,18,32-34] by a factor being in the range of 1.5-3 fold [31]. Our results were within this range, given that 65% of distal tumors showed moderate or high p53 expression while the corresponding proportion of proximal lesions was almost the half (33%). This site-specific pattern of p53 expression was an almost consistent finding among various patient groups. In this regard, other investigators found p53 alterations unrelated to any patient features with the exception of the tumor location (preferentially distal) [8,16,32], a fact that strongly supports the concept of an indigenous molecular differentiation between right and left-sided adenocarcinomas [31,33]. This is also suggested by the varying prognostic and predictive significance of p53 alterations according to tumor site [11,17,33].

As for Ki-67, our data indicated proximal superiority among lesions with lower expression values, distal predominance in the moderate staining group and absence of differences for tumors with higher expression, thus including previously reported conflicting trends regarding segmental differences in proliferative activity [10,11,21]. Notably, a similar pattern was recorded for p53 (Table 2), possibly attributable to existing correlations between these markers, as previously reported [11,18,23,35]. It remains to be ascertained whether the concurrent variation of the observed segmental differences according to staining level is reflective of a corresponding genetic variation, given that both markers have been associated with LOH (loss of heterozygosity) [7] or aneuploidy [11,14,17]. Indeed, segmental differences in the frequency of LOH have been found depended on its level [36], in a fashion similar to that seen in our study for Ki-67 and p53. Also, the frequency of tumors with high LOH status was found not substantially differing by site [37], which seems to fit well with our results for tumors with high Ki-67 or p53 expression.

Bcl-2 antiapoptotic protein has been generally found unrelated to tumor site [11,24-26,34], although such link was reported for other antiapoptotic markers [34]. In our study, specific predilections of Bcl-2 positivity for proximal or distal tumor location (though undetectable in the entire cohort) were particularly observed in males and females, respectively. Interestingly, segmental differences in p53 expression, being significant in both genders, were more striking in females, whereas those of Ki-67 were seen only in males. These findings possibly suggest that different hormonal influences on proximal and distal carcinogenic processes (implied by the reported different gender distribution by site [1,2,28] and the segmental predilection in the protective effect of female hormones on CRC) [8,31], may be specifically reflected in the expression of particular markers. Validation of this link could be useful in patients' selection for hormonal therapy (based on tumor site, gender and molecular status).

The tendency of proximal tumors to present at a higher stage, previously reported by ourselves [38] and others [1,2,28,39,40] was not observed in this study. However, a worse histological profile of them consistent with previous findings [9,28,33,38-41] was well documented. Impressively, at subgroup analysis both histological and molecular differences, retained their significance only in advanced disease (Figure 2, Tables 3,4). Reported associations of grade, Ki-67 and p53 with each other [10,11,18,23] and with higher stage [20,33,41], may partially account for these results. However, that similar segmental differences were previously found in early stage [11,38] is opposed to a connection of them with disease progression and warrants additional investigation.

The relatively small sample - though of similar size to other relevant studies [9,11,16,34,37] - is a limitation possibly affecting the validity of findings in some particular subsets. However, our cohort was clinicopathologically comparable to other large series [28,33] and results were generally consistent with previous data (from studies with similar methodology-Table 5) regarding overall [10,15,18,21,23,26,34] and segmental [9,11,14,18,34] molecular distribution. Moreover, the use of multiple thresholds (for Ki-67 and p53) revealed segmental differences selectively connected with particular staining levels, probably undetectable otherwise.

Confirmation of our findings in a larger sample could be combined by survival analysis, despite the existing controversy on the issue [9,13,33,39,40], possibly due to the wide methodological variability [27] and the conventional consideration of CRC as a single disease [31,33]. Such site-specific analysis preferentially focused on particular stages might be useful in determining patients at higher risk for relapse and best candidates for adjuvant treatment. Indeed, a large meta-analysis indicated that for Dukes C tumors with mutated p53, only those with proximal location had survival benefit from 5-FU chemotherapy [33]. The impact of tumor site on the prognostic significance of Ki-67 and Bcl-2 has been also reported [11,23,34].

Table 5. Results of relevant studies*

First author	*Number of cases*	*Stage*	*Antigen*	*Threshold (% staining level)*	*Immunohistochemical results (% positive cases)*			*Segmental difference*
					Ki-67	*p53*	*Bcl-2*	
Current study	119	I-IV	MIB-1 DO-7 Clone-124	5 5 5	100	70	46	Yes (for Ki-67 and p53)
Han 2006 [26]	81	II-IV	DO-7 Clone-124	5 5	–	63	40	No
Hilska 2005 [23]	363	I-IV	MIB-1 DO-7 Clone-124	1 1 5	91	74	68	Not reported
Krajewska 2005 [34]	106	II	MIB-1 DO-7 Clone-124	Any staining Any staining Any staining	87	70	Not reported	Yes (for p53)
Allegra 2002 [18]	465	II-III	MIB-1 DO-7	Any staining 10	100	60	–	Yes (for p53)
Buglioni 2001 [14]	94	II	DO-7	Any staining	–	52	–	Yes
Gervaz 2001 [9]	120	II	DO-7	10	–	47	–	Yes
Robinson 1999 [10]	100	I-IV	Anti-Ki-67 (polyclonal)	Any staining	100	–	–	Yes
Biden 1999 [25]	71	I-IV	DO-7 Clone-124	5 5	–	88	75	No

*Studies following similar methods (in terms of antigens and thresholds) with our work and - also - investigating segmental differences were included in this Table.

In conclusion, our study, although exploratory and retrospective, provided arguments in terms of histological and molecular differences, reinforcing the concept that proximal and distal cancers are distinct biological entities. However, these differences were found varying according to staining level, stage and gender, indicating a complicated interaction between these variables and tumor location and warranting further investigation to elucidate these correlations and to determine potential implications in the clinical course and treatment.

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