

Disparate clinicopathological correlations of p53 and Bcl-2 in colorectal cancer

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Abstract. p53 and Bcl-2 (both regulators of cell apoptosis) have been considered to be involved in the initiation and progression of the colorectal tumorigenic process, respectively. In this study, we investigated their association with tumor stage and grade –both substantially affecting prognosis. Immunohistochemical assessment of p53 and Bcl-2 was retrospectively conducted (using DO-7 and Ca-124 monoclonal antibodies, respectively) in 119 surgically resected colorectal carcinomas, and the results were correlated to tumor stage and grade. The proportion of tumors positively stained was 70% for p53 and 46% for Bcl-2, whereas co-expression of both markers was observed in 28% of cases. Tumors exhibiting the highest p53 staining (>60% stained cells) were more frequently found in disease stage IV ($p=0.03$), while Bcl-2 positivity showed a predilection for earlier stage ($p=0.02$) and better grade ($p=0.028$). The associations of both markers with stage, along with a reciprocal relationship between p53(+) and Bcl-2(+) tumors ($p=0.02$), stronger for cases with p53 staining >30% ($p=0.007$), remained significant only for distal tumors. The distinct correlations of p53 and Bcl-2 with disease progression and aggressiveness (being influenced by the extent of staining and tumor site) may be clinically useful in the determination of high-risk colorectal cancer cases.

Introduction

The high incidence and mortality rates of colorectal cancer (CRC) (1), particularly in the West, warrant increasing research regarding the mechanisms involved in CRC tumorigenesis. A step-wise genetic model (proposed by Fearon and Vogelstein in 1990) (2), comprising the genetic alterations of

certain oncogenes and tumor-suppressor genes through the major chromosomal instability (CIN) pathway (3), has been considered as the tumorigenic mechanism responsible for most (approximately 85%) of CRC cases.

Inactivation of the p53 tumor-suppressor gene is believed to be a late-event in this model, leading to disruption of the integrity of the genome (2-4). Normally, the intact gene (wild-type) activates the cell-apoptotic mechanism in response to extensive DNA damage, thus preventing the creation of potentially malignant cells (4,5). However, apoptosis is a complicated process, regulated by several genes, including the Bcl-2 proto-oncogene and other members of its family (6). Bcl-2 has been considered to participate in the early steps of CRC tumorigenesis, blocking cell apoptosis, thus facilitating the appearance of subsequent genetic events in this process (7-9).

Both markers, p53 and Bcl-2, have been extensively studied for their potential impact on the outcome of the disease (7-14). However, results have been inconclusive (14-16). One possible reason for this – apart from methodological factors (15,16) and the heterogeneity of CRC (11) – could be that the association between these markers and the conventional prognostic parameters remains unclear. p53 has been correlated with higher stage and poor grade (11,12,17,18); conversely, Bcl-2 has been linked to lower stage and better grade (12,17,19). Notwithstanding, these trends are generally inconsistent, as indicated by reviewing studies (6,10,14). However, defects in apoptosis contribute to tumor progression, allowing survival of malignant cells and facilitating metastasis (6,13). Therefore, apoptosis-related markers are theoretically expected to display an association with clinicopathological indicators of disease dissemination and aggressiveness (i.e., stage and grade).

Toward a better understanding of this relationship (if any), we conducted an immunohistochemical study of p53 and Bcl-2 gene products (i.e., proteins) in a series of CRC, including all disease stages.

Materials and methods

Study population. Hospital records of 147 cases that underwent surgery for CRC between 2000 and 2003 at the Second Surgical Department of Tzaneio Hospital of Piraeus were

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retrospectively inspected. After the omission of recurrences, hereditary cases and those with unclear pathology reports or insufficient tissue for analysis, 119 patients (69 males, 50 females; mean age 69.3 years) were finally enrolled in the study, incorporating a homogenous sample of primary, sporadic and untreated cases (without neoadjuvant therapy – not performed during the chosen study period).

Immunohistochemistry. Sections (5- μ m) were obtained from paraffin-embedded tissue blocks of primary tumor specimens. We performed the immunoperoxidase method in three steps, using the Envision DAKO kit (Dako, Glostrup, Denmark) and monoclonal antibodies DO-7 (dilution 1:100; Dako), and Bcl-2 clone 124 (dilution 1:80; Dako) for the assessment of p53 and Bcl-2, respectively. Diaminobenzidine (DAB; 0.6%) was used as chromogene, and tissues were counter-stained with hematoxylin. A tumor specimen with known p53 status and a normal lymph node served as positive controls for p53 and Bcl-2, respectively, whereas pre-immune rabbit serum was used as the negative control.

Staining interpretation. Immunoreactivity was independently evaluated by two observers (blinded to clinical information) and discrepancies between them were resolved by consensus. Any lesion with $\geq 5\%$ cells – showing distinctly visible staining (nuclear for p53 and cytoplasmic for Bcl-2) – was considered positive.

p53 expression was additionally scored as negative ($<5\%$), low (5-30%), moderate (31-60%) and high ($>60\%$). This classification was based on previous studies reporting prognostic, clinicopathological and molecular correlations of p53 using these thresholds (7,13,14,17-20). A similar categorization for Bcl-2 was deemed inappropriate because of the relatively lower proportion of positive staining (see Results). However, the chosen threshold (5%) was also found to reveal clinicopathological and molecular associations of this marker (17,19,21), whereas the previous use of multiple stratification did not provide any additional information (17,19). Staining intensity, considered as a less objective criterion, was not scored.

Clinicopathological classification. Cases were classified according to the results of the pathology report as stage I, II, III and IV (TNM classification), and Grade 1 (G1, well differentiated), 2 (G2, moderately differentiated) and 3 (G3, poorly differentiated), (WHO classification). The cases were also classified by site, as proximal and distal (in relation to the splenic flexure).

Statistical analysis. The distribution of p53 and Bcl-2 expression among various stage and grade categories was analyzed using the χ^2 test (with Yates correction when necessary) and Fisher's exact test (appropriate for categorical comparisons between small subsets). We also examined the inter-relationship between p53 and Bcl-2 using the same methods. All tests were two-sided, with p-values ≤ 0.05 considered as significant.

Results

General characteristics. Both clinicopathological and immunohistochemical features of the examined sample are listed

Table I. Clinicopathological and immunohistochemical features.

Feature	No. of patients (n=119) n (%)
Tumor site ^a	
Right	36 (30.0)
Left	83 (70.0)
Gender	
Male	69 (58.0)
Female	50 (42.0)
Age (years)	
<70	56 (47.0)
≥ 70	63 (53.0)
Mean	69.3
Median	71
Range	32-90
TNM stage	
I	12 (10.0)
II	50 (42.0)
III	44 (37.0)
IV	13 (11.0)
Grade	
Well (G1)	7 (6.0)
Moderate (G2)	103 (86.5)
Poor (G3)	9 (7.5)
Positive staining	
p53	84 (70.6)
Low expression	18 (15)
Moderate expression	36 (30)
High expression	30 (25)
Bcl-2	55 (46.2)
Combination of markers	
p53(+)/Bcl-2(+)	33 (28.0)
p53(-)/Bcl-2(-)	13 (11.0)
p53(+)/Bcl-2(-)	51 (43.0)
p53(-)/Bcl-2(+)	22 (18.0)

^aSplenic flexure was the division point. Rectal tumors (33 cases; 28%) were included in the distal group.

in Table I. Briefly, moderate grade (86.5%), distal tumor site (70%) and male gender (58%) were the prevailing characteristics, whereas cases were almost uniformly distributed between early (I-II) and advanced (III-IV) stage. The observed frequencies of positive staining were 70.6% for p53 and 46.2% for Bcl-2. Co-expression of markers was ascertained in 33 cases (28%), while the majority of tumors (62%) showed a reciprocal staining pattern [p53(+)/Bcl2(-) or p53(-)/Bcl-2(+)]. Typical immunostainings of both markers are presented in Fig. 1.

Staining distribution by stage and grade. As indicated in Table II, p53 expression values exceeding the defined thresholds (i.e., 5, 30 and 60% stained nuclei) were more frequently

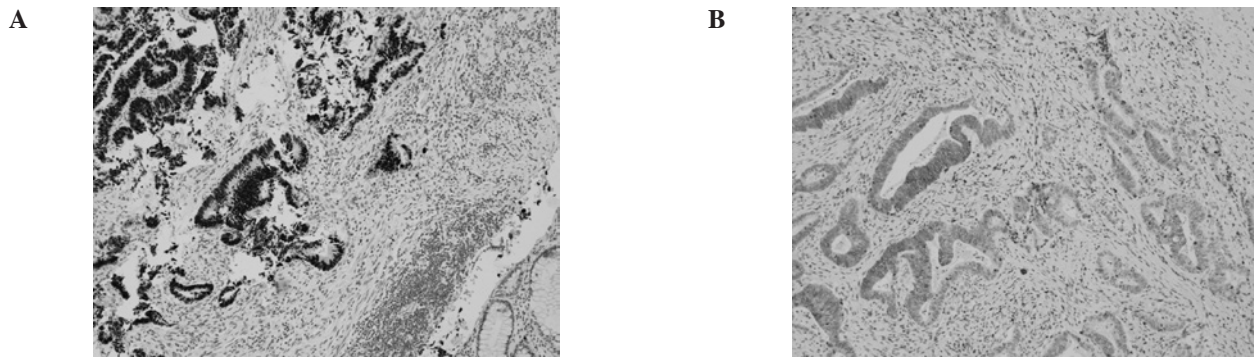


Figure 1. p53 and Bcl-2 immunostaining in CRC specimens. (A) Positive nuclear p53 immunoreactivity for DO-7 antibody (x400). (B) Positive cytoplasmic Bcl-2 staining for clone 124 antibody (x200).

Table II. p53 and Bcl-2 staining distribution by stage and by grade.

A, Stage						
Staining (% stained cells)	Total (n=119)	I (n=12)	II (n=50)	III (n=44)	IV (n=13)	p-value
p53						
>5%	84 (70%)	8 (66%)	36 (72%)	29 (65%)	11 (84%)	NS ^a
>30%	66 (55%)	7 (58%)	28 (56%)	21 (48%)	10 (77%)	NS (0.09) ^a
>60%	30 (25%)	5 (42%)	10 (20%)	8 (18%)	7 (54%)	0.03 ^a
Bcl-2(+)						
>5%	55 (46%)	3 (25%)	31 (62%)	15 (34%)	6 (46%)	0.02 ^b
B, Grade						
Staining (% stained cells)	Total (n=119)	G1 (well) (n=7)	G2 (moderate) (n=103)	G3 (poor) (n=9)		p-value
p53						
>5%	84 (70%)	4 (57%)	74 (72%)	6 (66%)		NS ^c
>30%	66 (55%)	3 (43%)	60 (58%)	3 (33%)		NS ^c
>60%	30 (25%)	2 (28%)	26 (25%)	2 (22%)		NS ^c
Bcl-2(+)						
>5%	55 (46%)	1 (14%)	53 (51%)	1 (11%)		0.03 ^c

^aStage IV vs. others. ^bp-value for overall Bcl-2 distribution by stage. Significant p-values for particular stages were 0.003 (stage II vs. others), 0.04 (stage III vs. others) and 0.049 (stage I-II vs. III-IV). ^cPoor grade vs. others. NS, not significant.

found in stage IV than in all other stages. However, this trend was significant only for tumors exhibiting p53 expression >60% (p=0.03). At subgroup analysis, the same trend was particularly observed in the distal subset and was found significant for tumors (of this location) exhibiting p53 staining >30 or >60% (p=0.026 and 0.008, respectively; Table III).

Bcl-2(+) expression (i.e., ≥5% stained cells) was found to widely vary by stage (p=0.02 for overall staining distribution; Table II). In particular, Bcl-2 positivity was more likely observed in tumors with disease stage II (p=0.003) and early stage (I-II) as well (p=0.049; details not shown). Conversely, tumors with stage III or with advanced disease (III-IV), were less likely to exhibit Bcl-2(+) staining (p=0.04 and 0.049, respectively). Notably (and similarly with the results for p53)

these findings remained either significant or marginal only for distal tumors (p=0.013 for stage II, 0.009 for stage III and 0.052 for early vs. advanced disease; Table III).

Regarding grade (Table II), there was lack of any significant difference in the observed p53 staining values between well, moderately and poorly differentiated tumors. By contrast, the proportion of Bcl-2(+) expression in poorly differentiated lesions was significantly lower than that in the other grade categories (11 vs. 49%; p=0.03).

Moreover, there was no significant relationship between the observed combinations of markers and disease stage (Fig. 2A). However, a total lack of tumors exhibiting the p53(-)/Bcl-2(+) staining pattern was recorded in stage IV (0 vs. 21% for stages I-III; p=0.054). A corresponding lack of tumors

Table III. p53 and Bcl-2 staining distribution by stage for distal tumors.^a

Staining (% stained cells)	Total (n=83)	Stage I (n=9)	Stage II (n=37)	Stage III (n=29)	Stage IV (n=8)	p-value
p53						
>5%	63 (76%)	6 (66%)	28 (75%)	21 (72%)	8 (100%)	NS ^b
>30%	54 (65%)	5 (55%)	24 (65%)	17 (59%)	8 (100%)	0.026 ^b
>60%	23 (27%)	3 (33%)	9 (24%)	5 (17%)	6 (75%)	0.008 ^b
Bcl-2(+)						
>5%	39 (47%)	3 (33%)	23 (62%)	8 (28%)	5 (62%)	0.013 ^c 0.009 ^d

^aA similar analysis for proximal tumors (36 cases) failed to yield significant findings. ^bStage IV vs. others. ^cStage II vs. others. ^dStage III vs. others. NS, not significant.

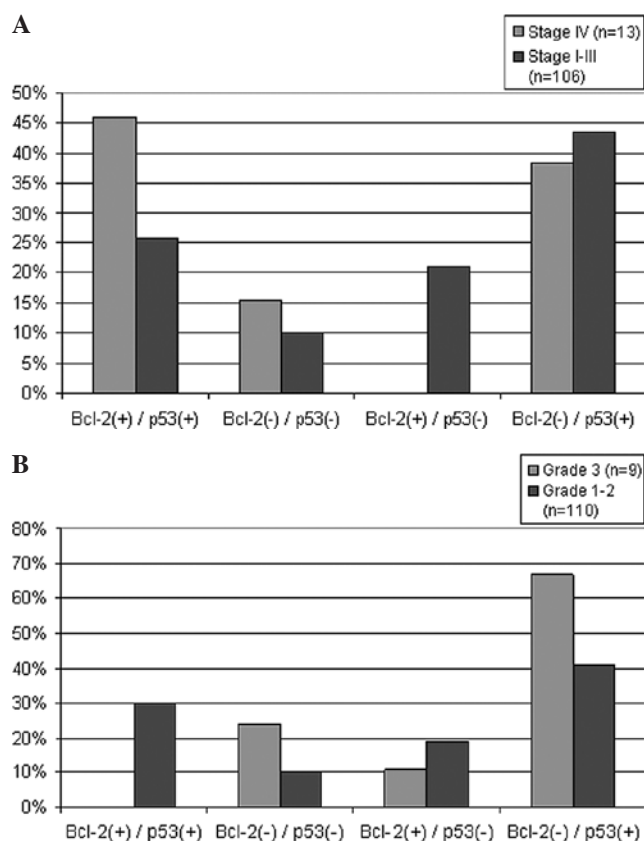


Figure 2. Distribution of various combinations of p53 and Bcl-2 by (A) stage and (B) grade. (A) Differences in the frequency of marker combinations between stage IV and stages I-III. None of them was significant despite the total lack of Bcl-2(+)/p53(-) lesions in stage IV ($p=0.054$; Fisher's exact test). (B) Corresponding differences of these combinations between G3 and G1-2. As indicated, among poorly differentiated tumors, no cases co-expressing both markers were observed ($p=0.047$; Fisher's exact test).

co-expressing both markers was noted among poorly differentiated lesions (0 vs. 30% for grade 1-2; Fig. 2B). This trend was significant ($p=0.047$).

There were no other clinicopathological correlations of the examining markers, with the exception of a strong predilection of tumors with p53 expression >30% for the distal segment ($p=0.0014$; data not shown).

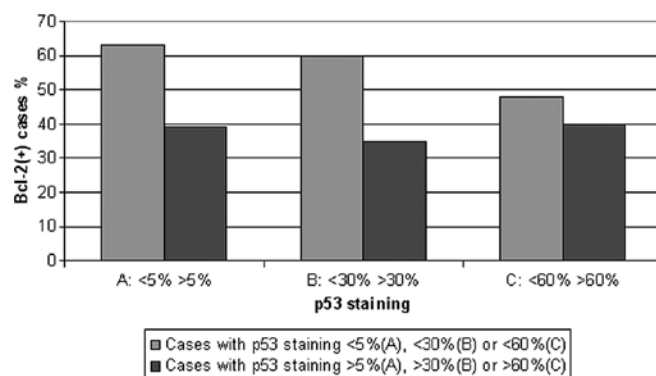


Figure 3. Relation between p53 and Bcl-2 expression. The fluctuation of Bcl-2 positivity according to p53 staining is presented. (A) p53 <5% (negative) vs. p53 >5% (positive), $p=0.02$. (B) p53 <30% (negative and low) vs. p53 >30% (moderate and high), $p=0.007$. (C) p53 <60% (negative, low and moderate) vs. p53 >60% (high), NS. p-value for the distribution of Bcl-2 among all p53 staining categories was 0.04 (χ^2 test).

Inter-relation between markers. Analysis of the incidence of Bcl-2 positivity among the various p53 staining groups (Fig. 3) revealed an inverse relationship between these markers; Bcl-2(+) expression was more likely observed among p53-negative rather than p53-positive tumors (63 vs. 39%; $p=0.02$). It was also more frequently detected in lesions displaying p53 expression <30% (negative and low) than in those with staining >30% (moderate and high) (60 vs. 35%; $p=0.007$). Notably, the significance of these findings was (again) maintained only in the distal subset ($p=0.02$ and 0.013, respectively; data not shown).

Table IV summarizes the main findings, providing a comprehensive picture of the ascertained clinicopathological and molecular correlations.

Discussion

The development of CRC has been considered a multistep process of genetic alterations providing selective advantage to abnormal cells, thus allowing tumor initiation and progression through the adenoma-carcinoma sequence (2,22). Bcl-2 overexpression has been connected with the former step, as implied by its higher detection rate in adenomas (6-8), whereas p53 overexpression has been associated with the latter ('late event') (2,4,5,7).

Table IV. Clinicopathological and molecular correlations of p53 and Bcl-2.

Marker/combination of markers	Clinicopathological/molecular correlation
p53(+)	Stage IV (for p53 staining >60%) ^a Distal site (for p53 staining >30%) Bcl-2 negative (for p53 staining >5% or >30%) ^a
Bcl-2(+)	Lower stage (II or I-II) ^a Better grade (well-moderate) ^b Negative or low p53 ^a
p53(+)/Bcl-2(+)	Better grade (well-moderate) ^c Trend for stage IV (NS)
p53(+)/Bcl-2(-)	Trend for poor grade (NS)
Bcl-2(+)/p53(-)	Trend for non-metastatic disease (I-III), marginally NS
p53(-)/Bcl-2(-)	-

^aAt subgroup analysis, these findings remained significant only for distal tumors. ^bSignificance was abolished in both sites. ^cSite-specific analysis was not performed for any marker combination, because of the small size of these subsets (see Table I). NS, not significant.

Our data seem to support the concept of distinct roles for p53 and Bcl-2 in CRC tumorigenesis; while the majority (70%) of carcinomas displayed p53 positivity, the corresponding proportion of positively stained tumors for Bcl-2 was only 46%. This disparity in the observed detection rate is consistent with previous findings (7,9,12,17,20).

Our results also demonstrated the distinct correlations of p53 and Bcl-2 with tumor stage and grade (12,17-19,23-25). Bcl-2(+) lesions showed a trend for earlier stage and better grade, a finding supporting the connection of this marker with early tumorigenic steps (7,12,17,19) and also with a favorable prognostic impact (8,12,13,19). That Bcl-2 negativity is linked with higher stage and poor grade could be clinically useful; Bcl-2(-) cases with stage II (but not stage III) CRC displayed a higher recurrence rate and shortened survival, being a high-risk subset within this particular stage (26).

On the other hand, the consistently recorded trend of tumors with stage IV for higher frequencies of p53 expression in all examined staining categories, being significant for lesions showing the highest immunoreactivity, may imply a connection of this marker with tumor dissemination and subsequently with worse outcome. Our observation that such an impact (if any) appears to be staining-dependent (i.e., stronger for tumors with high p53 expression) perhaps explains literature discrepancies on the issue (10,11,14), attributable (in part) to the wide variability in thresholds used to define p53 positivity. Among several studies using different cutoffs and reporting the presence (9,12,18,23) or absence (14,17,20) of a correlation between p53 and stage, only two (17,18) followed the multiple stratification of p53 staining (as we did). Therefore, despite the detected, in that way, significant associations of p53 with either higher stage (18) or poor grade (17), more research is required

to determine the clinicopathologically optimal categorization of p53 immunoreactivity.

The reported inverse relationship between p53 and Bcl-2 (7,9,12,25) was also ascertained here (Fig. 3); significantly higher frequencies of Bcl-2 positivity were recorded among tumors showing either negative or negative and low p53 staining. By contrast, lesions displaying either positive or higher than 30% staining values of p53 usually lacked Bcl-2 expression. Notably, at subgroup analysis, these findings along with the previously described associations of both markers with disease stage remained significant only for distal tumors. The loss of significance for proximal tumors (if not random – attributable to the smaller size of this subset) possibly reflects the existence of a segmental disparity in the biological behavior of CRC. That the overexpression of both p53 – more consistently (10-14) – and Bcl-2 – occasionally (27) – have been associated with distal tumor site, may contribute to these results. Such segmental predilection is expected for markers involved in certain steps of the adenoma-carcinoma sequence (7,8,12), the dominant tumorigenic mechanism in CRC (2,22), followed by the large majority of distal tumors. By contrast, a significant part of proximal tumors, evolving through the genetically different microsatellite instability pathway and probably arising from different precursor lesions (serrated polyps) (3,28), usually lacked p53 and Bcl-2 expression (19,27,28). This assumption is also suggested by the reported site-specific prognostic significance of p53 and Bcl-2 (9,13).

Investigation of the combined effect of Bcl-2 and p53 on disease progress (Fig. 2) revealed a total lack of the Bcl-2(+)/p53(-) combination in stage IV, suggesting a lower malignant potential for tumors with this molecular profile, in agreement with previous findings (7,12). By contrast, the reported aggressiveness (in terms of higher stage and grade) (7,12) for lesions with the alternative reciprocal combination [Bcl-2(-)/p53(+)] was not confirmed here. Notwithstanding, these tumors showed a trend for poor differentiation (67% for G3 vs. 41% for G1-2) which, although not significant, is suggestive of a less indolent behavior.

Moreover, the total lack of the Bcl-2(+)/p53(+) phenotype among poorly differentiated lesions (as anticipated by the favorable pattern accompanying Bcl-2 positivity) was in contrast to the higher frequency of this combination in stage IV (46 vs. 25% for all other stages), consistent with the establishment of a worse pattern for tumors with high p53 expression. That all p53(+)/Bcl-2(+) cases in stage IV belonged to this particular p53 category, possibly explains this result, suggesting that high p53 immunoreactivity may be connected with disseminated disease, even in the presence of Bcl-2 positivity. If so, elevated p53 staining could be clinically useful as a warning signal for metastatic predisposition even for CRC cases with lower stage (for instance stage II), warranting further and more detailed investigation and consistent follow-up (for the detection of occult metastases). Of note, the recurrence rate after curative resection for CRC was found higher in early stage p53(+) cases than in advanced stage p53(-) cases, indicating the importance of the p53 status (29).

The limitations of this study, apart from the relatively small sample [however comparable to other relevant reports (7,8,12,13,17-20)], are mostly related to the wide variability in the use of immunohistochemistry, particularly regarding

the interpretation of the results (15,16). However, our model (multiple stratification for p53 and single threshold for Bcl-2) has been previously validated, in terms of reproducibility and concordance between observers; as suggested by Zlobec *et al* (30), the high frequency and the wide range of p53 staining values – opposed to the relatively low rate and limited fluctuation of Bcl-2 positivity – warrant this scoring method. Moreover, the ascertained correlations of p53 with stage IV and Bcl-2 were (both) staining-dependent (recorded in particular expression levels). On the other hand, the lack of evaluation of staining intensity – in line with previous reports (12,27,31) – appears to have a rather minor impact on the results, considering that the intensity and the extent of staining were found to be associated with each other (12) and to show equal statistical correlation with tumor recurrence (32). It remains to be ascertained whether our stratification (with the chosen thresholds) is prognostically better than other previously used systems and cutoffs (8,9,13,26).

In conclusion, p53 and Bcl-2 were found related to CRC stage and grade; while the former marker emerged as a potential indicator of metastatic disease, the latter was connected with lower stage and better grade. These disparate relations seem to fit well with the ascertained reciprocity between p53 and Bcl-2. Moreover, the level of staining (at least for p53) appears to be essential in revealing the existing connection with stage. Finally, the observed impact of tumor location on almost all findings warrants further investigation in a larger sample. The utility of these findings, could, in the future, involve the ability to identify high-risk CRC cases on the basis of their molecular profile, with the assessment of a specific marker or combinations of markers.

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