

The pattern of epidermal growth factor receptor variation with disease progression and aggressiveness in colorectal cancer depends on tumor location

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Abstract. The role of epidermal growth factor receptor (EGFR) in colorectal cancer (CRC) prognosis remains unclear despite the recent development of anti-EGFR treatments for metastatic disease. The heterogeneity of CRC may account for this discrepancy; proximal and distal CRC has been found to be genetically and clinicopathologically different. The aim of this study was to investigate the effect of tumor location on the association of EGFR with the conventional prognostic indicators (stage and grade) in CRC. Immunohistochemical assessment of EGFR was retrospectively performed in 119 primary CRC specimens and data were correlated with tumor stage and grade in the proximal and distal tumor subset. The molecular combination of EGFR with p53 (previously assessed in this sample) was similarly analyzed. EGFR positivity was detected in 34, 30 and 35% of the entire cohort, proximal and distal tumors, respectively. The pattern of EGFR clinicopathological correlation was found to differ by site. A reduction in the frequency of EGFR(+) with progression of stage and/or worsening of grade was observed proximally, whereas an opposite trend was recorded distally. Proximal tumors with stage I or with indolent features (stage I, well-differentiated) exhibited a significantly higher proportion of EGFR positivity than other tumors of this location ($p=0.023$

and $p=0.022$, respectively) or corresponding distal tumors ($p=0.018$ and $p=0.035$, respectively). Moreover, the co-existence of EGFR and high p53 staining (accounting for 11% of cases) was found in a significantly higher proportion of stage IV tumors compared to other stages ($p=0.004$), although only for the distal subset. Proximal and distal tumors showed various patterns of EGFR variation with disease progression and aggressiveness. This disparity provides further support to the hypothesis that these particular subsets of CRC are distinct tumor entities. It may also be suggestive of a potentially different therapeutic approach according to tumor site, particularly regarding anti-EGFR targeted treatment.

Introduction

Colorectal cancer (CRC) is one of the most common malignancies and remains a major cause of cancer mortality in the West (1). It is also a multi-pathway disease with disparate subgroups exhibiting distinct genetic and clinicopathological features, and probably different outcomes (2). This may be the main reason for the variability in treatment response observed among patients of the same disease stage. Therefore, a combination of the conventional TNM staging classification (at present, the major prognostic indicator) with certain molecular markers involved in CRC tumorigenesis, with verified prognostic and predictive impact, is one of the main objectives of research worldwide (3).

Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein member of the tyrosine-kinase receptor family, encoded by the *c-erbB1* proto-oncogene and is considered as a major regulator of several distinct cellular pathways. Activation of EGFR promotes carcinogenesis, by increasing proliferation, cell migration, angiogenesis and apoptosis inhibition (4-6). On this basis, targeted therapies using anti-EGFR antibodies and tyrosine-kinase inhibitors are now an approved treatment in metastatic CRC (7,8). However, immunohistochemically assessed EGFR expression has not been validated as a predictor of response to this specific treatment. Moreover, the impact of EGFR expression on the outcome of CRC

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Abbreviations: EGFR, epidermal growth factor receptor; CRC, colorectal cancer; G1, Grade 1; G2, Grade 2; G3, Grade 3; MSI, microsatellite instability; CIMP, CpG island methylator phenotype; CIN, chromosomal instability

Key words: proximal and distal colorectal cancer, epidermal growth factor receptor, stage, grade

patients is generally unclear (3). Methodological variability, indicated by the wide range (18-97%) in the detected frequencies of EGFR in CRC (9-14), may be responsible for this effect.

Heterogeneity of CRC (2) should also be taken into account since EGFR expression may be discordant among primary tumors, lymph nodes and metastases (10,11). It may also be related to tumor stage and grade, although the reported results on this issue are inconsistent (9-14). However, there has been limited attention regarding the association of EGFR with tumor site, despite the considerable molecular and clinicopathological differences between proximal (right-sided) and distal (left-sided) CRC (15-18), suggesting the existence of two distinct disease entities with different outcomes and treatment responses (19,20).

In this study, differences regarding the immunohistochemically assessed EGFR expression rate were examined in a series of CRC cases previously investigated for segmental differences in other molecular markers (18). We analyzed the correlation of EGFR with stage and grade (i.e., the conventional prognostic indicators) in the entire cohort and in the proximal and distal tumor subsets. We also examined the correlation between EGFR and the previously assessed p53 (18), considering the central tumorigenic role of the latter marker along with its known predilection for the distal tumor site (2,15,16,18).

Materials and methods

Study population. Hospital records of 147 unselected cases that underwent surgery for CRC between 2000 and 2003 in the Second Surgical Department of Tzaneio Hospital of Piraeus were retrospectively examined. Following the omission of recurrences, hereditary cases, synchronous cancers of double location and those with unclear pathology reports or insufficient tissue for analysis, 119 patients (69 males, 50 females; mean age, 69.3 years; range, 32-90 years) were included in the study, providing a homogenous sample of primary, sporadic and untreated cases. None of the cases had undergone neo-adjuvant therapy, as it was not performed during the selected study period at this hospital. The study was approved by the Surgical department of the Athens Medical School.

Immunohistochemistry. Sections (5 μ m) were obtained from paraffin-embedded tissue blocks of primary tumor specimens. The immunoperoxidase method was performed in three steps, using an Envision Dako kit (Glostrup, Denmark). EGFR was assessed with anti-EGFR mouse monoclonal antibody (dilution 1:200, Dako). Diaminobenzidine (DAB, 0.6%) was used as a chromogen and tissues were counter-stained with hematoxylin. Normal epidermis with a known EGFR status served as a positive control, whereas pre-immune rabbit serum was used as a negative control.

Staining interpretation. Immunoreactivity was independently evaluated by two observers (blinded to clinicopathological information) and discrepancies between them were resolved by consensus. Any lesion with distinctly visible staining [membranous and/or cytoplasmic (9,11,12)] was considered positive.

Multiple cutoffs and the scoring of staining intensity (a less objective criterion), or complex scoring (i.e., combining percentages with intensity) were avoided as they all potentially

increase interobserver variability (21). Moreover, multiple stratification, used for other markers (including p53) in our previous study (18), was inappropriate due to the relatively low proportion of EGFR positivity (see Results). The selected threshold was similar to that used in previous studies (1%), revealing strong prognostic and clinicopathological correlations of EGFR (10,12,13).

Clinicopathological classification. Cases were classified according to the results of their pathology report as stage I, II, III or IV using the TNM classification, and Grade 1 (G1, well-differentiated), 2 (G2, moderately differentiated) or 3 (G3, poorly differentiated) using the WHO classification. The cases were also classified by site, as proximal (cecum, ascending, transverse) and distal (descending, sigmoid, rectum), in relation to the splenic flexure (15-18).

Moreover, considering the small size of certain subsets and the fact that we aimed to examine the combined effect of stage and grade on EGFR distribution, we stratified tumors into three additional categories, modifying the corresponding classification previously implemented by Resnic *et al* (14): i) cases with at least one indolent feature (stage I or G1), ii) cases with at least one unfavorable feature (stage IV or G3) and iii) cases with intermediate tumor characteristics (stages II-III with moderate grade). Given the absence of tumors with completely conflicting features (stage I/G3 or IV/G1) in our sample, there was no need for exclusion of such cases.

Statistical analysis. The distribution of EGFR expression among various clinicopathological variables was analyzed using the χ^2 test (with Yates correction when necessary) and Fisher's exact test (appropriate for categorical comparisons between small subsets). EGFR distribution by stage and grade (or their combination) was separately examined in the proximal and distal subsets using the same tests. Moreover, on the basis of the previously recorded data for p53 (18), the distribution of the various molecular combinations between EGFR and p53 was similarly analyzed, particularly focusing on the pattern of tumors combining EGFR with a high p53 expression level (>60%). Tests were two-sided, with p values ≤ 0.05 considered to indicate a statistically significant difference.

Results

Clinicopathological parameters and immunohistochemistry. Table I shows that moderate grade (86.5%), stage II-III (79%) and distal tumor location (70%) were the prevailing features in this sample. Positive EGFR expression was detected in 40 cases (34%), with typical immunostaining shown in Fig. 1. EGFR positivity was almost uniformly distributed among the various clinicopathological subsets. Even for tumor grade, the apparently considerable variation of EGFR was not significant. No association was observed between EGFR positivity and the previously assessed (18) high p53 staining found in 25% of cases.

Pattern of staining variation by stage and grade was different for each segment. EGFR expression was slightly higher in the distal compared to the proximal site tumors (35 vs. 30.5%), but the difference was insignificant (Table I). However, the

Table I. Clinicopathological and immunohistochemical features.

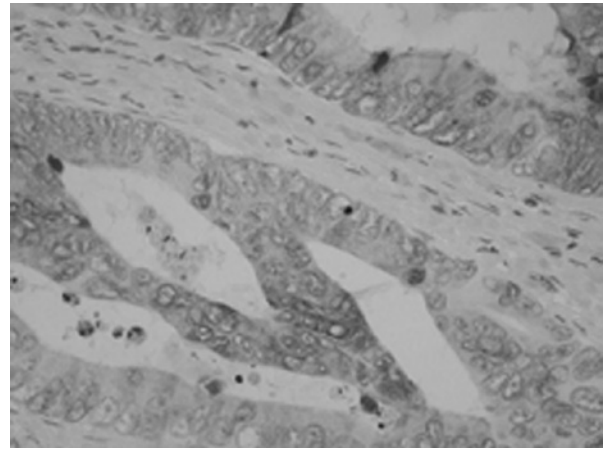
Parameters	Cases		EGFR(+)		P-value
	n	(%) ^a	n	(%) ^b	
TNM stage					NS
I	12	(10)	4	(33)	
II	50	(42)	16	(32)	
III	44	(37)	16	(36)	
IV	13	(11)	4	(31)	
Grade					NS
Well (G1)	7	(6)	4	(57)	
Moderate (G2)	103	(86.5)	35	(34)	
Poor (G3)	9	(7.5)	1	(11)	
Combined stage-grade					NS
Indolent ^c	16	(13.5)	6	(37.5)	
Intermediate ^d	82	(69)	29	(35)	
Unfavorable ^e	21	(17.5)	5	(24)	
Tumor site					NS
Proximal (right)	36	(30)	11	(30)	
Distal (left)	83	(70)	29	(35)	
Gender					NS
Male	69	(58)	25	(36)	
Female	50	(42)	15	(30)	
Age					NS
<70	56	(47)	19	(34)	
>70	63	(53)	21	(33)	
p53 staining					NS
High ^f	30	(25)	13	(43)	
Low ^g	89	(75)	27	(30)	
Total	119	(100)	40	(34)	

^aPercentages of the total number of cases. ^bPercentages of EGFR(+) for particular subsets. ^cStage I or G1. ^dStage II-III with G2. ^eStage IV or G3. ^fStaining level >60%. ^gStaining level ≤60%. EGFR, epidermal growth factor receptor. NS, not significant.

observed pattern of staining variation by stage and grade was markedly different for each particular segment (Table II); the frequency of EGFR in the proximal subset varied between 100% (stage I) and 0% (stage IV). Conversely, EGFR frequencies ranging from 11 (stage I) to 50% (stage IV) were observed distally. Subset analysis revealed: i) a significant difference of EGFR expression frequencies between stage I and the other stages (II, III and IV) of the proximal segment considered together ($p=0.023$) or separately ($p=0.04$, 0.07 , 0.02 , respectively); and ii) a significant difference of the EGFR staining frequency between proximal and distal tumors with stage I disease ($p=0.018$), whereas the corresponding segmental difference for stage IV did not reach the level of significance ($p=0.1$) (Table II, Fig. 2).

Moreover, a progressive reduction in EGFR frequency was observed with worsening of grade. This pattern was recorded for the overall series (from 57% in Grade 1 to 11% in Grade 3)

A



B

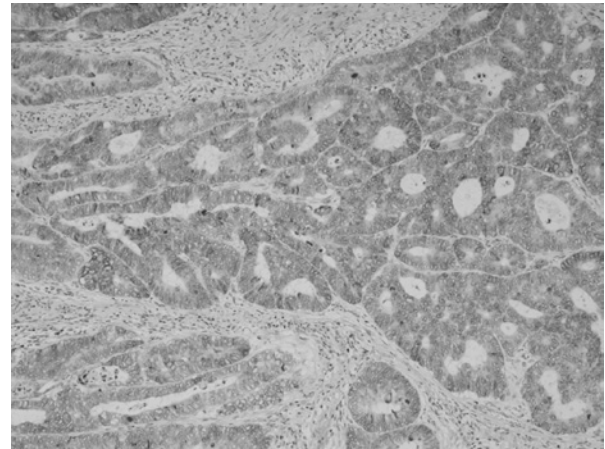


Figure 1. EGFR immunostaining. (A and B) Colorectal cancer specimens with representative immunostaining positive for EGFR monoclonal antibody (magnification, x200). EGFR, epidermal growth factor receptor.

and for the proximal subset (from 75% in Grade 1 to 14% in Grade 3) but was somewhat modified in the distal subset (Table II, Fig. 3). However, the observed differences of EGFR staining between particular grades (of the same segment), or between proximal and distal tumors of the same grade were not significant, although they approached the level of significance in certain comparisons within the proximal subset (G1 vs. G2-G3, $p=0.075$ and G1 vs. G3, $p=0.09$).

EGFR expression was examined in three additional tumor subsets including tumors with indolent (stage I or G1, 16 cases), unfavorable (stage IV or G3, 21 cases) and intermediate (stage II-III with moderate grade, 82 cases) clinicopathological features. The results of this analysis were similar to the previously ascertained findings regarding EGFR stage distribution; the indolent subset exhibited a significantly higher proportion of EGFR positivity compared with the other subsets (80 vs. 22%, $p=0.022$), although only for proximal cases. Additionally, the frequency of EGFR(+) was significantly higher proximally than distally for the indolent cases (80 vs. 37.5%, $p=0.035$; Table II and Fig. 4).

Molecular combinations were significantly elevated in stage IV. Although the tumor site was found to be unrelated to any molecular combination of EGFR with p53 (Table III),

Table II. EGFR segmental distribution by stage and grade.

	Proximal		Distal		P-value ^a
	n	EGFR(+) (%)	n	EGFR(+) (%)	
Stage					
I	3	3 (100)	9	1 (11)	0.018
II	13	3 (23)	37	13 (35)	NS
III	15	5 (33)	29	11 (38)	NS
IV	5	- (0)	8	4 (50)	0.1
Grade					
Well (G1)	4	3 (75)	3	1 (33)	NS
Moderate (G2)	25	7 (28)	78	28 (36)	NS
Poor (G3)	7	1 (14)	2	- (0)	NS
Combined stage - grade					
Indolent ^b	5	4 (80)	11	2 (18)	0.035
Intermediate ^c	20	6 (30)	62	23 (37)	NS
Unfavorable ^d	11	1 (9)	10	4 (40)	NS (0.12)
Total	36	11 (30.5)	83	29 (35)	NS

^aFor proximal vs. distal cases. ^bStage I or G1. ^cStage II-III with G2. ^dStage IV or G3. EGFR, epidermal growth factor receptor; NS, not significant.

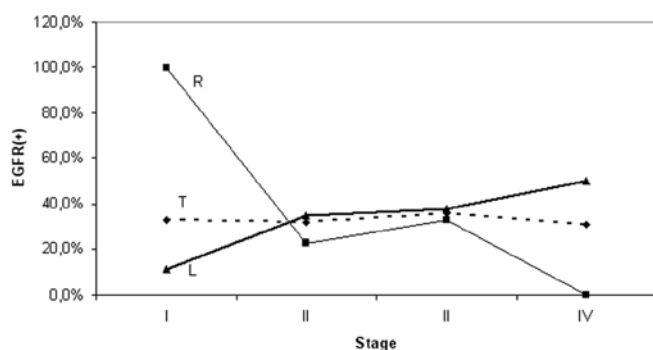


Figure 2. Variation of EGFR with stage. The frequency of EGFR positivity was reduced with disease progression for right-sided tumors (R). An opposite trend was shown by left-sided lesions (L). As a result, EGFR positivity was evenly distributed among stages of the total sample (T). Significant p-values of EGFR variation were recorded for right-sided stage I vs. other stages considered together (I vs. II-IV, $p=0.023$) or separately (I vs. II, III, IV/ $p=0.04$, 0.07 , 0.02 , respectively). EGFR, epidermal growth factor receptor.

cases with EGFR(+)/p53 high immunoreactivity (accounting for 11% of the total sample) were more frequently detected in stage IV than in other stages (31 vs. 8.5%, $p=0.051$). This trend was stronger and significant for distal tumors (50 vs. 8%, $p=0.004$) and completely absent for corresponding proximal tumors (Fig. 5).

Discussion

The involvement of EGFR activation in a number of cellular pathways promoting tumorigenesis may explain the benefit from the recently implemented anti-EGFR therapies (i.e., cetuximab or panitumab) (3,7,8). Nonetheless, the prognostic and predictive value of EGFR status in CRC remains uncertain (3). However, the effect of EGFR on prognosis and

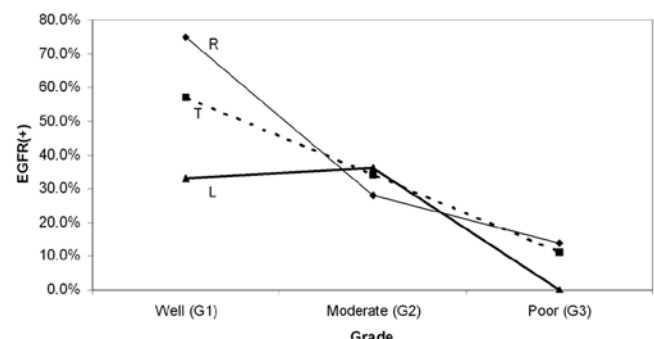


Figure 3. Variation of EGFR with grade. A progressive reduction of EGFR(+) cases with worsening of tumor grade is shown for the total sample (T) and the right-sided subset (R), with the two groups showing similar variation patterns. The corresponding pattern for left-sided tumors (L) was somewhat differential. None of the observed differences of EGFR positivity, between right and left subsets or between particular grades (G1, G2, G3) of the same category (R, L, T) were significant. However, the trend for EGFR(+) shown by the well-differentiated tumors approached significance for the right subset (G1 vs. G2-G3, $p=0.075$ and G1 vs. G3, $p=0.09$) and the total sample (G1 vs. G3, $p=0.08$). EGFR, epidermal growth factor receptor.

treatment response may vary among genetically different tumors; proximal and distal CRC have been considered to evolve through different genetic pathways [microsatellite instability/CpG island methylator phenotype (MSI/CIMP) and chromosomal instability (CIN), respectively] (15,16) with disparate clinicopathological features (17-19) and possibly different outcomes (19,20).

In the current study, we examined the impact of tumor site on EGFR distribution in particular clinicopathological variables. The observed variation in EGFR detection rate with disease progression (from stage I to IV) was found to differ between proximal and distal tumors, showing a reduction

Table III. Molecular combinations between EGFR and p53.

Marker	Total		Proximal		Distal		P-value ^a
	n	%	n	%	n	%	
EGFR(+)	40	(34)	11	(30)	29	(35)	NS
p53 high ^b	30	(25)	7	(19.5)	23	(28)	NS
Marker combination							
EGFR(+)/p53 high ^b	13	(11)	3	(8)	10	(12)	NS
EGFR(-)/p53 low ^c	62	(52)	21	(58)	41	(50)	NS
EGFR(-)/p53 high ^b	17	(14)	4	(11)	13	(15.5)	NS
EGFR(+)/p53 low ^c	27	(22.5)	8	(22)	19	(23)	NS
Total	119	(100)	36	(100)	83	(100)	

^aProximal vs. distal. ^bStaining level >60%. ^cStaining level ≤60%. EGFR, epidermal growth factor receptor; NS, not significant.

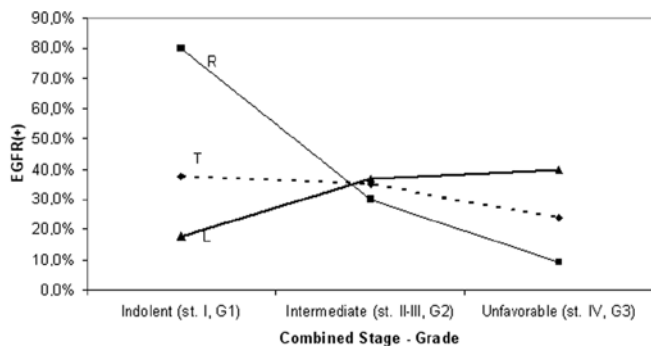


Figure 4. Variation of EGFR according to combined tumor characteristics (stage-grade). The pattern of EGFR variation with disease change from indolent to unfavorable was different for the right- (R) and the left- (L) sided lesions, revealing reductions and elevations of the EGFR detection rate, respectively. Corresponding EGFR variation in the total sample (T) was limited. Significant p-values were found for the observed variation in right-sided tumors (indolent vs. others, $p=0.022$ and indolent vs. unfavorable, $p=0.013$). EGFR, epidermal growth factor receptor.

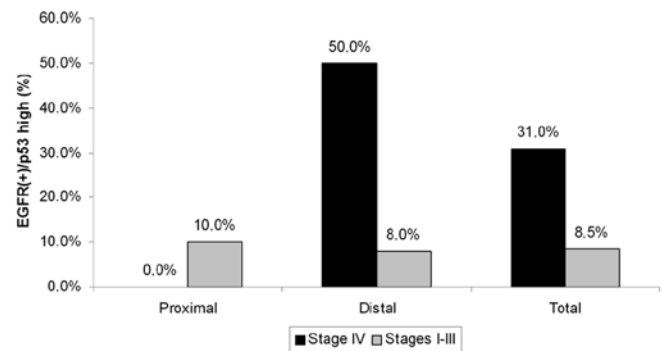


Figure 5. Variation of EGFR(+)/p53 high combination with stage. The frequency of the EGFR(+)/p53 high molecular combination was significantly higher in stage IV than in the other stages (I-III) for the total sample ($p=0.051$) and particularly for distal tumors ($p=0.004$). However, such a correlation was not observed in proximal tumors (actually revealing a lack of this combination among stage IV cases). EGFR, epidermal growth factor receptor.

and elevation of this rate, respectively. Proximal lesions also showed a similar decrease in the proportion of EGFR positivity with worsening of grade and, as expected, with the change of the combination of stage and grade from indolent to unfavorable. By contrast, for distally located tumors, the same change appeared to have the opposite effect (elevation).

Notably, these trends were not present in the entire cohort, with the exception of EGFR variation by grade, consistent with previous results (11). This lack of EGFR correlation with stage and grade has been also reported by other authors (9,14,22), whereas inconsistent findings are presented among studies suggesting such connections (10,12,13). Therefore, a separate investigation of proximal and distal CRC appears to be necessary for a more accurate determination of the effect of EGFR status on the progression, aggressiveness and, probably, the outcome of the disease. In this respect, the fact that EGFR status has failed to predict response in metastatic cases that underwent anti-EGFR therapy may be partially explained by the observed rarity of EGFR positivity in proximal metastatic

disease. Corresponding rarity of EGFR(+) in cases with poor grade may also be relevant (11); aggressive lesions are more commonly found in advanced stages, as well as at the proximal site (23).

Moreover, tumors with the EGFR(+)/p53 high molecular combination, exhibited a predilection for stage IV, which was particularly pronounced in the distal subset. This observation may be explained by the reported connection of p53 inactivation with both distal site (15,18,24) and higher stage (24), particularly stage IV (25). Nevertheless, the observed trend for metastatic disease (if validated) could be clinically useful, facilitating the selection of cases for chemotherapy and/or anti-EGFR therapy, based on combined EGFR/p53 status and tumor location. Notably, as recently reported, p53 mutation may predict response to cetuximab treatment (26), suggesting that p53 inactivation is likely one of the mechanisms leading to EGFR activation, as indicated by the 90% concordance between p53 mutations and the EGFR copy number increase (26).

Given that, at present, sufficient evidence of prognostic and predictive significance for any single marker is lacking, including EGFR and p53 (3,27), the potential usefulness of marker combinations appears to be a more promising approach (28,29). In this context, and as regards EGFR, it has recently been reported that the effectiveness of anti-EGFR therapy in metastatic CRC is decreased in cases with Ki-Ras (30) or BRAF and PTEN mutations (31). The impact of the tumor site on these findings should also be examined, considering reported associations of Ki-Ras mutations with metastatic disease and worse outcome, particularly detected in distal tumors (32,33).

A limitation of our study is that the main findings were detected in small subsets (stage I, IV - Grade 1, 3). However, despite the modest size of our sample, our results were similar to those of several relevant studies (9,10,12-14,22). Although we confirmed these results in the expanded additional subsets [created by combining stage and grade: interrelated features differentially representing tumor growth potential (34)], further investigation in a larger sample is necessary. Another limitation is the long-standing filing of paraffin blocks (7-10 years), which is shown to reduce EGFR immunoreactivity (35). Such an effect may explain the decreased EGFR(+) detection rate in our sample (34%) compared to those seen in other studies with similar thresholds (12,22,36), ranging from 50 to 97%. However, Galizia *et al* (13), using a similar cut-off value, found almost equal frequencies of EGFR(+) (35%), whereas other authors (10,37) reported even lower rates (18 and 21.5%, respectively).

However, the simplicity of our methodology in EGFR staining interpretation minimizes interobserver variability, facilitates reproducibility and is appropriate for samples of this size, with an observed EGFR(+) detection rate (34%). However, in larger samples, or in those with higher detection rates and a wide range in the observed percentages of positivity, the use of multiple thresholds or complicated scoring systems may provide better information (21).

Moreover, our data indicated the importance of separate segmental analysis in revealing clinicopathological correlations of EGFR; the uniform distribution of this marker among stages in the entire cohort was resulted (mostly) from the combined effect of the opposite trends in EGFR variation with disease progression recorded for proximal and distal subsets. Similarly, the counteraction between the indolent and the unfavorable tumor subsets, exhibiting different segmental predilections of EGFR positivity (for proximal and distal site, respectively), contributed to the observed lack of segmental difference in overall sample, perhaps explaining the reason for such differences having rarely been reported (38). Even more detailed analysis may be necessary; in particular, colon segments (cecum, ascending and sigmoid) have recently been found showing distinct clinicopathological features (39), possibly reflecting underlying molecular disparities.

In conclusion, in this exploratory study we found that the pattern of EGFR variation with disease progression and/or aggressiveness differed according to tumor location. Although these results support that proximal and distal CRC are different disease entities, their potential impact on prognosis and treatment should be investigated. Additional investigations may include: i) meta-analyses of selected EGFR immunohisto-

chemical studies with, preferably, similar methodology; ii) large retrospective site-specific analyses of EGFR predictiveness in patients receiving anti-EGFR treatment; and iii) corresponding prospective studies. In future, therapy decisions may be based on the combined clinicopathological and molecular tumor status, possibly including EGFR and tumor site.

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