

Immunohistochemical expression of the epidermal growth factor receptor (EGFR) in colorectal carcinoma: relation with clinicopathological parameters

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ABSTRACT: **Introduction:** The study of tissue immunostaining of the epidermal growth factor receptor (EGFR) may contribute with the understanding of its role in the prognosis of colorectal carcinoma. **Objective:** To analyze the immunohistochemical expression of EGFR in colorectal carcinoma tissues and transitional tumor-mucosa and mucosa adjacent to neoplasia, and its relation with cancer. **Method:** The study was conducted with 40 patients with colorectal carcinoma who had surgery with curative intent in order to analyze the immunoexpression of EGFR with anti-EGFR. We used parametric and nonparametric tests. **Results:** The immunohistochemical expression of EGFR in tumor samples showed a significant difference as to the level of immunostaining in tissue specimens of transitional tumor-mucosa ($p=0.01$) and the level of immunoreactivity in tissues of the adjacent mucosa ($p=0.04$). The immunoexpression of EGFR showed no significant relation with the size of the tumor, angiolymphatic invasion, neural invasion, cellular differentiation, level of carcinoma infiltration in the intestinal wall, lymph node metastases and liver metastases. **Conclusions:** The EGFR showed a more intense expression in the mucosa of colorectal carcinoma than in the transitional epithelium and adjacent non-neoplastic mucosa. The immunoexpression of EGFR did not correlate with pathological parameters of colorectal carcinoma and liver metastases.

Keywords: genes, erbB-1; receptor, epidermal growth factor; immunohistochemistry; colorectal neoplasms; neoplasm metastasis.

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INTRODUCTION

The incidence of colorectal carcinoma is increasing in western countries and in Brazil, colorectal carcinoma is the third most frequent^{1,2}. The colorectal neoplasm staging is still the most reliable prognostic factor; however, such information is not usually available at the preoperative period³.

In order to decide whether or not to submit the patient who had surgery for colorectal carcinoma to postoperative chemotherapy, it is important to select the patients with unfavorable prognosis, especially those with advanced lesions; in such cases, the expression of tumor markers at the neoplastic tissue may be useful for this purpose⁴⁻⁶.

Different tissue markers are described in literature, however, only a few are relevant in relation to the clinical treatment of the patient⁷⁻⁸. In colorectal neoplasms, ideally, tissue markers should be altered according to tumor staging, besides serving as prognosis and helping to define the need for complementary therapy^{7,9-12}. Even for those patients submitted to primary disease resection with curative intent, postoperative recurrence is a common cause of death¹³⁻¹⁵.

The protein family of the epidermal growth factor (EGF) includes groups of receptors and growth factors that are structurally related¹⁶⁻²⁰. Many of these proteins, which are highly expressed in human colon cancer cell line, are connected to EGF receptors (EGFR), and have an important role in the growth of colorectal carcinoma¹⁷⁻¹⁸.

EGFR tissue expression may be immunohistochemically determined by the connection of EGF with the tumor membrane^{17,21-25}. As to the gastrointestinal tract, EGFR expression is usually more intense for tumors than for regular tissues²⁶⁻²⁹.

EGFR tissue hyperexpression indicates an unclear clinical prognosis²⁸⁻²⁹, suggesting the evolution of the colorectal carcinoma and its metastatic potential²⁸⁻³¹. EGFR hyperexpression was reported in 25 to 82% of the patients with colorectal cancer, and proved to be predictive of distance metastases for patients with advanced staging²⁸⁻³². However, the impact of this finding in the prognosis is still controversial.

Studies failed to show the relation between the EGFR expression and the clinical efficiency of the target therapy³³⁻³⁶. Such difference brought to life some

explanations regarding this event, including tumor heterogeneity, low sensitivity to the method that detects EGFR and the lack of a standard methodology related to the studies^{33,37-38}.

It is important to define the factors that can be used to identify the patients who have favorable response to EGFR inhibitors, and so a treatment can be chosen³⁹.

The objectives of this study were to analyze EGFR expression by the immunohistochemical technique in the colorectal carcinoma tissue, in the transitional tumor-mucosa, and in the tissue of the mucosa adjacent to the neoplasm, and correlate it with clinicopathological aspects, clinical staging and metastases in patients who had surgery for colorectal carcinoma.

METHODS

This study was approved by the Research Ethics Committee of *Universidade Federal de São Paulo* (UNIFESP), protocol n. 0344/09, and the committee of *Instituto de Assistência Médica ao Servidor Público Estadual* (IAMSPE), protocol n. 056/08.

From October 2005 to March 2007, 40 patients presenting with colorectal carcinoma had surgery with curative intent at the Surgical Gastroenterology Service at *Hospital do Servidor Público Estadual de São Paulo* (IAMSPE). Out of these patients, 20 (50%) were males and 20 (50%) were females. Mean age was 68.7±11.6 years (44 to 90 years).

In this study, inclusion criteria were: the presence of colorectal carcinoma confirmed by histopathological analysis and lesion with curative intent.

Exclusion criteria were: patients aged less than 18 years, those with bowel inflammatory disease, neoplasm in other organs or lesion healing in a palliative way.

Preoperative staging was performed by complete clinical and Physical exam, serum determination of carcinoembryogenic antigen (CEA), colonoscopy with biopsy and histopathological analysis of the lesion, thoracic and abdominal CTscan.

The most common surgery was rectosigmoidectomy, performed in 24 patients (60%), followed by total colectomy, in 5 patients (12.5%), left colectomy, in 5 patients (12.5%), right colectomy in 5 patients (12.5%) and abdominoperineal rectal amputation in 1 patient (2.5%).

For the histopathological study, three specimens were obtained: one in the central area of the tumor, in order to avoid ulcers or necrosis; another in the transitional region between the neoplasm and the macroscopically non-tumoral area with microscopic confirmation of this transitional area, and a third sample of the adjacent mucosa located 10 cm from the lesion. All surgical specimens were previously fixed in formalin 10% and included in paraffin blocks.

Three 4 µm cuts were made in each block to obtain neoplastic areas, transitional area of mucosa neoplasm, macroscopically non-tumorous, and the area that is macroscopically neoplasm free. All specimens were stained with hematoxylin-eosin (HE) for the microscopic analysis and the verification of neoplastic compromise of resected lymph nodes and surgical margins.

Paraffin blocks were cut with 3 µm of width, and the blades were submitted to ABC immunohistochemistry (Avidin-Biotin-Peroxidase Complex) with anti-EGFR primary antibody in the dilution 1:30 (mouse monoclonal anti human epidermal growth factor receptor – EGFR, lot 3360, clone H11, Dako Cytomation, EUA).

A positive reaction to the EGFR antibody was considered when the color brown appeared on the cytoplasmic membranous area of the cell. Positive controls were normal cuts of the tonsil germinal center. For the blades used as negative control, the primary antibody reaction was removed.

An experienced pathologist analyzed the blades with a binocular microscope by Nikon, with planachromatic objectives. At first, the hot spots were selected with 100x zoom; afterwards, with 400x zoom, analyzing ten consecutive fields. EGFR immunoreaction represented by the color brown, both in the cytoplasm (Figure 1) and in the cytoplasmic membrane (Figure 2) of neoplastic cells, was analyzed in a semiquantitative way, according to the criteria proposed by Kountourakis et al.⁴⁰. In the cytoplasm, immunoreaction was classified as 0: without staining, or <10% of the neoplastic cells with low intensity staining; +: >10% of the cells with low intensity staining; ++: >10% of the cells with medium intensity staining; +++: >10% of the cells with strong intensity staining. At the cytoplasmic membrane, the immunoreaction was classified as 0: without staining; +: <10% of neoplastic cells with

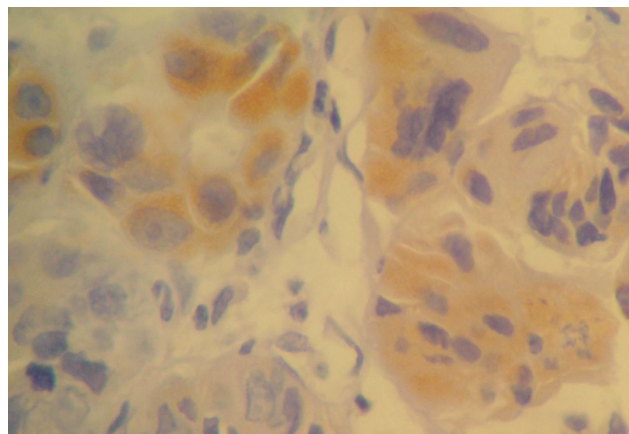


Figure 1. Immunoreaction of the epidermal growth factor receptor in neoplastic cells with medium intensity cytoplasmic pattern (immunohistochemistry; 400x).

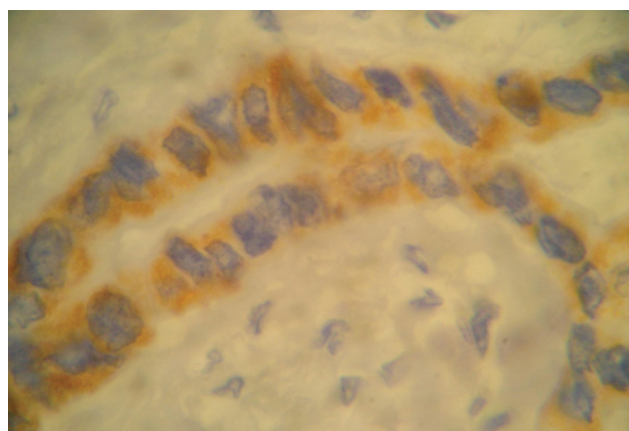


Figure 2. Immunoreaction of the epidermal growth factor receptor in neoplastic cells of membranous pattern and strong intensity (immunohistochemistry; 400x).

any rate of intensity, or <30% of the cells with weak intensity staining; ++: 10-30% of medium to strong intensity staining or 30-50% of cells with low to medium intensity staining; +++: >30% of the cells with strong intensity staining or >50% of neoplastic cells with any staining intensity.

EGFR immunosuppression was analyzed in the tumor, in the transitional non-neoplastic tumor-mucosa and in the mucosa that is 10 cm adjacent to the neoplastic lesion.

In order to analyze the results, patients were divided into two groups: group 0, which showed no EGFR immunoreaction, and group 1, with EGFR immunoreaction, regardless of its intensity.

Data referring to quantitative variables were presented by average. Categorical variables were analyzed by the Mann-Whitney test, and the correlation was observed by the Spearman test. The variance analysis of the samples of three or more groups was obtained by the Kruskal-Wallis test. Dicotomic variables were analyzed by the Fisher's exact test.

The statistical software used was Prism 4.0 (GraphPad Software Inc., USA), and the significance level was lower than 0.05 or 5%.

RESULTS

The mean size of colorectal neoplasm was 4.25 ± 2.1 cm (1.0 to 9.0 cm). The lesions of 21 patients had ≤ 5.0 cm in diameter, while 19 patients (47.5%) presented neoplasm with > 5.0 cm of diameter. Twelve (30%) patients presented neoplastic vascular invasion at clinicopathological examination, while 28 (70%) patients did not have vascular invasion. Fourteen (35%) patients presented lymphatic vascular invasion at clinicopathological examination, while 26 (65%) patients did not have lymphatic vascular invasion. Seven (17.5%) patients presented perineural invasion at clinicopathological examination, while 33 (82.5%) patients did not have neoplastic perineural invasion.

Lymph nodes were affected by the colorectal carcinoma in 19 patients (47.5%). For other 21 patients (52.5%), the lymph nodes were negative.

According to cell differentiation, 31 (77.5%) patients had moderately differentiated adenocarcinoma, 2 (5.0%) had a little differentiated adenocarcinoma, and 7 (17.5%) had a well differentiated carcinoma.

The lesion penetrated the serous (peritoneum) without the invasion of adjacent structures (spleen, stomach, liver, diaphragm, pancreas, abdominal wall, adrenal, kidney, small intestine and retroperitoneum) (T3) in 28 patients (70%); the lesion invaded the muscularis propria or subserosa (T2) in 10 patients (25%), and adjacent structures (T4) in 2 patients (5%). None of the patients presented only the invasion of lamina propria or submucosa (T1). Four patients (10%) had liver metastasis at the moment of surgery, while 36 (90%) patients did not present such condition.

The samples of tumorous tissue showed that EGFR immunoexpression was absent in 22 patients (55%) and present in 18 of them (45%). In relation to the samples of non-neoplastic transitional tumor-mucosa, EGFR immunoexpression was present in 14 patients (35%), while 26 (65%) tissue samples did not present EGFR immunoexpression. As to EGFR in the adjacent mucosa, it was observed that 11 patients (27.5%) had tissue samples with immunoexpression, and 29 (72.5%) did not present immunoexpression (Table 1).

The absence or presence of EGFR immunoexpression in neoplastic tissue samples, transitional tumor-mucosa and adjacent mucosa was not significantly different in relation to the location in the large intestine.

Table 1. Presence or absence of immunoexpression of the epidermal growth factor receptor in the samples of tumorous tissue, transitional tumor-mucosa and adjacent mucosa in patients with colorectal carcinoma.

Tissue	n	Immunoexpression of the present EGFR	Immunoexpression of the absent EGFR
		n (%)	n (%)
Tumorous tissue	40	18 (45.0)	22 (55.0)
Transitional tumor-mucosa	40	14 (35.0)	26 (65.0)
Adjacent mucosa	40	11 (27.5)	29 (72.5)

EGFR: epidermal growth factor receptor; n: number of patients

Table 2. Comparison between the presence of immunoexpression of the epidermal growth factor receptor in the tumorous tissue, transitional tumor-mucosa and the adjacent mucosa in patients with colorectal carcinoma.

Tissue	Tumorous tissue	Transitional tumor-mucosa	Adjacent mucosa	p
A) EGFR immunoexpression	n= 8 (45.0%)	n=14 (35.0%)	–	0.01*
B) EGFR immunoexpression	n=18 (45.0%)	–	n=11 (27.5%)	0.04*
C) EGFR immunoexpression	–	n=14 (35.0%)	n=11 (27.5%)	0.01*

EGFR: epidermal growth factor receptor; n: number of patients

*Mann-Whitney's test

tine, diameter of the lesion, vascular invasion, lymphatic vascular invasion, perineural invasion, lymph node metastasis, cell differentiation, staging classification of malignant tumors and liver metastases ($p > 0.05$).

EGFR immunoexpression in the neoplastic tissue was significantly higher ($p = 0.01$) than in the transitional tumor-mucosa. Likewise, EGFR immunoexpression was significantly more intense ($p = 0.04$) in the neoplastic tissue when compared to the adjacent mucosa. Also, EGFR immunoexpression in the transitional tumor-mucosa was significantly higher ($p = 0.01$) in relation to the adjacent mucosa (Table 2).

DISCUSSION

The EGFR marker was chosen to identify relevant information regarding the carcinogenesis of colorectal neoplasm. When the expression of this marker is increased in the tissue, there is a relation with a worse prognosis and the presence of liver metastases. However, the meaning of the increased expression of this marker is controversial in the literature^{30,32,33,35,37,39}.

The expression of the EGFR marker in this study was significantly higher in the neoplastic tissue than in specimens of transitional carcinoma/mucosa and the adjacent mucosa. EGFR is a receptor tyrosine-kinase, and is necessary to activate the system related with cell differentiation and multiplication. Goldstein and Arrmin²⁸ identified the hyperexpression of EGFR in the deeper layers of the tumor and its association with liver and lymph node metastases. These authors identified a higher expression of EGFR in the neoplastic tissue in relation to the non-neoplastic mucosa adjacent to the tumor. Also, this study observed that the measures of immunoexpression (positive index and intensity of expression) presented significant differences in the central region of the tumor, transitional tumor-mucosa and adjacent mucosa. Considering that the immunoexpression has a direct relation with the amount of EGFR in the sample tissue, this finding suggests that the content of EGFR is more intense in the neoplastic tissue when compared to the non-neoplastic transitional tumor-mucosa and to mucosa adjacent of the colorectal carcinoma.

Our study showed EGFR expression was similar to well, moderately and little differentiated colorectal carcinomas. Spano et al.²⁹ analyzed sample tissues of 150 specimens of colorectal carcinomas and did not

find an association between the hyperexpression of EGFR and cell differentiation. Galizia et al.³⁴ studied 49 specimens of colorectal neoplastic tissue and did not identify a relation between the intensity of EGFR expression and histological differentiation. The former authors also did not show a relation between the EGFR expression and histological differentiation; however, they identified the trend of association between EGFR hyperexpression in the deeper layers of carcinomas. On the other hand, in a study with 114 colorectal neoplastic tissue samples, McKay et al.²⁶ identified the association between EGFR hyperexpression in well or moderately differentiated tumors in relation to little differentiated tumors. In our study, the degree of cell differentiation of the neoplasm was not related to immunoexpression, which indicates that, unlike CEA, less differentiated neoplasms have the same intensity regarding EGFR than the more differentiated ones. Thus, this event would not depend on the degree of cell differentiation of the colorectal carcinoma.

In this study, EGFR expression was similar in tumors that presented or not the angiolymphatic and/or perineural invasion. Spano et al.²⁹ analyzed EGFR expression and its relation with angiolymphatic invasion, and did not observe a significant relation, as well as Baiocchi et al.⁴². On the other hand, Karameris et al.⁴¹ established an association between EGFR immunoexpression and angiolymphatic and neural compromise. As to morphological parameters, vascular invasion, lymphatic vascular invasion and neural invasion were not related to EGFR immunoexpression. It is possible to consider that vascular and lymphatic invasions are not the only mechanism to express the marker in other sites.

In the present study, EGFR expression presented no significant differences in relation to the clinical staging of colorectal neoplasm. Goldstein and Arrmin²⁸ analyzed EGFR expression in patients with stage IV colorectal carcinoma, according to the TNM classification of malignant tumors. Even though they found a more intense expression in tumors that were clinically more advanced, these authors did not find a significant difference between the hyperexpression of the marker and the most advanced stage of the disease. Spano et al.²⁹ identified a relation between EGFR hyperexpression and the more advanced stages of the TNM classification. These authors observed that EGFR hyperexpres-

sion in stage T3 was higher than the stage T4. Galizia et al.³⁴ identified a significantly higher EGFR expressions in stages C and D of the modified Duke's staging system (Turnbull) in relation to stages A and B, which suggests that the more advanced the clinical stage, the more intense the hyperexpression of EGFR. However, Baiocchi et al.⁴² and McKay et al.²⁶ did not find a relation between EGFR hyperexpression and the different stages of the Duke's system. These authors suggested the increased expression of EGFR had no relation with the colorectal carcinoma aggressiveness.

The depth degree of carcinoma invasion on the colorectal wall was not related to the indexes of EGFR immunoeexpression adopted in this study. The hyperexpression of EGFR would be expected in lesions of more advanced stages, as well as in deeper lesions of the tumor.

This study showed no significant difference to EGFR expression according to rectal location or carcinoma colic. The same finding was observed by Spano et al.²⁹, Baiocchi et al.⁴², McKay et al.²⁶ and Kountourakis et al.⁴⁰. This result may indicate that the immunoeexpression of EGFR does not depend on the morphological aspects of the different areas of the large intestine, when the colon and the rectum are compared.

In this study, the analysis of EGFR expression did not identify a significantly higher number of lymph nodes that were compromised by neoplasm in the patients with EGFR hyperexpression. Likewise, McKay et al.²⁶, Scartozzi et al.³³, Bralet et al.³² and Spindler et al.³⁸ demon-

strated that EGFR hyperexpression was not significantly related to the presence of lymph node metastases. Doger et al.³⁷ studied 60 specimens of colorectal carcinoma and did not observe a relation between EGFR hyperexpression and the presence of lymph node, liver and distance metastases. The immunoeexpression was not related to the neoplastic lymph node infiltration.

In the present study, no significant relation between EGFR expression and liver metastases was found. Likewise, Bralet et al.³² and Scartozzi et al.³³ analyzed the expression of EGFR in colorectal tumors, and could not find a relation between liver metastases and the expression of the marker. On the other hand, Italiano et al.³⁰ identified a more intense expression of EGFR in patients with liver and distance metastases. Khalifa et al.³¹ assessed the expression of EGFR in the tissue of 33 colorectal carcinomas and found a relation between liver and distance metastases with neoplasm recurrence.

CONCLUSIONS

EGFR immunoeexpression was more intense in the colorectal carcinoma mucosa than in the transitional tumor-mucosa epithelium and the non-neoplastic adjacent mucosa. Additional studies are important to analyze the relation between immunohistochemical expression of EGFR and the prognosis of colorectal carcinoma, and also if the immunohistochemical expression of EGFR can be used as a predictive marker so that the patient has positive results with chemotherapy.

RESUMO: Introdução: O estudo da imunoexpressão tecidual do receptor do fator de crescimento epitelial (EGFR) pode contribuir para o entendimento de seu papel no prognóstico do carcinoma colorretal. Objetivo: Analisar a expressão imuno-histoquímica do EGFR no carcinoma colorretal e nos tecidos da transição tumor-mucosa e da mucosa adjacente à neoplasia, e avaliar a relação com os aspectos anatomopatológicos da neoplasia. Método: Em 40 doentes com carcinoma colorretal operados com intenção curativa, estudou-se a imunoexpressão do EGFR com anticorpo anti-EGFR. Foram utilizados testes paramétricos e não paramétricos. Resultados: A imunoexpressão do EGFR nas amostras de tumores apresentou diferença significativa, em relação ao nível de imunoexpressão em espécimes de tecido da transição tumor-mucosa ($p=0,01$), e ao nível de imunoexpressão em tecidos da mucosa adjacente ($p=0,04$). A imunoexpressão do EGFR não apresentou relação significativa com o tamanho da neoplasia, invasão angiolinfática, invasão neural, grau de diferenciação celular, nível de infiltração do carcinoma na parede intestinal, acometimento linfonodal e metástase hepática. Conclusões: O EGFR apresentou maior imunoexpressão na mucosa do carcinoma colorretal do que no epitélio de transição e na mucosa adjacente não neoplásica. A imunoexpressão do EGFR não se relacionou com os parâmetros anatomopatológicos do carcinoma colorretal e com a presença de metástase hepática.

Palavras-chave: genes, erbB-1; receptor do fator de crescimento epidérmico; imuno-histoquímica; neoplasias colorretais; metástase neoplásica.

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