

Neutrophil gelatinase-associated lipocalin immunoexpression in colorectal carcinoma: A stage-specific prognostic factor?

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Abstract. TNM post-surgical staging is considered to be one of the most powerful prognosticators for colorectal carcinoma. Although patient survival mostly decreases concomitantly to stage increase, in a percentage of cases TNM stage appears only to express the anatomic extent of the neoplasia with no correlation with clinical outcome. Thus, the identification of additional prognostic markers for colorectal cancer is required. Neutrophil gelatinase-associated lipocalin (NGAL) is a 25-kDa protein that appears to play an important role in colorectal cancer progression. In order to evaluate whether NGAL expression may be considered as a predictor of colorectal cancer progression, we analyzed its correlation with clinicopathological characteristics, as well as with patient progression-free survival in a series of surgically resected colorectal carcinomas. A variable NGAL immunoexpression was found in 24 out of the 64 analyzed cases. When only the positive cases were considered, a significant association was found between a high NGAL expression and the presence of distant metastases or high tumor stage. In addition, the presence of NGAL was a significant negative prognostic marker correlated with a shorter progression-free survival in stage I colorectal carcinoma, but not in the remaining TNM stages. If our findings are confirmed in more extensive analyses on stage I colorectal carcinoma, NGAL assessment may be used in order to select those patients with a higher progression risk and to submit them to adjuvant therapies useful to prevent adverse outcome.

Introduction

Colorectal carcinoma is one of the leading causes of cancer-related death in Western Europe and the US (1). Post-surgical

TNM staging is a prognostic factor that is able to predict the clinical course of this neoplasia, with 5-year survival rates decreasing concomitantly to stage (2,3). For this reason, TNM staging is taken into consideration in the therapeutic protocols applied to colorectal cancer patients. Thus, stage I colorectal carcinoma is treated by surgery alone, with adjuvant chemotherapy being the choice of treatment in order to improve the survival of patients affected by higher stage carcinomas. Nevertheless, in certain instances TNM staging appears only to express the anatomic extent of the neoplasia with no evidence of correlation with patient survival (4). At present, histoprosthetic factors able to predict progression-free and overall survival of colorectal cancer in order to select patients for adjuvant therapies are not available. Thus, factors predicting progression risk, in addition to the currently used clinicalpathological staging system, are required.

Neutrophil gelatinase-associated lipocalin (NGAL) is also known as NRL (neu-related lipocalin), oncogene 24p3, uterocalin and lipocalin 2. It is a 25-kDa protein that is stored in the granules of human neutrophils (5). Additionally, it belongs to the lipocalin family which consists of more than 50 members, all of which are characterized by the ability to bind and transport small lipophilic substances (6). NGAL was shown to be involved in iron trafficking (7) and to increase the cytoplasmic levels of this mineral (8) by capturing and transporting the iron particles to the inner cell following interaction with specific membrane receptors (24p3, megalin). Its role in the delivery of iron to the cells underlies the multiple effects attributed to NGAL. Since it is released by activated neutrophils, this protein is involved in the iron depletion strategy exploited in immune defense against bacterial pathogens (9). Furthermore, NGAL appears to be involved in the growth, development and differentiation of a number of human tissues, as early as in the embryo, via its regulation of iron-responsive genes which are crucial in the differentiation of primordial cells (10,11).

Since NGAL synthesis is induced by factors promoting the development of neoplasias (6,11,12), it was suggested that this protein plays a role in the carcinogenesis and progression of human tumors (11). Accordingly, its overexpression has been found in various types of cancer, including colorectal (13-16). The role of NGAL in colorectal cancer progression (18) is evident due to the existence of a significant association between a high NGAL mRNA quantity and nodal metastases or advanced stage of this neoplasia (17). In addition, the

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induction of NGAL overexpression has been shown to decrease cell-cell adhesion, enhance cell-matrix attachment and increase cell motility and *in vitro* invasion in colorectal cancer cell lines (18). Subsequently, this study aimed to evaluate whether progression-free survival may differ on the basis of NGAL expression in colorectal cancer and whether this expression is a predictor of disease progression in this neoplasia.

Materials and methods

Materials. A total of 64 consecutive cases of surgically resected colorectal carcinomas, procured from an equal number of patients (29 female and 35 male patients; age range 36-89 years; mean age 68.09 ± 12.19), were obtained from the files of the Department of Human Pathology of the University of Messina, Italy. None of the patients had received pre-operative chemotherapy for their neoplasia or had colonic carcinoma *in situ*. Complete tumor resection had been achieved in all cases (R0). A total of 41 cases were located at the left colon (including rectum and sigma), while 23 were located at the right colon (including caecum, ascendant and transversus). Histological grade was established according to the World Health Organization classification system criteria; specifically, 24 cases were grade 1, 36 grade 2, while 4 cases were grade 3. Of all patients at the time of diagnosis, 20 had lymph nodes and 12 had liver metastases, with 5 of the latter being submitted to surgical resection for their metastases. Follow-up data, including information regarding progression-free survival, were available in all 64 cases. None of the patients with stage I cancer had been treated with adjuvant chemotherapy, while 3 out of 11 patients with stage II colorectal cancer had received chemotherapy (5-fluorouracil plus folinic acid) as had all the patients with stage III or IV cancers.

Methods. All 64 cases were formalin-fixed and paraffin-embedded. For each case, 4- μ m sections were cut from the corresponding paraffin block for subsequent immunohistochemical study.

Briefly, the endogenous peroxidase activity was blocked with 0.1% H_2O_2 in methanol for 20 min. Normal sheep serum was then applied for 30 min to prevent non-specific adherence of serum proteins. Sections were sequentially incubated at 4°C overnight with the primary antibody against NGAL (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:100). The bound primary antibody was visualized using the Dako EnVision peroxidase detection system (Dako Cytomation, Glostrup, Denmark), according to the manufacturer's instructions. For immunostaining, the sections were incubated in the dark for 10 min with 3,3'-diaminobenzidine tetra hydrochloride (Sigma Chemical Co., St. Louis, MO, USA), with the amount of 100 mg in 200 ml 0.03% hydrogen peroxide in phosphate-buffered saline (PBS). Nuclear counterstaining was performed by Mayer's haemalum. The binding specificity was assessed by omitting the primary antiserum or replacing it with normal rabbit serum or PBS (pH 7.4). Proximal tubules within samples of normal renal parenchyma were used as positive controls for the immunoreactions (15,19).

Assessment of the immunostained section was performed by two independent pathologists, who were blinded to the clinicopathological data. NGAL expression was based on

the presence of cytoplasmic and membranous staining. The intensity of staining (IS) was graded as negative (0), weak (1), moderate (2) and strong (3). The area of staining positivity (ASP), recorded as a percentage of neoplastic positive cells, was assessed using the values: 0 (<10%), 1 (11-25%), 2 (26-50%), 3 (51-75%) and 4 (>75%), according to the procedure previously described (16,21). An intensity distribution (ID) score was then generated for each case by multiplying the values of IS and ASP. Cases with an ID score of 0 were considered to be negative for NGAL.

For the statistical analyses, samples were subdivided into negative and positive tumors, according to the median NGAL ID score, which was 0. Moreover, when only positive cases were considered, low (ID score 1-2) and high (ID score >2) NGAL expression was defined on the basis of the median ID score of this subgroup (median ID score 2).

Statistical analysis. Fisher's exact and Chi-square tests were performed to assess the statistical correlations between NGAL expression and various clinicopathological characteristics, such as age, gender or the site, histological grade, depth of infiltration (T), presence of nodal (N) or distant metastases (M), as well as tumor stage.

Progression-free survival was assessed by the Kaplan-Meier method, with the date of primary surgery as the entry data. The end point was characterized as the length of survival to recurrent (local or distant) disease. The Mantel-Cox log-rank test was applied to assess the strength of association between survival time and each of the patient characteristics (age, gender, chemotherapeutic treatment, site, histological grade, TNM stage and NGAL immunoexpression of the tumor) as a single variable. Concomitantly, a multivariate analysis (Cox regression model) was utilised to determine the independent effect of each significant variable.

$P < 0.05$ was considered to be statistically significant. Data were analysed using the SPSS package version 6.1.3 (SPSS Inc., Chicago, IL, USA).

Results

Table I shows the clinicopathological and immunohistochemical data related to the 64 analyzed colorectal carcinomas. NGAL immunoexpression was found in 24 cases with variable ID scores. Immunostaining was present within the cytoplasm of the neoplastic cells (Fig. 1), whereas no immunolabelling was evident in the normal epithelium adjacent to the tumor. In addition, in positive cases immunoreactivity was always evident at the invasive front of the neoplasm.

No significant associations were found between NGAL presence and the various clinicopathological characteristics (Table II), but when only positive cases were examined, a significant association emerged between a high NGAL expression (ID score >2) and the presence of distant metastases at the initial diagnosis ($P = 0.017$) or high TNM stage ($P = 0.039$) (Table III).

Post-surgical follow-up ranged between 4 and 117 months. During the follow-up, 24 out of 64 patients developed recurrent disease, with distant metastases in 23 cases and local recurrence in 1 case. Patients who died of independent disease and those with no evidence of disease progression (3/64) were

Table I. Clinico-pathological characteristics and NGAL immunohistochemical expression in 64 colorectal carcinomas.

Case	Age	Gender	Site	Grade	T	N	M	Stage	NGAL ID score	Recurrence	Progression-free survival	Chemotherapy
1	63	M	Left colon	3	2	0	0	1	0	None	106	None
2	76	F	Right colon	2	2	0	0	1	0	None	105	None
3	86	F	Left colon	1	1	0	0	1	1	None	62	None
4	62	M	Left colon	1	2	0	0	1	0	None	96	None
5	58	M	Left colon	1	2	0	0	1	0	None	116	None
6	71	M	Right colon	1	2	0	0	1	0	None	88	None
7	55	M	Left colon	2	2	0	0	1	0	None	74	None
8	75	M	Right colon	2	2	0	0	1	0	None	86	None
9	62	M	Right colon	2	2	0	0	1	0	None	65	None
10	79	F	Left colon	2	2	0	0	1	0	None	78	None
11	83	F	Right colon	2	2	0	0	1	0	None	75	None
12	61	F	Left colon	2	2	0	0	1	0	None	61	None
13	62	M	Left colon	2	2	0	0	1	2	None	89	None
14	76	F	Left colon	2	2	0	0	1	0	None	90	None
15	64	F	Left colon	2	2	0	0	1	2	None	90	None
16	70	M	Left colon	2	2	0	0	1	0	None	82	None
17	79	M	Left colon	2	2	0	0	1	0	None	93	None
18	63	F	Right colon	3	2	0	0	1	0	None	77	None
19	74	M	Right colon	2	2	0	0	1	0	None	98	None
20	85	F	Left colon	2	2	0	0	1	0	None	65	None
21	53	F	Right colon	2	2	0	0	1	1	None	110	None
22	65	M	Left colon	2	2	0	0	1	0	None	106	None
23	86	M	Right colon	2	1	0	0	1	0	None	112	None
24	69	M	Left colon	2	2	0	0	1	0	None	117	None
25	66	F	Right colon	1	2	0	0	1	1	Distant	48	None
26	89	F	Right colon	1	2	0	0	1	2	Distant	30	None
27	71	F	Right colon	2	2	0	0	1	1	Distant	32	None
28	72	M	Left colon	2	2	0	0	1	0	None	106	None
29	47	M	Right colon	3	3	0	0	2	4	Distant	22	None
30	71	M	Right colon	2	4	0	0	2	0	None	84	Yes
31	81	F	Right colon	2	3	0	0	2	0	None	44	None
32	65	M	Left colon	2	4	0	0	2	2	Local	54	None
33	76	M	Left colon	3	3	0	0	2	0	Distant	32	None
34	37	F	Left colon	1	4	0	0	2	0	None	61	Yes
35	54	F	Left colon	2	4	0	0	2	1	Distant	20	Yes
36	86	M	Left colon	2	3	0	0	2	0	Distant	10	None

Table I. Continued.

Case	Age	Gender	Site	Grade	T	N	M	Stage	NGAL ID score	Recurrence	Progression-free survival	Chemotherapy
37	52	F	Left colon	2	3	0	0	2	1	None	60	None
38	82	M	Left colon	2	3	0	0	2	0	Distant	10	None
39	76	M	Left colon	2	3	0	0	2	4	None	35	None
40	64	M	Right colon	1	3	1	0	3	0	Distant	30	Yes
41	71	F	Left colon	2	2	1	0	3	0	Distant	12	Yes
42	88	F	Left colon	1	3	1	0	3	4	Distant	46	Yes
43	71	M	Right colon	2	3	1	0	3	0	Distant	58	Yes
44	63	F	Left colon	2	3	1	0	3	2	None	26	Yes
45	81	F	Right colon	1	2	1	0	3	1	Distant	34	Yes
46	36	F	Left colon	1	4	1	0	3	0	None	61	Yes
47	73	M	Left colon	1	4	1	0	3	1	None	76	Yes
48	68	F	Left colon	1	3	1	0	3	0	None	95	Yes
49	75	M	Left colon	3	4	1	0	3	0	Distant	20	Yes
50	46	F	Right colon	2	2	1	0	3	2	Distant	14	Yes
51	80	M	Left colon	2	2	1	0	3	1	None	78	Yes
52	75	M	Left colon	2	4	1	0	3	0	Distant	36	Yes
53	61	M	Right colon	1	4	1	1	4	2	Distant	25	Yes
54	77	M	Left colon	1	3	0	1	4	0	Distant	36	Yes
55	50	F	Left colon	1	4	1	1	4	0	Distant	24	Yes
56	63	F	Left colon	2	4	0	1	4	1	Distant	4	Yes
57	73	M	Right colon	2	3	1	1	4	0	Distant	14	Yes
58	78	F	Left colon	2	4	0	1	4	4	None	61	Yes
59	71	F	Left colon	2	3	1	1	4	1	Distant	29	Yes
60	62	M	Left colon	2	2	1	1	4	3	None	106	Yes
61	68	M	Left colon	2	3	1	1	4	0	None	80	Yes
62	42	F	Left colon	2	4	0	1	4	0	None	104	Yes
63	60	M	Right colon	3	4	1	1	4	0	None	76	Yes
64	60	M	Right colon	1	2	0	1	4	8	Distant	40	Yes

ID, intensity distribution; M, male; F, female; TNM, tumor node metastasis.

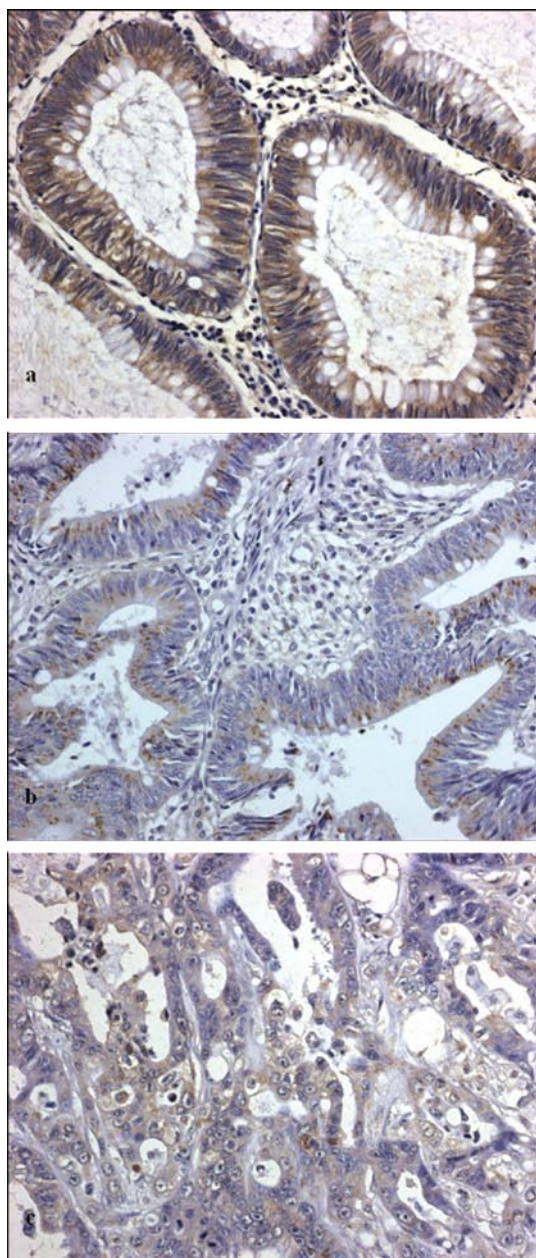


Figure 1. NGAL immunohistochemical expression in the cytoplasm of the neoplastic cells of a (a) well-differentiated (NGAL stain; original magnification, x100), (b) moderately differentiated (NGAL stain; original magnification, x100) and (c) poorly differentiated (NGAL stain; original magnification, x200) colorectal carcinoma.

censored. A total of 23 out of 24 patients with recurrent disease died of disease progression. Univariate survival analyses revealed TNM stage, depth of infiltration, nodal metastases, chemotherapy and NGAL immunoexpression to be significant prognostic factors for progression-free survival (Table IV). In particular, the length of survival to recurrent disease was significantly shorter in those patients with a colorectal carcinoma characterized by a high NGAL expression ($P=0.0305$) (Table IV). Nevertheless, the multivariate analysis suggested that NGAL expression was not an independent prognostic factor, whereas the application of chemotherapy was found to be an independent variable as compared to progression-free survival of patients (Table IV). When the prognostic value of

Table II. Statistical correlations between the NGAL immunoexpression and the various clinicopathological characteristics in 24 NGAL-positive colorectal carcinomas.

	NGAL ID score		P-value
	1-2	>2	
Gender			0.192
M	6	4	
F	12	2	
Age			0.665
<70 years	11	3	
>70 years	7	3	
Site			1.000
Left colon	11	4	
Right colon	7	2	
Grade			0.201
1	6	2	
2	12	3	
3	0	1	
T			0.418
1	1	0	
2	9	2	
3	3	3	
4	5	1	
N			1.000
0	11	4	
1-2	7	2	
M			0.017
0	16	2	
1	2	4	
Stage			0.039
1	7	0	
2	3	1	
3	6	1	
4	2	4	

ID, intensity distribution; M, male; F, female; TNM, tumor node metastasis.

NGAL expression was specifically investigated according to the TNM stage, the expression of this protein was evidenced as a negative prognostic factor that correlated with a shorter progression-free survival in stage I ($P=0.001$) (Fig. 2), but not in colorectal carcinomas of higher stages (Table V).

Discussion

NGAL overexpression and correlation with disease progression has been shown in malignancies of the gastro-intestinal tract, including esophageal, gastric and colorectal carcinomas (17,21,22). We investigated for the first time the relationship between NGAL prognostic value and progression-free survival of colorectal carcinoma. In particular, NGAL immunohistochemical expression was evaluated in a series of 64 colorectal carcinomas of different stages and correlated

Table III. Statistical correlations between NGAL immun-expression and the various clinicopathological characteristics of the 64 analyzed colorectal carcinomas.^a

	NGAL ID score		P-value
	0	>0	
Gender			0.066
M	27	10	
F	13	14	
Age			0.438
<70 years	18	14	
>70 years	22	10	
Site			1.000
Left colon	26	15	
Right colon	14	9	
Grade			0.055
1	9	8	
2	26	5	
3	5	1	
T			0.932
1	1	1	
2	21	11	
3	10	6	
4	8	6	
N			0.419
0	29	15	
1-2	11	9	
M			1.000
0	30	18	
1	10	6	
Stage			0.201
1	21	7	
2	4	4	
3	5	7	
4	10	6	

ID, intensity distribution; M, male; F, female; TNM, tumor node metastasis. ^aFisher's exact and Chi-square tests were used.

Table IV. Univariate and multivariate analyses for progression-free survival in 64 patients affected by colorectal carcinoma.

A, Univariate analysis.

Charateristics	χ^2	Df	P-value
Age	0.9862	1	0.3207
Gender	0	1	0.9935
Site	1.1595	1	0.2816
Grade	2.2580	2	0.3234
T	9.1260	3	0.0088
N	7.3205	1	0.0068
M	3.3093	1	0.0689
Stage	16.2523	3	0.0010
Chemotherapy	8.8210	1	0.0030
NGAL immunoexpression	4.6795	1	0.0305

B, Multivariate analysis.

Variable	β	SE	Exp (β)	P-value
Chemotherapy	1.2116	0.4348	3	0.005

df, degrees of freedom; SE, standard error.

Table V. NGAL univariate analysis for progression-free survival according to TNM staging of 64 colorectal carcinomas.

Univariate analysis (Characteristic: NGAL expression)	χ^2	Df	P-value
Stage			
I	10.5775	1	0.0011
II	0.0079	1	0.9290
III	0.2400	1	0.6242
IV	0.1811	1	0.6704

Df, degrees of freedom; TNM, tumor node metastasis.

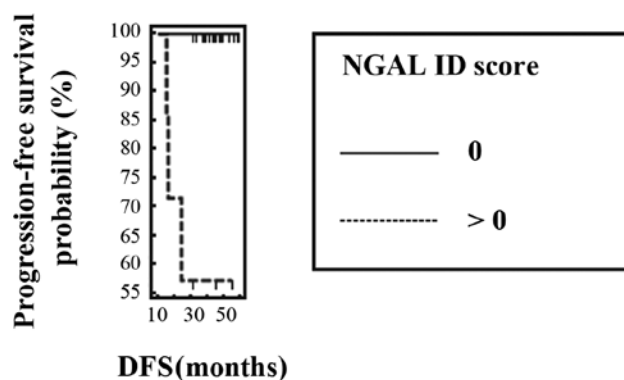


Figure 2. Kaplan-Meier-specific progression-free survival curve in relation to NGAL expression in stage I colorectal carcinomas. A significantly shorter progression-free survival was noted in cases with NGAL expression comparison to cases with no NGAL immunoexpression. DFS, disease-free survival.

with the various clinicopathological characteristics, as well as with progression-free survival of the patients.

A variable NGAL expression was found in 24 out of 64 cases. In accordance with previous reports (16-18), NGAL immunoexpression was found in the neoplastic cells of positive cases, but not in the adjacent normal epithelium, in line with its suggestive role in colorectal carcinogenesis. Immunostaining was localized at the cytoplasm of the neoplastic cells, as in the case of breast cancer (13). This localization suggests NGAL internalization to the inner cell following its binding to membrane receptors. Since NGAL is involved in iron uptake, we hypothesize that NGAL expression and cytoplasmic localization reflect augmented iron requirement by the neoplastic elements of colorectal carcinoma, as previously described (18). Notably, NGAL appears to play a role in carcinogenesis by

promoting iron uptake from the extracellular space within the malignant cells, a fundamental process for the maintenance of neoplastic cell multiplication (14,23). Besides, it is well-known that iron overload promotes carcinogenesis through the induction of oxidative DNA damage (24), whereas iron depletion leads to cell cycle arrest and apoptosis (25).

Notably, NGAL staining was mainly observed in the invasive front of the tumor in positive cases. This may be related to its ability to increase the invasive potential of tumor cells. Studies have shown that NGAL forms a complex with matrix metalloproteinase-9 (MMP-9), preventing MMP-9 degradation and resulting in increased MMP-9 enzyme activity *in vitro* (26). MMP-9 plays a role in cancer progression by degrading the molecular components of the basement membrane and extracellular matrix, releasing vascular endothelial cell growth factor (VEGF) from the extracellular matrix, and enabling angiogenesis, invasion and distant metastasis (26-28). Thus, NGAL may play a role in cancer progression by causing increased MMP-9 enzymatic activity, thereby promoting the invasive and metastatic potential of cancer cells *in vivo* and *in vitro* (28). However, NGAL was recently shown to increase invasiveness of colorectal cancer cells through an MMP-9-independent process (18). It has been shown that NGAL overexpression enhances the invasive and metastatic potential of colon cancer cells by decreasing e-cadherin-mediated cell-cell adhesion through an iron-dependent mechanism (18). In contrast to other authors (17), we failed to demonstrate any significant correlation between NGAL and the depth of infiltration of tumors. Nevertheless, the role of NGAL in the invasiveness of colorectal carcinoma was confirmed by a significant association between a high NGAL expression and the presence of distant metastases or a high TNM stage at the time of initial diagnosis of the analyzed colorectal carcinomas. Notably, NGAL involvement in the development of metastases had previously been hypothesized, since inhibition of this protein results in the blockage of lung metastases from breast carcinoma (29).

NGAL overexpression was previously considered to be a negative prognostic factor in colorectal cancer due to its significant association with the presence of adverse factors, such as lymph node metastases or venous invasion (17). However, its impact on specific survival in relation to neoplasia has yet to be investigated. Our results showed that patients with tumors characterized by NGAL immunohistochemical expression exhibited a significantly shorter progression-free survival in comparison to patients with absent NGAL staining. Thus, NGAL appears to act as a negative prognostic factor for colorectal cancer, as in the case of breast cancer (13). Notably, when the relationship between NGAL predictive value and disease-progression risk was evaluated according to the TNM stage of neoplasias, the presence of NGAL appeared to be a significant predictor of progression risk in stage I colorectal carcinoma, but not in the remaining TNM stages. Only the former group of patients had not been submitted to chemotherapy, which emerged as an independent prognostic factor for progression-free survival in our cohort.

In conclusion, the present study showed that NGAL immunoexpression is a negative prognostic marker associated with shorter disease-specific survival in stage I colorectal carcinomas. If our findings are confirmed on larger cohorts

of stage I colorectal carcinomas, NGAL assessment in colon cancer tissue may be used as a prognostic tool to determine tumors with a higher progression risk, so that selected patients may undergo adjuvant treatments in order to prevent adverse outcome. Moreover, the inhibition of NGAL expression or function may represent the target for novel therapeutic strategies in order to prevent disease progression in NGAL-positive colorectal carcinomas. Colorectal carcinomas showing NGAL expression may benefit from therapies with iron chelators as anti-cancer agents. Notably, since iron deprivation caused by iron chelators inhibits cell proliferation, iron and its corresponding transport proteins have become new targets in cancer therapy (30,31). Finally, since the efficacy of anti-NGAL antibodies has been shown by blocking cancer metastasis in mice (29), NGAL expression and involvement in colorectal cancer progression and metastatic disease may be exploited for such novel anti-cancer therapies.

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