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Up-regulation of Neutrophil Gelatinase-Associated Lipocalin in Colorectal Cancer Predicts Poor Patient Survival

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Abstract

Background Lipocalin-2 (Lcn-2) is expressed in human neutrophils and epithelial cells, particularly in the presence of inflammation or cancer. It was shown to be highly expressed in various human cancers. Increased protein levels were associated with decreased survival of patients with breast or gastric cancer. The main focus of this work was to analyze the implication of Lcn-2 up-regulation in the genesis of colon cancer.

Methods Expression of Lcn-2 was analyzed in colorectal carcinoma cell lines, paired colorectal carcinoma tissues, and regular mucosa by Western blot analysis. Lcn-2 immunohistochemical staining was performed in 192 colorectal carcinoma resection specimens and correlated with clinicopathologic parameters.

Results Western blot analysis of colorectal carcinoma tissues demonstrated Lcn-2 overexpression in carcinomas as compared with regular mucosa. Immunohistochemical staining revealed Lcn-2 expression in 179 (93.2 %) colorectal carcinoma tissues. Intense immunoreactivity was significantly correlated with metastasis ($p = 0.042$) and UICC stage ($p = 0.027$). Survival analysis according to the Kaplan–Meier method revealed a significant association between Lcn-2 overexpressing tumors and overall survival ($p < 0.001$) and disease-free survival ($p < 0.001$).

Conclusions Our data provide evidence that Lcn-2 expression is up-regulated with tumor progression and was found to be a predictor of overall survival.

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Introduction

Worldwide, colorectal cancer (CRC) is the fourth most common cancer, with approximately one million new cases annually [1]. It is now widely accepted that colorectal carcinogenesis is a multistep process involving inactivation of a variety of tumor suppressor and DNA repair genes and simultaneous activation of certain oncogenes. Consequently, it is now apparent that individual colorectal cancers may evolve along diverse molecular pathways [2].

Prognosis is best when the disease is detected early [3]. Nearly two-thirds of newly diagnosed cases of CRC have lymph node involvement or metastatic disease [3]. In recent years, the search for cancer-related biomarkers, especially cancers with a high incidence such as colon carcinoma, has become important. An increase in the expression of certain proteins in neoplastic cells was found. Among them, the latest studies focused on lipocalin-2 (Lcn-2), a glycoprotein with poorly explained function that

is suggested to play a role in colon cancer development and metastasis [4, 5].

Lcn-2 was first identified as a gene that is rapidly induced during SV40 tumor virus-triggered mitosis in quiescent primary murine kidney cells [6]. Lcn-2 expression was observed in human CRC [7–9], breast cancer [10], pancreatic cancer cells [11], ovarian cancer [12], and other human neoplastic tissues and cancer cell lines [13, 14].

Recent publications report that Lcn-2 promotes cancer progression. Yang et al. used an orthotopic breast cancer mouse model to show that Lcn-2 induces a poorly differentiated phenotype and increases local invasion and lymph node metastasis [15]. It has also been shown that the serum Lcn-2 concentration was higher in CRC patients than in the healthy population. High levels of the protein were associated with large neoplastic tissue volume [5].

In addition, the binding of Lcn-2 to matrix metalloproteinase-9 (MMP-9) protects this extracellular matrix remodeling enzyme from autodegradation [16]. It was shown that increased Lcn-2 expression of human breast cancer cells resulted in significant stimulation of tumor growth through this mechanism [17]. Lcn-2 may thus also play an important role in colon cancer by protecting and enhancing the enzymatic activity of MMP-9 and by facilitating angiogenesis and tumor growth.

In this study, we analyzed expression of Lcn-2 in 192 CRC patients with a 10-year follow-up.

Material and methods

Reagents and chemicals

Cell culture reagents (media, serum, antibiotics) were obtained from PAA Laboratories (Linz, Austria). Human anti-Lcn-2/neutrophil gelatinase-associated lipocalin (NGAL) and anti-MMP-9 monoclonal antibodies (mAB) were obtained from R&D systems (Minneapolis, MN, USA) and mouse anti-α-tubulin mAB from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The donkey anti-goat and the goat anti-mouse horseradish peroxidase (HRP)–immunoglobulin G (IgG) were purchased from Santa Cruz Biotechnology.

Cell culture

Experiments were conducted with established human colon cancer cell lines (HCT15, HRT18, HT29-19, HT29-21, CX-1) cultivated as suggested by the supplier (American Type Culture Collection, ATCC, Manassas, VA, USA). New cultures were reestablished from frozen stocks every 3 months. After reaching a confluency of about 50 %, cells were trypsinized, filtered through a 70-μm nylon mesh cell strainer (BD, Heidelberg, Germany) and plated, for further subcultivation, in a 75 mm^2 culture flask (Corning, Kaiserslautern, Germany).

Analysis of protein expression

Snap-frozen tissue samples were used for this analysis. Lysates were prepared in ice-cold cell lysis buffer (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 200 μM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, leupeptin1 μg/ml, 1 μM pepstatin A, 1 % Triton X-100). Tissue samples were homogenized, centrifuged, and the supernatant removed for immunoblots. Equivalent protein concentrations of 30 μg were resolved in 12 % sodium dodecyl sulfate (SDS) polyacrylamide gels on a Minigel apparatus (BioRad, Hercules, CA, USA) and transferred to a nitrocellulose membrane (Amerham Hypbid-P; GE Healthcare, Buckinghamshire, England). Membranes were incubated with the primary antibodies: anti-Lcn-2/NGAL (1:500, 0.2 μg/ml) or mouse anti-α-tubulin (1:2000). After the washing steps, membranes were incubated with IgG–HRP sheep anti-mouse antibody (1:1000). Protein–antibody complexes were detected by enhanced chemiluminescence (ECL, Amersham, Buckinghamshire, England). Relative quantification of Lcn-2/NGAL expression was determined using ImageJ software (http://rsb.info.nih.gov/ij/).

Patients and tissues

Formalin-fixed and paraffin-embedded samples from 192 CRC patients were obtained from the Department of Pathology, Innsbruck Medical University, Austria, from 1992 to 2001. Clinical and pathologic data were documented prospectively and entered into a specific tumor registry after surgery and follow-up. The patients were followed-up according to a standardized protocol of tumor follow-up care (quarterly during the first 2 years and half-yearly until year 5 postoperatively), each time including clinical assessment and tumor marker (carcinoembryonic enzyme, or CEA) tests, with colonoscopy and computed tomography (CT) scans at defined time points [18]. Table 1 shows their classification according to the TNM system, histologic tumor type, tumor localization, and patient sex. At that time, a neoadjuvant chemoradiation protocol was not yet being applied. In all, 43 patients [Union Internationale Contre le Cancer (UICC) III and IV] received adjuvant therapy.

Immunohistochemistry

Immunohistochemistry was performed as described elsewhere [19]. Briefly, antigen retrieval sections were

Table 1 Correlations of Lcn-2 and MMP-9 expression with sex, tumor localization, and various clinicopathologic parameters

Parameters	No. of patients	High Lcn-2 expression	p	No. of patients	High MMP9 expression	p
Age at diagnosis (years)						
≤65	71	20 (28 %)	0.607	70	57 (81 %)	0.881
>65	121	30 (25 %)		119	91 (76 %)	
Sex						
Female	84	20 (24 %)	0.195	84	61 (73 %)	0.353
Male	108	30 (28 %)		105	85 (81 %)	
Metastasis						
No	146	33 (23 %)	**0.042**	144	110 (76 %)	0.252
Yes	46	17 (37 %)		45	38 (84 %)	
Localization[a]						
1	66	27 (41 %)	**0.001**	67	56 (84 %)	0.403
2	71	16 (23 %)		66	49 (74 %)	
3	55	7 (13 %)		56	43 (77 %)	
T-stage						
1	16	0	**0.037**	14	10 (64 %)	0.063
2	24	4 (17 %)		25	19 (76 %)	
3	126	37 (29 %)		125	100 (80 %)	
4	26	9 (35 %)		25	19 (76 %)	
Lymph nodes						
No	88	17 (19 %)	0.051	87	69 (79 %)	0.757
Yes	104	33 (32 %)		102	79 (77 %)	
Recurrence						
No	155	34 (22 %)	0.058	154	121 (79 %)	0.735
Yes	37	16 (43 %)		35	27 (77 %)	
UICC						
I	33	4 (12 %)	**0.027**	33	26 (79 %)	0.380
II	48	9 (19 %)		47	38 (81 %)	
III	64	19 (30 %)		63	45 (71 %)	
IV	47	18 (38 %)		46	39 (85 %)	

Boldface type indicates significance

[a] *1* right hemicolon, *2* left hemicolon, *3* rectum

autoclaved in 10 mM sodium citrate (pH 6.0) at 121 °C for 10 min. Endogenous peroxidase activity was blocked by H_2O_2 treatment for 20 min. Thereafter, slides were incubated with anti-Lcn-2/NGAL (5 μg/ml; R&D Systems, Minneapolis, MN, USA) and anti-MMP-9 antibody (5 μg/ml; R&D Systems), respectively, in a humidified chamber for 60 min at room temperature. After washing, the slides were incubated with biotinylated rabbit anti-mouse IgG (Dako, Copenhagen, Denmark) at a dilution of 1:600 and detected with an ABC peroxidase kit (Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine as the substrate, according to the manufacturer's protocol. Sections were counterstained with Mayer's hemalaun (Merck, Darmstadt, Germany), washed in tap water, and mounted using Aquatex (Merck). Blocking controls were performed identically—except for the addition of native Lcn-2 protein to the primary antibody (10:1)—1 h before application of the antibody solution. Negative controls without primary antibody were included in each run.

Evaluation of Lcn-2/NGAL and MMP-9 expression

Two independent pathologists, blinded to the patients' clinical data, analyzed the Slides prepared from cancer specimens. Lcn-2 and MMP-9 immunostaining scores were calculated on the basis of a well-established proportion and intensity score [20]. The proportion score reflects the estimated percentage of positively stained tumor cells: score 1, <10 %; score 2, 10–49 %; score 3, 50–79 %; score 4, 80–100 %. The intensity score shows the estimated staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong). The selection of clinically important cutoff scores

for Lcn-2 and MMP-9 expression was based on receiver operating characteristic (ROC) curve analysis as described elsewhere [21]. For percentage and intensity of Lcn-2 staining, a ROC curve was generated for the outcome survival. The scores having the closest distance to the point (0.0; 1.0) on the curve were selected as the cutoff score. The cutoff scores were 2.5 for intensity and 45 % for percentage. Tumors with score 3 for staining intensity and more than 45 % positive tumor cells were classified into the Lcn-2 high-positive group. All others were placed in the Lcn-2 negative/low-positive group.

Statistical analysis

All calculations and statistical analyses were performed using SPSS 20 for Windows. A contingency table χ^2 test was performed to determine a possible association between Lcn-2 expression and age, sex, nodal status, T-stage, UICC, and histologic grade of the tumor. Kaplan–Meier curves were plotted to assess overall and disease-free survival. The survival curves were compared using the log-rank test. Follow-up time was censored if the patient was lost to follow-up.

Multivariate analysis was performed using a Cox proportional hazards model applied only to those markers that showed significance in the univariate analysis. A model was formulated using a forward selection procedure starting from the most significant prognostic variable in the univariate analysis and adding factors, retaining only significant variables each step. For all analyses, $p < 0.05$ was defined as statistically significant.

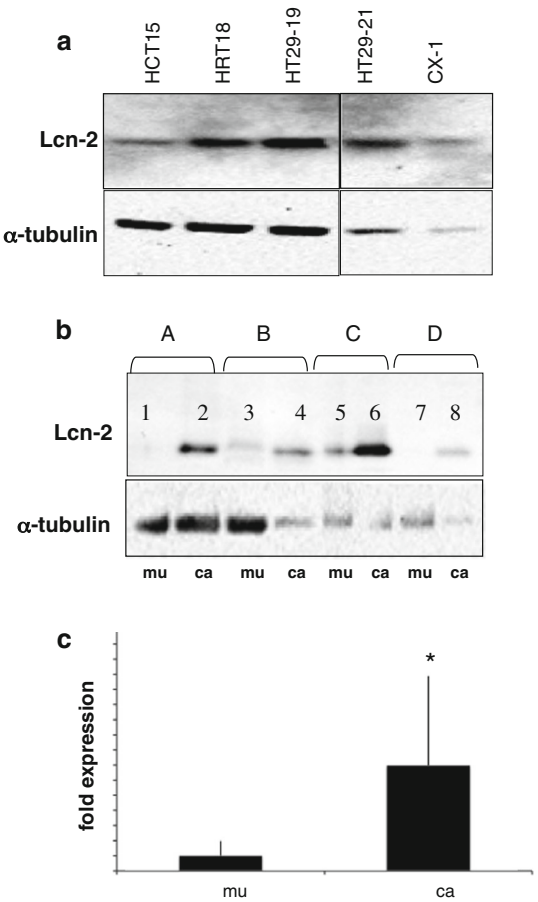

Fig. 1 **a** Lcn-2 Western blot of various colon cancer cell lines. **b** Lcn-2 Western blot of colon cancer tissue (*ca*) and normal mucosa (*mu*) from endoscopic biopsies of four patients. α-Tubulin serves as a control (**b**). **c** Intensity data of **b** (mean value) normalized by α-tubulin (*$p < 0.05$)

Results

Expression of Lcn-2 in colorectal carcinoma cell lines and tissues

To evaluate Lcn-2 expression in CRC, Western blots of five human CRC cell lines and four matched pairs of carcinoma and normal mucosa were performed. Lcn-2 was expressed in all CRC cell lines examined at the protein level. Strong Lcn-2 expression was shown in HRT-18, HT29-19, and HT29-19, whereas weak expression was observed in HCT-15 and CX-1 cells (Fig. 1a). Figure 1b shows higher Lcn-2 expression in all four human CRC tissues than in matched normal mucosa. Intensity data were obtained and used to quantify expression of Lcn-2 normalized by tubulin in various tumor samples. Only four tumor tissue samples and matched normal tissue were available from the local tissue repository. Lcn-2 expression was shown to be significantly higher in human carcinomas than in healthy normal mucosa (Fig. 1c).

Lcn-2 expression in human colorectal carcinoma tissue

To investigate the correlation of Lcn-2 expression with clinical prognostic features in vivo, we first determined the expression of Lcn-2 in 192 colon cancer tissue sections using immunohistochemistry. When the samples were obtained, no cancer patient had yet received neoadjuvant therapy. Lcn-2 was detected mainly in the cytoplasm of epithelial cells but also in some granulocytes and occasionally in endothelial cells. Of the 192 cancer sections, only 13 showed no Lcn-2 expression at all. Staining intensity was weak in 18 (9.4 %) patients, moderate in 78 (40.6 %), and strong in 83 (43.2 %) (Fig. 2). Distribution of the percentage scores (ranging from 1 to 4) was 11.5 % (22 patients), 34.3 % (66 patients), 18.8 % (36 patients), and 35.4 % (68 patients). Lcn-2 overexpression in tumors, as defined earlier, was found in 50 cases.

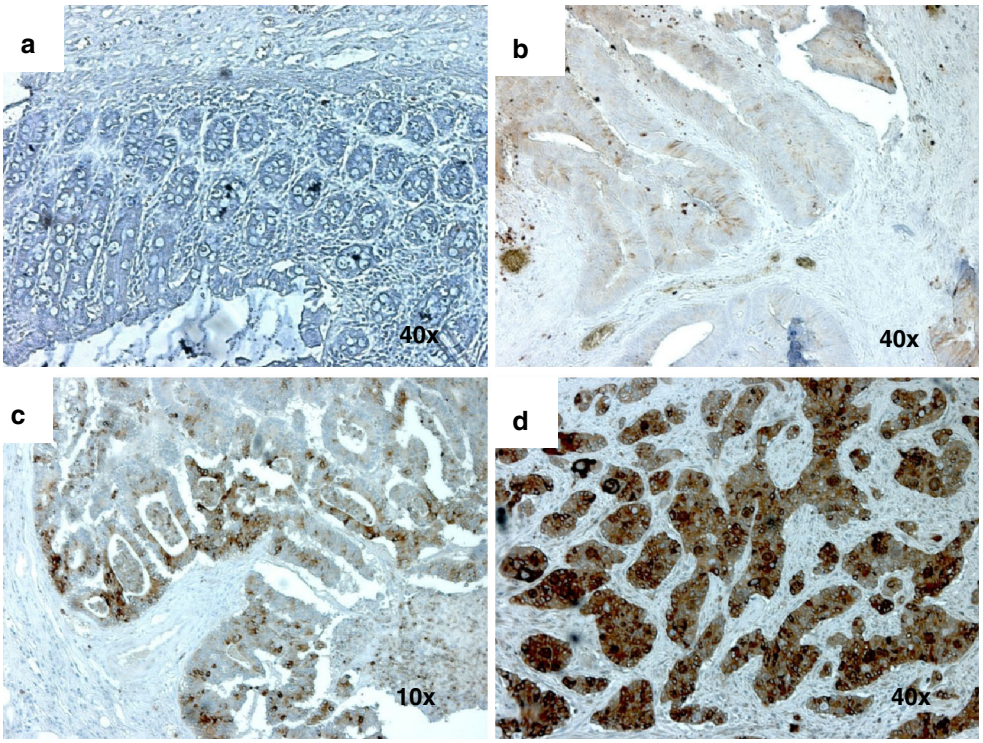

Fig. 2 Determination of Lcn-2 expression in colon cancer tissue using immunohistochemical staining. **a** Nontumor colon tissue. **b** Colon cancer tissue with low Lcn-2 expression. **c** Colon cancer tissue with moderate Lcn-2 expression. **d** Colon cancer tissue with high Lcn-2 expression

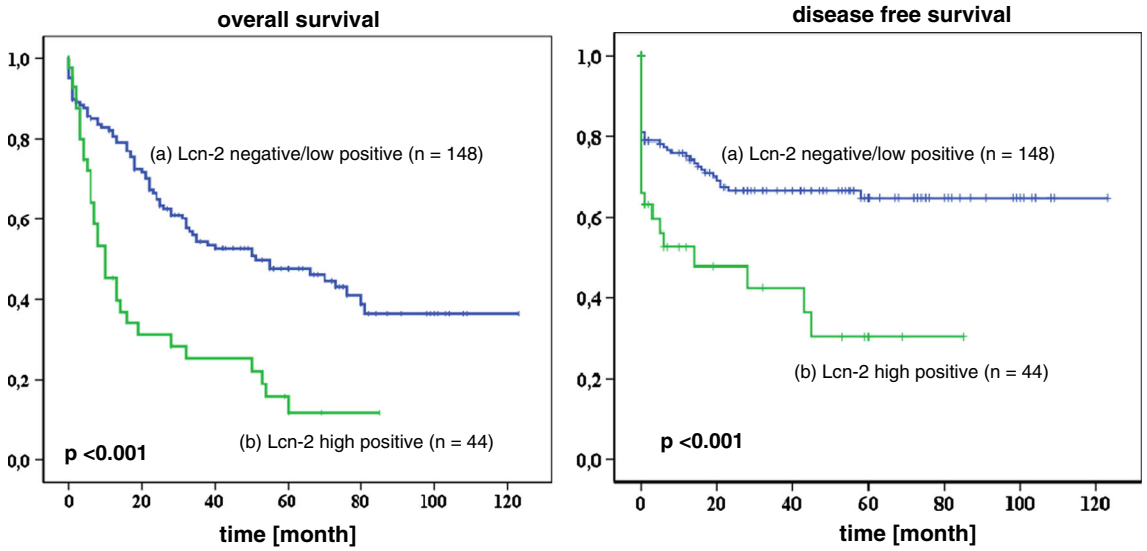

Fig. 3 Kaplan–Meier survival analysis shows a significant association between Lcn-2 expression and overall survival and disease-free survival ($p < 0.001$)

Clinicopathologic parameters of patients and expression of Lcn-2 are shown in Table 1. Univariate analyses revealed a significant correlation between Lcn-2 overexpression and T-stage ($p = 0.037$), tumor localization in the colon ($p = 0.001$), metastasis ($p = 0.042$), and UICC status ($p = 0.027$). Comparison of tumor sites in the colon showed that Lcn-2 overexpression was significantly higher in the right hemicolon and decreased in the left hemicolon and rectum. Metastases were more often seen in Lcn-2-overexpressing patients. These patients also had a higher UICC status and therefore poorer survival.

Table 2 Univariate and multivariate analyses to identify predictors of overall and disease-free survival

Variable	Univariate analysis				Multivariate analysis			
	OS		DFS		OS		DFS	
	HR (95 % CI)	p	HR (95 % CI)	p	HR (95 % CI)	p	HR (95 % CI)	p
UICC clinical stage [early (I, II) vs. advanced (III, IV)]	2.33 (1.83–2.96)	**<0.0001**	5.50 (3.57–8.44)	**<0.0001**	1.57 (1.19–2.08)	**0.002**	2.75 (1.70–4.45)	**<0.0001**
Metastasis (yes vs. no)	6.52 (4.17–10.20)	**<0.0001**	9.97 (5.60–27.62)	**<0.0001**	1.79 (0.62–5.20)	NS	2.96 (0.73–12.03)	NS
Lymph node status (negative vs. positive)	1.52 (1.27–1.82)	**<0.0001**	1.82 (1.45–2.27)	**<0.0001**	0.996 (0.756–1.307)	NS	1.09 (0.81–1.47)	NS
Lcn2 expression (high vs. low)	2.57 (1.69–3.92)	**<0.0001**	2.15 (1.30–3.57)	**0.003**	1.99 (1.31–3.04)	**0.001**	1.39 (0.83–2.32)	NS
Recurrence (yes vs. no)	2.50 (2.00–3.10)	**<0.0001**	4.08 (3.03–5.48)	**<0.0001**	1.84 (1.37–2.46)	**<0.0001**	2.46 (1.63–3.71)	**<0.0001**
T-stage (I:II:III:IV)	1.59 (1.31–1.93)	**<0.0001**	2.06 (1.60–2.65)	**<0.0001**	1.08 (0.77–1.51)	NS	0.90 (0.58–1.40)	NS

Multivariate analysis reveals Lcn-2 to be an independent prognostic parameter for overall survival

Boldface type indicates significance

OS overall survival, *DFS* disease-free survival, *CI* confidence interval, *HR* hazard ratio

Cumulative survival according to the Kaplan–Meier method (Fig. 3) revealed a significant association between Lcn-2-overexpressing tumors and overall survival (OS) ($p < 0.0001$). Median OS was 55 months in the Lcn-2 negative/low-positive group and 13 months in the Lcn-2 high-positive group. Patients were censored when the information about their survival time was incomplete. Likewise, high Lcn-2 expression correlates with low disease-free survival (DFS) ($p < 0.001$). Multivariate analysis (Table 2) showed that Lcn-2 is an independent prognostic indicator for OS. Patients with Lcn-2-overexpressing tumors had a higher risk for death than did patients with low-expressing tumors. However, Lcn-2 is not an independent prognostic factor for DFS.

Discussion

Lcn-2 is expressed in various carcinomas and was shown to be involved in tumor growth and metastasis [22, 23]. Results of The Cancer Genome Atlas (TCGA) data show significant up-regulation of Lcn-2 in colorectal tumors compared to control samples [24]. In this study, we analyzed whether Lcn-2 expression is up-regulated in colon cancer cells in vitro and in CRC samples. Only a few publications have reported on the role of Lcn-2 in CRC in a clinical setting. By screening the tissue of 192 CRC patients, we were able to present data from a well-documented cohort during a long-term follow-up.

Lcn-2-overexpressing colon carcinomas were significantly associated with poor survival according to the Kaplan–Meier analyses. These data are consistent with results of other authors. In breast carcinoma cells, for example, Lcn-2 expression was significantly associated with decreased disease-specific and disease-free survival [25]. Also, in gastric cancer, MMP-9–Lcn-2 complexes were highly associated with poorer survival [26]. Lcn-2 also interacts with LRP2, a protein that has been recently associated with pancreatic cancer [27]. According to our results, Lcn-2-overexpressing tumors showed significantly higher rates of metastasis. This is in contrast to the data reported by Lee et al. [9], in which Lcn-2 expression is inversely associated with the metastatic potential of various colon cancer cell lines, which suggests that Lcn-2 is a suppressor of colon cancer cell metastasis. Their hypothesis was based on in vitro experiments and on genetically modified cell lines transplanted into mice. Interestingly, similar findings have been made in pancreatic cancer, where Lcn-2 overexpression decreases invasion/adhesion and angiogenesis and reduces metastasis [28].

The spread of malignant tumor cells to form metastases at distant sites is responsible for the majority of deaths in affected humans. We suggest that Lcn-2 increases cell invasion by stabilizing or activating MMP-9, which promotes invasive tumor cells [29]. In vitro studies by Volpe et al., using human anaplastic thyroid carcinoma cell lines, outlined the mechanistic impact of Lcn-2 on MMP-9 activity. The authors showed that up-regulation of Lcn-2 other than that of MMP-9 is dependent on NF-κB activity during cancer development [30]. However, Hu et al. [30] showed that Lcn-2 expression can promote invasion of colon cancer cells

independently of MMP-9. Likewise, in our study we were not able to find a significant correlation between MMP-9 and Lcn-2 expression ($p = 0.781$) apart from a higher MMP-9 expression in higher T-stages even though not significantly different ($p = 0.063$, Table 1). The findings reported by Hu et al. [31] suggested that Lcn-2 contributes to colon cancer pathophysiology by altering Rac1 cellular distribution and subcellular localization of E-cadherin and catenins, decreasing E-cadherin-mediated cell–cell adhesion, enhancing cell–matrix attachment, and increasing cell motility and in vitro invasion.

Our protein analysis provided evidence that Lcn-2 is expressed in CRC cell lines and tissues, whereas there was no or only weak expression in normal colonic tissue. These findings are consistent with the study by Nielsen et al. [7], which already established the induction of Lcn-2 synthesis in neoplastic colorectal disease. However, they claimed that Lcn-2 expression is due only to inflammatory reactions, in which the protein may serve an important function as a scavenger of bacterial products. A recently published study demonstrated Lcn-2 overexpression in CRC cells and assumed that Lcn-2 contributes to colon cancer pathophysiology. Lcn-2 decreases cell–cell adhesion by altering subcellular localization of E-cadherin via Rac1, enhancing cell–matrix attachment and increasing cell motility and invasion [31].

Recently published data showed that Lcn-2 had no effect on colon cancer cell growth in vitro or in vivo [9, 31]. No significant association was seen between Lcn-2 expression and cell proliferation in esophageal cancer [32]. This finding parallels data from other authors evidence that Lcn-2 binds hepatocyte growth factor (HGF) to inhibit HGF-mediated c-met activation and thus MAPK and PI3K pathways in cancer cell lines [33]. In contrast, Fernandez et al. [17] showed a growth-stimulating effect of Lcn-2 overexpression in breast cancer. Accordingly, Wenners et al. [34] found Lcn-2 to be an independent prognostic factor for shorter DFS in primary human breast cancer. Such discrepancies may be related to the analysis of various tumor entities.

Our results showed significantly more Lcn-2-overexpressing tumors in the right than in the left hemicolon and rectum. These conclusions can also be drawn from former publications that proposed a division of colon cancer into two subsites [35]. Anatomic categorization of CRC in either a proximal or distal location relative to the splenic flexure seems simplified, but various authors have already provided evidence that this differentiation is still arguable. For example, there are differences in epidemiology, molecular genetics, and behavior between the two colon cancer subsites [35]. Reports on the influence of age, sex, or environmental factors on the subsite distribution of CRC have also been published [36, 37].

Questions remain to be answered by further in vivo and in vitro studies, such as the signaling pathways for tumor Lcn-2 expression at either an iron-dependent or iron-independent level [38]. Even though we demonstrated an association between Lcn-2 and advanced colon cancer, we were not able to elucidate how it contributes to tumor progression and invasion.

Conclusions

Our findings suggest that Lcn-2 is associated with poor differentiation, higher rate of metastasis, and poorer survival. Although the molecular mechanisms of Lcn-2 expression in cancer development are not known, we suggest that Lcn-2 promotes colon cancer progression and metastasis. The expression data will help us define benchmarks for clinical applications. These efforts might include biomarker studies and translational research for other immunologic questions such as prediction of graft survival and function in patients undergoing solid organ transplantation. We are expecting a correlation between long-term allograft function after 1 year and Lcn-2 expression during the early posttransplant period.

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Conflict of interest None.

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