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RESEARCH ARTICLE

Loss of MUC2 expression predicts disease recurrence and poor outcome in colorectal carcinoma

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Abstract Clinical staging and histological grading after surgery have been the "gold standard" for predicting prognosis and planning for adjuvant therapy of colorectal cancer (CRC). With the recent development of molecular markers, it has become possible to characterize tumors at the molecular level. This is important for stage II and III CRCs, in which clinicopathological features do not accurately predict heterogeneity, e.g., in their tumor response to adjuvant therapy. In the present study, archival samples from 141 patients with stage I, II, III, or IV CRC treated during 1981–1990 at Turku University Hospital (Finland) were used (as microarray blocks) to analyze MUC2 expression by immunohistochemistry. Altogether, 49.7 % of all tumors were positive for MUC2. There was no significant correlation between MUC2 expression and age ($P<0.499$), tumor invasion ($P<0.127$), tumor staging ($P<0.470$), histological grade ($P<0.706$), lymph node involvement ($P<0.854$), or tumor metastasis ($P<0.586$). However, loss of MUC2 expression was significantly associated with disease recurrence ($P<0.031$), tumor localization ($P<0.048$), and with borderline significance with gender ($P<0.085$). In univariate (Kaplan–Meier) survival analysis, positive MUC2 significantly predicted longer disease-free survival (DFS) and disease-specific survival (DSS) as well. However, in multivariate (Cox) survival analysis, MUC2 lost its power as an independent predictor of DFS and DSS. Our results implicate the value of MUC2 expression in predicting disease recurrence and long-term survival in CRC.

Keywords MUC2 expression · Colorectal cancer · Prognosis · Recurrence · Adjuvant therapy

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Introduction

Colorectal cancer (CRC) is the second most common type of cancer in both men and women worldwide [1]. Early diagnosis and treatment of the disease provides the best chance for survival. The best form of treatment for stage I and II tumors is surgical resection, which is curative in most cases. Stage III tumors receive adjuvant chemotherapy. Up to 25 % of patients with stage II cancers will present with relapse or metastatic disease, implicating that the biological behavior of stage II CRC is poorly predictable [2]. In the past 5 years, better understanding of the molecular aspects of colon carcinogenesis has resulted in increased survival rates of patients with metastatic disease by 100 % [3]. The need for informative molecular markers that provide prognostic information over and above that given by conventional pathological staging of CRCs has been repeatedly emphasized [4, 5].

Most epithelial tumors express various mucin glycoproteins on their cell surfaces. Mucins comprise a family of high

molecular weight glycoproteins with a large number of O-glycosylated tandem repeat domains varying in number, length, and extent of O-glycosylation [6–8]. Several human secretory mucin genes (MUC genes) have been identified, among which MUC1 gene encodes a membrane form of mucin-like O-glycoprotein or episialin, which is over-expressed in carcinoma of the breast and pancreas. The other MUC genes include MUC2 (prominent in the small and large intestine), MUC3 (predominant in the small intestine), MUC4 (universal for the epithelia), MUC5B (essentially in glandular acini in the submaxillary gland), MUC5C (present in the respiratory and gastric glands) [9, 10], MUC6 (prominent in the stomach and gall bladder) [11, 12], and MUC7 (mainly in the submandibular gland) [13].

Protein products of MUC genes have been studied in tumors arising from various organs, including the breast, colon, pancreas, and ovary [14–16]. Interesting relations between MUC2 expression and the pathogenesis of colorectal neoplasia have been disclosed [17]. MUC2 is expressed by adenomas and mucinous carcinomas. Downregulation of MUC2 is seen in non-mucinous adenocarcinoma arising within adenomas, whereas cancers considered to develop de novo do not express MUC2 [17]. However, little is known about the expression of glycoproteins encoded by MUC genes in CRC, and data on their potential prognostic value in these tumors are completely lacking. In the present study, we examined the expression of MUC2 mucins in 141 CRCs using immunohistochemistry (IHC) and correlated the results with the established clinicopathological factors of the disease.

Patients and methods

The present series consists of tissue samples obtained from 141 patients with stage I, II, III, or IV CRC who underwent bowel resection during 1981–1990 at Turku University Hospital (TUH, Finland), which were collected from the archives of the Department of Pathology. IHC staining was done at the Department of Pathology, Benghazi University, Benghazi, Libya. All pertinent clinical and histopathological data of the patients were collected from the patients' case records as summarized in Table 1. All patients have been prospectively followed up until death or when last seen alive on their clinical visit (March 2007), with the median FU time of 77 months (range, 2–263 months). The study was approved by the TUH Ethics Committee and was conducted in accordance with the endorsement of the National Authority for Medico-legal Affairs.

Table 1 Clinicopathological characteristics of the patients

Characteristic	Number of patients (%)
Gender	
Male	55 (39 %)
Female	86 (61 %)
Age (years)	
<65 years	63 (45 %)
>65 years	78 (55 %)
Primary tumor status	
T1	3 (2 %)
T2	12 (9 %)
T3	86 (61 %)
T4	40 (28 %)
LN involvement	
No	99 (70 %)
Yes	42 (30 %)
Metastasis	
No	126 (89 %)
Yes	15 (11 %)
Stage	
I	14 (10 %)
II	85 (60 %)
III	26 (19 %)
IV	16 (11 %)
Histological grade	
Grade I	20 (14 %)
Grade II	103 (73 %)
Grade III	18 (13 %)
Localization	
Right colon	54 (38 %)
Left colon	41 (29 %)
Rectum	46 (33 %)
Recurrence during the follow-up	
Yes	54 (39 %)
No	71 (50 %)
Unknown	16 (11 %)
Status at the end of follow-up	
Alive	40 (28 %)
Dead as result of disease	66 (47 %)
Dead from other cause(s)	35 (25 %)

Tissue microarray

Archival paraffin-embedded samples were used to build up tissue microarray (TMA) blocks for IHC staining. Areas of invasive tumor with the lowest degree of differentiation, abundant in cells with the highest number of mitoses, were chosen from the original blocks. Necrotic and autolytic areas and areas containing predominantly stromal tissue were excluded. For tumors producing abundant intra- or extracellular mucin, invasive areas with the highest number of epithelial cells were chosen. These representative areas were marked by an experienced pathologist on hematoxylin and eosin-stained slides from selected paraffin blocks, and a cylinder of tissue (1 mm in diameter) was cut with a TMA instrument (Beecher

Instruments, Sun Prairie, WI, USA) into a new paraffin block. This size of tissue section (1-mm wide) was equal to the often used three cores, 0.6-mm wide (20-23). As the core was larger than usual, sampling differences were less in 0.6-mm cores. Serial 4-μm sections were then cut from the TMA paraffin blocks. The sections were mounted on ChemMate™ Capillary Gap plus Slides (gray) by Dako, Glostrup, Denmark. Normal colorectal mucosa was selected adjacent to but at least 2 mm apart from the malignant tissues of the section. If available, another normal sample was obtained from normal colorectal mucosa at either of the resection margins in the surgical specimens. On average, two normal controls were available. Lymphatic follicles and hyperplastic and inflamed areas were avoided. To obtain enough mucosa for tissue array, all tangentially cut areas were avoided.

MUC2 immunostaining

IHC analysis was done using the automatic system (BenchMark XT, Ventana Medical Systems, Inc., Tucson, AZ, USA). This fully automated processing of bar code-labeled slides included baking of the slides, solvent-free deparaffinization, antigen retrieval in a cell conditioning buffer CC1 (mild, 36 min conditioning, and standard, 60 min conditioning), and incubation with the monoclonal anti-MUC2 antibody (clone MRQ-18; Ventana Medical Systems) for 32 min at 37 °C. Application of ultraView™ Universal DAB Inhibitor, ultraView Universal DAB Chromogen, ultraView Universal DAB H2O2, and ultraView Universal DAB Copper. Counterstaining with hematoxylin (2021) took 4 min, and post-counter staining with bluing reagent (2037) took 4 min as well. After staining, the sections were dehydrated in ethanol, cleared in xylene, and covered with Mountex and cover slips.

Evaluation of MUC2 expression

The evaluation of the staining of all TMAs was performed with a light microscope at ×40 magnification and with the evaluator blinded to the information on tumor grade, stage, or clinical outcome. The typical expression patterns of MUC2 are illustrated in Fig. 1. Three different grading (A, B, and C) systems were applied to assess the patterns of MUC2 expression in tumor cells. In system A, the cytoplasmic staining was graded into four categories: (0) no expression (no detectable staining), (1) weak staining, (2) moderate staining, and (3) strong staining intensity.

In system B, cytoplasmic staining was graded in two categories: (1) no/weak expression and (2) moderate/strong expression. Finally, in system C, MUC2 expression was categorized simply as negative or positive. All three systems were statistically tested, and the negative/positive grading (C) seemed to provide the most meaningful correlates of MUC2 with the clinically relevant data.

In calculating the staining indexes, cytoplasmic index, the intensity of staining and the fraction of positively stained cells were taken into account using the following formula:

$$I = 0 \times f0 + 1 \times f1 + 2 \times f2 + 3 \times f3$$

where I is the staining index, and f0–f3 are the fractions of the cells showing a defined level of staining intensity (from 0 to 3). Theoretically, the index could vary between 0 and 3 [18].

Statistical analysis

Statistical analyses were performed using the IBM SPSS® Statistics (IBM Company, New York, NY, USA) and STATA (StataCorp, TX, USA) software packages (IBM SPSS Statistics for Windows, version 20.0.1 and STATA/SE 12.1). Frequency tables were analyzed using the chi-square test, with likelihood ratio or Fischer's exact test being used to assess the significance of the correlation between the categorical variables. Odds ratio and their 95 % confidence intervals (95 % CI) were calculated where appropriate using the exact method. Differences in the means of continuous variables were analyzed using nonparametric tests (Mann–Whitney or Kruskal–Wallis) for two and multiple independent samples, respectively. Analysis of variance was only used for deriving the mean values (and their 95 % CI) of each individual stratum. Univariate survival analysis for the

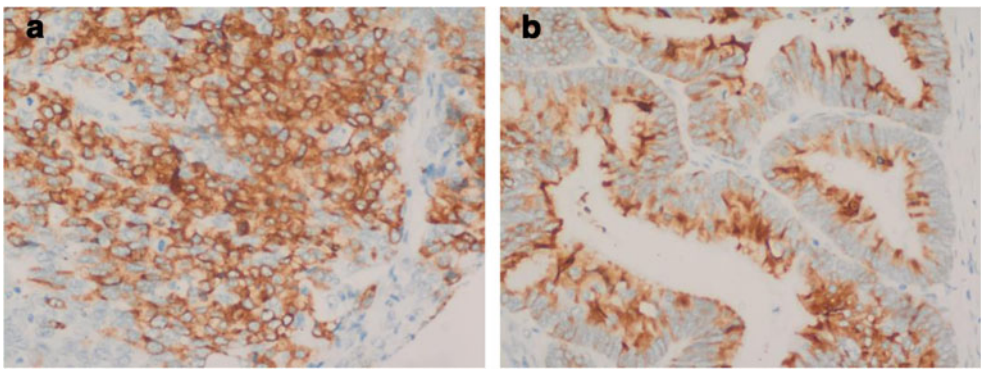

Fig. 1 Moderate diffuse perinuclear **(a)** and apical cytoplasmic **(b)** MUC2 expression in colorectal adenocarcinoma cells (×40)

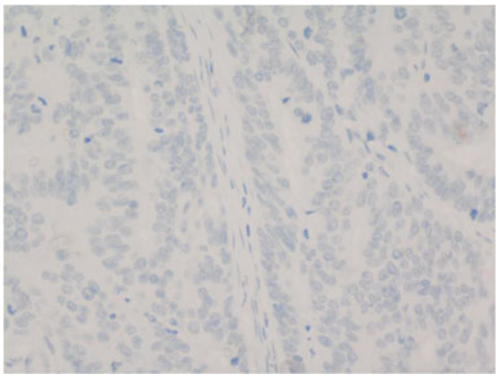

Fig. 2 Negative MUC2 expression in colorectal adenocarcinoma cells (×40)

outcome measures (disease-specific survival (DSS) and disease-free survival (DFS)) was based on the Kaplan–Meier method with logrank (Mantel–Cox) comparison test. To assess the value of MUC2 as an independent predictor, multivariate survival analysis was performed using the Cox proportional hazards regression model, controlling for the confounding by the following variables: age, sex, tumor localization, primary tumor status (T), grade (for DFS), and recurrence as additional variable (for DSS). In all tests, the values $P<0.05$ were regarded statistically significant.

Results

Description of MUC2 expression patterns

The expression pattern of MUC2 was predominantly perinuclear and cytoplasmic in normal colonic epithelium and in the tumor area as well. Examples of the staining patterns of MUC2 are illustrated in Figs. 1a, b and 2. Of the 141 tumors, 71 (50.3 %) were considered negative (staining intensity 0; Fig. 2), whereas 70 (49.7 %) were considered

Table 2 Correlation between MUC expression and clinicopathological features of CRC

Features	Number of cases (%)	MUC expression		*P* value
		<Mean	>Mean	
Gender				0.53
Male	55 (39 %)	45 (82 %)	10 (18 %)	
Female	86 (61 %)	66 (77 %)	20 (23 %)	
Age group (years)				0.54
<60	63 (45 %)	48 (76 %)	15 (24 %)	
>60	78 (55 %)	63 (81 %)	15 (19 %)	
Lymph node involvement				0.82
Yes	42 (30 %)	34 (81 %)	8 (19 %)	
No	99 (70 %)	77 (78 %)	22 (22 %)	
Distant metastasis				0.73
Yes	15 (11 %)	13 (87 %)	2 (13 %)	
No	126 (89 %)	98 (78 %)	28 (22 %)	
Tumor stage				0.44
I	14 (10 %)	13 (92.9 %)	1 (7.1 %)	
II	85 (60 %)	64 (75.3 %)	21 (24.7 %)	
III	26 (19 %)	20 (76.9 %)	6 (23.1 %)	
IV	16 (11 %)	14 (87.5 %)	2 (12.5 %)	
Tumor grade				0.94
Well	20 (14 %)	16 (80 %)	4 (20 %)	
Moderate	103 (73 %)	80 (78 %)	23 (22 %)	
Poor	18 (13 %)	15 (83 %)	3 (17 %)	
Tumor location				0.53
Right colon	54 (38 %)	41 (76 %)	13 (24 %)	
Left colon	87 (62 %)	70 (80 %)	17 (20 %)	
Recurrence				0.08
Yes	54 (39 %)	46 (85 %)	8 (15 %)	
No	71 (50 %)	51 (72 %)	20 (28 %)	
Unknown	16 (11 %)	Not studied	Not studied	

positive (staining intensity >1; Fig. 1a, b). Strong expression of MUC2 was noticed in normal colonic mucosa and in tubulovillous adenomas.

MUC2 expression correlates with the clinicopathological features

A significant correlation between MUC2 expression and tumor localization was found in that tumors arising in the colon express MUC2 more than tumors arising in the rectum (P= 0.04). Loss of MUC2 expression was more frequently detected in the left-sided and rectal carcinomas. Loss of MUC2 expression correlated significantly with disease recurrence after treatment as compared with positive MUC2 expression (P=0.03). MUC2 expression showed a borderline (P=0.08) correlation with gender in that tumors of the female patients expressed MUC2 more than tumors of the male patients. On the other hand, tumor grade, tumor invasion, age, and lymph node involvement had no significant relationship with the expression of MUC2 (Tables 2 and 3).

Survival analysis

In the Kaplan–Meier survival analysis, there was a significant difference in DFS between patients with MUC2-positive tumors (longer DFS) and those with negative tumors (Fig. 3). The same was true in DSS (Fig. 4), patients with MUC2-positive tumors living significantly longer. At 5-year follow-up, 30 % of the patients with MUC2-positive tumors showed recurrence as compared to 45 % of the patients with no MUC2 expression. The same was true in DSS patients with MUC2-positive tumors who had longer survival; 45 % of patients with MUC2-positive tumors were alive at 5 years as compared to 35 % of the patients whose tumors had no MUC2 expression.

The strength of MUC2 as an independent predictor of DFS and DSS was also tested in multivariate (Cox) proportional hazards regression models, where available prognostic factors,

Table 3 Correlation between MUC expression and clinicopathological features of the patients and their tumors

Features	Number of cases (%)	MUC expression		P value
		Negative (0)	Positive (1, 2, 3)	
Gender				0.085
Male	55 (39 %)	33 (60 %)	22 (40 %)	
Female	86 (61 %)	38 (44 %)	48 (56 %)	
Age group (years)				0.499
<60	63 (45 %)	34 (54 %)	29 (46 %)	
>60	78 (55 %)	37 (47 %)	41 (53 %)	
Lymph node involvement				0.854
Yes	42 (30 %)	22 (52 %)	20 (48 %)	
No	99 (70 %)	49 (49 %)	50 (51 %)	
Distant metastasis				0.58
Yes	15 (11 %)	9 (60 %)	6 (40 %)	
No	126 (89 %)	62 (49 %)	64 (51 %)	
Tumor stage				0.47
I	14 (10 %)	9 (64 %)	5 (36 %)	
II	85 (60 %)	40 (47 %)	45 (53 %)	
III	26 (19 %)	12 (46 %)	14 (54 %)	
IV	16 (11 %)	10 (63 %)	6 (37 %)	
Tumor grade				0.70
Well	20 (14 %)	12 (60 %)	8 (40 %)	
Moderate	103 (73 %)	50 (48 %)	53 (52 %)	
Poor	18 (13 %)	9 (50 %)	9 (50 %)	
Tumor location				0.04
Colon	95 (67 %)	42 (44 %)	53 (56 %)	
Rectum	46 (33 %)	29 (63 %)	17 (37 %)	
Recurrence				0.031
Yes	54 (39 %)	32 (59 %)	22 (41 %)	
No	71 (50 %)	29 (41 %)	42 (59 %)	
Unknown	16 (11 %)	No statistical data		

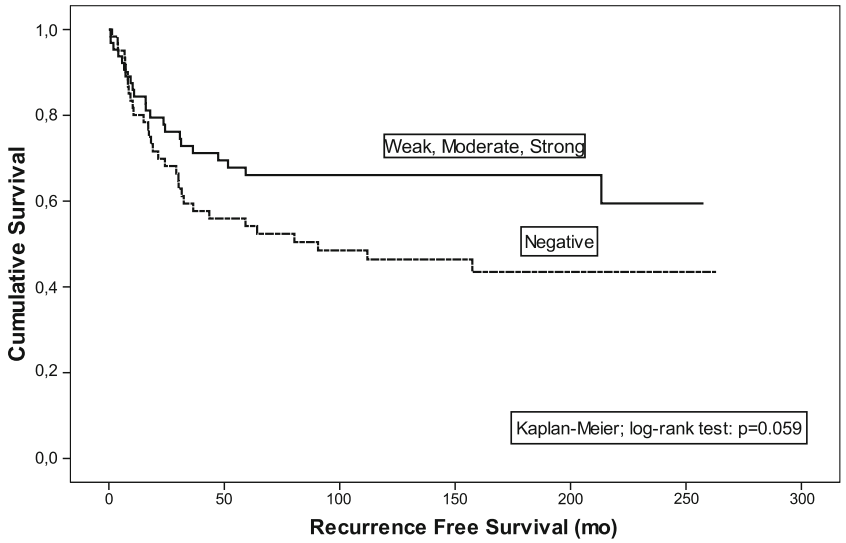

Fig. 3 MUC2 expression (negative/positive) as determinant of disease-free survival in univariate (Kaplan–Meier) analysis

age, sex, tumor, localization, T, grade (for DFS), and recurrence as additional variable (for DSS), were entered in a stepwise backward approach. Of the variables entered in the model, tumor localization and primary T were the only independent predictor of DFS, with HR=2.12 (95 % CI 1.21–3.70; P=0.008) and P=0.049 (for collective T), respectively. In a similar model for DSS, the three independent predictors are age (P=0.025), T (P=0.043), and recurrence (P=0.0001). In both models, MUC2 was removed from the model when adjusted for the other variables.

Discussion

This study is a continuation of our efforts to further elucidate the biology of CRC and to identify more effective prognostic factors than the traditional staging system to aid therapeutic decision making [19–21]. The aim of the present study was to cast further light on the issues related to prognostication of CRC while assessing the value of quantitative MUC2 expression profiles as predictive and prognostic factor. In this study, we focused on stage I–IV diseases, where molecular and other markers may help in pinpointing a subgroup of patients who would eventually benefit from the use of adjuvant therapy for their disease. This important decision involves careful weighing of the risks of toxicity and complications against the potential curability of the disease [22]. We used different approaches to analyze the expression of MUC2. On the basis of the present results, we do believe that the grading system classifying CRCs as MUC2-positive or -negative is the clinically most relevant approach.

In the present series, the loss of MUC2 expression was found in 50.3 % of CRCs, being consonant with some earlier findings reporting reduction of MUC2 in 53 % of CRCs [23]. We thus consider that the loss of MUC2 expression may be important for the occurrence and progression of these tumors. Several pathways may be included in the adenoma-to-carcinoma sequence in the colon and rectum.

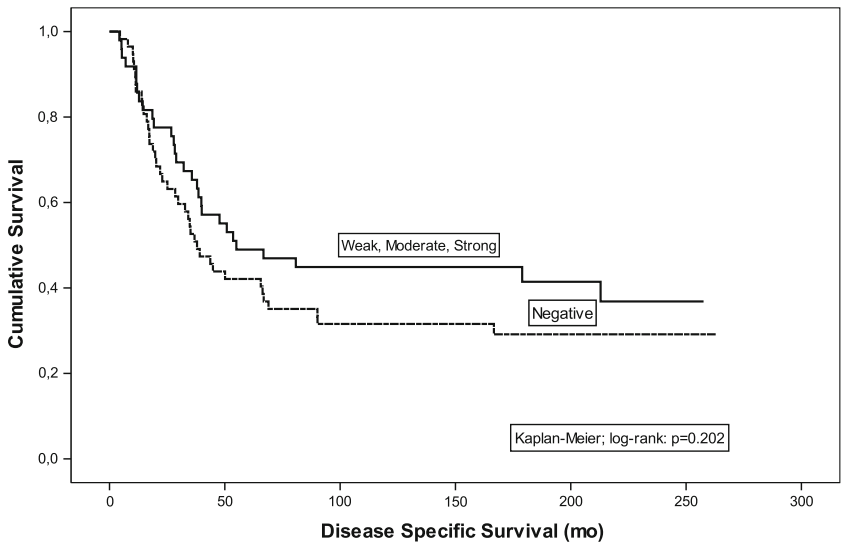

Fig. 4 MUC2 expression (negative/positive) as determinant of disease-specific survival in univariate (Kaplan–Meier) analysis

Regarding the regulation of MUC2 expression, Yamamoto et al. [24] have demonstrated that CDX2 interacts with the MUC2 promoter and activates MUC2 transcription. However, a recent study [25] shows that all cases of adenomas and cancers had CDX2 expression, while a decrease of MUC2 expression was observed in about half of the cases.

Suppression of the MUC2 gene in colon carcinoma cells is associated with methylation of the promoter region [26], and Ookawa et al. [27] have shown that p53 directly activates transcription of the MUC2 gene in many cell lines in vitro. Vincent et al. demonstrated that among the four 11p15 mucin genes, MUC2 and MUC5B are highly subjected to DNA methylation and histone modifications, whereas MUC5AC is rarely influenced by epigenetic regulation and MUC6 is not [28]. MUC2 repression by methylation is the result of site-specific methylation within its promoter, associated with establishment of a repression histone code. However, a recent study [29] showed that MUC2 gene methylation predominantly regulates its expression not only in a cell line study but also in a tissue study.

MUC2 downregulation observed in carcinomas, however, seems to be related to carcinomatous transformation of the intestinal epithelium, which loses its ability to express the native mucin type due to defective glycosylation observed in the late stages [30, 31]. Expression of MUC2 is frequently decreased with progression [17] and with an increase in the grade of epithelial dysplasia [23]. Interestingly, similar to the previous observations [30–32], the present study showed that downregulation of MUC2 is associated with progression and metastasis in CRC.

Interestingly, we observed a close association between MUC2 expression and tumor localization; negative expression of MUC2 was significantly associated with left-sided (distal) tumors of the colon. This was also clearly associated with different long-term survivals of these two groups (Fig. 3). Similar to our study, Lugli et al., [33] found that right-sided CRCs were associated with MUC2 expression, whereas MUC2 expression loss was more frequently detected in left-sided carcinomas. This suggests that there may be differences between the normal right and left colonic segments that could favor malignant transformation through different molecular pathways. Such differences are probably related to different molecular profiles of the tumors, microsatellite instability, and methylator phenotypes being associated with right-sided tumors as well as chromosomal instability with left-sided tumors [34, 35]. Some studies have shown that the biology of the rectum is close or nearly close to left-side colon and consider both of them, i.e., the left colon (descending colon and sigmoid colon) and the rectum as a representative of the left-side colon [36, 37]. We suggest that the higher levels of MUC2 expression associated with proximal tumors may be due to these divergent genetic pathways present in the left-sided and right-sided tumors. However, this remains only speculative at this stage, and future molecular studies are necessary to confirm this hypothesis [38–40]. Importantly, tumor localization was one of the significant independent predictors of DFS in the present multivariate models, further emphasizing the molecular differences associated with localization of CRC. Although not an independent prognostic factor for DFS or DSS in multivariate analysis, MUC2 might well represent an important constituent of these molecular pathways, making distinction between left- and right-colon carcinomas.

Taken together, the present results support the previously presented notion [25, 39] suggesting that the loss of MUC2 expression may be associated with disease recurrence and worse survival in CRC. Additional studies are warranted to assess, e.g., whether this predictive value is related to the stage of CRC, here analyzed collectively for stage I–IV diseases to accumulate enough cases for adequate strength of the study.

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Conflicts of interest None

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