Expression of cyclooxygenase-2 and mucin 1 in colorectal cancer

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Abstract. In colorectal cancer (CRC), pathological factors that correlate with negative prognosis include, among others, overexpression of cyclooxygenase-2 (COX-2) and abundant expression of mucin 1 (MUC1). COX-2 overexpression may therefore be associated with MUC1 overexpression. The aim of the present study was to investigate the possible correlation between COX-2 and MUC1 expression and to assess the correlation between their individual expression and the clinicopathological features of patients, paying particular attention to survival. The following data was collected from the 170 patients with CRC included in the present study: Age, sex, tumour localization, disease stage and survival. Tumour samples were immunostained with antibodies against COX-2 and MUC1. Protein expression was scored, relative to reference staining, and correlated with the clinicopathological data of patients. The results revealed no correlation between the expressions of COX-2 and MUC1, or with any of the studied clinicopathological variables. In addition, the expression of the two proteins were not associated. Neither of the proteins demonstrated prognostic value for survival. The present study did not confirm a direct relationship between the expressions of COX-2 and MUC1, or between the expression of either protein and the clinicopathological features of patients, including survival.

Introduction

According to epidemiological data gastrointestinal cancer is one of the largest problems within the field of oncology (1). In 2018, colorectal cancer (CRC) was the cause of 881,000 deaths (2). However, treatment and management of CRC have considerably changed over the last years.

Advances in systemic treatment, mainly targeted therapy and, in limited cases immunotherapy, have significantly changed the prognosis of patients with metastatic or recurrent CRC (3,4). Second and subsequent lines of treatment can slow the disease and, in effect, change it into a chronic disease. However, the aim of modern oncology is to definitively eliminate the danger of disease recurrence, which might be achievable by individualization of the treatment.

Chronic inflammation is a cancer-promoting factor that leads to genetic instability (5). Cyclooxygenase-2 (COX-2) is an enzyme that catalyses the formation of prostaglandins and therefore participates in the regulation of the consecutive stages of inflammatory processes. In CRCs overexpression of COX-2 has been shown to correlate with poor survival and metastasis (6).

Mucins are a family of glycoproteins form a protective gel layer on the surface of the mucosa under normal circumstances. Their expression varies in different areas of the digestive tract and is altered in tumours. Mucins in cancer cells are dysregulated on a genetic level and atypically glycosylated in posttranscriptional setting (7,8). Their quantitative and qualitative alterations impair their functions, heavily affect the properties of the mucus layer and presumably weaken the penetration of chemotherapeutic agents. Mucin 1 (MUC1) is a structural membrane-bound mucin that is normally present only on the apical borders of the secretory epithelium. In tumours, the polarization of MUC1 is lost and the protein is overexpressed at high levels over the entire cell surface (9). The positive correlation between abundant expression of MUC1 and tumour invasiveness, metastasis and poor prognosis has been reported in CRC (9,10).

The overexpression of COX-2 and the overexpression of MUC1 might be related and this relationship has already been studied in pancreatic cancer (11). The authors concluded that the intracellular tail of MUC1 participates in the activation of proapoptotic genes and indirectly upregulates COX-2 expression (11). Hypothetically, this relationship may also apply to other gastrointestinal cancers, including CRC. Perhaps this could be easily observed as altered COX-2 and MUC1 expression. In addition, MUC1 has been proposed as an alternative target for blocking COX-2 overexpression (11). In CRC, where abundant expression of COX-2 occurs frequently, such a discovery might be a substantial finding from the perspective of future targeted therapy. The significance of the present study

was to confirm the presented hypotheses in CRC. The direct aim was to investigate the correlation between COX-2 and MUC1 expression patterns and clinical-pathological factors in CRC, with particular attention to survival.

Materials and methods

Tissue samples. CRC samples were collected from the resources of the Histopathology Department of the Independent Clinical Hospital No. 1 (SPSK 1) in Lublin (Poland) in the form of paraffin-embedded tissue blocks. The studied material encompassed sections of colorectal adenocarcinomas from 170 patients treated in the Department of Surgical Oncology (Medical University of Lublin) between August 2004 and January 2014. Only samples that contained adenocarcinomas were included. Rectal tumour samples from patients who had undergone neoadjuvant radiotherapy were excluded from further analysis.

The clinicopathological data of patients were collected from the Medical Records Department and Outpatient Clinics of the Hospital. In the analysed population 102 (60%) patients were male, the median age was 62 years, 126 (74%) of the tumours were rectal cancers and the remaining 44 cases (26%) were colon cancers. Among the disease stages there were: 8 (5%) cancers *in situ* and 31 (18%) stage I, 51 (30%) stage II, 58 (34%) stage III and 22 (13%) stage IV cancers. Detailed data concerning the studied population are presented in Table I.

Antibodies. Immunostaining was performed using two antibodies for both COX-2 and MUC1. These were: Anti-COX-2 rabbit monoclonal antibody from Abcam (clone SP21, cat. no. ab16701) and anti-COX 2 mouse monoclonal antibody from Dako (clone CX-294); anti-Mucin 1C-term rabbit monoclonal antibody from antibodiesonline.com (clone G22-L, cat. no. ABIN371859) and anti-MUC1 mouse monoclonal antibody from Dako (EMA-human epithelial membrane antigen, clone E29, cat. no. M0613). The antibodies were stepwise optimized and tested at dilutions from 1:100 to 1:800 and for one antibody (anti-mucin 1 C-term clone G22-L, cat. no. ABIN371859 from antibodies online.com) even at 1:3200. The staining was comparatively performed with DAB and Bright-DAB and then the sections were counterstained with haematoxylin.

In effect, two antibodies and two staining protocols were chosen as the methods that provide the best sensitivity at the lowest background noise: Anti-COX-2 from Abcam (clone SP21, cat. no. ab16701), at a dilution of 1:200, incubated for 1 h and stained with DAB and anti-Mucin 1 C-term from antibodies-online.com (clone G22-L, cat. no. ABIN371859) diluted to 1:1,000, incubated overnight and stained with Bright-DAB (Table II).

Immunohistochemistry. Preparation of microscope slides, immunostaining and scoring were performed at University Medical Center Utrecht in the Netherlands. Sections of tissues (4 μm) were first deparaffinized and blocked for endogenous peroxidase activity by immersion in 0.3% H_2O_2 in methanol for 20 min. Then, antigen retrieval was performed in sodium citrate buffer (0.01 M/pH 6.0) for 20 min at 100°C. Nonspecific binding sites were blocked using Protein Block

Table I. Characteristics of the study population.

Characteristic	Value
Age, years	
≤55, n (%)	50 (29.41)
56-69, n (%)	74 (43.53)
≥70, n (%)	46 (27.06)
M ± SD	64.48±10.74
Me (Q1-Q3)	62 (54-70)
Min-Max	32-87
Sex, n (%)	
Male	102 (60.00)
Female	68 (40.00)
Localization, n (%)	
Colon	44 (25.88)
Rectum	126 (74.12)
Stage, n (%)	
0	8 (4.71)
1	31 (18.23)
2	51 (30.00)
3	58 (34.12)
4	22 (12.94)

M, mean; Me, median; Q1, lower quartile; Q3, upper quartile; Min, minimum score; Max, maximum score.

Serum Free (DAKO, X0909) for 10 min, followed by primary antibody incubation (anti-COX-2 at room temperature for 1 h and anti-MUC1 at 4°C overnight). Antibody binding was visualized using the BrightVision+poly-HRP detection system (VWR International, cat. no. VWRKDPVB110HRP), with 3,3-diamino-benzidine as chromogen (DAB, Sigma D5637 for COX-2 and Bright-DAB, VWR International VWRKBS04-110 for MUC1). Finally, the sections were counterstained with haematoxylin, dehydrated and coverslipped using Pertex (Table II).

Analysis of immunostaining intensity was conducted at 20x original objective magnification in the area that encompassed tumour cells with the strongest staining. Scoring systems for COX-2 and MUC1 based on the references (12,13). Scoring was performed independently by two researchers, the discrepancies were reanalysed by the expert and scored in accordance with his judgement.

The study was reviewed and approved by the Bioethics Committee of Medical University of Lublin (number of approval KE-0254/48/2015 from 26.02.2015).

Statistical analysis. The Statistica 9.1 software package was used for statistical analysis. The analysed data concerned the level of COX-2 and MUC1 expression with respect to age and sex of patients as well as the localization and stage of the tumour. The information regarding particular tests is included in the description of the tables.

The analysis regarding the interplay between the COX-2 and MUC1 expression levels was performed as a Spearman's rank correlation. The results were verified by the Chi-square test

Table II. Immunohistochemistry methods.

Antibody	Source	Dilution	Antigen retrieval	Antibody incubation	Chromogen
COX-2, clone SP21, cat. no. ab16701	Abcam	1:200	Sodium citrate buffer (0,01 M/pH 6.0) 20 min at 100˚C	1 h	DAB
MUC1 C-term, clone G22-L, cat. no. ABIN371859	Antibodies Online	1:1,000	Sodium citrate buffer (0,01 M/pH 6.0) 20 min at 100˚C	Overnight	Bright-DAB

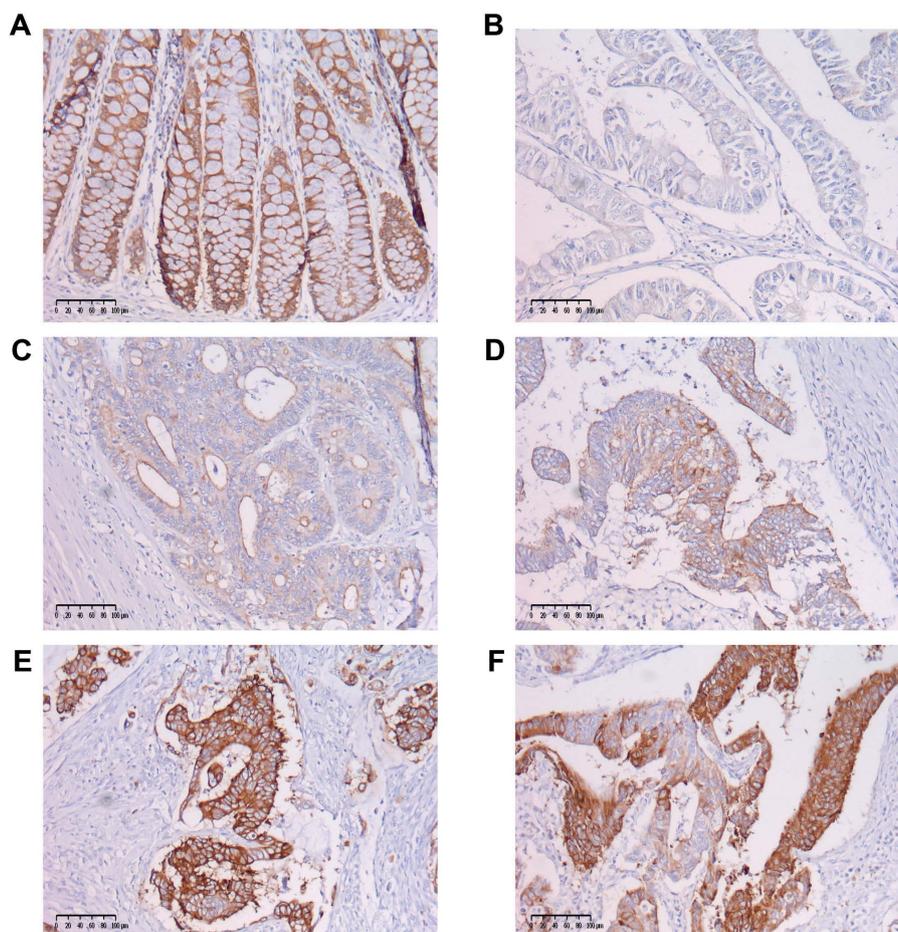COX-2, cyclooxygenase-2; MUC1, mucin 1.

Figure 1. Immunohistochemical COX-2 staining at x200 magnification. (A) Normal colon epithelium and representative colorectal cancer samples. (B) Category 0, no staining. (C) Category 1, weak diffuse cytoplasmic staining. (D) Category 2, moderate to strong granular cytoplasmic staining in 10-50% of cancer cells. (E) Category 3, >50% of tumor cells stained with a strong intensity. (F) patchy, heterogenic staining. Categories 0 and 1 were considered to demonstrate a low expression of COX-2 and scores 2 and 3, as well as patchy staining, was considered to demonstrate a high expression of COX-2. COX-2, cyclooxygenase-2.

comparing the incidence of cases with high COX-2 expression and high MUC1 expression, compared to the cases with low COX-2 expression and low MUC1 expression. An analogous analysis was performed comparing the cases with high COX-2 expression and low MUC1 expression compared to cases with low COX-2 expression and high MUC1 expression.Survival analysis was performed for high and low COX-2 expression and for high and low MUC1 expression, using MedCalc software. The results are presented as Kaplan-Meier curves, and the comparison of survival curves was performed using the log-rank test. P-values <0.05 were considered significant.

Results

COX-2 and MUC1 expression. COX-2 expression was categorized as follows: 0, no staining; 1, weak diffuse cytoplasmic staining; 2, moderate to strong granular cytoplasmic staining in 10-50% of cancer cells; and 3, >50% of tumour cells stained with strong intensity (Fig. 1). Samples with scores of 0 and 1 were further categorized as low COX-2 expression, and those with scores of 2 and 3 were categorized as high COX-2 expression. The cases of patchy, heterogenic COX-2 staining were distinguished and included as stained with strong intensity.

Table III. COX-2 expression and clinicopathological features of patients.

Characteristic	COX-2 expression		Statistics
	Low, n (%)	High, n (%)	
Age, years			H (2.170)=1.109; P=0.574
≤55	10 (20.0)	40 (80.0)	R=0.415; P=0.678
56-69	14 (18.9)	60 (81.1)	
≥70	11 (23.9)	35 (76.1)	
Sex			Z=0.068; P=0.945
Male	22 (21.6)	80 (78.4)	
Female	13 (19.1)	55 (80.9)	
Localization			Z=0.006; P=0.995
Colon	7 (15.9)	37 (84.1)	
Rectum	28 (22.2)	98 (77.8)	
Stage			H (4.170)=1.203; P=0.878
0	2 (25.0)	6 (75.0)	R=0.084; P=0.276
1	8 (25.8)	23 (74.2)	
2	10 (19.6)	41 (80.4)	
3	12 (20.7)	46 (79.3)	
4	3 (13.6)	19 (86.3)	
Total	35 (20.6)	135 (79.4)	-

P-value, statistical significance; Z, Mann-Whitney U test score; H, Kruskal-Wallis test score; R, Spearman's rank correlation coefficient; COX-2, cyclooxygenase-2.
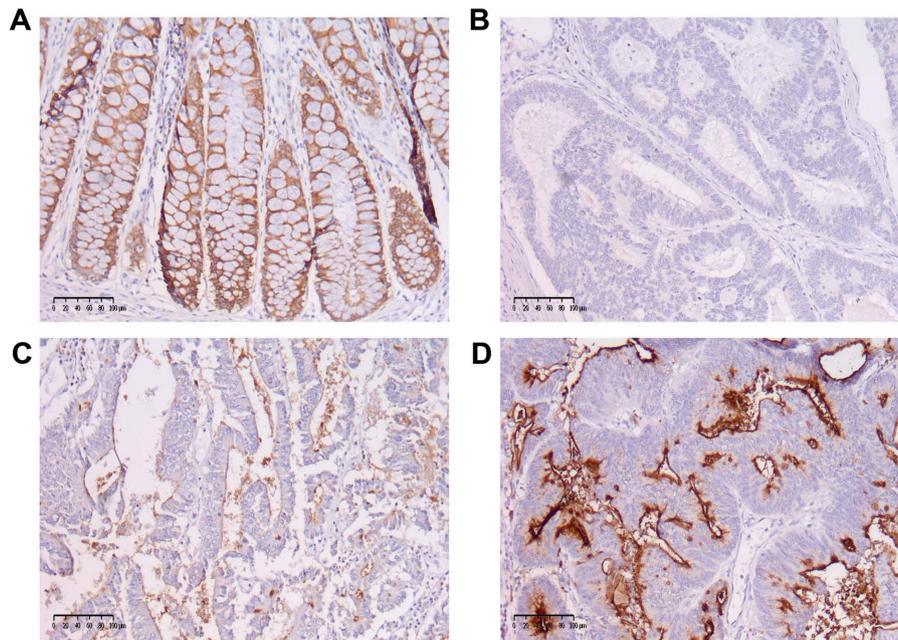

Figure 2. Immunohistochemical mucin 1 staining at x200 magnification. (A) Normal colon epithelium and representative colorectal cancer samples. (B) Category 0, no staining. (C) Category 1, weak staining. (D) Category 2, moderate to strong staining.

The expression level of COX-2 and its statistical correlation with: Age, sex, localization and disease stage are presented in Table III.

MUC1 staining was categorized as: 0, no staining; 1, weak staining; 2, strong staining and further categorized as low MUC1 expression (scores of 0 and 1) or high MUC1 expression (score 2) (Fig. 2). The correlations with clinical data are presented in Tables III and IV.

Neither the COX-2 expression level nor the MUC1 expression level correlated with any of the clinicopathological features.

Table IV. MUC1 expression and clinicopathological features of patients.

Characteristic	MUC1 expression		Statistics
	Low (0-1), n (%)	High (2), n (%)	
Age, years			H (2.170)=1.965; P=0.375
≤55	26 (52.0)	24 (48.0)	R=0.107; P=0.164
56-69	32 (43.2)	42 (56.8)	
≥70	18 (39.1)	28 (60.9)	
Sex			Z=0.658; P=0.510
Male	44 (43.1)	58 (56.9)	
Female	32 (47.1)	36 (52.9)	
Localization			Z=-0.554; P=0.580
Colon	22 (50.0)	22 (50.0)	
Rectum	54 (42.9)	72 (57.1)	
Stage			H (4.170)=2.630; P=0.622
0	3 (37.5)	5 (62.5)	R=-0.048; P=0.535
1	16 (51.6)	15 (48.4)	
2	19 (37.2)	32 (62.7)	
3	28 (48.3)	30 (51.7)	
4	10 (45.4)	12 (54.6)	
Total	76 (44.7)	94 (55.3)	-

P-value, statistical significance; Z, Mann-Whitney U test score; H, Kruskal-Wallis test score; R, Spearman's rank correlation coefficient; MUC1, mucin 1.

Table V. Ratio of COX-2 to MUC1 expression.

COX-2	MUC1		Total
	Low, n (%)	High, n (%)	
Low	17 (10.0)[a]	18 (10.6)[b]	35 (20.6)
High	59 (34.7)[c]	76 (44.7)[d]	135 (79.4)
Total	76 (44.7)	94 (55.3)	170 (100.0)

[a,d]Chi-squared test=36.172, P<0.001; [b,c]Chi-squared test=20.779, P<0.001. Spearman's rank correlation R-0.121, P=0.117. COX-2, cyclooxygenase-2; MUC1, mucin 1.

Figure 3. Ratio of COX-2 expression to MUC1 expression. COX-2, cyclooxygenase-2; MUC1, mucin 1.

The ratio of COX-2 to MUC1 expression is shown in Fig. 3. There was no linear correlation between the expression levels of these proteins (P=0.117). However, there were significantly more cases with the simultaneous occurrence of high COX-2 and MUC1 expression compared to those in which the level of COX-2 and MUC1 expression was low (P<0.001). There were also significantly more cases with high expression of COX-2 and low expression of MUC1 than cases with simultaneous low expression of COX-2 and high expression of MUC1 (P<0.001) (Table V).

Survival analysis. There was no statistical correlation between survival and the levels of COX-2 or MUC1 expression (Figs. 4 and 5).
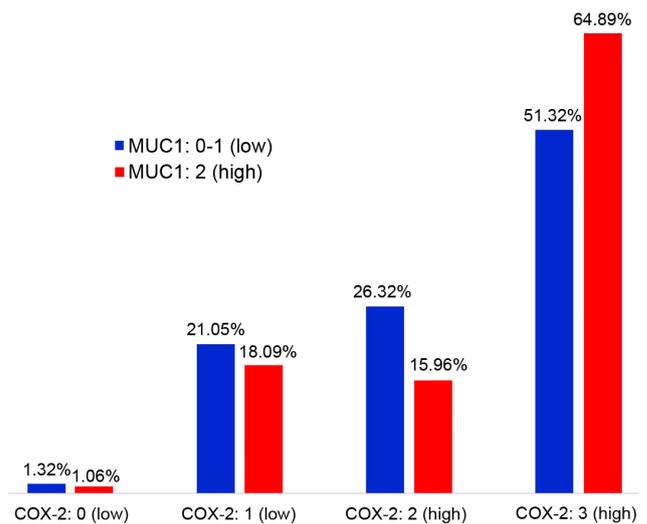
Discussion

There are many reports of the importance of COX-2 overexpression in patients with CRC. Studies carried out so fa, on populations of different races, have shown that COX-2 overexpression slightly worsens overall survival (6). In a recently published paper, Kim *et al* presented that in a group of Korean patients elevated COX-2 expression was not a prognostic factor, but COX-2 expression might have been an
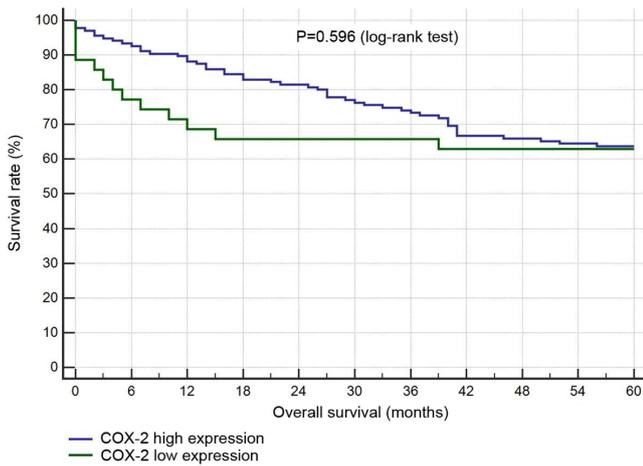
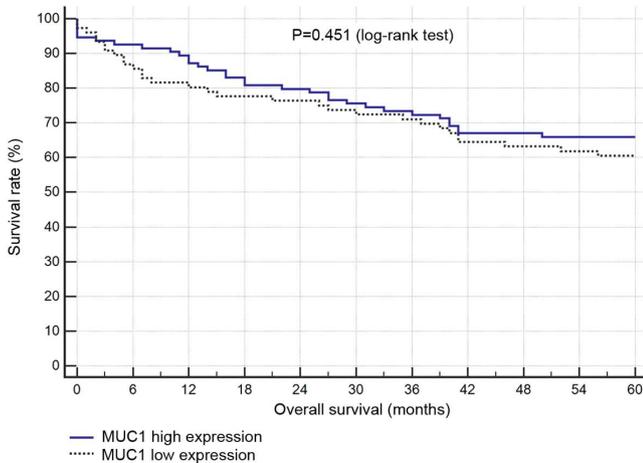Figure 4. Survival analysis in reference to the level of COX-2 expression. COX-2, cyclooxygenase-2

Figure 5. Survival analysis in reference to the level of MUC1 expression. MUC1, mucin 1.

independent predictive marker of late recurrence for patients with stage I to III CRC (14). Our studies showed no correlation between the expression of COX-2 and any studied clinical variables, or prognosis. The conclusions of Kim *et al* (14). seem to be consistent with ours, however, our analysis was narrower, because it did not relate to recurrence rates and it was also carried out among Caucasian patients so the results cannot be directly compared.

Our research did not confirm the correlation between the expression of MUC1 and clinical variables, including stage of the disease and survival. In a similar study performed independently at the same time as ours, there was no correlation between MUC1 expression and clinicopathological variables of the patients, but there was a significant increase in *MUC1* mRNA expression in CRC compared to healthy tissues (15). This could mean that in tumours, the level of MUC1 changes with the progression of the disease. In the cited study MUC1 expression was more often detected in patients with CRC with synchronic lymph node metastases, than in those without them (15). Duncan *et al*, in a study on a population of 462 patients showed that MUC1 expression can be considered

an independent marker of poor prognosis, which is in contrast to our results. However, they did not confirm the correlation of MUC1 with any of the clinicopathological variables including tumour grade and stage, vascular invasion and tumour type, which coincides with our results (16). Betge *et al* also showed a correlation of MUC1 expression with various clinicopathological variables as well as disease progression and lymph node metastasis. However, their study did not confirm a correlation between MUC1 expression and survival in patients with CRC (17). It is interesting that all these cited studies concerned similar populations, i.e., Caucasian patients were recruited consecutively for CRC surgery. MUC1 overexpression occurs in CRC with lymph node invasion (18). Therefore, hypothetically, the negative results of our and other authors' work may result from a small number of patients with lymph node metastases.

Nevertheless, the quality assessment of the MUC1 expression level based only on immunohistochemistry is limited in credibility. This is because mucins are alternatively glycosylated in tumours (7). Evaluation of the MUC1 expression level may be understated due to the specificity of the chosen antibody. In one of the larger earlier studies, MUC1 was detected only in 32.5% of CRC specimens (19). Therefore, we take into account that in our study, COX-2 level assessment is more reliable than MUC1. The expression of mucins and associated O-glycans differ in colorectal polyp subtypes (20). Most likely, it also applies to pathological subtypes of CRC. The distribution of goblet cells, which produce mucins, increases along the entire length of the digestive tract (21). These observations might explain the discrepancies in our and other cited studies, because all of them differed in the number of pathological subtypes of CRC and the number of tumours from different localizations of the colon. In most of the papers about MUC1 in CRC, parts of the colon were not distinguished. In our study, there was a similar number of MUC1-low and MUC1-high expression in the colon, but there were definitely more cases of MUC1-high expression in the rectum. The latter result is more reliable because most of the examined tissues were cancers of the rectum.

The present study does not confirm a direct relationship between the intensity of expression of COX-2 and MUC1 or between the expression of either of them and the clinicopathological characteristics of patients with CRC. In addition, neither protein had prognostic value for survival, which contradicts some previous reports. This issue needs further investigation based on larger sample analysis or stratified analysis.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MS, RS and RM concieved and designed the current study. RM, RS, FM, MS, MB and JM acquired the data. MS, JM, GJAO and WPP analyzed and interpreted the data. MS and RS drafted the manuscript. All authors critically revised the manuscript, with major contributions from GJAO and WPP. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The board of the Bioethics Committee of the Medical University of Lublin approved the present study protocol (approval no. KE-0254/48/2015 from February 26, 2015). Informed consent was obtained at the time of original tissue collection.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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