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ORIGINAL ARTICLE

Decreased ERβ expression and high cyclin D1 expression may predict early CRC recurrence in high-risk Duke's B and Duke's C stage

Aleksandra Babic[1], Nenad Miladinovic[2], Jelena Milin-Lazovic[3], Sanja Milenkovic[2]

[1]Department of Medical Oncology, University Clinical Hospital Center Zemun, Belgrade [2]Department of Clinical Pathology, University Clinical Hospital Center Zemun, Belgrade [3]Institute for medical statistics and informatics, Medical Faculty, Belgrade

Summary

Purpose: *Despite many known risk factors for the colorectal cancer (CRC) recurrence, significant differences in disease-free survival (DFS) impose the need to look for new explanations. This study aimed to determine the degree of expression of ERa, ERβ, PR, Cyclin D1, and Bcl-2 and their association with early CRC relapse.*

Methods: *This retrospective study included 101 radically operated CRC patients in high-risk Duke's B and Duke's C stage. Tissue samples were retrieved from paraffin blocks and clinical and diagnostic data from medical records obtained during further clinical treatment and follow up. Patients were divided into DFS≤24 months group and DFS≥48 months group. Immunostaining of ERa, ERβ, PR, Cyclin D1, and Bcl-2 was performed and analyzed.*

Results: *ERa was not expressed in all patients. ERβ moderate expression was present in 25% of all patients, more often in the DFS≥48 group (p=0.001). PR and Bcl-2 showed only moderate expression in 1/5 and 1/3 of the patients, respectively, without significant difference between groups (p=0.145;p=0.566). Cyclin D1 was expressed in the whole sample of patients with strong expression statistically more often in DFS≤24 group (p=0.011) and had 5.2 higher odds of having DFS<24 months. Moderate expression of ERβ was joined with 79.2% smaller odds for shorter DFS. Advanced T stage had 11.3 times higher odds of having DFS<24 months.*

Conclusion: *Early recurrence of CRC in high-risk Duke's B and Duke's C stage relates with reduced ERβ expression and the high cyclin D1 expression, so they could be considered independent prognostic factors, especially in patients in advanced T stage.*

Key words: *colorectal cancer, early recurrence, ERβ, Cyclin D1*

Introduction

According to the GLOBOCAN database, colorectal cancer (CRC) is the third most common cancer in men and the second in women. It is the second most deadly cancer worldwide [1]. About 70% of the patients do not have metastatic disease at diagnosis and undergo surgery, with or without adjuvant treatment, with curative intent. However, around 27% of the patients with CRC in stages II and III experience local disease recurrence or metastasis and approximately 73% of those die [2]. Therefore, the CRC recurrence is one of the most important factors influencing patient survival. Most recurrences occur in the first two years after surgery [3]. Despite the extensive knowledge about the risk factors for CRC recurrence, its occurrence cannot be fully explained. Researchers are permanently focusing on different, promising, potentially clinically applicable molecular profiles [4] that could play a role in this process and could be involved in future therapeutic strategies.

Corresponding author: Dr.Aleksandra Babic. Department of Medical Oncology, University Clinical Hospital Center Zemun, Vukova 9, 11080 Zemun, Belgrade.
Tel +381 638120622, Email: slaville2003@gmail.com
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In recent years, there has been growing evidence that the expression of estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), as well as progesterone receptor (PR), may be factors that influence CRC prognosis. It was found that the ERβ has a prominent role in the biological mechanisms of sex steroid action on colorectal tissue [5]. The loss of its expression in tumor tissue is associated with advanced cancer stages and is a poor prognostic sign [6]. However, the connection between ERβ expression and the CRC recurrence rate in "high risk" patients has not been pointedly investigated so far. The role of ERα and PR has not been previously emphasized in the pathogenesis of CRC. ERα was found in the colon at the mRNA level, but not by immunohistochemistry [7]. The results of subsequent studies examining the expression of estrogen and progesterone receptors on CRC tissue are contradictory in terms of large variations in expression levels and correlations with the clinical outcome of the disease [6-10]. Cyclin D1 is crucial for cell progression from G1 to the S phase of the cell cycle. It was found that in most tumors, overexpression of this marker is associated with a poor clinical outcome [11], and encirclement of its activity via blockade cyclin-dependent kinases (CDKs) has found purpose in the treatment of breast cancer [12]. However, studies that have addressed the role of cyclin D1 in the pathogenesis of CRC, as well as other tumors, and its value as a prognostic marker have yielded entirely contradictory results [13-15]. Bcl-2 (B-cell lymphoma 2) is an anti-apoptotic protein whose final effect is disabling of the caspase cascade process and blocking of cell apoptosis. The results of research on the importance of Bcl-2 expression in the prognosis of CRC were contradictory. Some of the results suggest that high expression of Bcl-2 is associated with favorable prognosis in CRC patients from Europe and America, but differences in expression depended on the use of neoadjuvant therapy [16].

The aim of this study was to examine the expression levels of ERα, ERβ, PR, Cyclin D1 and Bcl-2 on CRC tissue in high risk Duke's B and C stages, and to determine whether the expression correlates with early CRC recurrence within two years after surgery.

Methods

Study design and study population

The study was designed as a retrospective study in the field of examining the underlying pathogenetic mechanisms of CRC. We used 101 specimens of CRC, taken from the archive of Clinical Pathology Department of the University Clinical Hospital Center Zemun, as well as clinical and diagnostic data from medical records obtained during patients further clinical treatment and follow up. Only CRC patients at high risk for recurrence were included in the study. These were patients in Duke's C and high-risk Duke's B stages (stage pT4 and/or N2; a poorly differentiated tumor; perforation; lymphovascular invasion; perineural invasion; <12 examined lymph nodes; positive margins after surgery).

Surgical treatment carried out at the Surgery Clinic of the University Clinical Hospital Center Zemun and the additional adjuvant therapy and follow up were conducted at the Department of Medical Oncology of the University Clinical Hospital Center Zemun from April 1st, 2008. to January 1st, 2018. The study received ethical approval from the Local Research Ethics Committee of University Clinical Hospital Center of Zemun.

Immunohistochemical staining and evaluation

The tissue samples were obtained from resected specimens. The tissues were fixed in 10% buffered formalin (pH 7) and embedded in paraffin blocks. Representative tissue sections were made on the rotating microtome (Leica RM2125RT) 3-4μm thick. After transfer to Super frost + slides, the tissue was deparaffinized through a series of xylenes (4 times for 5 min), and then rehydrated by immersion in decreasing concentration alcohol (100%, 96%, 70%, and 50%) 3 times for 5 min. This process was followed by unmasking the antigen in citrate buffer (pH 6.0) and blocking endogenous peroxidase for 20 min in a 3% hydrogen peroxide solution (H2O2). After washing in PBS and overnight incubation at 4°C with primary antibodies (antihuman ERα, ERβ, PR, cyclin D1, and Bcl-2 antibodies) in a humid chamber, the labeled antigens were incubated with biotinylated antibody (Vectastain Elite ABC kit) 1 h at room temperature. This biotinylated antibody reacted with avidin molecules conjugated to horseradish peroxidase (HRP). Flushing in PBS was followed by visualization with 3,3'-diaminobenzedine (DAB), which marks the antigen-antibody reaction sites brown, supported by contrast staining with Mayer's hematoxylin and dehydration in a growing batch of alcohol (70%, 96%, 100%) and xylene. The stain was examined by two pathologists independently. Semiquantitative assessment of the expression of all immunohistochemical markers was performed based on the intensity of immunohistochemical staining, taking into account the number of stained tissue structures and the intensity of immunohistochemical staining according to the assessment system given separately for certain types of markers. A) ERα, ERβ, PR. It was used three-stage "scoring" system according to the recommendations of Konstantinopoulos et al [9] and consists of estimating the intensity of staining and the percentage of cell nuclei positivity. Tumors were classified as negative for the expression of ERα, ERβ, PR if less than 10% of cell nuclei showed positive staining. Moderate expression was defined as weak positive staining of more than 50% of cell nuclei or strong positive staining of 10-50% of cell nuclei. High receptor expression was defined if more than 50% of cell nuclei show strong positive staining. B) Cyclin D1 and Bcl-2. The intensity of nuclear expression of cyclin D1

and Bcl-2 is expressed as: no expression, weak, moderate, strong and very strong. The proportion of positive tumor cells is expressed as follows: 0=0 to 1%, 1=2 to 25%, 2=26 to 50%, 3=51 to 75% and 4=>75%.

Treatment

After radical surgical treatment and histopathological confirmation of the tumor, all patients treated with adjuvant chemotherapy followed the indications of the Ordinance on standards for the cytostatic use of the Republic Health Insurance Fund of the Republic of Serbia, and were monitored by the National Guidelines for treatment and follow up of CRC patients. Adjuvant chemotherapy was given within 4-8 weeks after the surgery at the following doses: 5FU - 425mg/m^2, LV -20mg/m^2, on day 1-5, every four weeks for six cycles (Mayo reg), or 5FU -400mg/m^2 + LV 200mg/m^2 on day 1, 5FU 600mg/m^2 for 22h on days 1 and 2, every two weeks for six cycles (de Gramont). None of the patients were treated with neoadjuvant therapy.

Follow up

The first evaluation was after 3 or 4 cycles of chemotherapy. If the first assessment showed relapse of the disease, the chemotherapy was stopped, and the patient was referred to the multidisciplinary team for further treatment. After the adjuvant treatment, the patients were routinely evaluated every three months for the next two years, then every six months for the next three years, and finally, once annually up to 10 years. Colonoscopy was performed within six months from the completion of surgical treatment, and then every 2-3 years. Computed tomography (CT) of the abdomen, pelvis, and chest were performed once a year during the first five years of

Table 1. Baseline characteristics of patients

	All (n=101)	*DFS ≤ 24 months (n=51)*	*DFS ≥ 48 months (n=50)*	*p*
Age*	62.7 ± 9.8	64.3±9.9	61.1±9.5	0.105
Gender, n (%)				
male	60 (59.4)	28 (54.9)	32 (64)	0.352
female	41 (40.6)	23 (45.1)	18 (36)	
Tumor localization, n (%)				
rectum	24 (23.8)	11 (21.6)	13 (26.0)	0.601
transversal	3 (3)	0 (0)	3 (6)	0.054
left	74 (73.3)	42 (82.4)	32 (64)	
right	24 (23.8)	9 (17.6)	15 (30)	
Duke's stage, n (%)				
B	31 (30.7)	11 (21.6)	20 (40)	0.045
C	70 (69.3)	40 (78.4)	30 (60)	
T stage, n (%)				
2	4 (4)	1 (2)	3 (6)	0.037
3	88 (87.1)	42 (82.4)	46 (92)	
4	9 (8.9)	8 (15.7)	1 (2)	
N stage, n (%)				
0	34 (33.7)	14 (27.5)	20 (40)	0.294
1	48 (47.5)	25 (49)	23 (46)	
2	19 (18.8)	12 (23.5)	7 (14)	
Number of positive nodes, n (%)**	1 (0-2)	2 (0-3)	1 (0-2)	
Lymphovascular invasion, n (%)	93 (93.9)	49 (98)	44 (89.8)	0.135
Perivascular invasion, n (%)	45 (48.4)	26 (56.5)	19 (40.4)	
Histological grade, n (%)				
1.0	7 (7)	6 (12)	1 (2)	0.087
2.0	73 (73)	34 (68)	39 (78)	0.120
3.0	20 (20)	10 (20)	10 (20)	
Hypertension, n (%)	59 (58.4)	30 (58.8)	29 (58)	0.141
Diabetes, n (%)	18 (17.8)	11 (21.6)	7 (14)	
HgB	122.5 ± 14	123.4 ± 12.5	121.6 ± 15.4	
CRP	3.5 (1.4-11)	6.6 (2.57-13)	1.9 (0.8-5.8)	0.933
NLR	2 ± 1.1	2.2 ± 1.3	1.9 ± 0.9	0.320
PLR	142.7 ± 63.4	134.8 ± 48.2	151.1 ± 76	0.517

Data are expressed as n (%); * data are expressed as mean ±SD; ** data are expressed as median (25-75 th percentile)

disease follow-up, and then every two years. In the case of new symptoms, patients were examined outside the schedule. Disease recurrence was confirmed by CT, and local relapse by colonoscopy, with biopsy and pathohistological confirmation.

Statistics

The sample size was calculated based on data on the expression of beta estrogen receptors in colorectal cancer. Data were obtained from a study by Rudolf et al [6]. According to data from the literature, the absence of beta estrogen receptor expression was detected in 48% of subjects with CRC. The study sample was calculated assuming that α=0.05, precision 0.1, and study power 1-β=0.95 (95%). Using the program G Power v.3.0.10, we determined that for the given criteria, a sample of at least 96 patients was necessary.

Descriptive statistics were calculated for the baseline demographic and clinical features, as well as treatment outcomes. Categorical variables were presented as number and percentage. Continuous data distribution was tested with mathematical and graphical methods. Continuous variables were presented as mean with standard deviation (SD) or median with 25-75 th percentile, according to data distribution. Differences between groups were analyzed using Student's t-test (or Mann Whitney U test) for continuous variables and Pearson's Chi-squared test for categorical variables. Multiple logistic regression was used for analysis of predictors for colorectal recurrence. Significant demographic and clinical features ($p<0.05$) were included and forward conditional model selection was used to avoid model overestimation. Hosmer-Lemeshow test was used for goodness of fit for logistic regression model. Nagelkerke coefficient was used as a proportion of variance in the dependent variable associated with the predictor (independent) variable. For all statistical calculations, the significance level (α) was 0.05. For statistical processing of the obtained results, we used the SPSS software package (version 23.0, SRSS Inc., Chicago, IL).

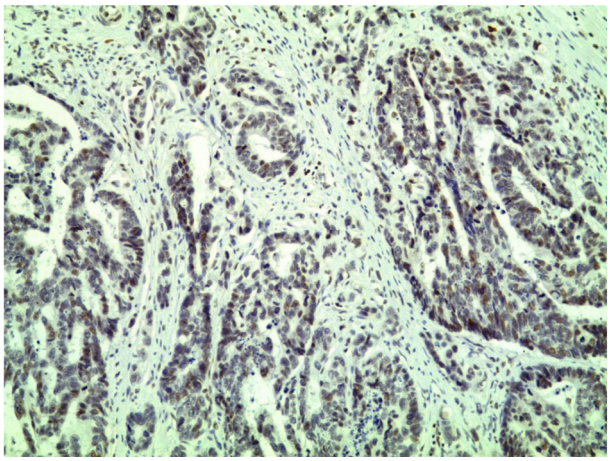

Figure 1. Immunohistochemical staining for ERβ in colorectal cancer tissue: moderate nuclear expression (magnification, ×10).

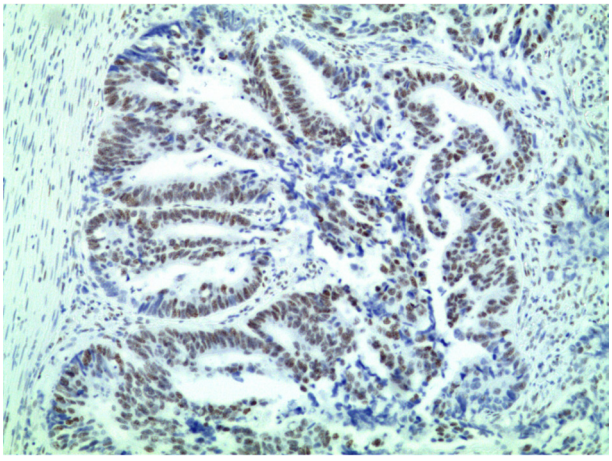

Figure 2. Immunohistochemical staining for PR in colorectal cancer tissue: moderate nuclear expression (magnification, ×10).

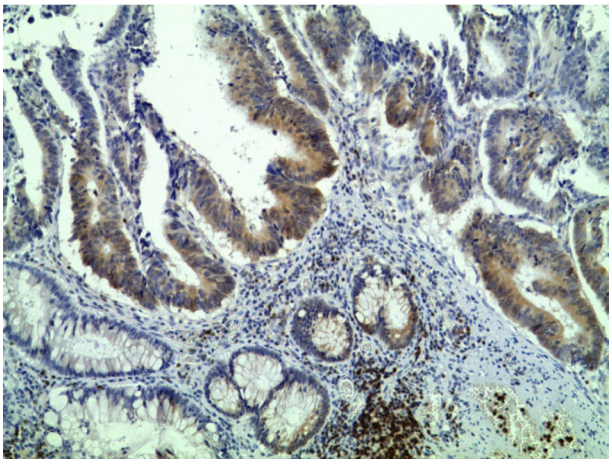

Figure 3. Immunohistochemical staining for Bcl-2 in colorectal cancer tissue: moderate nuclear expression (magnification, ×10).

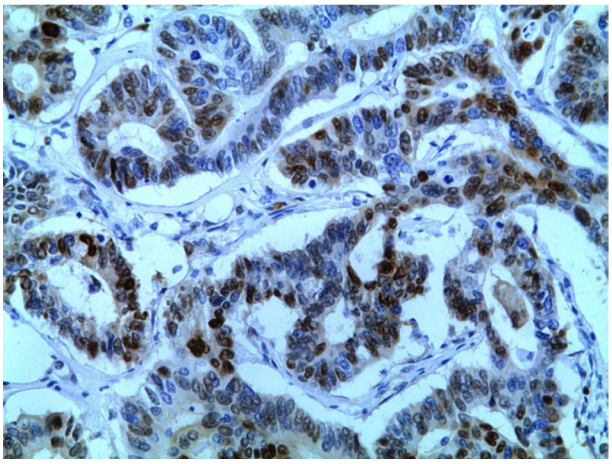

Figure 4. Immunohistochemical staining for cyclin D1 in colorectal cancer tissue: strong nuclear expression (magnification, ×20).

Results

Results

This study included 101 patients with "high risk" B and C stage of CRC. Baseline characteristics of the patients are presented in Table 1. Mean age was 64.3±9.9 years (DFS≤24 group) and 61.1±9.5 (DFS≥48 group) without significant difference between groups (p=0.105). Median follow up in DFS≤24 group was 10.0 (3.0-24.0) and in DFS≥48 was 69.3 (48.0-115.0) months. Frequency of male and female was similar between groups, no significant difference was observed. There was no significant difference in rectal localization between groups. Left side of the colon was more often affected in DFS≤24 group, difference was close to conventional level of significance (p=0.054). Duke's stage C and advanced T stage were statisticaly more frequent in DFS≤24 group (p=0.045; p=0.037). There was no significant difference in N stage and number of positive lymph nodes between groups. Lymphovascular invasion was more often present in DFS ≤ 24 group, difference was close to conventional level of significance (p=0.087). There was no difference between perivascular invasion and histological grade between groups. Frequency of hypertension and diabetes were similar between groups.

Overall, ERα was not expressed in all patients. ERβ moderate expression was present in 25% of all patients, more often in DFS≥48 group when compare to DFS≤24 group (p=0.001). Positive ERβ were present in 21 (28.4%) patients with left colon tumors, and in 4 (16.7%) with right side of the colon, and the difference was not significant (p=0.253) (Figure 1). Moderate PR expression was present in 1/5 of patients, and strong expression was present only in two patients, without significant difference between groups (p=0.145) (Figure 2.). Bcl-2 was not expressed in more than 2/3 of the patients; 1/3 of the patents had moderate expression of this receptor, with a similar expression between groups (p=0.566). (Figure 3). Cyclin D1 was expressed in the whole sample of patients, 15 (14.9) had a weak, 37 (36.6) moderate, 30 (29.7) strong, and 19 (18.8) very strong expression. Strong expression was sta-

Table 2. Tumor expression of ERα, ERβ , PR, Cyclin D1 and Bcl-2 between groups

	All (n=101) *n(%)*	*DFS ≤ 24 months (n=51)* *n (%)*	*DFS ≥ 48 months (n=50)* *n (%)*	*p*
ERα, n (%)				-
No expression	101 (100)	51 (100)	50 (100)	
ERβ, n (%)				0.001
No expression	75 (74.3)	45 (88.2)	30 (60)	
Moderate expression	26 (25.7)	6 (11.8)	20 (40)	
PR, n (%)				0.187
No expression	77 (76.2)	42 (82.4)	35 (70)	
Moderate expression	22 (21.8)	9 (17.6)	13 (26)	
Strong expression	2 (2)	0 (0)	2 (4)	
Cyclin D1, n (%)				0.021
0-1%	0 (0)	0 (0)	0 (0)	
2-25%	15 (14.9)	5 (9.8)	10 (20)	
26-50%	37 (36.6)	17 (33.3)	20 (40)	
51-75%	30 (29.7)	22 (43.1)	8 (16)	
>75%	19 (18.8)	7 (13.7)	12 (24)	
Cyclin D1, n (%)				0.011
2-50%	52 (51.5)	22 (43.1)	30 (60)	
51-75%	30 (29.7)	22 (43.1)	8 (16.0)	
>75%	19 (18.8)	7 (13.7)	17 (24)	
Bcl-2, n (%)				0.566
0-1%	67 (66.3)	34 (66.7)	33 (66)	
2-25%	30 (29.7)	16 (31.4)	14 (28)	
26-50%	4 (4)	1 (2)	3 (6)	
51-75%	0 (0)	0 (0)	0 (0)	
>75%	0 (0)	0 (0)	0 (0)	

Table 3. Multivariate logistic regression with DFS ≤ 24 months as depended variable

	OR (95%CI)	*p*
Cyclin D1		0.019
Cyclin D1(1)	0.939 (0.255-3.461)	0.925
Cyclin D1 (2)	5.234 (1.17-23.42)	0.030
T stage	11.348 (1.858-69.318)	0.009
ERβ	0.208 (0.063-0.684)	0.010
CRP >7	5.904 (1.927-18.092)	0.002

tistically more often in DFS≤24 group than in the DFS≥48 group (p=0.011) (Figure 4). Tumor expression of ERα, ERβ, PR, Cyclin D1 and Bcl-2 markers between groups is shown in Table 2.

Levels of hemoglobin were similar between groups. CRP was significantly higher in DFS≤24 group (p<0.001). There was no difference in NLR and PLR between groups. Level of albumins was significantly higher in DFS ≥ 48 group.

Multivariate logistic regression with lower disease survival (DFS≤24 months) as a dependent variable is presented in Table 3. Hosmer Lemeshow test confirmed that the model was well fitted (x^2=3.454, p=0.840). Nagelkerke coefficient R2 was 0.315, and model accuracy was 78%.

Patients with a strong expression of Cyclin D1>75% had 5.2, and an advanced T stage had 11.3 times higher odds of having a shorter DFS less than 24 months. Moderate expression of ERβ was joined with 79.2% smaller odds for shorter DFS. Higher levels of CRP (above referent range) were joined with 5.9 higher odds for shorter survival.

Discussion

The results of our study showed that only expressions of ERβ and cyclin D1 were related with the occurrence of early recurrence of CRC in high-risk Duke's B and C stage.

Since previous examinations showed that ERα was minimally expressed in normal and malignant colon, most studies have focused on the role of ERβ in the carcinogenesis of CRC. Konstantinopoulos et al revealed that colon adenocarcinoma cells display significantly lower ERβ expression than normal colonic mucosa, which parallels the loss of their differentiation [9]. RT-PCR and Western blot analyses showed lack of expression of the classical ERα and the high expression of the ERβ subtype in the four CRC cell lines [17]. Further research showed that adenocarcinomas exhibited positive protein staining for ERα and exhibited significantly increased Era mRNA expression, supporting the idea that ERα activity could be increased in the colon tumors [18]. In the our study, ERα was not expressed in any of the tumor samples. This is in contrast to the results of Ye et al [8] in which all of the 148 tumor samples in stages I-III showed some level of ERα expression: high expression showed 12.8% of samples and low expression 87,2% of samples. They also showed that patients with ERα tumor expression had poorer survival than those without ERα expression.

On the other hand, in our study, ERβ moderate expression was expressed in 1/4 of all patients, and strong expression was not observed on any tumor sample. Such expression of ERβ differs from the results of Rudolph et al study [6]. They included 1101 CRC samples, 34.6% showed ERβ moderate expression, 16.8% high expression and 48.6% showed no ERβ expression. It was noticed that ERβ expression was often unsuccessfully measured in tumours treated with neoadjuvant therapy. Our study demonstrated that radically operated CRC in high risk Duke's B and C stages, relapsed in the first two years, had a higher incidence rate of moderate ERβ expression than those who did not relapsed for at least 4 years. This correlates with previous findigs [5] that the loss of ERβ expression was associated with CRC progression, and an increased risk of dying.

The left-right dichotomy observed in CRC is suggested to be possibly a result of estrogen; cases affecting females tend to be right-sided and proximal while those that affect males tend to be distal and rectal [19]. In the study of Jassam et al [20] the decrease in ERβ expression was profound in proximal CRC i.e. 21% compared to distal CRC i.e. 7% . In our study, the left side of the colon was more often affected in DFS≤24 group; the difference was close to the conventional level of significance. Moderate expression of ERβ positive tumor tissues was showed in 21 (28,4%) patients with left colon tumor localisation, and in 4 (16,7%) with right colon tumors, difference was not significant (p=0.253).Our results can be explained by low expression of beta receptors in our sample.

We showed moderate PR expression in 1/5 of patients, and strong expresion was present only in 2 patients, without significant difference between groups. Moderate and strong PR expression were significantly more common in right colon tumors. The results of comparable studies revealed contradictory data. Qasim et al [21] analyzed 33 CRC tissue samples, 12 (36.36%) showed PR positivity and 14 (42.42%) ER positivity. On the other hand, Slattery et al [10] analyzed immunohistochemically 156 colonic carcinomas, none were positive for ER, and only one case was reactive for PR. In the study of Ye et al [8] 32 (21.6%) tumor samples

showed high PR expression, and 116 (78.4%) low PR expression; the level of expression was not associated with survival outcomes.

Most studies have confirmed that increased cyclin D1 expression is an unfavorable prognostic factor associated with poor survival outcome [22]. Its overexpression is especially crucial in stage II patients since 25-30% of those patients have a poor prognosis despite being node-negative [12]. Wangefjord et al [14] revealed that the prognostic value of cyclin D1 was only evident in male but not in female CRC patients. In contrast, Al-Maghrabi et al [15] showed that cyclin D1 immunoexpression could not be used as a predictor of survival in CRC. In their study, 90 (76.9%) of primary tumors showed low expression and 27 (23.1%) high expression. In our study, cyclin D1 was expressed in the whole sample of patients, 1/7 had a weak, more than 1/3 moderate, 1/3 strong, and 1/5 of patients had a very strong expression. Strong expression was statistically more often in DFS≤24 months group when compared with DFS≥48 months group.

A few decades ago it was shown that the high expression of Bcl-2 protein is associated with freedom from recurrence in moderately differentiated Dukes' B CRC [23]. According to the meta analyses of Huang et al [16] the beneficial effect of Bcl-2 expression on CRC patients' overall survival was insignificant in the subgroup receiving preoperative treatment. Although none of our patients received preoperative therapy, the expression between groups was simmilar. Bcl-2 was not expressed in more than 2/3 tumor samples, and in 1/3 it showed moderate expression.

In this study, we also compared the standard histopathological characteristics of tumors between groups. The occurrence of early disease recurrence was associated with advanced T stage and Duke's C stage. Lymphovascular invasion was more often present in DFS≤24 group, and the difference was close to the conventional level of significance.

Study limitations

Due to the current regulations (limitations) on the use of cytostatics of Serbian Republic Health Insurance Fund, patients in the Duke's C stage did not receive Oxaliplatin in addition to fluoropyrimidines. Therefore, not all current standards for CRC treatment have been met [24,25], which could have a lesser effect on patient DFS. One-quarter of patients underwent surgery for rectal cancer without prior neoadjuvant chemoradiotherapy, which could be an independent cause of earlier disease recurrence. But, the comparison of incidence rates of rectal cancer patients between groups showed no statistically significant difference. On the other hand, the non-application of neoadjuvant therapy in these patients made it possible to avoid its negative effect on the immunohistochemical expression of some markers [6,16].

Conclusion

Our results demonstrate that early recurrence of CRC in high-risk Duke's B and Duke's C stage is in relationship with reduced ERβ expression and the high cyclin D1 expression. The expression of ERα, PR, and Bcl-2 on tumor tissue did not correlate with early recurrence. These findings indicate the high expression of cyclin D1 and reduced expression of ERβ are indicators of poor prognosis in CRC, especially in patients with advanced T stage. Cyclin D1 and ERβ could, therefore, be considered significant, independent prognostic factors in CRC patients and possible therapeutic targets.

Conflict of interests

The authors declare no conflict of interests.

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