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Expression of ERα, ERβ and co-regulator PELP1/MNAR in colorectal cancer: Prognostic significance and clinicopathologic correlations

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Abstract. *Background*: Estrogen receptor β (ERβ) is abundantly expressed in colorectal tissue, but its role in colorectal carcinogenesis remains elusive. ER novel co-regulator, proline-, glutamic acid- and leucine-rich protein 1 (PELP1/MNAR) has been characterized, but its expression in colorectal carcinomas has not been investigated.

Methods: ERα, ERβ and PELP1/MNAR protein expression were evaluated by immunohistochemistry in colorectal normal mucosa, adenomas and adenocarcinomas from 113 patients with colorectal cancer.

Results: ERα expression is extremely rare in colorectal tissue and its expression does not appear to be associated with colorectal carcinogenesis. ERβ and PELP1/MNAR were detected in the nucleus of epithelial, endothelial, inflammatory, smooth muscle cells and myofibroblasts. When intensity of staining was taken into account, the expression of both proteins was significantly increased in epithelial cells of carcinomas compared to normal mucosa. ERβ expression in epithelial cells was correlated with decreased disease progression – free survival. PELP1/MNAR overexpression in epithelial cells was found to be an independent favorable prognostic factor. Additionally, the expression of both proteins was significantly increased in stromal myofibroblasts of carcinomas compared to adenomas and normal mucosa.

Conclusion: ERβ and PELP1/MNAR appear to be involved in colorectal tumorigenesis and might have prognostic significance.

Keywords: Colorectal cancer, estrogen receptor α/β, immunohistochemistry, PELP1/MNAR, tumorigenesis

1. Introduction

Colorectal cancer is the fourth most common malignancy in the Western world and the second most frequent cause of cancer-related mortality. At the post-genomic era, thorough understanding of intra/intermolecular interactions in tumor cell microenvironment could contribute to the identification of putative key-proteins that could be candidates for molecular targeting in cancer.

Sex and age variations in colorectal cancer suggest a potential role of sex steroid receptors. Estrogen receptor (ER) α and β have been identified both in colorectal cancer cell lines and in colorectal tissue, with ERβ being the predominant type [1,2]. ERβ gene is located on chromosome 14q. Alternative splicing of the last coding exon results in five different isoforms, with β1 being the originally cloned sequence, previously described as ERβ [3]. ERβ1 and 2 are the two predominant isoforms in colorectal tissue [4].

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ERs are nuclear receptors harboring a N-terminal domain for inter/intramolecular interactions, a DNA binding domain for binding to specific DNA sequences, a hinge domain for receptor dimerization, a ligand binding domain for estrogen binding and an F domain which modulates transcriptional activity [5]. Upon estrogen binding, ERs bind to DNA and modulate target gene transcription through context-specific interactions with various coregulators [6]. There is data that support promoter-specific differential activities of ERβ isoforms, suggesting their role in differentially modulating estrogen action [7].

ER activity seems to be facilitated by specific coregulators that integrate certain subcellular events in response to the presence of estrogen [8]. Recently, a novel coregulator, proline-, glutamic acid- and leucine-rich protein 1 (PELP1), also known as modulator of non-genomic activity of ER (MNAR) has been characterized [9]. PELP1/MNAR acts as a scaffolding protein that couples diverse signaling complexes with nuclear receptors, mediating both genomic and non-genomic functions [10]. PELP1/MNAR gene is located on chromosome 17p13.3 and encodes for a 160 Kd protein which is expressed in several tissues such as mammary gland, lungs, brain, skeletal muscles and testes [9]. PELP1/MNAR expression is upregulated by ERβ in various cell lines [11]. It is associated with chromatin remodeling activity via histone H1 displacement in cancer cells and also undergoes cyclic association and dissociation to the endogenous pS2 promoter in an E2 dependent manner [12]. It also acts as a co-repressor for other nuclear hormone receptors and transcription factors, such as AP-1 and NF-kB, blocking histone acetylation and thus gene transcription [13]. Although it is predominant in the nucleus, it has also been localized in the cytoplasm [9], presumably mediating ER non-genomic activity. It appears to serve as an adaptor protein between ER and Src leading to rapid activation of mitogen activated protein kinase (MAPK) signaling cascade [14]. It also appears to interact with phosphotidyl inositol 3 kinase (PI3K), and signal transducers and activators of transcription 3 (STAT3) [15,16].

PELP1/MNAR expression is deregulated in several malignancies, such as breast, endometrial, prostate and ovarian carcinomas interacting with various oncogenes. In breast tumors, its tumorigenic activity involves interaction with the tumor suppressor retinoblastoma (Rb) protein [17]. It has also been associated with androgen-independent prostate adenocarcinomas [18]. ERβ and PELP1/MNAR overexpression has been correlated with aggressive grade 3 endometrial adenocarcinomas [19], implicating the synergistic action of these proteins in a context-dependent manner during adenocarcinoma pathogenesis.

A substantial number of studies on ERβ in colorectal cancer was conducted with RT-PCR on small cohorts, so information regarding its distribution and association with clinicopathological parameters is still required. Furthermore, the role of PELP1/MNAR – a significant ER co-regulator – has not been investigated in colorectal adenocarcinomas.

To shed light into the role of ERβ and PELP1/MNAR in colorectal carcinogenesis, we studied their protein expression by immunohistochemistry in colorectal normal mucosa, adenomas and adenocarcinomas. Potential correlations with clinicopathologic parameters including survival were also explored.

2. Patients and methods

2.1. Patient selection and evaluation

The present study comprised 113 surgical specimens of primary colorectal adenocarcinomas. Of these, 109 were sequential colectomy specimens resected from an equal number of patients at the University Hospital of Patras, Greece, between 1994 and 2006 and four were biopsy specimens. Adjacent non-neoplastic mucosa was also evaluated in 88 cases. Additionally, 30 adenomas from the same cohort of patients were included in the study. Eleven adenomas were stained only for ERβ due to inadequate tissue. Patients who had received neoadjuvant chemo/radiation therapy were excluded from the study. Data from the pathology records and clinical follow up were readily available for all patients. This study got ethical approval from the Local Research Ethics Committee at University Hospital of Patras, according to the principles laid down by Declaration of Helsinki.

The patients' clinicopathologic characteristics are displayed in Table 1. The median follow up period was 56 months (7-153). During this period of time 34 deaths and 44 relapses were observed. The 3-year survival rate was 82.1% and the 5-year survival rate was 62.2%.

Table 1
Patients' clinicopathological characteristics

Feature	n (%) (113)
TNM stage	
I	8 (7)
II	36 (31.9)
III	63 (55.8)
IV	6 (5.3)
Grade	
I	21 (18.6)
II	81 (71.7)
III	11 (9.7)
Gender	
M	71 (62.8)
F	42 (37.2)
Histologic type	
Non-mucinous	101 (89.4)
Mucinous	12 (10.6)
Primary site	
Right colon	31 (27.4)
Left colon	45 (39.9)
Rectal	37 (32.7)
Age median (range)	66 (30−90)

2.2. Immunohistochemistry

As described previously [20], immunohistochemistry was performed on serial 4 μm thick formalin-fixed, paraffin-embedded tissue sections mounted on gelatin-coated glass slides. Antigen retrieval for ERα and ERβ was performed in a microwave oven using 10 mM citrate buffer pH6 for 15 min. No antigen retrieval was required for PELP1/MNAR, in accordance to the manufactures' instructions. Mouse monoclonal antibody against ERα (1:30, NCL-L-6F11, Novocastra, UK) and polyclonal antibodies against ERβ (ERb88, prediluted, BioGenex, CA, USA) and PELP1/MNAR (1:700, Novus Biologicals, CO, USA) were used for the primary reaction. The antibody used for ERβ detection recognizes the β1 isoform. The sections were incubated with primary antibodies for 1 h at room temperature, followed by sequential 30 min incubation with Dako EnVision Labelled Polymer (Dako, CA, USA). Diaminobenzidine (Dako, CA, USA) was used as the chromogen. Nuclei were counterstained with Harris hematoxylin. Sections from breast carcinoma were used as positive control. Furthermore, consistent positive staining of lymphocytes, which was present homogeneously in all slides, was also used as internal positive control. In negative control slides, the same method was performed and the primary antibody was substituted by 1% TBS.

2.3. Staining evaluation

Each slide was individually evaluated and scored in a blind fashion by two independent observers. Discrepancies in scoring between the observers were supposed to be resolved by review of the slides under a double-headed microscope until a consensus was reached. However, no discrepancies in staining evaluation were noticed. ERα expression was rare ($\leqslant$1% epithelial cells). Both ERβ and PELP1/MNAR were consistently stained in the nucleus. Areas with the highest density of positive cells were selected at low power (×40) magnification. The number of positive stained cells and the total number of cells was determined by visual inspection of six different fields per section at 400× magnification. For each field, a percentage of positive cells was calculated and the average of those was taken.The intensity of staining was also evaluated and scored as 1+ (weak), 2+ (moderate) and 3+ (strong). The % percentage of positive cells was then multiplied by the intensity score and a single value representative of ERβ and PELP1/MNAR expression for each patient was obtained (range: 0–300). To allow comparisons with previous immunohistochemical studies of ERβ in colorectal cancer [2,21–23] and PELP1/MNAR [24], a percentage of cells >10% with strong staining, corresponding to a histoscore >30 was considered positive. For statistical purposes PELP1/MNAR values equal or more than the median (170) were considered overexpression. Since there was no variation in the intensity of staining in stromal cells, staining evaluation was based on the % percentage of positive stromal cells.

Intensity evaluation might be influenced by the larger size or the more conspicuous chromatic pattern of the nuclei in adenomas and carcinomas compared to normal nuclei. Immunohistochemistry, a subjective morphological method, has the intrinsic potential limitation of this observer bias. However, the two morphologists attempted to minimize both these potential caveats.

As mentioned above, this study evaluated the ERβ1 isoform. This was decided on a number of reasons. First, ERβ1 is the predominant isoform in colorectal tissue, corresponding to the previously characterized ERβ, which has been evaluated in the vast majority of earlier studies, and thus allowing comparisons. Furthermore, only ERβ1 can activate estrogen response elements (ERE) in reporter assays. Therefore, many of the ways in which ER has been described to mediate estrogenic activity most likely represent β1-based activation, while differences in overall ERβ levels between

normal and malignant tissue appear to parallel by alterations in β1 levels [7]. Additionally, induction of ERβ2 did not appear to alter gene expression patterns and did not affect cellular proliferation in the presence of estradiol or tamoxifen [25]. In order to clarify the role of endogenous estrogens, hormone replacement therapy (HRT) and selective ER modulators (SERMs) in colorectal carcinogenesis, investigation of ligand activation of ERβ-mediated function was pursued. Therefore, potential selective chemopreventive approaches in colorectal cancer rely mostly on ligand activation of ERβ1. Moreover, both β1 and β2 isoforms increase as total ERβ increase in cancer. There appears to be a positive correlation in the expression pattern of these two isoforms, which usually follow the same pattern of biological behavior, especially in a cohort of men and postmenopausal women [4].

2.4. Statistical analysis

Intergroup comparisons, regarding correlation of clinicopathologic parameters with expression levels of ERβ and PELP1/MNAR were performed using Kruskal–Wallis and Mann–Whitney non-parametric tests for continuous or ordinal variables, and Chi square test for nominal variables. Because of multiple comparisons, these tests were followed by a post hoc Bonferroni test. Comparisons between related groups were performed using Wilcoxon paired samples test. Spearman rank correlation was used to detect any potential correlations between ERβ and PELP1/MNAR protein levels. Kaplan–Meier procedure was used to compare the survival and the time to disease progression rates. Cox hazard regression model was applied to assess the prognostic value of ERβ and PELP1/MNAR protein levels in conjunction with clinicopathologic parameters. Data were analyzed using the SPSS statistical package (SPSS©, Release 14.0.1, Chicago, IL, USA). The level of significance was set at p-value <0.05.

The methodological framework of the study and presentation of the data were based on the REMARK criteria [26].

3. Results

3.1. ERα is rarely expressed while ERβ and PELP1/MNAR are consistently expressed in the nucleus of colorectal cells

ERα, ERβ and PELP1/MNAR protein expression was assessed by immunohistochemistry in 113 carcinomas, 88 adjacent normal mucosa samples and 30 adenomas. ERα expression was noted in $\leqslant$1% of epithelial cells in two normal mucosa samples, 1 adenoma and 3 adenocarcinomas. Thus, ERα expression was rare in colorectal tissue in our cohort and no further statistical analysis was performed for this marker. ERβ and PELP1/MNAR proteins were detected in the nucleus of epithelial cells. Additionally, positive staining for both proteins was observed in endothelial cells, inflammatory cells, smooth muscle cells and myofibroblasts. Both epithelial and stroma staining were evaluated in our study. Regarding epithelial staining, of normal mucosa specimens, 80 (90.9%) were stained positive for both proteins, three (3.4%) only for PELP1/MNAR, two (2.3%) only for ERβ and three (3.4%) were double negative. Of carcinomas, 97 (85.8%) co-expressed the two proteins, four (3.5%) expressed only ERβ, nine (8%) only PELP1/MNAR, while three (2.7%) were double negative. All adenomas were positive for both proteins (Table 2(a)). ERβ and PELP1/MNAR co-expression status (positive or negative) did not differ between paired normal and malignant specimens. The median scores and standard deviations of both proteins expression in epithelial cells of normal mucosa, adenomas and carcinomas are displayed in Table 2(b).

Table 2(a)
ERβ and *PELP1/MNAR* positive staining in epithelial cells

	n total	ERβ positive n (%)	*PELP1/MNAR* positive n (%)
Normal	88	82 (93.2%)	83 (94.3%)
Adenomas	30/19	30 (100%)	19 (100%)
Carcinomas	113	101 (89.4%)	106 (93.8%)

Table 2(b)
ERβ and *PELP1/MNAR* immunohistochemical (IHC) expression in epithelial cells – median IHC scores and standard deviations

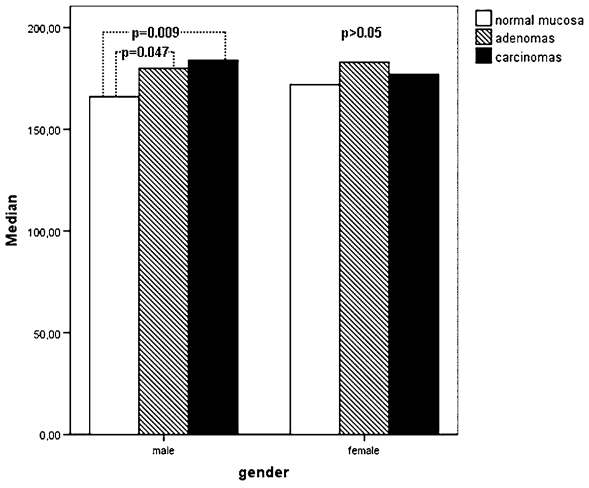
	ERβ median (SD)	*PELP1/MNAR* median (SD)
Normal	170 (51.7)	80 (47.3)
Adenomas	180 (19.4)	175 (46.5)
Carcinomas	180 (62.8)	170 (68.9)

Table 2(c)
ERβ and *PELP1/MNAR* immunohistochemical expression in stromal cells – mean % positive cells and standard deviations

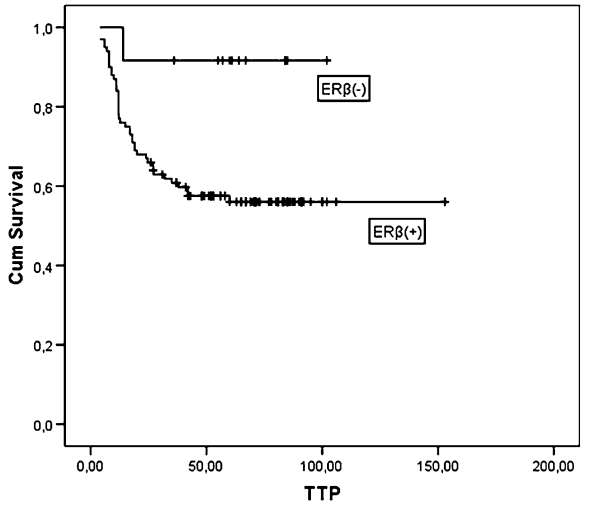
	ERβ mean % cells (SD)	*PELP1/MNAR* mean % cells (SD)
Normal	30 (17)	58 (22)
Adenomas	52 (20)	60 (25)
Carcinomas	82 (24)	90 (20)

3.2. ERβ expression in epithelial cells becomes more intense during colorectal tumorigenesis in male patients, while ERβ expression correlates with increased risk of relapse

ERβ expression was significantly lower in normal tissue than in adenomas ($p = 0.01$) and carcinomas ($p < 0.02$). In subgroup analysis, this was observed in males ($p < 0.05$ and $p = 0.01$, respectively), but not in females ($p > 0.05$) (Fig. 1(A)). ERβ expression did not differ significantly between adenomas and carcinomas ($p = 0.46$). Statistical analysis revealed no correlation between ERβ expression in carcinomas and age, stage, grade, gender, primary site, histologic type and overall survival (Table 3(a)). Furthermore, ERβ expression was not associated with local tumor invasion, lymph node involvement or the presence of distant disease (Table 3(b)). However, positive staining for ERβ expression in carcinomas was correlated with decreased time to disease progression as analyzed by Kaplan–Meier procedure (log rank test: $p = 0.03$, Fig. 1(B)) and greater probability of disease relapse compared to ERβ negative carcinomas (Fig. 2(A)) (43% *vs.* 8.3%, $p = 0.02$). Representative images of ERβ in normal mucosa, adenomas and carcinomas are depicted in Fig. 2(C–E).



(A)



(B)

Fig. 1. (A) Gender and median immunohistochemistry (IHC) levels (0–300) of ERβ in normal mucosa, adenomas, carcinomas. (B) ERβ expression and time to disease progression (TTP).

Table 3(a)
Clinicopathologic parameters in ERβ negative (−) and positive (+) carcinomas

Feature	$n = 113$	ERβ		p value
		(−) $n = 12$	(+) $n = 101$	
TNM stage				
I	8	0	8	0.6
II	36	5	31	
III	63	7	56	
IV	6	0	6	
Grade				
I	21	1	20	0.59
II	81	10	71	
III	11	1	10	
Gender				
Male	71	6	65	0.33
Female	42	6	36	
Histologic type				
Non-mucinous	101	12	89	0.2
Mucinous	12	0	12	
Primary site				
Right colon	31	3	28	0.73
Left colon	45	6	39	
Rectal	37	3	34	
Age median (range)		66 (45–77)	66 (30–90)	0.56

Further correlation analysis between ERβ protein expression in carcinomas and clinicopathologic parameters was performed separately in the two genders. No correlation between ERβ expression in carcinomas and age, stage, grade, gender, primary site, histologic type and overall survival was observed in either males or females. In males, however, positive staining for ERβ expression in carcinomas was associated with greater probability of disease relapse compared to ERβ negative carcinomas (41.5% *vs.* 0%, $p = 0.045$). There was also an apparent, but not statistically significant correlation between positive ERβ staining in

Table 3(b)
TNM stage and ERβ negative (−) and positive (+) carcinomas

Feature $n = 113$	ER β		p value
	(−) $n = 12$	(+) $n = 101$	
T stage			
T1	0	0	0.19
T2	0	13	
T3	12	79	
T4	0	9	
N stage			
N0	5	39	0.57
N1	6	36	
N2	1	26	
M stage			0.38
M0	12	95	
M1	0	6	

T1: localization in mucosa/submucosa; T2: extension into the thick inner muscle layer, but not complete invasion through muscle layer; T3: complete invasion through muscle layer, no extension to surrounding tissues/organs; T4: complete invasion through the wall of the colon/rectum and into surrounding tissues/organs; N0: no lymph nodes; N1: 1–3 regional lymph nodes; N2: 4 or more regional lymph nodes; M0: No metastasis; M1: liver or other distant metastasis.

Table 3(c)
Clinicopathologic parameters and PELP1/MNAR overexpression in ERβ positive carcinomas

Feature	$n = 101$	PELP1/MNAR		p value
		(−)* $n = 31$	(+)* $n = 70$	
TNM stage				
I	8	3	5	0.4
II	31	7	24	
III	56	18	38	
IV	6	3	3	
Grade				
I	20	5	15	0.35
II	71	21	50	
III	10	5	5	
Gender				
Male	65	21	44	0.63
Female	36	10	26	
Histologic type				
Non-mucinous	89	25	64	0.12
Mucinous	12	6	6	
Primary site				
Right colon	28	11	17	0.2
Left colon	39	8	31	
Rectal	34	12	22	
Age median (range)		66 (30–83)	66 (44–90)	0.66

*(+): overexpression, (−): no overexpression.

Table 3(d)
TNM stage and PELP1/MNAR overexpression in ERβ positive carcinomas

Feature $n = 101$	PELP1/MNAR		p value
	(−) $n = 31$	(+) $n = 70$	
T stage			
T1	0	0	0.8
T2	5	8	
T3	23	56	
T4	3	6	
N stage			
N0	10	29	0.1
N1	8	28	
N2	13	13	
M stage			
M0	28	67	0.29
M1	3	3	

carcinomas and decreased time to disease progression as analyzed by Kaplan–Meier procedure (log rank test: $p = 0.069$). No such correlations were observed in females.

3.3. PELP1/MNAR protein expression in epithelial cells increases during colorectal tumorigenesis

PELP1/MNAR levels were significantly lower in normal tissue than in adenomas ($p < 0.01$) and carcinomas ($p < 0.01$). This was still observed when only males were analyzed ($p < 0.01$, $p < 0.01$, respectively). In females, PELP1/MNAR levels increased significantly from normal mucosa to carcinomas ($p < 0.01$), but the increase from normal mucosa to adenomas did not reach the level of statistical significance ($p = 0.28$). There was no significant difference in PELP1/MNAR expression between adenomas and carcinomas ($p = 0.62$) (Table 2). Representative images of PELP1/MNAR expression in normal mucosa, adenomas and carcinomas are depicted in Fig. 3. The same results were obtained when the intensity of staining was not taken into account. Occasionally, PELP1/MNAR staining was intense in the nucleolus of epithelial cells in adenomas and carcinomas (Fig. 3(C) and (E)).

3.4. PELP1/MNAR overexpression in epithelial cells correlates with prolonged overall survival

Statistical analysis revealed no correlation between PELP1/MNAR expression in carcinomas and any of the aforementioned clinicopathologic parameters un-

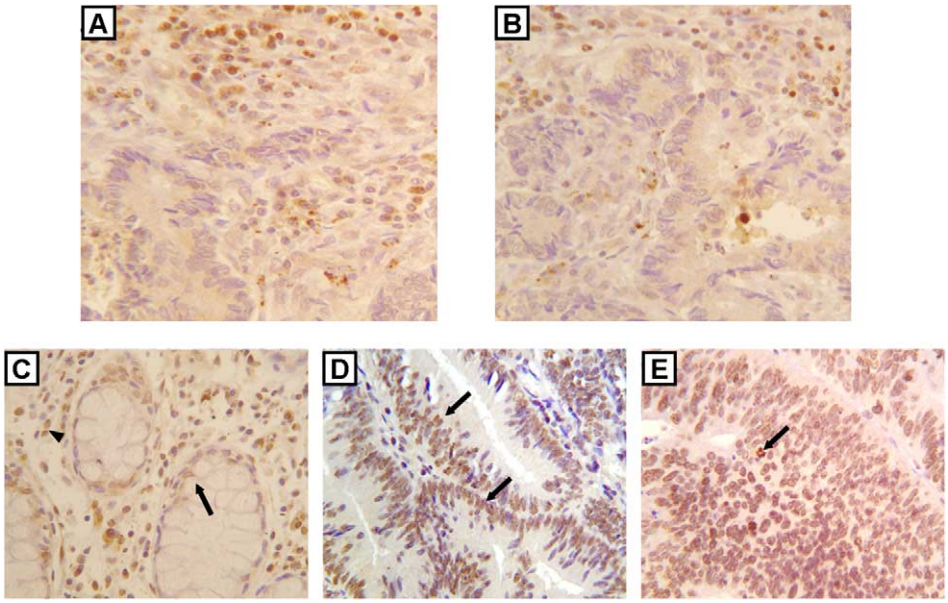


Fig. 2. (A) Expression of ERβ in rare epithelial cells in a case of non-recurrent colorectal carcinoma. Tumor associated lymphocytes exhibit intense immunostaining and serve as positive internal control (×400). (B) Expression of PELP1/MNAR in rare epithelial cells in a case of carcinoma associated with low overall survival (7 months). Tumor associated lymphocytes show intense immunostaining (×400). (C) Moderate expression in epithelial cells of normal colonic mucosa (arrows). Positive lymphocytes in the lamina propria (arrowheads) (×400). (D, E) Strong expression of ERβ in the nucleus of epithelial cells (arrows) of adenomas (D) and carcinomas (E). A cell in mitosis is stained positively (arrow in C) (×400).

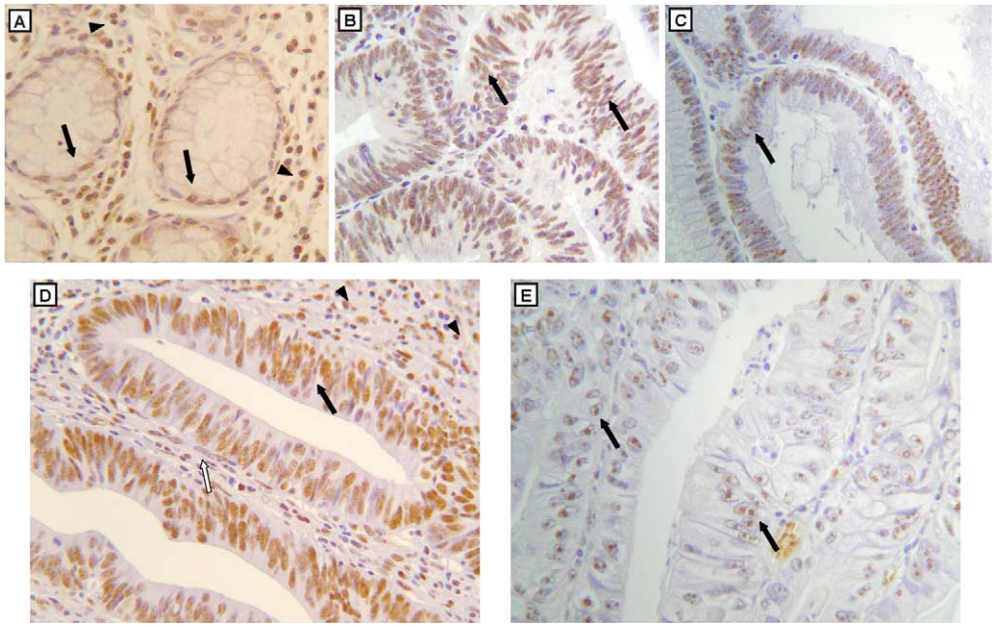


Fig. 3. PELP1/MNAR expression in normal mucosa (A), adenomas (B, C) and adenocarcinomas (D, E) (×400). (A) PELP1/MNAR in the nuclei of epithelial cells (arrows), lymphocytes (arrowhead) and endothelial cells (white arrow) of normal colon. (B) Strong nuclear expression (arrows) of PELP1/MNAR in epithelial cells of colonic adenoma. (C) Nucleolar accentuation (arrows) of PELP1/MNAR immunostaining in a case of colonic adenoma. (D) PELP1/MNAR is strongly expressed in the nuclei of malignant colonic cell (arrows). Lymphocytes (arrowhead) and stromal myofibroblasts (white arrow) exhibit moderate to strong immunostaining. (E) Nucleolar expression (arrows) of PELP1/MNAR in a case of adenocarcinoma.

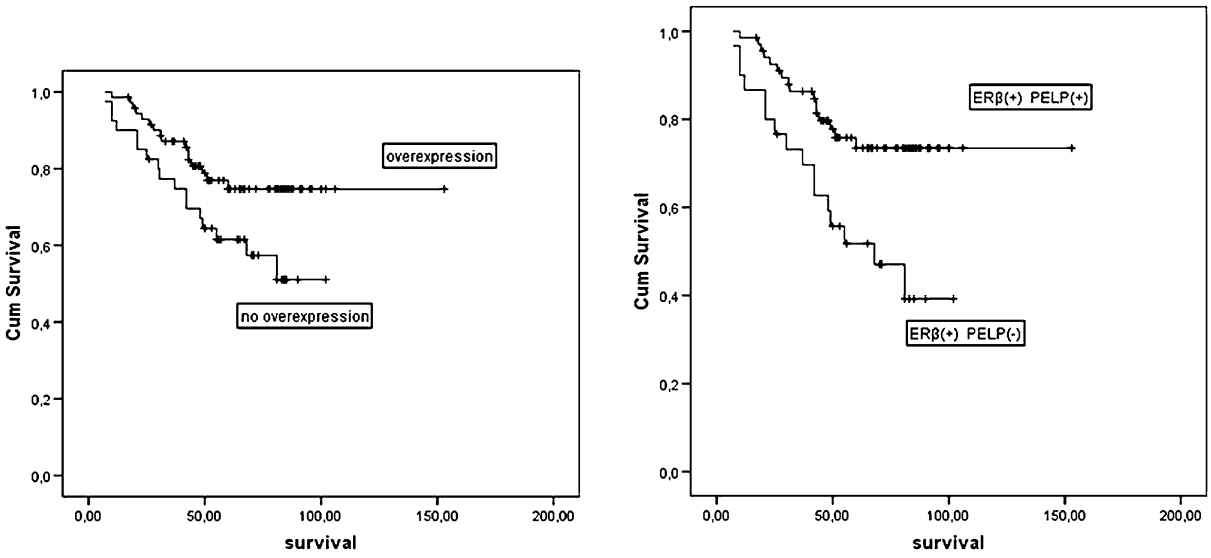


Fig. 4. (A) PELP1/MNAR overexpression and overall survival. (B) PELP1/MNAR overexpression (+) *vs.* no overxpression (−) and overall survival in ERβ positive adenocarcinomas.

der evaluation (data not shown). However, PELP1/MNAR overexpressing carcinomas were correlated with increased overall survival (log rank test: $p < 0.03$, Fig. 4(A)) and lower probability of death compared to carcinomas without PELP1/MNAR overexpression (Fig. 2(B)) (44.7% *vs.* 23.3%, $p = 0.02$). This finding was confirmed in univariate analysis based on Cox proportional-hazards model ($p < 0.05$, 95% CI: 1.01–3.94), and in multivariate Cox analysis when PELP1/MNAR overexpression was analyzed along with other clinicopathologic parameters and ERβ expression ($p = 0.03$, 95% CI: 1.095–5.092). In multivariate analysis, apart from PELP1/MNAR overexpression, stage ($p < 0.01$) and grade ($p < 0.01$) were also found to be independent prognostic factors for overall survival (Table 4).

3.5. *ERβ correlates with PELP1/MNAR protein expression in epithelial cells of carcinomas and PELP1/MNAR overexpression has prognostic value in the subset of ERβ positive carcinomas*

Despite that in normal tissue and adenomas ERβ was not correlated with PELP1/MNAR expression ($p > 0.05$), there was a moderate correlation in the expression of these two proteins in carcinomas ($r = 0.2$, $p = 0.03$). Taken into account that PELP/MNAR is an ERβ co-regulator, we also examined potential correlations of PELP/MNAR overexpression in the subsets of ERβ positive and negative carcinomas with clinicopathologic parameters, including overall survival. PELP1/MNAR was not found to be associated with survival or any other clinicopathologic parameters in the subset of ERβ negative adenocarcinomas. No correlation between PELP1/MNAR overexpression in ERβ positive carcinomas and any of the aforementioned clinicopathologic parameters was observed (Tables 3(c) and 3(d)). However, the presence

Table 4
Multivariate analysis based on Cox hazard regression model. Prognostic significance of clinicopathologic parameters, ERβ protein expression and PELP1/MNAR protein overexpression in epithelial cells of carcinomas

Parameter	*p* value	Exp(B)	95%CI
Gender	0.051*	2.6	0.994−6.847
Stage	<0.001	0.02	0.005−0.082
Age	0.9	0.99	0.958−1.039
Grade	0.007	0.064	0.009−0.465
Primary site	0.9	0.978	0.357−2.68
Histologic type	0.037**	0.22	0.054−0.912
ERβ expression	0.25	1.004	0.997−1.01
PELP1/MNAR overexpression	0.035	2.388	1.095−5.092

*Gender was not found to be a prognostic marker when univariate Cox analysis and Kaplan–Meier procedure were performed. **Histologic type was not found to be a prognostic marker when univariate Cox analysis and Kaplan–Meier procedure were performed.

of PELP1/MNAR overexpression was correlated with prolonged overall survival (log rank test: $p < 0.01$, Fig. 4(B)) and lower probability of death (53.3% *vs.* 24.6%, $p < 0.01$) in ERβ positive carcinomas. This finding was confirmed in stepwise regression analysis based on the Cox proportional-hazards model ($p < 0.04$, 95% CI: 1.05–4.92). Apart from PELP1/MNAR overexpression, stage and grade were also found to be independent prognostic factors for overall survival in ERβ positive carcinomas ($p < 0.01$, $p = 0.02$, respectively).

3.6. ERβ and PELP1/MNAR protein expression in stromal cells increases during colorectal tumorigenesis

ERβ protein was more frequently expressed in cancer-associated myofibroblasts compared to normal mucosa and adenomas ($p < 0.001$). Additionally, PELP1/MNAR protein expression was enhanced in cancer-associated myofibroblasts compared to myofibroblasts of normal mucosa and adenomas ($p < 0.001$). These differences were also noted when males and females were separately analyzed. In males, ERβ protein expression in myofibroblasts was significantly higher in carcinomas compared to adenomas and normal mucosa ($p < 0.001$, $p < 0.001$, respectively) and was also lower in normal mucosa compared to adenomas ($p = 0.002$). PELP1/MNAR protein expression in myofibroblasts was significantly higher in carcinomas compared to adenomas and normal mucosa ($p = 0.001$, $p < 0.001$, respectively), but did not differ between adenomas and normal mucosa ($p = 0.2$). In female patients, ERβ protein expression in myofibroblasts was significantly higher in carcinomas compared to adenomas and normal mucosa ($p < 0.001$, $p = 0.012$, respectively) and was also lower in normal mucosa compared to adenomas ($p = 0.011$). PELP1/MNAR protein expression in myofibroblasts was significantly higher in carcinomas compared to adenomas and normal mucosa ($p = 0.028$, $p < 0.001$, respectively), but did not differ between adenomas and normal mucosa ($p = 0.78$).

It should be mentioned that all statistical tests were repeated without the 12 mucinous cases of our cohort. The results were very similar to those presented and no significant discrepancies were observed when mucinous cases were excluded.

4. Discussion

Although there have been data regarding the tumor-suppressive activity of ERβ in oncogenic signaling, its definitive role in colorectal carcinogenesis remains elusive. In this study, expression of ERα, ERβ and co-regulator PELP1/MNAR was evaluated in 113 colorectal carcinomas, 88 adjacent normal mucosa specimens and 30 adenomas.

ERα expression was extremely rare in colorectal tissue in our cohort and its expression did not appear to be associated with colorectal carcinogenesis.

With regard to ERβ staining in epithelial cells, we observed positivity in 89.8% of normal mucosa samples, in all adenomas and in 89.4% of carcinomas. These results are in accordance with previous studies [2,22]. ERβ was consistently stained in the nucleus, while distinct strong cytoplasmic staining was not observed. This is consistent with previously presented data [21,23], while Witte et al. reported the presence of cytoplasmic ERβ immunoreactivity in carcinomas compared to normal mucosa, suggesting differential localization of the receptor in carcinogenesis.

ERβ protein expression was also observed in stromal myofibroblasts. A significant increase of ERβ levels from normal mucosa to carcinomas was found in both males and females. Stromal cells are more than just innate bystanders of the multi-step process of carcinogenesis. They should be considered to be active participants of cancer initiation and progression. Myofibroblasts are the predominant cells in cancer microenvironment and orchestrate the stromal response to the epithelial malignant component [27]. Increasing evidence suggests that estrogen mediate their effects through paracrine actions [28–31]. These data along with our findings imply that the potential involvement of myofibroblasts in colorectal tumorigenesis might include estrogen mediated effects.

A significant increase of ERβ levels in epithelial cells from normal mucosa to carcinomas was also observed, supporting a possible role of this receptor in tumorigenesis. This finding is inconsistent with previously published data. It has been speculated that the putative protective role of estrogen in colorectal cancer is exerted by ERβ [2,21,32,33], which generally has been considered a tumor suppressor gene [34]. Data has suggested that loss of ERβ leads to hyperproliferation, loss of differentiation, and decreased apoptosis in the cells of the colonic epithelium suggesting a pivotal role for ERβ in the organization and architectural maintenance of the epithelial barrier [35]. Nev-

ertheless, the precise *in vivo* mechanism of estrogen impact on colorectal carcinogenesis is not fully clarified. A functional role for ERβ in mediating *in vitro* hormonal effects on cell growth was reported [1]. In a previous study [21], ERβ mRNA levels in most samples were found to be increased in tumors compared to adjacent normal mucosa. Corresponding results were published by Cavallini et al. [4], suggesting oncogenic potential of ERβ. In that study, both β1 and β2 isoforms increased from normal to malignant mucosa, with the latter showing a greater increase. Furthermore, increase in ERβ1 levels may predispose to microsatellite instability, a common phenomenon in colorectal carcinogenesis [23]. These data taken together with our findings regarding ERβ1 might imply a possible role of this isoform in colorectal tumorigenesis.

Interestingly, data presenting a significant positive association between endogenous estradiol levels and colorectal cancer incidence was published recently. This finding was entirely unaffected by controlling for all known risk factors [36]. Several *in vitro* studies have shown that estrogen may have mitogenic and possibly tumorigenic effects on colorectal cells [37–40]. In human colorectal cancer cell lines, estradiol activates mitogen-activated protein kinase cascade, a key pathway in the stimulation of DNA and protein synthesis that can induce cell growth and excess proliferation [41,42]. There is also evidence of decreased metabolic inactivation of estradiol in colorectal malignant compared with normal tissue, suggesting that colon cancer cells may be exposed to higher estradiol levels than non-malignant cells [43,44]. The protective effect of hormone replacement therapy may be explained by the differential activity of estrone *vs.* estradiol in colorectal tissue [45]. Taken into account that ERβ is the predominant estrogen receptor in colorectal tissue, these data might suggest a ligand-dependent role of estrogen receptor signaling in colorectal tumorigenesis.

The incremental trend of ERβ protein expression in epithelial cells during malignant transformation was observed in male patients. This finding merits further investigation, taking into account the different gender associated incidence and site distribution of colorectal cancer [46]. Previous studies reported that loss of ERβ expression in colorectal carcinomas differed in the two genders [21,33]. Estrogen signaling might differ in males and females, reflecting distinct levels of circulating sex hormones and the presence of various co-regulators in the subcellular microcontext. Furthermore, potential premenopausal status and/or HRT may alter ER expression in colorectal tissue of females and modify estrogen-mediated signaling even before carcinogenesis occurs. In our study, only one patient was potentially in the premenopausal period (47 years old), while information regarding HRT before surgery was not obtainable. Last but not least, the number of female patients was smaller than that of male (42 *vs.* 71), so it might not provide enough statistical power to reach the level of significance.

Potential correlations of ERβ status and clinicopathologic parameters were not revealed in our evaluation. No associations between ERβ status and clinicopathologic characteristics were reported by Cavallini et al. [4] and Witte et al. [22]. Correlation between ERβ expression, stage and site of the tumor was presented by Jassam et al. [21]. In another study, higher ERβ2 expression was associated with right-sited tumors and lymph node metastasis, while lower ERβ1 levels were related to higher tumor grade and more advanced local disease [23]. Discordant results among studies may be explained by the presence of distinct ERβ isoforms with opposing effects on target genes resulting in diverse clinical outcomes. In our study, ERβ1 expression was associated with decreased time to disease progression and greater probability of disease relapse in male patients. In our knowledge, only one study has investigated the impact of ERβ in colorectal cancer prognosis and found no association between its expression and survival [22]. Since, this is the first study that demonstrates a correlation between ERβ1 and disease progression – free survival, further data is warranted to confirm such a potential prognostic significance. It is also worth noting that ERβ1 expression has been found to act as a predictive factor of disease relapse in breast cancer [47]. Furthermore, variations in receptor levels and/or subnuclear localization may affect ERβ genomic activity and thus its role in colorectal tumorigenesis. Variations in biologic samples from different populations, technical considerations and methodological diversity might also account for discrepancies in the results from various studies. Last but not least, the selection of a specific cut-off value might also introduce measurement bias. In this study, the selected cut-off for the positive status was based on previous descriptive data.

PELP1/MNAR an important ERβ co-regulator was also evaluated in this study. Regarding epithelial staining, it was found to be expressed in 94.3% of normal mucosa samples, all adenomas and in 93.8% of carcinomas. It was found consistently in the nucleus, corresponding to its localization in other cell types, although cytoplasmic immunoreactivity has been reported in ap-

proximately 50% of PELP1/MNAR positive breast, as well as endometrial tumors [9,19]. Nuclear localization seems to be implicated in genomic ER signaling. Interestingly, PELP1/MNAR staining was found to be more intense in the nucleolus of cells in adenomas and carcinomas. The biological significance of this observation remains elusive. In our study, PELP1/MNAR expression was found to increase significantly from normal mucosa, through adenomas to carcinomas, suggesting a potential involvement in colorectal tumorigenesis. Corresponding findings were reported in breast [9,10,48,49], endometrial [19], ovarian [10] and salivary gland adenocarcinomas [24]. PELP1/MNAR protein expression was also observed in stromal myofibroblasts. A significant increase of PELP1/MNAR levels from normal mucosa to carcinomas was found in both genders, suggesting a potential role of this co-regulator in the paracrine signaling during colorectal tumorigenesis.

Correlations of PELP1/MNAR status and clinicopathologic parameters were not observed in our assessment. However, PELP1/MNAR overexpression was associated with prolonged overall survival and decreased probability of death. In multivariate analysis it was found to be an independent prognostic factor along with disease stage and grade. To the best of our knowledge, this is the first study to suggest a prognostic significance of this protein in colorectal cancer.

The concomitant increase in the levels of ERβ and its coregulator PELP1/MNAR might indicate a potential role of these molecules in colorectal carcinogenesis. Although there have been no data in colorectal cancer, corresponding results were presented [24] in salivary duct adenocarcinomas. In our study, the prognostic role of PELP1/MNAR was further evaluated in the subsets of ERβ positive and negative tumors. PELP1/MNAR overexpression was found to be an independent prognostic factor, associated with prolonged overall survival and decreased probability of death in carcinomas expressing ERβ. These findings might suggest that PELP1/MNAR could be implicated in ERβ oncogenic signaling resulting at the same time in less aggressive tumors with more favorable outcomes. Nevertheless, PELP1/MNAR was associated with undifferentiated more invasive breast adenocarcinomas [49], a finding which could probably imply a distinct prognostic role in different tissues. Furthermore, nuclear *vs.* cytoplasmic localization as well as cross-talk with different signaling pathways may affect its functional role. Cytoplasmic localization of PELP1/MNAR appears to increase sensitivity to TNFα-induced apoptosis in breast cancer cells. Cytoplasmic PELP1/MNAR overexpression correlated with less anti-apoptotic protein Bcl-2 and NF-kB DNA-binding, as well as with increased cyclin E expression [50]. Noteworthy, PELP1/MNAR could also act as a co-repressor of specific nuclear receptors apart from ERβ [13] in a dose-dependent manner, acting either as a favorable or as an adverse prognostic factor. No association between PELP1/MNAR and survival was noted in ERβ negative carcinomas, but this finding warrants further investigation, since the number of ERβ negative cases was limited.

To the best of our knowledge, this is the first study revealing an independent prognostic role of PELP1/MNAR in colorectal adenocarcinomas. The great complexity of ER activity, the dynamic interplay and cross – talk among various signaling pathways render ER co-regulator targeting a very challenging endeavor. Larger cohorts of patients need to be studied and novel techniques should be implemented to illuminate further the significance of ERα, ERβ isoforms and PELP1/MNAR interactions in colorectal malignancies and to confirm our hypothesis generation results.

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