Sex-Dependent Regulation of Estrogen Receptor *β* in Human Colorectal Cancer Tissue and its Relationship With Clock Genes and *VEGF-A* Expression

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Summary

The incidence of colorectal cancer (CRC) shows a sex-dependent difference in humans. The aim of this study was to analyze estrogen receptor *β* mRNA (*ERβ*) expression in patients with CRC with respect to their gender and clinicopathological features. Since cancer progression is accompanied by tumor vascularization, VEGF-A (vascular endothelial growth factor A) transcription was analyzed along with *ERβ* mRNA. *ERβ* mRNA was also correlated with the expression of clock genes, which are known to influence the cell cycle. *ERβ* mRNA expression in females with CRC showed an inverse association with increasing tumor staging that was not observed in males. Lower levels of *ERβ* mRNA were observed in females with a higher clinical stage compared with those with earlier-stage tumors. *ERβ* mRNA expression showed a significant positive correlation with mRNA of clock genes period 2 and cryptochrome 2 in healthy but not in cancerous tissue in males. Expression of *VEGF-A* mRNA showed a negative correlation with *ERβ* mRNA after splitting of the cohort according to gender and nodus involvement. We propose that gender differences in *ERβ* mRNA expression in tumors during the early stages of CRC can partially explain the lower occurrence of CRC in females compared with males.

Key words

PER2 • *CRY1* • *CRY2* • Angiogenesis • Circadian

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Introduction

Epidemiological data clearly indicate that the incidence of colorectal cancer (CRC) is higher in males than in females. A sex-dependent difference in CRC incidence was reported at all anatomic subsites, with the male-to-female incidence rate ratio increasing progressively across the colon from the cecum to the rectum (Murphy *et al.* 2011, Torre *et al.* 2012, Siegel *et al.* 2015).

The higher frequency of CRC diagnosis in males is usually attributed to sex steroid hormones. Estrogen in particular is proposed to exert beneficial effects as a hormone-replacement therapy in postmenopausal females and is associated with a reduced risk of CRC and increased survival (Symer *et al.* 2018, Nie *et al.* 2018). In humans, estrogen exerts its effects *via* estrogen receptor α and β (*ERα* and *ERβ*, respectively). While ERα-mediated effects have been studied extensively with respect to several types of cancer (Clocchiatti *et al.* 2016), the functions of ERβ have not been completely elucidated. In the colon, mRNA expression of *ERβ* is much more abundant than that of *ERα* (Foley *et al.* 2000, Kennelly *et al.* 2008, Williams *et al.* 2016); therefore, effects of estradiol exerted in this tissue are usually attributed to *ERβ* signaling (Clocchiatti *et al.* 2016).

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It has been shown that overexpression of *ERβ* results in inhibition of proliferation and G1 phase cell cycle arrest in SW480 colon cancer cells. Overexpression of *ERβ* was also accompanied by downregulation of Myc proto-oncogene protein expression (Hartman *et al.* 2009). *ERβ* knockout in mice was associated with decreased apoptosis, an increased proliferation rate and abnormalities in the structure of intercellular junctions (Wada-Hiraike *et al.* 2006). Estrogen has been reported to play an important role in maintaining the function of the gastrointestinal epithelial barrier, e.g. by increasing occludin and junctional adhesion molecule-A expression (Nie *et al.* 2018). In mice that spontaneously develop intestinal adenomas because of a nonsense mutation in the Apc gene, deletion of *ERβ* leads to an increased size and number of adenomas (Giroux *et al.* 2008), and a selective *ERβ* agonist reversed this effect (Giroux *et al.* 2011).

Tumor growth is accompanied by production of novel vascularization, which is stimulated by VEGF (vascular endothelial growth factor) signaling (Claesson-Welsh and Welsh 2013). Expression of *VEGF-A* mRNA was increased in colorectal carcinoma compared with normal mucosa, and higher levels of *VEGF-A* mRNA were observed in tumors with lymph node metastases compared with non-metastatic tumors (George *et al.* 2001). An association between negative or low expression of VEGF in tumor tissue and better survival was also observed in patients with CRC (Bendardaf *et al.* 2017).

Angiogenesis that accompanies tumor expansion is inhibited by ERβ. In mice with implanted T47D breast cancer cells, an ERβ-mediated decrease in VEGF release and a reduction in intratumoral vascularization and tumor growth were observed (Hartman *et al.* 2006). In the colon estrogen has been shown to reduce the multiplicity and volume of dimethylhydrazine-induced polyps and decrease VEGF expression (Yang *et al.* 2013). These effects are probably mediated *via* estrogen response elements present in the VEFG sequence (Hyder *et al.* 2000).

Another modulation of CRC progression is associated with the functioning of the circadian system (Yang *et al.* 2017). The molecular principle of circadian oscillator function is based on clock gene expression creating a basic feedback loop. In humans a negative component of the feedback loop is created by period 1-3 (*PER)* genes and cryptochrome 1-2 (*CRY*) genes, while the positive arm is composed of transcriptional factors BMAL1 (brain and muscle ARNT-Like protein 1) and CLOCK (circadian locomotor output cycles kaput). Genes involved in basic loop functioning are able to mediate oscillations *via* several regulatory domains (including abundantly spread E-box) into the transcriptome (Honma 2018). *ERβ* mRNA expression is under circadian system control *via* an E-box present in its promoter (Cai *et al.* 2008), and *VEGF* shows a rhythmic pattern in several types of *in vivo* implanted tumor cell lines and livers of mice (Koyanagi *et al.* 2003). Clock genes have also been shown to influence cell cycle progression (Gaucher *et al.* 2018). A role for clock genes in tumor development was supported by a study showing that polymorphic variants of core clock genes of the circadian oscillator are associated with several types of cancer, including colorectal cancer (Valenzuela *et al.* 2016).

ERβ mRNA expression has been previously shown to exhibit a sex-dependent association with patient survival (Hasakova *et al.* 2018). A sex-dependent role of the circadian system in tumorigenesis was demonstrated in a study pointing out different survival rates in males and females depending on administration of chronomodulated chemotherapy (Giacchetti *et al.* 2012). Therefore, we focused on sex-dependent regulation of *ERβ* expression in CRC patients and analyzed it with respect to clock and *VEGF-A* expression.

Methods

The cohort consisted of 64 patients of both genders that underwent surgery for colorectal cancer. Detailed clinicopathological characteristics of patients and tumors are provided in Table 1. All patients were exposed to standard hospital practice with lights on from 6:00 a.m. to 9:00 p.m. (The First Surgery Department, University Hospital, Comenius University, Bratislava). Tumor, proximal (≥10 cm from tumor) and distal (≥2 cm from tumor) tissues were dissected during the surgery performed in a 2-h time window (10:00-12:00 a.m.). Sample collection was performed from 2008 to 2014. Follow-up lasted until 3 April 2018. Excised tissues were examined by a hospital pathologist to determine grading and staging. Tissue samples intended for gene expression analysis were frozen in liquid nitrogen and stored at -70 °C until mRNA extraction. All patients signed an informed consent, and the experimental protocol was approved by the Ethics committee.

Table 1. Characteristics of the patient cohort.

All patients		**n=64**	**%**
Gender	*Male*	38	59.4
	Female	26	40.6
Age	*Mean ± SEM (years)*	69 ± 1.4	
Tumor location	*Right-side*	25	39.1
	Left-side	39	60.9
Grading stage	*Well differentiated*	11	17.2
	Moderately differentiated	49	76.6
	Poorly differentiated	4	6.3
Clinical stage	*I*	4	6.3
	IIA, IIB	29	45.3
	IIIA, IIIB	15	23.4
	IVA, IVB	16	25.0
TNM classification			
Primary tumor invasion	*T1-T2*	4	6.3
	T3	48	75.0
	T4	12	18.8
Regional lymph node	*N0*	35	54.7
	N1	13	20.3
	N2	16	25.0
Distant metastasis	*M0*	48	75.0
	M1	16	25.0

n – number, T – tumor invasion, N – nodal status, M – distant metastasis, SEM – standard error of the mean.

RNA from tumor and proximal and distal healthy tissues (70 mg) was extracted with the use of RNAzol according to the manufacturer's instructions (MRC, USA). Synthesis of complementary DNA was performed with the ImProm-II Reverse Transcription System (Promega, USA) as described earlier (Herichova *et al.* 2014). To analyze gene expression QuantiTect SYBR Green PCR Kit with Sybr-green chemistry (QIAGEN, Germany) was used. Quantification was done by StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). Primers for the detection of *PER2*, *CRY1*, *CRY2*, *VEGF-A*, *ERβ* and *U6* (U6 small nuclear RNA) were:

PER2 (NM_022817.1)
sense 5'-AATGCCGATATGTTTGCGGT-3',
antisense 5'-GCATCGCTGAAGGCATCTCT-3';

CRY1 (NM_004075.4)
sense 5'-CCGTCTGTTTGTGATTCGTG-3',
antisense 5'-AAGTTAGAGGCGGTTGTCCA-3';

CRY2 (NM_001127457.2)
sense 5'-GGAGGCTGGTGTGGAAGTAG-3',
antisense 5'-CGTAGGTCTCGTCGTGGTTC-3';

VEGF-A (NM_001171623.1)
sense 5'-AGAAGGAGGAGGGCAGAATC-3',
antisense 5'-CATCAGGGGCACACAGGAT-3';

ERβ (NM_001437.2)
sense 5'-TGAGGGGAAATGCGTAGAAGG-3',
antisense 5'- CGTTCAGCAAGTGAGCCAG-3';

U6 (NR_004394.1)
sense 5'-GCTTCGGCAGCACATATACTAA-3',
antisense 5'-AAAATATGGAACGCTTCACGA-3'
(Zeman *et al.* 2008, Hasakova *et al.* 2018).

Primers used for measurement of *ERβ* mRNA expression were designed to detect isoforms *ERβ*1. Raw data were analyzed by software StepOne Software v2.0 designed for StepOnePlus™ Real-Time PCR System (both provided by ABI, USA). We performed arbitrary quantification using a standard curve generated by

logarithmic dilution of a sample with high expression. The default threshold was 10 standard deviations above the mean fluorescence generated during baseline cycles (in most cases baseline was established from cycles 3-15). The threshold value was used to calculate the Ct values for each sample in the run for every gene separately.

Real time PCR conditions were as follows: activation of hot start polymerase at 95 °C for 15 min followed by 35-45 cycles at 94 °C for 15 s, 49-53 °C for 30 s (49 °C for *cry1*, *cry2*, *per2;* 52 °C for *U6*; 53 °C; *vegf-a*, *ERβ*) and 72 °C for 30 s. Melting curve analysis and sequencing were used for validation of PCR product specificity. Nuclear RNA *U6* was used for gene normalization.

Statistical analysis

The normality of the data distribution was confirmed by the Kolmogorov-Smirnov test. To evaluate *ERβ* mRNA expression with respect to tumor staging, the male and female parts of the cohort were split into three groups according to TNM (Tumor, Nodes, Metastases) classification. Group 1 consisted of patients without nodus involvement and distant metastases (T1-4N0M0), group 2 was composed of patients with nodus involvement and without distant metastases (T3-4N1-2M0) and group 3 involved patients with distant metastases (T3-4N0-2M1). Analysis of variance (ANOVA) with Tukey's *post hoc* test was performed to compare the three groups.

Regression analysis was performed to analyze the association of *ERβ* mRNA expression with the expression of clock genes and *VEGF-A* mRNA. Data are provided as mean ± standard error of the mean (SEM). The threshold for significance was set at $P<0.05$.

Results

Males comprised 59 % and females 41 % of the cohort. Mortality during the follow-up period was 53 % in males and 54 % in females.

The expression of *ERβ* mRNA in cancer tissue was evaluated with respect to TNM staging after splitting of the cohort into three groups. Group 1 consisted of patients without nodus involvement and distant metastases, group 2 was composed of patients with nodus involvement and without distant metastases and group 3 involved patients with distant metastases.

In females from group 1, expression of *ERβ* mRNA in the cancer tissue did not differ from that measured in proximal and distal tissues. The decrease in *ERβ* mRNA expression in cancer tissue compared with healthy tissues was significant in groups 2 and 3 of the female part of the cohort (ANOVA, $P<0.05$, n=7-10). When the expression of *ERβ* mRNA in cancer tissue among groups 1-3 was compared, there was a significant difference between group 1 and group 3 (ANOVA, $P<0.05$, n=7-10, Fig. 1).

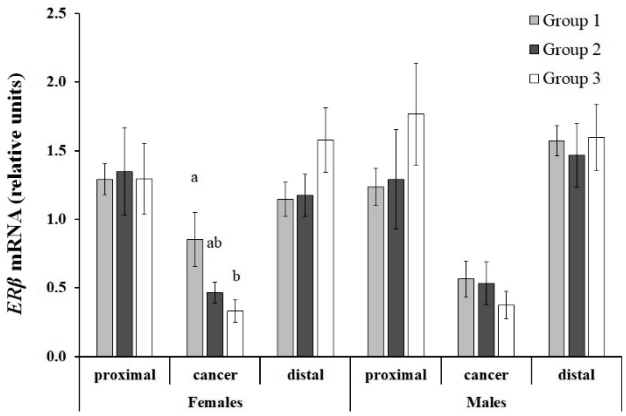

Fig. 1. Expression of estrogen receptor β (*ERβ*) in patients with colorectal cancer clustered into groups according TNM stage. Group 1 – patients without nodus involvement and distant metastases (gray columns); group 2 – patients with nodus involvement and without distant metastases (dark gray columns); group 3 – patients with distant metastases (white columns). Estrogen receptor β (*ERβ*) mRNA expression in tumors was compared among the three groups by ANOVA followed by Tukey's *post hoc* test (n=7-10). Columns labeled with different letters (a, b) indicate a significant difference between groups (Tukey's *post hoc* test, $P<0.05$). Data are provided as mean ± SEM.

Expression of *ERβ* mRNA in cancer tissue compared with proximal and distal tissues was significantly different between group 1 and group 3 (ANOVA, $P<0.05$, n=9-24) in males. The difference between cancer and healthy tissues achieved $P=0.063$ (ANOVA, n=5) in group 2 of the male part of the cohort. Comparison of *ERβ* mRNA expression in cancer tissue among groups 1-3 did not show a significant difference (ANOVA) in males.

ERβ mRNA expression in healthy tissues was not significantly different in comparisons among groups 1-3 or between males and females (Fig. 1).

In females, decreasing values of *ERβ* mRNA expression were detected with increasing extent of nodus involvement. When the female part of the cohort was split according to the presence of nodus metastases, ANOVA showed significant differences ($P<0.05$, n=6-10, Fig. 2A). A *post hoc* test indicated a difference between

females without nodus involvement and those with nodus metastases and/or distant metastases with P=0.053 and 0.059, respectively.

There was a significant difference between female clusters created according to clinical stage, implicating a negative correlation between *ERβ* expression and clinical stage (ANOVA, P<0.05, n=7-10, Fig. 2B). Dependence of *ERβ* on nodus involvement and clinical stage was not observed in men; however, there was a trend showing decreased *ERβ* expression in patients with high tumor size (data not shown).

We observed a positive correlation between the expression of *ERβ* and that of clock genes *PER2* and *CRY2* in male proximal tissue (regression analysis, P<0.05, n=38, Table 2). There was no such correlation in cancer tissue.

The expression of *ERβ* in cancer tissue was significantly correlated with *VEGF-A* expression after clustering of the cohort according to gender and nodus involvement. There was a significant correlation between *ERβ* and *VEGF-A* expression in females without nodus involvement (regression analysis, P<0.05, n=26, Table 3) that diminished in the presence of metastases. In males a negative correlation between *ERβ* and *VEGF-A* was present in patients with nodus metastases (regression analysis, P<0.05, n=38, Table 3) and not in an earlier stage of CRC. A negative correlation between *ERβ* and *VEGF-A* was not observed in healthy tissues (regression analysis, data not shown).

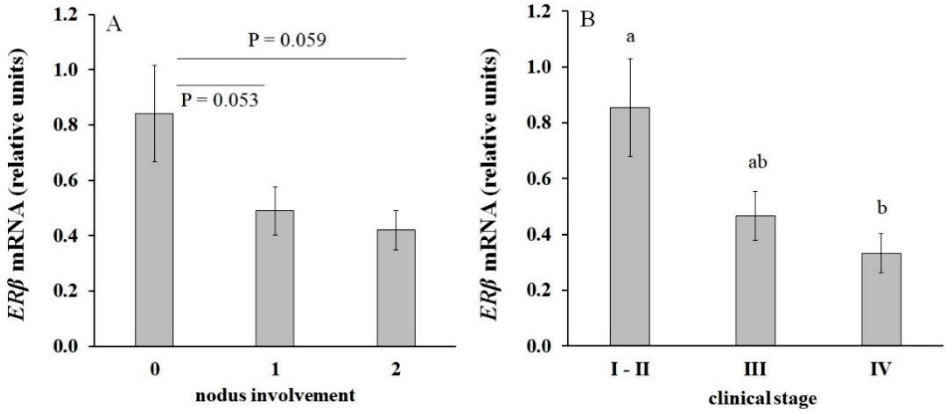

Fig. 2. Expression of estrogen receptor β (*ERβ*) in female patients with colorectal cancer clustered according to (**A**) nodus involvement and (**B**) clinical stage. Numbers below the x-axis indicate either presence/absence of nodus involvement (A, n=6-10) or clinical stage (B, n=7-10). In part B, clinical stages 1 and 2 were joined because of the low number of patients. *ERβ* mRNA expression in tumors was compared among the three groups by ANOVA followed by Tukey's *post hoc* test. Columns labeled with different letters (a, b) implicate a significant difference between groups (Tukey's *post hoc* test, P<0.05). Data are provided as mean ± SEM.

Table 2. Association between the expression of estrogen receptor β mRNA and that of clock genes and *VEGF-A* mRNA.

			PER2	***CRY1***	***CRY2***	***VEGF-A***
Females	*Tumor tissue*	*beta*	0.042	-0.120	0.052	-0.781
		R	0.100	-0.094	0.116	-0.375
		P	ns	ns	ns	0.059
	Proximal tissue	*beta*	0.039	0.007	0.045	0.006
		R	0.171	0.012	0.163	0.022
		P	ns	ns	ns	ns
Males	*Tumor tissue*	*beta*	-0.005	0.000	0.008	-0.004
		R	0.224	0.168	0.123	-0.255
		P	ns	ns	ns	ns
	Proximal tissue	*beta*	0.162	0.100	0.142	0.100
		R	0.422	0.116	0.349	0.222
		P	**0.008**	ns	**0.032**	ns

PER – period, CRY – cryptochrome, VEGF – vascular endothelial growth factor, beta – slope of the regression line, R – regression coefficient, ns – not significant, P – probability value.

Table 3. Association between the expression of *ERβ* and VEGF-A mRNA in tumor tissue clustered according gender and nodus involvement.

			Tumor Tissue		
			beta	R	**P**
VEGF-A	*Females*	*N0*	-0.935	-0.737	**0.015**
		N1-2	-0.874	-0.136	ns
	Males	*N0*	-0.342	-0.140	ns
		N1-2	-1.709	-0.560	**0.047**

VEGF – vascular endothelial growth factor, N0 – without nodus involvement, N1-2 – lymph node metastasis present, beta – slope of the regression line, R – regression coefficient, ns – not significant, P – probability value.

Discussion

The most important finding of the present study is that *ERβ* mRNA expression in colorectal cancer shows sex-dependent regulation. Unlike in males, in females with CRC with neither nodus involvement nor distant metastases, *ERβ* mRNA expression in cancer tissue is not significantly decreased compared with healthy tissues. On the other hand, a progressive decline of *ERβ* mRNA expression in the cancer tissue begins with higher stages of disease in females. In males, the expression of *ERβ* mRNA in cancer tissue shows a similar decrease in CRC compared with healthy tissues in all stages of disease. This finding is in agreement with our observation that *ERβ* mRNA expression correlates with survival in females but not in males (Hasakova *et al.* 2018).

Since the discovery of ERβ, several studies have investigated its expression in cancer tissue compared with adjacent tissues. While early studies did not reach a definitive conclusion concerning this issue, lately, a consensus has begun to arise. A decrease in *ERβ* expression in colorectal cancer compared with adjacent tissues was observed previously in males and females (Mostafaie *et al.* 2009) at the protein and mRNA levels (Williams *et al.* 2016, Ya *et al.* 2017). Sex-dependent changes in ERβ protein expression in CRC have been reported (Nüssler *et al.* 2008) in agreement with our present findings. High *ERβ* mRNA expression was associated with better survival in females but not males (Hasakova *et al.* 2018). On the contrary, sex-dependent differences in ERβ expression associated with survival were not observed in the pathology atlas (Uhlen *et al.* 2017). We suppose that discrepancies with respect to *ERβ* expression in CRC may be caused by alternative splicing of *ERβ* (Castiglione *et al.* 2008, Hua *et al.* 2018) and by race-dependent differences in CRC incidence and mortality (Alshareef *et al.* 2019).

The reported decrease in *ERβ* mRNA expression in CRC in females is in accordance with the finding that *ERβ* expression decreased progressively in female patients with nodus involvement and more advanced clinical stages. In males we only observed a decreasing trend in *ERβ* expression in patients with large tumors. Our data are in accordance with a previously observed inverse correlation between *ERβ* expression with increasing tumor staging (Rudolph *et al.* 2012, Williams *et al.* 2016). In addition, a study using the gene signature-based BinReg approach to semiquantitatively analyze estrogen receptor (ER) pathway activity in multiple CRC cohorts indicated that the ER pathway is significantly inversely associated with CRC recurrence in patients in stage II (Liu *et al.* 2016).

The reason why *ERβ* expression in CRC is decreased compared with adjacent tissues has not been completely elucidated. Regulation of ERβ is complex and involves alternative splicing, transcription factors and post-transcriptional regulation (Hua *et al.* 2018). miRNA-mediated regulation was implicated as the reason for decreased *ERβ* in CRC, as dual luciferase reporter assays pointed to *ERβ* as a direct target gene of miR-129, which was shown to inhibit *ERβ* expression and thereby enhance colon cancer cell proliferation and migration (Ya *et al.* 2017). In addition to the above mentioned *ERβ* regulatory factors, a circadian system also contributes to regulation of *ERβ* expression *via* E-box (Cai *et al.* 2008). *ERβ* shows a rhythmic pattern of expression in cultured cells and mouse tissues, and the daily rhythm disappears in BMAL1 knockout mice (Swedenborg *et al.* 2009).

In our study clock gene expression also exerted sex-dependent regulation. We observed a positive correlation between *PER2* and *CRY2* mRNA and *ERβ* in the proximal healthy tissue in male patients. No such correlation was observed in cancer tissue. Interestingly, we did not observe a correlation between any analyzed

clock genes and *ERβ* expression in female patients in either cancer or proximal tissue. Recent findings are in accordance with a previous report of sexual dimorphism in gene expression in CRC. Core clock genes *PER2* and *CRY2* exerted a more pronounced decrease in cancer tissue than in healthy tissues in males compared with females (Hasakova *et al.* 2018). Similarly, expression of miRNAs miR-16 and miR-21 showed sex-dependent differences in expression in cancer compared with healthy tissue (Hasakova *et al.* 2017). The reason why a circadian oscillator shows a tighter relationship with *ERβ* expression in males in comparison with females is not clear, but it is in accordance with the previous observation that chronomodulated chemotherapy is more beneficial for male than for female patients (Giacchetti *et al.* 2012).

ERβ also mediates estrogen effects related to tumorigenesis *via* inhibition of *VEGF-A* expression (Hartman *et al.* 2006, Yang *et al.* 2013), which can contribute significantly to its tumor suppressor capacity. Our results indicate that this regulation can occur at the transcriptional level, as we observed a negative correlation between *ERβ* and *VEFG-A*. This is possible because the *VEFG* gene contains estrogen response elements in its sequence (Hyder *et al.* 2000). Surprisingly, *ERβ* showed a negative correlation with *VEGF-A* expression in females without nodus involvement and in men with more advanced cancer stages. A possible explanation for this observation is that androgen signaling, which also differs between the two genders, influences angiogenesis in a sex-dependent way and is more efficient in males than in females (Sieveking *et al.* 2010). This observation is in accordance with the finding that *VEGF-A* expression in colorectal cancer tissue was associated with worse survival in males but not in females (Hasakova *et al.* 2018).

Limitations of the present study issue mainly from scarce information about sex hormones (and particularly estrogen) in the circulation of patients. It is difficult to estimate levels of sex hormones from references about the adult population because the average age of patients undergoing surgery for CRC is above 65 years (Uhlen *et al.* 2017), an age at which pronounced endocrine changes occur. A whole spectrum of sex hormones, their metabolites and their corresponding receptors would certainly improve our knowledge about sex-hormone-induced changes in CRC and its progression.

To conclude, in female patients, expression of *ERβ* mRNA decreased with increasing TNM stage. This dependence of *ERβ* expression on TNM staging was not observed in males. TNM staging was not associated with the expression of *ERβ* in healthy tissues. *ERβ* expression correlated with clock gene expression in the proximal tissues in male but not female patients, and this correlation was lost in cancer tissue. *ERβ* mRNA expression was also analyzed with respect to *VEGF-A* mRNA expression since sex steroid hormones are known to influence angiogenesis, which always accompanies tumor growth. We observed a significant negative correlation between *ERβ* and *VEGF-A* expression only in cancer tissue (not in healthy tissues) in females without nodus involvement and without distant metastases, whereas in male patients this negative correlation was observed in patients with more advanced stages of disease. Our data indicate that during tumorigenesis, *ERβ* expression shows sex-dependent differences. In CRC *ERβ* seems to mediate tumor suppressor effects; therefore, sex-dependent changes in its expression can probably contribute to the frequently reported differences in incidence and survival between males and females with this disease.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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