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Expression of oestrogen receptor β and prognosis of colorectal cancer

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BACKGROUND: Previous studies suggest that sex steroids influence colorectal cancer (CRC) carcinogenesis. The oestrogen receptor β (ERβ) is the predominantly expressed ER in the colon and loss of ERβ in CRC has been associated with advanced cancer stages.
METHODS: Information on vital status by the end of 2009 was obtained for 1262 CRC patients recruited between 2003 and 2007. The ERβ expression was immunohistochemically measured and associations of ERβ scores with overall survival (OS), disease-specific survival (DSS) and disease-free survival (DFS) were evaluated using Cox proportional hazard models adjusted for prognostic factors, such as tumour stage and second primary tumours.
RESULTS: Of the 1101 tumour samples with successful measurement, 535 were ERβ negative (48.6%), 381 (34.6%) showed moderate and 185 (16.8%) showed high ERβ expression. Compared with high ERβ expression, lack of ERβ was associated with higher cancer stages as well as greater tumour extent. In multivariate analyses, ERβ negativity was associated with an increased hazard ratio for death (HR = 1.61, 95% CI 1.09–2.40, $P = 0.02$), death attributed to CRC (HR = 1.54, 95% CI 0.99–2.39, $P = 0.06$) as well as a poorer DFS (DFS HR = 1.64, 95% CI 1.23–3.36, $P = 0.04$). The associations were stronger in stage I-III patients (OS HR = 2.20, 95% CI 1.28–4.06, $P = 0.007$, DSS HR = 2.38, 95% CI 1.20–5.39, $P = 0.02$, respectively).
CONCLUSIONS: Lack of ERβ expression is associated with advanced cancer stages and independently associated with poor survival.
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The role of oestrogen signalling in colorectal cancer (CRC) remains unclear (Kennelly *et al*, 2008), although incidence rates are lower in women than in men (Ferlay *et al*, 2010). Exposure to exogenous hormones through menopausal hormone therapy has consistently been found to be associated with a reduced risk for CRC in postmenopausal women (Hoffmeister *et al*, 2009; Lin *et al*, 2012). An abundantly expressed hormone receptor in the normal colonic mucosa is the oestrogen receptor β (ERβ) (Papaxoinis *et al*, 2010) and ERβ is thought to have a prominent role in the biological mechanisms of sex steroid action on colorectal tissue (Kennelly *et al*, 2008; Hartman and Gustafsson, 2010). On the other hand, the ERα, which has a major role in breast cancer development (Cuzick *et al*, 2011), treatment and prognosis (Davies *et al*, 2011), can be found only at very low levels in normal colorectal tissue (Kennelly *et al*, 2008). Results of previous studies showed that loss of ERβ expression in CRC is associated with poorer differentiation of tumours and more advanced cancer stages (Konstantinopoulos *et al*, 2003; Jassam *et al*, 2005; Elbanna *et al*, 2012).

Only one previous study investigated the prognostic implications of ERβ expression (Fang *et al*, 2010). In 423 patients with incident CRC, ERβ-positive tumours were associated with a better overall survival (OS) as well as CRC-specific survival in univariate analyses, but not after adjusting for additional prognostic factors (Fang *et al*, 2010).

The aim of this study was therefore to evaluate whether ERβ expression is an independent prognostic factor for overall as well as disease-specific survival (DSS) and disease-free survival (DFS) in a large population-based cohort of CRC patients. At the same time, the associations of ERβ expression with tumour and clinical characteristics were assessed.

MATERIALS AND METHODS

Study design and study population

The DACHS study is an ongoing population-based case–control study located in southwest Germany (Lilla *et al*, 2006; Brenner *et al*, 2011). Patients with a histologically confirmed first CRC diagnosis as of 1st January 2003 were eligible for recruitment if they were at least 30 years old, physically and mentally able to participate, sufficiently proficient in German and resident in the study region. Written informed consent was given by every study participant. The study was approved by the ethics committee of the University of Heidelberg and the medical boards of

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Baden–Wuerttemberg and Rhineland–Palatinate. The study population for this investigation comprised cases recruited between 1st January 2003 and 31st December 2007.

In June 2007, formalin-fixed paraffin-embedded (FFPE) surgical specimens of 1564 patients were requested from the pathology departments of the cooperating clinics and transferred to the tissue bank of the National Center for Tumor Diseases in Heidelberg. Samples of 1329 (85%) patients were obtained and 1262 (81%) contained sufficient tumour tissue to be successfully incorporated into tissue microarray (TMA) blocks.

Patients diagnosed with any other cancer (except benign diseases, squamous and basal skin cancer) before their first diagnosis of CRC ($N=114$), patients who died within 30 days after diagnosis whose death may be related to surgery ($N=3$) and patients without follow-up information ($N=2$) were excluded from the current survival analysis (Figure 1). Associations with DFS were evaluated in patients with non-metastatic disease (stage I–III). Therefore, we excluded stage IV patients ($N=141$) from analyses with DFS as the outcome. Also patients with unknown date of recurrence ($N=5$) had to be excluded from these analyses (Figure 1).

The study was sufficiently powered (80%) with a type I error probability of 5% to detect a true hazard ratio (HR) of 1.35 for 400 ERβ-negative cases relative to 600 ERβ-positive cases. The power was calculated assuming a recruitment period of 5 years, an additional follow-up period of 3 years and a median survival time of ERβ-positive cases of 7 years.

Data collection and follow-up

The patients gave information during a face-to-face interview conducted by a trained interviewer. The scope of the standardised questionnaire included sociodemographic data, life style and reproductive factors, as well as the family history and medical history of the patients. In addition, discharge letters and pathology reports were collected.

On average 3 years after diagnosis, a questionnaire was sent to the treating physicians of the patients to collect information on CRC therapy, newly diagnosed concomitant diseases and recurrences of CRC. Additional information including again newly diagnosed diseases and recurrences was collected from patients on average five years after diagnosis. After vital status was ascertained, a questionnaire was sent to all patients alive, except those who had denied further contacts. Data on vital status and date of death were obtained from the population registries and the cause of death was verified by death certificates obtained from the health authorities in the Rhein–Neckar–Odenwald region. New diagnoses and cancer recurrences were verified through medical records of the attending physicians.

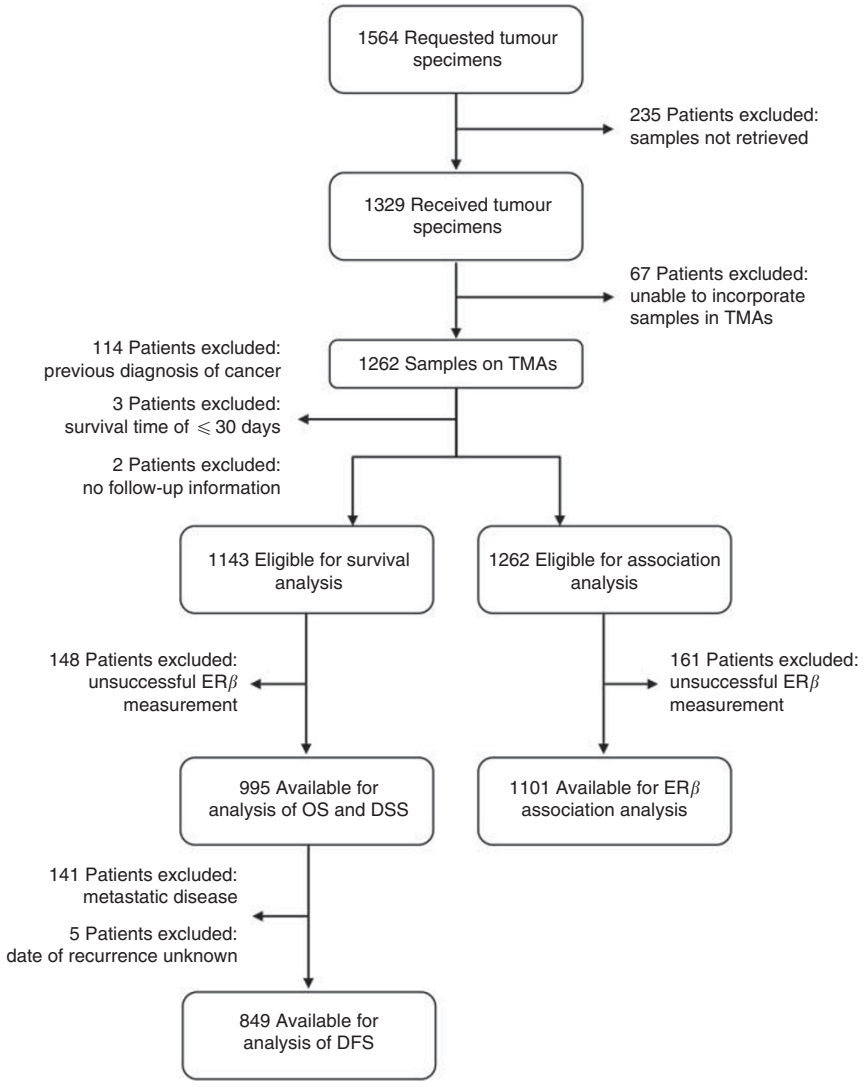

Figure 1 Diagram for colorectal cancer patients eligible for association and survival analyses.

Study end points

Follow-up time was used as the time variable, and calculated as the time between the date of diagnosis and the date of event or censoring. Death from any cause was the primary end-point. Death attributed to CRC (ICD 10: C18-20) as well as DFS were the secondary end-points. Events of interest with respect to DFS were either recurrent disease or death. Second primary tumours were not counted as events in the DFS analysis. For patients without any event of interest, censoring occurred at the date of last follow-up or 31st December 2009, whichever came first.

Immunohistochemistry

After collecting the requested FFPE samples, they were integrated into TMA blocks, which took place in June 2009. From each surgical specimen four 0.6-mm cores (two cores each from tumour and adjacent non-neoplastic tissue) were punched and integrated into TMA blocks. The 5-μm thick TMA sections were mounted on to superfrost slides. Staining for ERβ was performed in July 2010. The anti-ERβ antibody (primary mouse monoclonal, 14C8, Abcam, Cambridge, UK) was applied at a dilution of 1/50 at room temperature for 30 min. After the incubation with the appropriate biotinylated secondary antibody (Dako antimouse, 1/200 dilution, Dako, Glostrup, Denmark) at room temperature for 15 min and an incubation with the streptavidin avidin–biotin complex kit (Dako), antigen retrieval was performed following endogenous peroxidase blocking. The antibody reactions were revealed using the Dako EnVision + System-HRP. The ERβ expression was visualised with 3,3'-diaminobenzidine (Vector, Peterborough, UK). The lymphocytes in the lamina propria as well as the cores of adjacent non-neoplastic tissue were used as positive control and standard. Sections after the omission of the primary antibody or incubation with the appropriate blocking peptide were used as negative controls. The staining was performed on an autostainer (Dako) based on the avidin–biotin complex method. The sections were counterstained with haematoxylin, dehydrated and coverslipped.

The expression of ERβ in the CRC tissue was independently analysed by two pathologists (CT, WR) blinded to the patient's outcome. In 96.8% of the cases, results of the scoring were identical. Discrepancies were resolved by an additional joint review of the respective sample. A three-level scoring system (based on Konstantinopoulos *et al* (2003)) was applied that involved the staining intensity as well as the percentage of positivity in the cancer cell nuclei (Figure 2). Tumours were regarded as negative for ERβ expression, if $<10\%$ of the cell nuclei showed positive staining. A moderate expression was defined as weak positive staining of $>50\%$ of the cell nuclei or strong positive staining in 10–50% of the nuclei. High expression of ERβ was assigned if $>50\%$ of the cell nuclei showed strong positive staining.

Statistical analysis

All analyses were conducted using SAS, version 9.2 (SAS Institute, Cary, NC, USA). Two-sided tests were performed and a *P*-value of <0.05 was used as significance threshold. Pearson's χ^2 test and the Kruskal–Wallis test were applied to test for differences of clinical parameters and tumour characteristics between patients according to ERβ expression score.

To assess the association of ERβ expression scores with clinical parameters and tumour characteristics, unconditional multinomial logistic regression was carried out. The model was determined using backward selection, retaining variables with a *P*-value of $\leqslant 0.2$. The initial set of variables included tumour extent (T1, T2, T3, T4), nodal status (N0, N1, N2), distant disease (M0, M1), sex, tumour location (colon, rectum), age (in 5-year increments) and former neoadjuvant treatment (yes/no). Samples with missing values in any of the predictor variables or the outcome variable were excluded from the analyses.

Median follow-up time was computed using the reverse Kaplan–Meier method (Schemper and Smith, 1996). To evaluate the association of the ERβ expression scores with OS, DSS, and DFS, regression analyses based on the Cox proportional hazards model were applied. As some patients were interviewed several months after diagnosis, we accounted for possible survival bias by left truncation of the follow-up period. The validity of the model assumptions were assessed by examining plots of Schoenfeld residuals and score processes as well as by including a time-dependent component for each explanatory variable in univariate and multivariate models.

The multivariate models were adjusted for the established prognostic factors such as tumour extent, nodal status, distant disease, age as well as year of diagnosis and stratified by histological grade (well/moderate, poor/undifferentiated). Stratification was performed for all variables showing a time-dependent effect on OS. Again, final models were determined using backward selection, retaining variables with a *P*-value of $\leqslant 0.2$. The final model was additionally stratified for treatment with adjuvant chemotherapy (yes/no) and CRC detection by screening (yes/no)

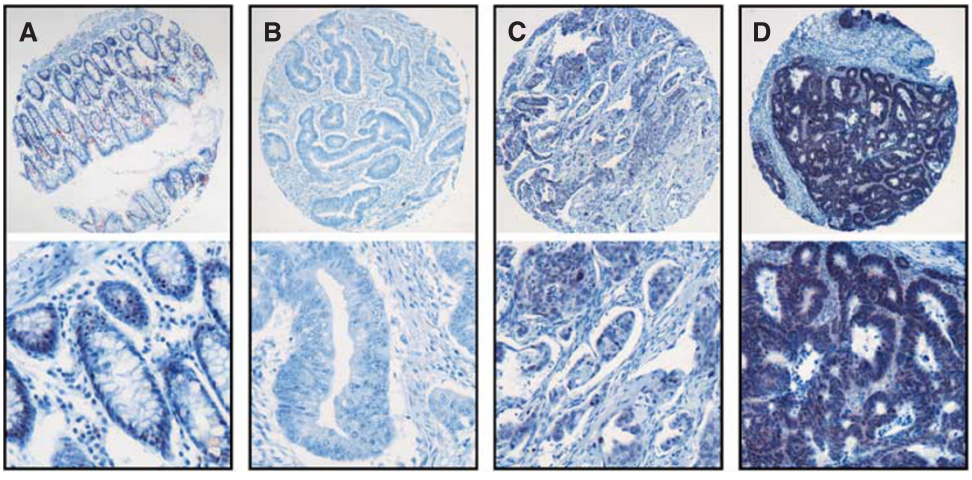

Figure 2 Photomicrographs showing the typical staining for the anti-ERβ monoclonal antibody. (**A**) ERβ expression in adjacent non-neoplastic colonic mucosa; (**B**) adenocarcinoma negative for ERβ ($<10\%$ of nuclei positive); (**C**) adenocarcinoma showing moderate ERβ expression (10–50% nuclei with strong positive staining or $>50\%$ of nuclei with weak positive staining); (**D**) adenocarcinoma showing high ERβ expression ($>50\%$ nuclei with strong positive staining).

and adjusted for diagnosis of other cancers after CRC diagnosis (yes/no) and BMI ($kg\,m^{-2}$, continuous). Kaplan–Meier curves as well as survival curves adjusted (Zhang *et al*, 2007) according to the final model were used to illustrate the association of ERβ expression scores with OS, DSS, and DFS. Patients with missing values were excluded.

Two sensitivity analyses were performed. First, patients who had received neoadjuvant therapy were excluded. In the second sensitivity analysis, patients with advanced disease (stage IV) were excluded. Owing to the limited number of events when assessing associations with OS and DSS in this latter analysis, Firth's penalised likelihood approach was applied (Firth, 1993; Heinze and Schemper, 2001).

To evaluate the predictive ability and the validity of the final model, we calculated the concordance probability estimate and R^2 and reported the mean values and 95% confidence intervals (CIs) from 1000 bootstrap samples (Nagelkerke, 1991; Gönen and Heller, 2005). We produced receiver-operating characteristic (ROC) curve plots and calculated the area under the ROC curve (AUC) by applying the methods described by Chambless *et al* (2011).

RESULTS

Measurement of ERβ expression was successful in 1101 of 1262 available surgical samples on TMA (87.2%). Reasons for unsuccessful measurements were an uninformative positive control and loss of cores. Samples with unsuccessful ERβ measurement were more often derived from tumours that were treated with neoadjuvant therapy (15.5% *vs* 6.0%, $P < 0.0001$) and tumours of T1 and T4 category (T1 9.9% *vs* 5.9% and T4 17.4% *vs* 10.9%, $P = 0.01$) than successfully measured samples. Of the 1101 samples with successful measurement, 535 were ERβ negative (48.6%), 381 (34.6%) showed moderate ERβ expression and 185 (16.8%) showed high ERβ expression.

The mean age of the participants was 68.7 years (s.d.: 10.4 years), 57.1% of them were male and 42.9% female. The population characteristics according to ERβ expression score are displayed in Table 1. Tumours negative for ERβ were of higher stage ($P = 0.003$), greater tumour extent ($P < 0.001$) and less often detected by screening ($P < 0.0001$).

The ERβ expression score was still significantly associated with UICC cancer stage and tumour extent after multivariate adjustment (Table 2). Compared with high ERβ expression, negative expression of ERβ was associated with advanced tumour stages (stage II *vs* I OR = 2.45, 95% CI 1.54–3.91, $P = 0.0002$; stage III *vs* I OR = 2.49, 95% CI 1.56–3.98, $P = 0.0001$; stage IV *vs* I OR = 1.79, 95% CI 1.03–3.12, $P = 0.04$) and greater tumour extent (T2 *vs* T1 OR = 2.23, 95% CI 1.10–4.52, $P = 0.03$; T3 *vs* T1 OR = 4.16, 95% CI 2.18–7.92, $P < 0.0001$; T4 *vs* T1 OR = 3.66, 95% CI 1.66–8.08, $P = 0.001$). Similar but weaker associations were found comparing moderate ERβ expression with high ERβ expression.

The median follow-up time of the 1143 patients included in the survival analyses was 4.9 years. During the follow-up period, 346 deaths occurred, including 265 deaths that were attributed to CRC. Further causes of death were other cancers ($N = 19$), cardiovascular disease ($N = 37$) and other causes ($N = 21$). For four participants, the cause of death could not be obtained.

We evaluated the association of the ERβ expression score with OS, DSS and DFS (Table 3). Kaplan–Maier curves as well as survival curves that illustrate the unadjusted and adjusted survival probabilities regarding OS, DSS and DFS are displayed in Figure 3. Compared with having a tumour with high ERβ expression score, the HR associated with having a tumour negative for ERβ expression was 1.61 (95% CI 1.09–2.40, $P = 0.02$) for death from any cause and 1.54 (95% CI 0.99–2.39, $P = 0.06$) for death attributed to CRC. Oestrogen receptor β negativity was also associated with a poorer DFS (HR = 1.64, 95% CI 1.23–3.36, $P = 0.04$). The associations for the moderate score of ERβ expression were similar but weaker for OS and DSS (HR for death from any cause: 1.50, 95% CI 0.99–2.27, $P = 0.06$; HR for death attributed to CRC: 1.43, 95% CI 0.89–2.28, $P = 0.14$). However, there was no association of moderate ERβ expression with DFS (HR = 1.16, 95% CI 0.71–1.92, $P = 0.55$).

The first sensitivity analysis excluding patients who received neoadjuvant treatment yielded results comparable to those of the main analysis (Table 3). The respective HRs associated with ERβ-negative tumours were similar to those obtained using the whole data set (OS HR = 1.62, 95% CI 1.09–2.43, $P = 0.02$; DSS HR = 1.56, 95% CI 0.99–2.45, $P = 0.06$ and DFS HR = 1.64, 95% CI 1.01–2.67, $P = 0.05$). In the second sensitivity analysis restricted to patients with stage I–III disease, stronger associations than in the main analysis were observed (Table 3). Compared with patients with tumours showing high ERβ expression, patients with ERβ-negative tumours had a significantly associated HR for death of any cause of 2.20 (95% CI 1.28–4.06, $P = 0.007$) and of 2.38 (95% CI 1.20–5.39, $P = 0.02$) for death attributed to CRC.

The multivariate model had a high discriminatory power (CPE = 0.73, 95% CI 0.70–0.76). R^2 as an additional measure for model validity was 0.47 (95% CI 0.38–0.57), hence the variables in the full model explained 47% of the variance in OS in this study. The inclusion of the ERβ expression score in the model improved the predictive ability of the model slightly (Figure 4A). The AUC value was 0.799 for the model excluding the ERβ expression score and 0.806 for the model including the ERβ expression score. The improvement in predicting OS by including the ERβ score was greater in the subgroup of stage I–III patients, as can be seen by the comparison of the ROC curves (Figures 4A and B). In this patient group, the AUC value was 0.740 for the model without the ERβ expression score and 0.758 for the model including the ERβ expression score.

DISCUSSION

In this prospective patient-cohort study, we found that, in comparison with tumours with high ERβ expression, tumours negative for ERβ were associated with advanced cancer stages. Stage III cancers were 2.5 times more likely to be ERβ-negative in comparison with stage I cancers. Also, tumours of greater extent (T4) were 3.5-fold more likely to show ERβ negativity than T1 tumours. Patients with ERβ-negative tumours had an associated significantly poorer OS with a 61% increased risk of dying compared with patients whose tumours showed high ERβ expression, even after accounting for tumour extent and other prognostic factors.

Lower levels of ERβ mRNA and protein in tumour tissue compared with non-neoplastic tissue have been found consistently in previous studies on CRC, with the percentage of tumours classified as ERβ-negative ranging from 21 to 38% (Foley *et al*, 2000; Campbell-Thompson *et al*, 2001; Konstantinopoulos *et al*, 2003; Jassam *et al*, 2005; Wong *et al*, 2005). Results of our study are in line with studies that associated ERβ-negative tumours with advanced tumour stages (Jassam *et al*, 2005; Elbanna *et al*, 2012). We did not observe a significantly different expression of ERβ in relation to tumour differentiation as reported by Konstantinopoulos *et al* (2003). However, sample sizes of previous studies were usually small and ranged from 11 to 91 samples (Foley *et al*, 2000; Campbell-Thompson *et al*, 2001; Konstantinopoulos *et al*, 2003; Jassam *et al*, 2005; Wong *et al*, 2005). One relatively large study by Fang *et al* (2010) including 423 CRC patients also investigated the association of ERβ expression with overall and CRC-specific mortality and reported findings consistent with those from our study. The study population was restricted to patients with stage I-III CRC and the median follow-up time was 86 months. The criteria used to define a tumour as negative for ERβ

Table 1 Distribution of selected covariates in 1262 colorectal cancer patients with univariate HRs for overall survival of 1143 colorectal cancer patients eligible for survival analysis

	Total		ERβ negative		ERβ moderate		ERβ high			ERβ unknown		Univariate		
Characteristic	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	***P***	**No.**	**%**	**HR**	**95% CI**	***P***
Total no.	1262	100.0	535	42.4	381	30.2	185	14.6		161	12.8			
Deaths ($N=1143$)	346	30.3	165	47.7	100	28.9	35	10.1	0.003	46	13.3			
Events DFS ($N=975$)[a]	273	28.0	136	49.8	75	27.5	22	8.1	0.0001	40	14.7			
Median age (years)[b]	69.0		69.0		69.0		68.0		0.51	69.0		1.17	1.11–1.24	<0.0001
Median BMI ($kg\,m^{-2}$)	26.0		26.0		25.7		26.3		0.33	26.2		0.94	0.91–0.96	<0.0001
Sex														
Female	541	42.9	234	43.7	163	42.8	81	43.8	0.95	63	39.1	1.00	(Ref.)	
Male	721	57.1	301	56.3	218	57.2	104	56.2		98	60.9	0.83	0.67–1.03	0.09
Tumour localisation														
Colon	807	64.0	337	63.0	250	65.6	124	67.0	0.53	96	59.6	1.00	(Ref.)	
Rectum	455	36.0	198	37.0	131	34.4	61	33.0		65	40.4	0.89	0.71–1.11	0.31
Cancer stage														
UICC stage I	244	19.2	81	15.1	72	19.0	53	28.6	0.003	38	23.6	1.00	(Ref.)	
UICC stage II	416	33.0	191	35.7	123	32.3	53	28.6		49	30.4	1.72	1.10–2.69	0.02
UICC stage III	426	33.8	187	35.0	137	36.0	50	27.0		52	32.3	2.81	1.83–4.30	<0.0001
UICC stage IV	176	14.0	76	14.2	49	12.9	29	15.8		22	13.7	13.77	9.02–21.0	<0.0001
Tumour extent														
T1	81	6.4	21	3.9	22	5.8	22	11.9	0.0005	16	9.9	1.00	(Ref.)	
T2	232	18.4	86	16.1	78	20.5	42	22.7		26	16.1	0.96	0.47–1.97	0.91
T3	800	63.4	365	68.2	244	64.0	100	54.1		91	56.5	2.66	1.41–5.00	0.003
T4	148	11.7	63	11.8	37	9.7	20	10.8		28	17.4	6.25	3.23–12.1	<0.0001
Unknown	1	0.1	0	0.0	0	0.0	1	0.5		0	0.0			
Nodal status														
N0	683	54.1	282	52.7	201	52.8	112	60.5	0.18	88	54.7	1.00	(Ref.)	
N1	319	25.3	133	24.9	103	27.0	43	23.2		40	24.8	2.01	1.53–2.63	<0.0001
N2	245	19.4	114	21.3	74	19.4	26	14.1		31	19.3	4.70	3.64–6.06	<0.0001
Unknown	15	1.2	6	1.1	3	0.8	4	2.2		2	1.2			
Distant metastasis														
M0	1086	86.0	459	85.8	332	87.1	156	84.3	0.65	139	86.3	1.00	(Ref.)	
M1	176	14.0	76	14.2	49	12.9	29	15.7		22	13.7	7.05	5.66–8.80	<0.0001
Grade of differentiation														
Well/moderate	875	69.3	365	68.2	263	69.0	134	72.4	0.70	113	70.2	1.00	(Ref.)	
Poor/undifferentiated	357	28.3	157	29.3	110	28.9	49	26.5		41	25.5	1.77	1.42–2.20	<0.0001
Unknown	30	2.4	13	2.4	8	2.1	2	1.1		7	4.3			
Microsatellite stability														
MSS	950	75.3	387	72.3	316	82.9	154	83.2	0.46	93	57.8	1.00	(Ref.)	
MSI	109	8.6	49	9.2	31	8.1	21	11.4		8	5.0	0.74	0.48–1.15	0.18
Unknown	203	16.1	99	18.5	34	8.9	10	5.4		60	37.3			
Diagnosis of other cancer after CRC diagnosis														
No	1214	96.2	510	95.3	369	96.8	178	96.3	0.50	157	97.5	1.00	(Ref.)	
Yes	48	3.8	25	4.7	12	3.2	7	3.7		4	2.5	1.70	1.12–2.60	0.01
CRC detected by screening														
No	996	34.6	446	83.4	290	76.1	128	69.2	<0.0001	132	82.0	1.00	(Ref.)	
Yes	263	64.8	86	16.0	91	23.9	57	30.8		29	18.0	0.40	0.28–0.57	<0.0001
Unknown	3	0.3	3	0.6	0	0.0	0	0.0		0	0.0			
Neoadjuvant therapy														
No	1171	92.8	498	93.1	360	94.5	177	95.7	0.39	136	84.5	1.00	(Ref.)	
Yes	91	7.2	37	6.9	21	5.5	8	4.3		25	15.5	0.90	0.60–1.35	0.62
Adjuvant chemotherapy														
No	687	54.4	281	52.5	203	53.3	116	62.7	0.05	87	54.0	1.00	(Ref.)	
Yes	564	44.7	248	46.4	175	45.9	68	36.8		73	45.4	1.92	1.55–2.39	<0.0001
Unknown	11	0.9	6	1.1	3	0.8	1	0.5		1	0.6			
Adjuvant radiotherapy														
No	1138	90.2	483	90.3	339	89.0	171	92.4	0.44	145	90.1	1.00	(Ref.)	
Yes	114	9.0	47	8.8	39	10.2	13	7.0		15	9.3	1.05	0.72–1.51	0.81
Unknown	10	0.8	5	0.9	3	0.8	1	0.5		1	0.6			
Other adjuvant therapy[c]														
No	1228	97.3	515	96.2	372	97.6	183	99.0	0.11	158	98.1	1.00	(Ref.)	
Yes	30	2.4	18	3.4	9	2.4	1	0.5		2	1.2	1.96	1.13–3.41	0.02
Unknown	4	0.3	2	0.4	0	0.0	1	0.5		1	0.6			

Abbreviations: BMI = body mass index; CRC = colorectal cancer; CI = confidence interval; ERβ = oestrogen receptor β; HR = hazard ratio. [a]Excluding stage IV patients. [b]HR per 5-year increments. [c]Includes herbal therapies (e.g. mistletoe therapy), vitamin preparations and therapies given in the setting of clinical trials.

Table 2 Association of ERβ expression scores with selected tumour and clinical characteristics of 1101 colorectal cancer patients

	ERβ negative		ERβ moderate		ERβ high
	OR (95% CI)[a]	*P*	OR (95% CI)[a]	*P*	OR (95% CI)
Age					
In 5-year increments	1.06 (0.97–1.15)	0.18	1.05 (0.96–1.14)	0.28	1.00 (Ref.)
Sex					
Male *vs* female	1.03 (0.73–1.45)	0.88	1.07 (0.74–1.53)	0.73	1.00 (Ref.)
Tumour localisation					
Rectum *vs* colon	1.39 (0.96–2.01)	0.08	1.17 (0.79–1.72)	0.43	1.00 (Ref.)
Cancer stage					
UICC stage II *vs* I	2.45 (1.54–3.91)	0.0002	1.73 (1.07–2.81)	0.03	1.00 (Ref.)
UICC stage III *vs* I	2.49 (1.56–3.98)	0.0001	2.04 (1.26–3.29)	0.004	1.00 (Ref.)
UICC stage IV *vs* I	1.79 (1.03–3.12)	0.04	1.28 (0.71–2.29)	0.41	1.00 (Ref.)
Tumour extent					
T2 *vs* T1	2.23 (1.10–4.52)	0.03	1.90 (0.94–3.84)	0.07	1.00 (Ref.)
T3 *vs* T1	4.16 (2.18–7.92)	<0.0001	2.55 (1.34–4.85)	0.004	1.00 (Ref.)
T4 *vs* T1	3.66 (1.66–8.08)	0.001	1.94 (0.86–4.38)	0.11	1.00 (Ref.)
Nodal status					
N1 *vs* N0	1.04 (0.68–1.58)	0.87	1.24 (0.80–1.93)	0.33	1.00 (Ref.)
N2 *vs* N0	1.41 (0.85–2.33)	0.18	1.47 (0.87–2.50)	0.15	1.00 (Ref.)
Distant metastasis					
M1 *vs* M0	0.75 (0.46–1.24)	0.27	0.75 (0.44–1.28)	0.29	1.00 (Ref.)
Grade of differentiation					
G3/G4 *vs* G1/G2	1.10 (0.74–1.63)	0.63	1.14 (0.75–1.71)	0.54	1.00 (Ref.)
Neoadjuvant therapy					
Yes *vs* no	1.32 (0.57–3.04)	0.52	1.16 (0.48–2.82)	0.74	1.00 (Ref.)
Microsatellite stability					
MSI *vs* MSS	0.98 (0.55–1.73)	0.93	0.74 (0.40–1.36)	0.33	1.00 (Ref.)

Abbreviations: CI = confidence interval; ERβ = oestrogen receptor β; OR = odds ratio. [a]Models adjusted for age, tumour extent and tumour localisation. The model used to assess association with cancer stage was adjusted for age and tumour localisation.

expression were comparable to ours, although staining of the whole cell rather than the cell nuclei was scored and a different antibody was used. Of the 423 analysed tumour samples, 32.4% were defined as being ERβ-negative and 67.6% as ERβ-positive (including both moderate and high level of ERβ expression). In univariate analyses, Fang *et al.* found that ERβ-positive tumours were associated with OS (HR = 0.58, 95% CI 0.40–0.84, $P = 0.004$) and CRC-specific survival (HR = 0.53, 95% CI 0.36–0.79 $P = 0.001$), but the associations were no longer significant after accounting for further prognostic factors. This can be attributed in part to the smaller sample size of the study, yet the magnitude of the reported associations is comparable to that in our study for stage I-III CRC.

We performed a sensitivity analysis excluding patients who had received neoadjuvant therapy, as ERβ expression was less often successfully measured in tumours treated with neoadjuvant therapy. The results did not differ substantially from those obtained based on the whole data set (Table 3). Exclusion of stage IV patients from the analysis yielded larger estimated HRs. Also the improvement of the predictive ability of the model by the ERβ expression score was higher among this subgroup (Figure 4). This is most likely due to a reduction in heterogeneity in the remaining study population with stage I-III patients. The identification of a distant metastasis always leads to a stage IV classification of the tumour, irrespective of its size and differentiation. This heterogeneity in tumour properties of stage IV disease could explain why ERβ negativity was not as strongly associated with stage IV disease as with stage II and stage III disease (Table 2). Furthermore, compared with patients with non-metastatic disease, the survival probability of patients with stage IV disease is very poor overall, and the mostly very short survival times are influenced by additional factors, such as surgical treatment of metastasis (Dahabreh *et al*, 2011).

We also assessed the association of the ERβ score with DFS in patients with non-metastatic disease. Having a tumour negative for ERβ was associated with a greater risk for disease recurrence or death (Table 3). The association was weaker compared with the association with OS in the same group of stage I–III patients, but similar to that observed with OS in the whole patient group. Hence, the results of the DFS analysis support the associations observed with OS.

Studies on colon cancer cells suggest that ERβ has a role in the regulation of cell proliferation by control of key cell cycle modulators (Martineti *et al*, 2005; Hartman *et al*, 2009). In ERβ-knockout mice, cells of the colonic epithelium showed increased proliferation rates, decreased apoptosis as well as less differentiation and cellular adhesion (Wada-Hiraike *et al*, 2006). Also, an increased incidence of precancerous lesions (aberrant crypt foci) in ERβ-knockout mice has been reported (Saleiro *et al*, 2010). In a study of $Apc^{Min/+}$ mice, upregulation of ERβ through a diet containing ERβ-agonists increased the apoptosis rate and normalised the proliferation in the intestinal mucosa (Barone *et al*, 2010). Taken together, the expression of ERβ seems to be important for the maintenance of the physiologic proliferation of the colonic epithelium, which provides biological plausibility to our results.

Our study had certain strengths and weaknesses. The events of interest were verified by death certificates and medical records, therefore misclassification is unlikely. We were able to account for

Table 3 Association of ERβ expression scores with overall survival, disease-specific survival and disease-free survival

	Overall survival			Disease-specific survival			Disease-free survival[a]		
Variable	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
Univariate model, all cases (N = 995, DFS: N = 849)									
ERβ expression score									
High	1.00	(Ref.)		1.00	(Ref.)		1.00	(Ref.)	
Moderate	1.36	0.93–2.00	0.12	1.29	0.84–1.99	0.25	1.42	0.88–2.29	0.15
Negative	1.68	1.17–2.42	0.005	1.66	1.10–2.49	0.02	2.02	1.28–3.18	0.002
Multivariate model (N = 995, effective N = 934, DFS: N = 849, effective N = 780)[b]									
ERβ expression score									
High	1.00	(Ref.)		1.00	(Ref.)		1.00	(Ref.)	
Moderate	1.50	0.99–2.27	0.06	1.43	0.89–2.28	0.14	1.16	0.71–1.92	0.55
Negative	1.61	1.09–2.40	0.02	1.54	0.99–2.39	0.06	1.64	1.02–2.64	0.04
Covariates in the multivariate model									
Age (5-year increments)	1.14	1.07–1.22	<0.0001	1.08	1.01–1.16	0.02	1.14	1.05–1.22	0.0008
Tumour extent									
T1	1.00	(Ref.)		1.00	(Ref.)		1.00	(Ref.)	
T2	0.61	0.27–1.39	0.24	0.41	0.14–1.23	0.11	1.02	0.44–2.37	0.97
T3	1.21	0.58–2.50	0.62	1.16	0.46–2.90	0.75	1.45	0.67–3.17	0.35
T4	1.33	0.60–2.92	0.48	1.42	0.54–3.74	0.48	2.58	1.09–6.12	0.03
Nodal status									
N0	1.00	(Ref.)		1.00	(Ref.)		1.00	(Ref.)	
N1	1.93	1.32–2.81	0.0007	1.98	1.27–3.10	0.003	1.43	0.93–2.19	0.10
N2	3.00	2.05–4.39	<0.0001	3.46	2.24–5.34	<0.0001	2.71	1.73–4.26	0.00001
Distant metastasis									
M0	1.00	(Ref.)		1.00	(Ref.)		NA	NA	NA
M1	4.86	3.55–6.64	<0.0001	5.05	3.61–7.07	<0.0001	NA	NA	NA
Diagnosis of other cancer after CRC diagnosis									
No	1.00	(Ref.)		1.00	(Ref.)		1.00	(Ref.)	
Yes	2.49	1.56–3.98	0.0001	1.62	0.81–3.22	0.17	2.04	1.23–3.36	0.005
BMI ($kg\,m^{-2}$)	0.94	0.91–0.97	0.0001	0.94	0.90–0.97	0.0003	0.96	0.93–1.00	0.03
Year of diagnosis	0.96	0.86–1.07	0.48	0.94	0.83–1.07	0.34	0.97	0.85–1.11	0.67
Multivariate model, cases not treated with neoadjuvant therapy (N = 931, effective N = 885, DFS: N = 791, effective N = 737)[b]									
ERβ expression score									
High	1.00	(Ref.)		1.00	(Ref.)		1.00	(Ref.)	
Moderate	1.46	0.95–2.23	0.08	1.39	0.86–2.25	0.19	1.12	0.67–1.88	0.66
Negative	1.62	1.09–2.43	0.02	1.56	0.99–2.45	0.06	1.64	1.01–2.67	0.05
Multivariate model, cases with early stage disease (stage I–III) (N = 854, effective N = 801)[b]									
ERβ expression score									
High	1.00	(Ref.)		1.00	(Ref.)		NA	NA	NA
Moderate	1.71	0.97–3.20	0.08	1.74	0.85–3.99	0.16	NA	NA	NA
Negative	2.20	1.28–4.06	0.007	2.38	1.20–5.39	0.02	NA	NA	NA

Abbreviations: BMI = body mass index; CI = confidence interval; CRC = colorectal cancer; ERβ = oestrogen receptor β; HR = hazard ratio. [a]Patients with metastatic disease were excluded for this end point. [b]Model stratified for grade of differentiation (well/moderate, poor/undifferentiated), CRC detected by screening (yes/no) and treatment with adjuvant chemotherapy (yes/no) and adjusted for tumour extent (T1, T2, T3, T4), nodal status (N0, N1, N2), distant metastasis (M0, M1), diagnosis of other cancer after CRC diagnosis (yes/no), BMI ($kg\,m^{-2}$, continuous), age and year of diagnosis.

many factors that are thought to be associated with mortality in CRC patients, including adjuvant therapy and certain co-morbidities. The discriminatory power of the multivariate model was high. However, sufficient data on further potentially important factors such as physical activity (Meyerhardt *et al*, 2006) and use of NSAIDs (Chan *et al*, 2009) after diagnosis was not available.

As we did not attempt to repeat the ERβ measurement for samples with unsuccessful measurement, the ERβ score was missing for a relatively large proportion of patients (12.8%). This proportion of failed measurements due to loss of cores and other reasons is not uncommon for immunohistochemistry using TMAs (Jourdan *et al*, 2003). Our study size was sufficient to compensate the loss of power due to exclusion of samples with missing ERβ score. By using available tissue samples, patient selection might have occurred. However, we did not observe OS to be different for patients with and without TMA samples (HR = 1.11, 95% CI 0.74–1.67, $P = 0.61$).

Another common concern with the use of TMAs has been the representativeness of the punched tissue. Using two cores to represent the tumour has been shown to result in sufficient concordance for many different tissue types, including CRC (Jourdan *et al*, 2003; Giltnane and Rimm, 2004). The antibody 14C8 used in this study recognises most ERβ variants, including the full-length form, and has been shown to be a useful tool for the immunohistochemical assessment of ERβ expression in paraffin-embedded tissue (Skliris *et al*, 2002; Carder *et al*, 2005; Speirs *et al*, 2008). However, splice-variants of ERβ are thought to differ in function from the wild-type ERβ,

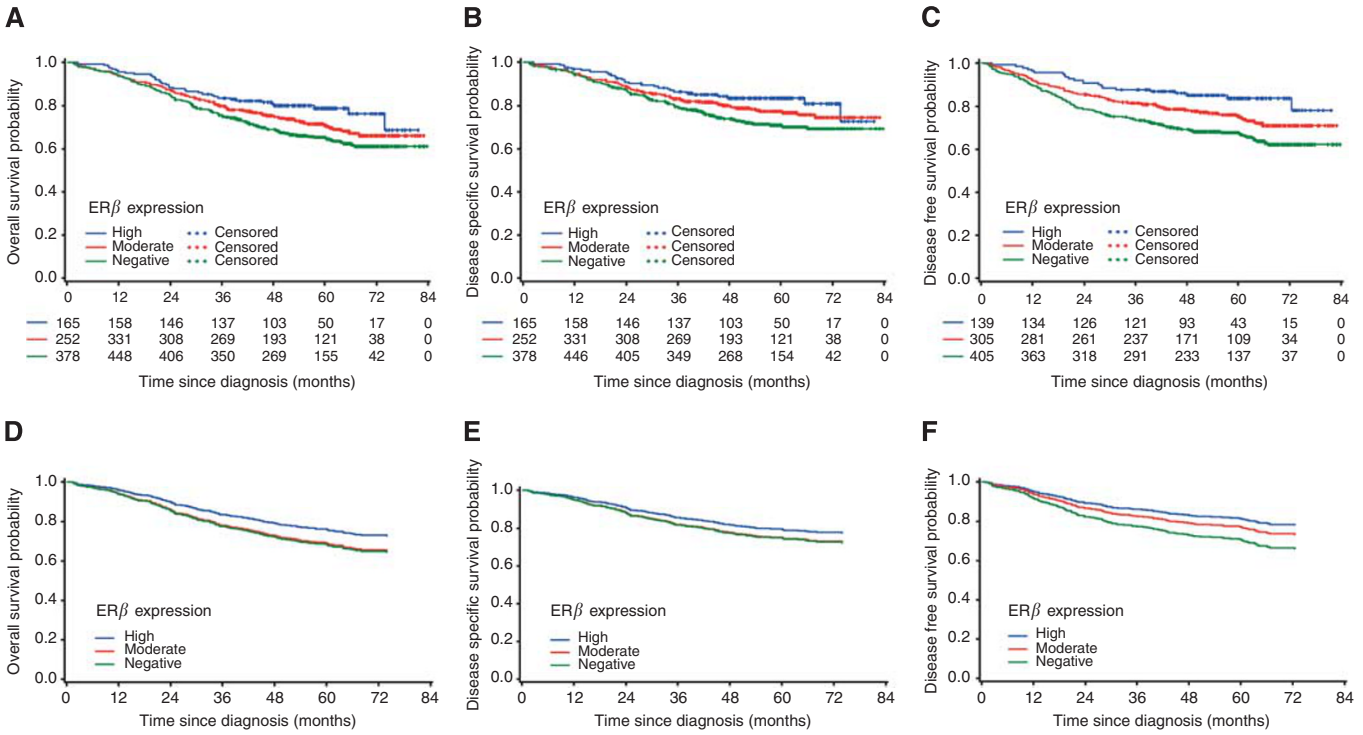

Figure 3 Kaplan–Meier curves for OS (**A**), DSS (**B**) and DFS (**C**) with numbers of patients at risk according to ERβ expression scores as well as directly adjusted survival curves for OS (**D**), DSS (**E**) and DFS (**F**).

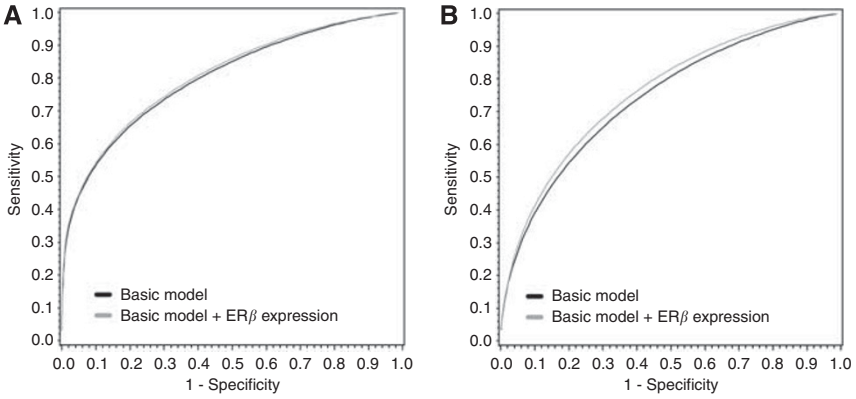

Figure 4 ROC curves comparing the basic model with the extended model including the ERβ expression score using data of all patients eligible for survival analysis (**A**) and data of stage I–III Patients (**B**).

which is the only form that has shown transcriptional activity (Peng *et al*, 2003; Leung *et al*, 2006). The roles of ERβ splice-variants in CRC deserve further research and future studies could potentially gain more detailed insight by using variant-specific antibodies.

To conclude, this is the first study that reports a potential independent prognostic value of ERβ expression in CRC. Results of our study suggest that the loss of ERβ expression is related to CRC progression, and that it is associated with an increased risk of dying, also due to the cancer itself. Additional investigations in prospective patient-cohorts of sufficient size are needed to confirm our findings and to further evaluate the role of ERβ variants in CRC. If the role of ERβ expression in CRC prognosis is confirmed, the prognosis of patients could potentially be improved by therapies aimed at inducing ERβ expression.

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