Combined Estrogen Alpha and Beta Receptor Expression Has a Prognostic Significance for Colorectal Cancer Patients

Geriolda Topi 1, Souvik Ghatak 1, Shakti Ranjan Satapathy 1, Roy Ehrnström 2, Marie-Louise Lydrup 3 and Anita Sjölander 1*

1 Division of Cell Pathology, Skåne University Hospital, Lund University, Malmö, Sweden, 2 Division of Pathology, Department of Translational Medicine, Skåne University Hospital, Lund University, Malmö, Sweden, 3 Division of Surgery, Department of Clinical Sciences, Skåne University Hospital, Lund University, Malmö, Sweden

We reported that high estrogen receptor beta (ERβ) expression is independently associated with better prognosis in female colorectal cancer (CRC) patients. However, estrogen receptor alpha (ERα) is expressed at very low levels in normal colon mucosa, and its prognostic role in CRC has not been explored. Herein, we investigated the combined role of ERα and ERβ expression in the prognosis of female patients with CRC, which, to the best of our knowledge, is the first study to investigate this topic. A total number of 306 primary CRCs were immunostained for ERα and ERβ expression. A Cox regression model was used to evaluate overall survival (OS) and disease-free survival (DFS). The combined expression of high ERβ + negative ERα correlates with longer OS (HR = 0.23; 95% CI: 0.11–0.45, P < 0.0001) and DFS (HR = 0.10; 95% CI: 0.03–0.26, P < 0.0001) and a more favorable tumor outcome, as well as significantly higher expression of antitumorigenic proteins than combined expression of low ERβ + positive ERα. Importantly, we found that low ERβ expression was associated with local recurrence of CRC, whereas ERα expression was correlated with liver metastasis. Overall, our results show that the combined high ERβ + negative ERα expression correlated with a better prognosis for CRC patients. Our results suggest that the combined expression of ERα and ERβ could be used as a predictive combination marker for CRC patients, especially for predicting DFS.

Keywords: estrogen receptor beta, estrogen receptor alpha, colorectal cancer, CRC disease-free survival, CRC overall survival

INTRODUCTION

The physiological effects of estrogens are mediated by two main receptors, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), which belong to the nuclear receptor family and are encoded by two different genes, ESR1 (ERα) and ESR2 (ERβ) (1, 2). These receptors are implicated in different types of cancer, including colorectal cancer (CRC) (1–3).

ERβ is the predominant ER in normal colon mucosa, and its expression is reduced during tumor progression (4). Previous research has reported association of ERβ expression with CRC survival (5, 6). We recently reported that high nuclear ERβ expression is independently associated with
better prognosis in female CRC patients and associated with hormonal status but not with lifestyle indicators (7). Furthermore, we investigated the antitumor effects of ERβ induction in colon cancer cells and in an in vivo zebrafish xenograft model (8). On the other hand, ERα is expressed at very low levels in normal colon mucosa (1, 2), and few studies have reported its prognostic role in CRC survival (9–11). Evidence shows that the manipulation of estrogen signaling to inhibit ERα and stimulate ERβ may have preventive and therapeutic effects for obesity-associated colon cancer (12, 13). However, the relationships among estrogen hormones, reproductive factors, and CRC remain unclear and await further investigation (14).

Many mutations and proteins have been implicated in CRC progression. KRAS mutation status is reported to be an important prognostic and treatment marker in CRC, and screening for KRAS mutations is now mandatory for metastatic colon cancer before treatment with therapies that target the EGFR pathway (15–17). Furthermore, the activation of the Wnt/β-catenin pathway plays a crucial role in CRC development and progression (18). In addition, high cyclooxygenase-2 (COX-2) expression in CRC correlates with poor prognosis via the effect of prostaglandin E$_2$ (PGE$_2$) (19). 15-Hydroxyprostaglandin dehydrogenase (15-PGDH) is the key enzyme in PGE$_2$ catabolism and is often downregulated in CRC, while its upregulation has been shown to lead to a better prognosis in CRC (20–22). The G protein-coupled receptors cysteinyl leukotriene receptors 1 and 2 (CysLT$_1$R and CysLT$_2$R), the receptor for LTD$_4$ respectively LTC$_4$ are implicated in the prognosis of CRC (23). Patients with low CysLT$_1$R and high CysLT$_2$R expression levels have better survival than those with high CysLT$_1$R and low CysLT$_2$R expression levels (23).

In this study, we aimed to investigate the prognostic significance of the combined expression of ERα and ERβ in female CRC patients and to explore their correlations with other tumor promoter or suppressor proteins and hormonal status.

**MATERIALS AND METHODS**

**Study Populations**
The study included a cohort of female patients who were diagnosed with CRC and operated between January 1, 2008, and June 30, 2012. This investigation included 269 patients with available data on clinical information, tumor characteristics, hormonal status as well as ER, ER, KRAS, CysLT$_1$R, CysLT$_2$R, COX-2, 15-PGDH, β-catenin, Mucin-2 and PGD2 synthase expression in CRC tissue. The study population is briefly described in the Supplementary Materials. Details about the study design, patient follow-up and data collection are provided elsewhere (7).

**Immunohistochemistry (IHC)**
Tumor samples were retrieved and incorporated into tissue microarray (TMA) blocks based on the protocol described earlier (7). The tissues were stained with specific antibodies for the expression of ERα, ERβ and other proteins of interest (Supplementary Material). Two independent investigators (GT and RE), blinded to the patient and tumor characteristics, evaluated the staining immunoreactivity using the immunoreactive score (IRS) with a range 0–9, which was calculated as a multiplication of staining intensity (0 = negative, 1 = weak, 2 = moderate and 3 = strong) with percentage of positive stained cells (1 = <10%, 2 = 11–50% and 3 = >50%) (7). The staining intensity was determined based on the criteria of Konstantinopoulos et al. (4), which are described in the Supplementary Materials. For ERα and ERβ expression, only the nuclear staining intensity was taken into consideration, based on which they were also scored as categorical variables, respectively low/high and negative/positive expression (Figure 2A). Briefly, negative and weak ERβ staining were grouped as low expression and moderate and strong ERβ staining as high expression (7). Because ERα is very little expressed in the normal colonic mucosa (1, 2), we defined its expression as positive if more than 10% of the nuclei were stained, regardless the staining intensity. All the other tumor samples that had <10% of the nuclei stained, regardless the staining intensity, were considered to have negative ERα expression. Each tumor sample was in duplicate. Cores with loss of tissue or with only stromal tissue were excluded from the analysis.

**Acquisition of Gene Expression and Clinical Data From the Cancer Genome Atlas (TCGA) Dataset**
Normalized RNA sequencing data in transcripts per million (TPM), reverse phase protein array (RPPA) data, and the associated clinical information of the colon adenocarcinoma (COAD) samples were downloaded from the TCGA dataset (https://portal.gdc.cancer.gov/; https://tcga火portal.tcgaportal.org/ctca; ≤June 20, 2020). Out of 361 patients, 12 patients missing pathological information, 16 patients with a follow-up period of ≤30 days, and 52 patients with metastasis (stage IV) were eliminated. Thus, 282 patients with clinical information were included in the study. Normalized gene expression and protein expression data from the TCGA-COAD dataset were log2-transformed for further analysis.

**Identification of Independent Prognostic Parameters of Colon Cancer**
To identify independent prognostic parameters and to validate the independent prognostic value of ERα and ERβ, univariate and multivariate Cox regression analyses were performed in the TCGA-COAD dataset on the ERα and ERβ gene and protein signature and clinicopathological parameters. Parameters with $P < 0.05$ in the univariate analysis were further included in the multivariate Cox regression analysis. The TCGA samples were divided into high- and low-risk groups according to the optimal cutoffs determined by the Youden Index association criteria and analyzed using Circos visualization package (24).

**Abbreviations**: ERβ, estrogen receptor beta; ERα, estrogen receptor alpha; CC, colon cancer; CRC, colorectal cancer; DFS, disease-free survival; OS, overall survival.
Statistical Analysis
The variables were compared between the group of interest using Pearson’s \( \chi^2 \) test or Fisher’s exact test for categorical variables and the Mann-Whitney U test or t-test for continuous variables. Survival curves, generated via the Kaplan-Meier method, were compared between the groups using the log-rank test. Univariate and multivariate Cox proportional hazards regression models were applied, and hazard ratios (HRs) together with 95% confidence intervals (CIs) were calculated to determine the risk of death or cancer recurrence. Receiver operating characteristic (ROC) curves were used to calculate the area under the curve (AUC) to determine the predictive ability of the final model with combined ER\( \beta \) + ER\( \alpha \) expression compared to models with only one ER expression or the basic model. Binary logistic regression model was used to determine the odds ratios (ORs) of having a metastatic event for each unit increase in ER\( \alpha \) and ER\( \beta \) intensity. The estimates with their corresponding 95% CIs were used to build forest plots by the ggplot2 package in R. Statistical analyses were performed using SPSS version 23.0 (SPSS, IBM, Armonk, NY, USA) and GraphPad Prism version 8.0a (GraphPad Software, Inc., San Diego, CA, USA). A two-sided \( P < 0.05 \) was considered statistically significant.

RESULTS
Evaluation of ER\( \alpha \) and ER\( \beta \) Expression in Female CRC Patients
We had 306 primary CRC samples available for the evaluation of ER\( \alpha \) and ER\( \beta \) expression. Fourteen patients, who were previously operated and treated for breast cancer, were excluded from the study due to the risk of ER\( \alpha \) alterations from the anti-estrogen therapies (Figure 1). We successfully evaluated ER\( \beta \) in 300 CRC patients and ER\( \alpha \) in 270 CRC patients. Based on the staining intensity assessed with IHC, ER\( \beta \) expression was categorized as low and high, while ER\( \alpha \) expression was categorized as negative and positive (Figure 2A). We next compared the expression of these receptors between normal and matched cancer tissues and found that compared to ER\( \alpha \) expression levels, ER\( \beta \) expression levels were higher in both normal and cancer tissues (Figure 2B). However, compared to normal tissues, a downregulation of ER\( \beta \) and an upregulation of ER\( \alpha \) were observed in the matched CRC tissues (Figure 2B, see violin bar graph). Since we previously reported that high ER\( \beta \) expression correlated with better prognosis in CRC (7), we investigated the distribution of ER\( \alpha \) expression in patients with low and high ER\( \beta \) expression. We grouped the patients into four categories based on ER\( \alpha \) and ER\( \beta \) expression (Figure 2C). We found that 79% of patients with high ER\( \beta \) expression had also negative ER\( \alpha \) expression compared with 63% in the low ER\( \beta \) group (Figure 2D). Likewise, the percentage of patients with positive ER\( \alpha \) expression was higher in the low ER\( \beta \) expression group (37%) than in the high ER\( \beta \) expression group (21%) (Figure 2D). For representative IHC images of matched pairs of patients for both ER\( \alpha \) and ER\( \beta \) expression, see Supplementary Figure 1A.

Next, we used ESR1 (ER\( \alpha \)) and ESR2 (ER\( \beta \)) mRNA levels from the TCGA-COAD database to investigate the differential expression of ER\( \alpha \) and ER\( \beta \) in CRC patients with TNM stage I disease and TNM stage IIIc+IV disease. Compared to those with stage I disease, a smaller percentage of patients with stage IIIc+IV disease had upregulated ESR2 mRNA levels (Figure 2E). Additionally, ESR2 levels were lower in patients with stage IIIc+IV disease than in those with stage I disease (Figure 2E). Furthermore, ESR1 mRNA levels were obviously higher in patients with stage IIIc+IV disease than in those with stage I disease (Figure 2E).

The Specificity of the ER\( \alpha \) Antibody
Because the role of ER\( \alpha \) expression in CRC is very little studied and all our results are based on antibody staining, we tested the specificity of the antibodies we used, in order to validate the antibodies. First, we stained the normal breast tissue, which is known to abundantly express ER\( \alpha \) (positive control), and normal kidney, prostate, and skin tissues, which are known to lack ER\( \alpha \) expression (negative controls, Supplementary Figure 1B) (25–27). Next, the same tissues were also stained with another anti-ER\( \alpha \) antibody, D12 (Supplementary Figure 1C), which is widely used for the detection of ER\( \alpha \) expression (28–30). We randomly stained 59 patients from the Female cohort with the D12 antibody. As shown in Supplementary Figure 1D the distribution of the IRS for nuclear ER\( \alpha \) expression for each patient (\( n = 59 \)) was the same for both antibodies. Likewise, when the patients were grouped as positive and negative nuclear ER\( \alpha \) expression, no significant difference was observed between the two antibodies (\( P = 0.11 \), Supplementary Figure 1E). Out of 59 patients randomly stained with D12 antibody, 13 patients (22%) were positive for ER\( \alpha \) expression, while 19 patients (32%) were detected as positive using the cocktail antibody (Supplementary Figure 1E). This could be explained by the fact that the cocktail antibody 1D5 + 6F11 was created by mixing two monoclonal antibodies that detect two different epitopes (31, 32). Representative IHC images of matched-pair CRC tissues for both antibodies are shown in the Supplementary Figure 1F.

Correlation of ER\( \alpha \) and ER\( \beta \) Expression With KRAS Mutation Status
Out of 252 patients with successful staining for the KRAS mutation, only 31 (12.3%) had positive staining (Figure 2F). Patients with a KRAS mutation had a significantly higher intensity of ER\( \alpha \) expression (\( P < 0.05 \)) and a tendency to have lower ER\( \beta \) expression (\( P = 0.06 \)) than patients with wild-type (WT) KRAS (Figure 2F). Additionally, we observed that 19% of patients with positive ER\( \alpha \) expression had KRAS mutations, while 9% of patients with negative ER\( \alpha \) expression had KRAS mutations (Figure 2G). An opposite tendency was observed when looking at the distribution of KRAS mutations in patients with low and high ER\( \beta \) expression. While 15% of patients with low ER\( \beta \) expression had KRAS mutations, only 7% of patients with high ER\( \beta \) expression had KRAS mutations (Figure 1G). However, no statistical significance was reached. To further validate these findings, we used mRNA data from the TCGA-COAD public database and found a strong and significant positive correlation between the mRNA levels of ESR1 (ER\( \alpha \)) and the Mann-Whitney U test or t-test for continuous variables. Survival curves, generated via the Kaplan-Meier method, were compared between the groups using the log-rank test. Univariate and multivariate Cox proportional hazards regression models were applied, and hazard ratios (HRs) together with 95% confidence intervals (CIs) were calculated to determine the risk of death or cancer recurrence. Receiver operating characteristic (ROC) curves were used to calculate the area under the curve (AUC) to determine the predictive ability of the final model with combined ER\( \beta \) + ER\( \alpha \) expression compared to models with only one ER expression or the basic model. Binary logistic regression model was used to determine the odds ratios (ORs) of having a metastatic event for each unit increase in ER\( \alpha \) and ER\( \beta \) intensity. The estimates with their corresponding 95% CIs were used to build forest plots by the ggplot2 package in R. Statistical analyses were performed using SPSS version 23.0 (SPSS, IBM, Armonk, NY, USA) and GraphPad Prism version 8.0a (GraphPad Software, Inc., San Diego, CA, USA). A two-sided \( P < 0.05 \) was considered statistically significant.
and KRAS mutations, while no correlation was found with ESR2 mRNA levels (ERβ) (Figure 2H).

### Evaluation of the Prognostic Relevance of ERα and ERβ Expression in CRC Patients
Previously we reported that high nuclear ERβ expression is independently associated with better OS and DFS in female CRC patients (7). Herein, we report that CRC patients with negative nuclear ERα expression have 19% lower risk for 5-years overall mortality (HR = 0.81; 95% CI, 0.68-0.94; P = 0.042, Figure 3A). Likewise, in the TCGA-COAD cohort, low ERα protein expression (HR = 0.73; 95% CI, 0.62-0.92; P = 0.035, Figure 3B) and high ERβ protein expression (HR = 0.78; 95% CI, 0.68-0.89; P = 0.001, Figure 3C) are associated with better prognosis of CRC patients. Additionally, we investigated the predicting ability of ERα and ERβ expression in our female patient’s cohort calculating the ROC curves. We found that

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**FIGURE 1 | Consort diagram of colorectal cancer patients involved in the study.**

- **320 primary CRC samples available on TMAs**
- **14 patients operated and treated previously for breast cancer**
- **306 CRC samples available for ERβ and ERα staining**
- **6 (1.9%) patients with unreadable tissue**
- **300 CRC samples with successful evaluation of ERβ**
- **36 (11%) patients with unreadable tissue**
- **270 CRC samples with successful evaluation of ERα**
- **269 patients with combination of ERβ and ERα expression:**
  - High ERβ+Negative ERα = 81
  - Low ERβ+Negative ERα = 105
  - High ERβ+Positive ERα = 21
  - Low ERβ+Positive ERα = 62
- **143 patients for correlation analysis:**
  - High ERβ +Negative ERα = 81
  - Low ERβ+Positive ERα = 62
- **Follow up until 31 August 2016**
- **269 patients for OS analysis**
  - **15 patients with TNM stage IV**
  - **22 patients with unknown state or date of recurrence**
  - **232 patients for DFS analysis**
FIGURE 2 | Expression levels of ERα and ERβ in CRC tissue. (A) Representative IHC images showing the nuclear expression of ERα and ERβ in CRC tissue. (B) Representative IHC images of ERα and ERβ expression in normal and matched cancer tissues, and violin plots showing the distribution of IRSs for ERα and ERβ expression in normal and matched cancer tissues. (C) IHC images of CRC tissue in four subgroups of patients with combined ERα and ERβ expression levels. (D) The percentage of CRC patients with negative and positive ERα expression according to low and high ERβ expression. (E) Waterfall plots of the mRNA expression levels of ESR1 (ERα) and ESR2 (ERβ) in the subgroups of CRC patients with TNM stage I (n = 49) and TNM stage IIc + IV (n = 58) from the TCGA-COAD public database. (F) Intensity of ERα and ERβ expression in patients with wild-type (WT) and KRAS mutations, together with representative IHC images for KRAS status. The arrows (Continued)
ERα expression predicts the 5-years OS with higher specificity (AUC = 0.720, Sensitivity = 65.22 and Specificity = 79.37, Figure 3D), while ERβ expression with higher sensitivity (AUC = 0.674, Sensitivity = 71.05 and Specificity = 49.42, Figure 3E). When we combined the ERα and ERβ expression, the predicting ability for 5-years OS in CRC patients was significantly improved with higher sensitivity and higher specificity (AUC = 0.842, Sensitivity = 71.53 and Specificity = 82.90, Figure 3F). Next, we looked at the risk score profile with TNM-stage and 5-years OS event by combining the ERα and ERβ expression in four groups as described above (Figure 2C). As shown in Figure 3G, the subgroups with positive ERα expression had the highest risk score profile, while the patients with negative ERα expression had the lowest risk score profile, despite the ERβ expression levels.

**Association of Combined ERα and ERβ Expression With OS and DFS in CRC Patients**

Next, we investigated the combined role of ERα and ERβ expression in CRC OS and DFS (Figure 4). The Cox regression analysis showed that patients with combined high ERβ + negative ERα expression were independently associated with better OS and had a 77% reduction in overall mortality (Figures 4A,B, Supplementary Table 1), as well as better DFS with a 90% reduction in cancer recurrence (Figures 4C,D, Supplementary Table 1) after adjustment for age, TNM stage and tumor vascular invasion, compared to patients with combined low ERβ + positive ERα expression, which were taken as the reference group. This finding was consistent even for the subgroups of patients with stage I-III cancer (Figure 4E), patients with colon cancer (Supplementary Figures 2A,B) and patients who did not receive adjuvant treatment (Figure 4F and Supplementary Figure 2C). In the second group of patients with low ERβ expression, even though the expression of ERα remained negative, the risk was increased by 14% for overall mortality and 33% for cancer recurrence compared to patients with combined high ERβ negative ERα expression (Supplementary Table 1). In addition, in the third group of patients with positive ERα expression, even though the expression of ERβ was high, the increase in the risks of overall mortality and cancer recurrence was much lower than that in the first group with combined high ERβ + negative ERα expression (3 and 22% lower, respectively: Supplementary Table 1, multivariate analysis). It is difficult to draw any conclusions about the subgroup of patients with rectal cancer due to the very small number of patients in each category, especially the category with combined high ERβ + positive ERα expression that has only one patient, n = 1 (Supplementary Table 1, Supplementary Figures 2D,E). These results clearly show that CRC patients with combined high ERβ + negative ERα expression have the best prognosis and that the subgroup with combined low ERβ + positive ERα expression has the worst prognosis.

**Predictive Ability of Combined ERα and ERβ Expression**

To further investigate the role of the combined ERα and ERβ expressions in predicting CRC prognosis, we evaluated the ROC curves for the basic model (adjusted for age, TNM stage and tumor vascular invasion), the model extended with only ERβ expression, the model extended with only ERα expression, and the model that included the combined ERβ + ERα expressions. As shown in Figures 4G,H, the AUC was significantly higher for the model with the combined ERβ + ERα expressions than for all the other models for both OS and DFS. However, the predictive ability of the combined ERβ + ERα extended model was higher for DFS (AUC = 0.812, Figure 4H) than for OS (AUC = 0.801, Figure 4G). The same results were obtained using the TCGA-COAD external cohort, where the combined expression of ERs had the best predictive ability for DFS compared with the other models (Figures 4I,J). These results clearly show that the combined expression of ERα and ERβ plays an important role in predicting the prognosis of CRC patients.

**Distribution of Clinical Parameters and Tumor Characteristics in Patients With Combined High ERβ + Negative ERα Expression VS. Patients With Combined Low ERβ + Positive ERα Expression**

We aimed to evaluate the distribution of clinical parameters and tumor characteristics between patients with combined high ERβ + negative ERα expression, considered to be the best prognostic group, and those with combined low ERβ + positive ERα expression, considered to be the worst prognostic group. As shown in Table 1, patients with combined high ERβ + negative ERα expression had a significantly lower number of overall deaths and cancer recurrence events, smaller tumor extent, fewer tumor metastases in the regional lymph nodes and distant organs, predominantly stage I and II disease, and were less likely to receive adjuvant treatment after the operation. Additionally, tumors with combined high ERβ + negative ERα expression had a higher frequency of the mucinous type of COAD and a never smoking status (Table 1).

**Correlation of Combined ERα and ERβ Expression With Hormonal Characteristics in Female Patients With CRC**

We explored the hormonal characteristics of CRC female patients in relation to the combined ERα and ERβ expression. We found that female patients with combined high ERβ +
negative ERα expression had a lower number of pregnancies (mean ± standard error of the mean, 1.8 ± 0.13, \( P = 0.04 \); Figure 5A) and shorter breastfeeding times (calculated as the total breastfeeding months for all the children a woman had; 8.2 ± 0.95, \( P = 0.08 \); Figure 5B) than female patients with combined low ERβ + positive ERα expression (2.2 ± 0.14 and 10.8 ± 1.2, respectively). No significant differences were observed between the two groups regarding the age of menopause and frontiers in medicine | www.frontiersin.org 7 March 2022 | Volume 9 | Article 739620
FIGURE 4 | Association of concomitant ERβ and ERα expression with CRC patient survival. Kaplan-Meier survival curves for OS: (A) univariate model, n = 269; (B) multivariate model adjusted for age, TNM stage and tumor vascular invasion, n = 214; (C) multivariate model for patients with stage I-III cancer, n = 180.

(Continued)
The association with Mucin-2 expression known to be reduced in CRC tissues compared to the normal mucosa (35, 36). We found that patients with combined high ERβ + negative ERα expression had significantly higher IRSs for Mucin-2 expression (35, 36). We found that patients with combined high ERβ + negative ERα expression had significantly higher IRSs for Mucin-2 expression (35, 36).

**Association of ERα and ERβ Expression With Metastasis in Patients With CRC**

We investigated the risk of having a metastatic event for each unit increase in the ERβ and ERα staining intensity, evaluated by IHC. We found that for each unit increase in the ERβ intensity, the risk of having a metastatic event were significantly and independently decreased by 60% after adjustment for age, TNM stage and tumor vascular invasion (OR = 0.40; 95% CI: 0.19–0.82; P = 0.012; Figure 7A). In addition, for each unit increase in the ERα intensity, the risk of having a metastatic event increased almost 2.5-fold (OR = 2.47; 95% CI: 1.15–5.32; P = 0.021; Figures 7A,B). The ERα intensity was strongly associated with liver metastasis, where for each unit increase in the ERα intensity, the risk of liver metastasis independently increased almost 4-fold (OR = 3.48; 95% CI: 1.38–8.77; P = 0.008) disappeared after adjustment for other confounding factors (OR = 3.05; 95% CI: 0.99–9.42; P = 0.052; Figure 7A). Importantly, each unit increase in the ERβ intensity significantly and independently decreased the risk of local recurrence and abdominal metastasis by 79% (OR = 0.21; 95% CI: 0.06–0.67; P = 0.009; Figures 7A,B). These results were summarized graphically using the forest plots, where the increased risk is shown in red, and the decreased risk is shown in blue (Figure 7B).

**DISCUSSION**

CRC is one of the most common malignancies worldwide. Despite the current technologies for early detection and targeted therapies, the risk of recurrence in patients with stage II and III cancer remains high (37). Prognostic markers are needed to predict the recurrence risk with higher precision. Herein, we demonstrate the prognostic significance of the combined high ERβ + negative ERα expression in CRC patients.
TABLE 1 | Distribution of clinical parameters and tumor characteristics in 143 CRC patients according to subgroups with combined high ERα-negative ERα and combined low ERβ-positive ERα expressions.

| Characteristics               | Total          | High ERβ Negative ERα | Low ERβ Positive ERα | P    |
|-------------------------------|----------------|-----------------------|----------------------|------|
| Patients no.                  | 143 (100)      | 81 (56)               | 62 (44)              |      |
| Deaths                        | 48 (34)        | 14 (29)               | 34 (71)              | <0.0001* |
| DFS events*                   | 24 (19)        | 4 (17)                | 20 (83)              | <0.0001* |
| Age (mean, years)             | 70.9           | 71.8                  | 69.8                 | 0.198 |
| BMI (mean, kg/m²)             | 26.1           | 25.9                  | 26.2                 | 0.931b |
| Tumor extent T ≤ T2           | 41 (29)        | 30 (73)               | 11 (27)              | 0.011* |
| Tumor extent T > T2           | 102            | 51 (50)               | 51 (50)              |      |
| Lymph node metastasis N0      | 90 (63)        | 60 (67)               | 30 (33)              | 0.002a |
| Lymph node metastasis N1/N2   | 53 (37)        | 21 (40)               | 32 (60)              |      |
| Distant metastasis diagnosis | 128 (89)       | 80 (63)               | 48 (37)              | <0.0001* |
| TNM stage                     |                |                       |                      |      |
| I                             | 30 (21)        | 21 (70)               | 9 (30)               | <0.0001* |
| II                            | 55 (39)        | 38 (69)               | 17 (31)              |      |
| III                           | 42 (29)        | 20 (48)               | 22 (52)              |      |
| IV                            | 15 (11)        | 1 (7)                 | 14 (93)              |      |
| TNM stage Missing             |                |                       |                      |      |
| Tumor intravascular invasion  |                |                       |                      |      |
| No                            | 83 (72)        | 53 (64)               | 30 (36)              | 0.174a |
| Yes                           | 32 (28)        | 16 (50)               | 16 (50)              |      |
| Missing                       | 28             |                       |                      |      |
| Tumor differentiation         |                |                       |                      |      |
| Low                           | 21 (15)        | 14 (67)               | 7 (33)               | 0.354a |
| Moderate/High                 | 120            | 67 (56)               | 53 (44)              |      |
| Missing                       | (85)           | 2                     |                      |      |
| Tumor localization            |                |                       |                      |      |
| Colon                         | 106 (74)       | 58 (55)               | 48 (45)              | 0.431a |
| Rectum                        | 37 (26)        | 25 (62)               | 14 (38)              |      |
| Tumor histological type       |                |                       |                      |      |
| Non-mucinous AC*              | 110 (77)       | 57 (52)               | 53 (48)              | 0.079a |
| Partly Mucinous AC            | 22 (15)        | 15 (68)               | 7 (32)               |      |
| Mucinous AC                   | 11 (8)         | 9 (82)                | (18)                 |      |
| Neoadjuvant treatment         |                |                       |                      |      |
| No                            | 124 (87)       | 70 (57)               | 54 (43)              | 0.906a |
| Yes                           | 19 (13)        | 11 (58)               | 8 (42)               |      |
| Adjuvant treatment            |                |                       |                      |      |
| No                            | 99 (71)        | 63 (64)               | 36 (36)              | 0.016a |
| Yes                           | 41 (29)        | 17 (42)               | 24 (58)              |      |
| Missing                       | 3              |                       |                      |      |
| Smoking status                |                |                       |                      |      |
| Ever smokers                  | 5 (11)         | 1 (20)                | 4 (80)               | 0.059a |
| Never smokers                 | 39 (89)        | 25 (64)               | 14 (36)              |      |
| Missing                       | 99             |                       |                      |      |
| Alcohol use                   |                |                       |                      |      |
| Yes                           | 19 (43)        | 9 (47)                | 10 (53)              | 0.168a |
| No                            | 25 (57)        | 17 (68)               | 8 (32)               |      |
| Missing                       | 99             |                       |                      |      |

*Patients with TNM stage IV are excluded. *aPearson chi-square test. *bMann-Whitney U test.
†AC, Adenocarcinoma; BMI, Body Mass Index.
FIGURE 5 | Correlation of hormonal status with subgroups of female CRC patients with both ERβ and ERα expression. Hormonal characteristics for (A) number of full-term pregnancies, where 0 refers to women who never had children; (B) total breastfeeding time for all the children a woman had, where 0 refers to women who never breastfed; (C) age at menopause; and (D) age at menarche. Percentage of female CRC patients with combined high ERβ + negative ERα expression or combined low ERβ + positive ERα expression who never or ever used (E) hormonal contraception (HC); (F) combined (estrogen and progesterone) HC; (G) progesterone HC; (H) hormonal replacement therapy (HRT); (I) combined (estrogen and progesterone) HRT; or (J) estrogen HRT. The data are presented as the mean ± SEM (A–D). *P < 0.05, unpaired t-test; χ² test or Fisher’s exact test as indicated.
FIGURE 6 | Correlation of subgroups of patients with ERβ and ERα expression with proteins important for CRC progression and development. (A) Mean IRS for CysLT1R, COX-2, membrane and nuclear β-catenin, CysLT2R, 15-PGDH, Mucin-2, and PGD2 synthase expression levels evaluated with IHC in subgroups of CRC. (Continued)
ERα and ERβ expression in female patients with CRC and explore their correlations with other prognostic markers and hormonal status.

We found that in cancer tissues, ERβ expression was downregulated while ERα expression upregulated, compared to the normal matched pair tissues (Figure 2B). We previously reported that high ERβ expression is associated with better OS and DFS (7), and in this investigation we showed that most of the patients with high ERβ expression were negative for ERα expression, while the majority of patients with low ERβ expression were positive for ERα expression. Many have reported the downregulation of ERβ during tumor progression (2–4, 7), while others have shown that ERs protein levels significantly increase in men but not in women with CRC (38). Herein, we showed that ERα expression levels are increased in cancer tissues compared to matched normal tissues in females with CRC. A previous report detected ERα and ERβ protein levels in CRC and they found no significant difference of ERβ expression levels between normal and cancer colon tissues (39). Another report showed that ERα expression is rare in CRC tissue and its expression does not correlate with colon carcinogenesis, while ERβ expression was upregulated in CRC tissues and correlated with poor DFS (40). It is worth noting that both studies had a small number of patients and included in their studies even colon adenomas (41). Moreover, both studies used polyclonal antibodies and the antibody used from Grivas et al., recognizes only the β1 isofrom (40).

Furthermore, we investigated the correlation of ERα and ERβ expression with KRAS mutation, which plays an important role in the prognosis and treatment of CRC (15). In 4,411 CRC patients, KRAS mutations were independently associated with shorter relapse times, survival after recurrence and OS in patients with MSS but not MSI tumors (16). Additionally, treatment with anti-EGFR is ineffective in CRC patients with KRAS mutations (17). Interestingly, we found that patients with positive ERα expression, which were associated with shorter OS (Figures 3A, B), had a higher frequency of KRAS mutations than patients with negative ERα expression. This result was further supported by mRNA data from the TCGA-COAD cohort, where we found a significant positive correlation between the mRNA levels of ESR1 (ERα) and KRAS mutations. This finding can provide new opportunities for patients with KRAS mutations, where ERα-selective antagonists might be an alternative to improve their prognosis. No correlations were observed between KRAS status and ERβ expression at either expression level detected by IHC or mRNA levels from the TCGA-COAD cohort.

Next, we evaluated the prognostic role of the combined ERα and ERβ expression in CRC patient survival. Patients with combined high ERβ - negative ERα expression had the best OS and DFS, with a reduction in overall mortality by 77% and cancer recurrence by 90%. Patients with combined low ERβ - positive ERα expression, taken as the reference category, had the worst OS and DFS. The model with the combined expression of ERs had the highest predicting ability compared to all the other models taken into consideration. Moreover, we found that each unit increase in the ERα intensity independently increased the risk of liver metastasis almost 4-fold, while each unit increase in the ERβ intensity reduced the risk of local recurrence and abdominal metastasis by 79%. These results imply an important role of the combined ERα and ERβ expression as a future prognostic marker in patients with CRC. Reports show that CysLT2R, COX-2 and nuclear β-catenin expression levels are linked to CRC development and prognosis (42). High levels of 15-PGDH and PGD2 synthase in CRC are reported to have antitumor properties (20–22, 33, 34). Wc found that patients with combined high ERβ - negative ERα expression had significantly lower IRSs of tumor-promoting proteins, such as CysLT2R, COX-2 and nuclear β-catenin, and higher IRSs of anti-tumorogenic proteins such as CysLT2R, membrane β-catenin, 15-PGDH and PGD2 synthase, compared to patients with combined low ERβ - positive ERα expression. To validate our findings, we used protein data from the TCGA-COAD cohort and found that compared to patients with combined low ERβ - high ERα expression, patients with combined high ERβ - low ERα expression had a better tumor profile and a more favorable prognosis (Figure 7C).

Interestingly, we found that patients with combined high ERβ - negative ERα expression had significantly smaller tumors, fewer regional and distant metastases, predominantly TNM stage I and II and were less likely to receive adjuvant treatment. In addition, patients with combined high ERβ - negative ERα expression were more likely to have a never smoking status, which is an established risk factor for CRC (43), and a higher frequency of mucinous adenocarcinoma, which also correlated with higher IRS for Mucin-2 expression. High Mucin-2 levels are linked to colon cell differentiation (36, 44). Previous studies have shown that ERs are implicated in the obesity-associated CRC (12, 13), however we found no correlation between BMI and the combined ERα and β expression.

We previously found that high ERβ expression in female CRC patients was associated with a lower number of pregnancies, shorter breastfeeding times, a longer time of combined HC use, and a longer time of HRT use (7). Many studies have suggested a lower risk of CRC incidence among women who use HRT (45). However, none of them took into consideration the combined expression of ERα and ERβ in CRC tissue. Herein, we showed that in female CRC patients, combined high ERβ - negative ERα expression correlated with lower pregnancy number, shorter breastfeeding times, non-use of HC and long-term use of HRT, both estrogen monotherapy and combined HRT.
FIGURE 7 | Correlation of ERβ and ERα expression with CRC metastasis. (A) Binary logistic regression model showing the odds ratios (ORs) and 95% confidence intervals (CIs) for total metastatic events; liver metastasis; lung metastasis; other metastases; ocal recurrences; abdominal metastasis and bone metastasis. (B) Forest plots showing the respective estimates for the corresponding metastatic events for the patients included in the study. (C) Distributions of each clinical factor and associated protein expression pattern in the combined high ERβ + low ERα or combined low ERβ + high ERα expression groups in the TCGA-COAD cohort. The data were visualized via Circos software. The area of each colored ribbon depicts the frequency of the samples. *P < 0.05, **P < 0.01.
An important issue to address is the antibody used in IHC. The use of TMAs in cancer research raises the concern whether the chosen core tissue is representative of the whole tumor. However, the use of two cores to represent the tumor has shown sufficient concordance for many cancer types, including CRC (46). The clone 14C8 of the anti-ERβ antibody that we used, recognizes most of ERβ variants including ERβ wild-type, and is shown to be useful for the assessment of ERβ expression in paraffin-embedded tissues (47). In a recent publication for the validation of ERβ antibodies in 44 different tissues, 14C8 antibody showed in CRC IHC the same intensity band as PPZ0506, which was reported to be the most specific anti-ERβ antibody, and that correlated with ERβ mRNA levels detected in the CRC tissue [Figure 3, see reference (48)]. Because ERα is low expressed in the colon tissue, we used a cocktail antibody (1D5 + 6F11) created by mixing two monoclonal antibodies that target ERα. Human normal tissues verified for ERα expression levels were used as positive and negative controls to test the antibody specificity (25–27). To validate the IHC staining, 59 randomly selected patients from the cohort were stained with another ERα monoclonal antibody D12, widely used for the detection of ERα (28–30). The same control tissues that were stained positive for ERα expression using the cocktail antibody, were also stained positive with D12 antibody but the staining intensity was weaker. This was the reason that we identified more patients with positive ERα expression using the cocktail antibody, which might be missed using the monoclonal D12 antibody (32). It is important to highlight that we validated our findings by using protein expression data from the TCGA-COAD cohort, which was used as an external cohort and includes both female and male patients.

To the best of our knowledge, this is the first study to investigate the prognostic significance of combined ERα and ERβ expressions in CRC patients. Our results suggest that patients with combined high ERβ + negative ERα expression have a better outcome with longer OS and DFS. Interestingly, ERβ intensity was important for the local recurrence of CRC, while the ERα intensity was important for the liver metastasis. ERβ expression levels are found significantly decreased in CC tissues of both males and females compared to the matched normal mucosa, and ERα/ERβ protein ratio are altered in both male and female CRC tissues (38). Therefore, we believe that our results are applicable to both female and male CRC patients. In summary, our results highlight the role of combined expression of ERα and ERβ as important prognostic and treatment markers in CRC patients.

**DATA AVAILABILITY STATEMENT**

The datasets used and analyzed in the current study are available from the corresponding author upon request.

**ETHICS STATEMENT**

The studies involving human participants were reviewed and approved by Lund University Ethical Committee Approval 3/2006. The patients/participants provided their written informed consent to participate in this study.

**AUTHOR CONTRIBUTIONS**

GT and AS: conception and design. GT, SG, and M-LL: development of methodology. GT, SG, RE, and AS: analysis and interpretation of data. RE and ML-L: administrative and/or material support. GT, AS, SG, and SS: writing and review of the manuscript. All authors have read, reviewed, and approved the final version of the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.739620/full#supplementary-material

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