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Original Article

Prognostic significance of full-length estrogen receptor beta expression in stage I-III triple negative breast cancer

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Abstract: Triple negative breast cancer (TNBC) is an aggressive breast cancer subtype for which there is a need to identify new therapeutic targets. Full-length estrogen receptor beta (ERβ1) may be a possible target given its antiproliferative effects on breast cancer cells. The prognostic significance of ERβ in breast cancer subtypes has remained elusive, and disparate results observed across previously published reports might be due to the detection of multiple ERβ isoforms, the lack of specific antibodies and the use of different cutoffs to define ERβpositivity. The objective of this retrospective study was to determine the association between ERβ1 expression and disease-free and overall survival, as well as Ki67 expression, in non-metastatic TNBC. Immunohistochemical protocols were optimized using xenograft tissues obtained from a breast cancer cell line with inducible ERβ1 expression. ERβ1 localization and expression were assessed in two cohorts of TNBC using the VECTRA™ platform. There was a close relationship between nuclear and cytoplasmic ERβ1 expression. ERβ1 was expressed in a subset of TNBCs, but its expression was significantly associated with Ki67 in only one of the cohorts. There was no significant association between ERβ1 expression and disease-free and overall survival in either cohort. Although these results suggest that ERβ1 expression alone may not be informative in TNBCs, this study provides a new strategy for optimizing and objectively measuring ERβ1 expression in tissues, which may provide a standard for ERβ1 immunohistochemistry in future large-scale clinical studies aimed at better understanding the role of ERβ1 in breast cancer.

Keywords: Estrogen receptor beta, breast cancer prognosis, immunohistochemistry, VECTRA, Ki67

Introduction

The expression of two hormone receptors, progesterone receptor (PR) and estrogen receptor alpha (ERα), in breast cancers is extremely informative for determining patient prognosis and response to endocrine therapies such as aromatase inhibitors or tamoxifen. Approximately 70% of breast cancers are hormone receptor positive and may respond to these treatments. The human epidermal growth factor receptor, or HER2, is another prognostic and predictiveindicator for breast cancer. However, approximately 10%-15% of breast cancers have poor expression of all three receptors and have been characterized as triple negative breast cancers (TNBCs). This breast cancer subtype has worse disease-free and overall survival, associates with poor clinical outcomes, and treatments are currently limited to chemotherapy [1]. Thus, there is a critical need to identify new therapeutic targets for TNBC.

Two estrogen receptors (ERs) are expressed in mammary epithelial cells, ERα and ERβ. For decades, ERα was thought to be the sole ER

and was found to mediate the proliferative actions of estrogens in breast cancers. With the identification of ERβ in 1996 [2], significant effort has been put forth to elucidate the role of ERβ in breast cancer. ERα and ERβ are both members of the nuclear receptor superfamily of transcription factors and share some structural similarities. Within the DNA binding domain, ERα and ERβ share 97% homology and can bind similar DNA sequences. As such, the receptors can regulate some common target genes, although they have been found to also regulate unique sets of target genes [3-5]. In addition, several *in vitro* studies have shown that the two ERs regulate proliferation in opposite manners. While ERα stimulates proliferation in response to estrogens, ERβ expression and activation by estrogens has been shown to inhibit the growth of both ERα-positive and ERα-negative breast cancers [6-10]. Because of the consistent *in vitro* data demonstrating the antiproliferative activity of ERβ, it has been suggested that ERβ may act as a tumor suppressor and could be a possible therapeutic target for cancers such as TNBC.

ERα and ERβ are expressed from unique genes on separate chromosomes. The *ESR2* gene can encode several different ERβ isoforms. The full-length isoform, ERβ1, is the only isoform that has a high affinity for 17β-estradiol (E2) and can transactivate gene expression in response to ER ligands [11, 12]. Four additional ERβ isoforms, ERβ2-ERβ5, have been identified in human tissues [13]. These isoforms have unique C-terminal sequences that arise from alternative splicing from the seventh exon of the *ESR2* gene. Although these isoforms do not have high affinity for ER ligands, ERβ2-ERβ5 have the capacity to dimerize with ERβ1 to enhance transactivation in response to ER ligands [12]. Because ERβ1 is the only isoform with the capacity to bind ligands with high affinity, this receptor would be the primary isoform to mediate gene expression and growth inhibition in response to E2 or ERβ-selective ligands. Indeed, only the full length ERβ1 isoform inhibited the growth of ERα-negative breast cancer cells *in vitro* [3, 8].

In light of the consistent *in vitro* evidence suggesting that ERβ1 is antiproliferative, several studies have aimed to assess the clinical significance of ERβ1 expression in breast cancers; however, the data have been inconclusive [14-21]. The receptor should ideally be detected at the protein level, as a poor correlation between ERβ1 mRNA and protein has been observed in breast cancers [22]. However, the antibodies and cutoffs used to determine ERβ1 expression have been inconsistent across studies [23], and there is a need to stringently confirm the specificity of ERβ1 immunohistochemical protocols. In this report, we describe a system to optimize immunohistochemistry (IHC) for full length ERβ using xenograft tissue obtained from breast cancer cell lines with inducible expression of ERβ1 [10]. We used these protocols to assess the subcellular localization of ERβ1 in two cohorts of patients with Stage IIII TNBC. We also determined the associations between ERβ1 and the proliferative marker Ki67, as well as tumor grade, tumor stage, and survival. This study provides a new strategy for optimizing ERβ1 IHC and objectively detecting the nuclear localization of the receptor, which may prove useful for future clinical studies aimed at determining the importance of full length ERβ expression in breast cancers.

Materials and methods

Cell lines and reagents

HEK293 cells were cultured in cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Turbofect and SuperSignal West Pico enhanced chemiluminescent reagent were obtained from Thermo Scientific (Rockford, IL). Blasticidin S and Zeocin were purchased from Research Products International (Mount Prospect, IL), and doxycycline was purchased from Clontech (Mountain View, CA). Athymic nude mice were purchased from Harlan Laboratories (Madison, WI). All the reagents for immunohistochemistry were purchased from Biocare Medical (Concord, CA).

A rabbit polyclonal antibody for ERα (HC-20) and a rabbit polyclonal antibody raised against the N terminus of ERβ (H150) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A polyclonal antibody raised against a peptide corresponding to the C terminus of ERβ1 (PA1-313) was purchased from Thermo Scientific (Rockford, IL). A mouse monoclonal antibody for β-actin was obtained from Sigma-

Aldrich (St. Louis, MO). A mouse monoclonal antibody for Ki67 (Clone SP6) was obtained from Dako (Carpinteria, CA) and Biocare Medical.

Validation of the PA1-313 antibody

For initial characterization by western blotting, HEK293 cells were transfected with 2 μg of the following expression vectors: CMX-YFP, pcDNA4/TO-ERα, pcDNA4/TO-ERβ1, and pcDNA5/TO-ERβcx. After 48 hr, the cells were collected by trypsinization and lysed as previously described [24]. Thirty micrograms of protein were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then incubated with antibody for ERα (HC-20), ERβ (H150), ERβ1 (PA1-313), or β-actin. After successive washes and incubation with secondary HRP-conjugated antibodies, membranes were visualized using ECL.

To validate the PA1-313 antibody for IHC, MDA-MB-468-ERβ1 breast cancer cells with inducible ERβ1 expression were used to generate tumor tissues with or without ERβ1 expression as previously described [10]. Once tumors reached a palpable size, the water was supplemented with or without 2 mg/mL doxycycline (Dox) in 1% sucrose. After 5 days of treatment, the tumors were collected and fixed in 10% neutral buffered formalin for 48 hr. Subsequently, the tissues were paraffin embedded and sectioned for IHC optimization. All animal work was performed in accordance with protocols approved by the Animal Care and Use Committee of the University of Wisconsin-Madison.

For successful IHC with the xenograft tissues, slides were de-paraffinized and rehydrated in a series of xylene and ethanol gradients. Antigen retrieval was performed by microwaving the samples for 20 min in 10 mM citrate buffer, pH 6.0. Slides were then incubated with Peroxidazed followed by a protein blocking step with Background Punisher for 10 min. After a biotin-avidin block according to the manufacturer's protocol, the slides were incubated with PA1-313 antibody diluted 1:200 (5 μg/mL) in DaVinci Green diluent for 16 hr at 4°C. Slides were then washed in TBS-T (0.05 M Tris, 0.15 M NaCl and 0.05% Tween 20, pH 7.4) and incubated with goat anti-rabbit biotinylated secondary antibody, followed by incubation with streptavidin HRP. The slides were then incubated with Betazoid DAB followed by a light counter stain with CAT Hematoxylin. For the pre-absorption control, the PA1-313 antibody was incubated for 16 hr at 4°C prior to IHC with 2 mg of the following peptide: EDSKSKGSQNPQS.

Patient population

Two resources were utilized for tumor tissue analysis. From the Marshfield Clinic Cancer Registry, 79 subjects with Stage I-III, hormone receptor poor (defined as ERα and PR expression of < 5% and HER2 negative by IHC or fluorescence *in situ* hybridization) breast cancer diagnosed between 1/1998-6/2007 and with adequate follow-up data regarding recurrence and survival were identified [25]. Tumor blocks were evaluated for adequate tumor tissue and five fresh unstained slides were cut from areas of block with representative tumor. Slides were marked with a non-identifiable study ID number, placed in an air-tight container, and shipped with cold packs to the University of Wisconsin (UW) research staff within 48 hours. The slides were stored at -20°C until ERβ1 and Ki67 staining were performed. Three of the Marshfield slides did not have adequate tissue to measure ERβ1 expression, and these samples were excluded from further analyses.

The second resource was a tissue microarray (TMA) that included tumor tissue from breast cancer patients diagnosed between 1999 and 2009 identified through the UW Hospital and Clinics Tumor Registry. Stage I-III breast cancer cases were included if adequate excess tumor tissue was available, as well as complete clinical follow-up or recurrence or death within 5 years after diagnosis. Tumor registry data regarding receptor status, treatments rendered, recurrence, and survival were associated with tumor specimens. Available medical records were also manually reviewed for receptor status and recurrence of cancer and death. ERα, PR, and HER2 IHC were performed on the TMA samples and interpreted by a breast pathologist. Construction of the TMA and its associated coded clinical dataset was approved for this study by the Institutional Review Board, as well as for future research use. For this analysis, only the ERα-, PR- and HER2-negative Stage I-III breast cancer cases from the TMA were included (n = 50). Summaries of the patient characteristics of each cohort are presented in **Table 1**.

Table 1. Patient characteristics

	Marshfield Cohort (n = 76)	UWCCC Cohort (n = 50)	Overall (n = 126)
	n (%)	n (%)	
Median Age (range)	60.8 (29-95)	52 (35-88)	
Menopausal status at diagnosis*			
Pre-	19 (25)		42 (33)
Post-	53 (70)	23 (46)	80 (63)
Unknown	4 (5)	27 (54)	4 (3)
Stage			
I	36 (47)	6 (12)	42 (33)
II	34 (45)	33 (66)	67 (53)
III	6 (8)	11 (22)	17 (13)
Grade			
1	1 (1)	0 (0)	1 (1)
2	12 (16)	10 (20)	22 (17)
3	57 (75)	39 (78)	96 (76)
Unknown	6 (8)	1 (2)	7 (6)
Chemotherapy			
Yes	60 (80)	40 (80)	100 (79)
No	16 (20)	10 (20)	26 (21)

*UWCCC Cohort defined menopause as age > 50 at diagnosis.

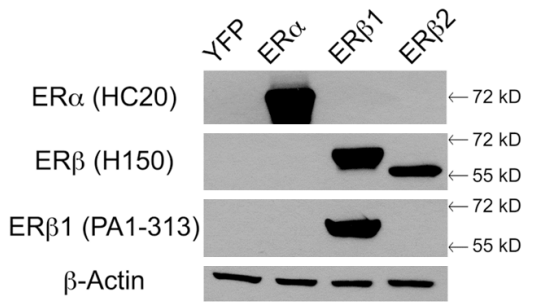

Figure 1. PA1-313 specifically detects ERβ1 in western blots. HEK293 cells were transfected with expression vectors for YFP, ERα, ERβ1, or ERβcx. Total proteins were resolved using SDS-PAGE and antibodies for ERα (HC20), the N terminus of ERβ (H150), and ERβ1 (PA1-313), and β-actin were used to confirm the specificity of the PA1-313 antibody.

Automated IHC and pathology review of clinical samples

Automated IHC using a Ventana Autostainer Benchmark XT (Ventana Medical, Inc, Tucson, AZ) and the pathology review were performed at the University of Wisconsin-Madison Translational Research Initiatives in Pathology (TRIP) lab. The Marshfield slides were stained with the PA1-313 ERβ1 antibody using the protocol described above with the following modifications to the reagents: 1) Background Sniper (Biocare Medical) was used for the blocking step; 2) the antibody was diluted in Van Gogh Yellow (Biocare Medical); and 3) the secondary antibody was a biotin-free Mach3 rabbit probe followed by Mach3 rabbit HRP polymer (Biocare Medical). For both cohorts, a second slide was stained with Ki67 (Clone SP6) antibody.

After staining, ERβ1 and Ki67 expression were analyzed using VECTRA™ (Caliper Life Sciences, Inc, Hopkinton, MA), which merges automated slide-handling, multispectral imaging technology, and unique pattern-recognition-based image analysis to accurately measure protein expression after labeling with immunohistochemical stains on a per-tissue, per-cell, and by cellular localization. The slides were scanned with the VECTRA™ platform, and the data analysis was performed using Nuance and in Forms1.4 software (Caliper Life Sciences, Hopkinton, MA). If the section size was larger than the imaging area, each section was divided into several quadrants for imaging. Images with poor tissue quality were eliminated from the analysis, and the remaining images were averaged to obtain the mean optical density per unit area (mean OD/unit area), which represents the average expression in each section normalized by the total cells analyzed. For the Marshfield cohort, the percentage of cells that showed negative (0+), weakly positive (1+), moderately positive (2+), and strongly positive (3+) nuclear staining was also determined in order to compare the different scoring strategies. The images were analyzed by a pathologist to determine the optical density thresholds for defining negative, weak, moderate, and strong staining. In order to combine the data from the two cohorts for the analysis of associations with Ki67 and progression-free and over-

all survival, the data were normalized by determining a z-score using the following calculation: [(OD value)-(mean of all OD values in cohort$_x$)]/(standard deviation of all OD values in cohort$_x$), where x is a given cohort (either Marshfield or UW Carbone Cancer Center [UWCCC]). This approach has been previously established for comparing IHC data from two experiments [26, 27]. The 25th percentile of the z-scores was used as a cutoff to define ERβ1 positive status for performing the survival analysis for the combined data sets.

Statistical analysis

All statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC, USA). Overall survival (OS) was defined as the time of diagnosis to the date of death, and disease-free survival (DFS) was defined as the time of diagnosis to the date of disease recurrence or breast cancer-related death. Statistical associations between ERβ1 expression and clinical characteristics were assessed between the negative and positive patients using Fisher's exact test for categorical variables, Student's t-test for numerical variables, and log rank test for time to event data. The survival distribution for DFS and OS were estimated using Kaplan-Meier method. Association between two numerical variables was assessed using Spearman's rank correlation analysis to account for nonlinear relation.

Results

Validation of ERβ1 antibody specificity

In order to specifically detect full length ERβ1, a polyclonal antibody raised against a synthetic peptide corresponding to residues 459 through 477 of human ERβ1 was selected for characterization. First, a western blot was performed to demonstrate the specificity of the antibody for detecting only the full length isoform. ER-negative HEK293 cells were transfected with expression vectors for yellow fluorescent protein (YFP, negative control), ERα, ERβ1, or ERβ2. Protein lysates were separated by SDS-PAGE, and the expression of ERα, total ERβ, and ERβ1 was determined. As shown in **Figure 1**, a polyclonal antibody raised against the first 150 amino acids of ERβ could detect both ERβ1 and ERβ2. In contrast, the PA1-313 rabbit polyclonal antibody raised against the C-terminus of the receptor only detected the full length isoform. The ERβ antibodies did not react with lysates from cells transfected with ERα.

Because the PA1-313 antibody specifically detected full length ERβ in western blots, this antibody was used to optimize IHC protocols for detecting ERβ expression in formalin-fixed tissues. MDA-MB-468-ERβ cells, which have inducible expression of ERβ1 after treatment with Dox [10], were used to generate xenograft tissues in which ERβ1 was absent (-Dox) or present (+Dox). The cells were injected into the mammary fat pads of nude mice and allowed to form palpable tumors. The mice were then separated into two groups and one group was given a diet containing Dox while the other group served as the control. After 5 days, the tumors were collected, fixed in formalin, and paraffin-embedded to replicate clinical tissue sample preparation. As shown in **Figure 2**, the xenograft tissue from mice exposed to Dox showed strong reactivity towards the PA1-313 antibody, as indicated by the brown nuclear staining (**Figure 2D**). In the control tissue, the PA1-313 antibody showed very little reactivity (**Figure 2A**). To further confirm the specificity of the staining, IHC was performed after pre-absorption with a peptide corresponding to the epitope used to generate the antibody (**Figure 2B**, **2E**). This pre-absorption control showed minimal reactivity in the Dox-treated tissues. Similar results were observed when the primary antibody was omitted entirely (**Figure 2C**, **2F**). These data demonstrate the utility of the xenograft tissues for optimizing IHC to detect ERβ1.

Scoring strategy and localization of ERβ1 in TNBCs

An initial analysis of ERβ1 expression and localization was performed using the Marshfield cohort to determine the best way to quantify and score ERβ1 expression. First, the relationship between the proportion of ERβ1-positive nuclei and the mean OD was assessed to determine which quantification method was most appropriate. The proportion of ERβ1-positive cells has been used as a way to measure ERβ1 expression, but this strategy does not take into account the variable staining intensity that can occur across samples. Scoring methods that do consider the staining intensity are often subjective and depend on the interpretation of individual pathologists. The mean OD determined using the VECTRA™ platform is a quantitative

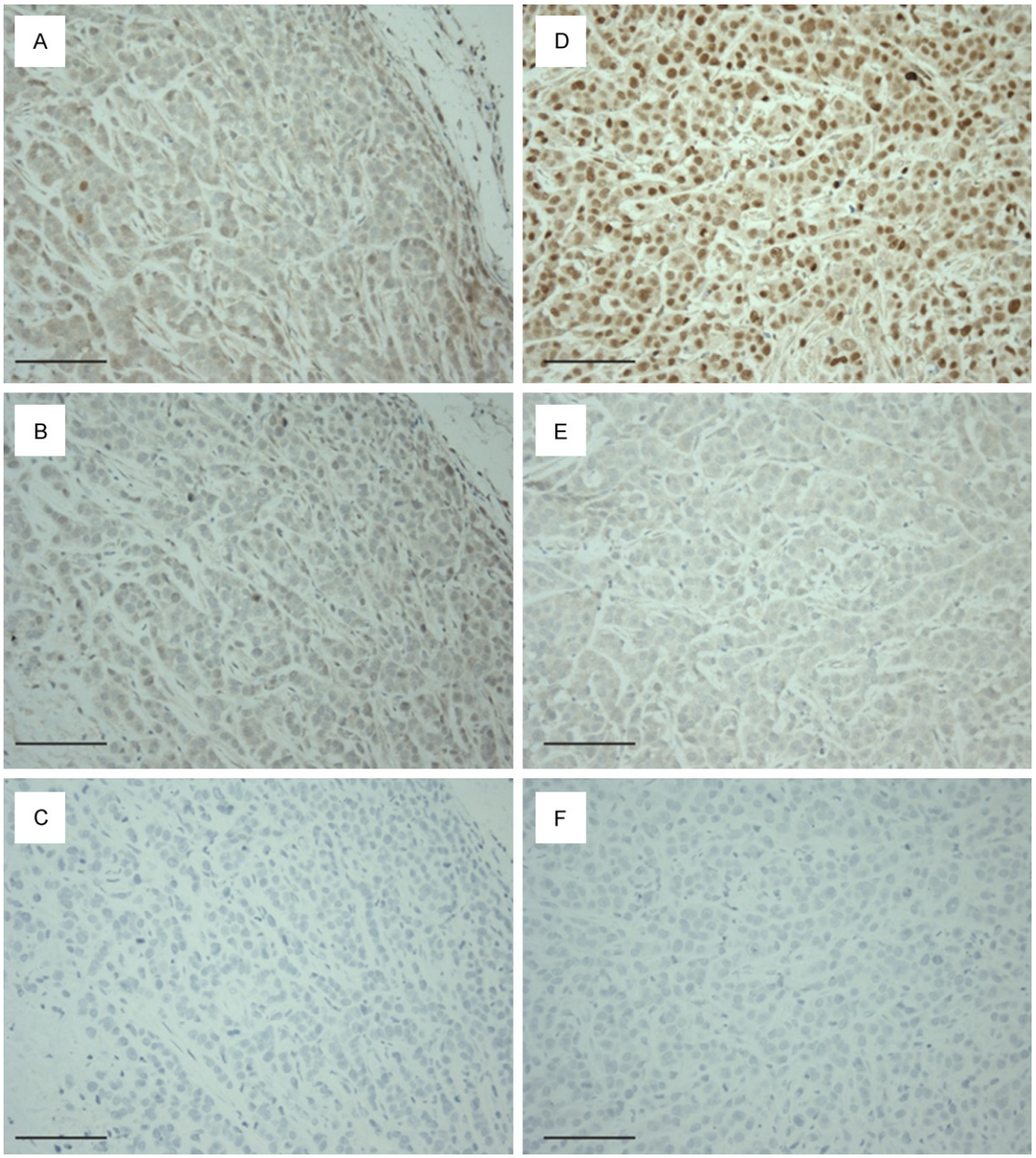

Figure 2. Xenograft tissues with inducible ERβ1 expression are useful for optimizing ERβ1 IHC. MDA-MB-468-ERβ1 cells were injected into the mammary fat pads of nude mice. After tumors formed, mice were treated with either vehicle (1% sucrose) (A-C) or Dox (D-F). IHC was performed with the PA1-313 antibody (A, D) as described in the Methods section. For controls, the antibody was pre-absorbed with ERβ1 peptide (B, E) or the primary antibody was excluded entirely (C, F). The brown staining that indicates reactivity toward ERβ1 is only observed in tissues from mice exposed to Dox (+ERβ1) (A).

measure that accounts for both the number of ERβ1-positive cells and the staining intensity of those cells. In the Marshfield cohort, there was a very close relationship between the mean OD and the percent of nuclei with ERβ1 staining intensity of 1+ or greater (**Figure 3A**, $R^2 = 0.89$). Only in samples in which many (> 60%) of the nuclei showed detectable expression of ERβ1 did the relationship between the mean OD and proportion of ERβ1-positive nuclei deviate from linearity. These data suggest that at lower cut-offs (such as 20% ERβ1-positive nuclei) incorporating staining intensity in the measurement of ERβ1 expression does not provide additional

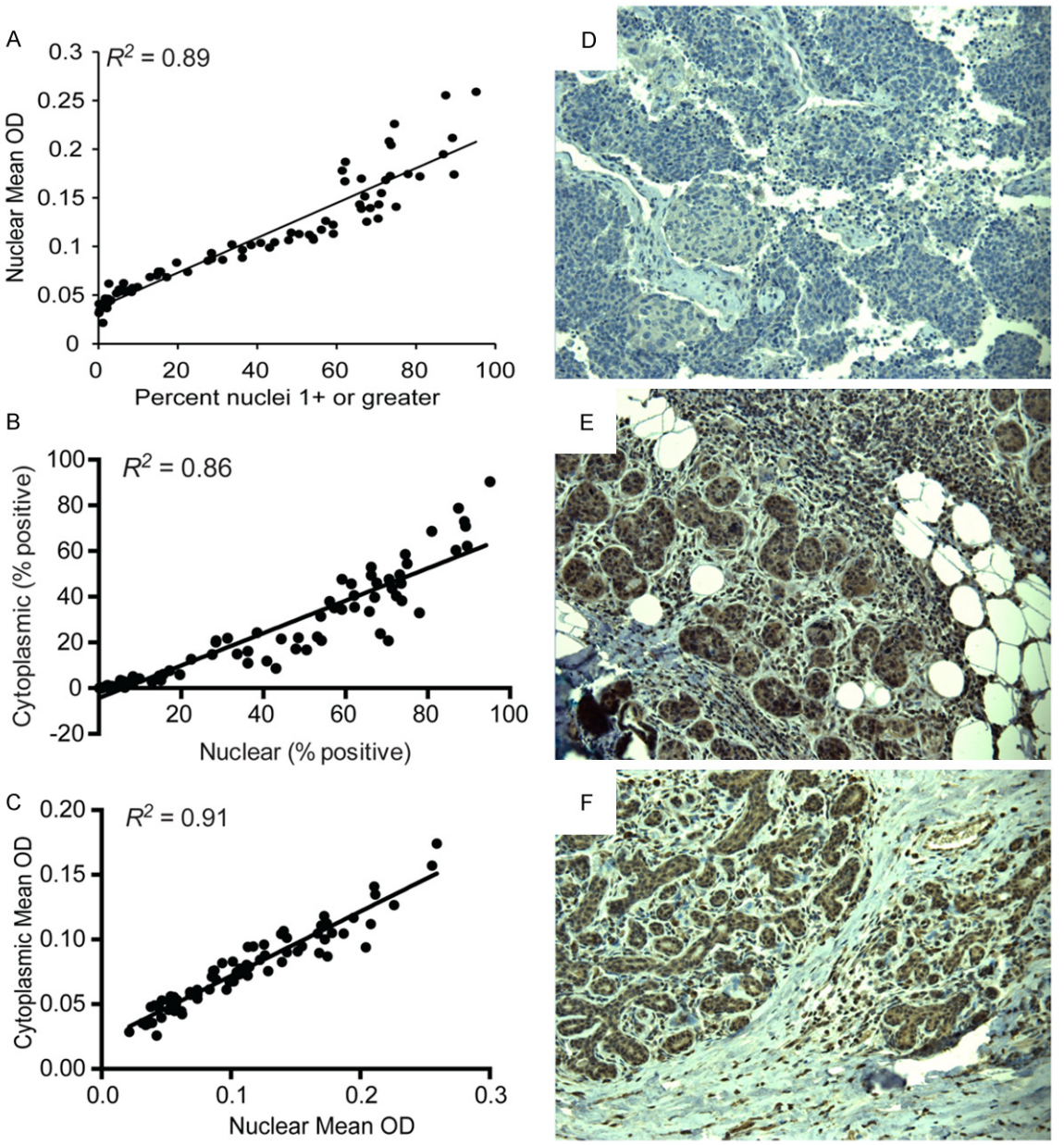

Figure 3. Relationships between ERβ1 subcellular localization and scoring strategies in the Marshfield cohort. A. A comparison of the nuclear mean OD and percent ERβ1-positive nuclei. B. Relationship between the nuclear and cytoplasmic ERβ1-positive cells. C. Relationship between the nuclear and cytoplasmic mean OD values. D. Representative image of ERβ1-negative tumor tissues (0.33% 1+ or higher nuclei). E and F. Representative images of ERβ1-positive tumor tissues (66.2% and 73.2% 1+ or higher nuclei, respectively). Scale bars = 200 μm.

information about the level of ERβ1 expression in the samples.

To determine if localization of ERβ1 might provide some unique insight, we assessed the relationship between the cytoplasmic and nuclear expression of ERβ1. There was a linear relationship between the nuclear and cytoplasmic levels of ERβ1, as determined by both the percent positivity (**Figure 3B**, R^2 = 0.86) and the mean OD (**Figure 3C**, R^2 = 0.91). These results suggest that tumors with nuclear ERβ1 nuclear expression will also exhibit cytoplasmic staining of the receptor and that scoring methods that utilize nuclear staining should sufficiently capture ERβ1 expression in this cohort of TNBCs. Representative images of ERβ1 IHC results are presented in **Figure 3D-F**.

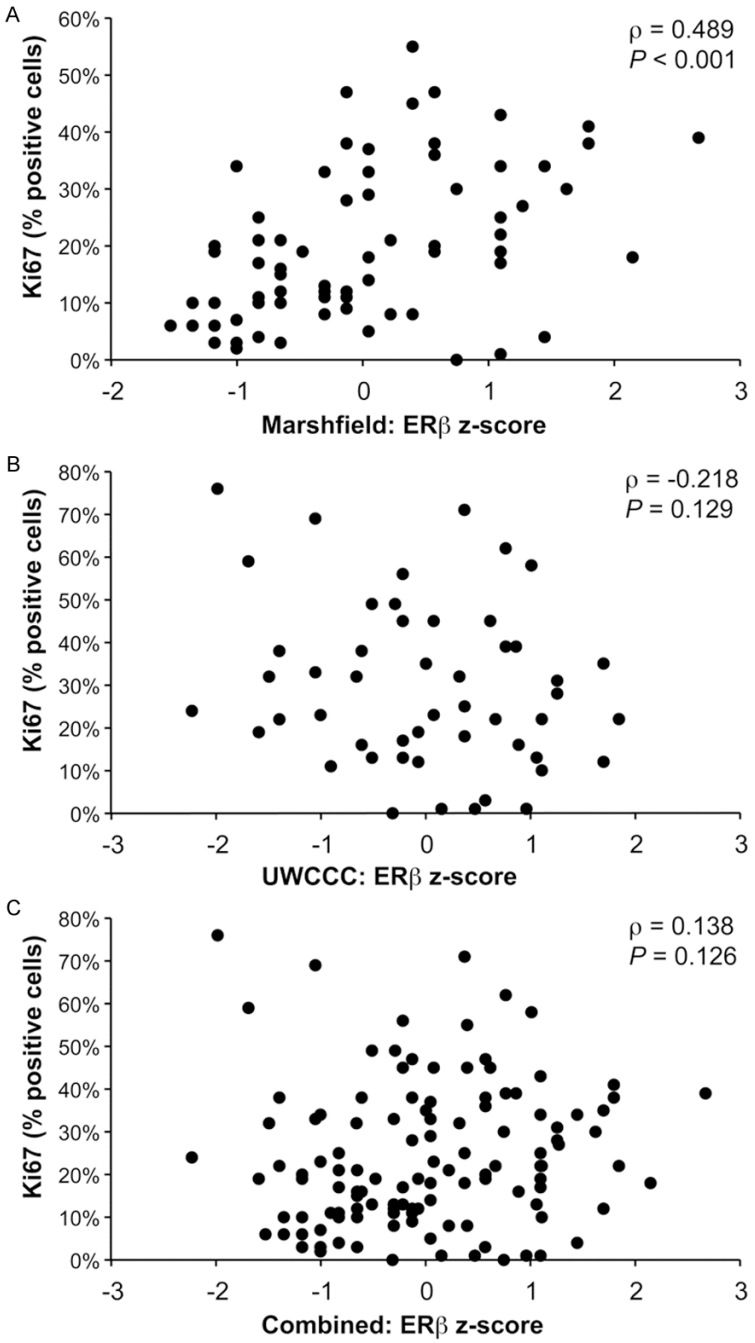

Figure 4. Association between ERβ1 and Ki67 expression in TNBCs. A. In the Marshfield cohort, the ERβ1 z-score was significantly associated with Ki67 ($P < 0.0001$). B. In the UWCCC cohort, there was no significant association between the ERβ1 z-score and the percent Ki67-positive cells ($P = 0.129$). C. In the combined data set, there was no significant association between the percent positive Ki67 cells and the Eβ1 z-score $P = 0.126$).

Associations between ERβ1 expression and Ki67

Based on the previous results, we decided to use the mean OD of nuclear ERβ1 expression to perform further analyses of the potential clinical implications of ERβ1 expression. First, we determined the association between the mean OD of ERβ1 and the percent Ki67-positive cells. In order to increase the power of our study and validate our findings in the Marshfield cohort, we incorporated a second cohort of 50 TNBCs available through the UWCCC. The nuclear mean OD data from each cohort were normalized by calculating a z-score (see Methods) using an approach that has been previously established for comparing quantitative IHC data from two experiments [26, 27]. As shown in **Figure 4A**, there was a significant positive association between the ERβ1 z-score and the percent positive Ki67 cells (Spearman's rank correlation coefficient $p = 0.489$, $P < 0.001$), although this positive association was not observed in the UWCCC cohort (Spear man's rank correlation coefficient $p = -0.218$, $P = 0.129$). When the data from the two cohorts were combined, there was no significant association between the ERβ1 z-score and the percent positive Ki67 cells (Spearman's rank correlation coefficient $p = 0.138$, $P = 0.126$).

Associations between ERβ1 expression and clincopathologic characteristics

In order to assess if ERβ1 expression was associated with other clinicopathologic characteristics, patients from both cohorts were classified into two groups (ERβ1 low and ERβ1 high) using a cutoff of the 25th percentile for the ERβ1 z-scores. The results of these analyses are presented in **Table 2**. Overall, there were no significant associations between nuclear ERβ1 sta-

Table 2. Contingency table of ERβ1 expression and clinicopathologic characteristics in the TNBC cohorts. Patients were classified into two groups based on ERβ1 expression: Low (< 25th percentile) or High (≥ 25th percentile). The numbers of patients in each category are shown. *P* values were determined using Fisher's exact test

Cohort		Marshfield (n = 76)			UWCCC (n = 50)			Overall (n = 126)		
ERβ1 Status		Low	High	*P value*	Low	High	*P value*	Low	High	*P value*
		n (%)	n (%)		n (%)	n (%)		n (%)	n (%)	
Menopausal status	Post	18 (34)	35 (66)	*0.775*	3 (10)	26 (90)	*0.036*	21 (26)	61 (74)	*0.519*
	Pre	5 (26)	14 (74)		8 (38)	13 (62)		13 (32)	27 (68)	
AJCC Stage	I	9 (25)	27 (75)	*0.454*	1 (17)	5 (83)	*1*	10 (24)	32 (76)	*0.672*
	II-III	14 (35)	26 (65)		10 (23)	34 (77)		24 (29)	60 (71)	
Grade	I-II	2 (15)	11 (85)	*0.326*	3 (30)	7 (70)	*0.405*	5 (22)	18 (78)	*0.794*
	III-IV	18 (31)	40 (69)		7 (18)	32 (82)		25 (26)	72 (74)	
Nodal Status	Neg	16 (30)	37 (70)	*0.777*	7 (25)	21 (75)	*0.734*	23 (28)	58 (72)	*0.517*
	Pos	5 (25)	15 (75)		4 (18)	18 (82)		9 (21)	33 (79)	
Recurrence	No	17 (31)	38 (69)	*0.762*	6 (19)	26 (81)	*0.494*	23 (26)	64 (74)	*1*
	Yes	4 (24)	13 (76)		5 (28)	13 (72)		9 (26)	26 (74)	

tus (low versus high) and tumor stage, grade, menopausal status, lymph node involvement, or recurrence. Finally, a survival analysis was performed to determine if DFS and OS were different between the ERβ1-low and ERβ1-high groups (**Figure 5**). No significant difference in DFS ($P = 0.176$) or OS ($P = 0.239$) was observed between the two groups. Similar results were observed when the cohorts were analyzed separately (data not shown).

Discussion

Several studies have aimed to determine the clinical significance of ERβ expression in breast cancers [14-21], but the results have been inconsistent. Some authors have suggested that the discrepant conclusions may be due to a lack of standardized detection methods, poorly validated antibodies, inconsistent cutoffs for defining ERβ1 positive cancers, and variable tissue preparation and processing methods [23, 28]. In order to address issues of specificity, xenograft tissues in which ERβ1 expression was regulated by a Dox-inducible system were used to optimize IHC for ERβ1 using a polyclonal antibody raised against the C-terminus of the full length receptor. This antibody showed specificity for full length ERβ in western blots and specifically reacted with MDA-MB-468-ERβ xenograft tissue from mice that had been exposed to Dox to induce expression of the receptor. This reactivity was blocked by pre-absorption with a peptide corresponding to the immunogenic epitope, thereby confirming the specificity of the IHC protocols. These xenograft tissues provide a useful tool for optimizing ERβ1 IHC and could be useful for standardizing ERβ1 IHC at different laboratories.

The data presented here are a quantitative assessment of ERβ1 expression in TNBC. VECTRA™ technology allows for a high throughput quantitative determination of biomarker expression in different cellular and tissue compartments in an objective and reproducible manner [26]. Using this technology, the expression of ERβ1 was quantified in the nuclear and cytoplasmic compartments of TNBCs and given as a mean optical density (mean OD), which incorporates both the proportion of cells that express ERβ1 and the staining intensity. Interestingly, there was a close relationship between the proportion of ERβ1-positive nuclei and the mean OD, particularly in samples with less than 60% ERβ-positive nuclei. These data indicate that cutoffs based on the percent of ERβ1-positive cells may be just as informative as those that utilize a scoring method that incorporates staining intensity. In addition, the nuclear and cytoplasmic expression levels of ERβ1 were very similar, suggesting that nuclear scoring methods should be sufficient for determining ERβ1 expression in TNBCs. ERα can also be detected in the cytoplasmic compartment [29], but current guidelines solely recommend the use of ERα nuclear staining [30]. In addition, quantitative immunofluorescence for

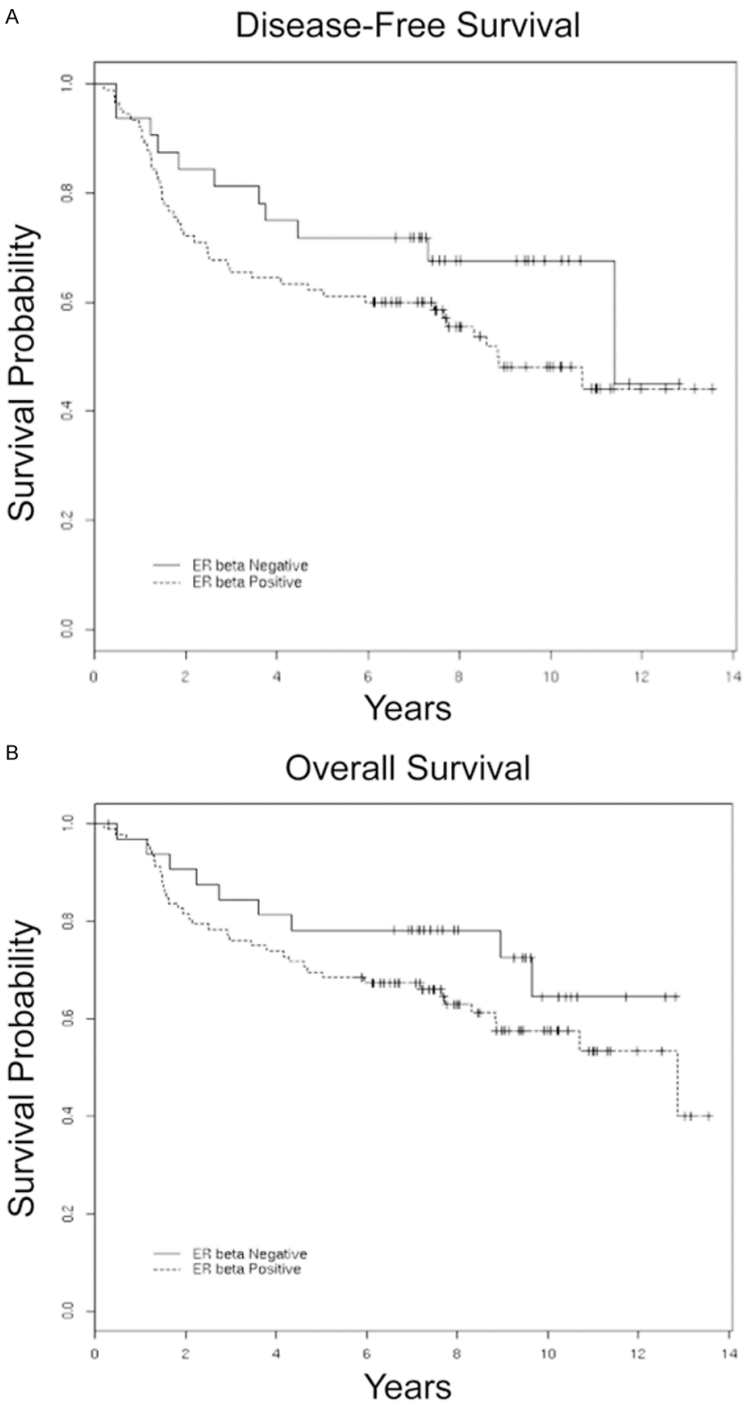

Figure 5. Association between ERβ1 and survival in TNBCs. A. There was no significant association between the ERβ1 z-score and disease-free survival (P = 0.176). B. There was no significant association between the ERβ1 z-score and overall survival (P = 0.239).

ERα has been found to be more objective and accurate for defining ERβ status, especially in cases in which the receptor expression is lower [31]. For ERβ1, it will be necessary to objectively determine the proportion of ERβ1-positive nuclei using technology such as VECTRA™, which can quantitatively determine the protein expression in specific cellular and tissue compartments, in order to standardize the assessment of ERβ1 expression across different laboratories.

Several ERβ antibodies have been used for IHC, including those that detect all ERβ isoforms, such as 14C8 [32] and MC10 [28]. In this study, the PA1-313 antibody was selected to specifically detect ERβ1 expression in TNBCs and was found to be specific for ERβ1 in western blots. Previously, Skliris and colleagues evaluated seven ERβ antibodies for IHC and western blotting and found that the PA1-313 antibody gave more intense and specific staining in frozen tissues when compared to the PPG5/10 clone, which has been used extensively to detect full length ERβ in previous studies [32]. Only one previous study utilized PA1-313 to assess ERβ1 expression in breast cancers [18]. In a cohort of 92 breast cancers, ERβ1 expression was associated with ERα expression, and 60% of the cancers were ERβ1-positive using a 20% positive cellular staining as a cutoff [18].

The relationship between ERβ1 expression and clinicopathologic breast cancer characteristics has remained elusive and may not be the same in the various breast cancer subtypes. Although the *in vitro* evidence suggests that ERβ1 may act as a potential tumor suppressor [3, 6, 8-10, 33], in the TNBCs

we analyzed, ERβ1 expression was only found to be associated with the proliferative marker Ki67 in the Marshfield cohort. A similar relationship was observed by Skliris and colleagues in a cohort of over 200 ERα-negative breast cancers, and this association was specific for ERβ1 since ERβ2 expression was not associated with Ki67 [17]. In addition, Jensen and colleagues found that Ki67-positive cells often co-expressed ERβ in a small set of ERα-negative ERβ-positive primary breast cancers [34]. These results suggest that ERβ1 is more highly expressed in proliferative cancers and could therefore play a role in the proliferation of breast cancers that lack ERα expression. In support of this conclusion, O'Neill and colleagues also found that ERβ1 expression in ERα-negative breast cancers was associated with increased Ki67 [22], and total ERβ expression was associated with a high S-phase fraction in a cohort of ERα-negative breast cancers from Sweden [35]. However, these data were not validated in the UWCCC cohort, suggesting that this weakly positive association may not occur in all cohorts. It is unclear why the two cohorts showed different relationships between ERβ1 and Ki67. In addition, there were no significant associations with tumor grade or stage in any of these studies or the cohort analyzed in this study.

Whether or not ERβ1 expression holds prognostic value also remains unclear. Several studies have shown that ERβ expression in ERα-negative breast cancers is beneficial when patients are treated with tamoxifen, which is not the standard modern day approach to treat these patients. In a Swedish cohort, total ERβ expression was associated with both improved DFS and OS in ERα-negative cancers [35]. In a Japanese cohort, ERβ1 expression was associated with improved OS in tamoxifen-treated patients diagnosed with TNBC. Yan and colleagues also found that patients with breast cancers expressing nuclear ERβ1 were more responsive to endocrine therapy [19]. These studies suggest that endocrine therapy may be beneficial for improving the outcomes of patients diagnosed with ERβ1-positive/ERα-negative breast cancers, although more work needs to be done to determine if ERβ1 is mediating the antiproliferative effects of tamoxifen.

Several other studies have found that ERβ1 expression is uninformative in ERα-negative breast cancers. For example, O'Neill and colleagues found that ERβ1 expression was not associated with outcome in a cohort of patients diagnosed with ERα-negative breast cancer and treated with adjuvant hormone therapy [22], and Shabaan and colleagues similarly found that nuclear ERβ1 expression was uninformative while the subcellular localization of ERβ2 was differentially associated with survival [16]. A more recent study by Wimberly et al. showed that other ERβ isoforms may be informative, although ERβ1 is not [21]. In support of the possible anti-proliferative function of ERβ1, ERβ1-negative tumors in the Nurse's Health Study were larger in size, higher grade and stage, and more likely to be lymph node positive [14]. However, ERβ1 expression did not associate with improved outcomes in ERα-negative breast cancers [14]. Similarly, ERβ1 expression was not associated with improved survival in the cohort analyzed in the present study.

Overall, this study provides a strategy for optimizing ERβ1 IHC using xenograft tissues in which ERβ1 expression can be regulated by Dox treatment. It also provides an objective quantification of ERβ1 nuclear and cytoplasmic expression. By utilizing VECTRA™ technology, these results demonstrate that the proportion of ERβ1-positive nuclei corresponds well with the mean OD for ERβ1 expression and the cytoplasmic expression of the receptor, indicating that scoring methods that incorporate staining intensities or subcellular localization may not be more informative than scoring the proportion of positive nuclei. Finally, ERβ1 expression was found to be associated with Ki67 expression in one cohort of TNBCs. However, this result could not be confirmed in a separate cohort suggesting that future work should aim to better characterize the significance of ERβ1 expression, possibly through the use of larger cohorts comparing different treatment strategies.

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Abbreviations

TNBC, triple negative breast cancer; ER, estrogen receptor; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; HER2, human epidermal growth factor 2; PR, progesterone receptor; Dox, doxycycline; IHC, immunohistochemistry; UW, University of Wisconsin; UWCC, University of Wisconsin Carbone Cancer Center; TMA, tissue microarray; OS, overall survival; DFS, disease free survival; OD, optical density.

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