Validation of Antibodies to Estrogen Receptor β and Quantitative Assessment of ERβ1 and ERβ5 Expression in Breast Cancer

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ABSTRACT (updated)

INTRODUCTION: Estrogen receptors are members of the nuclear hormone receptor family that play an important role in breast carcinogenesis and response to endocrine therapy. Though the role of ERα in breast cancer has been studied extensively, little is known about the alternative isoform ERβ. ERβ has significant sequence homology to Erα, but is located on a different chromosome and maintains both overlapping and unique functional attributes. Five variants resulting from alternative splicing of the C-terminal region of ERβ exist. The relevance of ERβ variants in breast cancer outcomes and response to therapy is difficult to assess because of conflicting results in the literature, likely due to variable methods used to assess ERβ in patient tumors.

METHODS: Antibodies against ERβ variants (ERβ1: ThermoScientific PPG5/10; ERβ2/cx: Serotec Clone 57/3; ERβ5: Serotec Clone 5/25) were validated for staining specificity by siRNA knockdown of ESR2 as well as staining reproducibility on FFPE tissue by quantitative immunofluorescence (QIF) using AQUA technology (HistoRx). QIF staining of validated antibodies was then assessed on two separate breast cancer cohorts.

RESULTS: ERβ1 and ERβ5, but not ERβ2/cx, antibodies were found to be sensitive, specific and reproducible, as shown by reduction in signal after siRNA knockdown in cell lines and reproducible QIF scores on a set of breast cancer control cases. The distribution of both ERβ1 and ERβ5 is similar in two breast cancer cohorts and QIF scores are significantly associated in both cohorts. When patients are stratified into low and high ERβ5 groups based on the median QIF score, high ERβ5 is a trending marker of worse prognosis in (1) ERα positive patients on one of the cohorts examined (p=0.0201) and (2) in patients treated with adjuvant tamoxifen and/or chemotherapy (p=0.0195). ERβ1 does not appear to provide any prognostic information on either cohort.

CONCLUSIONS: Rigorous validation of ERβ antibodies is required for accurate measurement of expression. Assessment of two breast cancer cohorts using validated reagents show that ERβ5, but not ERβ1, is a trending marker of worse prognosis in ERα positive patients and patients treated with adjuvant tamoxifen and/or chemotherapy.

BACKGROUND & METHODS

To assess specificity of ERβ variants, commercially available antibodies against unique regions of the C-terminus were obtained and stained on cell lines and tissue microarrays (TMAs) to qualitatively assess specificity to ERβ after knockdown with ESR2 siRNA, as well as staining reproducibility on the TMAs by quantitative immunofluorescence (QIF) using Automated QUantitative Analysis (AQUA). **Figure 1** illustrates the basis of AQUA technology. Briefly, TMAs are stained with pan-cytokeratin, DAPI and target of interest, all on different fluorophores. Cytokeratin is used to mark epithelium, which serves as a tumor mask for breast cancer. Localization of DAPI can then be used to establish subcellular compartments within the tumor mask. The sum of the intensity of pixels of the target divided by the total compartment pixel area then gives a relative intensity, denoted AQUA score.[1]

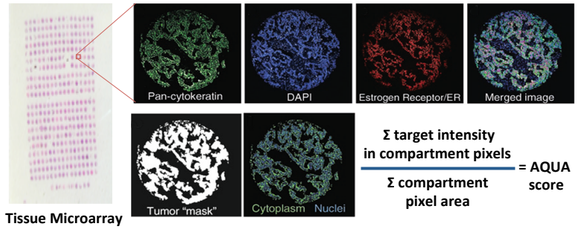

Figure 1. Illustration of AQUA, in which pan-cytokeratin marks breast tumor epithelium and DAPI enables subcellular compartmentalization within the tumor mask. The target of interest, ER shown here, can then be measured quantitatively within the desired compartment using the depicted algorithm (image adapted from Dolled-Filhart et al., *Methods in Molecular Biology* 2010[2]).

BACKGROUND & METHODS

ERβ1 and ERβ5 expression was quantified using AQUA on two breast cancer patient cohorts from Yale. Cohort 1, YTMA 49, consists of 649 patients diagnosed between 1962-1989 and cohort 2, YTMA 130/201, consists of 536 patients diagnosed between 1976-2005.

	ERβ and survival			ERβ and response to therapy		
	Better survival	Worse survival	No connection	Sensitivity	Resistance	No connection
ERβ	2	3	2	4	-	-
ERβ1	9	1	5	5	-	4
ERβ2	8	4	2	2	1	2
ERβ5	3	3	-	-	-	-

Table 1. Survey of ERβ literature illustrating discrepancies in reports on ERβ variant correlations with breast cancer patient survival and response to therapy, with numbers representing the number of published accounts, in total accounting for 17 publications.

RESULTS

Antibody Validation

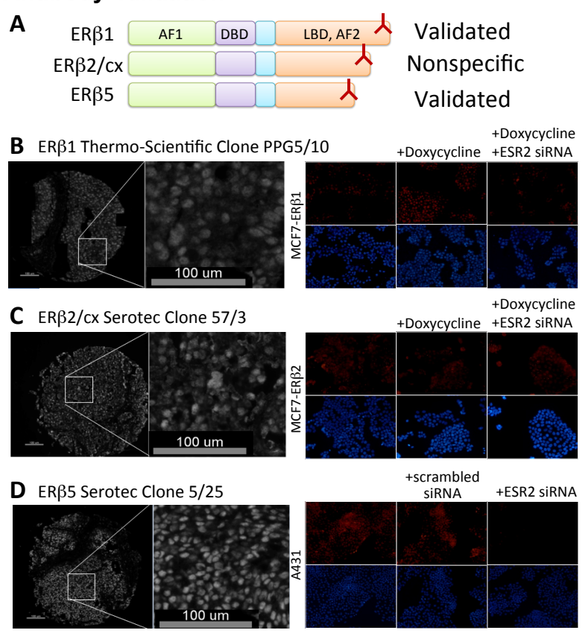

Figure 2. Specificity of three commercially available ERβ antibodies. (**A**) Illustration of ERβ splice variants with location of antibodies specific to each variant indicated. (**B**) Left, example of immunofluorescent (IF) staining on breast cancer histospot; right, specificity of ERβ1 illustrated by IF of MCF7-ERβ1 cell line[3] with doxycycline-inducible ERβ1 grown on coverslips and +/- doxycycline and +/- ESR2 siRNA. (**C**) Left, example of IF staining on breast cancer histospot; right, lack of specificity of ERβ2 illustrated by IF of MCF7-ERβ2 cell line[3] with doxycycline-inducible ERβ2 grown on coverslips and +/- doxycycline and +/- ESR2 siRNA. Successful induction and knockdown of ERβ2 construct was confirmed by IF and Western blot using anti-Xpress antibody (data not shown). (**D**) Left, example of IF staining on breast cancer histospot; right, specificity of ERβ5 illustrated by IF of A431 grown on coverslips and +/- ESR2 siRNA. QIF of ERβ1 and ERβ5 was performed twice on a TMA containing panel of breast cancer control cases and AQUA scores regressed to assay QIF reproducibility, resulting in $R^2 > 0.8$ (data not shown).

RESULTS

ERβ1 and ERβ5 on 2 Breast Cancer Cohorts

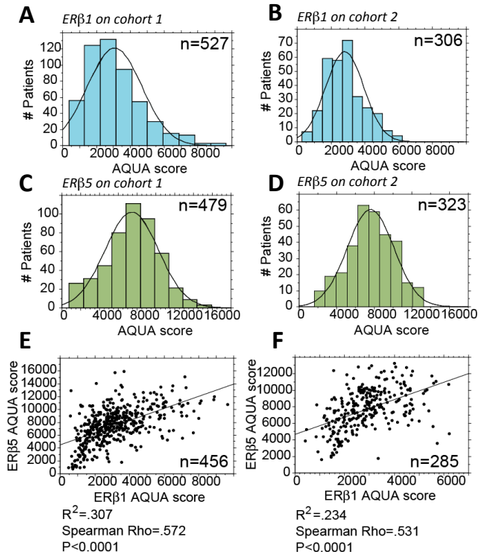

Figure 3. Distributions of ERβ1 (**A,B**) and ERβ5 (**C,D**) and regression of ERβ1 and ERβ5 AQUA scores (**E,F**) on breast cancer patient cohorts 1 on the left and cohort 2 on the right.

ERβ5 and Patient Survival

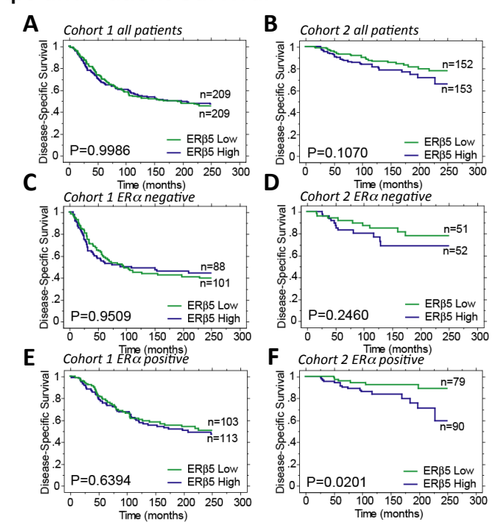

Figure 4. Kaplan-Meier curves illustrating Disease-Specific Survival (DSS) in which patients are grouped into ERβ5 low and high according to the median AQUA score for cohort 1 (**A**) and for cohort 2 (**B**). Also shown is separation of the two cohorts into ERα negative (**C,E**) and ERα positive subgroups (**D,F**), as defined by IHC, with cohort 1 on the left and cohort 2 on the right.

RESULTS

ERβ5 and Patient Survival in Treatment Groups

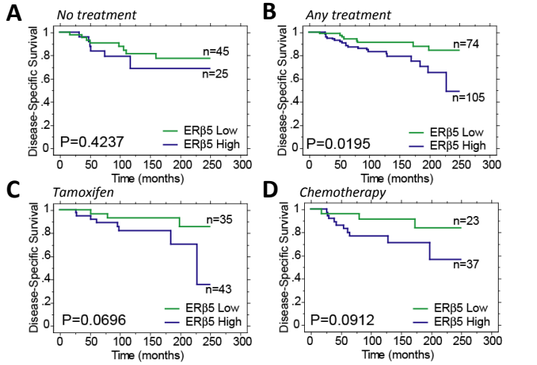

Figure 5. Kaplan-Meier curves illustrating DSS in patients on cohort 2 grouped into ERβ5 low and high according to the median AQUA score, separated according to adjuvant treatment received. **(A)** No treatment; **(B)** Any adjuvant treatment which includes either tamoxifen, chemotherapy or both; **(C)** Tamoxifen only; **(D)** Chemotherapy only.

ERβ1 and Patient Survival

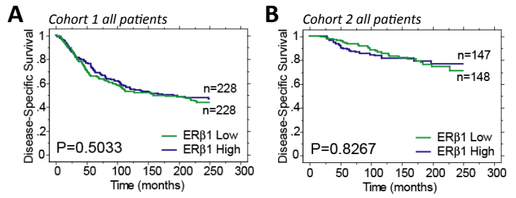

Figure 6. Kaplan-Meier curves illustrating DSS in which patients are grouped into ERβ1 low and high according to the median AQUA score for cohort 1 **(A)** and for cohort 2 **(B)**. ERβ1 did not stratify DSS in ERα negative or ERα positive subgroups in either cohort or in patients receiving different adjuvant treatment regimens in cohort 2 (data not shown).

CONCLUSIONS

- Rigorous antibody validation is required for measurement of ERβ.
- ERβ1 and ERβ5 AQUA scores show similar distributions in two independent breast cancer cohorts and have correlated expression in both.
- ERβ1 expression is not prognostic in either cohort.
- ERβ5 expression is associated with worse prognosis in:
  1) ERα positive patients in cohort 2 (current cohort)
  2) Patients treated with adjuvant tamoxifen and/or chemotherapy

REFERENCES

1. Camp RL, Chung GC, Rimm DL. *Nature Medicine*. Nov 2002; 8(11):1323-1328.
2. Dolled-Filhart M, Gustavson M, Camp RL, et al. *Methods in Molecular Biology*. 2010; 664:151-162.
3. Murphy LC, Peng B, Lewis A, et al. *Journal of Molecular Endocrinology*. Apr 2005;34(2): 553-566.