Estrogen Receptor mRNA Levels in Breast Cancer Predicts Response to Tamoxifen

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ABSTRACT

PURPOSE: Quantification of mRNA has historically been done by reverse transcription polymerase chain reaction (RT-PCR). Recently, a robust method of detection of mRNA utilizing in situ hybridization has been described that is linear and shows high specificity with low background. Here we describe the use of the AQUA method of quantitative immunofluorescence (QIF) for measuring mRNA in situ using *ESR1* (the estrogen receptor alpha gene) in breast cancer to determine its predictive value compared to Estrogen Receptor a (ER) protein.

METHODS: Messenger RNA for ER (*ESR1*) and Ubiquitin C (*UbC*) were visualized using RNAscope probes and levels were quantified by quantitative in situ hybridization (qISH) on two Yale breast cancer cohorts on tissue microarrays. *ESR1* levels were compared to ER protein levels measured by QIF using the SP1 antibody.

RESULTS: *ESR1* mRNA is reproducibly and specifically measurable by qISH on tissue collected from 1993 or later. *ESR1* levels were correlated to ER protein levels in a non-linear manner on two Yale cohorts. High levels of *ESR1* were found to be predictive of response to tamoxifin.

CONCLUSION: Quantification of mRNA using qISH may allow assessment of large cohorts with minimal formalin fixed, paraffin embedded tissue. Exploratory data using this method suggests that measurement of *ESR1* mRNA levels may be predictive of response to endocrine therapy in a manner that is different from the predictive value of ER. Further studies are underway using tissue microarrays from other institutions to determine how tissue age affects this assay.

BACKGROUND

Despite the usefulness of estrogen receptor (ER) as a predictive marker for endocrine therapy 50% of ER positive patients still recur, indicating a need for additional predictive biomarkers for endocrine therapy [1]. Assessment of mRNA expression signatures allows for the comparison of thousands of genes at a time. As a result, mRNA expression-based signatures have shown that better patient stratification can be achieved by looking at many genes [2,3]. However, Paik and colleagues have suggested that even looking at the mRNA from a single gene could show predictive power [4]. Recently a novel mRNA *in situ* hybridization (ISH) technique called RNAscope (Advanced Cell Diagnostics, Inc.) has been developed that can be used to detect RNA transcripts on formalin-fixed paraffin embedded (FFPE) tissue [5]. Here we modified the AQUA method for quantitative measurement of protein to combine it with the RNAscope method to quantify ER mRNA *in situ* and to compare to ER protein levels determined by quantitative immunofluorescence (QIF) on two breast cancer cohorts.

MATERIALS & METHODS

Two tissue microarray (TMA) cohorts of archival breast cancer samples from Yale were used in this study. The Yale Sentinel Node Cohort, called YTMA 128 (patients diagnosed from 2002-2006, n = 238) and an independent and non-overlapping cohort, called YTMA 130, from patients diagnosed from 1976-2005, (n = 524). Clinico-pathologic characteristics of both cohorts are found in Table 1.

Table 1: Cohorts Characteristics and Post-1993 Subset from YTMA-130

	YTMA 128	YTMA 130	YTMA 130 Subset	
Characteristic	No. (%)	No. (%)	No. (%)	p-value
All patients	238	524	226	
Age, years				
<50	75 (31.5)	152 (29.0)	73 (32.3)	
≥50	147 (61.8)	293 (55.9)	153 (67.7)	0.3151
Unknown	16 (6.7)	79 (15.1)	0 (0)	
Nodal status				
Positive	72 (30.3)	72 (13.7)	42 (18.6)	
Negative	151 (63.4)	253 (48.3)	146 (64.6)	0.4804
Unsampled/unknown	15 (6.3)	199 (38.0)	38 (16.8)	
Tumor size, cm				
<2	143 (60.1)	267 (51.0)	145 (64.2)	
2-5	72 (30.3)	124 (23.7)	69 (30.5)	0.4469
>5	10 (4.2)	2 (0.4)	0 (0)	
Unknown	13 (5.5)	131 (25.0)	12 (5.3)	
ER (IHC)				
Positive (1-3)	162 (68.1)	220 (42.0)	125 (53.3)	
Negative (0)	39 (16.4)	169 (32.3)	87 (38.5)	0.4823
Unknown	37 (15.5)	135 (25.8)	14 (6.2)	
PgR (IHC)				
Positive (1-3)	142 (59.7)	37 (7.1)	28 (12.4)	
Negative (0)	59 (24.8)	349 (66.6)	182 (80.5)	0.0804
Unknown	37 (15.5)	138 (23.3)	16 (7.1)	
Her2 (IHC)				
Positive (2-3)	56 (23.5)	39 (7.4)	26 (11.5)	
Negative (0-1)	140 (58.8)	344 (65.6)	186 (82.3)	0.2179
Unknown	42 (17.6)	141 (26.9)	14 (6.2)	
Follow-up, months				
Median	49	81	64.5	
Range	1-340	2-327	3-169	

RNAscope Assay and AQUA Quantification

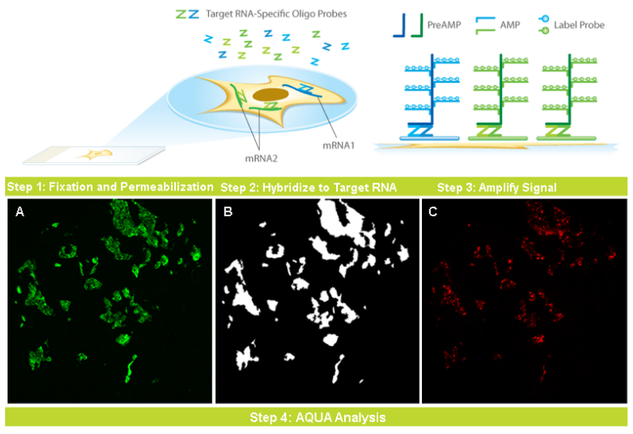

Figure 1. Schematic of the RNAscope assay and AQUA quantification. In step 1, tissues are fixed and permeabilized to allow for target probe access. In step 2, target RNA-specific oligonucleotide probes (Z) are hybridized in pairs (ZZ) to multiple RNA targets. In step 3, multiple signal amplification molecules are hybridized, each recognizing a specific target probe, and each unique label probe is conjugated to a different fluorophore or enzyme. In step 4, for each histospot an in and out-of-focus image were obtained for each fluorescence channel, DAPI (nuclei), Alexa 546 (cytokeratin), or Cy5 (target probe). The cytokeratin signal **(A)** was binarized to create the tumor mask **(B)** and target probe expression (C, *ESR1*) was quantified only in the tumor. AQUA scores were calculated for a given target within the tumor mask by dividing the signal intensity by the area of the tumor mask within the histospot.

RESULTS

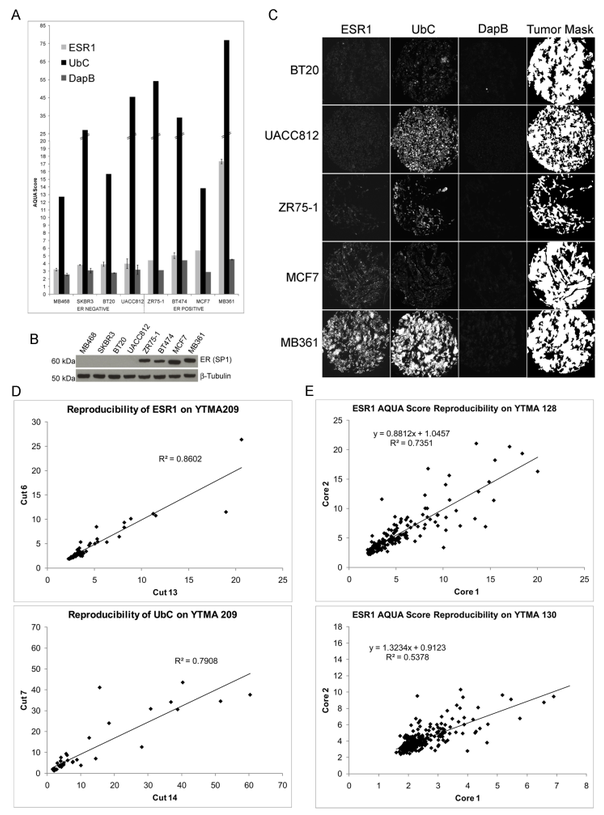

Figure 2. Validation of the RNAscope assay. **(A)** Average AQUA score distributions of the RNAscope assay for *ESR1*, *UbC*, and *DapB* performed on serial sections of the control TMA are shown in order of increasing *ESR1*. Error bars represent standard deviation. **(B)** 20 μg total cell lysate were probed with ER SP1 antibody. β-Tubulin served as a loading control. **(C)** Representative images are shown for *ESR1* negative cell lines BT20 and UACC812 and positive cell lines ZR75-1, MCF7 and MDA-MB-361 with the corresponding *UbC* positive control, *DapB* negative control, and tumor mask generated using cytokeratin. **(D)** Reproducibility of the assay between serial sections of the same TMA core is shown on the breast index array for *ESR1* and *UbC*. **(E)** Reproducibility of *ESR1* between 2 patient cores on YTMA 128 (top) and YTMA 130 (bottom).

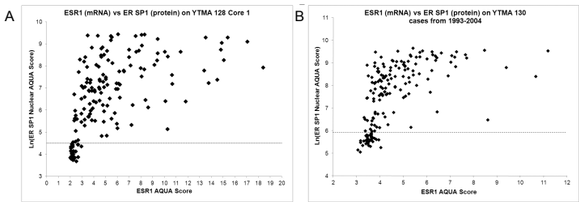

Figure 3. Comparison between *ESR1* and ER protein. The natural log of the nuclear ER AQUA score determined by QIF using SP1 is shown on the y-axis and the AQUA score for *ESR1* determined by qISH is on x-axis for YTMA 128, n = 167 **(A)** and YTMA 130 1993-2005 Subset, n = 195 **(B)**. The dotted line represents the threshold for ER protein positivity.

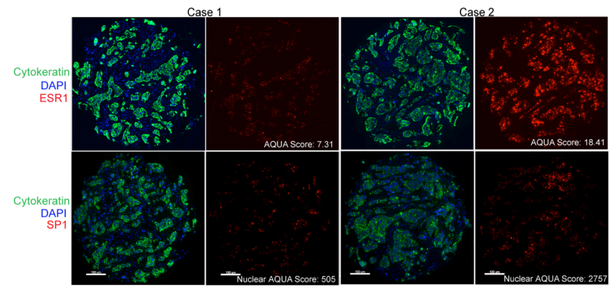

Figure 4. Representative images from 2 cases on YTMA 128 for cytokeratin (green), DAPI (blue), and *ESR1* or ER SP1 (red).

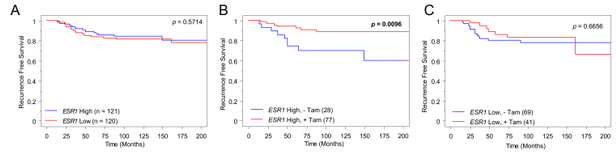

Figure 5. *ESR1* predicts response to tamoxifen on YTMA 130 Subset. Kaplan-Meier curves all show 10-year recurrence-free survival. **(A)** Cases from the YTMA 130 Subset were split by the median *ESR1* AQUA score and shows no prognostic value. **(B)** *ESR1* high (AQUA > 4.13) cases from the YTMA 130 Subset were split by tamoxifen treatment status. *ESR1* high patients who received tamoxifen had a statistically significant reduced risk of recurrence. **(C)** *ESR1* low (AQUA < 4.13) cases from the YTMA 130 Subset were split by tamoxifen treatment status and show no statistically significant trend. All p values were calculated using the log-rank test.

CONCLUSIONS

- The RNAscope assay combined with AQUA quantification specifically and reproducibly measures *ESR1* in FFPE breast cancer tissues.
- ER mRNA and ER protein measured by AQUA have a non-linear relationship.
- High *ESR1* predicts tamoxifen response whereas low *ESR1* does not.
- *ESR1* levels were not prognostic at any cutpoint.

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