Estrogen receptor (ER) mRNA expression and molecular subtype distribution in ER negative/Progesterone Receptor positive breast cancers.

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Running title

Molecular distribution in ER negative/PR positive breast cancers.

Key words

Estrogen receptor; Progesteron receptor; cDNA microarray; breast cancer; hormone therapy

Abbreviations

ER: Estrogen receptor; PR: Progesterone receptor; IHC: immunohistochemistry; HR: hormone receptor; HER2: Human Epidermal Growth Factor Receptor 2; DLDA: diagonal linear discriminant analysis; SET: Sensitivity to Endocrine Therapy.

Abstract (272 words)

Purpose: We examined estrogen receptor (ER) mRNA expression and molecular subtypes in stage I-III breast cancers that are progesterone receptor (PR) positive but ER and HER2 negative by immunohistochemistry (IHC) or fluorescent in situ hybridization.

Patients and Methods: The ER, PR and HER2 status was determined by IHC as part of routine clinical assessment (N=501). Gene expression profiling was done with the Affymetrix U133A gene chip. We compared expressions of *ESR1, MKI67* mRNA, distribution of molecular subtypes by the PAM50 classifier, the sensitivity to endocrine therapy index and the DLDA30 chemotherapy response predictor signature among ER/PR positive (n=223), ER positive/PR negative (n=73), ER negative/PR positive (n=20), and triple-negative (n=185) cancers. All patients received neoadjuvant chemotherapy with an anthracycline and taxane and had adjuvant endocrine therapy

only if ER or PR $\geq$ 10% positive.

Results: *ESR1* expression was high in 25% of ER negative/PR positive, in 79% of ER positive/PR negative, in 96% of ER/PR positive, and in 12% of triple negative cancers by IHC. The average *MKI67* expression was significantly higher in the ER negative/PR positive and triple-negative cohorts. Among the ER negative/PR positive patients, 15% were luminal A, 5% Luminal B, and 65% basal like. The relapse free survival rate of ER negative/PR positive patients was equivalent to ER positive cancers and better than the triple-negative cohort.

Conclusion: Only 20-25% of the ER negative/PR positive tumors show molecular features of ER positive cancers. In this rare subset of patients (i) a second RNA based assessment may help identifying the minority of ESR1 mRNA-positive, luminal type cancers and (ii) the safest clinical approach may be to consider both adjuvant endocrine and chemotherapy.

(1,483 words)

Introduction

Estrogen (ER) and progesterone receptor (PR) are routinely assessed in all primary breast cancers by immunohistochemistry (IHC) [1] and adjuvant endocrine therapy is recommended if either of these receptors is positive (i.e. > 1% by IHC) [2-4]. The expression of PR is activated by ERα via an estrogen responsive element–containing gene promoter. Therefore, it has been proposed that PR expression indicates the presence of functional ERα[5] and loss of PR expression potentially defines a subpopulation of patients with inferior benefit from tamoxifen compared to PR receptor persisted cancers.[6] In this model, the existence of ER negative/PR positive cancers represents an enigma. It has even been suggested that the majority of ER negative but PR positive cancers may represent false negative IHC results for ER.[7] After reevaluation of the tumor slides and control tissues, most cases of ER negative/PR positive cases changed their original phenotype. [7] The more, Hefti et al also reported that ER negative/PR positive cases showed no significant reproducibility by integrated gene expression microarray and clinico-pathological data.[8]

The prognostic value of PR expression independent of any endocrine therapy and its interaction with benefit from endocrine therapy in ER positive cancers has been documented by several studies. In ER positive cancers, patients with PR positive disease have lower risks of recurrence and mortality compared to PR negative cancers both with and without adjuvant endocrine therapy.[9] Prat et al reported that PR expression adds to the prognostic value of luminal A classification and can further sub-stratify patients among luminal cancers.[10] Viale et al also showed that PR expression predicts for adjuvant chemotherapy benefit among pre- and peri-menopausal

but not post-menopausal patients with ER positive cancers.[11] The predictive and prognostic value of PR expression in ER negative cancers is much less understood, mostly because of the rarity of this disease subset.

Approximately 3 % of all breast cancers are ER negative and PR positive.[12] Some data suggests that cancers may not significantly benefit from adjuvant endocrine therapy.[9] In 2010, joint guidelines by the American Society of Clinical Oncology (ASCO) and the American College of Pathologists recommended that hormone receptor (HR) status should be considered positive if 1% or more of tumor cells demonstrate positive nuclear staining of either ER or PR with an IHC assay[1]. Historically, many investigators and clinicians considered 10% or greater IHC staining as the threshold for defining HR positive status and therefore eligibility for endocrine therapy. We have previously showed that the majority of ER borderline, 1-9% IHC positive, cases had molecular features similar to ER negative cancers.[13]

In the current study, we examined *ESR1* mRNA expression and molecular subtype distribution among ER negative but PR positive cancers and assessed hormone and chemotherapy sensitivity markers in these cancers.[14, 15] The purpose of these analyses was to determine whether ER negative/PR positive cancers show the same global gene expression patterns and molecular subtypes as ER positive cancers do or if they are more similar to ER negative cancers.

Patients and Methods

Five hundred one patients were included in this study who participated in a prospective institutional review board approved biomarker discovery study at MD Anderson Cancer Center in Houston, TX and signed informed consent for molecular analysis of their pretreatment cancer biopsy and had routine marker and gene expression data available. The ER, PR and HER2 status was assessed on diagnostic core needle biopsies in the routine pathology laboratory. Clinical ER status was determined by IHC using the ERalfa antibody 6F11 (Novocastra/Vector Laboratories, Burlingame, CA) and PR status was determined by using the antibody 1A6 (Novacastra Laboratories Ltd., Burlingame, CA). The cut-off for ER or PR positivity for this analysis was ≥1% tumor cells with nuclear staining. HER2 status had been assessed either by fluorescence in situ hybridization or by IHC (Dako North America, Inc., Carpinteria, CA, USA). HER2 positivity had been defined as either HER2 gene amplification if immunohistochemical score was 2+ or an immunohistochemical score of 3+. Two hundred and twenty three patients were ER and PR positive, 73 were ER positive but PR negative, 20 were ER negative but PR positive and 185 were ER and PR negative. All patients received neoadjuvant chemotherapy containing a taxane and anthracycline based regimen, and patients with ER or PR positive tumors defined as ≥ 10% staining also received adjuvant endocrine therapy.

Gene expression profiling with Affymetrix U133 gene chips were performed on fine needle aspirations obtained before any therapy and independent of the diagnostic core needle biopsy used for routine ER, PR and HER2 determination. Gene expression data is available under GEO (Gene Expression Omnibus) accession number GSE 25066 and methods were described in a previous a publication.[16] Expression data were

normalized with the MAS5 algorithm, mean centered to 600 and log2 transformed. Probe set 205225_at was used as the measure of *ESR1* mRNA expression, and a log2-transformed expression value of ≥ 10.18 was considered as ER positive by mRNA based on of a threshold established and validated in previous publications.[13, 17, 18] We also assessed PR mRNA expression by probe set 208305_at and Ki67 (*MKI67*) expression by probe set 212021_s_at. An ER metagene was calculated as the average log2 transformed expression values of *ESR1*, *PR*, *BCL2* and *SCUBE2* as measure of endocrine sensitivity (based on OncotypeDX ER score). The PAM50 classifier, the sensitivity to endocrine therapy (SET) index and the DLDA30 chemotherapy response predictor signature were also applied to the data as previously described.[14-16, 19] The Wilcoxon test was used to determine statistical significance of gene expression differences between IHC subsets. We also plotted survival curves with the log-rank test by ER and PR status based on IHC. Statistical analyses were performed using the BRB-ArrayTools v 4.1.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html) and the R software v 2.7.2 (http://www.r-project.org). Two sided p values< 0.05 were considered statistically significant.

Results

Patients characteristics are shown in Table 1. Sixty three percent of tumors were hormone receptor (HR) positive (ER and/or PR ≥ 1%[1]) by IHC. Among the IHC ER negative/PR positive, ER positive/PR negative, ER/PR positive, and ER/PR negative patients, 25% (= 5/20), 79% (= 58/73), 96% (= 213/223) and 12% (= 22/185) were also positive by *ESR1* mRNA expression, respectively (Table 2). Among the ER negative/PR positive patients, 15% were luminal A, 5% were Luminal B, and 65% were

basal like; among the ER positive/PR negative patients, 59% were luminal type (Table 2). An additional 22 patients who were IHC ER/PR negative (12% of ER/PR negative cases) were positive by *ESR1* mRNA gene expression and may be considered as false negative IHC results (Table 2). The Sensitivity to Endocrine Therapy (SET) index assigned low sensitivity to 90% of the ER negative/PR positive cancers (Table 2). The chemotherapy sensitivity gene score, DLDA30, predicted high chemotherapy sensitivity for 60% of the ER negative/PR positive patients and for 21% of ER positive/PR negative patients (Table 2). Only 5 % (12/233) of the ER/PR positive patients were assigned to the high chemotherapy sensitivity group.

Figure 1 shows the relationship between ER/PR protein expression and *ESR1*, *PR* and *MKI67* mRNA gene expression and the ER metagene. The associations between IHC ER/PR subtypes and the mRNA gene expression level (*ESR1*, *PR* and ER-related genes) were similar and consistent, indicating that they were highly correlated each other. The majority of the ER negative/PR positive patients (75%) showed low *ESR1* mRNA, low PR and low ER metagene expression, and were assigned to ER negative status by these metrics. In contrast the majority of ER positive/PR negative cases showed high *ESR1* and ER metagene expression that were consistent with ER positive status. The average *MKI67* expression was also significantly higher in the ER negative/PR positive and ER/PR negative cancers compared to other subtypes. (Fig. 1)

Among the ER negative/PR positive, ER positive/PR negative, ER/PR positive and ER/PR negative, 40% (8/20), 16% (12/73), 8% (18/223) and 32% (60/185) achieved pathological complete response that was defined as absence of any residual invasive cancer in the breast and absence of any metastatic cells in the regional lymph nodes after neoadjuvant chemotherapy. The relapse free survival rate of ER negative/PR

positive patients who received chemotherapy (and nine of them received additional adjuvant endocrine therapy) was equivalent to ER/PR positive or ER positive/PR negative cases that received both endocrine and chemotherapy, and significantly better than the relapse free survival of ER/PR negative cancers. (Fig. 2)

Discussions

ER negative/PR positive breast cancers are rare; this and previous studies indicate that approximately 3 to 4% if all breast cancers fall into this category.[12] Because it represents a rare receptor subtype, it is unlikely that a prospective clinical trial would ever be conducted to define the optimal adjuvant treatment strategy for this disease. ER negative/PR positive status may arise from testing artifacts, including false positive IHC results in a truly HR negative tumor[20] or erroneously ER negative results in a truly ER positive tumor. It may also indicate the presence of tumor heterogeneity as a small PR positive subpopulation of cells within a larger both ER/PR negative tumor. In our study IHC ER/PR status defined by the routine analysis has been done on a fixed core needle biopsy, whereas the molecular profiling has been realized on another frozen samples by fine needle aspirations. Discordance form the distinct methods of the sampling and the possibilities false positive or negative results may be inevitable.[21] mRNA based methods to assess hormone receptor status may help resolve some of these uncertainties.[22] We assessed gene expression profiling data in 501 primary breast cancer to find out how often ER negative/PR positive patients by IHC showed molecular features of ER positive disease.

The minority (25%) of ER negative/PR positive tumors and the majority (79%) of ER positive/PR negative tumors showed ER positive status by *ESR1* mRNA gene expression data and had high expression of ER related genes. Five of twenty patients

with ER negative/PR positive cancers by IHC were ER positive by *ESR1* mRNA and ER metagene expression. Four of these 5 cancers were also classified as luminal subtypes by the PAM50 classifier and therefore likely represent false negative ER IHC results. On the other hand, 15 of the 20 ER negative/PR positive cancers showed low *ESR1* mRNA and ER metagene expression and all of these cancers were classified as non-luminal subtypes by a PAM50. This suggests that the majority of ER negative but PR positive cancers may not be endocrine sensitive. However the mRNA measurements represent bulk expression results for heterogeneous tissue. It is possible that small truly PR positive and endocrine sensitive subpopulation of cells may exist within a larger ER/PR negative tumor and signal from these cells is not apparent in the global expression data from the whole tissue.[11, 23, 24]

In these series, twenty ER negative/PR positive patients who received chemotherapy (and about half of them received additional adjuvant hormone therapy) have equivalent prognosis to ER/PR positive or ER positive/PR negative that received both chemo and hormone therapies. Overall, the expected benefit from hormone therapy is small in ER negative/PR positive tumors because majority of these tumors tend to be ER negative by *ESR1* mRNA (75%), show low predicted hormone sensitivity by the SET gene signature (90%), and are predominantly non-luminal class (85%). On the other hand 60% of the ER negative/PR positive cancers were predicted to have high chemotherapy sensitivity by the DLDA30 predictor.

This study has limitations. The number of ER negative/PR positive patients in this analysis is low. No prior study examined the molecular features of this rare disease subset and this study has the advantage of using centrally reviewed IHC results and a uniformly performed gene expression analysis. The molecular analysis yielded

generally consistent results for different RNA-based methods to assess ER status, and hormone and chemotherapy sensitivities. Another limitation should be that gene expression analysis does not necessarily imply protein expression. Elevated mRNA may not be indicative of elevated protein expression. Therefore, the potential false positive that can be obtained through IHC, there is equaling uncertainty on whether the mRNA levels in these samples translates to protein expression. The uneven samples size for the four ER/PR subgroups, different types of adjuvant hormone therapy used, and different TNM stages across cohorts limit the interpretation of the survival analysis.

In summary, only 20-25% of the ER negative/PR positive tumors show molecular features of ER positive cancers (i.e high ER mRNA expression, luminal molecular class). These cancers also have higher proliferation rate than ER positive cancers, higher predicted chemotherapy sensitivity and lower predicted hormone sensitivity. We concluded that in this rare subset of patients (i) a second RNA based assessment may help identifying the minority of *ESR1* mRNA-positive, luminal type cancers and (ii) due to the substantial uncertainty about endocrine sensitivity and high chemotherapy sensitivity in this IHC ER negative/PR positive cancer population, the safest clinical approach may be to consider both adjuvant endocrine and chemotherapy.

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Figure legends (2 Tables and 2 figures)

Fig. 1 *ESR1*, *Progesterone receptor (PR)*, ER metagene and *MKI67* mRNA gene expression in four distinct immunosittochemistry (IHC) groups. **I**HC groups were defined by the percentage of cells of that were positive of nuclear ER and PR staining. (A) Expression distribution of *ESR1* mRNA. (B) Expression distribution of *PR* mRNA. (C) ER-related genes refers to the average expression of 4 probe sets that are highly coexpressed with *ESR1*.[25] (D) Expression distribution of *MKI67* mRNA. *P* values were calculated with the Wilcoxon test.

Fig. 2 Kaplan-Meier relapse free survival curves by estrogen receptor (ER) /progesterone receptor (PR) immunohistochemistry status. Immunohistochemistry groups were defined by the percentage of ER/PR positive cells. P: Positive; N: Negative; HR: Hazard ratio; CI: Confidential interval.

Table 1 Patient characterisctics*	
N° of Pt. (%)	501
Age	
Average	49.8
(mini.-max)	(24 - 75)
ER by IHC	
Positive/Negative	296 (59.1)/ 205(40.1)
PR by IHC	
Positive/Negative	243(48.5) / 258(51.5)
HER2 by IHC and/or FISH	
Positive/Negative	6 (1.2)/ 483(96.4)
NA	12(2.4)
T	
0-2/3-4	284(56.7) / 217(43.3)
N	
Positive/Negative	155(30.9) / 346(69.1)
Grade	
I / II / III	31(6.2)/ 178(35.5) / 256(51.1)
NA	36(7.2)

* ER: Estrogen receptor; PR: Progesterone receptor; HER2: Human epidermal growth factor receptor 2; T: Clinical tumor size; N: Clinical lymph node status; NA: Not available.

Table 2 Breast cancer subtypes and Genomic markers*

ER / PR by IHC		Pos/Pos		Pos/Neg		Neg/Pos		Neg/Neg	
N° of Pt. (%)		223	44.5%	73	14.6%	20	4.0%	185	36.9%
ER by GE	Positive	213	95.5%	58	79.5%	5	25.0%	22	11.9%
	Negative	10	4.5%	15	20.5%	15	75.0%	163	88.1%
Molecular subtypes	LumA	131	58.7%	21	28.8%	3	15.0%	2	1.1%
	LumB	51	22.9%	22	30.1%	1	5.0%	4	2.2%
	Her2	12	5.4%	7	9.6%	2	10.0%	15	8.1%
	Basal	13	5.8%	13	17.8%	13	65.0%	147	79.5%
	Normal	16	7.2%	10	13.7%	1	5.0%	17	9.2%
SET index	High	21	9.4%	0	0.0%	1	5.0%	1	0.5%
	Intermediate	33	14.8%	3	4.1%	1	5.0%	3	1.6%
	Low	169	75.8%	70	95.9%	18	90.0%	181	97.8%
DLDA30	pCR	12	5.4%	15	20.5%	12	60.0%	154	83.2%
	RD	211	94.6%	58	79.5%	8	40.0%	31	16.8%

*ER: Estrogen receptor; PR: Progesterone receptor; Pos: sitive; Neg: Negative; GE: Gene expression; SET index: Symmans et al 2010 JCO; DLDA30; Lee et al 2010 CCR; pCR: Pathological complete response; RD: Residual disease;