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BRIEF REPORT

Genomic predictor of residual risk of recurrence after adjuvant chemotherapy and endocrine therapy in high risk estrogen receptor-positive breast cancers

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Abstract A subset of early stage estrogen receptor (ER)-positive breast cancers considered "high risk" for recurrence with endocrine therapy alone by current genomic prognostic predictors, such as Oncotype DX, is no longer high risk after receiving adjuvant chemotherapy. We hypothesized that a recently described gene expression-based outcome predictor adjuvant chemotherapy and endocrine therapy sensitivity (ACES) could re-stratify these patients into high and low risk groups for relapse when treated with both chemo- and endocrine therapies. ACES involves four separate modules (endocrine sensitivity, chemotherapy sensitivity, chemotherapy resistance, and survival prediction) that yield a prediction for good or poor outcome with current standard of care multimodality therapy. ACES was applied to Affymetrix gene expression data from 2 retrospectively collected ER-positive and HER2-negative patient cohorts that were uniformly treated with adjuvant endocrine and chemotherapy ($n = 250$). Each sample was first risk stratified by a genomic surrogate of Oncotype DX, and the high risk patients ($n = 76$) were re-stratified by ACES. Recurrence-free survival (RFS) was evaluated with ACES risk categories. The Oncotype DX high risk but ACES good prognosis patients ($n = 24$, 32 %) had an RFS of 95 % compared to 76 % in the poor prognosis group ($n = 52$; log-rank $p = 0.033$) at 5 years. ACES risk category remained an independent predictor in multivariate analysis after adjusting for age, T-stage, and lymph node involvement at diagnosis (hazard ratio 0.15; $p = 0.072$). Tertiary risk prediction that takes into account chemotherapy and endocrine sensitivity, and baseline prognosis may help identify high risk ER-positive patients who have excellent survival after chemotherapy.

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Abbreviations

ACES	Adjuvant chemotherapy and endocrine sensitivity
ARR	Absolute risk reduction
DLR	Diagnostic likelihood ratio
ER	Estrogen receptor
GGI	Genomic grade index
HR	Hazard ratio
NPV	Negative predictive value
OR	Odds ratio
PAM50	Prediction analysis of microarray 50-gene signature
pCR	Pathologic complete response
PPV	Positive predictive value
RCB	Residual cancer burden
REMARK	Reporting documentation for tumor marker prognostic studies
RFS	Recurrence-free survival
RS	Recurrence score
SET	Sensitivity to endocrine therapy

Introduction

Several prognostic gene signatures are now routinely used in the clinic to help improve adjuvant treatment decisions for stage I–II estrogen receptor-positive (ER) breast cancers. These tests categorize tumors into low (or intermediate) and high risk for recurrence at the time of diagnosis, if treated with surgery alone (primary prognostic predictor) or with surgery and adjuvant endocrine therapy (secondary prognostic predictor) [1]. MammaPrint (by Agendia) is an example of a primary prognostic predictor [2, 3]. Secondary residual-risk predictors include Oncotype DX (Genomic Health) [4], PAM50 molecular subtype classifier (Prosigna by NanoString) [5], and the Genomic Grade Index (GGI) (MapQuant DX by Ipsogen/Qiagen) [6]. These latter tests such as Oncotype DX were developed in studies of patients who received adjuvant endocrine therapy but not chemotherapy [4, 7–9]. Currently, the most widely used clinical test in the U.S is Oncotype DX, a 21-gene signature related to breast cancer proliferation, invasion, and ER transcriptional activity, which calculates a risk of recurrence score (RS) [1, 10, 11]. High RS has a highly significant association with breast cancer relapse and death in patients who receive adjuvant endocrine therapy alone, and therefore high RS identifies ER-positive patients who may benefit from adjuvant chemotherapy [4, 12, 13].

High risk ER-positive cancers that are considered appropriate candidates for adjuvant chemotherapy include those with high RS [12, 14], Luminal B molecular subtype [15, 16], and high GGI score [17]. These cancers typically have high proliferation rates and are also more sensitive to chemotherapy [16]. Several retrospective studies have demonstrated that while low or intermediate RS tumors receive no additional benefit from adjuvant chemotherapy, high RS patients treated with adjuvant chemotherapy in addition to endocrine therapy demonstrate close to 30 % absolute reduction in distant recurrence, compared to treatment with endocrine therapy alone [12, 14, 18]. Because of the consistency of these results, ER-positive patients considered to be high risk by the above secondary prognostic predictors routinely receive adjuvant chemotherapy. However, the residual risk after completion of all therapy remains uncertain. It is likely that many initially "high risk" patients revert to low risk.

Adjuvant chemotherapy and endocrine therapy sensitivity (ACES) is a tertiary multi-gene residual-risk predictor which identifies patients who have excellent distant recurrence-free survival (DRFS) after both endocrine therapy and chemotherapy. The development and validation of ACES has previously been published [19]. ACES captures information on sensitivity and resistance to endocrine therapy and chemotherapy and is a combination of four multi-gene scores [19]. One component is the sensitivity to endocrine therapy (SET) index, a 165-gene set associated with ER, which can predict survival after endocrine therapy or after combined endocrine therapy and chemotherapy [20]. A second component predicts the probability of extensive residual cancer (RCB-III) after neoadjuvant chemotherapy using 73 genes, while another component includes 33 genes that predict death or relapse within 3 years of diagnosis. These latter two components measure chemotherapy resistance. The fourth module is a 39-gene signature that predicts pathologic complete response (pCR/RCB-0) or minimal residual cancer burden (RCB-I) and captures chemotherapy sensitivity [19].

The complementary residual-risk re-stratification value of ACES for ER-positive cancers assigned to "high risk' category by secondary prognostic predictors such as Oncotype DX has not been studied. We hypothesize that ACES, which measures sensitivity to both endocrine and chemotherapies, will be able to re-stratify the currently "high risk" ER-positive cancers that receive appropriate multimodality systemic adjuvant therapy into good and poor outcome groups. We applied the ACES predictor to gene expression data from two ER-positive patient cohorts who received both endocrine and chemotherapy to addresses the following clinical question: for which high risk ER-positive patient is a standard of care adjuvant multimodality therapy sufficient, and which patient remains high risk?

Patients and methods

Study design

In this retrospective cohort study, ACES was applied to assess residual risk in ER-positive breast cancers classified as high risk (i.e., high RS) by a genomic surrogate version of Oncotype DX and treated with systemic adjuvant endocrine and chemotherapies. This tertiary risk re-stratification was applied to the cohort used previously to validate ACES [19] and to a new blinded independent validation cohort. We calculated that in order to have 80 % power to detect a hazard ratio (HR) of 0.2 between the good and poor prognosis groups, a minimum sample size of 60 high risk ER-positive cases would be required with at least 12 events (i.e., relapses or deaths). To achieve this sample size, the two independent validation cohorts that were not included in the development of ACES were pooled. The main outcome measures were relapse-free survival (RFS), including any local (in breast) or regional (regional lymph nodes) or distant recurrence, and absolute risk reduction (ARR) at 5 years after diagnosis. The study strategy is shown in Fig. 1. Analysis was performed according to the guidelines of Reporting recommendations for tumor MARKer prognostic studies (REMARK) [21].

Patients and gene expression data

Validation Cohort 1 includes all 123 ER-positive/HER2-negative tumors from the original ACES cohorts [19]. Patients prospectively provided written informed consent to participate in an institutional review board-approved research protocol [19]. Patients received neoadjuvant taxane–anthracycline-based chemotherapy and endocrine therapy. Detailed cohort characteristics have been previously published [19]. Gene expression data were generated from baseline fine needle aspiration specimens using Affymetrix Human Gene U133A GeneChips [19]. Validation Cohort 2 ($n = 155$) was obtained from Goethe-University, Frankfurt, Germany and the University Hospital Hamburg-Eppendorf, Germany [22, 23]. Informed consent was obtained from all patients for use of tissue materials as approved by the local ethics committees (Ethik-Komission der Goethe-Universität Frankfurt and Ethik-Komission der Ärztekammer Hamburg). These samples were identified as ER-positive/HER2-negative tumors that were treated with combined endocrine and chemotherapy and had follow-up information available. Tumors were considered ER-positive if at least 10 % of the cells had positive ER staining. Biopsies were snap-frozen in liquid nitrogen. RNA extraction and gene expression profiling from tissue biopsies were performed with Affymetrix U133A gene chips using standard protocols in a different laboratory. Gene expression data was provided blindly to C.H and S.S.K. Of these, 127 ER-positive/HER2-negative cases were selected for the analysis for which microarray-based ER and HER2 status determination based on previously defined cutoffs [19] matched the clinical ER and HER2 status provided. Eighteen patients who where HER2 positive by microarray gene expression were excluded from the analysis. The 127 patients that remained in Cohort 2 all received endocrine therapy, 22 % received combined taxane and anthracycline-based regimen, 68 % had an anthracycline but not taxane, and 10 % received non-anthracycline or taxane chemotherapy. Gene expression data have been deposited into the Gene Expression Omnibus database (GSE46184, GSE4611), and GSM accession numbers are given in Supplementary Table S7.

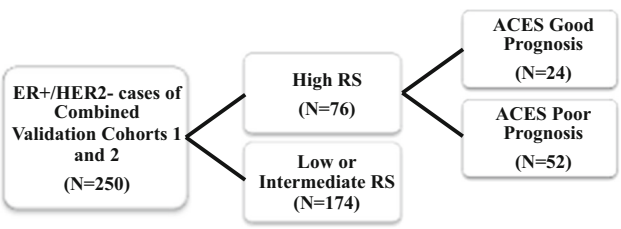

Fig. 1 Analysis strategy for secondary stratification of high recurrence score ER-positive tumors by ACES outcome predictor

Data analysis and generation of predictions

All raw data from microarrays were processed using Bioconductor (www.bioconductor.org) and R (www.r-project.org, 2.10.1) using custom R programs. Since sample preparation and microarray protocols differed between validation cohort 1 and 2, outcome-blinded normalization was performed to make measurements comparable [24]. Cases in validation cohort 2 were stratified by T-stage, and classification thresholds were adjusted so that the frequency of the predicted ACES subcomponent classes matched the frequency in the previous cohorts (combined validation cohort 1 and ACES discovery cohort). Further details on the normalization strategy are provided in the Supplementary Methods. "High risk" cases were defined as high RS classification by Oncotype DX, using the previously published genomic proxy-version of the test [19, 25]. ACES was applied to high RS cases, and the resulting ACES predicted good prognosis and poor prognosis groups were compared with Kaplan–Meier survival analysis using the log-rank test. Distant relapse was the outcome measure for validation cohort 1 and any relapse for validation cohort 2. Multivariate Cox regression models were used to adjust the risk associated with ACES for other clinical prognostic variables that included age, T-stage, and nodal status at time of diagnosis. "In addition to RS," luminal B subclass by PAM50 and high GGI were also used to define "high

risk". Results for these are presented in the Supplement. For the sake of completeness, we also performed the same tertiary risk stratification analysis on the original ACES discovery data set, and these results are included in Supplementary Table S1-6 and Figure S1.

Results

Patient characteristics

Patient demographics and tumor characteristics for the two validation cohorts are presented in Table 1. Validation cohort 2 had more T1 ($p < 0.001$) and lower grade (grade 1 or 2) tumors ($p < 0.001$) and fewer lymph node-positive cases ($p = 0.005$) compared to validation cohort 1. We used a normalization method to adjust for the impact of these imbalances in the distribution of clinical prognostic variables on RFS. Thirty percent ($n = 76$) of cases in the combined validation set (pooled cohorts 1 and 2, $n = 250$) had high RS (Table 1).

ACES prediction of residual risk

ACES classified 24 (32 %) of the high RS cases as good prognosis in the pooled validation cohorts. This proportion did not differ from the proportion of all ER-positive tumors regardless of RS, predicted by ACES to have good prognosis. The Kaplan–Meier plot for risk stratification by the ACES predictor of the high RS tumors is shown in Fig. 2. Corresponding plots for additional risk stratifiers are shown in Figure S1. The median follow-up time was 3 years (range 0.1–7 years) for validation cohort 1, 5 years (range 0.4–10 years) for validation cohort 2, and 4 years (range 0.1–10 years) for the combined cohort. A significant difference in RFS was seen between the ACES predicted good and poor prognosis groups among the high RS cases of the combined validation cohort (log-rank test $p = 0.033$). The 5-year RFS in the ACES good prognosis and poor prognosis groups were 95 % (95 % CI 72–99 %) and 76 % (95 % CI 59–87 %), respectively (Table S3). The absolute risk reduction (ARR) at 5 years for the treatment sensitive group is 19 % (95 % CI 2–36 %).

Factors associated with RFS

We evaluated the independent prognostic information of ACES in multivariate Cox Proportional Hazards regression analysis (Table 2). The model included ACES along with patient age, T-stage of the cancer, and nodal status at diagnosis. Among the high RS ER-positive cases, the ACES risk predictor showed a strong association with RFS, which was only borderline insignificant (HR 0.15, $p = 0.072$). Being predicted as good prognosis by ACES indicated a more than 6-fold reduction in relapse risk than if predicted poor prognosis. As expected, nodal involvement was also significantly associated with RFS (HR 5.35, $p = 0.047$).

Performance of ACES algorithm

Table 3 presents the predictive performance of ACES in the high RS group for predicting patient relapse status at 5 years. The sensitivity of 91 % (95 % CI 59–100 %) and negative predictive value (NPV) of 95 % (95 % CI 87–100 %) were higher compared to the specificity of 35 % (95 % CI 24–48 %) and positive predictive value (PPV) of 24 % (95 % CI 9–37 %) in the combined validation cohorts. The negative diagnostic likelihood ratio (DLR−) was significant at 0.22 (95 % CI 0.01–0.83), indicating a one-fifth fold reduction in relapse among the ACES predicted sensitive group relative to the predicted insensitive group. The positive diagnostic likelihood ratio (DLR+) was 1.48 (95 % CI 0.60–2.80), but it did not reach significance. The odds ratio (OR) (DLR+/DLR−) was 6.64 (95 % CI 1.17–248). These performance measures did not differ from those previously reported for all ER-positive cases, indicating that ACES performed equally well in both low and high risk ER-positive patients [19].

Subcomponents of ACES

We also examined what response status the subcomponents of ACES assigned to the high RS cases. Compared to all ER-positive cases, a lower proportion of high RS tumors were predicted to have high or intermediate sensitivity to endocrine therapy by the SET index ($p = 0.037$; Table 4), and more cases were predicted to have relapse or death within 3 years of diagnosis ($p = 0.011$). The pathologic response predictors did not show a different distribution between only high RS cases and all ER-positive cases. The dominant subcomponents of ACES that drive the re-stratification of high RS cases into better and worse outcome groups are the SET Index and the gene signature for early relapse or death. When high RS cases were stratified by SET Index alone, none of the 7 cases predicted to have high/intermediate SET class had relapsed. In contrast, 19 % of the tumors predicted to have low sensitivity to endocrine therapy had relapse. When high RS cases were stratified by the early relapse/death gene signature, there was a trend toward lower RFS among the group predicted to have early relapse or death. However, these trends were not statistically significant. Stratification by the RCB-0/I or RCB-III subcomponent predictors did not show difference in RFS.

Table 1 Patient pre-treatment characteristics of estrogen receptor-positive cohorts used in this study

Characteristic	Validation cohort 1 *N* (%)	Validation cohort 2 *N* (%)	Odds ratio[a]	*p* value[a]
Cohort size	123	127	–	–
Age				
<50	64 (52)	63 (50)	1.10	0.706
≥50	59 (48)	64 (50)	–	–
Mean (SD)	50 (10)	51 (10)	–	–
T-stage				
1	8 (7)	43 (34)	0.14	**<0.001**
2	54 (44)	69 (54)	–	–
3	41 (33)	9 (7)	–	–
4	19 (15)	6 (5)	–	–
Unknown	1 (1)	0	–	–
Nodal status				
Negative	46 (37)	69 (54)	2.05	**0.005**
Positive	77 (63)	57 (45)	–	–
Unknown	0	1 (1)	–	–
AJCC stage				
I	1 (1)	–	–	–
II	51 (41)	–	–	–
III	45 (37)	–	–	–
Unknown	26 (21)	127 (100)	–	–
Grade				
1	10 (8)	12 (9)	0.35	**<0.001**
2	53 (43)	83 (65)	–	–
3	54 (44)	32 (25)	–	–
Unknown	6 (5)	0	–	–
PR status				
Negative	30 (24)	–	–	–
Positive	93 (76)	–	–	–
Unknown	0	127 (100)	–	–
Oncotype DX				
High RS	39 (32)	37 (29)	1.13	0.682
Intermediate	9 (7)	31 (24)	–	–
RS	75 (61)	59 (46)		–
Low RS	–	–	–	–

[a] Odds ratio and *p* value reflect the results of Fisher's exact test when age, T-stage, lymph node involvement, and pathologic grade at time of diagnosis were compared between the validation cohort 1 and 2

SD standard deviation, *AJCC* American Joint Committee on Cancer, *PR* progesterone receptor

Bold face indicates significance at the 95 % level

Discussion

We demonstrated that it is possible to identify a group of currently "high risk" ER-positive cancers that are no longer high risk for recurrence after completion of adjuvant chemotherapy in addition to surgery and endocrine therapy. A statistically significant difference in RFS was found between the ACES predicted good and poor prognosis groups among cancers that all had high RS using a genomic surrogate for Oncotype DX (log-rank test p = 0.033). We also applied two other secondary risk stratification methods including the PAM50 luminal B class and the GGI high-grade class to define baseline "high risk" status. These results are presented in the supplementary files, along with assessment within the original ACES development cohort, which also support a tertiary risk stratification function for ACES.

Cox regression analysis showed a sizeable but statistically borderline insignificant effect for ACES (HR 0.15,

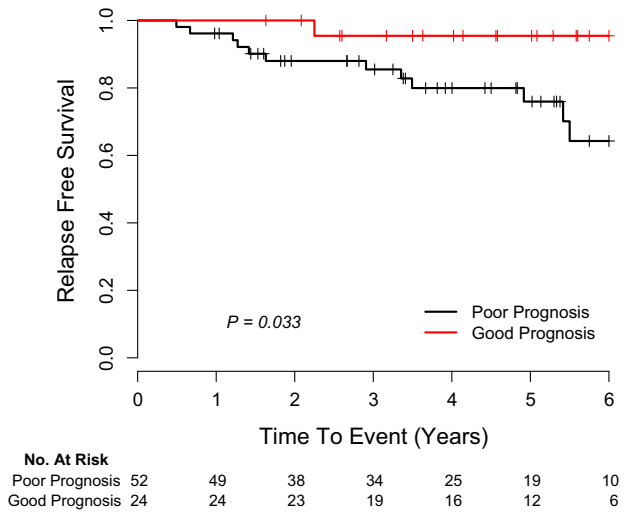

Fig. 2 Kaplan–Meier estimator of relapse-free survival in ACES stratified high recurrence score cases in the combined validation cohort. Validation cohort 2 was normalized as described in "Methods" section. *Vertical marks* indicate censored observations. *P* value is from the log-rank test

Table 2 Multivariate cox proportional hazards analysis of association with relapse-free survival for high recurrence score estrogen receptor-positive tumors

Prognostic variables	High RS cases in validation cohorts 1 and 2[a] ($N = 76$)	
	HR[b] (95 % CI)	*p* value
Age	1.53 (0.6–4.3)	0.415
T-stage	1.46 (0.4–5.0)	0.554
Nodal status	**5.35 (1.0–28)**	**0.047**
ACES	**0.15 (0.0–1.2)**	**0.072**

[a] Normalized

[b] The hazard ratio (HR) measures the relative risk of relapse

Bold face indicates significance at the 95 % level

$p = 0.072$) after adjusting for patient age, T-stage, and nodal status at time of diagnosis. It should be noted that the power of this analysis is severely limited to demonstrate significant effect in a multivariate analysis due to the small sample size which was determined by the availability of samples. Due to low numbers of high risk ER-positive cases, two independent validation cohorts, not included in the development of ACES, were pooled to increase the power of the study, which required additional normalization and scaling steps to adjust for the heterogeneous cohorts. Small differences in sample collection and preparation protocols can also lead to large deviations in microarray results [26]. Blinded assessment of different normalization strategies showed that standardization of the distributions of ACES subcomponents within strata defined by T-stage was the most successful to make the two

Table 3 Performance of ACES in predicting a patient's relapse-free status at 5 years in high recurrence score, endocrine receptor-positive tumors ($N = 76$)

Measures	High RS cases in validation cohorts 1 and 2[a] Value (95 % CI)
Sensitivity	0.91 (0.59–1)
Specificity	0.35 (0.24–0.48)
PPV	0.24 (0.09–0.37)
NPV	0.95 (0.87–1)
DLR+	1.48 (0.60–2.80)
DLR−	**0.22 (0.01–0.83)**
OR	6.64 (1.17–248)

[a] Normalized

Bold face indicates significance at the 95 % level

validation cohorts compatible. Validation cohort 2 also differed in the types of chemotherapeutic agents used; some patients received only anthracycline-based chemotherapy, and few received neither taxane nor anthracycline chemotherapies. ACES has previously been shown to predict well in combined taxane–anthracycline-containing regimen [19]. The outcome metric for validation cohort 2 was any relapse-free survival, while it was distant relapse-free survival in validation cohort 1.

Despite these limitations of the data sets and the modest sample size of the combined validation cohort, the sensitivity and negative predictive value of ACES in predicting RFS among high RS tumors were high: 91 % (95 % CI 59–100 %) and 95 % (95 % CI 87–100 %) respectively. The clinical relevance of this study is that with further validation of ACES on a significantly larger data set, patients with low residual risk by ACES can be safely treated with current adjuvant chemotherapies and reassured about their prognosis, while patients who remain at substantial risk for relapse or death despite receiving the current standard of care multimodality systemic adjuvant therapies, can be encouraged to enroll into clinical trials that aim to improve the efficacy of current therapies. Further molecular characterization of these truly high risk ER-positive cancers could also lead to the discovery of new drug targets for the very patient population who needs novel therapies in order to improve survival. Costs may be saved if research trials are enrolling only the specific subset of patients for whom improvements in therapy are needed and by avoiding over-treatment with therapeutic regimens of limited value [27, 28].

This study placed ACES, the first tertiary residual-risk predictor for ER-positive patients who receive both endocrine and chemotherapies, into the context of the most commonly used current predictor of risk in the clinic, Oncotype DX. Retrospective analysis of two independent validation cohorts in this study provides initial evidence to suggest that ACES may further risk stratify high RS tumors into those with low

Table 4 Distribution of sub-components of ACES algorithm in the pooled validation cohort

ACES subcomponent	High RS cases in validation cohorts 1 and 2[a] *n* (% of $N = 76$)	All ER-positive cases in validation cohorts 1 and 2[a] *n* (% of $N = 250$)
SET-high/intermediate	7 (9)	50 (20)
Predicted early relapse/death	34 (45)	71 (28)
Predicted RCB-III	30 (39)	112 (45)
Predicted RCB-0/I	40 (53)	112 (45)
ACES Rx sensitive	24 (32)	91 (36)

[a] Normalized

and high residual risk after adjuvant chemotherapy and endocrine therapy. These results indicate that future independent validation of ACES should be pursued in an adequately powered prospective–retrospective study.

Conclusions

This study demonstrates the feasibility of developing tertiary risk stratifiers that estimate the residual risk of recurrence for early stage estrogen receptor (ER)-positive breast cancers who receive both adjuvant endocrine and chemotherapies and were initially considered high risk for recurrence if treated with endocrine therapy alone. Primary prognostic predictors that estimate prognosis in the absence of any systemic therapy (e.g., MammaPrint) and secondary residual-risk predictors that estimate prognosis after receiving adjuvant endocrine therapy (e.g., Oncotype DX) have been developed. ACES is the first example of a tertiary residual-risk predictor which estimates residual risk after both endocrine and chemotherapies. "High risk" patients by primary and secondary prognostic predictors who are found to have low risk of recurrence after re-stratification by ACES are more likely to have favorable prognosis with current best therapies. Those with continued high risk after standard chemo-endocrine treatment may benefit from prolonged endocrine treatment [29] or could be encouraged to explore alternate therapeutic options such as combination of aromatase inhibitors with ovarian suppression, if pre-menopausal [30], or other novel therapeutic strategies .

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Conflict of interest The authors declare that they have no conflict of interest.

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