“A novel MAPK-microRNA signature is predictive of hormone-therapy resistance and poor outcome in ER-positive breast cancer”

Philip C. Miller\textsuperscript{3,4}, Jennifer Clarke.\textsuperscript{1,2,3,4,5} Tulay Koru-Sengul,\textsuperscript{2,3} Joeli Brinkman\textsuperscript{3}, and Dorrya El-Ashry\textsuperscript{1,3,4}.

University of Miami Miller School of Medicine, Miami, FL 33136

Departments of Medicine\textsuperscript{1}, Epidemiology and Public Health\textsuperscript{2}, Sylvester Comprehensive Cancer Center\textsuperscript{3}, Braman Family Breast Cancer Institute\textsuperscript{4}, and University of Nebraska-Lincoln\textsuperscript{5}

Corresponding Author: Dorrya El-Ashry, Department of Medicine, University of Miami Miller School of Medicine, Sylvester Comprehensive Cancer Center and Braman Family Breast Cancer Institute, Miami, FL 33136 305-243-4721 del-ashry@med.miami.edu

Abbreviations used:
MAPK – Mitogen Activated Protein Kinase

EGFR – Epidermal Growth Factor Receptor

hMAPK - hyperactive MAPK

ERα – Estrogen Receptor Alpha

PR - Progesterone Receptor

miRNA – microRNA

RPPA – Reverse Phase Protein Array
Translational Relevance

Activated signaling through the ERK1/2 MAPK axis is associated with estrogen receptor (ER) negativity and poor outcome in breast cancer. microRNA profiling is a powerful tool complementing gene expression analysis, and provides additional insight into the biodiversity of breast cancer, helping to further stratify existing breast cancer classifications into more discrete and coherent subtypes. Our study defines a microRNA signature associated with hyperactive ERK1/2 MAPK signaling and investigates associations with genetic and molecular alterations, pathological features and clinical outcomes among primary breast tumors. We report that a majority of ER-negative tumors possess this microRNA signature, as well as a group of ER+ tumors which exhibit molecular and genetic characteristics similar to ER- tumors, and which display significantly poorer clinical outcomes, including poorer responses to hormone therapy. This suggests that these microRNAs may be important in facilitating ER negativity and poor outcomes in breast cancer, and may be predictive of resistance to hormone therapy.
ABSTRACT

Purpose: Hyperactivation of ERK1/2 MAPK (hMAPK) leads to loss of estrogen receptor expression and poor outcome in breast cancer. microRNAs play important regulatory roles and serve as biomarkers of disease. Here we describe molecular, pathological, and clinical outcome associations of an hMAPK-microRNA expression signature in breast cancer.

Experimental Design: An hMAPK-microRNA signature was identified, and associations of this signature with molecular and genetic alterations, gene expression, pathological features, and clinical outcomes were determined in primary breast cancers from training data and validated using independent data sets. Univariate and multivariate analyses identified subsignatures associated with increased disease recurrence and poorer disease survival among ER-positive patients, respectively.

Results: High-hMAPK-microRNA status significantly correlated with ER-negativity, enrichment for Basal and HER2-subtypes, and reduced recurrence-free and disease-specific survival in publicly available data sets. A robust determination of a recurrence-signature and a survival-signature identified hMAPK-miRNAs commonly associated with poor clinical outcome, and specific subsets associated more closely with either disease recurrence or disease survival, especially among ER+ cancers of both luminal A and luminal B subtypes. Multivariate analyses indicated these recurrence and survival signatures significantly associated with increased risk of disease-specific death and disease recurrence in ER+ cancer and ER+ cancers treated with hormone therapy.
Conclusions: We report an hMAPK-microRNA signature and two subsignatures derived from it which associate significantly with adverse clinical features, poor clinical outcome, and poor response to hormone therapy in breast cancer, thus identifying potential effectors of MAPK signaling, and novel predictive and prognostic biomarkers or therapeutic targets in breast cancer.
Introduction

Treatment decisions in breast cancer are informed not only by tumor grade, stage, and lymph node status, but also by genomic biomarkers, estrogen receptor (ER) and HER2. Increasingly, multi-gene assays such as Oncotype DX and Mammaprint are being used in prognostic capacities for breast cancer patients (1, 2). ER+ tumors have better overall prognosis and response to hormonal therapy, while ER- tumors are more aggressive, resistant to hormonal therapies, and frequently display elevated expression of growth factor receptors such as erbB family members, EGFR or HER2 (3-5). Overexpression or amplification of HER2 occurs in approximately 25% of breast cancers, while EGFR overexpression occurs in up to 30% of breast cancers. Both are poor prognostic indicators independent of ER status; EGFR expression has an inverse correlation with ER and is more common in triple negative and basal breast cancers (6),(7, 8), while HER2 amplification tends to correlate with ER-negativity or lower levels of ER expression. Downstream effector pathways activated by erbB family signaling include the RAS/RAF/MEK/ERK, PI-3K/AKT/MTOR, and the MEKK1/SEK1/JNK pathways. Over the past decade, prominent roles for PI-3K/AKT/MTOR signaling in the biology of breast cancer have been established, in particular downstream of HER2 (ERBB2: gene product: HER2 protein product) signaling and in tamoxifen resistance mechanisms (9-11).

We have previously examined the role of hyperactivation of ERK1/2 MAPK (hMAPK) occurring downstream of EGFR or HER2 in breast cancer. We established that hMAPK induces loss of ER expression, and that inhibition of hMAPK in ER- breast cancer cell lines and primary cultures from ER- breast tumors restores ER expression, suggesting that hMAPK plays a direct
role in establishing the ER- phenotype in breast cancer (12-14). mRNA expression profiling of these hMAPK cell lines defined an “hMAPK gene expression profile” which strongly correlates with ER- status in clinical samples, establishing a link between hMAPK and the transcriptomes of ER- breast tumors (15).

MicroRNAs play critical regulatory roles in a wide range of biological and pathological processes, and microRNA expression profiling reveals substantial differences between cancer and normal tissue, and among different cancer types (16-18). In the context of breast cancer, microRNA expression has been correlated with numerous biopathological features such as tumor grade, ER, PR, and HER2 status (19-21), TP53 mutation status, or proliferation status (22), and has also been used to classify the breast cancer subtypes identified by gene expression profiling (23). MicroRNA expression studies have demonstrated the utility for microRNA profiles as disease classifiers and prognostic tools in breast cancer (24, 25). Functional studies indicate that microRNAs regulate important genes and processes germane to breast cancer pathology, and are effectors of disease-associated signaling pathways (26-30).

In the present study, we identify microRNAs associated with hyperactivation of ERK1/2 MAPK signaling and investigate their involvement in facilitating gene expression, adverse clinical features, poor clinical outcome, and poor response to hormone therapy. Analysis of gene and protein expression, molecular alterations, and pathway enrichment of gene targets of these hMAPK-microRNAs identifies microRNAs which may potentially act as important regulators of MAPK signaling and downstream pathways, and which may serve as novel biomarkers or therapeutic targets in the treatment of breast cancer. Multivariate analysis
indicates that discrete signatures made up of subsets of these hMAPK-microRNAs are independently associated with increased risk of disease specific death and disease recurrence, particularly among patients with ER+ disease and in ER+ cancers treated with hormone therapy.

Materials and Methods

Clinical datasets

The following publicly available breast cancer datasets were used in this study: GSE22220 (Buffa dataset, accessed 4/2012)(31), GSE19536 (Enerly dataset, accessed 4/2012)(22), The Cancer Genome Atlas (TCGA, accessed 4/2012)(32), Lyng (GSE37405 accessed 5/2012)(33), and the METABRIC breast cancer dataset (accessed 7/2013)(34, 35). Patient populations have been previously described in detail (22, 31-35). Enerly and Lyng cohorts have previously been employed as validation or comparison datasets for identification of novel roles of microRNAs in breast cancer(35-40).

Gene and microRNA expression datasets

Gene and/or microRNA expression data for Buffa, Enerly, and Lyng datasets were retrieved from NCBI Gene Expression Omnibus. Gene expression, DNA copy number, RPPA protein expression, microRNA expression, and mutation status for breast cancers from the TCGA dataset were obtained using the CGDS-R package v 1.1.30. microRNA expression for the METABRIC dataset was retrieved from the European Genome-phenome Archive.

Signature generation and statistical analysis
mRNA expression and microRNA expression values were standardized by scaling expression of an individual mRNA/microRNA within each individual dataset to have mean 0, standard deviation 1. Buffa and TCGA datasets were used as training datasets for generating the hMAPK microRNA signature. Cancers from each dataset were classified as high-hMAPK-mRNA or low-hMAPK-mRNA by Pearson's correlation to an idealized hMAPK mRNA signature as performed by Creighton et al. (15). Briefly, for the ideal signature genes upregulated in the signature were given an arbitrary value of "1", and genes downregulated in the signature given an arbitrary value of "-1"; Pearson's correlation between the ideal signature and z-score standardized gene expression for breast cancers from the training and validation datasets were determined and cancers with positive Pearson's correlation were classified as "high-hMAPK-mRNA" while cancers with negative Pearson's correlation were classified as "low-hMAPK-mRNA".

MicroRNAs significantly differentially expressed ($p \leq 0.05$) between breast cancers from training datasets classified as hMAPK-mRNA or not-hMAPK-mRNA were identified by student’s t-test. P-values for expression differences between groups were permutation-adjusted by randomly assigning each sample to a group (creating a random group assignment for each sample) and performing a Student’s t-test for between-group differences for each gene; this was repeated 1000 times, and the rank of the p-value for each individual gene from the non-permuted analysis was divided by 1001, resulting in the permutation-adjusted p-value (for example: if a non-adjusted p-value of $p=0.022$ ranked 34$^{th}$ lowest, the adjusted p-value would = $34/1001 = 0.0339$) (41). This procedure is effectively a Westfall-Young correction for multiple comparisons (42). Only microRNAs that were commonly differentially expressed between
hMAPK-mRNA tumors and not-hMAPK-mRNA tumors from both the Buffa and TCGA training datasets comprised the total hMAPK-microRNA signature.

Correlation to ideal hMAPK-microRNA signature was performed for all available primary breast cancers from both training and validation datasets, as described for hMAPK-mRNA signature above. Differential mRNA expression between high-hMAPK-microRNA and low-hMAPK-microRNA cancers from TCGA training dataset, and likewise from METABRIC validation dataset, was analyzed by student’s t-test.

MicroRNA target prediction was performed using the miRWalk database(40), a database that uses 10 different microRNA target prediction programs to identify putative microRNA targets. Genes were considered putative targets of hMAPK-microRNAs if they were predicted to be a target by 3 or more of the following prediction programs: DIANA-mt, miRanda, miRDB, miRWalk, RNAhybrid, PICTAR4, PICTAR5, PITA, RNA22, or TargetScan. Pearson’s correlation of hMAPK-microRNA target expression to hMAPK-microRNA expression was performed to identify regulatory relationships that may differ between cancers classified as high-hMAPK-microRNA and those classified as low-hMAPK-microRNA. Standard statistical tests were performed, as described in supplemental methods. All statistical analyses were performed using R statistical software v 2.15.2 (43). Microsoft Excel 2007 and R statistical software v 2.15.2 were used for figure generation. All t-tests performed were 2-sided.

To generate a hMAPK-microRNA recurrence signature, univariate analyses were first performed on the total hMAPK-microRNA signature to determine if expression of individual microRNAs (median cutoff) was associated with increased rate of disease recurrence in the Buffa training dataset. Breast cancers from the Buffa dataset were then partitioned into 2
groups based on median expression of every hMAPK-microRNA on an individual basis. Using this grouping as a classifier, Kaplan-Meier survival curves were generated for disease recurrence, and logrank test p-values were determined; 22 microRNAs for which this logrank test p-value was ≤ 0.1 were included in the recurrence signature. Correlation to an ideal hMAPK-recurrence signature was determined as described above for mRNA signature analysis.

To generate an hMAPK-microRNA survival signature, primary Breast Cancers from the METABRIC dataset were first randomized into 2 populations. A leave-one-out analysis was then performed, sequentially removing single individual microRNAs from the hMAPK microRNA signature, and determining whether removal of each microRNA improved the ability of the signature to predict poor survival events in ER+ population at 5 years by Cox Proportional Hazards analysis, separately for each randomized population. The analysis was limited to the ER+ population due to the fact that the overwhelming majority of the ER-negative cancers were already classified as "high hMAPK". miRNAs whose removal improved the model in both randomized populations from the METABRIC dataset were tabulated. This randomization and leave-one-out analysis was repeated in 1000 random permutations of the dataset to ensure robustness and serve as a self-validation. MicroRNAs whose removal improved the model in more than 20% of randomizations were removed from the signature, establishing a 21-member refined hMAPK-microRNA signature. Correlation to an ideal hMAPK-microRNA survival signature was determined as described above for mRNA signature analysis.

**microRNA expression array and qRT-PCR analysis**
microRNA expression array and qRT-PCR analysis of cell lines were performed according to standard practices, and are described in Supplemental Materials and Methods.

Results

hMAPK-mRNA signature associates with adverse features of breast cancer and poor clinical outcome

We previously observed that the hMAPK-mRNA signature is present in a majority of ER-breast cancers and a small subset of ER+ breast cancers (13, 14). We confirmed that the hMAPK-mRNA signature was similarly represented in the primary breast tumor datasets with paired mRNA-microRNA expression data analyzed in this study [The Cancer Genome Atlas breast cancer samples: “TCGA” (training cohort); Gene Expression Omnibus (GEO) GSE22220: “Buffa dataset” (training cohort); GEO GSE19536: “Enerly dataset” (validation cohort); METABRIC microRNA dataset: "METABRIC" (validation cohort); datasets previously described (22, 31, 32, 34, 35); clinical characteristics in Supplemental Table 1]. Tumors in each of these datasets classified as “high-hMAPK-mRNA” according to our hMAPK-mRNA signature(15) exhibited a significant association with ER-negativity in training and validation datasets (Figure 1A,1B). hMAPK-mRNA classification associated with a trend towards increased incidence of disease recurrence in the training dataset (Figure 1C), and increased incidence of disease recurrence and poorer disease-specific survival in the validation datasets (Figure 1D).

Generation and characterization of a breast cancer hMAPK-microRNA signature
We identified 16 commonly underexpressed and 41 commonly overexpressed microRNAs in tumors classified as hMAPK-mRNA from both training cohorts (p-value ≤ 0.05, See Supplemental Figure 1 for study overview), establishing a 57 member hMAPK-microRNA signature (Supplemental Table 2). Primary breast cancers from training and validation datasets were classified as "high-hMAPK-microRNA" or "low-hMAPK-microRNA" according to this microRNA signature. To confirm that this hMAPK-microRNA signature identifies tumors with activated ERK1/2 MAPK signaling, we analyzed the expression of several genes regulated by ERK1/2 in breast cancer in the TCGA training cohort. Genes known to be downregulated by ERK1/2 signaling (ESR1, CDKN1B, TOB1, PDCD4) are underexpressed in a higher proportion of high-hMAPK-microRNA tumors and, similarly, genes upregulated by ERK1/2 (ETV5, RPS6KA4, CREB1, SRF, ANGPTL4, ETV1, ELK4, ATF4, RPS6KA1) are overexpressed in a higher proportion of high-hMAPK-microRNA cancers compared to low-hMAPK-microRNA cancers (Figure 2A). We also investigated mutational status, alterations in DNA copy number, and expression of upstream regulators of ERK1/2 signaling (Figure 2B). Alterations indicative of activation of ERK1/2 MAPK signaling pathways, such as enhanced expression of EGFR, ERBB2, SOS1, NRAS, RAF1, and MAPK1 and lower expression of ERBB3 and RKIP, are seen in a higher proportion of high-hMAPK-microRNA cancers compared to low-hMAPK-microRNA cancers. Gene set enrichment analysis of predicted targets of hMAPK-microRNAs identifies substantial enrichment for genes involved in the MAPK signaling pathway (Supplemental Figure 2A-B), suggesting that a number of these hMAPK-microRNAs may not only be regulated by MAPK signaling, but also contribute to sustaining hyperactivation of ERK1/2 MAPK signaling. Analysis of protein expression data from the TCGA dataset available for breast cancers with microRNA
expression data revealed significant differences in expression and phosphorylation of numerous proteins between high-hMAPK-microRNA and low-hMAPK-microRNA tumors, including proteins involved in or regulated by ERK1/2 MAPK signaling, as well as EMT proteins, protein markers of basal-like breast cancer, and proteins involved in mediating tamoxifen resistance (Supplemental Table 3).

To study the impact that altering MAPK signaling has on miRNA expression in established models of hyperactive MAPK signaling in breast cancer we altered MAPK signaling in our stable hMAPK cell lines (15) and investigated effects on select microRNAs from the hMAPK-microRNA signature that had been previously implicated in breast tumor biology, or that have predicted targets in genes validated by Creighton et al. to be regulated by MAPK signaling (15). In particular, we treated ca-RAF/MCF-7 (which exhibit constitutive activation of MAPK signaling) with a MEK inhibitor to abrogate MAPK signaling, and in a complementary approach we stimulated MAPK signaling in EGFR/MCF7 cell line (which overexpresses EGFR and is a ligand-inducible model for hyperactivation of MAPK signaling). Several hMAPK-microRNAs were altered in their expression by abrogating or inducing hMAPK (Supplemental Figure 3A-B): overexpressed hMAPK-microRNAs significantly decreased following abrogation of MAPK signaling with U0126 treatment (hsa-miR-221, hsa-miR-222) or increased following EGF treatment (hsa-miR-378). Likewise, underexpressed hMAPK-microRNAs were increased following abrogation of MAPK signaling with U0126 treatment (hsa-let-7a, hsa-miR-30a, hsa-miR-30a*, hsa-miR-125a-5p, hsa-miR-375) or decreased following EGF treatment (hsa-let-7a, hsa-let-7e, hsa-miR-29c, hsa-miR-30c), indicating that these microRNAs are regulated by MAPK signaling. We observed that target genes of these validated hMAPK-microRNAs are
differentially expressed between tumors classified as high-hMAPK-microRNA compared to those classified as low-hMAPK-microRNA in the TCGA training dataset (Figure 2C-D), and verified these relationships in the METABRIC validation dataset (Supplemental Figure 4A-F). Additionally, we observed differential protein expression of targets of hMAPK-microRNAs in breast cancers from the TCGA dataset classified as “high-hMAPK” vs “low-hMAPK” by our microRNA signature, and inverse relationships between expression of microRNAs and protein expression of selected gene targets in the TCGA dataset, suggesting that differential expression of these microRNAs may have a regulatory impact on the protein expression of their predicted gene targets (Supplemental figure 5 A-H).

**hMAPK-microRNA signature is associated with adverse clinical features of breast cancer**

Cancers classified as high-hMAPK-microRNA in the TCGA and Buffa training data were enriched for ER-negative status (TCGA p-value = 4.308e-16, Buffa p-value = 8.508e-14, Supplemental Figure 6A), high tumor grade (Buffa p-value= 7.711e-11, Supplemental Figure 6B), and basal-like or HER2+ PAM50 molecular subtypes (TCGA p-value < 2.2e-16, Figure 3A, top). High-hMAPK-microRNA cancers also displayed significantly earlier disease recurrence among all patients (Buffa p= 0·00242, Figure 3B); segregation of patients by cancer ER status revealed significant trends for earlier disease recurrence among patients with ER+ disease (p-value = .0835) and ER- disease (p-value = 0.167)(Figure 3B).

In the Enerly and METABRIC validation datasets, the hMAPK-microRNA signature was significantly associated with cancers that are ER-negative (p-values: Enerly = 2.728e-08; METABRIC < 2.2e-16, Supplemental Figure 7A), higher grade (p-values: Enerly = 1.198e-09;
METABRIC < 2.2e-16, Supplemental Figure 7B), classified by a high proliferation metric (described by Enerly et al. (22); p-value = 2.218e-05, Supplemental Figure 7C), HER2+ (p-values: Enerly = 0.02115, METABRIC < 2.2e-16, Supplemental Figure 7D), and Basal and HER2+ PAM50 subtypes (p-values: Enerly = 9.621e-16, METABRIC < 2.2e-16, Figure 3A, middle and bottom). Survival analyses indicate that, among all patients, cancers classified as high-hMAPK-microRNA demonstrated significantly increased disease recurrence (p-value: Enerly = 0.0119, Figure 3C, top) and decreased disease-specific survival (p-values: Enerly = 0.00194; METABRIC = 6.28e-06, Figure 3C, top) at 5 years. Stratifying these analyses by ER status indicates that classification as high-hMAPK-microRNA identifies a population of ER+ cancers with significantly increased disease recurrence (Enerly: p-value = 0.0059, Figure 3C, bottom) and significantly poorer disease specific survival (Enerly: p-value = 0.0191; METABRIC p-value = 0.0818, Figure 3C, bottom).

**hMAPK-microRNAs associated with recurrence identify a hMAPK-microRNA recurrence signature**

By limiting the signature to microRNAs whose individual expression was significantly associated with reduced recurrence-free survival on univariate analysis among patients from the Buffa training dataset, we identified a 22 member hMAPK-microRNA recurrence signature (Supplemental Table 4). Cancers from the Buffa dataset classified as high-hMAPK-microRNA by this recurrence signature demonstrated significantly reduced recurrence-free survival at 5 years in all patients (p-value = 5.52 e-06, Supplemental Figure 8) and in ER+ and ER- specific cohorts (ER-positive p-value = 0.00117; ER-negative p-value = 0.0177, Supplemental Figure 8). In the
Enerly validation dataset, classification by this recurrence signature significantly associated with reduced recurrence-free survival among all patients and patients with ER+ disease (p-values = 0.015 and 0.0523, respectively, Figure 4A). In the Enerly and METABRIC datasets, high-hMAPK-microRNA status also significantly associated with shorter disease-specific survival in all patients (p-values: Enerly = 0.00121, Figure 4B; METABRIC = 4.49e-10, Figure 4C) and patients with ER+ disease (p-values: Enerly = 0.0672, Figure 4B; METABRIC = 0.00144, Figure 4C).

Cancers from the Lyng ER+ dataset (GSE37405) classified as high-hMAPK-microRNA by this recurrence signature demonstrated significantly earlier disease recurrence among breast cancers treated with adjuvant tamoxifen monotherapy (p= 0.00348, Figure 4D). ER+ cancers from the METABRIC dataset receiving hormone therapy, either alone or in conjunction with chemotherapy or radiotherapy, that were classified as high-hMAPK by the hMAPK-microRNA recurrence signature demonstrated significantly poorer disease specific survival (p-value = 0.00301 Figure 4D).

**Multivariate Analysis of hMAPK-microRNA recurrence signature**

Multivariate analysis of the hMAPK-microRNA recurrence signature in the METABRIC dataset using Cox Proportional Hazards analyses revealed that among all patients, high-hMAPK-microRNA status is associated with a significant increase in risk of breast cancer specific death within 5 years from diagnosis (multiplicative hazard factor: 1.48, 95% CI: 1.002-2.175, p-value = 0.04886, Supplemental Table 5A). Because we observed that classification as high-hMAPK-microRNA by the hMAPK-microRNA recurrence signature was significantly associated with poor outcome in ER+ breast cancers, and ER+ breast cancers displayed a large variation in correlation
values with this recurrence signature ranging from very positive to very negative (in contrast to ER- breast cancers, whose correlations were almost entirely positive) we sought to determine if an incremental change in correlation with the hMAPK-microRNA recurrence signature (represented as 1% increase in Pearson correlation coefficient) associated with change in risk of disease specific death. In the ER+ METABRIC cohort, multivariate analysis indicated that incremental increase in Pearson correlation with the ideal hMAPK-microRNA recurrence signature carries a significant correspondingly incremental increase in risk of breast cancer specific death within 5 years from diagnosis (multiplicative hazard factor: 1.01, 95% CI = 1.001-1.011, p-value = 0.0185, Supplemental Table 5B).

Similarly, incremental increased Pearson correlation with the ideal hMAPK-microRNA recurrence signature is also associated with a significant incremental increase in risk of breast cancer specific death within 5 years from diagnosis among patients with ER+ cancer receiving hormone therapy, either alone or in conjunction with chemotherapy or radiotherapy (multiplicative hazard factor: 1.008, 95% CI = 1.002-1.014, p-value = 0.006285, Supplemental Table 5C). Multivariate analysis of the Lyng ER+ cohort indicated a significant association with high-hMAPK-microRNA status according to the hMAPK-microRNA recurrence signature and increased risk of disease recurrence within 5 years (multiplicative hazard factor: 2.109, 95% CI = 1.1992-3.7085, p-value = 0.009583, Supplemental Table 5D). Additionally, multivariate analysis indicated that increased Pearson correlation with the ideal hMAPK-microRNA recurrence signature associates with a significant increase in risk of breast cancer specific death within 5 years from diagnosis among ER+ patients from METABRIC dataset stratified by adjuvant therapy...
received (multiplicative hazard factor: 1.01, 95% CI = 1.0006-1.011, p-value = 0.029455, Supplemental Table 6A).

**Leave-one-out analysis of hMAPK-microRNA signature identifies a subset associated with poor disease-specific survival outcomes in ER-positive population**

We hypothesized that microRNAs from the hMAPK-microRNA signature may contribute to de novo or acquired resistance to hormone therapy, and may be associated with poor survival outcomes in patients with ER-positive disease. In order to test this hypothesis, we performed a leave-one-out analysis in the large METABRIC dataset. Briefly, microRNAs were individually excluded from analysis of the 57-member hMAPK-microRNA signature, and Cox Proportional Hazards analysis for hazard of disease-specific death event within 5 years among patients with ER-positive disease from the METABRIC dataset was performed (see Methods for additional details). Individual microRNAs whose removal improved the ability of the hMAPK-microRNA signature to predict poor survival events in the ER+ population at 5 years by Cox Proportional Hazards analysis were tabulated (see methods), and the 21 retained microRNAs make up a “hMAPK-microRNA survival signature” (see supplemental table 7). As expected, this survival signature has substantially improved the ability to predict poor disease survival in Kaplan-Meier survival analysis and Cox univariate hazard analysis of patients in the METABRIC dataset, in both total patient population (p= 4.12e-17; univariate HR = 3.7[2.67-5.13]) and in the ER-positive patient population (p=1.44 e-08, univariate HR = 2.87[1.96-4.21]) (Figure 5A) compared to either the total hMAPK-microRNA signature or the hMAPK-microRNA recurrence signature. Breast cancers of the Luminal A and Luminal B subtypes that were classified as “high-hMAPK”
by this hMAPK-microRNA survival signature exhibited significantly poorer 5-year survival compared to those classified as “low-hMAPK” (Luminal A: p = 0.0151, univariate HR: 2.28[1.15-4.52]; Luminal B: p = 0.000614, univariate HR = 2.48[1.45-4.25]) (Figure 5A). Breast cancers treated with any hormone therapy that were classified as “high-hMAPK” by this survival signature were at significantly higher risk for poorer disease survival than those classified as “low-hMAPK” (p = 1.7 e-06, univariate HR = 2.74[1.78-4.23])(Figure 5B). Multivariate analysis of all breast cancers and ER-positive breast cancers in the METABRIC dataset indicated that high-hMAPK status, as determined by our hMAPK-microRNA survival signature, is an independent risk factor for reduced disease survival at 5 years among all patients (p= 0.0031, multiplicative hazard factor = 1.89 (1.24-2.89) and especially among patients with ER-positive disease (p = 0.0027, multiplicative hazard factor = 1.95 (1.26-3.02) (Table 1).

In order to validate this hMAPK-microRNA ER+ survival signature, we assessed its association to disease survival in all patients and patients with ER+ disease in the Enerly dataset (p= 0.00689, 0.0538, respectively)(Figure 5C). We additionally assessed association of this survival signature with disease recurrence in all patients and patients with ER-positive disease in the Buffa (p= 1 e-07, 0.000245, respectively)(Figure 5D) and Enerly datasets (p= 0.00393, 0.0167, respectively)(Figure 5D), and in ER+ patients treated with tamoxifen monotherapy from the Lyng dataset (p = 0.0371)(Figure 5B). This survival signature was vastly superior to the total hMAPK-microRNA signature, and performed comparably to or better than the hMAPK-recurrence signature in the ability to predict poor outcome in patients classified as “high-hMAPK” vs “low-hMAPK”, even in the smaller publicly available datasets. This strongly suggests
that the microRNAs retained in the survival signature may drive the poor survival outcome associated with the hMAPK-microRNA signature.

Discussion

Using paired mRNA and microRNA expression data from primary breast tumor datasets (TCGA, Buffa), we report a microRNA signature that associates significantly with the ERK1/2 hMAPK-mRNA expression signature. Classification of primary breast cancers using this hMAPK-microRNA signature indicates significant association with ER negative status, high tumor grade, increased proliferation, basal and HER2 molecular subtypes, and poor clinical outcomes. This signature identifies a population of ER+ primary breast cancers classified as high-hMAPK-microRNA that not only exhibit gene expression patterns similar to ER- primary breast cancers, but also exhibit significantly poorer clinical outcomes than ER+ cancers classified as low-hMAPK-microRNA. These observations were validated in independent, publicly available cohorts (Enerly, METABRIC). We report a subsignature composed of 22 microRNAs, an hMAPK-microRNA recurrence signature, which retains significant associations with poor clinical outcome among breast cancer patients and significantly associates with poor response to hormone therapy in two independent validation datasets by multivariate analysis (Lyng and METABRIC datasets). Finally, we report a subsignature of this hMAPK-microRNA signature, consisting of 21 microRNAs, that is significantly associated with poorer disease-specific survival outcome in patients with ER-positive disease; an hMAPK-microRNA survival signature.
The hMAPK-microRNA signature (Supplemental Table 2) contains numerous microRNAs with previously reported roles in breast cancer etiology. Several hMAPK-microRNAs are significantly differentially expressed between basal/HER2+ subtype and Luminal A type cancers, between ER- and ER+, and between high grade vs low grade cancers (overexpressed: hsa-miR-150, -142-3p, -142-5p, -148a, -155, -135b; underexpressed: hsa-miR-30a-3-, -30a-5p, let-7a, -342) (35). miR-29c, underexpressed in the hMAPK-microRNA signature, correlates with expression of GATA3, a luminal identity promoting transcription factor, and the miR-29 microRNA family facilitates GATA3 mediated maintenance of luminal identity (44). The let-7 family of microRNAs, underexpressed in the hMAPK-microRNA signature, negatively regulates RAS (45), and represses self-renewal and tumorigenicity of breast cancer stem cells (30). The EMT-promoting transcription factor SNAI1 has been shown to be a direct target of miR-30a (46). Overexpression of the hMAPK-downregulated microRNAs miR-29c, miR-30c, and miR-342 has been reported to significantly associate with good prognosis (35), and we observe significantly lower expression of GATA3 as well as miR-29c in ER+ tumors classified as hMAPK-microRNA, and higher expression of basal markers and genes associated with poor clinical outcome, suggesting poor outcomes associated with these tumors may be due to loss of luminal identity and acquisition of ER-negative characteristics. A number of microRNAs upregulated in the hMAPK-microRNA signature (miR-142-5p/3p, miR-146a, miR-150, and miR-155) (35) are associated with markers of lymphocytic infiltrate and may reflect altered host immune or inflammatory responses associated with this hMAPK-microRNA signature. Thus the downregulation and upregulation of these family members, respectively, in the hMAPK-
microRNA signature may contribute to the breast cancer aggressiveness predicted by this profile.

A number of hMAPK-microRNAs regulate the estrogen receptor (28-30, 47, 48). The hMAPK-upregulated miR-221/222 family is overexpressed in ER- cancers (49), directly targets ER (49), is upregulated in HER2 amplified versus non-amplified breast cancers (29), and mediates tamoxifen resistance in MCF-7 cells by repression of CDKN1B, a cell cycle regulator that is underexpressed in breast cancers classified as high-hMAPK by our microRNA signature (see figure 2) (28, 29). Six underexpressed microRNAs in the hMAPK-microRNA signature (hsa-miR-125a, -let7e, -30d, -30a-5p, -30a-3p, -149) are underexpressed in treatment naïve ER+ primary cancers which expressed lower ESR1 mRNA levels, higher ERBB2 mRNA levels, and which had higher proportions of basal and HER2 subtype tumors than ER+ tumors with higher expression of these microRNAs (50). Elevated expression of miR-30 family members (hsa-miR-30a, -3p, -30c) is significantly associated with tamoxifen sensitivity (51), while repression of miR-375, underexpressed in the hMAPK-microRNA signature, is associated with resistance to tamoxifen in an ER+ breast cancer model (52). Analysis of the Lyng dataset containing only ER+ tumors treated with adjuvant tamoxifen monotherapy and the hormone therapy treated cohort of the METABRIC dataset demonstrates that this hMAPK-microRNA signature identifies a subset of ER+ tumors which exhibit a higher incidence of recurrence and decreased disease-specific death following hormone therapy, suggesting an association of this hMAPK-microRNA signature with tamoxifen resistance and agreeing with previous observations linking activated MAPK signaling and resistance to endocrine therapy (53). The Lyng dataset consists of only ER+ cancers treated with adjuvant tamoxifen monotherapy, selected such that half of the patients...
exhibited disease recurrence over the course of 10-year follow-up (33). Lyng et al. were not able to identify a microRNA signature which consistently predicted disease recurrence among ER+ breast tumors, and suggested that as numerous mechanisms contribute to hormone therapy resistance there may not be a single microRNA profile able to predict response to tamoxifen therapy (33). In our approach, we identified a microRNA signature indicative of a common biology, that of hMAPK signaling, and queried whether there was an association with clinical outcome, rather than taking patients with discrete clinical outcomes and searching for a microRNA signature that would predict those outcomes. That our hMAPK-recurrence and survival signatures show predictive value in the Lyng dataset reinforces the idea that activation of MAPK signaling represents a significant biological event in the establishment of estrogen independence and tamoxifen insensitivity and that these hMAPK-microRNAs may mediate these effects. The hMAPK-microRNA recurrence signature and the hMAPK-microRNA survival signature identified here significantly associate with increased early risk of recurrence and poor disease survival in ER-positive patient populations, supporting the notion that these microRNAs contribute to an ERK1/2 MAPK mechanism of tamoxifen resistance.

Multivariate analysis of the breast cancers from METABRIC and Lyng datasets indicates that classification as high-hMAPK-microRNA by the hMAPK-microRNA recurrence signature significantly contributes to increased risk of breast cancer specific death and disease recurrence within 5 years post diagnosis. While we report that the majority of ER- breast cancers fall under the classification of high-hMAPK-microRNA, we did not identify ER status as a significant covariate in our multivariate analysis, indicating that increased risk associated with hMAPK-microRNA status is independent of ER-status. We observed a significant increase in hazard
associated with incremental increase in correlation to the idealized hMAPK-microRNA recurrence signature in ER+ breast cancers. Importantly, the hMAPK-microRNA survival signature demonstrated prognostic capability in both luminal A and luminal B subtypes of breast cancer from the METABRIC dataset. This indicates that hMAPK-microRNA survival signature is not just a surrogate for luminal B breast cancers from the ER+ group, which often exhibit higher proliferation rates, more activated growth factor signaling, and less reliance upon estrogen signaling than luminal A breast cancers. Additionally, multivariate analysis of ER+ breast cancers from the METABRIC and Lyng datasets receiving hormone therapy, either alone or in combination with chemotherapy and/or radiotherapy, revealed that classification of ER+ breast cancers as high-hMAPK-microRNA by the recurrence or survival signature was significantly associated with increased risk of disease recurrence (Lyng) and disease specific mortality (METABRIC). These results reinforce the observations we made from survival analysis of the hMAPK-microRNA signatures in the other training and validation datasets, and suggest that these hMAPK-microRNA signatures may have predictive value regarding response to Tamoxifen therapy for patients with ER+ disease.

Focusing the analysis on the 12 microRNAs in common between the hMAPK-recurrence and hMAPK-survival microRNA signatures reduced the ability to prognosticate increased disease recurrence and decreased disease survival in all patient cohorts compared to the recurrence and survival signatures, but was still more significant than the total 57-member hMAPK microRNA signature (data not shown). This suggests that, while there are hMAPK-microRNAs common to both the recurrence and the survival signature that may be useful biomarkers or potential drivers of general poor outcome (i.e low expression of miR-29c, miR-30
family, high expression of 221/222, etc), alterations in particular hMAPK-microRNAs associate specifically with either increased disease recurrence or poor disease-specific survival (i.e.- in the survival signature miR-22 and miR-224 are overexpressed, while let-7a is underexpressed), especially among ER-positive breast cancers.

The data presented here suggest that molecular and behavioral characteristics of tumors with activated growth factor signaling through the ERK1/2 MAPK axis are in part coordinated by aberrant microRNA expression associated with such activated MAPK signaling. The reported functions of members of the hMAPK-microRNA signature, together with the survival and recurrence data from the Enerly, METABRIC, and Lyng validation datasets (Figure 4,5, Table 1), suggest that the microRNAs identified in our hMAPK-microRNA signature contribute to endocrine resistance associated with activated ERK1/2 MAPK signaling, and raise the provocative possibility that the microRNAs contained within this signature may ultimately prove to have value as prognostic indicators of clinical outcome and as predictors of ER+ breast cancers with de novo endocrine resistance. These findings support further evaluation of the predictive potential of members of this hMAPK-microRNA signature for hormone therapy resistance and suggest that a subset of these microRNAs may also prove to be potential therapeutic targets. The identification of sub-signatures of hMAPK-microRNAs that significantly associate with increased disease recurrence and reduced disease-specific survival outcomes, particularly in patients with ER+ breast cancer, indicates that stratification of patients according to expression of these microRNAs may ultimately provide important information related to disease prognosis and response to therapy; these data indicate that the clinical application of these signatures warrants further investigation prospectively in large patient cohorts where
patients tumors would be arrayed for microRNA expression of these 21 hMAPK-microRNAs, and patients then stratified by high hMAPK-microRNA classification or low hMAPK-microRNA Such classification by this signature could then inform decisions on Tamoxifen treatment alone or more aggressive therapy, such as in combination with MEK or other signaling inhibitors, as informed by the targets of these microRNAs. Such prospective studies could be performed in tandem with current predictive gene expression assays (such as Oncotype DX or Mammaprint) to determine the added value of incorporating microRNA analysis with existing mRNA-based assays in predicting response of ER+ breast cancer to targeted therapy.

Acknowledgements

We thank Dr. Joyce Slingerland (University of Miami), Dr. Dan Hayes (University of Michigan), Dr. James Rae (University of Michigan), Dr. Jennifer Richer (University of Colorado), and Dr Marc Lippman (University of Miami) for critical review of the manuscript; Dr. Marc Lippman and members of the El-Ashry and Lippman lab group for thoughtful discussion; The University of Miami Sylvester Comprehensive Cancer Center Oncogenomics Core Facility for technical assistance and generation of microarray data. Funding for this research was provided by NIH grant: NIH 1R01 CA113674 and by Bankhead Coley Foundation 09BW-04.

Author Contributions

P.M. and D.EA. planned the experiments; P.M and J.B. carried out the experiments; P.M., J.C., and T.KS. analyzed data; P.M., J.C., and D.EA. wrote and revised the manuscript.
Figure Legends

Figure 1. Breakdown of ER-status of primary breast cancers with paired mRNA and microRNA expression data from the 2 training and 2 validation datasets classified according to the hMAPK-mRNA signature established by Creighton et al. (A) Association of hMAPK-mRNA status with ER status in the TCGA and Buffa training datasets. (B) Association of hMAPK-mRNA status with ER status in the Enerly and METABRIC validation datasets. (C-D) Kaplan-Meier analysis of clinical outcome in patients with tumors classified according to hMAPK-mRNA signature; (C) Recurrence-free survival in Buffa dataset, (D) Recurrence-free survival (left) and disease-specific survival in Enerly dataset (middle); disease specific survival in METABRIC dataset (right). Bar graphs: white = not hMAPK-mRNA, grey = hMAPK-mRNA; Kaplan-Meier curves: dashed= low-hMAPK-mRNA, solid= high-hMAPK-mRNA. p-values from logrank tests are indicated.

Figure 2: (A) Expression of genes downregulated (ESR1, CDKN1B, TOB1, PDCD4, RPS6KA5) and upregulated (ETV5, RPS6KA4, CREB1, SRF, ANGPTL4, ETV1, ELK4, ATF4, RPS6KA1) [underlined and bolded genes reported to be downregulated or upregulated in hMAPK mRNA signature (15)] by activated MAPK signaling was examined in primary tumors from the TCGA dataset which were classified as high-hMAPK-microRNA (top) or low-hMAPK-microRNA (bottom) by our hMAPK-microRNA signature. Proportions of tumors with a given alteration ≥ 1.5 fold are reported. Blue: gene expression downregulation; Green: gene expression upregulation. Alterations in copy number, mutational status, gene expression, and protein expression of numerous genes in the EGFR-ERK signaling pathway found in cancers form TCGA dataset.
classified by our hMAPK-microRNA signature. (B) Proportion of tumors classified as high-hMAPK-microRNA with alterations for a given gene (top); proportion of tumors classified as low-hMAPK-microRNA with alterations for a given gene (bottom). Red: copy number amplification; Light Blue: homozygous deletion; Green: gene expression upregulation; Dark Blue: Gene expression downregulation; Pink: protein expression increased (RPPA); Grey: protein expression decreased (RPPA). Gene and protein expression increases considered significant if expression z-score > 1.5 fold different from dataset average. Gene set enrichment analysis for predicted and validated gene targets of microRNAs in the hMAPK-microRNA signature reveals significant enrichment of genes that fall into numerous pathways associated with proliferation, signal transduction, survival, differentiation, and cancer. (C) Expression of genes that are predicted targets of microRNAs overexpressed in the hMAPK-microRNA signature validated to be upregulated by MAPK signaling. (D) Expression of genes that are predicted targets of microRNAs underexpressed in the hMAPK-microRNA signature validated to be downregulated by MAPK signaling. Yellow boxplot: high-hMAPK-microRNA cancers, Blue boxplot: low-hMAPK-microRNA cancers, Red circle: ER+ sample, Green circle: ER- sample, Grey circle: ER status for sample not available.

Figure 3. Molecular subtyping and Kaplan-Meier survival analysis of disease recurrence and survival in patients with tumors classified according to hMAPK-microRNA signature from training (Buffa, TCGA) and validation datasets (Enerly, METABRIC). (A) Association of hMAPK-microRNA status with PAM50 molecular subtypes in TCGA (top), Enerly (middle) and METABRIC (bottom) datasets. (B) Kaplan-Meier survival analysis of disease recurrence in patients with
tumors classified according to the hMAPK-microRNA signature from training (Buffa dataset); disease recurrence among all patients, patients with ER+ disease, patients with ER- disease (Buffa). (C) Disease recurrence and disease survival among all patients (Enerly) and disease survival among all patients from METABRIC dataset (top), disease recurrence and disease survival among patients with ER+ disease (Enerly) and disease survival among patients from METABRIC dataset with ER+ disease (bottom). Bar graphs: white = low-hMAPK-microRNA, grey = high-hMAPK-microRNA; p-values given are for chisquare test or Fisher's exact test, as indicated. Kaplan-Meier curves: dashed= low-hMAPK-microRNA, solid= high-hMAPK-microRNA; logrank test p-values are indicated.

Figure 4. 5-year outcome analysis of tumors from validation datasets (Enerly, METABRIC, Lyng) classified according to the hMAPK-microRNA recurrence signature. (A) Kaplan-Meier survival analysis of disease recurrence among all patients and patients with ER+ disease (Enerly dataset). (B) Disease survival among all patients and patients with ER+ disease (Enerly dataset). (C) Disease survival among all patients and patients with ER+ disease (METABRIC dataset). (D) Disease recurrence among patients from Lyng dataset treated with tamoxifen monotherapy and patients from METABRIC dataset with ER+ disease who received any hormone therapy. Kaplan-Meier curves: dashed= low-hMAPK-microRNA, solid= high-hMAPK-microRNA. Logrank test p-values are indicated.

Figure 5. 5-year outcome analysis of tumors from training (Buffa) and validation datasets (Enerly, METABRIC, Lyng) classified according to the hMAPK-microRNA survival signature. (A) Kaplan-Meier survival analysis of disease recurrence in patients with tumors classified according
to hMAPK-microRNA survival signature from METABRIC dataset; disease survival among all patients, patients with ER+ disease (top), Disease survival among patients with Luminal A and Luminal B type breast cancer (bottom). (B) Disease recurrence among patients from Lyng dataset treated with tamoxifen monotherapy and patients from METABRIC dataset with ER+ disease who received any hormone therapy. (C) Disease survival among all patients and patients with ER+ disease (Enerly dataset). (D) Disease recurrence among all patients and patients with ER+ disease (Enerly dataset and Buffa datasets). Kaplan-Meier curves: dashed=low-hMAPK-microRNA, solid= high-hMAPK-microRNA. Logrank test p-values are indicated.

Table 1

Multivariate analysis of hMAPK-microRNA survival signature in METABRIC dataset.

(A) Cox Proportional Hazards analysis of all patients. Cancers from METABRIC dataset were classified as high-hMAPK-microRNA or low-hMAPK-microRNA by hMAPK-microRNA survival signature, and this hMAPK-microRNA classification was included in the Cox Proportional Hazards analysis along with standard clinical covariates including tumor grade, stage, HER2 status, Progesterone Receptor status (PGR), lymph node status, and tumor PAM50 molecular subtype. (B) Cox Proportional Hazards analysis of METABRIC ER positive breast cancers only, including otherwise identical covariates. Only covariates with significant hazard ratios are indicated.
References


2A 2B

**Proportion of samples with alterations**

- **high-hMAPK-microRNA**
- **low-hMAPK-microRNA**

**Targets of hMAPK upregulated microRNAs**

- ESR1
  - p = 9.44e-24
  - t = -11.8
- CDKN1B
  - p = 0.000934
  - t = -3.34
- GATA3
  - p = 2.15e-19
  - t = -10.2
- SMAD3
  - p = 3.95e-07
  - t = -5.21

**Targets of hMAPK downregulated microRNAs**

- CCNB1
  - p = 2.1e-07
  - t = 5.33
- SNAI1
  - p = 6.33e-14
  - t = 7.93
- CDH3
  - p = 3.33e-13
  - t = 7.68
- NOTCH1
  - p = 8.02e-13
  - t = 7.58

**Parameters**

- UP
- DOWN
- HOMDEL
- RPPA-overexpressed
- MUT
- RPPA-underexpressed

**Authors**

Research.

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4A  Enerly All patients: Disease Recurrence

% Event Free

Time to event (months)

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4B  Enerly All patients: Disease Survival

% Event Free

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4C  METABRIC All patients: Survival

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4D  METABRIC ER+ cancers with any hormone therapy: Survival

% Event Free

Time to event (months)

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4B  Enerly ER+ cancers: Disease Survival

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4B  Enerly ER+ cancers: Disease Survival

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4B  Enerly ER+ cancers: Disease Survival

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### Table 1 A

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<td>Positive LN(2)</td>
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<td>*</td>
</tr>
<tr>
<td>PR-positive</td>
<td>0.67 (0.46-0.99)</td>
<td>*</td>
</tr>
</tbody>
</table>

**Significance codes:** 0 '***'; 0.001 '***'; 0.01 '**'; 0.05 '*'; 0.1 '.

Baseline: low hMAPK, grade 1, stage 0, 0 positive LN, ERBB2 negative, PR negative, ER-positive, Luminal A

### Table 1B

#### ER+ cancers:

<table>
<thead>
<tr>
<th>Covariate</th>
<th>HR (95% CI)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive LN (3+)</td>
<td>2.81 (1.62-4.86)</td>
<td>***</td>
</tr>
<tr>
<td>Positive LN(2)</td>
<td>2.16 (1.05-4.45)</td>
<td>*</td>
</tr>
<tr>
<td>high-hMAPK</td>
<td>1.95 (1.26-3.02)</td>
<td>**</td>
</tr>
<tr>
<td>Luminal B</td>
<td>1.64 (1.04-2.56)</td>
<td>*</td>
</tr>
<tr>
<td>PR-positive</td>
<td>0.59 (0.39-0.87)</td>
<td>**</td>
</tr>
</tbody>
</table>

**Significance codes:** 0 '***'; 0.001 '***'; 0.01 '**'; 0.05 '*'; 0.1 '.

Baseline: low hMAPK, grade 1, stage 0, 0 positive LN, ERBB2 negative, PR negative, Luminal A
A novel MAPK-microRNA signature is predictive of hormone-therapy resistance and poor outcome in ER-positive breast cancer

Philip Miller, Jennifer Clarke, Tulay Koru-Sengul, et al.

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