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MST1R (RON) expression is a novel prognostic biomarker for metastatic progression in breast cancer patients

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Abstract

Purpose: This study evaluates the prognostic significance of MST1R (RON) expression in breast cancer with respect to disease progression, long-term survival, subtype and association with conventional prognostic factors.

Methods: The approach includes interrogation of survival and tumor staging with paired MST1R RNA expression from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) datasets. Protein expression evaluation was performed using immunohistochemistry (IHC) staining of MST1R on breast cancer tissue samples from the Cancer Diagnosis Program Breast Cancer Progression tissue microarray and locally obtained breast tumor tissue samples analyzed with paired survival, metastasis, and subtype.

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- Conflicts of Interest
  - The authors declare that they have no conflicts of interest.
- Research involving Human Participants and/or Animals
  - Human participants: Samples obtained from human subjects were anonymized and de-identified prior to our acquisition
  - Animals were not used in this study
- Informed consent: As is common practice at the University of Cincinnati and the Cooperative Human Tissue Network, Institutional Review Board (IRB) review took place and IRB protocol deemed unnecessary due to the anonymized and de-identified nature prior to acquisition of samples

Statement of human rights
Retrospective and archived sample studies presented herein comply with ethical standards implemented by the University of Cincinnati according to the specific requirements for human subjects testing in the United States.

Results: Data from TCGA (n=774) show poorer relapse-free survival (RFS) in patients with high MST1R expression (P=0.32) and no difference in MST1R expression based on tumor stage (P=0.77) or nodal-status (P=0.94). Patients in the GEO-derived Kaplan-Meier Plotter microarray dataset demonstrate association of MST1R and poorer overall survival (n=1402, P=0.018), and RFS in patients receiving chemotherapy (n=798, P=0.041). Patients with high MST1R expression display worse overall survival (P=0.01), and receiver-operator characteristic (ROC) analysis demonstrate the predictive capacity of increased MST1R with early death (P=0.0017) in IHC stained samples. Paired IHC stained breast tumor samples from the primary versus metastatic site show MST1R expression is associated with metastatic progression (P=0.032), and ROC analysis support the predictive capacity of MST1R in metastatic progression (P=0.031). No associations of MST1R with estrogen receptor (ER), progesterone receptor (PR), both ER and PR, HER2 positivity or triple negativity were found (P=0.386, P=0.766, P=0.746, P=0.457, P=0.947 respectively).

Conclusions: MST1R expression has prognostic value in breast cancer with respect to survival and metastatic progression. MST1R expression is not associated with tumor stage, nodal status, or subtype.

Keywords

MST1R; RON; survival; metastasis; subtype; biomarker

BACKGROUND

Since 1980, the breast cancer (BC) mortality rate has been reduced by nearly 40% due to significant progress in clinical detection and refined treatment strategies [1,2]. Despite this success, more than 40,000 individuals in the United States were predicted to die of BC in 2019 [1], and since 2010, the BC mortality rate has only been reduced by less than 2% [2]. Thus, while our current understanding of BC and treatment has largely improved, significant barriers remain in the effective treatment of BC. Currently, assessment of expression of hormone receptors (estrogen receptor, ER, and progesterone receptor, PR) and the human epidermal growth factor receptor 2 (HER2) determine the first-line treatment with respect to targeted approaches that accompany chemotherapy, surgery, and radiation. BC subtypes indicated by the presence or absence of these biomarkers stratify BCs into discrete biologic entities with different prognoses; however, distant metastatic recurrence occurs throughout all subtypes [3]. Public health campaigns and screening guidelines have led to earlier detection with more early stage cancers detected [4]. As a consequence, overtreatment of early stage BC has also been a significant public health issue with some questioning the necessity of adjuvant chemotherapy in ensuring cure given the significant impairments in quality of life [4]. While modern guidelines indicate adjuvant chemotherapy for the treatment of node-positive BC [5], causative or associated factors prognostic for BC recurrence BC remain poorly understood. Moreover, this remains a challenging area of study, as patients must be followed for years to decades to document recurrences, and many databases are limited to initial biopsies or lack molecular characterization including the TCGA BRCA dataset mined for part of this study.

The recent conclusion of the TAILORx trial assessing the 21 gene panel Oncotype Dx recurrence risk scoring system shows promising results in providing means of assessing recurrence risk, and is generally accepted as the standard-of-care approach to identifying the need for adjuvant chemotherapy in early stage BC patients [6]. This quantitative real-time polymerase chain reaction (qRT-PCR)-based assay is performed in a central lab and involves submission of samples in addition to what is used for standard on-site pathology laboratory analyses. Oncotype Dx additionally uses a scoring algorithm dependent on expression of ER and HER2 and respective target genes potentiating inherent bias of specific subtypes, as well as proliferation and invasion genes that are generally supportive of cancer independent of subtype and recurrence [7]. Thus, a recurrence biomarker independent of subtype, proliferation, and invasion would show significant clinical utility independently. Moreover, addition of such a biomarker to existing recurrence scoring algorithms has potential to produce more robust recurrence scores to positively impact BC patient outcomes.

The MST1R (also known as RON) receptor tyrosine kinase (RTK) is a member of the c-Met family of RTKs that is expressed in epithelial cells and terminally-differentiated macrophages [8–12]. MST1R is overexpressed in numerous solid tumors including breast, prostate, pancreas, and more with strong oncogenic function elicited through various signaling mechanisms [8–10,13–21]. MST1R overexpression incidence and examination of its prognostic features with respect to survival and metastatic progression in BC have been reported, but are limited by subtype, sample size, technology, access to clinical information, or are limited to node-negative samples lacking overall survival data [8,12,21–24]. The purpose of this study is to evaluate MST1R as a prognostic biomarker of breast cancer recurrence and survival and association with conventional prognostic factors. In order to robustly evaluate the role of MSTR1, we utilized publicly available datasets, locally-obtained patient samples, and a BC progression tissue microarray (TMA) with long-term clinical data. Preclinical studies of MST1R in BC have demonstrated strong oncogenic function *in vitro* and *in vivo* including roles in BC stem cell phenotypes, production of angiogenic factors, endocrine therapy resistance, and metastasis, providing significant rationale for MST1R to have a distinct role in supporting human BC [15–17,20,23,25–29].

METHODS

Datasets:

The Cancer Genome Atlas (TCGA) RNA sequencing data was used with individuals with incomplete data in the categories of interest excluded from analysis. Additionally, the present study focused on women BC patients and excluded 5 male patients from analyses as well as patients with incomplete survival data. Demographics include age, presence of cancer invaded lymph nodes, pathologic T stage, and race (Table 1). The Gene Expression Omnibus (GEO)-derived Kaplan-Meier Plotter microarray data was also used as previously published [30].

Immunohistochemistry:

Tissues used in this study were formalin-fixed, paraffin-embedded tumor samples obtained from patients from the University of Cincinnati and from the Cancer Diagnosis Program

Breast Cancer Progression tissue microarray (University of Virginia). De-identified clinical and demographic patient data was provided for each tissue sample (Table 2). Slides were stained in a blinded fashion with an established MST1R staining protocol using a polyclonal anti-MST1R β-chain antibody (Santa Cruz, C-20 clone, Lot#B2316) [16,23,29]. Scoring occurred in a blinded fashion by a single individual (that did not perform the staining procedure) using a tissue positivity score (0–100) and intensity score (0–3) that were multiplied together to obtain a histology score (H-Score; 0–300) [14,16,31,32]. A total of 186 samples were used for analyses.

Statistical analysis:

Survival analyses were performed with Kaplan-Meier curves with Cox proportional hazards and log-rank or Gehan-Breslow tests as specified using R statistical software (The R Foundation) to examine significant differences in long-term and short-term survival, respectively. Hazard ratio (HR), P-value, and sample size (n) are provided for each plot. Mann-Whitney rank-sum tests were employed for ordinal-nominal associations with medians (Mdn), P-value, and sample size (n) provided for each plot using R statistical software or Prism 5.0 (Graphpad) Receiver-operator characteristic (ROC) curve analyses were utilized to examine MST1R H-Score as a continuous variable in predicting dichotomous outcome with Sigmaplot v14 (Systat). Area-under-curve (AUC), P-value, and sample size (n) are provided for each plot. Spearman's correlation analyses were employed to ascertain correlation between MST1R H-Score and survival as continuous variables using Prism 5.0. Correlation coefficient (R), P-value, and sample size (n) are provided for each plot. Multivariate factor analysis of independent correlation performed using NCSS Statistical Software.

Transcriptomic-based subtype assignment:

TCGA RNA expression data (RNA sequencing) of the PAM50 genes [33] were extracted and applied against reference data of median gene expression to bin each sample into probable subtypes using hierarchical clustering with average linkage and (one minus) Pearson Correlation distance [33]. From these assigned subtypes, MST1R RNA expression of each subtype was compared against one another using ANOVA. Sample sizes of each subtype (n) are provided for each group.

RESULTS

To interrogate the clinical features of MST1R in BC, data was first mined from The Cancer Genome Atlas (TCGA) BRCA dataset. Clinical and demographic information of the TCGA data analyzed is provided in Table 1. To view the sample distribution of MST1R expression in this dataset, MST1R gene expression, as measured via RNA sequencing of 774 independent samples, was plotted as a histogram (Fig 1A) with MST1R expression displaying a non-normal distribution with data skewed to the right of the graph. A cut-off value of 800 (top 5%) was selected for subsequent analyses to represent MST1R overexpression based on the histogram distribution. Survival analysis of samples with MST1R expression above 800 compared to that below 800 show poorer survival in high MST1R expressing samples with a hazard ratio (HR)=1.42 (0.91–2.13) measuring out to 5

years (60 months). However, the data were not significant (P=0.32; Fig 1B). Further analyses demonstrated no difference in MST1R expression when stratifying samples by tumor stage and nodal-positivity (P=0.77 and P=0.94 respectively; Fig 1C). While clinical subtype information was not provided in the TCGA dataset mined, we employed a gene expression-based approach to assign subtypes to individual samples and assess association between BC subtypes and MST1R expression. We used the PAM50 gene set which requires gene expression data of 50 genes to cluster samples into subtypes [33]. With hierarchical clustering arranged in a heat map of Figure 2A, we assigned subtypes with known MST1R RNA expression values compared against one another (Figure 2B). ANOVA analysis shows statistically significant variance between only basal-like and HER2-enriched, but not between the remaining three (Luminal A, Luminal B, normal-like) or between these three and basal-like or HER2. Taken together, the TCGA data are suggestive, albeit weakly supportive, of the hypothesis that increased MST1R expression is associated with poor clinical outcomes.

Given the limitations of the TCGA dataset (initial biopsies only, mostly node positive, limited follow up), MST1R expression was also analyzed in additional publicly available datasets. Derived from several Gene Expression Omnibus breast cancer datasets, the Kaplan-Meier Plotter (www.kmplot.com/analysis) data has been previously published [30]. Using microarray-based RNA expression of the MST1R gene in this dataset, we stratified between the upper quartile and lower three quartiles of MST1R expression. Patients in the upper quartile of MST1R expression experience worse overall survival outcomes with HR=1.33 (1.05–1.69), log-rank P=0.02; Gehan-Breslow P=0.01 measuring out to 10 years (120 months; Fig 3A). Pertinent to the hypothesis that MST1R is associated with BC recurrence is the availability of relapse-free survival (RFS) data in a sufficient number of patients receiving chemotherapy in this dataset. These patients likely fall into the category of poorer initial prognosis due to subtype and/or nodal positivity. We therefore examined the RFS data in MST1R expression upper quartile-versus lower three quartile-stratified samples. Of patients receiving chemotherapy, those with lower MST1R expression are at increased chance of RFS with HR=1.35 (1.01–1.83), log-rank P=0.04; Gehan-Breslow P=0.02 measuring out to 10 years (120 months; Fig 3B). These analyses strongly support the hypothesis that MST1R expression is associated with poor clinical outcomes and in particular, that high MST1R expression is associated with recurrent disease.

Based on our *in silico* data suggesting a role for MST1R in prognosticating recurrence, we sought to prospectively validate our findings in human tissue samples. We hypothesized that the immunohistochemistry (IHC) staining-derived histo-score (H-score) is associated with poor survival and metastatic disease. To test this hypothesis, we performed IHC for MST1R on archived breast cancer samples with paired patient clinical outcomes. Sections were stained and scored in a blinded fashion for tissue positivity (0–100) and intensity (0–3). Individual scores were multiplied together to generate a H-score and then data was released for analysis with sample-matched clinical parameters. Demographics of stained samples are found in Table 2. Representative H-score samples are displayed in Fig 4A. MST1R H-score was stratified above or below the median H-Score (180) and then overall survival was analyzed. Fig 4B shows that samples above the median for MST1R expression have a significantly worse 5-year overall survival HR=1.42 (0.91–2.13), P=0.01. To evaluate if

MST1R is a predictor of early death, we employed receiver-operator characteristic (ROC) analyses. Rather than stratifying samples based on MST1R H-score (leaving MST1R H-score as a continuous variable) and instead stratifying between early death (events before 24 months post diagnosis modeling after previously published work but retaining a large enough sample size [34]) or not (events after 24 months post diagnosis), ROC analysis suggests MST1R to be a good predictor of early death of BC patients (AUC=0.75; P=0.0017; Fig 4C). Next, to ascertain whether MST1R H-score associates with progression to metastatic disease, we focused on samples taken from patients with distant metastases compared to that of patients without metastasis. We found a significant increase of MST1R H-score in samples from patients with metastases (Non-metastatic MED=150, Metastatic MED=205, P=0.0323; Fig 4D). To determine if MST1R is a predictor of metastatic progression, we employed ROC analysis with MST1R H-score as a continuous variable and found predictive value of MST1R H-score (AUC=0.63; P=0.031; Fig 4E) using the same patient-matched tumor samples as above. Multivariate analysis to examine the correlation of independent clinical factors and MST1R expression was performed specifically evaluating the MST1R H-score, estrogen receptor (ER) status, progesterone receptor (PR) status, age at diagnosis, human epidermal growth factor receptor 2 (HER2) status, tumor stage, nodal status, and number of nodes positive (Figure 4F). MST1R H-score showed highest, however weak, correlation with age at diagnosis and a lack of association with other independent factors. ER status and PR status were strongly correlated as well as N stage with number of nodes positive, as is expected. These results support the TCGA data where MST1R expression was found to be an independent factor of existing prognostic factors T stage, N stage, and subtype.

To ascertain whether a relationship exists between MST1R expression and existing BC subtypes, we divided samples into groups based on expression of the estrogen receptor (ER), the progesterone receptor (PR), ER/PR co-expression, HER2 amplification, or lack thereof (triple negative). The MST1R H-scores of each individual group were then compared with the remaining counterparts in the other groups using rank-sum tests. There were no significant differences in MST1R H-scores between any subgroups (Fig 5A) providing evidence that MST1R overexpression occurs independent of subtype. We also examined MST1R H-score stratified by subtype biomarker with respect to overall survival and employed Spearman's test to deduce correlations between these variables in (Fig 5B). An inverse correlation between MST1R H-score and overall survival is seen in ER-negative (R= −0.226, P=0.111), PR-negative (R= −0.253, P=0.021), ER/PR negative (R= −0.2501, P=0.0243), and triple negative (R= −0.4377, P=0.05) samples.

To further analyze subpopulations of interest, we focused on the PR-negative population as this population showed a significant negative inverse correlation with sufficient sample size and removal of $HER2^{High}$ samples. We employed survival analysis with Kaplan-Meier plots and ROC analysis to view the predictive potential of MST1R H-score on early death (using a 30-month cutoff as sample size was limited when using a 24-month cutoff; Fig 5C). Patients with $MST1R^{High}$, PR-negative biopsies have decreased survival relative to $MST1R^{Low}$, PR-negative counterparts HR=0.59 (0.32–1.1) Gehan-Breslow P=0.03; log-rank P=0.08 and MST1R H-score is predictive of early death in PR-negative patients (AUC=0.71, P=0.007).

As the PR-negative subpopulation demonstrated a significant inverse correlation between MST1R H-Score and survival outcomes, we further analyzed overall survival with respect to MST1R H-score of PR-negative samples. Within the PR-negative samples, particularly evident was a cluster of samples with limited months of survival and high MST1R H-score (circled in Fig 5D). We confirmed that the $MST1R^{High}$ subgroup was distinct from the $MST1R^{Low}$ group using rank-sum tests based on H-score ($P<0.0001$) and also found a significant reduction in overall survival in the $MST1R^{High}$ group relative to the $MST1R^{Low}$ group (rank-sum test ($P=0.008$; Fig 5E))[18].

DISCUSSION

In recent years, the boom in cancer biomarkers has seen few successes largely due to lack of rigorous validation and limitations in the resources required to perform these validations [37,38]. Publicly available datasets such as TCGA are valuable hypothesis-generating tools with relatively larger numbers of patients, but are limited in scope as certain clinical parameters that may over time become integrated with standard-of-care (such as recurrence scores, and hormone receptor status) are not always available in addition to limited follow-up. Identifying putative biomarkers beginning with TCGA database can result in false negative findings based on initial tests that are confined by limited clinical parameters. In the context of this study, MST1R would have been screened out if the TCGA database was our sole consideration. However, based on a number of other studies as well as our understanding of the molecular mechanisms underlying MST1R, further exploration was warranted. Thus, it remains critically important that multiple, non-overlapping datasets be consulted evaluated for interrogation of biomarkers to inform clinical decision making, with this approach being corroborated by an emerging body of literature [35,36,39,40].

Multiple preclinical studies have enumerated the oncogenicity of MST1R [15–17,20,23,26,27,29,41,42]. MST1R function is diverse and has been shown to support tumorigenesis and progression through cell survival, growth, migration, angiogenesis, anti-inflammatory cytokine production, and more. In breast cancer, we have shown MST1R activity supports breast cancer stem-like/tumor-initiating cell properties which were dependent on activation of NF-kB and β-catenin. These studies suggest NF-kB and β-catenin signaling downstream of MST1R may support cancer recurrence by impacting tumor repopulation, drug resistance, and/or dormancy properties of this subpopulation [15,16]. Moreover, additional signaling pathways including MAPK and Akt have been shown to be important for MST1R dependent cancer progression [20,29,43]. Thus, several downstream effectors of MST1R have been and continue to be studied as a means to target this pathway. Interestingly, the mechanism by which MST1R becomes overexpressed in breast cancer is unknown. While the closest family member of MST1R, Met, is found to be a recurrently mutated/amplified gene in several cancer types, MST1R is not. It is possible that a mechanism involving protein synthesis or stability impacting MST1R protein levels exists which may explain why MST1R protein expression seems to show better prognostic resolution that RNA levels. Future studies to examine mechanisms upstream to MST1R overexpression may prove to be valuable knowledge for cancer therapeutics and cancer biology in general.

While these preclinical studies have included interrogation of MST1R function in murine and human BC cell lines and tissue samples, MST1R expression has been shown to prognosticate relapse-free survival in node-negative BC patients [24]. Adding to this existing body of knowledge, this current study is the first to present empirical data on the tumor stage independent, nodal stage independent, and subtype independent prognostic ability of MST1R expression in BC with respect to overall survival, relapse-free survival, and progression to metastatic disease. Biomarkers are most meaningful when they guide clinical practice [35,36], however, rigorous testing of clinical applicability of a given biomarker requires significant effort and coordination between scientists and clinical teams to obtain samples with defined clinical parameters, treatment course, and experimental evaluation of the variable of interest. The data presented herein extends the large body of preclinical evidence into patients in a prognostic sense but does not predict response to a given therapy or treatment strategy. Future prospective studies to evaluate predictivity of MST1R expression on a given therapy will be required to refine the clinical utility of MST1R as a biomarker. Moreover, the complementary datasets employed in this study include MST1R expression measured through RNA and IHC-staining for protein. While our data suggest MST1R protein expression to show higher prognostic resolution, future studies would benefit from evaluating protein and RNA expression from matched samples to definitively determine prognostic resolution.

Here, we focus on the development of recurrent disease as it significantly limits improvements in BC survival. We have intentionally utilized datasets that have sufficient survival follow-up time (at least 5 years). This significantly narrowed the field as many studies do not necessarily have this information and MST1R expression information. Of most significant value to this study was the extensive follow-up time of the tissue used for IHC in large enough quantity for statistical power to test our primary objectives. Limitation of tissue availability with matched clinical parameters often confounds use of archived samples whereas *de novo* acquisition of patient samples presents its own issues with respect to time for sufficient sample collection and clinical endpoints like recurrence to occur [37,44]. With respect to interrogating recurrence outcomes in patient samples and/or datasets, we found limited resources to perform robust testing as biobanking of recurrent samples is still in its infancy. This is a major limitation in the assessment of recurrence using cross-sectional approaches. Moreover, nuanced parameters such as Oncotype Dx recurrence scores are only recently being utilized en masse and are not typically found in retrospective chart reviews further limiting existing datasets/bioarchives. Further, Oncotype Dx scores are calculated based on several groups of genes including that of proliferation, invasion, estrogen responsiveness, and HER2. Thus, these genes are dependent on expression of genes that are important for normal cellular function and are not recurrence specific. It is important to note that the conclusion and publication of the TAILORx trial which included longitudinal investigation of patients receiving Oncotype Dx testing showed adequate ability to prognosticate recurrence [6]. Therefore, Oncotype Dx recurrence scores could be used as a tool to test predictive capacity of MST1R expression on breast cancer recurrence prior to performing a longitudinal study, and one could envision refinement of Oncotype Dx through inclusion of MST1R (and gene target) signatures due to its independence of subtype, tumor stage, and nodal-positivity. While expression of Oncotype Dx genes may be found in RNA

sequencing datasets, recurrence scores are typically calculated from RT-qPCR methods that involve data normalization processes that are atypical for RNA sequencing. To understand the relationship between RT-qPCR generated and RNA sequencing generated values, each technique will need to be employed on concordant patient samples and evaluated for low risk, mid risk, and high risk patients with defined recurrence outcomes.

It has been speculated, but never empirically tested, that MST1R overexpression occurs independent of BC subtype. While our data support that MST1R expression occurs independent of subtype based on the expression of ER, PR, and HER2, further examination of MST1R expression associations with more conventional subtyping strata (Luminal A, Luminal B, basal-like, etc) should be performed on prospective samples in a study with MST1R protein expression and subtype associations as the primary objective. We leveraged TCGA RNA sequencing data to make comparisons of MST1R RNA expression which supports our clinical marker data failing to find subtype association, however, our data suggest protein expression to show greater prognostic resolution which could not be tested. Based on the data obtained, we hypothesize that MST1R is associated with recurrent disease irrespective of subtypes. Overlaying survival parameters on top of MST1R expression between subtype biomarker-stratification, we observe MST1R levels to be inversely correlated with survival. In these sub-analyses, PR-negativity, ER-negativity, and triple-negativity demonstrate this relationship with consideration to the few samples that comprise these groups analyzed. The PR-negative subpopulation demonstrates this relationship most strongly.

An emerging body of literature reports prognostic features of PR-negativity in BC in general [45,46] and more specifically with respect to BC recurrence [47–50]. Our analyses support poor survival for PR-negative samples and further show distinct groups of high MST1R and low MST1R expression with poorer survival outcomes in the PR-negative, $MST1R^{High}$ population. Further stratifying these PR-negative samples shows this effect persists in only ER-positive samples, which have been associated with shorter survival and recurrence times, similar to those of TNBC [47,48,50]. While TNBC samples also have a PR-negative, $MST1R^{High}$ subpopulation, sample variability in our study was too high to make conclusions regarding survival. With current clinical classifications built on ER/PR/HER2 status, typically ER/PR are combined to describe the hormone receptor positivity. Our data suggests that MST1R expression may provide prognostic insight into the response of ER-positive, PR-negative breast tumors to conventional treatment, and provide evidence for a role of PR independent of ER that requires further investigation.

The results of this study support a prognostic function of MST1R expression in BC. Whereas established biomarkers in BC strongly indicate a predictive response to therapeutics targeting the biomarker itself (hormone therapies targeting ER/PR and trastuzumab/pertuzumab targeting HER2), the predictive capacity of MST1R expression for MST1R-targeted therapeutics has not yet been investigated. Currently, there are no FDA-approved inhibitors for MST1R on the market; however, a Phase I trial has been completed on the BMS777607 (formerly ASLAN002) compound showing promising results on MST1R-mediated effects in preclinical models of breast and prostate cancers [14,25]. Based on our findings, a prospective study to ascertain the predictive potential of MST1R protein

expression on response to BMS777607 should be encompassed in the scientific aims of a future Phase II trial.

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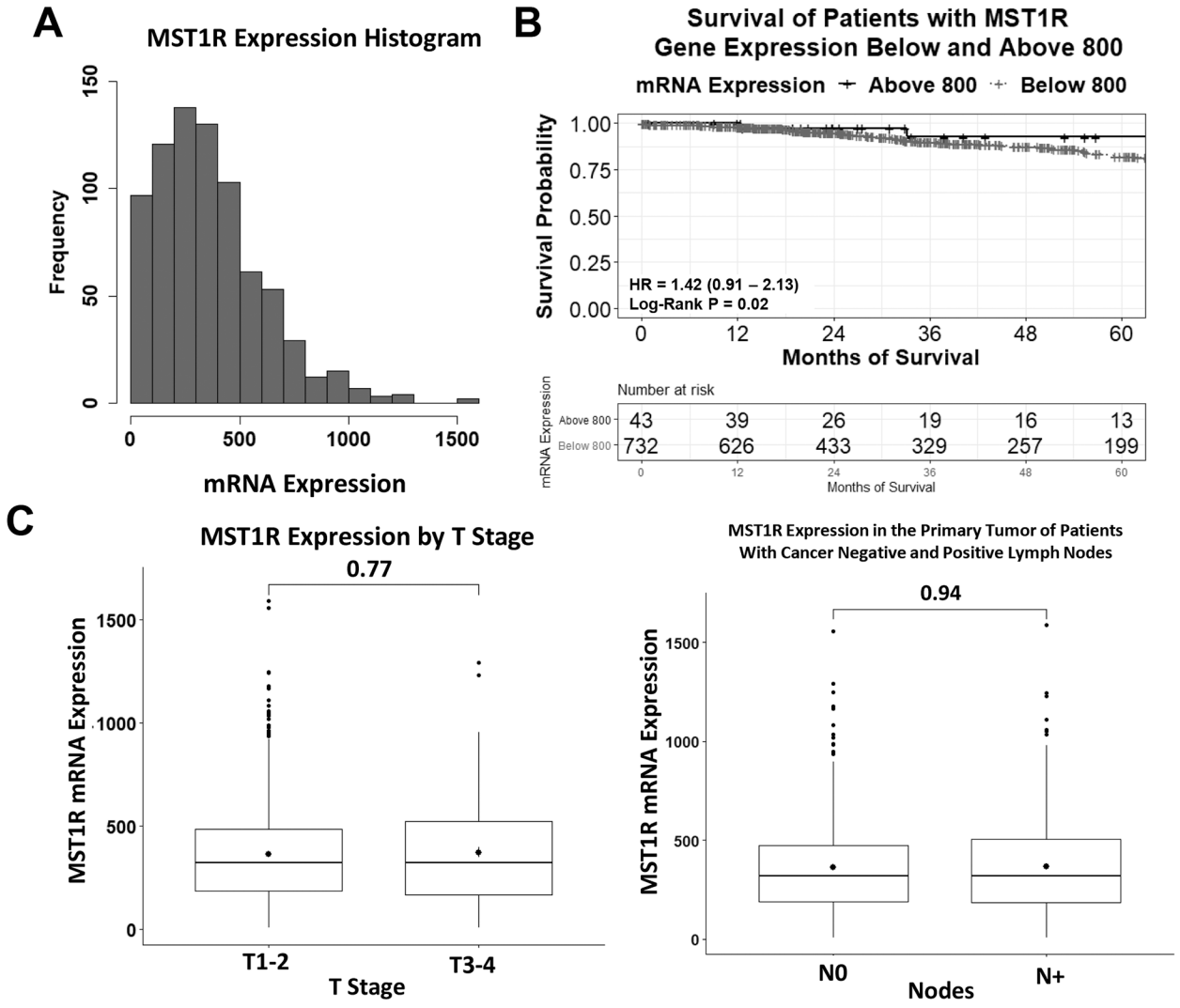

Figure 1:

TCGA BC data are weakly suggestive of association between MST1R and poor clinical outcomes in BC patients. Histogram depicting MST1R mRNA expression in the BRCA TCGA database (A). Kaplan-Meier survival curve comparing survival between patients with MST1R expression above or below 800 (B). Hazard Ratio (HR) calculated with Cox proportional hazards model, and log-rank to calculate P-value. MST1R mRNA expression compared with respect to tumor stage (T Stage) and nodal positivity (Nodes) using box-whisker plots with rank-sum test (C).

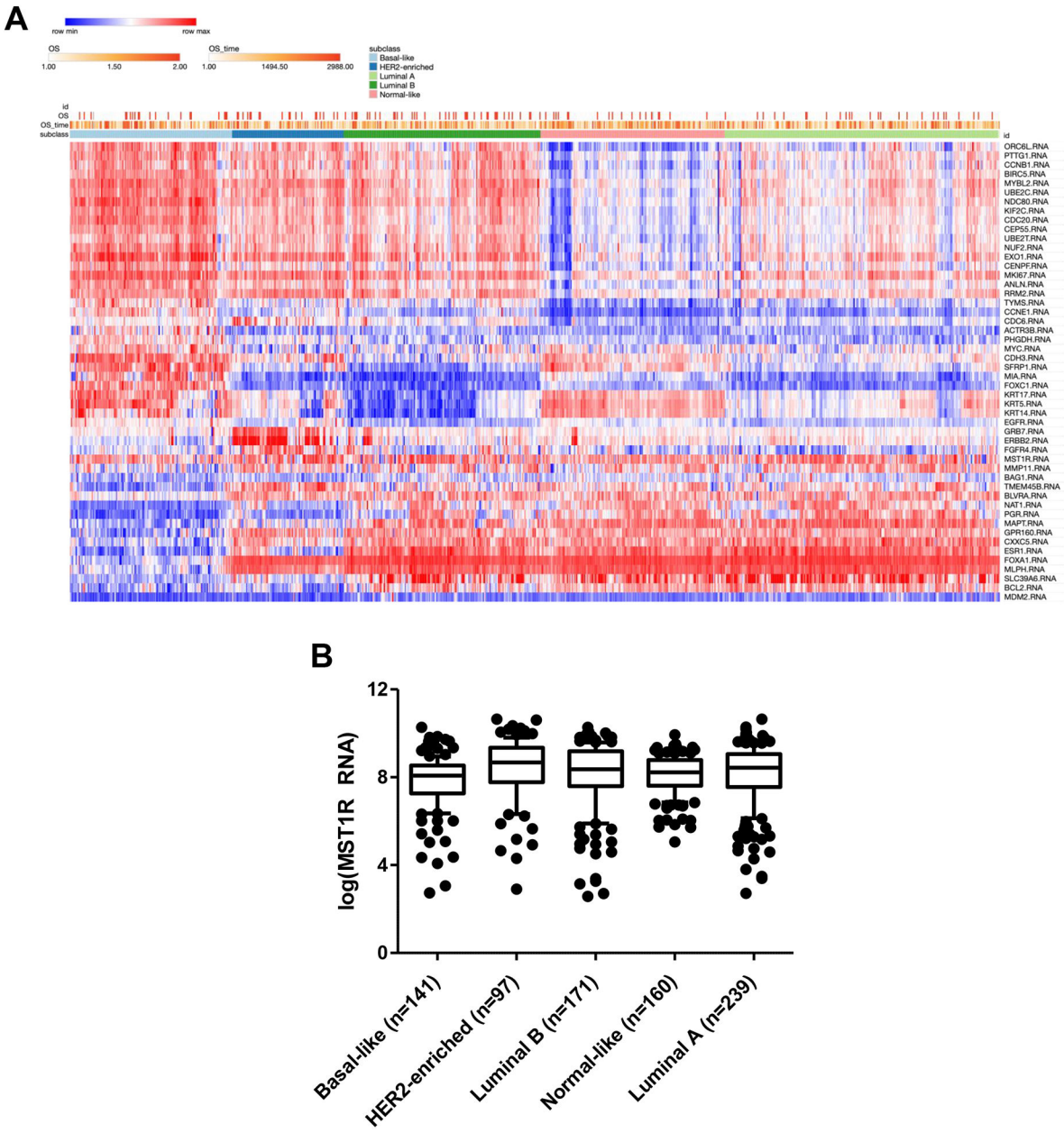

Figure 2:

MST1R RNA expression is not associated with PAM50-based molecular subtypes. PAM50 gene expression hierarchical clustering used for subtype assignment of TCGA samples (n=808) (A). MST1R RNA expression in PAM50-assigned subtypes of TCGA samples (B).

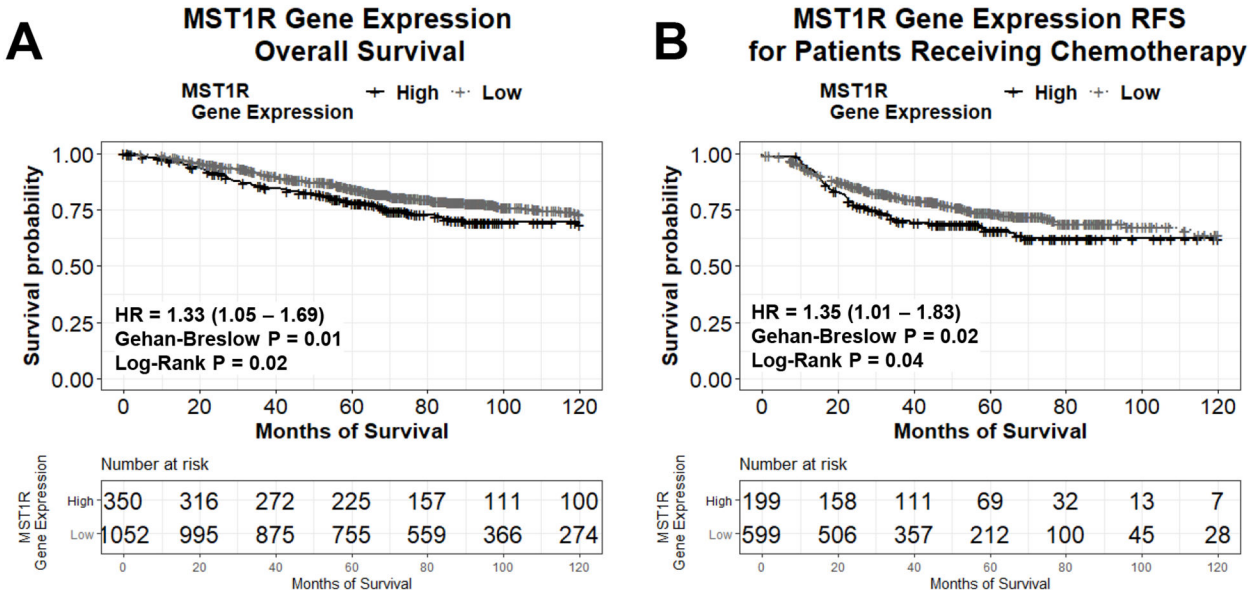

Figure 3:

High MST1R expression in breast tumors is associated with worse overall survival and relapse-free survival in patients receiving chemotherapy. Gene Expression Omnibus (GEO)-derived Kaplan-Meier Plotter data [30] was used to compare survival of the upper quartile of MST1R RNA expression versus the lower three-quartiles of expressing using Kaplan-Meier curves. Overall survival (A) and relapse-free survival (RFS) in patients receiving chemotherapy (B) were examined. HR calculated using Cox proportional hazards model and P-value calculated with log-rank and Gehan-Breslow tests.

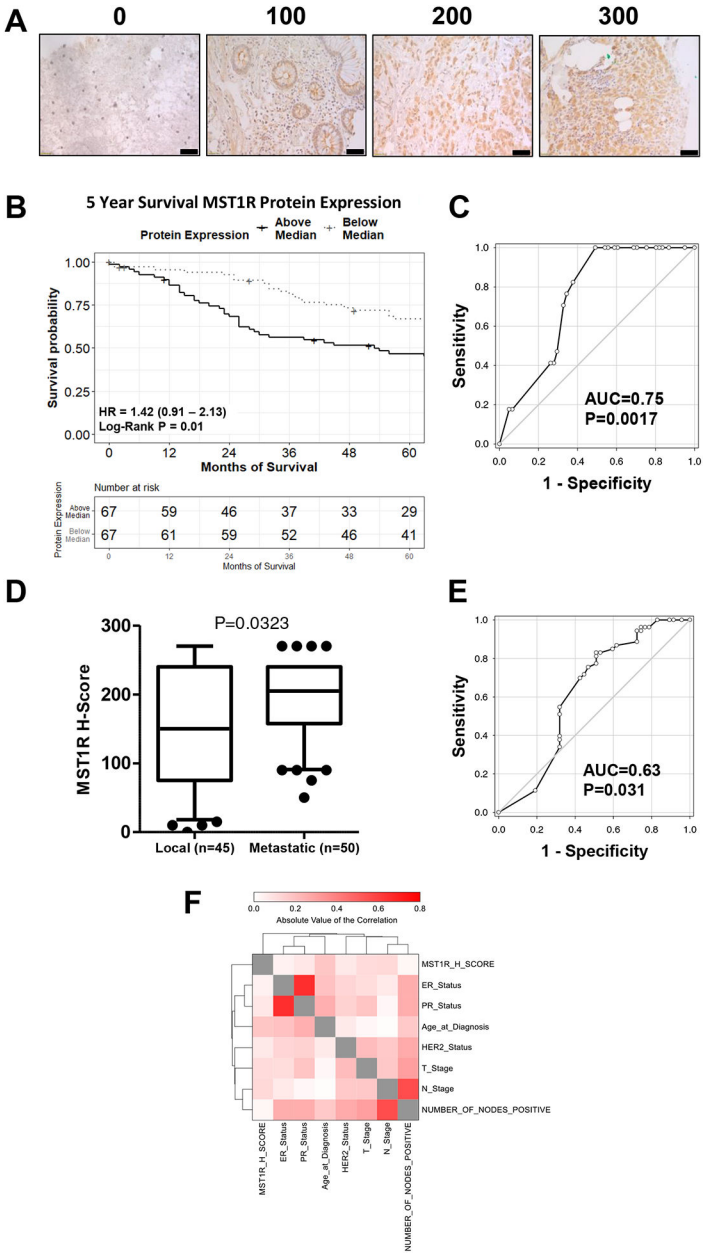

Figure 4:

MST1R expression is associated with reduced survival and metastatic disease progression in breast cancer. MST1R H-Score generated from immunohistochemistry staining of human breast cancer samples from the CDP Breast Cancer Progression tissue microarray and locally-obtained samples. Representative scoring images of 0, 100, 200, and 300 MST1R H-Score (A). Overall survival of patients taken from samples stratified using median MST1R H-Score analyzed by Kaplan-Meier survival curves (B). Statistical significance was determined using Gehan-Breslow test and the hazard ratio (HR) was calculated using Cox proportional hazards analysis on 134 independent samples. Receiver-operator characteristic (ROC) analysis on samples from B stratified as being early death (<24 months post diagnosis) or not (>24 months post diagnosis) to examine predictive potential of the MST1R

H-score to early death (C). H-Scores of metastatic and locally confined samples were compared with box-whisker plots and the rank-sum test (D). ROC analysis of metastatic and locally confined samples from D to examine the predictive potential of the MST1R H-score for metastatic progression (E). Multivariate analysis of correlation between MST1R H-score and other prognostic factors (F).

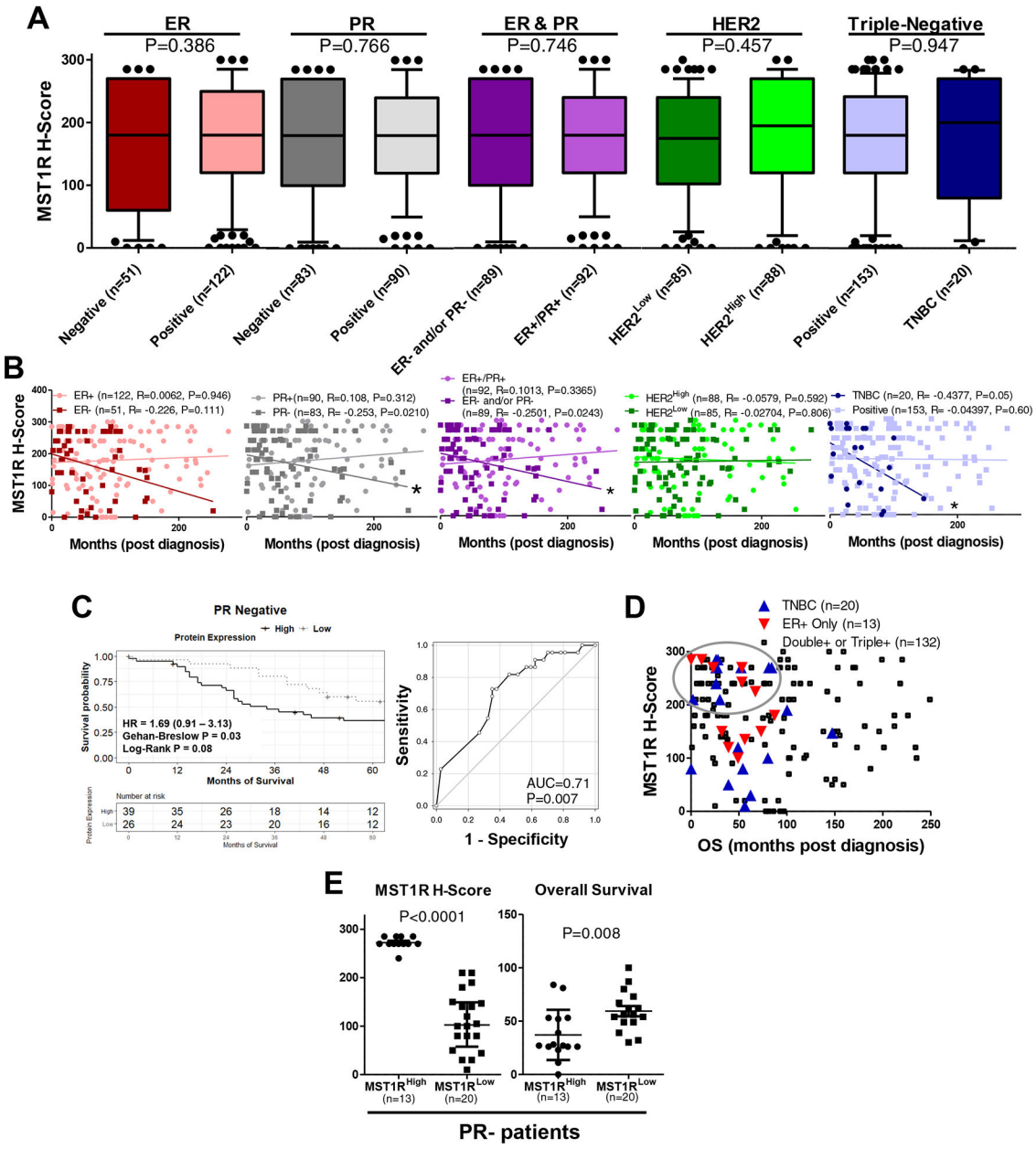

Figure 5:

MST1R expression is not associated with a particular BC subtype, but is associated with poor prognosis in samples with fewer subtype markers. MST1R H-score generated from IHC of human BC samples from the CDP Breast Cancer Progression Tissue Microarray and locally-obtained tissues. Stratification of samples into ER-negative or –positive, PR-negative or –positive, ER/PR double positive or not, HER2 high/amplified or not, and triple negative (TNBC) or not for comparison of MST1R H-score using box-whisker plots with rank-sum tests (A). Overall survival versus MST1R H-score plotted with Spearman's correlation analysis to analyze relationships of MST1R H-score and survival in BC subtypes (B). Samples stratified into above and below the MST1R H-score median of specified subtypes showing an inverse correlation with survival in B and plotted using Kaplan-Meier survival

curves (C). Cox proportional hazards used for HR and log-rank and Gehan-Breslow tests for P-value. ROC analysis with PR-negative samples using MST1R H-score as a continuous variable but stratified for early death (<30 months post diagnosis) or not (>30 months) to examine the predictive value of MST1R H-score on early death in the specified subtype (C). Overall survival versus MST1R H-score plotted with PR-negative (TNBC and ER+ Only) samples of particular interest: High MST1R expressing samples are circled to note the subpopulation to be analyzed (D). PR-negative (PR^{-}) samples stratified into $MST1R^{High}$ (circled in A) or $MST1R^{Low}$ (not circled in D) compared for MST1R H-score (left) and overall survival (right) using box-whisker plots with rank-sum tests (E)

Table 1:

Demographics for patients in the BRCA TCGA transcriptome database including PAM50 subtype, pathologic T stage, presence of cancer positive lymph nodes, and race.

	Count	% (n=808)
PAM50 Subtypes		
Basal-like	141	17.45
HER2-enriched	97	12.00
Luminal B	171	21.16
Normal-like	160	19.80
Luminal A	239	29.58
Stage		**% (n=775)**
T1–2	664	85.79
T3–4	108	13.95
Tx	2	0.26
Nodes		
N0	366	47.29
N+	395	51.03
Nx	13	1.68
Race		
American Indian or Alaska Native	1	0.13
Asian	39	5.08
African American	58	7.55
White	602	78.39
Unreported	74	9.64

Table 2:

Clinical and demographics data of the patients included in the stained breast cancer tissue including subtypes, pathologic T stage, presence of cancer positive lymph nodes, and race.

	Count	% (n=186)
Subtype Markers		
ER+	122	65. 59
PR+	90	48.38
HER2-enriched	85	45.69
Triple negative	20	10.75
Stage		
T1–2	167	89. 78
T3–4	19	10.22
Tx	0	0
Nodes		
N0	33	17. 74
N+	90	48.39
Nx	63	33.87
Race		
American Indian or Alaska Native	0	0
African American	22	11.83
White	163	87.63
Unreported	1	0.54