The neighbor to BRCA1 gene 1, NBR1, is differentially expressed in human breast cancers and its expression positively associates with survival.

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Breast cancer is a leading cause of cancer death for women in the United States (1) and metastasis, or spread of the disease beyond the breast is the central reason women with breast cancer die (2). We utilized a systems-level approach to discover genes associated with breast cancer survival by comparing primary and metastatic tumor transcriptomes based on survival greater or less than 24 months using published datasets (3, 4). We found that the autophagy cargo receptor Neighbor to BRCA1 gene, NBR1 was among the genes most significantly differentially expressed between the tumors of women with disease-free survival more or less than 24 months (3). Analysis of a separate dataset (4), here comparing metastatic tissues of women with breast cancer based on overall survival, again identified NBR1 among the genes whose expression was most significantly different, genome-wide, between women surviving more than 24 months as compared to women who expired before 24 months. NBR1 was recently reported as a regulator of metastasis in mouse models of breast cancer (5). Our findings reveal that NBR1 is expressed at significantly higher levels in women who survive stage IV metastatic breast cancer greater than 24 months as compared to those who will expire from the disease before 24 months, and are in distinct opposition with recently published mechanistic studies in mice suggesting that NBR1 inhibition is anti-metastatic (5). The fact that NBR1 is among the genes whose expression is most significantly higher in the primary and metastatic tumors of patients based on survival outcomes greater than 24 months and expressed significantly higher among survivors, suggests that NBR1 inhibition will not be effective in halting metastasis and should be approached with caution.

Keywords: NBR1, autophagy, Neighbor to BRCA1 gene, breast cancer, systems biology of breast cancer, tumor transcriptome analysis, targeted therapeutics in breast cancer

Each year, tumors that originate in the breast tissues, breast cancer, affects over quarter of a million women in the United States and 42,000 of these women will die from the disease (6). The major reason women die from breast cancer is the spread of the tumor from the breast to a foreign site such as the liver, lungs, bone and brain (2, 7). There are limited targeted therapeutics available for the treatment of metastatic breast cancer (8) and an enhanced understanding of the transcriptional behavior that distinguishes gene expression of tumors in women that survive the disease versus tumors in women that will rapidly expire from the disease is required.

In this study we performed such an analysis, comparing global gene expression profiles of both primary and metastatic tumors from women with breast cancer using separate microarray datasets (3, 4). This systems-level comparative transcriptome analysis revealed that the Neighbor to BRCA1 gene, NBR1 is uniquely expressed in women that survive primary and metastatic breast cancer greater than 24 months. A recent genetic analysis of the cellular autophagic machinery in mouse models of breast cancer argued that NBR1 is a critical regulator of metastatic disease in murine breast cancers (5). Our findings contradict those data, revealing that NBR1 expression is significantly correlated with positive survival outcomes in humans with breast cancer, both primary and metastatic, and suggest that NBR1 inhibition can be dangerous in women with breast cancer, highlighting the importance of using human tissues for the study of human diseases.

Methods

We used GSE48391 (3) and GSE124647 (4) for this analysis in conjunction with GEO2R.

GSE48391 (3) was generated using Affymetrix Human Genome U133 Plus 2.0 Array technology with n=11 for primary tumors of the breast from women with disease-free survival greater than 24 months and an n=70 for primary tumors of the breast from women with disease-free survival less than 24 months.

1 GSE124647 (4) was generated using Affymetrix Human Genome U133A Array from
2 biopsies from patients with stage IV metastatic breast cancer with n=70 for metastatic tumors
3 from women surviving greater than 24 months and n=70 for metastatic tumors from women
4 surviving less than 24 months.

5 The Benjamin and Hochberg method of p-value adjustment was used for ranking of
6 differential expression but raw p-values were used for assessment of statistical significance of
7 global differential expression. Log-transformation of data was auto-detected, and the NCBI
8 generated category of platform annotation was used.

9
10 A statistical test was performed to evaluate the significance of difference between mRNA
11 expression levels of NBR1 in the primary tumors of women surviving less than 24 months as
12 compared to the primary tumors of women surviving greater than 24 months, as well as in the
13 metastatic tissues of women surviving less than 24 months as compared to the metastatic
14 tissue of women surviving greater than 24 months using a two-tailed, unpaired t-test with
15 Welch's correction. Only p-values less than 0.05 were considered statistically significant. We
16 used PRISM for all statistical analyses (Version 8.4.0)(455).
17
18
19

20 **Results**

21 We performed systems-level, unbiased comparative transcriptome analysis with paired
22 survival data to discover genes differentially expressed in the primary and metastatic tumor
23 tissues of women with breast cancer, based on survival at 24 months, using independent
24 published datasets (3, 4)
25

26 NBR1 is differentially expressed in the primary breast tumors of patients when comparing
27 tumors transcriptomes based on disease-free survival greater than 24 months.

28 We identified the autophagy cargo receptor NBR1 (Neighbor to BRCA1 gene) as among
the genes whose expression was most quantitatively difference when comparing the global

gene expression profiles of primary tumors from women with breast cancer surviving less than 24 months with that of primary tumors from women with breast cancer surviving greater than 24 months using a published dataset (3). When sorting each of the transcripts detected in the microarray dataset analyzed here based on magnitude of change in the tumors of women surviving greater or less than 24 months, NBR1 ranked 70 out of 54674 transcripts. Differential expression of NBR1 was statistically significant (Table 1; $p=0.00070602$).

NBR1 is differentially expressed in the metastatic tumor tissues of patients when comparing metastatic tumors transcriptomes based on overall survival greater than 24 months.

We used a separate dataset (4) to compare the global gene expression profiles of metastatic tissues from women with breast cancer based on survival greater or less than 24 months. We again identified IL6ST as one of the genes whose expression changed most significantly in the metastatic tumor transcriptomes of patients based on survival at 24 months. When sorting each of the transcripts detected in the microarray dataset analyzed based on magnitude of change in the metastatic tumor tissue of women based on survival at 24 months, a transcript encoded by the NBR1 gene ranked 229 out of 22283 transcripts. Differential expression of NBR1 in metastatic tissue samples based on survival at 24 months was statistically significant (Table 2; $p=1.07E-03$).

NBR1 is expressed at significantly higher levels in the primary breast tumors of patients with disease-free survival greater than 24 months.

Next, we extracted exact mRNA expression values for NBR1 in the primary tumors of women whose transcriptomes we compared based on disease-free survival at 24 months. We also performed a statistical test to evaluate whether the difference in expression of NBR1 was statistically significant. NBR1 was expressed at higher levels in the primary breast tumors of patients surviving greater than 24 months. The difference in expression of NBR1 between the

primary tumors of patients surviving more or less than 24 months approached the level of statistical significance (Figure 1; $p=0.0575$).

NBR1 is expressed at significantly higher levels in the metastatic tumor tissues of patients with overall survival greater than 24 months.

We also extracted exact mRNA expression values for NBR1 in the metastatic tumor tissues of women and compared NBR1 expression based on disease-free survival at 24 months. We also performed a statistical test to evaluate whether the difference in expression of NBR1 was statistically significant. NBR1 expression was higher in the metastatic tumor tissues of patients surviving greater than 24 months than in the metastatic tumor tissues of patients surviving less than 24 months. This difference in NBR1 expression was statistically significant (Figure 2; $p<0.0001$).

Thus, we found that NBR1 was expressed at higher levels in both the primary and metastatic tumor tissues of patients with breast cancer who survived greater than 24 months when compared to that of patients surviving less than 24 months. In both primary tumors from patients with breast cancer and in the metastatic tumors from the lungs, the liver, the brain, the bones and soft tissues from patients with stage IV metastatic breast cancer, the difference in NBR1 expression was statistically significant. These data, in direct opposition with recently published findings from mechanistic studies in mice (CITATION) arguing that NBR1 promotes metastasis, indicate that NBR1 expression promotes survival in humans, in both primary and advanced metastatic breast cancers.

Discussion

Women diagnosed with metastatic breast cancer are presented with a 5-year survival rate of 27% (9). In this study, we used a method termed differential gene expression analysis to mine published datasets (3, 4) and compare the transcriptomes of primary and metastatic tumors from women with breast cancer in order to identify genes that were expressed most uniquely when comparing these women based on survival outcomes greater or less than 24

1 months. In our analysis of primary breast tumors (3), we identified NBR1 as among the genes
2 most differentially expressed in the tumors of women who with disease-free survival greater
3 than 24 months, at significantly higher levels in survivors. In our analysis of metastatic tissues
4 from women with stage IV metastatic breast cancer (4), we identified NBR1 as among the
5 genes most differentially expressed in the metastases of women who survived greater than 24
6 months, and again, we found that NBR1 was expressed at significantly higher levels in the
7 metastatic tissues of survivors. NBR1 is a receptor for ubiquitinated cargo in the autophagy
8 pathway, contains both an LC3 interaction region (LIR) and a ubiquitin-binding domain, and is
9 degraded during autophagy (10).

11 A recently published study performing genetic analyses of autophagic machinery in pre-
12 clinical mouse models of breast cancer came to the conclusion that expression of NBR1
13 promoted metastases in murine breast cancers after finding that inhibition of NBR1 reducing
14 by short hairpin depletion reduced metastasis size (5). Our data, obtained by unbiased and
15 systems-level analysis of published transcriptome data from human tissues, both primary
16 tumors and metastatic tumors, argue that NBR1 expression is rather positively associated with
17 survival outcomes greater than 24 months. NBR1 was expressed at significantly higher levels
18 in the primary tumors of women with disease-free survival greater than 24 months. Most
19 importantly, NBR1 was expressed at significantly higher levels in metastatic tissues from
20 multiple organ sites in women that survived stage IV metastatic breast cancer greater than 24
21 months. There are several reasons why our data might come to the opposite conclusion of
22 Marsh et al (5). Our study analyzed gene expression data from the tumors and metastatic
23 tissues of humans with breast cancer (3, 4), as opposed to Marsh et al. who performed elegant
24 genetic studies of autophagic machinery in breast cancer but using mice (5). We discovered
25 NBR1 by blindly asking which genes were expressed most differently in the transcriptomes of
26 metastatic tissues of women who survived stage IV metastatic breast cancer, rather than
27 studying NBR1 by experimental assays through expression and depletion in mice to elucidate
28 more clearly the role of autophagy in metastatic progression (5). Moreover, the data we mined

for this analysis were associated with survival outcomes from women with breast cancer, either disease-free survival (3) or overall survival (4).

In our comparative transcriptome analysis of primary tumors from patients with breast cancer based on disease-free survival, we used transcriptome data from women from breast cancer across multiple cancer types: hormone receptor positive as well as hormone receptor negative (HR+ and HR-), cancers that expressed normal levels of HER2 as well as cancers over-expressing HER2, cancers that were both positive and negative from estrogen receptor (ER+ and ER-), and from cancers with molecular subtypes including both Group 1 (luminal A), Group 2 (luminal B), Group 3 (HER2), and Group 4 (basal-like) (3) (11). In our comparative transcriptome analysis of metastatic tumor tissue from patients with stage IV metastatic breast cancer (4), we used transcriptome data from women diagnosed with HR+/HER2- breast cancers, from metastases to the liver, the lungs, the skin, the soft tissues, the lymph nodes, and the bones, meaning that our findings were not restricted to metastases to any one site. Thus, across multiple molecular subtypes of breast cancers, in both primary and metastatic tumors from women with breast cancer, across metastases to multiple organ sites, and across ethnically distinct cohorts of women, increased expression of NBR1 was associated with enhanced survival outcomes in breast cancer. (3, 4) Nevertheless, these data should be confirmed in separate cohorts of patients with breast cancer, in patients with HR- and HER2+ metastatic breast cancers, and at the protein level. NBR1 inhibition as a therapeutic strategy in metastatic breast cancer should be viewed with caution. We argue that our findings are one example of why research and development of therapeutics in breast cancer and cancer in general should start with target discovery using human data and subsequently utilize pre-clinical murine models, to gain mechanistic insights of targets discovered through analysis of data from humans, and/or for pre-clinical screening of compound efficacy.

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Rank	ID	p-value	t	B	Gene	Gene name
70	201384_s_at	0.00070602	3.52	-0.91283	NBR1	NBR1, autophagy cargo receptor

Table 1: NBR1 is differentially expressed in the primary tumors of patients with breast cancer based on disease-free survival at 24 months.

The rank of differential expression globally, out of 54674 transcripts in total, the probe ID, the p-value of global differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the two groups compared, the gene and gene name are all listed in this chart.

Rank	ID	p-value	t	B	Gene	Gene name
229	201384_s_at	1.07E-03	3.34018604	-0.89788	NBR1	NBR1, autophagy cargo receptor

Table 2: NBR1 is differentially expressed in the metastatic tumor tissues of patients with breast cancer based on overall survival at 24 months.

The rank of differential expression globally, out of 22283 transcripts in total, the probe ID, the p-value of global differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the two groups compared, the gene and gene name are all listed in this chart.

Figure 1: NBR1 is expressed at significantly higher levels in the primary tumors of breast cancer patients with disease-free survival greater than 24 months.

The expression of NBR1 in the primary tumors of patients with breast cancer based on disease-free survival at 24 months is shown on the left by patient with the mean value marked and p-value listed, and on the right as a violin plot with the median value, 25th and 75th percentiles marked and p-value listed.

Figure 2: NBR1 is expressed at significantly higher levels in the metastatic tumors of breast cancer patients with overall survival greater than 24 months.

The expression of NBR1 in the metastatic tumor tissues of patients with breast cancer based on overall survival at 24 months is shown on the left by patient with the mean value marked and p-value listed, and on the right as a violin plot with the median value, 25th and 75th percentiles marked and p-value listed.