The zinc finger E-box binding homeobox transcriptional repressor ZEB1 is differentially expressed in the metastases of patients with breast cancer.

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Metastasis is a major problem in patients with breast cancer (1). We analyzed published microarray (2) and multiplexed mRNA quantification (3) datasets to identify transcription factors whose expression changed most significantly between primary tumors in patients with breast cancer and metastatic tissues. We identified ZEB1 as differentially expressed when comparing the transcriptomes of primary tumors of the breast to the metastases they generate (2). Analysis of a separate multiplexed mRNA quantification dataset uncovered similar differential expression of ZEB1 in metastases compared to primary breast tumors (3). ZEB1 expression has been reported as important for metastases and for the epithelial to mesenchymal transition in cancer (4-9); we found in this study, however, that ZEB1 was expressed at significantly lower levels in metastases across tissue type when compared to primary breast tumors.

Keywords: breast cancer, metastasis, metastatic breast cancer, ZEB1, deltaEF1, systems biology of breast cancer, tumor transcriptome analysis.

1 Patients diagnosed with metastatic breast cancer are faced with a 5-year overall survival
2 rate of 27% (10). An enhanced understanding of the metastatic process in patients with breast
3 cancer is required in order to design novel therapeutics to halt, reverse or delay metastases in
4 patients with breast cancer. Systems-level analysis of the metastatic transcriptome as
5 compared to primary tumors of the breast can facilitate this understanding by revealing how
6 gene expression is most significantly different in metastatic tissues and help identify therapeutic
7 targets for patients with metastatic breast cancer. In this study we performed such an analysis,
8 mining published transcriptome data to compare global transcriptional profiles of liver
9 metastases from patients with breast cancer to primary tumors of the breast. We
10 complemented this analysis using a separate and more limited dataset generated using
11 NanoString nCounter mRNA quantification technology of metastatic tissues from multiple organ
12 sites compared to that of primary tumors of the breast. Across both datasets, we identified the
13 zinc-finger E-box binding homeobox transcription factor and transcriptional repressor ZEB1 as
14 among the most differentially expressed genes in metastases when compared to primary
15 tumors of the breast. It is documented that ZEB1 expression facilitates metastases and enables
16 the epithelial to mesenchymal transition that is thought to accompany the metastatic process by
17 repression of genes such as E-cadherin (4-9); however, we found that ZEB1 was expressed at
18 significantly lower levels in the metastatic tissues of patients with breast cancer as compared to
19 primary tumors of the breast.
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22 **Methods**

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24 We used GSE124648 (2) and GSE92977 (3) for this differential gene expression
25 analysis in conjunction with GEO2R. GSE124648 was generated using Affymetrix Human
26 Genome U133A array technology with $n=235$ primary tumors of the breast and $n=16$ liver
27 metastases. These primary tumors included stage IA, IIA, IIB, IIIA and IIIC or of a stage not
28 indicated. Tissue sample types included surgical tissue, fine needle aspiration, or core biopsy.

1 The types of breast cancers whose expression was profiled in the dataset utilized for this
2 analysis included hormone receptor positive cancers (ER+/HER2-, ER+/HER2+, HR+/HER2-,
3 HR+/HER2+), as well as a group of breast cancers without immunohistochemical subtype.
4 GSE92977 was generated using was generated using NanoString nCounter Nano105 Gene
5 Expression technology using a custom CodeSet, with $n=123$ for primary breast tumors and
6 $n=123$ for metastases from multiple organ sites. The types of breast cancers whose expression
7 was profiled in the dataset utilized for this second analysis included tumors of the molecular
8 subtype luminal A, luminal B, basal, normal, and HER2+. The organ sites from which
9 metastases were obtained included the bones, the lungs, the ovaries, the skin, the lymph nodes
10 and the pleura.
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13 The Benjamini and Hochberg method of p-value adjustment was used for ranking of
14 differential expression but raw p-values were used for assessment of statistical significance of
15 global differential expression. Log-transformation of data was auto-detected, and the NCBI
16 generated category of platform annotation was used.
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18 To evaluate the statistical significance of difference in ZEB1 expression between primary
19 and metastatic tumor tissues, we used a two-tailed, unpaired t-test with Welch's correction,
20 using PRISM (8.4.0)(455).
21

22 **Results**

23 We sought to further understand the transcriptional behavior of breast cancer
24 metastases using human data at the systems-level in an unbiased fashion. Thus, we mined
25 published transcriptome (1) and multiplexed mRNA quantification data (2) to identify
26 transcription factors whose expression was most significantly different between primary tumors
27 of the breast and metastases they generate.
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ZEB1 is differentially expressed in metastases to the liver when compared to primary tumors of the breast.

We found that the zinc-finger E-box binding homeobox transcription factor ZEB1 was among the genes most differentially expressed in metastases to the liver from patients with breast cancer when compared to primary tumors of the breast. When sorting all of the mRNAs detected by microarray based on change in expression between liver metastases and primary tumors of the breast, ZEB1 ranked 58 out of 22283. Differential expression of ZEB1 in liver metastases when compared to primary tumors from patients with breast cancer was statistically significant (Table 1; $p=1.18E-34$).

ZEB1 is differentially expressed in metastases to multiple organ sites when compared to primary tumors of the breast.

Analysis of a more limited mRNA expression dataset generated using multiplexed mRNA qualification by NanoString nCounter revealed that ZEB1 was also among the genes most differentially expressed in metastases to multiple organ sites from patients with breast cancer when compared to primary tumors of the breast. These organ sites and tissues included the bones, the lungs, the ovaries, the skin, the lymph nodes and the pleura. When sorting all of the mRNAs detected by microarray based on change in expression between metastatic tissues and primary tumors of the breast, ZEB1 ranked 4 out of 105. Differential expression of ZEB1 in metastases to multiple organ sites when compared to primary tumors from patients with breast cancer was statistically significant (Table 1; $p=4.75E-08$).

ZEB1 is expressed at significantly lower levels in metastases to the liver when compared to primary tumors of the breast.

We extracted exact mRNA expression levels of ZEB1 from each liver metastasis sample and from each primary breast tumor sample to compare expression of ZEB1 between metastases and primary tumors. We also performed a statistical test to determine if the

1 difference in ZEB1 expression between primary and metastatic tumor tissues was statistically
2 significant. ZEB1 expression was significantly lower in liver metastases when compared to
3 primary tumors of the breast in patients with breast cancer (Figure 1; $p < 0.0001$). We calculated
4 a fold change of 0.59 in expression of ZEB1 between metastases and primary tumors (Table 1).

5
6 ZEB1 is expressed at significantly lower levels in metastases to multiple organ sites when
7 compared to primary tumors of the breast.

8 Again, we extracted exact mRNA expression levels of ZEB1 from each metastasis
9 sample and from each primary breast tumor sample to compare expression of ZEB1 between
10 metastases and primary tumors. We also performed a statistical test to determine if the
11 difference in ZEB1 expression between primary and metastatic tumor tissues was statistically
12 significant. ZEB1 expression was significantly lower in metastases to multiple organ sites in
13 patients with breast cancer when compared to primary tumors of the breast (Figure 2;
14 $p < 0.0001$). We calculated a fold change of 0.79 in expression of ZEB1 between metastases
15 and primary tumors (Table 2).

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17 Thus, we found that ZEB1 was differentially expressed in metastases to the liver and
18 multiple organ sites when compared to primary tumors from patients with breast cancer, and
19 that it was expressed at significantly lower levels in metastases when compared to primary
20 tumors of the breast.

21 22 **Discussion**

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24 In this study we mined published transcriptome (2) and multiplexed gene expression
25 data (3) from the metastatic tissues and primary tumors of patients with breast cancer to identify
26 transcription factors differentially expressed between metastases and primary tumors. We
27 identified ZEB1 as among the genes whose expression most significantly changed between
28 metastatic tumor tissues from the liver and from multiple organ sites as compared to primary

1 breast tumors. ZEB1 was expressed at significantly lower levels in metastases when compared
2 to primary tumors from patients with breast cancer.

3 ZEB1 has been shown to promote metastasis and its expression has been
4 demonstrated to correlate with metastatic potential (4, 5). ZEB1 induces the expression of
5 genes associated with the epithelial-mesenchymal transition, thought to be important for the
6 metastatic process (5-8). Specifically, ZEB1 (also known as deltaEF1) functions as a
7 transcriptional repressor of the cell adhesion molecule E-cadherin (7). Intertwined in this
8 process is the non-coding RNA microRNA miR-200, which bears a reciprocal relationship with
9 ZEB1 (8, 9). miR-200 represses expression of ZEB1, which represses expression of E-cadherin
10 (8, 9). Down-regulation of miR-200 relieves repression of ZEB1, which induces E-cadherin
11 repression and acts to promote metastasis, while up-regulation of miR-200 inhibits ZEB1
12 expression and thereby relieves ZEB1 repression of E-cadherin, limiting the metastatic process
13 (8, 9). Despite the fact that ZEB1 expression has been demonstrated to enhance the metastatic
14 process through its enabling of the epithelial-mesenchymal transition, we found that ZEB1 was
15 expressed at significantly lower levels in metastatic tissues in the liver and across multiple organ
16 sites when compared to primary tumors of the breast from patients with breast cancer. One
17 study has documented higher expression of ZEB1 in primary tumors correlated with distant
18 metastasis and that ZEB1 was expressed at significantly higher levels in primary breast tumors
19 than in normal breast tissues (4). ZEB1 expression should be thoroughly assessed in larger
20 and separate cohorts of patients with breast cancer, in primary tumors and in metastatic tissues
21 to determine if the findings here can be validated. While activation of EMT is important for
22 changes in the plasticity of epithelial cells and for induction of the mesenchymal program,
23 theoretically, repression of EMT should be similarly important once metastatic cells have
24 colonized a foreign tissue to enable metastatic tumor growth in the foreign site (10), and down-
25 regulation of ZEB1 in metastases is one mechanism by which EMT inhibition to promote
26 metastatic colonization of a foreign site might be achieved.
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Rank	ID	p-value	t	B	Fold change	Gene	Gene name
58	212758_s_at	1.18E-34	-14.383152	68.050659	0.59	ZEB1	zinc finger E-box binding homeobox 1

Table 1: ZEB1 is differentially expressed in metastases to the liver when compared to primary tumors of the breast.

The rank of differential expression, probe/transcript ID, the p-value of global differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the two groups, the fold change in mRNA expression between the metastases and primary tumors, the gene and gene name are listed in this chart.

Rank	ID	p-value	t	B	Fold change	Gene	Gene name
4	110/ NM_001128128.1	4.75E-08	-5.6358	7.966	0.79	ZEB1	zinc finger E-box binding homeobox 1

Table 2: ZEB1 is differentially expressed in metastases to multiple organ sites when compared to primary tumors of the breast.

The rank of differential expression, probe/transcript ID, the p-value of global differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the two groups, the fold change in mRNA expression between the metastases and primary tumors, the gene and gene name are listed in this chart.

Figure 1: ZEB1 is expressed at significantly lower levels in metastases to the liver when compared to primary tumors of the breast.

The mRNA expression level of ZEB1 is shown in primary tumors of the breast (left) and liver metastases (right). The mean mRNA expression levels in each group are marked and the result of a statistical test evaluating the significance of difference in expression of ZEB1 between groups, a p-value, is listed.

Figure 2: ZEB1 is expressed at significantly lower levels in metastases to multiple organ sites when compared to primary tumors of the breast.

The mRNA expression level of ZEB1 is shown in primary tumors of the breast (left) and liver metastases (right). The mean mRNA expression levels in each group are marked and the result of a statistical test evaluating the significance of difference in expression of ZEB1 between groups, a p-value, is listed.