1 **Expression of zinc-finger protein X-linked, ZFX, is associated with enhanced survival in**
2 **patients with breast cancer.**

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7 We mined published tumor transcriptome data with paired survival data to discover genes
8 associated with survival outcomes in breast cancer (1, 2). We found that the zinc-finger protein
9 X-linked ZFX (3, 4) was among the genes most differentially expressed in both primary and
10 metastatic tumor tissues when comparing tumor transcriptomes based on survival at 24
11 months. ZFX expression was significantly higher in the metastatic tumors of patients surviving
12 greater than 24 months, suggesting that increased ZFX expression confers a survival benefit to
13 patients with stage IV metastatic breast cancer.

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21 Keywords: breast cancer, metastatic breast cancer, ZFX, targeted therapeutics in breast
22 cancer, tumor transcriptome analysis, systems biology of breast cancer.

1 Patients with metastatic breast cancer possess unacceptable survival outcomes, with a
2 5 year overall survival (OS) rate of 27% (5). To facilitate therapeutic target discovery and an
3 understanding of the basic transcriptional nature of metastatic breast cancer we mined
4 published microarray data from the primary and metastatic tumors of patients with breast
5 cancer with paired survival outcome data (1, 2). By comparing the transcriptomes of
6 metastatic tumor tissues from patients with breast cancer, we found that the ZFX was among
7 the genes whose expression most significantly differed between those surviving more or less
8 than 24 months. Analysis of a separate dataset revealed that ZFX expression was also
9 associated with disease-free survival at 24 months when comparing the transcriptomes of
10 primary breast tumors. These data indicate that increased ZFX expression is associated with
11 enhanced survival in human breast cancer and are at odds with published data suggesting
12 higher ZFX expression is associated with poorer survival outcomes in patients with breast
13 cancer.
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15 16 **Methods**

17 We used datasets GSE48391 (1) and GSE124647 (2) in conjunction with GEO2R for this
18 comparative differential gene expression analysis of the breast cancer transcriptome.
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20 GSE48391 (1) was generated using Affymetrix Human Genome U133 Plus 2.0 Array
21 technology with $n=70$ for primary tumors of the breast from women with disease-free survival
22 greater than 24 months and an $n=11$ for primary tumors of the breast from women with
23 disease-free survival less than 24 months.

24 GSE124647 (2) was generated using Affymetrix Human Genome U133 Array from
25 biopsies from patients with stage IV metastatic breast cancer with an $n=70$ for metastatic
26 tumor tissues from women surviving greater than 24 months and an $n=70$ for metastatic tumor
27 tissues from women surviving less than 24 months.
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The Benjamin and Hochberg method of p-value adjustment was used for ranking of
differential expression but raw p-values were used for assessment of statistical significance of

1 global differential expression. Log-transformation of data was auto-detected, and the NCBI
2 generated category of platform annotation was used.

3 A statistical test was performed to evaluate the significance of difference between
4 mRNA expression levels of ZFX in the primary tumors of women surviving less than 24 months
5 as compared to the primary tumors of women surviving greater than 24 months, as well as in
6 the metastatic tissues of women surviving less than 24 months as compared to the metastatic
7 tissue of women surviving greater than 24 months using a two-tailed, unpaired t-test with
8 Welch's correction. Only p-values less than 0.05 were considered statistically significant. We
9 used PRISM for all statistical analyses (Version 8.4.0)(455).
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12 **Results**

13 We mined published microarray data to perform a comparative transcriptome analysis
14 of tumors from patients with breast cancer and discover genes associated with enhanced
15 survival outcomes.
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17 ZFX is differentially expressed in the primary breast patients with breast cancer that have
18 disease-free survival greater than 24 months when compared to those with disease-free
19 survival less than 24 months.
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22 We identified ZFX as one of the genes most differentially expressed in the tumors of
23 patients when comparing transcriptomes based on disease-free survival at 24 months.
24 When sorting each transcript measured by microarray based on change in expression between
25 tumors from patients with disease-free survival less than 24 months versus tumors from
26 patients with disease-free survival greater than 24 months, a transcript encoded by the ZFX
27 gene ranked 229 out of 54674 transcripts. Differential expression of ZFX in primary breast
28 tumors from patients with disease-free survival greater than 24 months was statistically
significant (Table 1; $p=0.00221466$).

ZFX is differentially expressed in the metastatic tumor tissues of patients with breast cancer that have overall survival greater than 24 months when compared to those with overall survival less than 24 months.

We further analyzed a separate microarray dataset, this containing transcriptome data from metastatic tissues from multiple organ sites from patients with stage IV metastatic breast cancer. Again, we identified ZFX as among the most differentially expressed genes in tumor metastases when comparing tumor transcriptomes based on overall survival at 24 months (Table 2).

When sorting each transcript measured by microarray based on change in expression between metastatic tumors from patients with overall survival greater than 24 months versus tumor metastases from patients with overall survival less than 24 months, a transcript encoded by the ZFX gene ranked 137 out of 22283 transcripts. Differential expression of ZFX in primary breast tumors from patients with disease-free survival greater than 24 months was statistically significant (Table 2; $p=5.11E-04$).

ZFX is expressed at significantly higher levels in the primary tumors of patients with breast cancer with disease-free survival greater than 24 months as compared to the primary tumors of patients with breast cancer with disease-free survival less than 24 months.

We extracted exact mRNA expression levels of ZFX from each patient tumor and performed a statistical test to determine whether the difference in expression between survival groups was statistically significant. ZFX was expressed at significantly higher levels in the primary tumors of breast cancer patients with disease-free survival greater than 24 months as compared to that of patients with disease-free survival less than 24 months, and this difference was statistically significant (Figure 1; $p=0.0151$). We calculated a fold change of 1.05 between survival groups (Table 1).

ZFX is expressed at significantly higher levels in the metastatic tumor tissues of patients with breast cancer with overall survival greater than 24 months as compared to the primary tumors of patients with breast cancer with overall survival less than 24 months.

We also extracted exact mRNA expression levels of ZFX from each metastatic tumor tissue and performed a statistical test to determine whether the difference in expression between survival groups was statistically significant. ZFX was expressed at significantly higher levels in the metastatic tumor tissues of stage IV metastatic breast cancer patients with overall survival greater than 24 months as compared to that of patients with overall survival less than 24 months, and this difference was statistically significant (Figure 2; $p=0.0011$). We calculated a fold change of 1.19 between survival groups (Table 2).

Thus, we identified ZFX as a differentially expressed in human breast cancer, both in primary tumors and in metastatic tumors, when comparing patients based on survival outcomes at 24 months. ZFX was expressed at significantly higher levels in primary tumors from breast cancer patients with disease-free survival greater than 24 months. In patients with stage IV metastatic breast cancer, ZFX was expressed at significantly higher levels in metastatic tumors from patients with overall survival greater than 24 months.

Discussion

The ZFX gene is a gene encoded on the X chromosome, possesses 13 zinc fingers and is identical to a similar gene on the Y chromosome, ZFY, except for 10 amino acids out of 393 amino acids in total (3, 4). ZFX is critical for the self-renewal of embryonic stem cells (ESC) (6). ZFX-deficient embryonic stem cells cannot self renew but their differentiation capacity is intact. ZFX is also critical for hematopoietic stem cell (HSC) function and survival (6). ZFX deficiency in Tie2-Cre mice resulted in loss of adult HSC, while homing, egress, and erythromyeloid progenitor function was normal in remaining knock-out HSC (6). Complete knock-out was rare,

1 however, suggesting that there is strong selection against loss of ZFX in the HSC (6). Primitive
2 hematopoiesis in the yolk sac of Tie2-Cre Zfx-deficient mice was normal, indicating that ZFX is
3 selectively required for adult but not primitive hematopoiesis (6). After injection with plpC, mice
4 reconstituted with Mx-Cre Zfx-deficient bone marrow lose the ability to produce B-cells, T-
5 cells, granulocytes, dendritic cells, and most NK cells, and there was a significant increase in
6 Annexin-V positive apoptotic HSC, demonstrating that ZFX is required for HSC self-renewal,
7 maintenance and survival *in vivo* (6). ZFX is a unique molecule that has the ability to control the
8 properties of both embryonic and hematopoietic stem cells (6, 7).

10 Elsewhere in the hematopoietic system, ZFX has been reported as important for BCR-
11 induced B-cell proliferation and B-cell survival (8). ZFX was also found to be an inhibitor of the
12 differentiation of acute T-lymphoblastic leukemia and myeloid leukemias (9), and important for
13 imatinib resistance and proliferation of chronic myeloid leukemia cells (10).

14 With respect to non-hematopoietic cancers, ZFX has been widely reported to be
15 important for tumor progression and tumor cell growth in a variety of solid tumors. These
16 include gastric cancer, gliomas and glioblastomas, squamous cell carcinomas of the tongue,
17 pancreatic cancer, renal carcinoma, gallbladder cancer, and colorectal cancer (11-19).

19 Three separate studies have come to the conclusion that increased ZFX expression, in
20 general, is pro-tumorigenic and/or facilitates tumor growth in breast cancer (20-22). One of
21 these studies found that ZFX silencing resulted in decreases in breast cancer cell proliferation
22 (20). Another study found that increased ZFX expression was linked with metastatic
23 progression (21). A third study found that the long non-coding RNA was important for triple
24 negative breast cancer cells through microRNA 218 silencing of ZFX (22). On the other hand,
25 our data demonstrate that higher ZFX expression is associated with enhanced survival
26 outcomes and that ZFX is among the genes most differentially expressed in those surviving
27 stage IV metastatic cancer greater than 24 months and is expressed at higher levels in the
28 primary and metastatic tumors of human patients with breast cancer with survival outcomes
greater than 24 months.

1 Mechanistically, one study concluded that ZFX was important for tumorigenesis by
2 activation of the Hedgehog pathway (23). The transcriptional targets of ZFX include histone
3 genes (24) and the MHC-Class I HLA-11 promoter (25).

4 Differential expression of ZFX in breast cancer should be validated at the protein level,
5 and in larger and separate cohorts of breast cancer patients. As we found that patients with
6 better survival outcomes, in general, expressed higher levels of ZFX, the effect of enforced ZFX
7 expression on tumor cell proliferation and viability should be evaluated in breast cancer cell
8 lines *in vitro* and the effect of enforced expression of ZFX expression in tumor growth,
9 metastasis and survival should be evaluated in xenograft breast cancer models *in vivo*.

11 In this study, we found that ZFX was among the genes whose expression was most
12 quantitatively different when comparing the tumors of patients with localized and metastatic
13 breast cancer based on survival outcomes at 24 months. ZFX was expressed at significantly
14 higher levels in the primary tumors of breast cancer patients with disease-free survival greater
15 than 24 months. ZFX was expressed at significantly higher levels in the metastatic tumor
16 tissues of patients with stage IV metastatic breast cancer with overall survival greater than 24
17 months. Together our findings indicate that ZFX expression is associated with enhanced
18 survival outcomes in patients with breast cancer, including patients with stage IV metastatic
19 breast cancer. Modulation of ZFX expression may be a rationale therapeutic strategy in breast
20 cancer.
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Rank	ID	p-value	t	B	Fold change	Gene	Gene name
229	214678_x_at	0.00221466	3.16	-1.71336	1.05	ZFX	zinc finger protein, X-linked

Table 1: ZFX is differentially expressed in the primary tumors of breast cancer patients with disease-free survival greater than 24 months as compared to that of patients with disease-free survival less than 24 months.

The rank of differential expression globally (out of 54674 total transcripts), 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 listed in this chart.

Rank	ID	p-value	t	B	Fold change	Gene	Gene name
137	217176_s_at	5.11E-04	3.55664279	-0.26492	1.19	ZFX	zinc finger protein, X-linked

Table 2: ZFX is differentially expressed in the metastatic tumor tissues of breast cancer patients with overall survival greater than 24 months as compared to that of patients with overall survival less than 24 months.

The rank of differential expression globally (out of 22283 total transcripts), 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 listed in this chart.

Figure 1: ZFX is expressed at significantly higher levels in the primary tumors of breast cancer patients with disease-free survival greater than 24 months as compared to that of patients with disease-free survival less than 24 months.

The mRNA expression level of ZFX in each patient tumor is graphically depicted with mean mRNA expression levels marked and p-value evaluating statistical significance of difference in ZFX expression listed. Patients with disease-free survival (DFS) less than 24 months: $n=11$. Patients with disease-free survival (DFS) greater than 24 months: $n=70$.

Figure 2: ZFX is expressed at significantly higher levels in the metastatic tumor tissues of breast cancer patients with overall survival greater than 24 months as compared to that of patients with overall survival less than 24 months.

The mRNA expression level of ZFX in each patient tumor is graphically depicted with mean mRNA expression levels marked and p-value evaluating statistical significance of difference in ZFX expression listed. Patients with overall survival (OS) less than 24 months: $n=70$. Patients with overall (OS) greater than 24 months: $n=70$.