Differential expression of fibronectin in cancers of the breast.

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Breast cancer affects women at relatively high frequency¹. We mined published microarray datasets^{2,3} to determine in an unbiased fashion and at the systems level genes most differentially expressed in the primary tumors of patients with breast cancer. We report here significant differential expression of fibronectin, encoded by FN1, when comparing primary tumors of the breast to the tissue of origin, the normal breast. FN1 expression in primary tumors of the breast was significantly higher than in normal breast tissue, and distant metastasis-free survival in breast cancer patients was significantly worse in patients with high tumor expression of FN1 than in patients with low tumor expression of FN1. FN1 may be of relevance to initiation, maintenance or progression of cancers of the female breast.

Keywords: breast cancer, fibronectin, FN1, systems biology of breast cancer, targeted therapeutics in breast cancer.

Invasive breast cancer is diagnosed in over a quarter of a million women in the United States each year¹ and in 2018, breast cancer was the leading cause of cancer death in women worldwide⁴. While patients with localized breast cancer are provided a 99% 5-year survival rate, patients with regional breast cancer, cancer that has spread to lymph nodes or nearby structures, are provided an 86% 5-year survival rate^{5,6}. Patients with metastasis to distant sites are provided a 27% 5-year survival rate^{5,6}. Understanding how primary tumors are most transcriptionally different from the tissue from which they originate, the breast, can facilitate development of novel diagnostic and therapeutics to promote early detection and enhanced treatment, and contribute to efforts to prevent progression to metastatic stages. We mined published microarray data^{2,3} to understand at the transcriptome level and in an unbiased fashion genes most differentially expressed in primary tumors of the breast as compared to normal breast tissue. FN1 emerged as among the most differentially expressed genes in cancer of the female breast.

Methods

We utilized datasets GSE124648² and GSE109169³ and for this differential gene expression analysis of female breast cancer. GSE124648 was generated using Affymetrix Human Genome U133A Array technology with $n=10$ normal breast tissue and $n=126$ tumors of the breast and; analysis was performed using platform GPL96. GSE109169 was generated using Affymetrix Human Exon 1.0 ST Array technology with $n=25$ normal breast tissue and $n=25$ tumors of the breast and; analysis was performed using platform GPL5175. The tissues from this dataset are paired tissues (25 tumors matching 25 breast tissues from 25 patients). The Benjamini and Hochberg method of p -value adjustment was used for ranking of differential expression but raw p -values were used to assess statistical significance of global differential

expression. Log-transformation of data was auto-detected, and the NCBI generated category of platform annotation was used. A statistical test was performed to evaluate whether FN1 expression was significantly between primary breast tumors and breast tissue using a two-tailed, unpaired t-test with Welch's correction. We used PRISM for all statistical analyses of differential gene expression in human breast cancer (Version 8.4.0)(455). For Kaplan-Meier survival analysis, we used the Kaplan-Meier plotter online tool⁷ for correlation of FN1 mRNA expression levels with distant metastasis-free survival (DMFS) in $n=1746$ breast cancer patients.

Results

We performed discovery of genes associated with breast cancer in females by mining two independently published microarray datasets^{2,3}.

FN1 is differentially expressed in primary tumors of the breast.

Comparison of 126 tumors of the breast to 10 normal breast tissues² revealed that the gene FN1 was among the genes most differentially expressed in tumors of the breast in female breast cancer (Table 1). When sorting each of the genes expressed in primary breast tumors based on significance of difference as compared to normal breast tissue, FN1 ranked 362 out of 22283 total transcripts, equating to 98.4% differential expression (Table 1). Differential expression of FN1 in female breast cancer was statistically significant (Table 1; $p=3.06E-12$).

Analysis of a second microarray dataset³, here comparing 25 tumors of the breast to 25 normal breast tissues again revealed significant differential expression of FN1 in tumors of the breast in female breast cancer (Table 2). When sorting each of the genes expressed in tumors of the breast based on significance of difference as compared to normal breast tissue,

1 FN1 ranked 125 out of 19076 total transcripts, equating to 99.3% differential expression (Table
2 2). Differential expression of FN1 in female breast cancer was again statistically significant
3 (Table 2; $p=1.04E+01$).
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6 **FN1 is expressed at significantly higher levels in breast tumors as compared to the** 7 **breast.**

8 We obtained exact mRNA expression levels for FN1 from normal breast tissue and from
9 breast tumors to understand the magnitude and direction of FN1 expression change in human
10 breast cancer. In both datasets, FN1 was expressed at higher levels in tumors of the breast as
11 compared to normal breast tissue (Figure 1). Increased expression of FN1 in primary breast
12 tumors was statistically significant (Figure 1: $p<0.0001$). We calculated a mean fold change of
13 1.2 ± 0.1 when comparing primary tumors of the breast to normal breast tissues (Table 2).
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17 **FN1 expression associates with survival outcomes in breast cancer.**

18 We performed Kaplan-Meier survival analysis in an attempt to correlate FN1 mRNA
19 expression levels with survival outcomes in 1746 breast cancer patients. We found a
20 statistically significant correlation between FN1 expression and distant metastasis-free survival
21 (DMFS) in patients with breast cancer (Figure 2; log rank p -value: 0.00021 for distant
22 metastasis-free survival, hazard ratio: 1.44 (1.19-1.76) (Fig. 2)). High FN1 mRNA levels were a
23 negative prognostic indicator in patients with breast cancer. Median DMFS was 127.2 months
24 for patients with low tumor expression of FN1 while median DMFS was 68.4 months for patients
25 with high tumor expression of FN1 (Table 3).
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27 Thus, through comparative transcriptome analysis of primary tumors of the breast and
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normal breast tissue, we found that differential expression and up-regulation of FN1 was among the most significant transcriptional features in primary tumors from patients with breast cancer. FN1 expression in primary tumors of the breast demonstrated a significant correlation with distant metastasis-free survival in patients with breast cancer, with low expression of FN1 correlating with improved survival.

Discussion

Fibronectin functions in binding to a multitude of proteins of the extracellular matrix⁸. Fibronectin is secreted as a disulfide-bonded dimer, and each monomer is composed of 12 type I repeats, 2 type II repeats, and 15-17 type III repeats⁸. The FN transcript is subject to alternative splicing⁹. FN exists both in soluble form in the plasma and as an insoluble component of the extracellular matrix. The tenth type III repeat of fibronectin possess a motif known as RGD which functions in integrin recognition¹⁰. Interaction partners for fibronectin include glycosaminoglycan, chondroitin sulfate, heparin, collagen, gelatin, talin and fibrin^{8,11-16}. Fibronectin is glycosylated⁸.

Fibronectin is important during the process of epithelial branching which occurs during organ morphogenesis of tissues such as the lung and kidney during embryonic development - specifically at initiation of epithelial branching for cleft formation, where fibronectin can support conversion of cell-cell adhesions to cell-matrix adhesions¹⁷. Tenascin can inhibit the cell-attachment functions of fibronectin¹⁸. An interesting function of fibronectin which has been reported is the activation of the toll-like receptor 4 (TLR) in response to stimulation with the fibronectin type II repeat extra domain A (EDA), an alternatively spliced exon¹⁹.

Multiple studies have described expression of and functions for fibronectin in human breast cancer, as well as correlation of FN1 expression with clinical or survival outcomes in

1 patients with breast cancer. One study described association of FN expression with lymph node
2 involvement and increased risk of mortality associated with high expression of FN²⁰. One study
3 reported a loss of fibronectin expression in invasive breast adenocarcinomas, described as a
4 “complete disappearance”²¹, in stark contrast to our findings from independent microarray data
5 demonstrating not only that FN1 is expressed in breast tumors, but that FN1 is expressed at
6 significantly higher level in breast tumors as compared to the normal breast. Another study
7 evaluated fibronectin expression in the stroma and cancer epithelial cells separately, in 1512
8 total patient tumors, describing no or weak expression of fibronectin in the tumor stroma in
9 23.9% of cases, and expression of fibronectin in the tumor epithelium in only 23.5% of cases²².
10 Patients with any level of fibronectin expression in the tumor epithelium were reported to
11 possess worse overall and disease-free survival than those with “negative” expression of
12 fibronectin, while fibronectin expression in the stroma was not significantly correlated with
13 patient outcomes²². Assembly of a viscous and unfolded fibronectin matrix by cancer
14 associated fibroblasts, subsequently remodeled into a thick and rich matrix containing collagen I
15 was reported in one study, with remodeling of the extracellular matrix performed by matrix
16 metalloproteinases²³. The localization of estrogen receptor alpha (ER α) internalized at the cell
17 surface was reported to be determined by the presence or absence of fibronectin in the ECM; in
18 the absence of FN, ER α was trafficked to lysosomes but not in the presence of FN; integrin- β 1
19 was bound to ER α at the cell membrane and accompanied ER α during its subcellular
20 trafficking²⁴. Autocrine production of fibronectin, *in vitro*, was reported to be involved in inhibition
21 of breast cancer metastasis - mesenchymal tumor cells that did not express an FN matrix were
22 unable to complete metastasis - however, analysis of METABRIC patient data revealed an
23 association between high FN expression and decreased patient survival²⁵. METABRIC analysis
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1 is consistent with our findings of significantly increased expression of FN1 in primary breast
2 tumors relative to normal breast tissue and significantly decreased distant metastasis-free
3 survival in patients with high tumor expression of FN1 as compared to patients with low tumor
4 expression of FN1. A gadolinium-based contrast agent named CREKA-Tris(Gd-DOTA)₃ which
5 specifically recognizes and labels fibrin-fibronectin could detect micro-metastases less than 0.5
6 millimeters in xenografted mice with 4T1 metastatic breast cancer using magnetic resonance
7 imaging²⁶. Finally, a study evaluating breast cancer cells entering and exiting dormancy
8 described a requirement for degradation of fibronectin by matrix metalloproteinase-2 for exit
9 from dormancy²⁷. Thus, there are conflicting descriptions of the expression of fibronectin in
10 primary tumors from patients with breast cancer and the progression and metastasis of breast
11 cancer in relation to fibronectin expression, with studies describing non-existent, or low/weak
12 expression of fibronectin in a substantial fraction of patient tumors which appears incompatible
13 with our findings, and both pro- and anti-metastatic functions elaborated for fibronectin, despite
14 findings from our analysis in 1746 breast cancer patients demonstrating that distant-metastasis
15 free survival is nearly 5 years greater in patients with low tumor expression of FN1 as compared
16 to patients with high tumor expression of FN1.

20 We found that FN1 was among the genes most differentially expressed transcriptome-
21 wide in human breast cancer when comparing primary tumors of the breast to the tissue of
22 origin, normal breast tissue. FN1 was expressed at significantly higher levels in
23 primary breast tumors than in the breast, and low tumor expression of FN1 was associated with
24 enhanced disease-free metastasis survival in patients with breast cancer. FN1 may be relevant
25 to the initiation or progression of human breast cancer.

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Rank	ID	p-value	t	B	Gene	Gene name
362	212464_s_at	3.06E-12	7.659071	17.42312	FN1	fibronectin 1

Table 1: FN1 is differentially expressed in human breast cancer.

Rank of differential expression, probe ID, p-value of global differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the groups compared, gene and gene name are listed in this chart.

Rank	ID	p-value	t	B	FC	Gene	Gene name
125	2598261	3.42E-14	1.04E+01	22.119311	1.2 ± 0.1	FN1	fibronectin 1 (FN1), transcript variant 1

Table 2: FN1 is differentially expressed in human breast cancer.

Rank of differential expression, probe ID, *p*-value of global differential expression, *t*, a moderated *t*-statistic, *B*, the log-odds of differential expression between the groups compared, fold change of FN1 in primary breast tumors as compared to normal breast, gene and gene name are listed in this chart.

Figure 1: FN1 is expressed at significantly higher levels in primary breast tumors as compared to normal breast tissue.

The mRNA expression level of FN1 in normal breast tissue (left) and in primary tumors of the breast (right) is graphically depicted with the result of a statistical test evaluating significance of difference in FN1 expression between normal breast tissue and primary tumors of the breast, a *p*-value, listed above.

Figure 2: FN1 expression correlates with distant metastasis-free survival in patients with breast cancer.

Depicted in this Kaplan-Meier plot is the probability of distant metastasis-free survival (DMFS) for $n=1746$ total patients stratified into two groups, based on low or high expression of FN1 in patient primary tumors. The log rank p -value denoting statistical significance of difference in distant metastasis-free survival when comparing the two groups, as well as hazard ratio for this comparison is listed above. Listed below is the number of patients at risk (number of patients alive) per interval, after stratification based on FN1 expression; in the first interval, number at risk is number of patients alive; in each subsequent interval, number at risk is the number at risk less those who have expired or are censored.

Low expression cohort (months)	High expression cohort (months)
127.2	68.4

Table 3: Median distant metastasis-free survival is superior in breast cancer patients with low primary tumor expression of FN1.

The distant metastasis-free survival of $n=1746$ breast cancer patients based on stratification into low or high tumor expression of FN1 is listed in this chart.