Differential expression of CD34 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 CD34 when comparing primary tumors of the breast to the tissue of origin, the normal breast. CD34 expression in primary tumors of the breast was significantly lower than in normal breast tissue, and relapse-free and overall survival in breast cancer patients was significantly worse in patients with low tumor expression of CD34 than in patients with high tumor expression of CD34. CD34 may be of relevance to initiation, maintenance or progression of cancers of the female breast.

Keywords: breast cancer, CD34, 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. CD34 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 first 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 CD34 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 CD34 mRNA expression levels with relapse-free survival (RFS) in $n=3951$ breast cancer patients and with overall survival (OS) in $n=1402$ breast cancer patients.

Results

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

CD34 is differentially expressed in primary tumors of the breast.

Comparison of 126 tumors of the breast to 10 normal breast tissues revealed that the gene CD34 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, CD34 ranked 150 out of 22283 total transcripts, equating to 99.3% differential expression (Table 1).

Differential expression of CD34 in female breast cancer was statistically significant (Table 1; $p=1.8E-15$)².

Analysis of a second microarray dataset³, here comparing 25 tumors of the breast to 25 normal breast tissues again revealed significant differential expression of CD34 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, CD34 ranked 91 out of 19076 total transcripts, equating to 99.5% differential expression (Table 2). Differential expression of CD34 in female breast cancer was again statistically significant (Table 2; $p=6.27E-15$).

CD34 is expressed at significantly lower levels in breast tumors as compared to the breast.

We obtained exact mRNA expression levels for CD34 from the breast and from breast tumors to understand the magnitude and direction of CD34 expression change in human breast cancer. In both datasets, CD34 was expressed at lower levels in tumors of the breast as compared to normal breast tissue (Figure 1). Decreased expression of CD34 in primary breast tumors was statistically significant (Figure 1: $p<0.0001$ and Figure 2: $p<0.0001$). We calculated a mean fold change of 0.8771 ± 0.0405 and 0.8593 ± 0.0388 when comparing primary tumors of the breast to normal breast tissues (Table 1 and Table 2, respectively).

CD34 expression associates with survival outcomes in breast cancer.

We performed Kaplan-Meier survival analysis in an attempt to correlate CD34 mRNA expression levels with survival outcomes in 3951 breast cancer patients. We found a statistically significant correlation between CD34 expression and relapse-free survival (RFS) in patients with breast cancer (Figure 3; log rank p -value: $4e-16$ for relapse-free survival, hazard ratio: $0.63 (0.57-0.71)$ (Fig. 3)). Low CD34 mRNA levels were a negative prognostic indicator in patients with breast cancer. Median RFS was 35 months for patients with low tumor expression of CD34 while median RFS was 76.02 months for patients with high tumor expression of CD34 (Table 3).

We also evaluated whether CD34 mRNA expression was correlated with overall survival in 1402 breast cancer patients. We found a statistically significant correlation between CD34 expression and overall survival (OS) in patients with breast cancer (Figure 4; log rank p -value: 0.027 for overall survival, hazard ratio: 0.78 (0.63-0.97) (Fig. 4)). Low CD34 mRNA levels were again a negative prognostic indicator in patients with breast cancer. Median OS was 77.4 months for patients with low tumor expression of CD34 while median OS was 135.88 months for patients with high tumor expression of CD34 (Table 4).

Thus, through comparative transcriptome analysis of primary tumors of the breast and normal breast tissue, we found that differential expression and down-regulation of CD34 was among the most significant transcriptional features in primary tumors from patients with breast cancer. CD34 expression in primary tumors of the breast demonstrated a significant correlation with both relapse-free and overall survival in patients with breast cancer, with high expression of CD34 correlating with improved survival.

Discussion

Expression of CD34, an 110 kilodalton glycoprotein was described at the protein level in hematopoietic progenitors, vascular endothelium, capillaries of most tissues and the umbilical artery. Interestingly, though endothelial cells proliferation in culture expressed CD34 RNA, the antibodies utilized by investigators in one study did not detect protein-level CD34 expression. While endothelial cell contacts with tight junctions were devoid of CD34 protein staining, CD34 expression was observed at membrane processes at adjacent endothelial cells⁸. Expression of CD34 has also been observed in the sinusoidal cells of the fetal liver, the fetal bone marrow, in the yolk sac, in endothelial cells of the adult lung and kidney and in cells of the nervous systems including the embryonic neural tube⁹. CD34 is important as a marker of cells in the

1 hematopoietic system, and its expression or lack thereof can signify functionality of
2 hematopoietic stem and progenitor cells. Transplantation of mice with CD34- bone marrow cells
3 gives rise to CD34+ progenitors with long-term engraftment potential in secondary
4 transplantation hosts¹⁰. Transplantation of a single CD34^{lo/-}, c-kit⁺ Sca-1⁺ cell negative for
5 lineage markers (Lin⁻) was capable of reconstituting lymphohematopoietic cells in 21% of
6 recipients¹¹. CD34 expressed on high endothelial venules can interact with L-selectin on
7 leukocytes to facilitate lymphocyte migration across the endothelium¹². Erythroid and myeloid
8 differentiation from CD34-deficient embryonic stem cells *in vitro* is delayed, demonstrating that
9 CD34 can support commitment to the erythroid and myeloid lineages, and colony formation
10 potential for these lineages in the yolk sacs and fetal livers of CD34-deficient embryos is
11 reduced¹³. While adult peripheral blood is apparently normal in CD34-deficient mice,
12 differentiation of progenitors isolated from CD34-deficient mice in liquid culture *in vitro* is
13 deficient¹³.

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17 CD34 has been described as a marker of tumor lymphatic endothelial cells in human
18 cancers¹⁴, and perturbed expression of CD34 has been described by multiple studies in human
19 cancers including gastric cancer¹⁵, small cell lung cancer¹⁶, and breast cancer¹⁷⁻¹⁹. In gastric
20 cancer, CD34 expression used a surrogate for microvessel density was correlated with
21 histological grade¹⁵. Two out of eleven small-cell lung cancer cell lines were positive for
22 expression of CD34¹⁶. One study found that fibroblasts around invasive breast carcinomas
23 were CD34 positive and negative for smooth muscle actin (SMA); while fibroblasts around
24 normal duct-lobule units were also CD34 positive and negative for SMA, fibroadenoma
25 fibroblasts were CD34 positive and SMA positive¹⁷. In ductal carcinoma *in situ*, loss of CD34
26 appeared to be more frequent in high grade tumors than in low and intermediate grade
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1 tumors¹⁷. Microvessel density, or Chalkley count, has been proposed as a means of evaluating
2 angiogenesis by immunohistochemical means in tumor tissues¹⁸, and one study presented a
3 promising immunohistochemical method of CD34 evaluation in tumor tissues from patients with
4 breast cancer¹⁹. Finally, one study demonstrated that changes in microvessel density as
5 assessed by CD34 staining could be observed in tumor tissue from patients with breast cancer;
6 while the authors argued that these changes were not biologically important because they were
7 small, the change in microvessel density displayed a trend towards correlation with patient
8 HER2 status. Thus, while it has been argued that solid tumors save for endothelial cancers
9 generally do not express CD34¹⁶, CD34 expression has been demonstrated in multiple cancers
10 including human breast cancer and investigators have attempted to measure CD34 expression
11 in patient tumors by immunohistochemical methods as a quantitative indicator of tumor
12 microvessel density.

15 We found that CD34 was among the genes most differentially expressed transcriptome-
16 wide in human breast cancer when comparing primary tumors of the breast to the tissue of
17 origin, normal breast tissue. CD34 was expressed at significantly lower levels in
18 primary breast tumors than in the breast, and high tumor expression of CD34 was associated
19 with enhanced relapse-free and overall survival in patients with breast cancer. CD34 may be
20 relevant to the initiation or progression of human breast cancer.

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Rank	ID	p-value	t	B	FC	Gene	Gene name
150	209543_s_at	1.8E-15	-8.988138	24.68541	0.8771 ± 0.0405	CD34	CD34 molecule

Table 1: CD34 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 CD34 in primary breast tumors as compared to normal breast, gene and gene name are listed in this chart.

Rank	ID	p-value	t	B	FC	Gene	Gene name
91	2453307	6.27E-15	-1.09E+01	23.789117	0.8593 ± 0.0388	CD34	CD34 molecule (CD34), transcript variant 1, mRNA.

Table 2: CD34 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.

Figure 1: CD34 is expressed at significantly lower levels in primary breast tumors as compared to normal breast tissue.

The mRNA expression level of CD34 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 CD34 expression between normal breast tissue and primary tumors of the breast, a *p*-value, listed above.

Figure 2: CD34 is expressed at significantly lower levels in primary breast tumors as compared to normal breast tissue.

The mRNA expression level of CD34 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 CD34 expression between normal breast tissue and primary tumors of the breast, a *p*-value, listed above.

Figure 3: CD34 expression correlates with relapse-free survival in patients with breast cancer.

Depicted in this Kaplan-Meier plot is the probability of relapse-free survival (RFS) for $n=3951$ total patients stratified into two groups, based on low or high expression of CD34 in patient primary tumors. The log rank p -value denoting statistical significance of difference in distant relapse-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 CD34 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)
35	76.02

Table 3: Median relapse-free survival is greater in breast cancer patients with high primary tumor expression of CD34.

The relapse-free survival of $n=3951$ breast cancer patients based on stratification into low or high tumor expression of CD34 is listed in this chart.

Figure 4: CD34 expression correlates with overall survival in patients with breast cancer.

Depicted in this Kaplan-Meier plot is the probability of overall survival (OS) for $n=1402$ total patients stratified into two groups, based on low or high expression of CD34 in patient primary tumors. The log rank p -value denoting statistical significance of difference in distant relapse-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 CD34 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)
77.4	135.88

Table 4: Median overall survival is greater in breast cancer patients with high primary tumor expression of CD34.

The overall survival of $n=1402$ breast cancer patients based on stratification into low or high tumor expression of CD34 is listed in this chart.