VEGFR-1 (Flt1) and VEGFR-3 (Flt4) are expressed at higher levels in the tumors of breast cancer patients treated with trastuzumab.

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Patients treated with trastuzumab experience a brain metastasis rate of up to 34%¹. While the mechanism of action of trastuzumab is thought to involve binding of the F_{ab} region of trastuzumab to the extracellular portion of the human epidermal growth factor receptor 2 (HER2)², the transcriptional consequences of such binding events are less well understood. We mined published microarray and multiplexed gene expression profiling datasets^{3,4} to understand in an unbiased fashion the genes most transcriptionally perturbed following trastuzumab treatment in patients with breast cancer. We identified transcriptome-wide differential expression of FLT1^{5,6} and FLT4⁷⁻¹⁰, also known as the vascular endothelial growth factor receptors VEGFR-1 and VEGFR-3. VEGFR-1 and VEGFR-3 were expressed at higher levels in the primary tumors of patients treated with trastuzumab, raising concerns over the use of trastuzumab in a patient population already battling evasion of growth control mechanisms through tumor growth factor signals.

Keywords: VEGFR-1, VEGFR-3, Flt1, Flt4, trastuzumab, breast cancer, systems biology of breast cancer, targeted therapeutics in breast cancer.

Cancers are defined by the inability to control cell division¹¹, and one central manner by which cancers fuel their growth is through growth factor signaling, aided by expression of growth factor receptors to respond to growth factor ligands¹². Patients treated with trastuzumab, a monoclonal antibody targeted against HER2, experience central nervous system metastases at a frequency of up to 34%¹. Growth factor signals can provide critical survival support to allow further colonization of distant sites, including the central nervous system. We present evidence here that the primary tumors of breast cancer patients treated with trastuzumab express significantly higher levels of the growth factor receptors Flt1 and Flt4 than patients not treated with trastuzumab.

Methods

We used datasets GSE38057³ and GSE130788⁴ for this global and multiplexed differential gene expression analysis in conjunction with GEO2R. GSE38057 was generated using DASL Human Cancer Panel by Probe technology with either $n=11$ primary breast tumors from patients treated not treated with trastuzumab and $n=36$ primary breast tumors from patients treated trastuzumab, both of which did not develop brain metastases, or $n=6$ primary breast tumors from patients treated not treated with trastuzumab and $n=35$ primary breast tumors from patients treated trastuzumab, both of which did develop brain metastases; analysis was performed using platform GPL5826. GSE130788 was generated using Whole Human Genome Microarray 4x44K G4112F technology with $n=110$ primary breast tumors from patients before trastuzumab treatment and $n=89$ primary tumors from patients after trastuzumab treatment, analysis performed using platform GPL6480. 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 or multiplexed 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 Flt1 (VEGFR-1) or Flt4 (VEGFR-3) gene expression was significantly between primary breast tumors of patients receiving trastuzumab or not 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).

Results

We mined published microarray and multiplexed mRNA quantitation data^{3,4} to understand in an unbiased fashion genes most transcriptionally perturbed in the primary tumors of patients treated with trastuzumab, a monoclonal antibody associated with 34% central nervous system metastasis in treated patients.

VEGFR-1 (Flt1) is among the genes whose expression changes most significantly in the primary tumors of breast cancer patients treated with trastuzumab.

We identified VEGFR-1^{5,6} among the genes most differentially expressed in the primary tumors of breast cancer patients to whom VEGFR-1 had been administered using a multiplexed mRNA gene expression profiling dataset³. When sorting each of the genes expressed in the primary breast tumors of patients treated with trastuzumab based on significance of change as compared to the primary breast tumors of patients not treated with trastuzumab, VEGFR-1 ranked 50 out of 1506 total transcripts (Table 1). Differential expression of VEGFR-1 was statistically significant (Table 1; $p=0.04561$).

We queried a second dataset⁴ to validate differential expression of VEGFR-1 in the primary tumors of breast cancer patients treated with trastuzumab. We found that VEGFR-1 was again among the genes most differentially expressed in the primary tumors of breast cancer patients to whom VEGFR-1 had been administered - in this dataset, transcriptome-wide. When sorting each of the genes expressed in the primary breast tumors of patients treated with trastuzumab based on significance of change as compared to the primary breast tumors of patients not treated with trastuzumab, VEGFR-1 ranked 24855 out of 41000 total transcripts (Table 2). Differential expression of VEGFR-1 was statistically significant (Table 2; $p=2.93E-02$).

VEGFR-3 (Flt4) is among the genes whose expression changes most significantly in the primary tumors of breast cancer patients treated with trastuzumab.

We identified a second fms related tyrosine kinase receptor, VEGFR-3⁷⁻¹⁰, among the genes most differentially expressed in the primary tumors of breast cancer patients to whom VEGFR-3 had been administered³. When sorting each of the genes expressed in the primary breast tumors of patients treated with trastuzumab based on significance of change as compared to the primary breast tumors of patients not treated with trastuzumab, VEGFR-3 ranked 48 out of 1506 total transcripts (Table 3). Differential expression of VEGFR-3 was statistically significant (Table 3; $p=0.036331$).

We queried a second dataset⁴ to validate differential expression of VEGFR-3 in the primary tumors of breast cancer patients treated with trastuzumab. We found that VEGFR-3 was again among the genes most differentially expressed in the primary tumors of breast cancer patients to whom VEGFR-3 had been administered - in this dataset, transcriptome-wide. When sorting each of the genes expressed in the primary breast tumors of patients treated with trastuzumab based on significance of change as compared to the primary breast tumors of patients not treated with trastuzumab, VEGFR-3 ranked 11371 out of 41000 total transcripts (Table 4). Differential expression of VEGFR-3 was statistically significant (Table 4; $p=1.45E-06$).

VEGFR-1 (Flt1) is expressed at higher levels in the primary tumors of breast cancer patients treated with trastuzumab.

We obtained exact mRNA expression levels of VEGFR-1 in the primary tumors of patients treated with trastuzumab and in the primary tumors of patients not treated with trastuzumab to determine magnitude and direction of change in VEGFR-1 expression in the primary tumors of trastuzumab-treated patients. VEGFR-1 was expressed at higher levels in the primary tumors of patients treated with trastuzumab, though this change was not deemed statistically significant by one measure (Figure 1; $p=0.1494$).

VEGFR-3 (Flt4) is expressed at higher levels in the primary tumors of breast cancer patients treated with trastuzumab.

We obtained exact mRNA expression levels of VEGFR-3 in the primary tumors of patients treated with trastuzumab and in the primary tumors of patients not treated with trastuzumab to determine magnitude and direction of change in VEGFR-3 expression in the primary tumors of trastuzumab-treated patients. VEGFR-3 was expressed at higher levels in the primary tumors of patients treated with trastuzumab, this change approached the level of statistical significance (Figure 2; $p=0.0717$).

1 Thus, through unbiased, systems level analysis of primary tumors from patients to which
2 trastuzumab had been administered, we found that trastuzumab treatment was associated with up-
3 regulation of two critical growth factor receptors that allow cancer cells to evade growth control
4 mechanisms: VEGFR-1 (Flt1) and VEGFR-3 (Flt4).

5 **Discussion**

6 Multiple studies have demonstrated dangerous properties associated with up-regulation of
7 VEGFR-1 and VEGFR-3 in human cancer. VEGFR-1 signaling in macrophages promoted metastasis in
8 breast cancer in one study⁵, and higher levels of VEGFR-1 in breast tumors was associated with worse
9 outcomes in patients with breast cancer⁸. Multiple studies have demonstrated association of VEGFR-3/
10 Flt4 with metastatic properties in patients with breast cancer^{7,8}; an alternative splice variant of Flt4 was
associated with metastasis to the lymph nodes in patients with breast cancer⁸ and suppression of
VEGFR-3 could impair metastasis to the lymph nodes in patients with gastric cancer¹⁰. Moreover,
VEGFR-3 expression was associated with tumor angiogenesis in breast cancer, or the growth of blood
vessels to supply nutrients to tumors⁹.

11 A central property of cancer cells is their ability to support their abnormal growth and cycling
12 properties through up-regulation of receptors for growth factor to transduce uncontrolled growth factor
13 signals. We provided evidence here that the use of trastuzumab, a monoclonal antibody targeted HER2,
14 in patients with breast cancer is associated with up-regulation of growth factor receptors VEGFR-1 (Flt1)
and VEGFR-3 (Flt4). These data should prompt critical evaluation of the use of a therapy that appears to
up-regulate expression of growth factor receptors critical for tumor survival and metastasis.

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Rank	ID	p-value	t	B	FC	Gene
50	GI_32306519-S-3	0.04561	2.058813	-4.35	1.47 ± 0.63	FLT1

Table 1: VEGFR-1 (Flt1) is differentially expressed in the primary tumors of breast cancer patients treated with trastuzumab.

Rank of differential expression, probe ID, *p*-value with respect to differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the two groups compared, fold change (FC) of Flt1 expression between the primary tumors of breast cancer patients treated with trastuzumab as compared to that of patients not treated with trastuzumab, and gene symbol are listed in this chart.

Rank	ID	p-value	t	B	Gene	Gene name
24855	A_24_P246125	2.93E-02	2.195847	-5.224488	FLT1	fms related tyrosine kinase 1

Table 2: VEGFR-1 (Flt1) is differentially expressed in the primary tumors of breast cancer patients treated with trastuzumab.

Rank of differential expression, probe ID, *p*-value with respect to differential expression, *t*, a moderated *t*-statistic, *B*, the log-odds of differential expression between the primary tumors of breast cancer patients treated with trastuzumab as compared to that of patients not treated with trastuzumab, gene symbol and gene name are listed in this chart.

Rank	ID	p-value	t	B	FC	Gene
48	GI_4503752-A-4	0.036331	2.15275	-3.83	1.28 ± 0.49	FLT4

Table 3: VEGFR-3 (Flt4) is differentially expressed in the primary tumors of breast cancer patients treated with trastuzumab.

Rank of differential expression, probe ID, *p*-value with respect to differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the two groups compared, fold change (FC) of Flt4 expression between the primary tumors of breast cancer patients treated with trastuzumab as compared to that of patients not treated with trastuzumab, and gene symbol are listed in this chart.

Rank	ID	p-value	t	B	Gene	Gene name
11371	A_23_P213394	1.45E-06	-4.969976	4.057927	FLT4	fms related tyrosine kinase 4

Table 4: VEGFR-3 (Flt4) is differentially expressed in the primary tumors of breast cancer patients treated with trastuzumab.

Rank of differential expression, probe ID, *p*-value with respect to differential expression, *t*, a moderated *t*-statistic, *B*, the log-odds of differential expression between the primary tumors of breast cancer patients treated with trastuzumab as compared to that of patients not treated with trastuzumab, gene symbol and gene name are listed in this chart.

Figure 1: VEGFR-1 (Flt1) is expressed at higher levels in the primary tumors of breast cancer patients treated with trastuzumab.

The mRNA expression level of VEGFR-1 (Flt1) in the primary tumors of breast cancer patients not treated with trastuzumab (left) and in the primary tumors of breast cancer patients treated with trastuzumab (right) is graphically represented here with mean VEGFR-1 (Flt1) levels marked and the result of a statistical test evaluating difference in mRNA expression level between patients treated with trastuzumab or not, a *p*-value, listed above.

Figure 2: VEGFR-3 (Flt4) is expressed at higher levels in the primary tumors of breast cancer patients treated with trastuzumab.

The mRNA expression level of VEGFR-3 (Flt4) in the primary tumors of breast cancer patients not treated with trastuzumab (left) and in the primary tumors of breast cancer patients treated with trastuzumab (right) is graphically represented here with mean VEGFR-3 (Flt4) levels marked and the result of a statistical test evaluating difference in mRNA expression level between patients treated with trastuzumab or not, a *p*-value, listed above.