FOXD2 is differentially expressed in brain metastatic human breast cancer.

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Metastasis to the brain is a clinical problem in patients with breast cancer¹⁻³. We mined published microarray data^{4,5} to compare primary and metastatic tumor transcriptomes for the discovery of genes associated with brain metastasis in humans with metastatic breast cancer. We found that the forkhead box D2, encoded by FOXD2, was among the genes whose expression was most different in the brain metastases of patients with metastatic breast cancer as compared to primary tumors of the breast⁴. FOXD2 was also differentially expressed in the tumor cells of patients with triple negative breast cancer⁵. FOXD2 mRNA was present at increased quantities in brain metastatic tissues as compared to primary tumors of the breast. Importantly, expression of FOXD2 in primary tumors was significantly correlated with patient recurrence-free survival. Modulation of FOXD2 expression may be relevant to the biology by which tumor cells metastasize from the breast to the brain in humans with metastatic breast cancer.

Keywords: breast cancer, metastasis, brain metastases, central nervous system metastases, the forkhead box D2, FOXD2, systems biology of breast cancer, targeted therapeutics in breast cancer.

One report described a 34% incidence of central nervous system metastases in patients treated with trastuzumab for breast cancer². More recently, the NEfERT-T clinical trial⁶ which compared administration of either neratinib or trastuzumab in conjunction with paclitaxel demonstrated that in a randomized, controlled setting, in breast cancer patients treated with neratinib, not only was the incidence of central nervous system recurrence significantly lower, the time to central nervous system metastasis was significantly delayed as compared to patients administered trastuzumab⁶. The alarmingly high rate of central nervous system metastasis described, as well as data, both anecdotal² and from a randomized, controlled setting⁶ illustrating that treatment with trastuzumab may be associated with these events demands an enhanced understanding of the transcriptional makeup of brain metastatic tissues to support identification of therapeutic targets, whether they are treatment related or not. We performed a global comparative analysis of primary and metastatic tumors in patients with brain metastatic breast cancer^{4,5}. We discovered differential and increased expression of the gene encoding the forkhead box D2, FOXD2, in brain metastatic tissues of patients with metastatic breast cancer.

Methods

We used datasets GSE10893⁴ and GSE38959⁵ for this global differential gene expression analysis of brain metastatic breast cancer in conjunction with GEO2R. GSE10893 was generated using Agilent-011521 Human 1A Microarray G4110A technology with $n=11$ primary breast tumors and $n=3$ brain metastases from patients with breast cancer; analysis was performed using platform GPL885. GSE38959 was generated using Agilent-014850 Whole Human Genome Microarray 4x44K G4112F with $n=13$ samples of normal mammary gland ductal cells and $n=30$ samples of primary tumor cells from patients with triple negative breast cancer; analysis was performed using platform GPL4133. 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 FOXD2 gene expression was significantly different between primary tumors of the breast and brain metastases in humans with breast cancer using a two-tailed t-test. For Kaplan-Meier survival analysis, we used the Kaplan-Meier plotter online tool⁷ for correlation of FOXD2 mRNA expression levels with recurrence-free survival in $n=3951$ breast cancer patients.

Results

We performed global comparative transcriptome analysis of metastatic tumor tissues of patients with metastatic breast cancer using published microarray data^{4,5} to describe the transcriptional landscape of brain metastasis in human breast cancer in an unbiased fashion and for the discovery of novel therapeutic targets.

FOXD2 is differentially expressed in the brain metastases of patients with brain metastatic breast cancer.

Through blind, systems-level analysis of published microarray data⁴, we identified forkhead box D2, encoded by FOXD2, as a differentially expressed gene in the breast metastatic tissues of humans with breast cancer (Table 1). When sorting each of the genes expressed in brain metastases based on significance of difference as compared to primary tumors of the breast

in patients with breast cancer, FOXD2 ranked 240 out of 17418 total transcripts (Chart 1), equating 98.6% differential expression. Differential expression of FOXD2 in the brain metastases of patients with metastatic breast cancer was statistically significant (Chart 1; $p=7.08E-04$).

To attempt to validate differential transcriptome-wide differential expression of FOXD2 in human breast cancer, we queried a second microarray dataset⁵, here comparing primary tumor cells from patients with triple negative breast cancer to normal mammary gland ductal cells. Again, we identified FOXD2 as a differentially expressed gene in the tumor tissues of patients with breast cancer (Chart 2). When sorting each of the genes expressed in triple negative breast cancer tumor cells based on significance of difference as compared to normal mammary gland ductal cells, FOXD2 ranked 5362 out of 45015 total transcripts (Chart 2), equating to 88.1% differential expression. Differential expression of FOXD2 in the brain metastases of patients with metastatic breast cancer was statistically significant (Chart 2; $p=6.14E-04$). Thus, differential expression of FOXD2, transcriptome-wide, in the tumor tissues of women with breast cancer was conserved across two independent microarray datasets, both when in primary and metastatic tumor tissues.

FOXD2 is expressed at higher levels in the brain metastases of patients with metastatic breast cancer.

We obtained exact mRNA expression levels for FOXD2, in primary tumors of the breast and in brain metastasis of patients with brain metastatic breast cancer to determine direction and statistical significance of change in FOXD2 expression in brain metastatic tissues. We observed significantly higher expression of FOXD2 in the brain metastases of patients with breast cancer as compared to primary tumors of the breast: FOXD2 was expressed at 0.29 ± 0.79 arbitrary units (AU) in primary tumors of the breast, while it was expressed at 1.99 ± 0.53 AU in brain metastatic tissues. The difference in FOXD2 mRNA levels between primary tumors of the breast and brain metastatic tissues was statistically significant (Figure 1; $p=0.001718$).

FOXD2 expression is significantly correlated with survival outcomes in human breast cancer.

We performed Kaplan-Meier survival analysis⁸ in 3951 breast cancer patients in total, to evaluate whether FOXD2 tumor expression was correlated with survival outcomes in human breast cancer. We observed a statistically significant correlation between primary tumor expression of FOXD2 and recurrence-free survival (RFS) in patients with breast cancer (Figure 2). Patients whose primary tumors expressed low levels of FOXD2 possessed median RFS of 38.4 months, while patients whose tumors expressed high levels of FOXD2 possessed median RFS of 65.04 months. This difference in RFS based on FOXD2 tumor expression in patients with breast cancer was statistically significant (Figure 2, Chart 3; logrank p -value: 6.9×10^{-9} ; hazard ratio: 0.72 (0.65-0.81)).

Thus, by mining published microarray data^{4,5} in an unbiased and systematic fashion, we identified forkhead box D2, encoded by FOXD2, as among the genes whose expression was most different, transcriptome-wide, in the brain metastases of patients with breast cancer, when compared to primary tumors of the breast and in the primary tumor cells of patients with triple negative breast cancer when compared to normal mammary gland ductal cells; we observed significantly increased expression of FOXD2 in brain metastases as compared to primary tumors

of the breast. Further, we found a significant correlation between FOXD2 expression and patient survival outcomes, as recurrence-free survival was significantly higher in patients whose primary tumors expressed higher levels of FOXD2 as compared to patients whose primary tumors expressed lower levels of FOXD2.

Discussion

We provided evidence here that the forkhead box D2, encoded by FOXD2, is among the genes whose expression is most different in the brain metastases of patients with brain metastatic breast cancer, that FOXD2 is also differentially expressed in the primary tumor cells of patients with triple negative breast cancer, that FOXD2 mRNA is present at significantly increased quantities in brain metastatic tissues as compared to primary tumors of the breast, and that primary tumor FOXD2 expression is significantly correlated with patient survival outcomes in human breast cancer. Evaluation of the effects of genetic depletion of FOXD2 in mouse models of metastatic breast cancer on metastasis to the central nervous system is merited. Modulation of FOXD2 expression may be relevant to the processes by which breast cancer cells exit the breast, enter the vasculature and/or lymphatics, reside in the lymph nodes, evade immune clearance, breach the blood-brain barrier and colonize the brain.

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Rank: 240
Probe ID: 13346
P-value: 7.08E-04
t: -4.19
B: -0.274521
Gene: FOXD2
Gene name: forkhead box D2

Chart 1: FOXD2 is differentially expressed in brain metastatic breast cancer when comparing brain metastases to primary tumors of the breast.

The rank of global differential expression, the probe/transcript ID, the *p*-value with respect to differential expression transcriptome-wide, *t*, a moderated *t*-statistic, *B*, the log-odds of differential expression between the groups compared, the gene and gene name are listed in this chart.

Rank: 5362
probe ID: 7582
p-value: 6.14E-04
t: -3.6830886
B: -0.789381
Gene: FOXD2
Gene name: forkhead box D2

Chart 2: FOXD2 is differentially expressed in the tumor cells of patients with triple negative breast cancer when compared to normal mammary gland ductal cells.

The rank of global differential expression, probe/transcript ID, the *p*-value with respect to differential expression transcriptome-wide, *t*, a moderated *t*-statistic, *B*, the log-odds of differential expression between the groups compared, the gene and gene name are listed in this chart.

Figure 1: FOXD2 is expressed at significantly higher levels in the brain metastases of patients with metastatic breast cancer when compared to primary tumors of the breast.

The mRNA expression level of FOXD2 in primary tumors of the breast (left) and in brain metastases of women with metastatic breast cancer (right) is graphically depicted; the result of a statistical test evaluating significance of difference in FOXD2 expression between primary tumors and brain metastasis, a p -value, is $p=0.001718$.

Figure 2: Significant correlation between FOXD2 primary tumor expression and recurrence-free survival in patients with breast cancer.

Depicted in this Kaplan-Meier plot is the probability of recurrence-free survival for $n=3951$ total patients stratified into two groups, based on low or high expression of FOXD2 in patient primary tumors. The log rank p -value denoting statistical significance of difference in recurrence-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 FOXD2 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 FOXD2 expression: 38.4 months
High FOXD2 expression: 65.04 months

Chart 3: Median recurrence-free survival is inferior in patients with low primary tumor expression of FOXD2.

The median RFS (recurrence-free survival) of patients with low primary tumor expression of FOXD2 and high primary tumor expression of FOXD2 is listed in this chart, for $n=3951$ breast cancer patients.