ANO1 is differentially expressed in lymph node metastasis in human breast cancer.

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Metastasis to the brain is a clinical problem in patients with breast cancer¹⁻³. Between the breast and the brain reside the secondary lymphoid organ, the lymph nodes. We mined published microarray data^{4,5} to compare primary and metastatic tumor transcriptomes for the discovery of genes associated with metastasis to the lymph nodes in humans with metastatic breast cancer. We found that anoctamin 1, ANO1, was among the genes whose expression was most different in the lymph node metastases of patients with metastatic breast cancer as compared to primary tumors of the breast. ANO1 mRNA was present at decreased quantities in lymph node metastases as compared to primary tumors of the breast. Importantly, expression of ANO1 in primary tumors of the breast was correlated with patient overall survival, in lymph node positive patients but not in lymph node negative patients. Modulation of ANO1 expression may be relevant to the biology by which tumor cells metastasize from the breast to the lymph nodes and the brain in humans with metastatic breast cancer.

Keywords: breast cancer, metastasis, brain metastasis, central nervous system metastasis, lymph node metastasis, anoctamin 1, ANO1, 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 and lymph node metastatic tissues to support identification of therapeutic targets, whether they are treatment related or not. The lymph nodes reside between the breast and the brain. We performed a global comparative analysis of lymph node metastatic tumor tissues in patients with metastatic breast cancer, compared to primary tumors and normal breast tissues^{4,5}. We discovered transcriptome-wide differential expression of the gene encoding anoctamin 1, ANO1, in lymph node metastatic tissues of patients with metastatic breast cancer.

Methods

We used datasets GSE10893⁴ and GSE124648⁵ 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 $n=71$ primary breast tumors and $n=7$ lymph node metastases from patients with breast cancer; analysis was performed using platform GPL887. GSE124648 was generated using Affymetrix Human Genome U133A array technology with $n=10$ normal breast tissues and $n=44$ lymph node metastases from patients with breast cancer; analysis was performed using platform GPL96. 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 ANO1 gene expression was significantly different between primary tumors of the breast and lymph node 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 ANO1 mRNA expression levels with overall survival in $n=726$ patients with lymph node positive breast cancer patients and for $n=452$ breast cancer patients with lymph node negative breast cancer.

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 metastasis in human breast cancer in an unbiased fashion and for the discovery of novel therapeutic targets.

ANO1 is differentially expressed in the lymph node metastases of patients with metastatic breast cancer.

Through blind, systems-level analysis of published microarray data⁴, we identified anoctamin 1, encoded by ANO1, as a differentially expressed gene in the lymph node metastatic tissues of humans with breast cancer (Table 1). When sorting each of the genes expressed in lymph node metastases based on significance of difference as compared to primary tumors of the breast in patients with breast cancer, ANO1 ranked 23 out of 18034 total transcripts (Chart 1), equating to 99.9% differential expression. Differential expression of ANO1 in the lymph node metastases of patients with metastatic breast cancer was statistically significant (Chart 1; $p=6.99\text{e-}05$).

We queried a second microarray dataset⁵ to attempt to validate transcriptome-wide differential expression of ANO1 in the lymph node metastases of patients with metastatic breast cancer. We again observed ANO1 to be among the genes expressed most quantitatively differently in the lymph node metastases of patients with metastatic breast cancer as compared to primary tumors of the breast. When sorting each of the genes expressed in lymph node metastases based on significance of difference as compared to normal breast tissues, ANO1 ranked 2342 out of 22283 total transcripts (Chart 2), equating to 89.5% differential expression. Differential expression of ANO1 in the lymph node metastases of patients with metastatic breast cancer was statistically significant (Chart 2; $p=1.49\text{e-}04$).

ANO1 is expressed at lower levels in the lymph node metastases of patients with metastatic breast cancer.

We obtained exact mRNA expression levels for ANO1, in primary tumors of the breast and in lymph node metastases of patients with metastatic breast cancer to determine direction and statistical significance of change in ANO1 expression in lymph node metastatic tissues. We observed significantly decreased expression of ANO1 in the lymph node metastases of patients with breast cancer as compared to primary tumors of the breast: ANO1 was expressed at 2.88 ± 1.00 arbitrary units (AU) in primary tumors of the breast, while it was expressed at 1.21 ± 1.29 AU in lymph node metastatic tissues (Figure 1). The difference in ANO1 mRNA levels between primary tumors of the breast and lymph node metastases was statistically significant (Figure 1; $p=0.000092$).

ANO1 expression is correlated with survival outcomes in human breast cancer.

We performed Kaplan-Meier survival analysis⁷ in $n=726$ lymph node positive breast cancer patients and $n=452$ lymph node negative breast cancer patients, to evaluate whether ANO1 tumor expression was correlated with survival outcomes in breast cancer and whether lymph node status influenced correlation of ANO1 tumor expression with survival outcomes in human breast cancer. We observed a correlation between primary tumor expression of ANO1 and overall survival (OS) in lymph node positive breast cancer (Figure 2). Lymph node positive patients whose primary tumors expressed low levels of ANO1 possessed median OS of 27.6 months, while lymph node positive patients whose tumors expressed high levels of ANO1 possessed median OS of 53.45 months. This difference in OS based on ANO1 tumor expression in lymph node positive patients with breast cancer was statistically significant (Figure 2, Chart 3; logrank p -value: 0.0089 (hazard ratio: 0.72 (0.56-0.92))). ANO1 primary tumor expression was not correlated with overall survival in lymph node negative patients (Figure 2, Chart 3; logrank p -value: 0.7; hazard ratio: 1.05 (0.82-1.34)).

Thus, by mining published microarray data^{4,5} in an unbiased and systematic fashion, we identified ANO1 as among the genes whose expression was most different, transcriptome-wide, in the lymph node metastases of patients with breast cancer, when compared to primary tumors; we observed significantly decreased expression of ANO1 in lymph node metastases as compared to primary tumors of the breast. Further, we found a correlation between ANO1 primary tumor expression and patient overall survival, in lymph node positive patients but not in lymph node negative patients.

Discussion

We provided evidence here that anoctamin 1, encoded by ANO1, is among the genes whose expression is most different in the lymph node metastases of patients with metastatic breast cancer as compared to primary tumors of the breast, that ANO1 mRNA is present at significantly decreased quantities in lymph node metastatic tissues as compared to primary tumors of the breast, and that

1 primary tumor ANO1 expression is correlated with patient survival outcomes in human breast cancer, in
2 lymph node positive patients but not in lymph node negative patients. Evaluation of the effects of genetic
3 depletion of ANO1 in mouse models of metastatic breast cancer on metastasis to the lymph nodes and to
4 the central nervous system is merited. Modulation of ANO1 expression may be relevant to the processes
5 by which breast cancer cells exit the breast, enter the vasculature and/or lymphatics, reside in the lymph
6 nodes, evade immune clearance, breach the blood-brain barrier and colonize the brain.
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Rank: 23
Probe ID: 5042
p-value: 6.99e-05
t: 4.1986137
B: 1.4607
Gene: ANO1
Gene name: anoctamin 1

Chart 1: ANO1 is differentially expressed in lymph node metastatic breast cancer when comparing lymph node 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: 2342
Probe ID: 218804_at
p-value: 1.49e-04
t: 4.073433
B: 0.347389
Gene: ANO1
Gene name: anoctamin 1

Chart 2: ANO1 is differentially expressed in lymph node metastatic breast cancer when comparing lymph node metastases to normal breast tissues.

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.

Figure 1: ANO1 is expressed at significantly lower levels in the lymph node metastases of patients with metastatic breast cancer when compared to primary tumors of the breast.

The mRNA expression level of ANO1 in primary tumors of the breast (left) and in lymph node metastases of women with metastatic breast cancer (right) is graphically depicted; the result of a statistical test evaluating significance of difference in ANO1 expression between primary tumors of the breast and lymph node metastases, a *p*-value, is $p=0.000092$.

Figure 2: ANO1 primary tumor expression is correlated with overall survival in human breast cancer: in lymph node positive patients but not in lymph node negative patients.

Depicted in these Kaplan-Meier plots is the probability of overall survival in $n=726$ lymph node positive patients (**left**) and $n=452$ lymph node negative patients (**right**), based on low or high expression of ANO1 in patient primary tumors. The log rank p -value denoting statistical significance of difference in overall 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 ANO1 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.

Lymph node positive patients

Low ANO1 expression: 27.6 months

High ANO1 expression: 53.45 months

Chart 3: In lymph node positive breast cancer patients, median overall survival is inferior in patients with low primary tumor expression of ANO1.

The median OS (overall survival) of patients with low primary tumor expression of ANO1 and high primary tumor expression of ANO1 is listed in this chart, for $n=726$ patients with lymph node positive breast cancer.