CD28 is differentially expressed in the lymph node metastases of patients with 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 the T-cell co-stimulatory receptor cluster of differentiation 28, CD28, 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. Analysis of a separate microarray dataset revealed that CD28 was also differentially expressed in brain metastatic tissues. CD28 mRNA was present at increased quantities in lymph node metastases as compared to primary tumors of the breast. Importantly, expression of CD28 in primary tumors of the breast was correlated with patient recurrence-free survival, in lymph node negative patients but not in lymph node positive patients. Modulation of CD28 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, metastatic breast cancer, cluster of differentiation 28, CD28, 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 cluster of differentiation 28, CD28, in lymph node metastatic tissues of patients with metastatic breast cancer.

Methods

We used datasets GSE10893⁴ and GSE52604⁵ 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. GSE52604 was generated using Agilent-014850 Whole Human Genome Microarray 4x44K G4112F with $n=10$ normal breast tissues and $n=35$ brain metastases from patients with breast cancer; analysis was 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 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 CD28 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 CD28 mRNA expression levels with recurrence-free survival in $n=3951$ breast cancer patients, $n=1133$ lymph node positive patients and $n=2020$ lymph node negative 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 metastasis in human breast cancer in an unbiased fashion and for the discovery of novel therapeutic targets.

CD28 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 cluster of differentiation 28, encoded by CD28, 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, CD28 ranked 147 out of 18034 total transcripts (Chart 1), equating to 99.2% differential expression. Differential expression of CD28 in the lymph node metastases of patients with

metastatic breast cancer was statistically significant (Chart 1; $p=1.54e-03$).

Differential gene expression analyses that utilize primary and metastatic tumor tissues to discover genes associated with metastasis can be challenging due to the relatively heterogeneous cellular composition and constitution of the tumor as compared to normal, untransformed (benign) tissues. Thus, to attempt to validate differential transcriptome-wide differential expression of CD28 in metastatic tissues in human breast cancer, we queried a second microarray dataset⁵, here comparing normal breast tissues rather than primary tumors of the breast, to brain metastases. Again, we identified CD28 as a differentially expressed gene in the metastatic tissues of humans with breast cancer, here, in the brain metastatic tissues of patients with breast cancer (Chart 2). When sorting each of the genes expressed in brain metastases based on significance of difference as compared to normal breast tissues, CD28 ranked 9278 out of 41093 total transcripts (Chart 2), equating to 77.4% differential expression. Differential expression of CD28 in the brain metastases of patients with metastatic breast cancer was statistically significant (Chart 2; $p=3.56e-03$). Thus, differential expression of CD28, transcriptome-wide, in the metastatic tissues of women with metastatic breast cancer was conserved across two independent microarray datasets, both in lymph node and brain metastases. This suggests that CD28 differential expression in human metastatic breast cancer is a *bona fide* biological process and that differential expression of CD28 in metastatic breast cancer is shared between the brain and the lymph nodes.

CD28 is expressed at higher levels in the lymph node metastases of patients with metastatic breast cancer.

We obtained exact mRNA expression levels for CD28, 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 CD28 expression in lymph node metastatic tissues. We observed significantly increased expression of CD28 in the lymph node metastases of patients with breast cancer as compared to primary tumors of the breast: CD28 was expressed at -2.18 ± 0.92 arbitrary units (AU) in primary tumors of the breast, while it was expressed at -0.96 ± 1.27 AU in lymph node metastatic tissues (Figure 1). The difference in CD28 mRNA levels between primary tumors of the breast and lymph node metastases was statistically significant (Figure 1; $p=0.00183$).

CD28 expression is correlated with survival outcomes in human breast cancer.

We performed Kaplan-Meier survival analysis⁷ in $n=3951$ breast cancer patients in total, to evaluate whether CD28 tumor expression was correlated with survival outcomes in breast cancer. We observed a correlation between primary tumor expression of CD28 and recurrence-free survival (RFS) in patients with breast cancer (Figure 2). Patients whose primary tumors expressed low levels of CD28 possessed median RFS of 191.21 months, while patients whose tumors expressed high levels of CD28 possessed median RFS of 228.85 months. This difference in RFS based on CD28 tumor expression in patients with breast cancer was statistically significant (Figure 2, Chart 3; logrank p -value: $1.1e-09$; (hazard ratio: 0.71 (0.64-0.79))).

We also performed Kaplan-Meier survival analysis⁷ in $n=1133$ lymph node positive breast cancer patients and $n=2020$ lymph node negative breast cancer patients, to evaluate whether lymph node status influenced correlation of CD28 tumor expression with survival outcomes in human breast cancer. We observed a statistically significant correlation between primary tumor expression of CD28 and recurrence-free survival (RFS) in lymph node negative breast cancer (Figure 2). Lymph node negative patients whose primary tumors expressed low levels of CD28 possessed median RFS of 216.66 months, while lymph node negative patients whose tumors expressed high levels of CD28 possessed median RFS of 228.85 months. This difference in RFS based on CD28 tumor expression in lymph node negative patients with breast cancer was statistically significant (Figure 2, Chart 3; logrank p -value: 0.036; (hazard

ratio: 0.83 (0.71-0.99)). CD28 primary tumor expression was not significantly correlated with recurrence-free survival outcomes in lymph node positive patients (Figure 2, Chart 3; logrank *p*-value: 0.68; hazard ratio: 0.96 (0.79-1.17)).

Thus, by mining published microarray data^{4,5} in an unbiased and systematic fashion, we identified CD28 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 increased expression of CD28 in lymph node metastases as compared to primary tumors of the breast. Further, we found a significant correlation between CD28 expression and patient survival outcomes, as recurrence-free survival was greater in patients whose primary tumors expressed higher levels of CD28 as compared to patients whose primary tumors expressed lower levels of CD28, and this correlation was significant in lymph node negative patients but not in lymph node positive patients.

Discussion

We provided evidence here that the T-cell co-stimulatory receptor cluster of differentiation 28, CD28, 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 CD28 is also differentially expressed in brain metastatic tissues in human breast cancer, that CD28 mRNA is present at significantly increased quantities in lymph node metastatic tissues as compared to primary tumors of the breast, and that primary tumor CD28 expression is significantly correlated with patient survival outcomes in human breast cancer, in all patients and in lymph node negative patients but not in lymph node positive patients. Evaluation of the effects of genetic depletion of CD28 in mouse models of metastatic breast cancer on metastasis to the central nervous system is merited. Modulation of CD28 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.

References

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Rank: 147
Probe ID: 3817
p-value: 1.54e-03
t: -3.280421
B: -1.1782
Gene: CD28
Gene name: cluster of differentiation 28

Chart 1: CD28 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: 9278
Probe ID: A_23_P91095
p-value: 3.56e-03
t: 3.0690148
B: -2.410055
Gene: CD28
Gene name: cluster of differentiation 28

Chart 2: CD28 is differentially expressed in brain metastatic breast cancer when comparing brain 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: CD28 is expressed at significantly higher 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 CD28 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 CD28 expression between primary tumors of the breast and lymph node metastases, a *p*-value, is $p=0.00183$.

Figure 2: CD28 primary tumor expression is significantly correlated with recurrence-free survival in lymph node negative patients but not in lymph node positive patients.

Depicted in this Kaplan-Meier plot (**above**) is the probability of recurrence-free survival for $n=3951$ total patients stratified into two groups, based on low or high expression of CD28 in patient primary tumors, in the upper survival quartile. 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 CD28 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. Below, Kaplan-Meier plots for probability of recurrence-free survival in $n=1133$ lymph node positive patients (**left**) and $n=2020$ lymph node negative patients (**right**).

All patients

Low CD28 expression: 191.21 months

High CD28 expression: 228.85 months

Lymph node negative patients

Low CD28 expression: 216.66 months

High CD28 expression: 228.85 months

Chart 3: Median recurrence-free survival is inferior in patients with low primary tumor expression of CD28.

The median RFS (recurrence-free survival) of patients with low primary tumor expression of CD28 and high primary tumor expression of CD28 is listed in this chart, for $n=3951$ breast cancer patients and $n=2020$ lymph node negative patients.