CCL21 is differentially expressed in the lymph nodes of patients with metastatic breast cancer.

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Metastasis to the brain is a clinical problem in patients with breast cancer¹⁻³. The lymph nodes reside between the breast and the brain. 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 CCL21 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. CCL21 mRNA was present at increased quantities in lymph node metastases as compared to primary tumors of the breast. Importantly, expression of CCL21 in primary tumors of the breast was significantly correlated with patient overall survival. Modulation of CCL21 expression may be relevant to the biology by which tumor cells metastasize from the breast to the brain in humans with metastatic breast cancer. CCL21 may function to support tissue infiltration in brain metastatic breast cancer in humans.

Keywords: breast cancer, metastasis, brain metastases, central nervous system metastases, C-C motif chemokine ligand 21, CCL21, 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, comparing to primary tumors and normal breast tissues, respectively^{4,5}. We discovered transcriptome-wide differential expression of the gene encoding the C-C motif chemokine ligand 21, CCL21, 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; with $n=11$ primary breast tumors and $n=4$ brain metastases from patients with breast cancer; analysis was performed using platform GPL885. 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 CCL21 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 CCL21 mRNA expression levels with overall survival in $n=1402$ 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 metastasis in human breast cancer in an unbiased fashion and for the discovery of novel therapeutic targets.

CCL21 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 the

C-C motif chemokine ligand 21, encoded by CCL21, 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, CCL21 ranked 25 out of 18034 total transcripts (Chart 1), equating to 99.9% differential expression. Differential expression of CCL21 in the lymph node metastases of patients with metastatic breast cancer was statistically significant (Chart 1; $p=8.59\text{e-}05$).

We queried a second microarray dataset to attempt to validate transcriptome-wide differential expression of CCL21 in the lymph node metastases of patients with metastatic breast cancer. We again observed CCL21 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, CCL21 ranked 1690 out of 22283 total transcripts (Chart 2), equating to 92.4% differential expression. Differential expression of CCL21 in the lymph node metastases of patients with metastatic breast cancer approached the level of statistical significance (Chart 2; $p=2.61\text{e-}05$).

CCL21 is not differentially expressed in the brain metastases of patients with metastatic breast cancer.

To ascertain whether transcriptome-wide differential expression of CCL21 in metastatic breast cancer was specific to the lymph nodes, we again queried microarray data⁴, here comparing primary tumors of the breast to brain metastases. We found here that CCL21 was not differentially expressed in the metastatic tissues of patients with breast cancer (Chart 3). When sorting each of the genes expressed in brain metastases based on significance of difference as compared to primary tumors of the breast, CCL21 ranked 17194 out of 17418 total transcripts (Chart 3), equating to 1.29% differential expression. Differential expression of CCL21 in the brain metastases of patients with metastatic breast cancer was not statistically significant (Chart 3; $p=9.78\text{e-}01$). Thus, differential expression of CCL21, transcriptome-wide, in the metastatic tissues of women with breast cancer was conserved across two independent microarray datasets, and was specific to the lymph nodes and not the brain.

CCL21 is expressed at higher levels in the lymph node metastases of patients with metastatic breast cancer.

We obtained exact mRNA expression levels for CCL21, 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 CCL21 expression in lymph node metastatic tissues. CCL21 was expressed at higher levels in the lymph node metastases of patients with breast cancer as compared to primary tumors of the breast, and this difference was statistically significant (Figure 1; $p=0.000099$).

CCL21 expression is significantly correlated with survival outcomes in human breast cancer.

We performed Kaplan-Meier survival analysis⁸ in $n=1402$ breast cancer patients in total, to evaluate whether CCL21 tumor expression was correlated with survival outcomes in breast

1 cancer. We observed a statistically significant correlation between primary tumor expression of
2 CCL21 and overall survival (OS) in patients with breast cancer (Figure 2). Patients whose
3 primary tumors expressed low levels of CCL21 possessed median OS of 70.8 months, while
4 patients whose tumors expressed high levels of CCL21 possessed median OS of 125.92 months.
5 This difference in OS based on CCL21 tumor expression in patients with breast cancer was
6 statistically significant (Figure 2, Chart 3; logrank p -value: 0.0031; hazard ratio: 0.72 (0.58-0.9)).

7 Thus, by mining published microarray data^{4,5} in an unbiased and systematic fashion, we
8 identified CCL21 as among the genes whose expression was most different, transcriptome-wide,
9 in the lymph node metastases of patients with breast cancer, when compared to primary tumors of
10 the breast and to normal breast tissues, respectively; we observed significantly increased
11 expression of CCL21 in lymph node metastases as compared to primary tumors of the breast.
12 Further, we found a significant correlation between CCL21 expression and patient survival
13 outcomes, as overall survival was significantly greater in patients whose primary tumors
14 expressed higher levels of CCL21 as compared to patients whose primary tumors expressed
15 lower levels of CCL21.

16 Discussion

17 We provided evidence here that the C-C motif chemokine ligand 21, CCL21, is among the
18 genes whose expression is most different in the lymph node metastases of patients with metastatic
19 breast cancer as compared to primary tumors of the breast and to normal breast tissues, that
20 CCL21 mRNA is present at significantly increased quantities in lymph node metastatic tissues as
21 compared to primary tumors of the breast, and that primary tumor CCL21 expression is
22 significantly correlated with patient survival outcomes in human breast cancer. Evaluation of the
23 effects of genetic depletion of CCL21 in mouse models of metastatic breast cancer on metastasis
24 to the central nervous system is merited. One study has described the importance of CCL21 in
25 facilitating recruitment of melanoma cells to the lymph nodes⁸. We suggest that in human
26 metastatic breast cancer, up-regulation of CCL21 by circulating tumor cells functions to support
27 recruitment to the lymph nodes and in the metastatic process functions specifically in homing to
28 the lymph nodes and not the brain. Modulation of CCL21 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. Inhibition of CCL21 represents one potential therapeutic approach to interfering with
brain infiltration of metastatic tumor cells by limiting progression of local breast cancer to
“regional”, or lymph node metastatic breast cancer.

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Rank: 25
Probe ID: 14986
p-value: 8.59e-05
t: -4.1411357
B: 1.2834
Gene: CCL21
Gene name: C-C motif chemokine ligand 21

Chart 1: CCL21 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: 1690
Probe ID: 204606_at
p-value: 2.61e-05
t: 4.586424
B: 2.017023
Gene: CCL21
Gene name: C-C motif chemokine ligand 21

Chart 2: CCL21 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.

Rank: 17194
probe ID: 14986
p-value: 9.78e-01
t: 2.83e-02
B: -6.140472
Gene: CCL21
Gene name: C-C motif chemokine ligand 21

Chart 3: CCL21 is not differentially expressed in brain metastatic breast cancer when comparing brain metastases to primary tumors of the breast.

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.

CCL21

Figure 1: CCL21 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 CCL21 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 CCL21 expression between primary tumors of the breast and lymph node metastases, a p -value, is $p=0.000099$.

Figure 2: Significant correlation between CCL21 primary tumor expression and overall survival in patients with breast cancer.

Depicted in this Kaplan-Meier plot is the probability of overall survival for $n=1402$ total patients stratified into two groups, based on low or high expression of CCL21 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 CCL21 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 CCL21 expression: 70.8 months
High CCL21 expression: 125.92 months

Chart 3: Median overall survival is inferior in patients with low primary tumor expression of CCL21.

The median OS (overall survival) of patients with low primary tumor expression of CCL21 and high primary tumor expression of CCL21 is listed in this chart, for $n=1402$ breast cancer patients.