CDKN2D (INK4D) is differentially expressed in the lymph nodes of patients with metastatic breast cancer.

Shahan Mamoor, MS¹

¹shahanmamoor@gmail.com

East Islip, NY USA

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 cyclin dependent kinase inhibitor 2D, CDKN2D, also known as INK4D, 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 CDKN2D was also differentially expressed in brain metastatic tissues. CDKN2D mRNA was present at increased quantities in lymph node metastases as compared to primary tumors of the breast. Importantly, expression of CDKN2D 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 CDKN2D 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, cyclin dependent kinase inhibitor 2D CDKN2D, CDKN2D, 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 the cyclin dependent kinase inhibitor 2D CDKN2D, CDKN2D, 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 CDKN2D 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 CDKN2D 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.

CDKN2D 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 cyclin dependent kinase inhibitor 2D, encoded by CDKN2D, 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, CDKN2D ranked 225 out of 18034 total transcripts (Chart 1), equating to 98.8% differential expression. Differential expression of CDKN2D in the lymph node metastases of patients with metastatic breast cancer was statistically significant (Chart 1; $p=2.74e-03$).

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 CDKN2D 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 CDKN2D 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, CDKN2D ranked 267 out of 41093 total transcripts (Chart 2), equating to 99.4% differential expression. Differential expression of CDKN2D in the brain metastases of patients with metastatic breast cancer was statistically significant (Chart 2; $p=6.93e-10$). Thus, differential expression of CDKN2D, 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 CDKN2D differential expression in human metastatic breast cancer is a *bona fide* biological process and that differential expression of CDKN2D in metastatic breast cancer is shared between the brain and the lymph nodes.

CDKN2D is expressed at higher levels in the lymph node metastases of patients with metastatic breast cancer.

We obtained exact mRNA expression levels for CDKN2D, 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 CDKN2D expression in lymph node metastatic tissues. We observed significantly increased expression of CDKN2D in the lymph node metastases of patients with breast cancer as compared to primary tumors of the breast: CDKN2D was expressed at 0.11 ± 0.83 arbitrary units (AU) in primary tumors of the breast, while it was expressed at 1.11 ± 0.80 AU in lymph node metastatic tissues (Figure 1). The difference in CDKN2D mRNA levels between primary tumors of the breast and lymph node metastases was statistically significant (Figure 1; $p=0.003159$).

CDKN2D expression is significantly correlated with survival outcomes in human breast cancer.

We performed Kaplan-Meier survival analysis⁷ in $n=313$ lymph node positive breast cancer patients and $n=594$ lymph node negative breast cancer patients, to evaluate whether CDKN2D tumor expression was correlated with survival outcomes in breast cancer, and to ascertain whether lymph node status influenced correlation of CDKN2D tumor expression with survival outcomes in human breast cancer. We observed a correlation between primary tumor expression of CDKN2D and overall survival (OS) in lymph node positive breast cancer (Figure 2). Lymph node positive patients whose primary tumors expressed low levels of CDKN2D possessed median OS of 38.57 months, while lymph node positive patients whose tumors expressed high levels of CDKN2D possessed median OS of 57.53 months. This difference in OS based on CDKN2D tumor expression in lymph node positive patients with breast cancer approached statistical significance (Figure 2, Chart 3; logrank p -value: 0.089; hazard ratio: 0.71 (0.48-1.05)). CDKN2D primary tumor expression was not significantly correlated with overall survival outcomes in lymph node negative patients (Figure 2, Chart 3; logrank p -value: 0.52; hazard ratio: 1.13 (0.78-1.64)).

Thus, by mining published microarray data^{4,5} in an unbiased and systematic fashion, we identified CDKN2D 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 CDKN2D in lymph node metastases as compared to primary tumors of the breast. Further, we found a significant correlation between CDKN2D expression and patient survival outcomes, as overall survival was significantly greater in patients whose primary tumors

expressed higher levels of CDKN2D as compared to patients whose primary tumors expressed lower levels of CDKN2D, and this correlation was significant in lymph node positive patients but not in lymph node negative patients.

Discussion

We provided evidence here that the cyclin dependent kinase inhibitor 2D CDKN2D, CDKN2D, 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 CDKN2D is also differentially expressed in brain metastatic tissues in human breast cancer, that CDKN2D mRNA is present at significantly increased quantities in lymph node metastatic tissues as compared to primary tumors of the breast, and that primary tumor CDKN2D expression is significantly correlated with patient survival outcomes in human breast cancer, in all patients and in lymph node positive patients but not in lymph node negative patients. Evaluation of the effects of genetic depletion of CDKN2D in mouse models of metastatic breast cancer on metastasis to the central nervous system is merited. Modulation of CDKN2D 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: 225
Probe ID: 21205
p-value: 2.74e-03
t: -3.0929177
B: -1.6601
Gene: CDKN2D
Gene name: cyclin dependent kinase inhibitor 2D

Chart 1: CDKN2D 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: 267
Probe ID: A_23_P89941
p-value: 6.93e-10
t: -7.7121186
B: 12.468636
Gene: CDKN2D
Gene name: cyclin dependent kinase inhibitor 2D

Chart 2: CDKN2D 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: CDKN2D 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 CDKN2D 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 CDKN2D expression between primary tumors of the breast and lymph node metastases, a p -value, is $p=0.003159$.

Figure 2: CDKN2D primary tumor expression is correlated with overall survival in all patients with breast cancer and in lymph node positive patients but not in lymph node negative patients.

Depicted in this Kaplan-Meier plot (**above**) is the probability of overall survival for $n=1402$ total patients stratified into two groups, based on low or high expression of CDKN2D 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 CDKN2D 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 overall survival in $n=313$ lymph node positive patients (**left**) and $n=594$ lymph node negative patients (**right**).

Lymph node positive patients

Low CDKN2D expression: 38.57 months

High CDKN2D expression: 57.53 months

Chart 3: Median overall survival is inferior in patients with low primary tumor expression of CDKN2D.

The median OS (overall survival) of patients with low primary tumor expression of CDKN2D and high primary tumor expression of CDKN2D is listed in this chart, for $n=1402$ breast cancer patients and for $n=313$ lymph node positive patients.