AXL receptor tyrosine kinase is differentially expressed in lymph node metastases in human breast cancer.

Shahan Mamoor, MS¹

¹shahanmamoor@gmail.com

East Islip, NY USA 11730

Breast cancer is the most frequently diagnosed cancer in women¹. Metastasis is the major cause of death in patients diagnosed with cancer². Prognosis for patients with breast cancer worsens as the number of axillary lymph nodes with metastasis increases³. We mined published microarray and multiplexed mRNA quantitation datasets^{4,5} to discover genes associated with metastasis to the lymph nodes, an early event in breast cancer metastasis. We found significant differential expression of the gene encoding AXL when comparing lymph node metastases to primary breast tumors from women diagnosed with breast cancer. In primary tumors, lower expression of AXL correlated with decreased disease-free survival in breast cancer patients. AXL may be of relevance to processes underlying metastasis to the lymph nodes.

Keyword: breast cancer, breast cancer metastasis, lymph node metastasis, AXL, systems biology of breast cancer, targeted therapeutics in breast cancer.

Invasive breast cancer is diagnosed in over quarter of a million women in the United States each year¹. Metastasis is the most common cause of death in humans diagnosed with cancer². The lymphatic system, along with the blood system, are the primary routes by which tumor cells traverse the body⁷. While patients with localized breast cancer are provided a 99% 5-year survival rate, patients with regional breast cancer, cancer that has spread to lymph nodes or nearby structures, are provided an 86% 5-year survival rate^{8,9}. Patients with metastasis to distant sites are provided a 27% 5-year survival rate^{8,9}. We mined published microarray and multiplexed gene expression datasets^{4,5} to discover genes associated with lymph node metastasis in breast cancer and identified significant differential expression of AXL. AXL may be of relevance to biological processes underlying breast cancer metastasis to the lymph nodes.

Methods

We used datasets GSE124648⁴, GSE92977⁵ and GSE109169⁶ for this differential gene expression analysis in conjunction with GEO2R. GSE124648, a microarray dataset, was generated using Affymetrix Human Genome U133A Array technology with $n=256$ primary breast tumors from patients with breast cancer and $n=67$ lymph node metastases from patients with breast cancer; platform GPL96 was used for this analysis. GSE92977, a multiplexed mRNA quantitation dataset, was generated using NanoString nCounter Nano105 Gene Expression Custom CodeSet technology with $n=123$ primary breast tumors from patients with breast cancer and $n=24$ lymph node metastases from patients with breast cancer; platform GPL17071 was utilized for this analysis. GSE109169 was generated using Affymetrix Human

Exon 1.0 ST Array technology with $n=25$ adjacent control breast tissue and $n=25$ primary breast tumors from patients with breast cancer; platform GPL5175 was used for this analysis. 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 AXL expression was significantly between primary breast tumors and lymph node metastases using a two-tailed, unpaired t-test with Welch's correction. We used PRISM for all statistical analyses of differential gene expression in human breast cancer (Version 8.4.0)(455). For Kaplan-Meier survival analysis, we used the Kaplan-Meier plotter online tool⁹ for correlation of AXL mRNA expression levels with disease-free survival in $n=3951$ breast cancer patients and overall survival in $n=1402$ breast cancer patients.

Results

We mined published microarray and multiplexed mRNA quantitation data^{4,5} to discover genes associated with metastasis of invasive breast cancer to the lymph nodes.

AXL is differentially expressed in lymph node metastases in invasive breast cancer.

We identified AXL as among the genes expressed most quantitatively differently, transcriptome-wide, when comparing primary tumors of the breast to lymph node metastases in women diagnosed with breast cancer⁴ (Table 1). When sorting each of the transcripts measured by microarray based on significance of change in expression between lymph node metastases

1 and primary tumors of the breast, AXL ranked 111 out of 22283 total transcripts (Table 1).
2 Differential expression of AXL in lymph node metastases in human breast cancer was
3 statistically significant (Table 1; $p=1.43E-49$).
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5 Analysis of a second mRNA multiplexed quantitation dataset⁵ again revealed differential
6 expression of AXL in lymph node metastases when compared to primary breast tumors (Table 2).
7 When sorting each of the transcripts measured by microarray based on significance of change in
8 expression between primary tumors of the breast lymph node metastases, AXL ranked 12 out of
9 105 transcripts measured, equating to differential expression more significant than 88.6% of
10 genes measured in this code set (Table 2). Differential expression of AXL in lymph node
11 metastases in human breast cancer in this second dataset was statistically significant (Table 2;
12 $p=3.88E-03$).
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17 AXL is expressed at significantly lower levels in lymph node metastases when compared to
18 primary tumors of the breast.

19 We obtained exact mRNA expression levels of the differentially expressed AXL transcript
20 to directly compare AXL expression between primary tumors of the breast and lymph node
21 metastases. We also performed a statistical test to evaluate whether the difference in AXL
22 expression between primary tumors of the breast and lymph node metastases was statistically
23 significant. AXL was expressed at lower levels in lymph node metastases as compared to
24 primary tumors of the breast; this difference in AXL mRNA expression was statistically
25 significant (Figure 1; $p<0.0001$; Figure 2: $p=0.0040$). We calculated a fold change of $0.9002 \pm$
26 0.0862 in AXL mRNA expression in lymph node metastases relative to primary breast tumors
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(Table 1).

AXL expression in primary breast tumors in patients with breast cancer correlates with disease-free survival in patients with breast cancer.

We performed Kaplan-Meier survival analysis¹⁰ using AXL mRNA expression in primary breast tumors coupled with paired disease-free survival data from each patient, in 3951 patients in total, to determine whether AXL tumor expression was correlated with survival outcomes in breast cancer. In the lowest survival quartile, patients whose primary tumors expressed lower levels of AXL possessed significantly shorter disease-free survival (DFS) than patients with high primary tumor expression of AXL (Figure 3). DFS of patients in the low expression cohort was 185.16 months, while DFS in patients in the high AXL expression cohort in this sub-group was 216.66 months (Table 3); this difference in DFS based on AXL tumor expression in breast cancer was statistically significant (Figure 3, Table 3; logrank p -value: 0.00012; hazard ratio: 0.81 (0.73-0.9); false discovery rate: 0.05). Overall survival (OS) also displayed a trend towards association with AXL expression in primary tumors from patients with breast cancer, with patients with lower AXL expression in primary tumors possessing shorter OS than patients with higher AXL expression in primary tumors (Figure 4). OS of patients in the low expression cohort was 81.87 months, while DFS in patients in the high AXL expression cohort in this sub-group was 135.84 months (Table 4). However, false discovery rate was unacceptably high for correlation analysis between AXL primary tumor expression and OS in patients with breast, at greater than 50%, despite a significant log rank p -value (0.0088) and hazard ratio (0.74; (0.59 - 0.93) similar to that observed in analysis when correlating DFS in patients with breast cancer

1 with primary tumors expression of AXL.

2
3 AXL is not differentially expressed when comparing primary tumors of the breast to normal
4 breast tissue.

5 AXL inhibitors have been developed and assume increased expression of AXL in primary
6 tumors or in metastases¹¹; we found decreased expression of AXL in lymph node metastases. We
7 queried a third microarray dataset⁶ to determine if AXL was differentially expressed when
8 comparing. While this would not indicate whether AXL was over- or under-expressed, it would
9 provide important information as to whether relative to all other transcripts expressed in the
10 primary tumor, AXL expression was significantly different in the primary tumors as compared to
11 the normal breast. We found that AXL was not significantly differentially expressed in primary
12 tumors of the breast when compared to normal breast tissue (Table 5). When sorting all genes
13 expressed in primary tumors of the breast from women diagnosed with breast cancer based on
14 significance of change in expression between primary tumors and normal breast tissue, AXL
15 ranked 16280 out of 19076 total transcripts, equating to 14.7% differential expression; this was
16 not statistically significant (Table 5; $p=6.16E-01$). This means greater than 85% of the measured
17 transcriptome changed expression more significantly than AXL when comparing breast tumors to
18 normal breast tissue.
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24 Thus, we identified AXL as among the genes whose expression was most significantly
25 different when comparing global gene expression of lymph node metastases from patients with
26 breast cancer to primary tumors; AXL expression in lymph node metastases was significantly
27 lower than in primary tumors, and lower expression of AXL in primary tumors of the breast in
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1 was significantly correlated with shorter disease-free survival. AXL was not differentially
2 expressed in primary tumors of the breast, in one dataset queried.
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4 **Discussion**

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6 The family of enzymes that includes the AXL tyrosine kinase includes three enzymes:
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8 AXL (Ufo), SKY (Tyro-3) and MER. These receptor tyrosine kinases, at the structural level, are
9 composed of an extracellular domain with immunoglobulin-like domains, along with two
10 fibronectin type 3-like domains¹²⁻¹⁴. The growth arrest-specific gene GAS6 is the ligand for
11 AXL. Our findings implicating the receptor tyrosine kinase AXL in human breast cancer are not
12 novel. There are multiple studies illustrating the importance of AXL in breast cancer
13 progression, metastasis, and the epithelial to mesenchymal transition associated with metastasis
14 in breast cancer¹⁵⁻¹⁸. However, our findings are different, demonstrating that AXL is among the
15 genes whose expression most significantly distinguishes lymph node metastases from patients
16 with breast cancer as compared to primary tumors of the breast at the transcriptome-level.
17 Moreover, we find decreased expression associated with decreased survival and in metastases as
18 compared to primary tumors, as opposed to increased expression of AXL associating with worse
19 patient outcomes.
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22 Genetic depletion of Axl resulted in complete block of metastasis to the lymph nodes
23 upon in the MDA-MB-231-D3H2LN NOD/SCID mouse model of breast cancer; all mice with
24 wild-type expression of AXL in MDA-MB-231-D3H2LN NOD/SCID mice developed prominent
25 metastasis to the lymph nodes¹⁵. An inhibitor of AXL, R428, now known as bemcentinib,
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proved effective in reducing metastasis in MB231 intracardiac and 4T1 orthotopic mouse models and proved efficacious as an add-on to cisplatin in reducing liver metastasis¹⁶. In mammary epithelial cells, AXL can induce the epithelial to mesenchymal transition (EMT) gene expression program, and AXL expression in ANV5 cells correlates with tumorigenicity of breast cancer stem cells as measured by tumor volume of sorted AXL⁺ and AXL⁻ stem cells¹⁷. In human embryonic vein endothelial cells (HUVEC), depletion of AXL resulted in down regulation of DKK3 and up-regulation of Ang-2; both molecules can influence angiogenesis¹⁸. AXL antibodies can impair tumor growth of the A549 non-small cell lung cancer cell line grafted in nude mice. However, use of an inhibitor is predicated upon logic that a gene is so differentially expressed when comparing a tumor to the tissue from which it originates, or a metastasis to the tumor from which it originates, that its differential and increased expression will result in inhibition being efficacious in blocking tumor growth or metastasis.

By mining independently published gene expression data^{4,5}, we found that AXL was among the genes most differentially expressed in lymph node metastases from patients with breast cancer when compared to primary tumors of the breast. Lower primary tumor expression of AXL correlated with disease-free survival in patients with breast cancer. AXL was not differentially expressed in primary tumors of the breast when compared to normal breast tissue, in one microarray dataset queried. AXL may be of relevance to processes underlying spread of breast cancer to the lymph nodes.

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Rank	ID	p-value	t	B	FC	Gene	Gene name
111	202686_s_at	1.43E-49	-17.710247	102.06486	0.9002 ± 0.0862	AXL	AXL receptor tyrosine kinase

Table 1: Differential expression of AXL in lymph node metastases in breast cancer.

Rank of differential expression, probe ID, p-value of global differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the groups compared, fold change of AXL in lymph node metastases as compared to primary breast tumors, gene and gene name are listed in this chart.

Rank	ID	p-value	t	B	Gene
12	6	3.88E-03	-2.9341	-2.7595	AXL

Table 2: Differential expression of AXL in lymph node metastases in breast cancer.

Rank of differential expression, probe ID, *p*-value of global differential expression, *t*, a moderated *t*-statistic, *B*, the log-odds of differential expression between the groups compared, gene and gene name are listed in this chart.

Figure 1: AXL is expressed at significantly lower levels in lymph node metastases as compared to primary tumors of the breast.

The mRNA expression level of AXL in primary tumors of the breast from women diagnosed with invasive breast cancer (left) and in lymph node metastases from women diagnosed with breast cancer (right) is graphically depicted with the result of a statistical test evaluating significance of difference in AXL expression between primary tumors and lymph node metastases, a *p*-value, listed above.

Figure 2: AXL is expressed at significantly lower levels in lymph node metastases as compared to primary tumors of the breast.

The mRNA expression level of AXL in primary tumors of the breast from women diagnosed with invasive breast cancer (left) and in lymph node metastases from women diagnosed with breast cancer (right) is graphically depicted with the result of a statistical test evaluating significance of difference in AXL expression between primary tumors and lymph node metastases, a *p*-value, listed above.

Figure 3: AXL expression in primary tumors is significantly correlated with disease-free survival in patients with breast cancer.

Depicted in this Kaplan-Meier plot is the probability of overall survival for $n=3951$ total patients stratified into two groups, based on low or high expression of AXL in patient primary tumors, in the lowest quartile. 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 AXL 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. The false discovery rate is 5%.

Low expression cohort (months)	High expression cohort (months)
185.16	216.66

Table 3: AXL expression in primary tumors from patients with breast cancer significantly correlates with disease-free survival.

Disease-free survival of $n=3951$ breast cancer patients based on stratification into low or high tumor expression of AXL is listed in this chart.

Figure 4: AXL expression in breast tumors is correlated with overall survival in patients with breast cancer but with high false discovery rate.

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 AXL 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 AXL 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. The false discovery rate is greater than 50%.

Low expression cohort (months)	High expression cohort (months)
81.87	135.84

Table 4: AXL expression in breast tumors correlates with overall survival but with high false discovery rate.

The overall survival of $n=1402$ breast cancer patients based on stratification into low or high tumor expression of AXL is listed in this chart.

Rank	ID	p-value	t	B	Gene	Gene name
16280	3834046	6.16E-01	-5.05E-01	-6.859742	AXL	AXL receptor tyrosine kinase

Table 5: AXL is not differentially expressed when comparing primary tumors of the breast to normal breast tissue.

Rank of differential expression, *p*-value of global differential expression, *t*, a moderated *t*-statistic, *B*, the log-odds of differential expression between the groups compared, gene and gene name are listed in this chart.