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Angiopoietin pathway gene expression associated with poor breast cancer survival

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Abstract

Purpose—Angiogenesis is one of the hallmarks of cancer and is essential for cancer progression and metastasis. However, clinical trials with vascular endothelial growth factor (VEGF) pathway inhibitors have failed to show overall survival benefit in breast cancer. Targeted therapy against the angiopoietin pathway, a downstream angiogenesis cascade, could be effective in breast cancer. This study investigates the association of angiopoietin pathway gene expression with breast cancer survival using a “big data” approach employing RNA sequencing data from The Cancer Genome Atlas (TCGA).

Methods—A total of 888 patients with adequate gene expression, disease-free survival (DFS) and overall survival (OS) data were selected for analysis. DFS and OS were calculated for patients with high and low expression of angiopoietin and VEGF pathway genes using TCGA data. Gene-specific thresholds to dichotomize patients into high and low expression were determined and survival plots were generated.

Results—The TCGA cohort was representative of national breast cancer patients with respect to stage, pathology and survival. High Ang2 gene expression was associated with not only decreased DFS ($p=0.05$), but also decreased OS ($p<0.05$). High co-expression of Ang2 and its receptor Tie2 was associated with both decreased DFS and OS ($p<0.05$). There was strong correlation between angiopoietin and VEGF pathway genes. While high expression of VEGFA alone was not associated with survival, high co-expression with Ang2 was associated with decreased OS.

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Conclusions—This study validates TCGA as a representative database providing genomic data and survival outcomes in breast cancer. Our TCGA data supports the angiopoietin pathway as a key mediator in the pathologic angiogenic switch in breast cancer.

Keywords

ANGPT; TEK; TCGA; The Cancer Genome Atlas; genomic; angiogenesis; outcomes

Background

Breast cancer is a leading cause of cancer-related death for women worldwide. It is the second most common cancer affecting women in the United States, with an estimated 231,840 new cases of invasive breast cancer and 40,290 deaths in 2015 [1]. The mortality from breast cancer closely follows stage of disease, and angiogenesis is one of the hallmark mechanisms contributing to advancement of stage in breast cancer [2, 3]. In tumor angiogenesis, there is a switch from normal recruitment of endothelial progenitor cells to pathologic sprouting of new blood vessels from existing vessels [4, 5]. The two major tyrosine kinase pathways that regulate angiogenesis and contribute to tumor angiogenesis include the angiopoietin pathway and the vascular endothelial growth factor pathway (VEGF).

Despite early encouraging results with VEGF pathway modulators in breast cancer, survival benefits have seldom been observed. Therefore, there has been increased interest in the angiopoietin pathway, a downstream cascade in normal and tumor angiogenesis [6-8]. As a result of promising animal and cell culture experiments, early phase clinical trials are currently underway investigating the efficacy of angiopoietin pathway blockade [7-10]. Trebananib is an investigational peptide-Fc fusion protein that inhibits the interaction of the Ang ligand with its Tie receptor. It has undergone early phase human clinical trials in several cancers, including metastatic breast cancer. However, it remains unclear whether inhibition of the angiopoietin pathway will affect survival outcomes in breast cancer [11-13].

Use of “big data” approaches could serve as a preliminary adjunct to prospective clinical trials and enable researchers to infer the likely impact of a therapeutic target [14]. One such “big data” resource in oncology is The Cancer Genome Atlas (TCGA). TCGA is a joint collaboration of the National Cancer Institute and National Human Genome Research Institute that collects de-identified clinical data and tumor samples for genome sequencing. TCGA, to the best of our knowledge, has yet to be used to characterize the associations between specific patterns of gene expression and survival in breast cancer. Such an approach may predict the likelihood for survival impact with therapeutic targeting of specific gene patterns.

In this study, we use a novel “big data” approach of analyzing the association between angiopoietin pathway gene expression and survival in a large cohort of TCGA breast cancer patients to infer whether angiopoietin pathway modulation might serve as a promising target for breast cancer treatment.

Methods

TCGA Data Acquisition and Pre-Processing

Level 3 RSEM RNA-seq data for 1092 solid tumor samples was downloaded on 12/18/14 from the TCGA Data Portal. Of these, 1058 had clinical data entries. Genes investigated for this study included Ang2, Tie1, Tie2, VEGFA and VEGFR1-3. Clinical XML and RNA-seq files were parsed, processed and merged using custom in-house python and R scripts [15]. The RSEM RNA-seq “raw counts” (rc) data were log2 transformed after rounding to the nearest whole number and adding one ($\log_2(\text{round}(\text{rc}) + 1)$) before being normalized across all samples using simple quantile normalization with the `normalizeQuantiles()` function in the `limma` R package [16].

Survival Analysis

A manual review of TCGA clinical records identified disease-free survival (DFS) and overall survival (OS) event times for each patient. Of the 1058 records with gene expression and clinical data, 848 had useable DFS data and 886 had useable OS data to determine event times, resulting in a cohort of 888 patients. OS was calculated as time in months from diagnosis to death, and DFS was calculated as time in months to recurrence of breast cancer or death from any cause. Patients who did not have an event were censored at the last date of follow-up or after 10-years. Survival analyses were performed using the `survival` R package for single genes and pairs of genes [17].

For survival analyses based on expression of a single gene, patients were classified as having high or low expression of the given gene using a gene-specific threshold. Patients were labeled as “high” if the expression of the interrogated gene was above the threshold and “low” otherwise. To determine the gene-specific threshold, a series of 20 cutoffs evenly spaced between the minimum and maximum expression value were investigated. For each cutoff that had at least 30 patients in both the high and low groups, a survival curve was generated. The cutoff that generated the lowest log-rank p-value, regardless of significance, was chosen as the gene-specific cutoff for the double-gene survival analyses (Supplementary Tables 1-8).

For the double-gene survival analyses, four patterns of expression were defined for a pair of genes (gene1-gene2) based on the gene-specific expression threshold described previously: high-high, high-low, low-low, low-high. Patients were classified into two groups, those that matched the specified pattern and those that did not. A plot was only generated if there were at least 5 patients in both the pattern matching and non-matching groups. For all analyses, log-rank p-values were calculated using R, and are considered significant at the $\alpha=0.05$ significance level.

Results

TCGA patient sample and data

There were 888 breast cancer cases with genomic and clinical data for analysis of DFS (n=848 cases) and OS (n=886 cases). The mean age of the cohort was 58.0 ± 13.2 years

(Table 1). At the time of diagnosis, 18.6% had Stage I breast cancer, 54.8% had Stage II, 22.8% had Stage III and 1.7% had Stage IV. Survival at 5-years by stage was 88.2% for Stage I, 81.0% for Stage II, 71.7% for Stage III, and 36.6% for Stage IV, respectively (Figure 1a). The majority, 72.9%, of patients had infiltrating ductal carcinoma (IDC), followed by 17.9% of patients with infiltrating lobular carcinoma (ILC). 5-year survival for patients with IDC and ILC was 76.8% and 94.6%, respectively (Figure 1b).

These data were in agreement with national cancer statistics collected and reported by the United States Surveillance, Epidemiology and End Results (SEER) program [18]. In the SEER database, 61% of patients had localized Stage I and Stage II disease, 32% had Stage III regional disease and 6% had Stage IV distant disease. Similar to our TCGA survival statistics, SEER reported 5-year survival of 98.6% for localized disease, 84.9% for regional disease and 25.9% for metastatic disease. Additionally, SEER reported that 73.2% of cancers were infiltrating ductal and 9.1% of cancers were infiltrating lobular on final pathology, which is similar to the TCGA cohort. The similarity between SEER and TCGA data validate TCGA as a representative cohort of breast cancer patients.

Association between breast cancer survival and Ang2 ligand-Tie2 receptor expression

In order to examine the associations between the angiopoietin pathway genes and survival, gene expressions were dichotomized as high and low using gene-specific expression cutoffs. Individual gene expression distributions and cutoffs are provided in Supplementary Figure 1.

In normal states, the Ang1 ligand and the Tie2 receptor help regulate vascular maturation [6-8]. In tumor angiogenesis, however, Ang2 antagonizes Ang1 and binds to the Tie2 receptor, increasing downstream signaling and resulting in pathologic angiogenesis [7-10]. We found that high Ang2 gene expression in breast cancer tumor tissues was associated with not only decreased DFS ($p=0.05$), but also decreased OS ($p=0.02$) (Figures 2a and 2b respectively). When we analyzed survival by stage based on Ang2 expression, higher stages of disease were associated with inferior DFS and OS irrespective of Ang2 expression (Supplementary Figure 2). Limitations in the number of patients with high Ang2 expression precluded stage-specific analysis of the effect of in high Ang2 expression on survival.

While high Tie2 expression alone did not have any significant association with survival, patients with combined high Ang2 expression and high Tie2 expression had both decreased DFS ($p<0.01$) and OS ($p<0.01$) (Figures 3a and 3b respectively). However, patients with low Ang2 and high Tie2 expression had increased DFS ($p=0.05$), further implicating high Ang2 expression as the determining factor in the angiogenic switch (Figure 3c).

Interplay between the angiopoietin and VEGF pathways in breast cancer

There is increasing evidence showing interplay between the angiopoietin and VEGF pathways in pathologic tumor angiogenesis [19]. A correlation matrix of gene expressions of Ang2, Tie1, Tie2, VEGFA and VEGFR1-3 indicated a positive correlation in gene expression between the angiopoietin pathways and VEGF pathways. The correlations were particularly strong between Ang2 and VEGFR1, Tie2 and VEGFR3, and Tie1 and VEGFR3, indicating potential options for dual targeting of the angiopoietin and VEGF pathways (Figure 4a).

In our analysis we found that high gene expression of VEGFA was not associated with decreased DFS and trended towards decreased OS, even though it did not reach statistical significance in this cohort ($p=0.06$) (Figures 4b). However, high Ang2 in conjunction with high VEGFA was associated with significantly decreased OS ($p=0.02$) (Figure 4c). Additionally, low Ang2 and low VEGFA expression was associated with increased DFS ($p<0.01$) (Figure 4d). This reiterates the relationship of the VEGF and angiopoietin pathways, and may suggest a stronger role for Ang2 in pathologic tumor angiogenesis.

Discussion

TCGA is a versatile tool that has been used for a variety of purposes, including gene profiling in specific disease states, and predicting responsiveness to chemotherapy based on gene mutation status [20, 21]. To our knowledge, TCGA has not been used to investigate associations between specific patterns of gene expression and survival in breast cancer. Our analysis of TCGA data provide compelling evidence that therapeutic targeting of angiogenesis through the angiopoietin pathway in breast cancer may result in improvements in survival.

In this analysis, we found that the TCGA cohort closely approximates national data for breast cancer stage distribution and survival, validating it as a representative cohort. Utilizing this “big data” resource, we demonstrated decreased survival among patients with high tissue gene expression of Ang2 and combined high Ang2 and high Tie2 gene expression. Additionally, we showed that although high VEGFA expression alone was not associated with survival, high VEGFA expression in conjunction with high Ang2 expression was associated with decreased OS.

Our results from TCGA mirror findings from SynTarget, a new online tool that interrogates the expression of multiples genes from microarray data sets for synergistic survival relationships [22, 23]. We utilized this tool to assess the synergistic relationships between Ang2, Tie2 and VEGFA within two breast cancer microarray data sets provided by SynTarget. These data sets were METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) [24], which contained expression data from 1,971 samples, and a dataset published by Hatzis C et al. that included expression data from 508 samples (GEO accession GSE25055) [25, 26]. In agreement with the relationships identified using RNA-seq data from TCGA, SynTarget identified decreased survival in those with high Ang2 expression (Supplementary Figure 3a). Similarly, those with combined low Ang2 expression and high Tie2 expression saw increased survival (Supplementary Figure 3b) [22, 23]. Additionally, SynTarget reported decreased survival with high Ang2 and high VEGFA expression, and improved survival with low Ang2 and low VEGFA expression (Supplementary Figure 3c and 3d). These data support our TCGA findings despite the technological differences between microarray and RNA-seq platforms.

The VEGF pathway was among the first angiogenesis pathways to be delineated, and while VEGF inhibitors, such as bevacizumab, have proven effective in some cancers, it has failed to show improvements in OS in breast cancer [27-32, 49]. In one of the original prospective studies of angiogenesis modulation in breast cancer, treatment with bevacizumab showed no

improvement in progression-free survival or OS in metastatic disease [33]. Subsequently, although NSABP-B40 showed improvement in survival in the neo-adjuvant setting, the GeparQuinto trial did not, making the role of neo-adjuvant bevacizumab in breast cancer unclear [34, 35]. Interestingly, GeparQuinto and other studies showed preferential benefit in terms of response for triple negative breast cancers, and the NSABP-B40 trial demonstrated preferential response and survival benefit for hormone responsive breast cancers, in agreement with a neoadjuvant study from Norway [36]. In this TCGA dataset, we found no association between increased VEGFA expression and survival. We did not search for associations between the expression of these angiogenesis markers and hormone receptor status in this dataset. Similarly, limitations in the dataset did not enable to investigate associations between the angiopoietin pathway and stromal components. It would be of interest to examine the expression of these genes in tissue collected prospectively in trials of anti-angiogenesis agents like bevacizumab (e.g., B40) to determine whether expression of any gene or combination of genes might predict the benefit of adding inhibitors of angiogenesis to standard therapy.

Several pre-clinical studies point to the potential efficacy of angiopoietin blockade. Tumors overexpressing Ang2 are associated with greater endothelial cell proliferation, vascular growth, and metastasis in cell culture and animal models [37-41]. In several human solid tumors, circulating Ang2 has been identified as a potential biomarker with prognostic significance [42- 46]. Among breast cancer patients, high Ang2 expression has been previously correlated with lymph node metastasis, lymphovascular invasion and poor disease-free survival [10, 41]. In our analysis of TCGA data, we found that Ang2 overexpression was associated with decreased OS and DFS, indicating that tumors with high Ang2 may be more aggressive, perhaps as a result of increased tumor angiogenesis. Furthermore, in xenografted mouse models, Ang2-Tie2 blockade has been associated with a decrease in distant metastasis, suggesting that down-regulation of the pathway may make the disease less aggressive [47]. We similarly found that tumors with low Ang2 and high Tie2 expression had increased DFS, suggesting the key role of Ang2 in the angiogenic switch that drives aggressive disease through activation of the angiopoietin pathway.

Our study revealed significant crosstalk between genes in the angiopoietin pathway and the VEGF pathway. Ang2 activation and secretion by endothelial cells occurs in part as a result of VEGF signaling, and dual targeting of VEGF and Ang2 has been shown in xenografted mouse models to potentiate the effectiveness of single pathway blockade [2, 19]. We found that while VEGFA alone did not correlate with survival, high Ang2 and high VEGFA co-expression did correlate with decreased overall survival. It is conceivable that blockade of VEGF signaling alone may be less effective against breast cancers that co-express high levels of both gene products or vice versa.

Trebananib is an investigational peptide-Fc fusion protein that modulates the angiopoietin pathway by inhibiting the interaction of the Ang ligand with the Tie2 receptor. To date, there are several phase I and phase II clinical studies evaluating trebananib as monotherapy and in combination with chemotherapy and anti-VEGF therapy in a number of cancer types [12, 48]. In breast cancer, trebananib has been evaluated in a phase II study with HER2-negative, locally recurrent and metastatic breast cancer. That study found no prolongation in

progression-free survival with the addition of trebananib to a regimen of paclitaxel and bevacizumab [11]. While this initial study appears to be at odds with our analysis, the study's patient sample consisted almost exclusively of patients with distant, metastatic disease, limiting the ability to extrapolate from this data. Like other targeted therapies, moreover, it may be that the combination of anti-angiogenic agents may only benefit certain subsets of patients, perhaps those whose cancers co-express high levels of both Ang2 and VEGFA.

Limitations of our current study include those inherent to genomic analyses and retrospective outcomes evaluations. Given the novelty of this genomic data, absolute cutoffs and normal ranges for specific gene expressions are not available, prompting us to generate cutoffs based on statistical distributions in the gene expressions. Additionally, the TCGA data set does not include gross pathologic features, and thus increases angiopoietin pathway expression could not be correlated with increased tumor vascularity. Further, it needs to be taken into account that TCGA exclusively sampled the parts of the tumor rich with viable cancer cells, i.e. it does not include any peritumoral tissue. As we previously described, it is commonly the peritumoral area where microvessel density are typically measured to evaluate angiogenesis [50, 51]. Therefore, the gene expression data from TCGA reflect the expression at cancer cell rich portions of the tumor and it may or may not be in agreement with actual microvessel density data. Although confounding variables and gene expressions would serve to limit the extrapolations of the impact of specific genes on survival, an advantage of this approach is that it examines the associations of specific gene expressions with outcomes in a true clinical milieu of a large cohort.

Conclusions

In conclusion, this study validates TCGA as a representative cohort of breast cancer cases. Using a “big data” approach with TCGA, we identified a strong association between high angiopoietin pathway gene expression and decreased breast cancer survival outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

TCGA cohort survival outcomes

Kaplan-Meier estimates of OS of breast cancer patients in TCGA cohort (A) Stage of breast cancer (B) Histology of breast cancer (IDC: Infiltrating ductal carcinoma, ILC: Infiltrating lobular carcinoma)

Figure 2.
High Ang2 gene expression and survival
Kaplan-Meier estimates of survival among TCGA breast cancer patients based on high gene expression of Ang2 (a) DFS (b) OS

Figure 3.

Ang2-Tie2 gene expression and survival

Kaplan-Meier estimates of survival among TCGA breast cancer patients (a) DFS among patients with high Ang2 and high Tie2 gene expression (b) OS among patients with high Ang2 and high Tie2 gene expression (c) DFS among patients with low Ang2 and high Tie2 gene expression **Figure 4** Angiopoietin and VEGF pathway gene expression and survival (a) Gene expression profiles of angiopoietin and VEGF pathways (Pearson's correlation coefficient represented on bottom axis) (b) Kaplan-Meier estimates of OS among breast cancer patients with high VEGFA expression (c) Kaplan-Meier estimates of OS among breast cancer patients with high Ang2 and high VEGFA expression (d) Kaplan-Meier estimates of DFS among breast cancer patients with low Ang2 and low VEGFA expression

Figure 4.

Angiopoietin and VEGF pathway gene expression and survival

(a) Gene expression profiles of angiopoietin and VEGF pathways (Pearson's correlation coefficient represented on bottom axis) (b) Kaplan-Meier estimates of OS among breast cancer patients with high VEGFA expression (c) Kaplan-Meier estimates of OS among breast cancer patients with high Ang2 and high VEGFA expression (d) Kaplan-Meier estimates of DFS among breast cancer patients with low Ang2 and low VEGFA expression

Table 1

Demographics and breast cancer characteristics

Patients (n=888)		
Mean Age, years (SD)	58.0 ± 13.2	
Histology (%)		
Infiltrating ductal carcinoma	646	(72.9%)
Infiltrating lobular carcinoma	158	(17.8%)
Mucinous Carcinoma	11	(1.2%)
Medullary Carcinoma	4	(0.5%)
Other	75	(7.1%)
Stage at Diagnosis (%)		
Stage I	165	(18.6%)
Stage II	486	(54.9%)
Stage III	202	(22.8%)
Stage IV	15	(1.7%)
Unknown	12	(1.4%)