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Original Article

Angiogenesis is associated with an attenuated tumor microenvironment, aggressive biology, and worse survival in gastric cancer patients

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Abstract: Angiogenesis is a cornerstone of cancer as it allows tumors to receive oxygen and nutrients. A high level of angiogenesis within a tumor may therefore be indicative of its aggressiveness. In this study, we examined this hypothesis in gastric cancer. Gene set variation analysis was used to measure the level of angiogenesis in tumors in 1,348 gastric cancer patients using the Hallmark_angiogenesis gene set to score tumor transcriptomes. As we predicted, there was a significant correlation between angiogenesis score and expression of angiogenesis-related genes. The score moderately correlated with abundance of vessel-related stromal cells, fibroblasts and chondrocytes in the tumor microenvironment (TME). Tumors with high score had low infiltration of T helper type 1 and 2 cells but a greater infiltration of M1 macrophages and dendritic cells. They also had enriched expression of gene sets for coagulation, hypoxia, epithelial mesenchymal transition (EMT), and TGF-β signaling. High angiogenesis score was significantly associated with advanced AJCC stage and higher T- but not N-parameters in the TNM staging system. Patients with a high score also had shorter survival. In conclusion, bulk tumor transcriptome-based quantification of tumor angiogenesis using a computational algorithm may serve to identify patients with worse survival in gastric cancer.

Keywords: Angiogenesis, EMT, gastric cancer, gene set, survival, GSVA, prognostic biomarker

Introduction

Across the world, gastric cancer ranks third in cancer-related deaths [1]. At diagnosis, eighty percent of patients have unresectable or metastatic disease [2]. Despite improvements in perioperative treatment, approximately half of the patients who undergo curative intent surgery eventually relapse. Though efforts have focused on identifying prognostic biomarkers that can be applied in clinical practice to improve the poor outcome [3, 4], we urgently need a predictive biomarker to help guide treatment decision-making to precisely address gastric cancer progression and metastasis.

Angiogenesis is the creation of de novo blood vessels from existing ones. Angiogenesis is a cornerstone of cancer as it paves the way for cancer progression and metastasis [5, 6]. Blood vessels allow oxygen and nutrients to be delivered to tumors and also carry cells and factors that promote immune resistance. Additionally, the new blood vessels generated can act as a gateway for cancer cells to enter the blood stream and metastasize distantly [7]. Cancer and stromal cells produce angiogenic factor such as vascular endothelial growth factor and endothelial cell growth factor in several types of cancers including gastric cancer [8, 9]. We have previously reported that there is an

association between abundant blood vessels and both infiltration of anti-tumor immune cells and increased survival in pancreatic adenocarcinoma [10]. Therefore, it is critical to study angiogenesis not only in cell culture and in vivo animal models, but also in large human cohorts to assess its clinical relevance.

Utilizing the Gene Set Variation Analysis (GSVA) method to computationally analyze tumor transcriptomes to score them for activity of various biological processes, we have demonstrated the clinical relevance of multiple gene pathways [11-13]. We previously reported that in breast cancer patients, angiogenesis score was significantly associated with metastasis and inflammation [14]. The main advantage of scoring is that it is quantifiable using the gene expression data of the existing bulk tumors in large patient cohorts. In this study, we hypothesized that intra-tumoral angiogenesis promotes gastric cancer aggressiveness.

Materials and methods

Collection of transcriptomic and clinical data in gastric cancer patients

Transcriptome data of The Cancer Genome Atlas Stomach Adenocarcinoma project (TCGA-STAD, *n* = 375) was obtained through the Genomic Data Commons data portal (GDC). Clinical outcomes of the project's patients were from the curated and filtered Pan-Cancer Clinical Data Resource of survival endpoints [15]. Normalized genomic and clinical data published by Paik et al. (GSE26253, *n* = 432) [16], Lee et al. (GSE26901, *n* = 432) [17], and Yoon et al. (GSE84437, *n* = 432) [18] were procured by the Gene Expression Omnibus (GEO) repository. Gene expression was $\log_2$-transformed for all analyses.

Hallmark pathway enrichment analysis

Using our previously published methodology [14], Gene Set Variation Analysis (GSVA) Bioconductor package [19] was used to measure the "HALLMARK_ANGIOGENESIS" in the MSigDB Hallmark gene sets collection [20].

Gene set enrichment analysis

Gene Set Enrichment Analysis (GSEA) was performed using the GSEA Java software (version 4.0) with MSigDB Hallmark gene sets [21] to study the enrichment of signaling pathways between low- and high-angiogenesis score groups [22-27]. For statistical significance in GSEA analysis, we used the recommended false discovery rate (FDR) of less than 0.25.

Tumor immune microenvironment analysis

As we previously reported [29], we estimated the relative abundance of 64 types of immune and stromal cells in tumors using the xCell algorithm from their transcriptome profile [28].

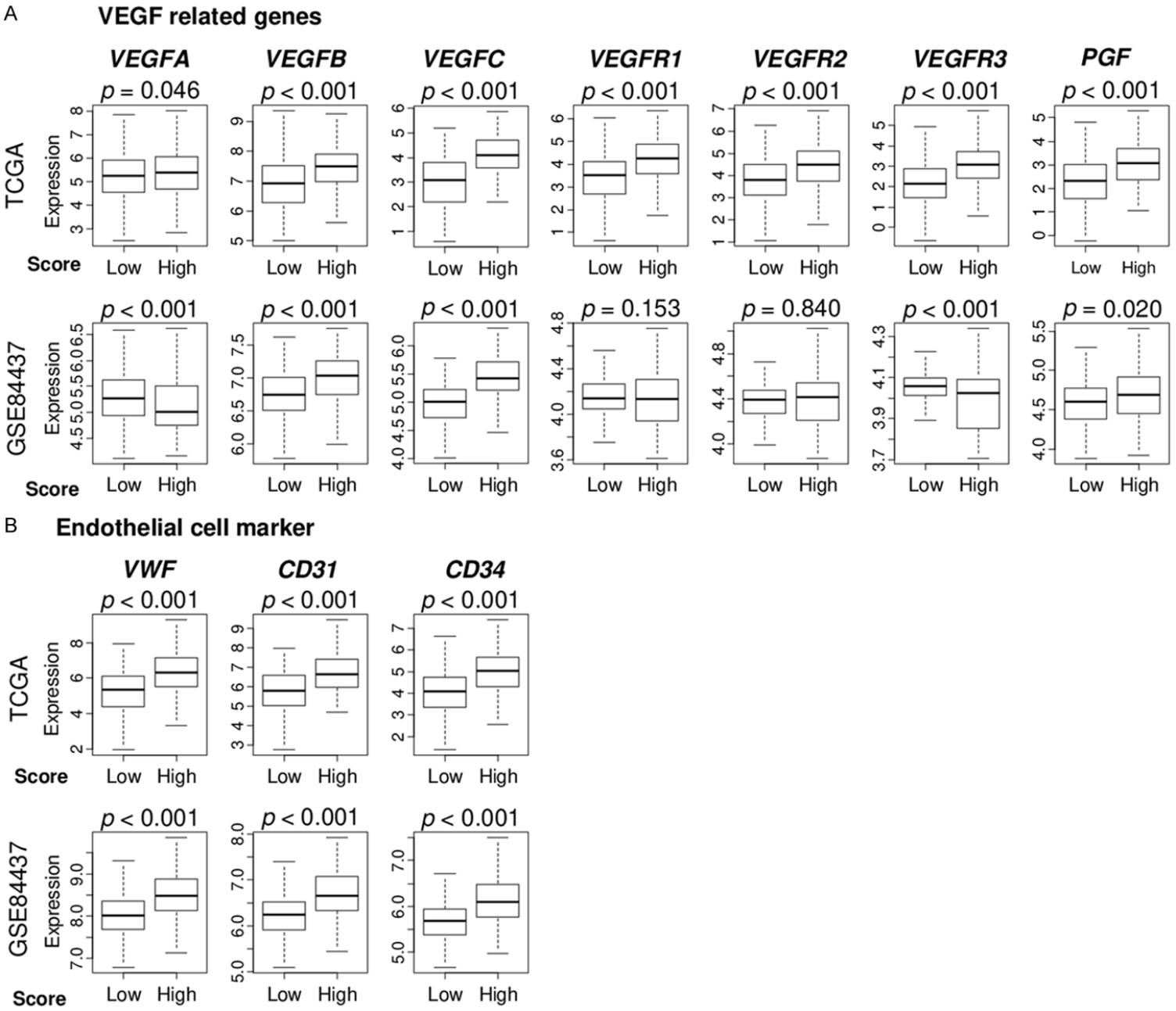
Other statistical analyses

R software (version 4.0.2) was utilized for all analyses. Boxplots that are used in the figures depict medians and interquartile range (IQR). *P* values for group comparisons were calculated by one-way ANOVA or Fisher's exact tests. The within-cohort median value was used to categorize the patients into low or high angiogenesis score groups. Survival curves were plotted using the Kaplan-Meier curve with log-rank test. Statistical significance was reached with *P*-values of less than 0.05.

Results

Angiogenesis-related genes are highly expressed in gastric cancers with high angiogenesis score

We calculated the angiogenesis score of tumors from 1,348 gastric cancer patients in multiple cohorts using the GSVA method we previously reported [14] to score tumor transcriptomes in the Hallmark angiogenesis gene set. First, to confirm that the angiogenesis score adequately captured intratumoral angiogenesis in gastric cancer, we examined the association between the score and multiple angiogenesis-related gene expression using the TCGA (*n* = 375) and GSE84437 (*n* = 432) gastric cancer cohorts. We used the median as a cutoff between the high vs low scores within cohorts because the angiogenesis score was distributed either normally or bimodally in the TCGA and GSE84437 cohorts (Figure S1). We chose *VEGF*-related (*VEGFA, VEGFB, VEGFR1, VEGFR2, and VEGFR3*), endothelial cell marker (*VWF and CD31*), and vascular stability-related (*ANGPT1, ANGPT2, TIE1, TIE2, VE-Cadherin, JAM2, and Claudin5*) genes as angiogenesis-related genes. All angiogenesis-related genes had increased expression in the high angiogenesis score group in the TCGA gastric cancer cohort (**Figure 1A-C**). These results were validated in the GSE84437


A
VEGF related genes
VEGFA
VEGFB
VEGFC
VEGFR1
VEGFR2
VEGFR3
PGF
p = 0.046
p < 0.001
p < 0.001
p < 0.001
p < 0.001
p < 0.001
p < 0.001
TCGA
GSE84437
Expression
Score
Low
High
p < 0.001
p < 0.001
p < 0.001
p = 0.153
p = 0.840
p < 0.001
p = 0.020
B
Endothelial cell marker
VWF
CD31
CD34
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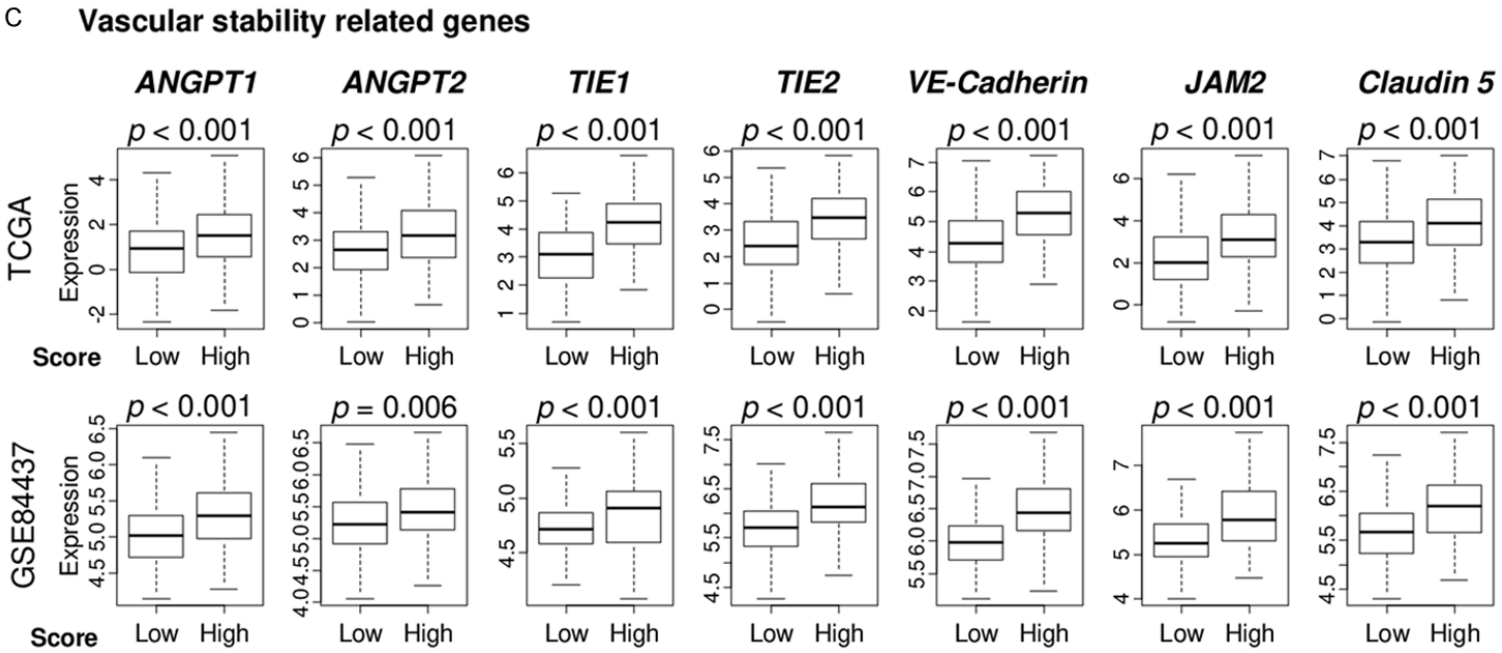

Figure 1. The angiogenesis score was associated with expressions of angiogenesis-related genes. Boxplots showing comparisons of the expression levels of (A) vascular endothelial growth factor (*VEGF*)-related genes, (B) endothelial cell marker-related genes; and (C) vascular stability-related genes, between low and high angiogenesis score groups in the TCGA and GSE84437 cohorts. We defined the VEGF-related genes as *VEGFA, VEGFB, VEGFC, VEGFR1 (FLT1), VEGFR2 (KDR), VEGFR3 (FLT4)*, and *PGF,* and the endothelial cell marker-related genes as *VWF*, *CD31 (PECAM1), and CD34,* and vascular stability-related genes as *ANGPT1, ANGPT2, TIE1, TIE2 (TEK), VE*-cadherin (*CDH5*), *JAM2* and *Claudin5 (CLDN5).*

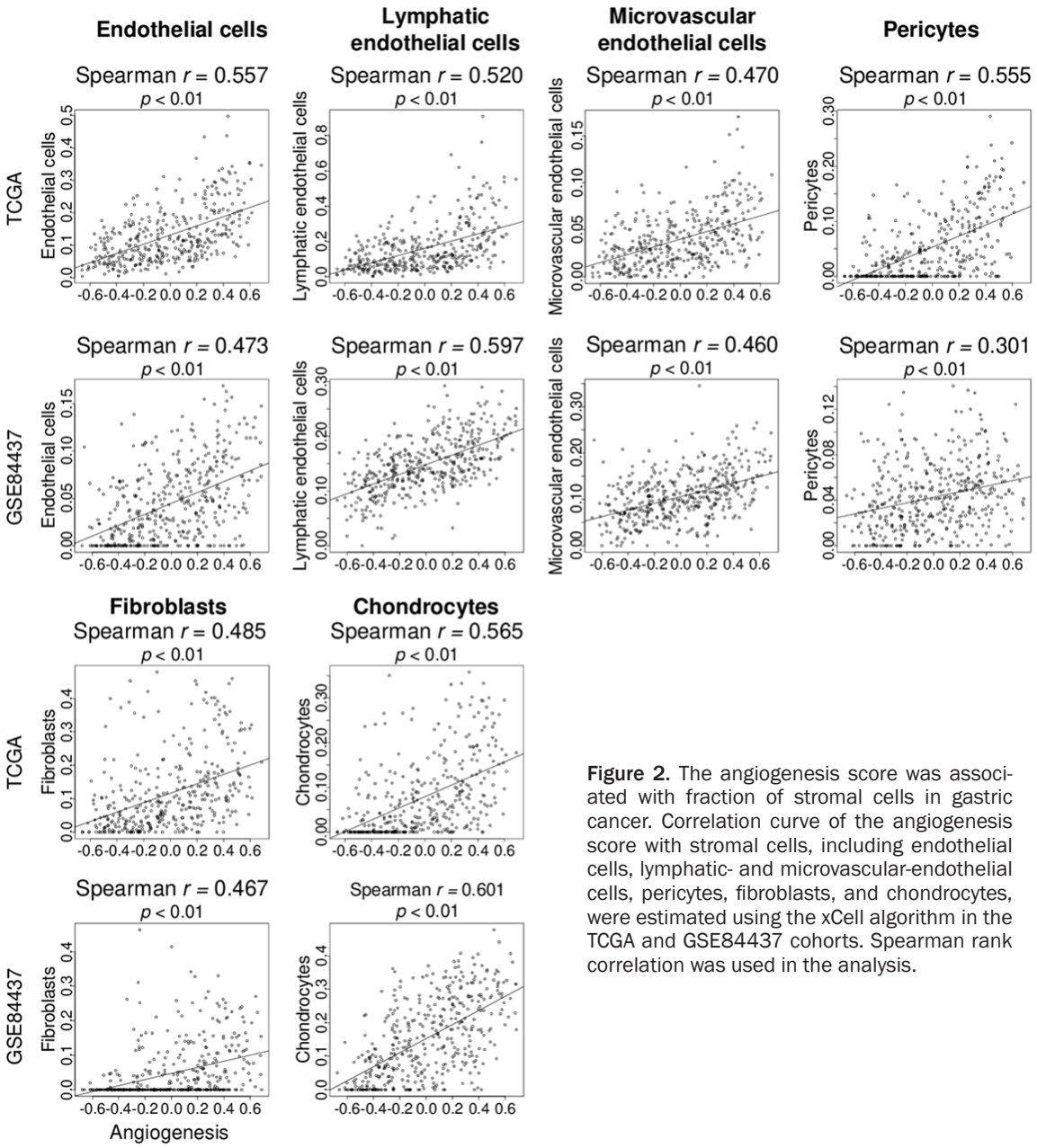

Figure 2. The angiogenesis score was associated with fraction of stromal cells in gastric cancer. Correlation curve of the angiogenesis score with stromal cells, including endothelial cells, lymphatic- and microvascular-endothelial cells, pericytes, fibroblasts, and chondrocytes, were estimated using the xCell algorithm in the TCGA and GSE84437 cohorts. Spearman rank correlation was used in the analysis.

cohort where most genes were highly expressed in the high angiogenesis score group except for *VEGFA, VEGFR1, VEGFR2* and *VEGFR3 genes.* There were no significant differences in the expression of *VEGFR1* and *VEGFR2* genes, and there was a decreased expression of *VEGFA* and *VEGFR3* genes in the high angiogenesis score group.

Angiogenesis score correlated with fibroblasts and chondrocytes in addition to epithelial cells and pericytes in gastric cancer

Both cancer and stromal cells in gastric cancer produce several angiogenic factors [30]. Thus, we examined the association of intratumoral angiogenesis with stromal cells. To determine the fraction of stromal cells in tumors, we used the xCell algorithm [28], which estimates the abundance of 64 cell subtypes in the tumor microenvironment (TME) from bulk tumor transcriptomic data [31-35]. The angiogenesis score significantly correlated with cells that constitute vessels, namely endothelial cells, lymphatic- and microvascular-endothelial cells, and pericytes as noted in both the TCGA and GSE84437 cohorts (**Figure 2**). Infiltration of other stromal cells, including fibroblasts and chondrocytes, correlated with angiogenesis in

gastric cancer. Furthermore, angiogenesis score correlated with fibroblast-related genes, αSMA (*ACTA2*) and FAPα (*FAP*) genes (Figure S2). These findings suggest tumors with a high angiogenesis score have a high fraction of not only vascular-related cells, but also fibroblasts and chondrocytes.

High angiogenesis score patients had low infiltration of T helper type 1 and 2 cells and high infiltration of dendritic cells and M1 macrophages

Based on the notion that the tumor immune microenvironment (TIME) is influenced by tumor angiogenesis, we studied the association between the angiogenesis score and TIME using the xCell algorithm in the TCGA and GSE84437 gastric cancer cohorts. High angiogenesis score tumors were infiltrated with a low fractions of type 1 T helper (Th1) and type 2 T helper (Th2) cells, but high fractions of dendritic cells (DC) and M1 macrophages consistently in both cohorts (**Figure 3**). High angiogenesis score tumors had infiltration with a low fractions of $CD8^+$ T cells, $CD4^+$ memory T cells, and a high fraction of M2 macrophages in the TCGA cohort, but this result was not validated in the GSE84437 cohort. Furthermore, we investigated the correlation of the angiogenesis score with inflammation-related scores (inflammatory response, TNF-α signaling, IL6/JAK/STAT3 signaling) and with immune response score (interferon (IFN)-γ response), which were calculated as the GSVA score (similarly to how the angiogenesis score was calculated). We found that angiogenesis was significantly and strongly correlated with inflammation-related pathways especially inflammatory response and IL6/STAT3 signaling, but not with immune response pathway (IFN-γ response) in both cohorts (Figure S3). This is consistent with the notion that inflammation promotes and immune response suppresses cancer.

High angiogenesis score tumors significantly enriched coagulation, epithelial mesenchymal transition (EMT), hypoxia, and transforming growth factor (TGF)-β signaling gene sets

To further understand the association between abundance of angiogenesis and gastric cancer aggressiveness, Gene Set Enrichment Analysis (GSEA) was used to perform pathway analysis with Hallmark gene sets in three large cohorts (TCGA, GSE84437, and GSE26253). In all three cohorts, high angiogenesis score tumors consistently and significantly enriched coagulation, epithelial mesenchymal transition (EMT), hypoxia, and transforming growth factor (*TGF)*-β signaling gene sets (**Figure 4**). High angiogenesis score tumors also significantly enriched KEGG extracellular matrix (ECM) receptor interaction gene set consistently in all cohorts, whereas none of the Hallmark defined metabolism-related gene sets (cholesterol homeostasis, fatty acid metabolism, glycolysis, oxidative phosphorylation, and xenobiotic metabolism) were enriched to angiogenesis (Figure S4).

Aggressive American Joint committee on cancer (AJCC) stage and worse survival in gastric cancer with high angiogenesis score

As we found that angiogenesis score was related to tumor aggressiveness, we wanted to see if the abundance of intratumoral angiogenesis was also associated with more aggressive disease in gastric cancer patients. We examined the relationship between the angiogenesis score and AJCC pathological stage and T- and N-categories using gastric cancer cohorts in the TCGA (n = 375), GSE26901 (n = 109), and GSE84437 (n = 432) cohorts. As we expected, abundance of angiogenesis was associated with higher AJCC stage in both TCGA and GSE26901 cohorts (**Figure 5A**; $P = 0.003$ and $P < 0.001$, respectively). The angiogenesis score also correlated with the T parameter in both TCGA and GSE84437 cohorts (both $P < 0.001$), but not with N parameter in the TCGA cohort.

Next, we studied how the angiogenesis score affects clinical outcome. Patients with high angiogenesis score gastric cancer had reduced overall survival in the TCGA and GSE84437 cohorts as well as worse disease-free survival in the GSE26253 (n = 432) cohort (**Figure 5B**; $P = 0.002$, $P < 0.001$, and $P = 0.011$, respectively). Our findings suggest that high compared to low angiogenesis score gastric cancer is clinically more aggressive and has worse survival.

Discussion

In this study, we used the angiogenesis score to study its association with clinical aggressiveness and as a potential biomarker in gastric cancer. Gastric cancer with high score highly expressed of multiple angiogenesis-related

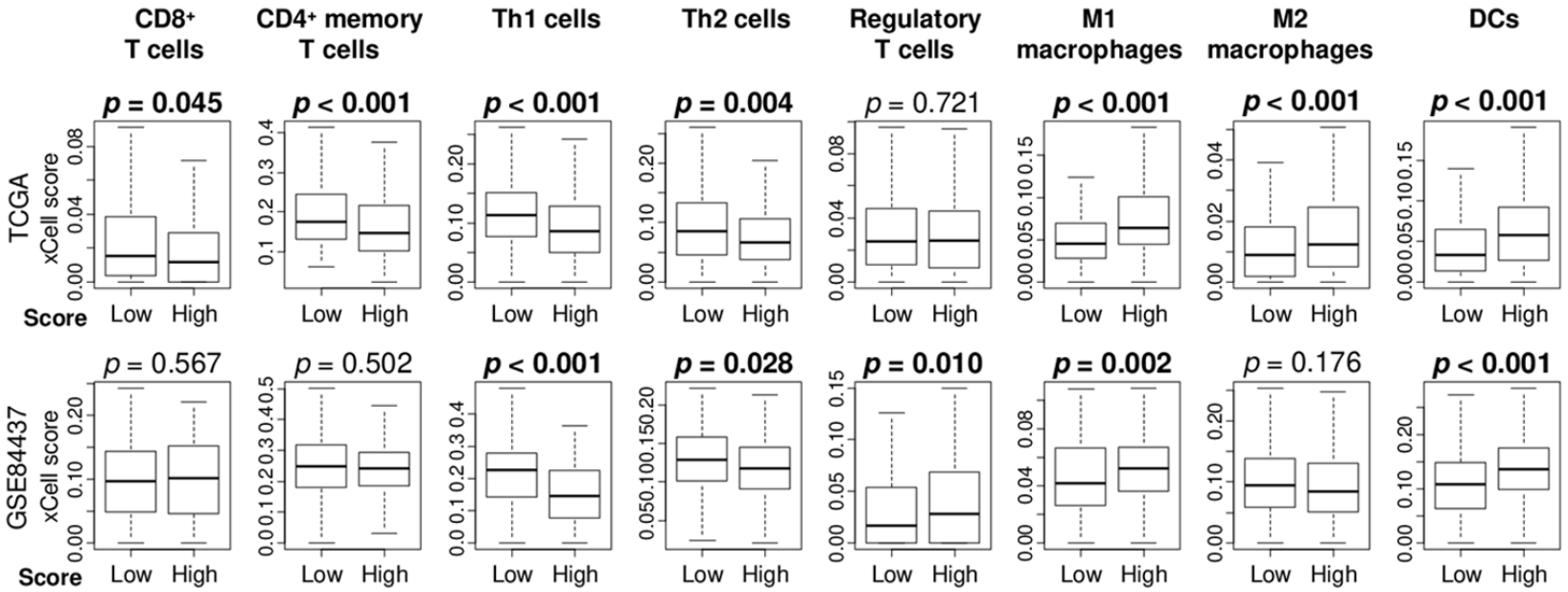

Figure 3. The angiogenesis score was associated with the fraction of immune cells in gastric cancer. Boxplots comparing immune cells including $CD8^+$ T cells, $CD4^+$ memory T cells, regulatory T cells, T helper cell type 1 (Th1), T helper cell type 2 (Th2) cells, M1 macrophages, M2 macrophages, and dendritic cells (DCs) by high and low angiogenesis score in TCGA and GSE84437 cohorts.

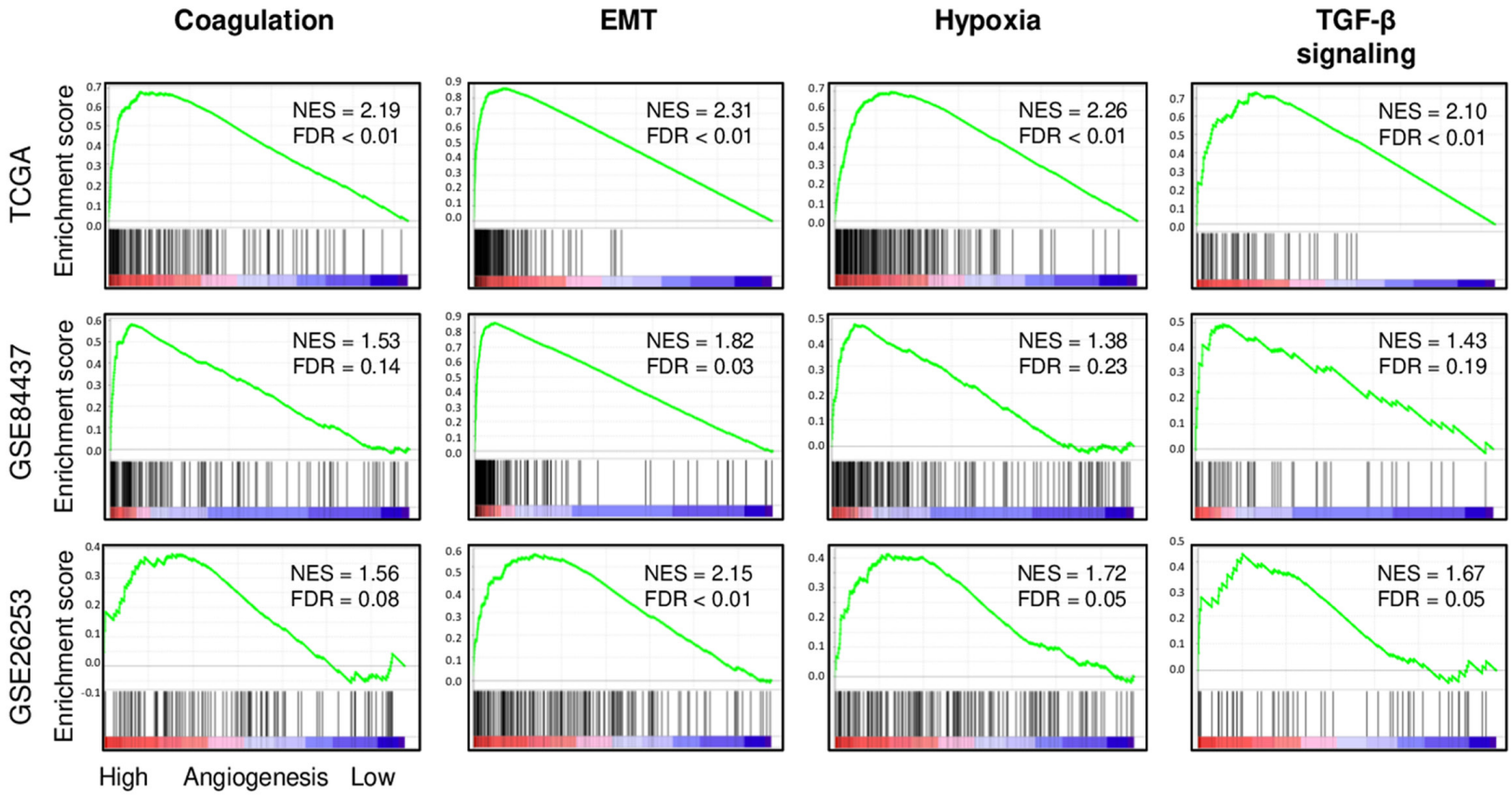

Figure 4. High angiogenesis score tumor groups enriched coagulation, epithelial mesenchymal transition (EMT), hypoxia, and transforming growth factor (TGF)-β signaling gene sets in the TCGA, GSE84437, and GSE26253 cohorts. Correlation curve of coagulation, epithelial mesenchymal transition (EMT), hypoxia, and *TGF*-β signaling gene sets with false discovery rate (FDR) and normalized enrichment score (NES) by gene set enrichment analysis.

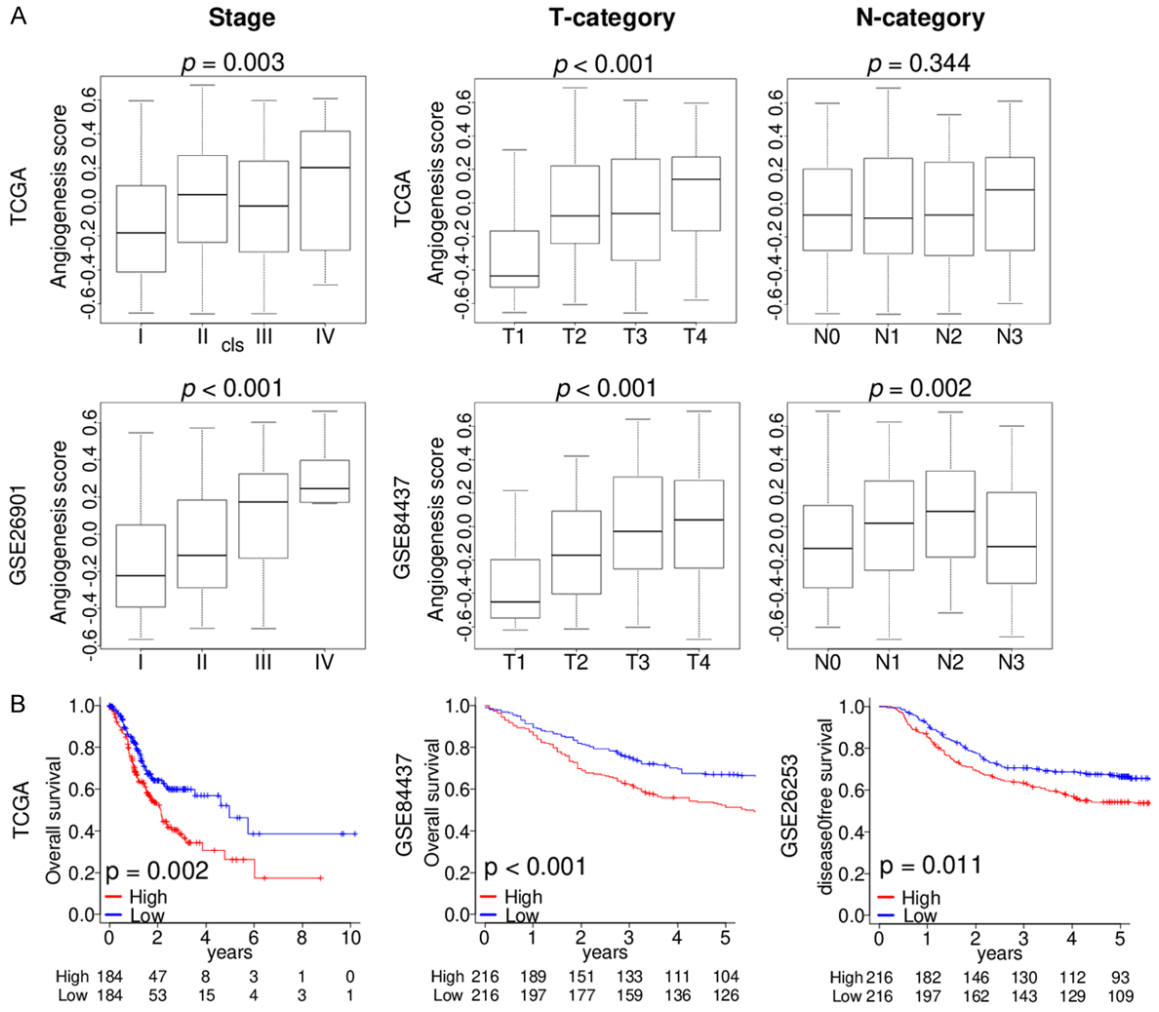

Figure 5. The angiogenesis score was associated with clinical cancer aggressiveness. Boxplots of the angiogenesis score by (A) AJCC stage, T- and N-category in the TCGA, GSE26901, and GSE84437 cohorts. (B) Kaplan-Meier curves of OS in the TCGA (n = 368) and GSE84437 (n = 432) cohorts, and DFS in the GSE26253 (n = 432) cohort of angiogenesis score high (red) and low (blue) in gastric cancer. AJCC, American Joint Committee on Cancer; disease-free survival, DFS; overall survival, OS.

genes including *VEGF*-related, endothelial cell marker-related, and vascular stability-related genes. Furthermore, the angiogenesis score was moderately correlated with not only vessel-related stromal cells, but also fibroblasts and chondrocytes in the TME. Gastric cancer with high angiogenesis score was significantly associated with a lower infiltration of Th1, Th2 cells, and DCs, and a higher infiltration of M1 macrophages compared with low angiogenesis score tumors. Gastric cancer with a high angiogenesis score was enriched with coagulation, epithelial mesenchymal transition (EMT), hypoxia, and *TGF*-β signaling gene sets. Interestingly, a high angiogenesis score was significantly associated with a higher AJCC stage and T-category, but not N-category, and also associated with shorter survival.

To quantify the activity of angiogenesis pathway, we defined the angiogenesis pathway score based on the gene set variation analysis (GSVA) [19] score of the Molecular Signatures Database (MSigDB) collection [20], similar to how we previously did in breast cancer [12, 36, 37]. The hallmark gene sets were determined by Liberzon et al. in Cell systems [20] using computational biological methods that identified overlaps between the other gene sets in the MSigDB and retained genes that display coordinate expression. The hallmark gene sets are widely accepted and recognized as repre-

senting specific and representing specific and distinct biological functions and exhibiting consistent expression including angiogenesis. The Hallmark angiogenesis gene set is constructed of 36 genes. Biological processes such as angiogenesis is regulated by multiple genes acting in concert. Hence, using multiple genes in a shared pathway to score gene expression is a more comprehensive approach than using individual genes. Employing gene sets allows consideration of gene regulation, decreased model complexity, and greater explanatory power of prediction models. For these reasons, results were obtained for cancer biology such as survival and gene enrichment analysis in independently different cohorts, although the relationship between expressions of certain single genes, such as *VEGFA* and *VGFR3*, and angiogenesis score was not always consistent between cohorts.

Increased deposition and remodeling of the extracellular matrix with collagen crosslinking drive fibrosis in gastric cancer. Fibrosis leads to stromal stiffening which in turn promotes tumor growth, migration, and mesenchymal transition [38, 39]. A dense extracellular matrix leads to poor vascularization and induces hypoxia which hinders drug delivery. In addition, a stiffer stroma and abundance of fibrosis is associated with more aggressive tumor and worse prognosis. In agreement, we found strong correlation between angiogenesis and fibroblast-related genes (αSMA (*ACTA2*) and FAPα (*FAP*)). Angiogenesis occurs when tumor and normal cells produce an imbalance of positive and negative angiogenic factors into the TME [30]. Various angiogenic factors, including *VEGF* and *EGF,* are released by cancer and stromal cells in gastric cancers [8, 9]. Hence tumors with increased tissue fibrosis and stromal stiffness are more aggressive and linked with worse prognosis [40].

We demonstrated that the intratumoral angiogenesis score is correlated with fraction of fibroblasts and chondrocytes using the gene expression profile of patient cohorts. Therefore, their mechanistic roles in gastric cancer need to be elucidated by in vitro or in vivo studies.

In this study, we demonstrated that the high angiogenesis score tumor was enriched with coagulation, hypoxia, EMT, and *TGF*-β signaling. Our analyses did not provide information on causality; however, we speculate that elevated coagulation levels may worsen hypoxia in TME and subsequently promote angiogenesis. On the other hand, our results may implicate that high angiogenesis gastric cancer has enhanced EMT and *TGF*-β signaling, which are both known to worsen cancer progression and metastasis [41]. Weidner et al. [42] reported that the number and density of blood vessels correlated with metastatic frequency in invasive breast cancer. Similar observations have been obtained for gastrointestinal tumors [43, 44]. We previously reported that in breast cancer, the angiogenesis score was not associated with survival [14]; however, in this study, we found that gastric cancer patients with a high angiogenesis score had worse OS and DFS. This may demonstrate the differences in clinical relevance of angiogenesis across cancer types.

Both TIME and tumor-associated macrophages (TAMs) play important roles in gastric cancer progression [45]. TAMs arise from monocytes circulating in the TME in response to chemoattractants. TAMs interact with cancer cells and play a major role in angiogenesis and tumor vascularity [46] and is thus associated with gastric cancer progression as related to depth of tumor invasion, metastasis to lymph nodes and clinical stage [47]. In this study, the fraction of infiltrated cells in the TME was quantified using the xCell algorithm. xCell algorithm allows the estimation of 64 types of immune and stromal cells in the TME from global gene expression data and is widely used throughout the world. We used the algorithm to demonstrate the clinical relevance of $CD8^+$ T cells [35], regulatory T cells [29], and dendritic cells [31] in breast cancer. In this study, M1 macrophages were highly infiltrated in tumors with high angiogenesis score, whereas M2 macrophages were not. M1 macrophages are usually associated with anti-cancer activities, which is somewhat puzzling given our finding indicating worse survival in high angiogenesis gastric cancer. We believe this seemingly contradictory result is due to how M1 macrophage has been defined transcriptomically. In our current study, we used xCell algorithm to estimate the fraction of immune cells including M1 macrophages. In our previous study in breast cancer patients, we reported that high infiltration of M1 macrophages defined by xCell was associated with

increased cancer aggressiveness and worse survival [48]. In other words, M1 macrophages defined by xCell are not associated with better survival. Although xCell algorithm is a useful tool, its limitation is that it can only compare scores between samples within the same cell type, but not between cell types due to the way it is standardized. To this end, the number of different types of cells including macrophages cannot be compared using xCell.

Furthermore, we found that the angiogenesis score was highly correlated with inflammation-related scores, including inflammatory response, TNF-α signaling, and IL6/JAK/STAT3 signaling score, but not with IFN-γ response score that reflects immune response pathway. This is consistent with the notion that angiogenesis is associated with inflammation that promotes cancer progression and not with immune response that suppresses it; thus angiogenesis is associated with poor prognosis.

Robust use of sequencing technology and mandated storage of genomic information in the public domain allow for a massive number of translational studies by many investigators including our group. Indeed, research on cancer-associated stromal cells as well as immune cells using transcriptome of a bulky tumor has become common [28, 49, 50]. In this current study, we demonstrated several associations between angiogenesis and clinical relevance as well as biological features. We consistently found that the angiogenesis score was a negative prognostic biomarker for gastric cancer survival in multiple large patient cohorts.

There are several limitations to this study. First, this is a retrospective study using multiple large cohorts, and the angiogenesis pathway score cannot be rigorously established as a prognostic biomarker without a prospective study. Second, detailed information on systemic treatment, which would have affected survival outcomes, was unavailable for all analyzed cohorts. Third, our study lacked experimental data demonstrating the mechanisms in which tumor angiogenesis contributes to tumor features and outcomes in gastric cancer.

In conclusion, the angiogenesis score can be a useful tool to assess intratumoral angiogenesis, which has clinical consequences in gastric cancer patients.

Acknowledgements

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Disclosure of conflict of interest

None.

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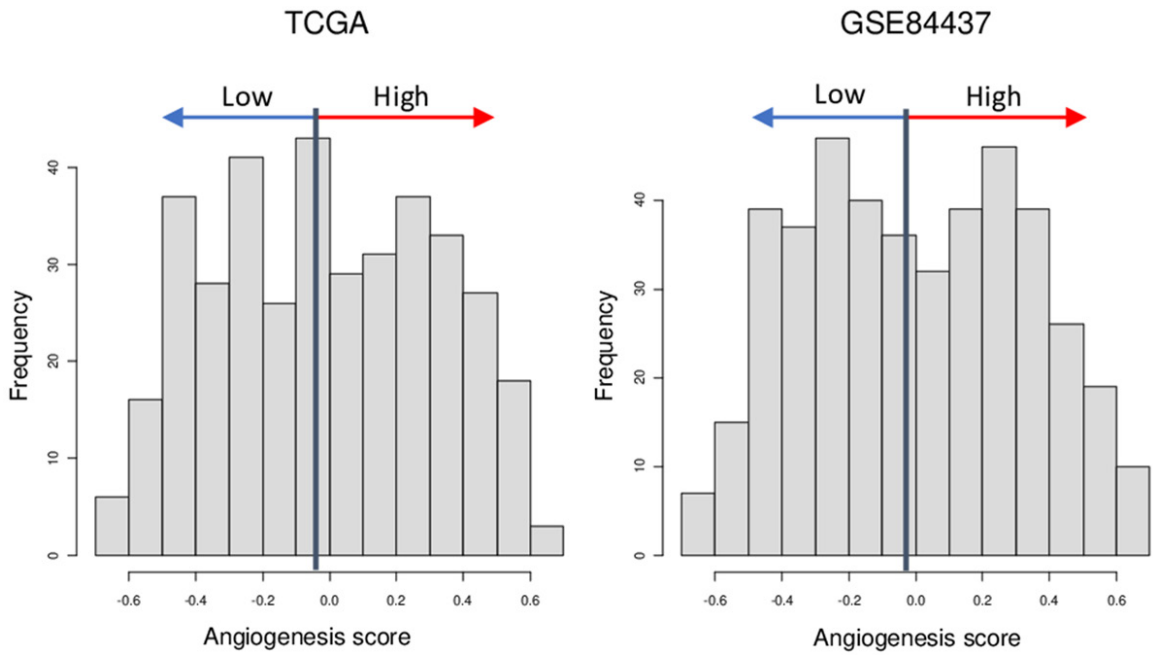

Figure S1. Histogram of the angiogenesis score in the TCGA and GSE84437 cohorts.

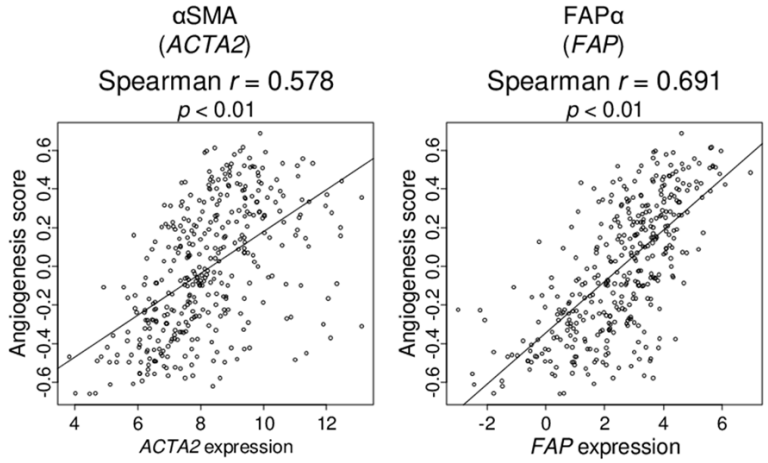

Figure S2. Correlation of the angiogenesis score with fibroblast-related genes in the TCGA cohort. Correlation plots of the angiogenesis score with *ACTA2* and *FAP* gene expression. Spearman rank correlation was used to the analysis.

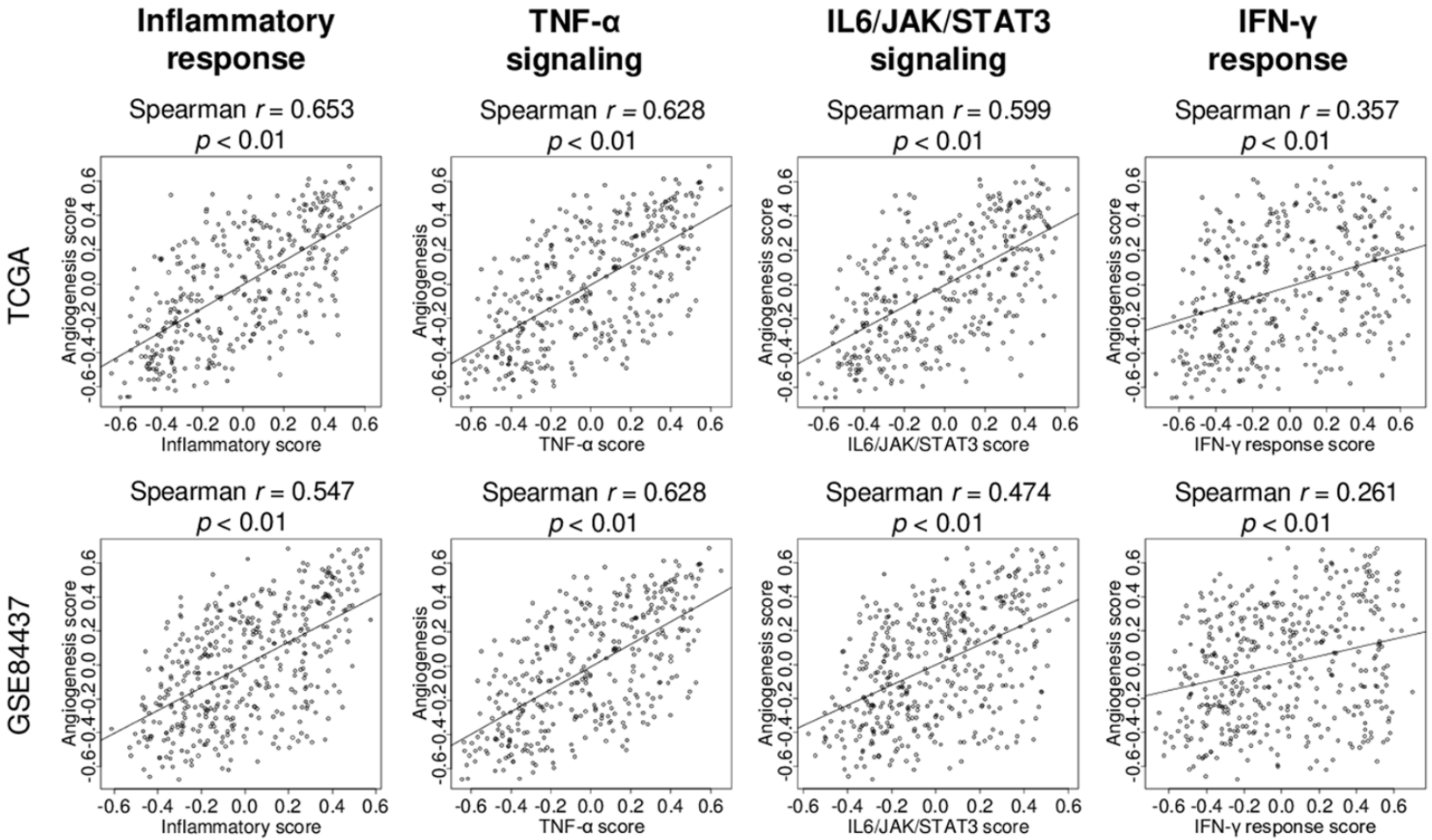

Figure S3. Correlation of the angiogenesis score with inflammation-related and immune response-related pathway scores in the TCGA and GSE84437 cohorts. Correlation curves of the angiogenesis score with inflammation-related pathway scores; inflammatory response, TNF-α signaling, IL6/JAK/STAT3 signaling, and immune response-related pathway score; Interferon (IFN)-γ response score in both cohorts. Spearman rank correlation was used to the analysis.

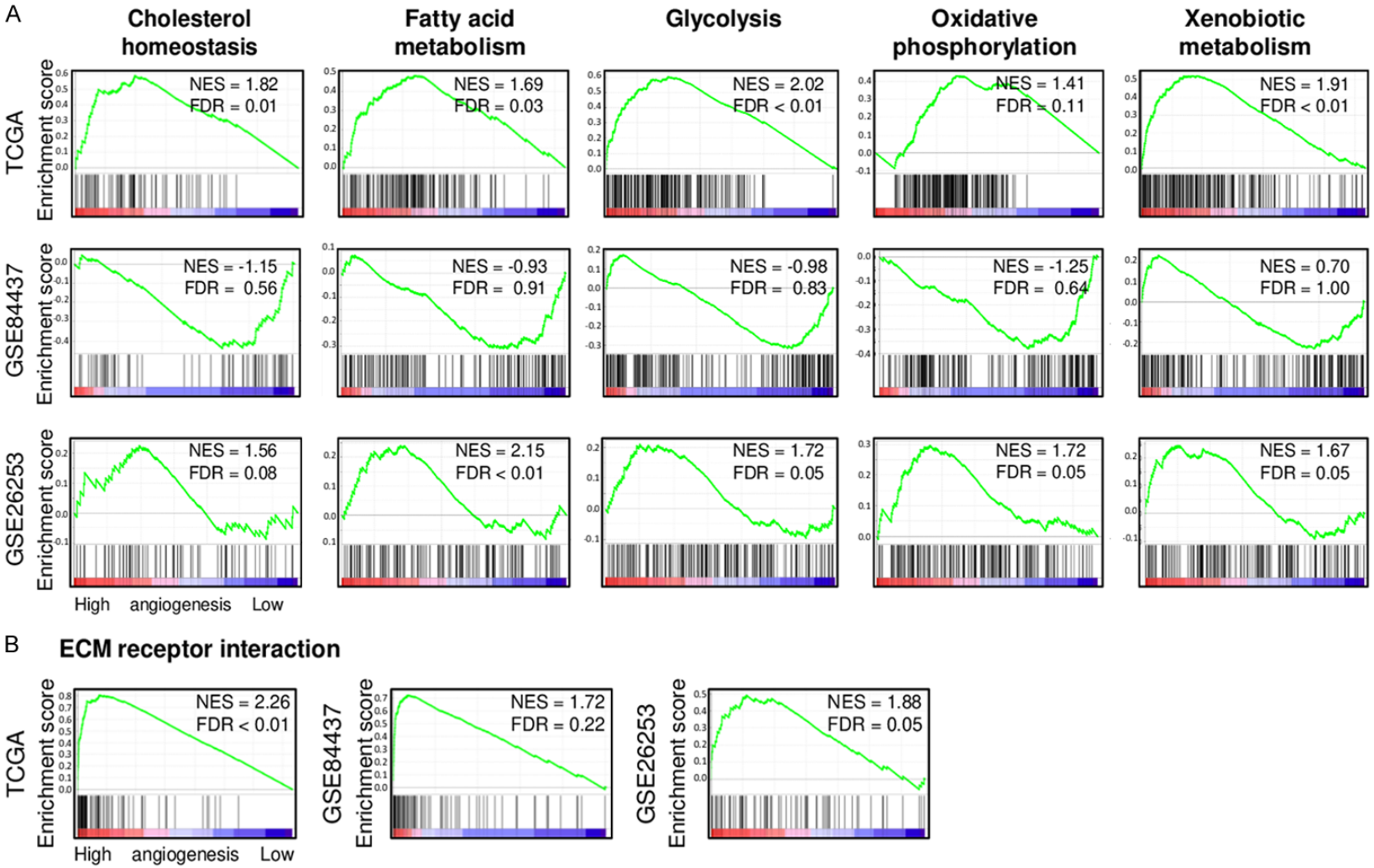

Figure S4. Gene Set Enrichment Analysis comparing high and low angiogenesis score tumors with metabolism-related Hallmark gene sets and KEGG extracellular matrix (ECM) receptor interaction gene set in the TCGA, GSE84437, and GSE26253 cohorts. Correlation curve of (A). cholesterol homeostasis, fatty acid metabolism, glycolysis, oxidative phosphorylation, and xenobiotic metabolism hallmark gene sets. (B) ECM receptor interaction KEGG gene set. FDR; false discovery rate, NES; normalized enrichment score.