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Original Article

World J Oncol. 2025;16(2):173-181

Angiogenesis Is Associated With Aggressive Biology That Counterbalances With Tumor Immunogenicity in Hepatocellular Carcinoma

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Abstract

Background: Hepatocellular carcinoma (HCC) is an arterialized tumor; thus, anti-angiogenesis targeted therapy is in clinical practice. Herein, we hypothesized that HCC with high angiogenesis is biologically aggressive with worse survival.

Methods: Angiogenesis score (AS) was derived from the Molecular Signatures Database (MSigDB) Hallmark Angiogenesis Gene Set, and median was used to divide high versus low groups. Transcriptome of HCC patients of The Cancer Genome Atlas (TCGA, n = 386) and GSE76427 (n = 115) cohorts were analyzed.

Results: High AS correlated with angiogenesis-related gene expressions. Both microvascular and lymphatic endothelial cell infiltrations were higher in high angiogenesis HCC. Surprisingly, no survival difference was seen with varying levels of angiogenesis. High angiogenesis significantly enriched tumor aggravating signaling pathways: glycolysis, Notch, Hedgehog, KRAS, epithelial mesenchymal transition, and transforming growth factor-beta (TGF-β) in Gene Set Enrichment Analysis (GSEA), but also infiltrated less $CD8^+$ T cells and T-helper 1 cells, and higher M1 macrophages and conventional dendritic cells (cDCs) with elevated cytolytic activity score in both cohorts. In agreement, immune response-related gene sets: inflammatory response, tumor necrosis factor-alpha (TNF-α) signaling, allograft rejection, interferon-alpha, and interferon-gamma were all enriched to high angiogenesis HCC. Programmed cell death protein 1 (PD1), programmed death ligand 1 (PD-L1), programmed death ligand 2 (PD-L2), and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) were higher in high angiogenesis HCC in TCGA, but not in GSE76427 cohort.

Conclusions: Angiogenesis quantified using transcriptome of HCC patients demonstrated that it is associated with aggressive biology but also with tumor immunogenicity and immune response that counterbalance and did not reflect in survival. Given high expression of immune checkpoint molecules, we cannot help but speculate that immunotherapy may be useful for high angiogenesis HCC patients.

Keywords: Hepatocellular carcinoma; Angiogenesis; Immunogenicity

Manuscript submitted December 9, 2024, accepted January 17, 2025
Published online January 25, 2025

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doi: https://doi.org/10.14740/wjon2009

Introduction

Despite the recent advances in hepatocellular carcinoma (HCC) treatment, mortality remains high making HCC the third leading cause of cancer-deaths worldwide [1]. In early-stage disease, resection remains the cornerstone of treatment, but for those with advanced or recurrent disease, therapeutic options become more limited. With the recent burgeoning of immunotherapy, the potential for a more efficacious treatment paradigm in HCC is promising. Yet the complex physiological role of the liver makes understanding the interplay between angiogenesis and the host immune response paramount to efficient patient selection and treatment application.

HCC is known to develop in a multinodular fashion within inflamed parenchyma and accounts for 90% of malignant tumors found in the liver [2]. It tends to display a relatively arterialized, angiogenic phenotype with the tumor relying on new blood vessel formation for establishment and persistence within the liver parenchyma [3, 4]. This mechanism has served as the basis for traditional anti-angiogenic therapy including systemic agents that target the vascular endothelial growth factor (VEGF) pathway and for direct action modalities such as transcatheter arterial chemoembolization (TACE). Further, the

liver is a key actor in host immune response and acts as a vast repository of immune cells and producers of pro-inflammatory cytokines, molecules, and proteins. The close relationship between a tumor's blood supply and the host's immune response has been demonstrated across tumor types and understanding this relationship using a large human cohort is critical to designing the next phase of HCC treatment [5].

Recent advance in bioinformatic analyses allows us to elucidate the clinical relevance of gene expressions [6-8] or cell infiltration [9-12] in cancer using transcriptomics. Genes Set Variation Analysis (GSVA) has been used to investigate a host of biological pathways as a way to understand the cumulative expression of genes (gene sets) as they apply to host effector function [12-19]. Using this methodology, an angiogenesis pathway score has quantified this exceedingly intricate physiological function as it takes places within the distinctly aberrant setting of HCC tumor establishment in a large cohort of human patients. In this investigation, angiogenesis is hypothesized to be a key driver of HCC immunogenicity and immune checkpoint expression.

Materials and Methods

HCC cohort

The Cancer Genome Atlas (TCGA) clinical and RNA-sequencing data for 386 patients with HCC were downloaded via the cBioportal as we described previously [4, 6, 9, 19, 20]. An additional validation cohort of 115 HCC patients (GSE76427) with clinical and RNA-sequencing data was acquired from the National Center for Biotechnology (NCBI) Gene Expression Omnibus (GEO). IRB exemption was granted from the Institutional Review Board at Roswell Park Comprehensive Cancer Center given the publicly available and de-identified nature of the data sets.

Gene Set Enrichment Analysis (GSEA) and GSVA

The Molecular Signatures Database (MSigDB) hallmark annotated gene set for angiogenesis (HALLMARK_ANGIOGENESIS) was used to derive the angiogenesis score (AS) as previously described [21, 22]. GSEA was carried out utilizing software from a joint project of UC San Diego and the Broad Institute [23]. Patients were stratified into high versus low score cohorts using a median AS value. It is known that receiver operating characteristic (ROC) curve is commonly used as an optimal cutoff to maximize the difference below and above the cutoff; however, we chose median as the cutoff because our objective of this study was not to report that our AS as a prognostic biomarker, but rather to investigate the biological differences between HCC with high vs. low angiogenesis. Stratification of AS based on median value is simple and reproducible. Its use as cutoff is also independent of clinical outcome, avoiding potential biases in selecting a cutoff based on specific dataset being analyzed. Its non-dependent nature allows better comparability and generalizability between different cohorts. A false discovery rate (FDR) of < 0.25 was deemed significant [24].

Cellular infiltration analysis and cytolytic activity (CYT) score

A gene signature-based method, xCell [25], was used to estimate the infiltration patterns of 64 immune and stromal cell types [26-31]. The immune CYT score was defined as a quantitative measure based on transcript levels of granzyme A (GZMA) and perforin (PRF1) [32].

Statistical analysis

The Kaplan-Meier method with the log-rank test was used to compare overall survival (OS) and disease-free survival (DFS) between the high and low AS groups. $P < 0.05$ was considered significant and all analysis was carried out using R [33] and Bioconductor [34].

Results

Elevated AS is associated with increased expression of angiogenesis-related genes

To investigate if the AS can serve as a reasonable proxy for intra-tumoral vasculogenic activity, we first examined the gene expression of known angiogenesis-related proteins (Fig. 1). In both the TCGA and GSE76427 cohorts, those tumors with high AS also had increased expression of vascular endothelial growth factor (VEGFC). Additionally, high AS tumors also demonstrated elevated endothelial cell markers: platelet endothelial cell adhesion molecule (PECAM-1, CD31) and von Willebrand factor (VWF). Finally, higher levels of vascular stability genes including tyrosine kinase with immunoglobulin-like and epidermal growth factor-like domains 1 and 2 (TIE1 and 2), angiopoietin 1 (ANGPT1), vascular endothelial cadherin (CD144, VE-cadherin), Claudin5 (CLDN5) and junction adhesion molecule 2 (JAM2) were found in tumors with high AS. The expression of these proteins was distinct from the genes which were used to derive the "HALLMARK_ANGIOGENESIS" annotated gene set, lending credence to the viability of the AS as a metric of intra-tumoral angiogenesis.

Next, the structural foundation of angiogenesis was investigated. Using the gene set derived infiltration signatures from the xCell method, both microvascular endothelial cell (MEC) and lymphatic endothelial cell (LEC) expression levels were measured in both the TCGA and GSE76427 cohorts. MEC and LEC infiltration were found to be significantly increased in tumors which had high AS (Fig. 2).

AS does not augment patient survival

In advanced, non-operable HCC, antiangiogenic therapy

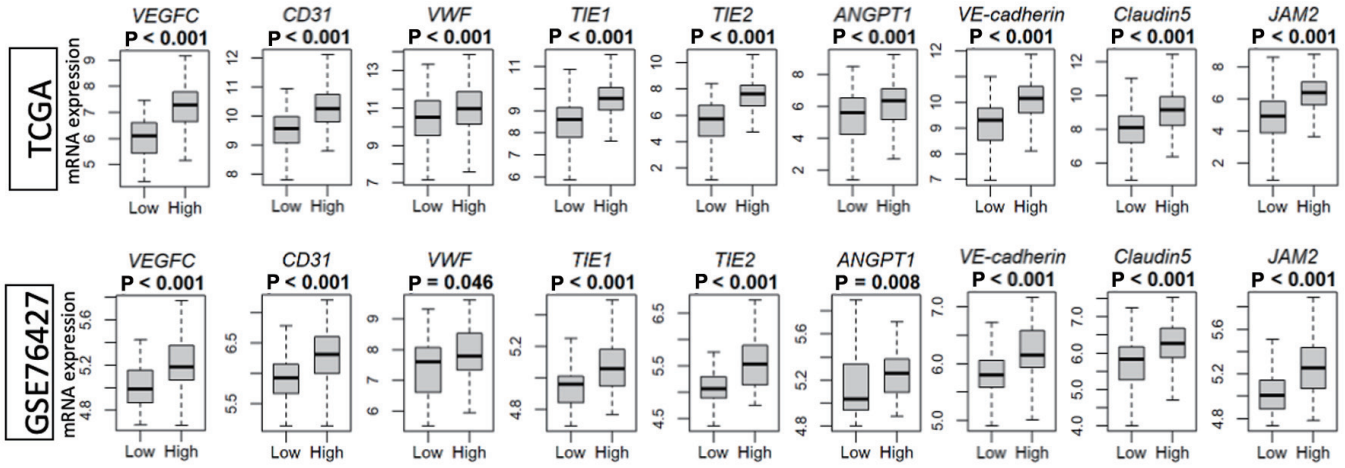

Figure 1. Intra-tumoral angiogenesis score correlates with the expression of VEGF (VEGFC)-, endothelial cell marker (CD31 and VWF)-, and vascular stability (TIE1 and 2, ANGPT1, VE-cadherin, CLDN5 and JAM2)-related genes in the TCGA and GSE76427 cohorts. High versus low, median angiogenesis pathway score. ANGPT1: angiopoietin 1; CD31/PECAM-1: platelet endothelial cell adhesion molecule; CLDN5: Claudin5; JAM2: junction adhesion molecule 2; TIE1 and 2: tyrosine kinase with immunoglobulin-like and epidermal growth factor-like domains 1 and 2; VE-cadherin/CD144: vascular endothelial cadherin; VEGF: vascular endothelial growth factor; VWF: von Willebrand factor.

forms a cornerstone of therapeutic approach. Mechanistically, aberrant and uncontrolled angiogenesis contributes to tumor sustenance and thus it could stand to reason that those tumors with more angiogenesis may indeed have worsened outcomes. To investigate this, the survival outcomes of HCC patients were examined (Fig. 3). No differences were appreciated between patients who had tumors with high versus low AS in terms of DFS and OS. However, early in the course of HCC within 3 years of resection, there appears to be an interval during which patients whose tumors had a higher AS did demonstrate a survival benefit that eventually abrogated as time progressed.

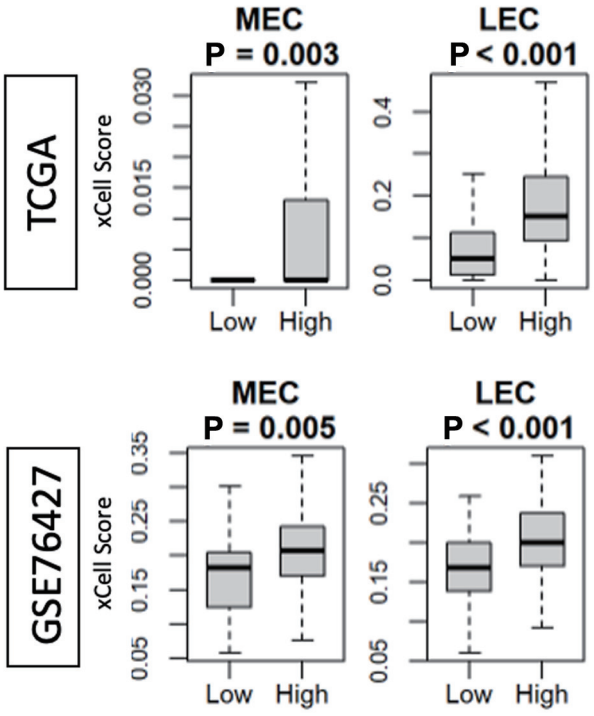

Figure 2. Intra-tumoral angiogenesis score correlates with the presence of MEC and LEC in the TCGA and GSE76427 cohorts. High versus low, median angiogenesis pathway score. MEC: microvascular endothelial cell; LEC: lymphatic endothelial cell.

A high AS significantly enriches pro-tumor gene sets

To further investigate the mechanistic role of angiogenesis in HCC, GSEA was performed to examine cellular pathway in both the TCGA and GSE76427 cohorts (Fig. 4). Tumors with a high AS enriched glycolysis and epithelial mesenchymal transition (EMT) gene sets. Additionally high AS tumors also significantly enriched Notch, Hedgehog, KRAS, and transforming growth factor-beta (TGF-β) signaling gene sets.

High angiogenesis HCC is associated with less lymphocytes and higher myeloid cells, and summative CYT score is significantly elevated

As tumor angiogenesis is not the sole drive of progression, we sought to examine one other major contributor to tumor persistence: the host immune response. To investigate the impact of AS on the tumor microenvironment (TME), the cellular infiltration patterns of tumors were examined using the xCell algorithm in both TCGA and GSE cohorts. Interestingly, the infiltration patterns were variable in terms of a pro- or anti-tumor environment (Fig. 5a). $CD8^+$ lymphocytes and T-helper 1 (Th1) cells were more prominent in tumors with low AS. Additionally macrophages, specifically M1 type, and conventional dendritic cells (cDC) were prevalent in tumors with high

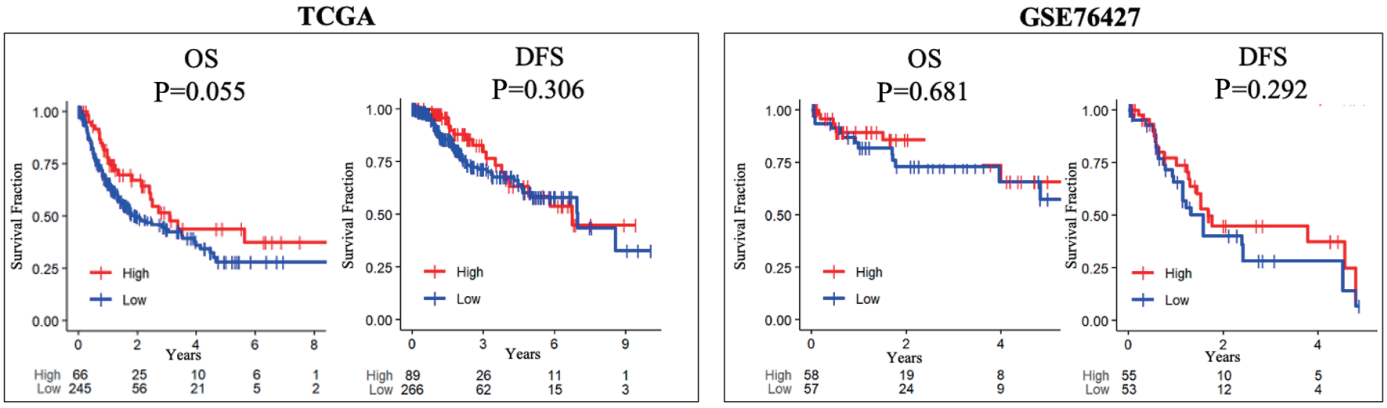

Figure 3. Intra-tumoral angiogenesis score does not correlate with OS or DFS in either the TCGA and GSE76427 cohorts. High versus low, median angiogenesis pathway score. DFS: disease-free survival; OS: overall survival.

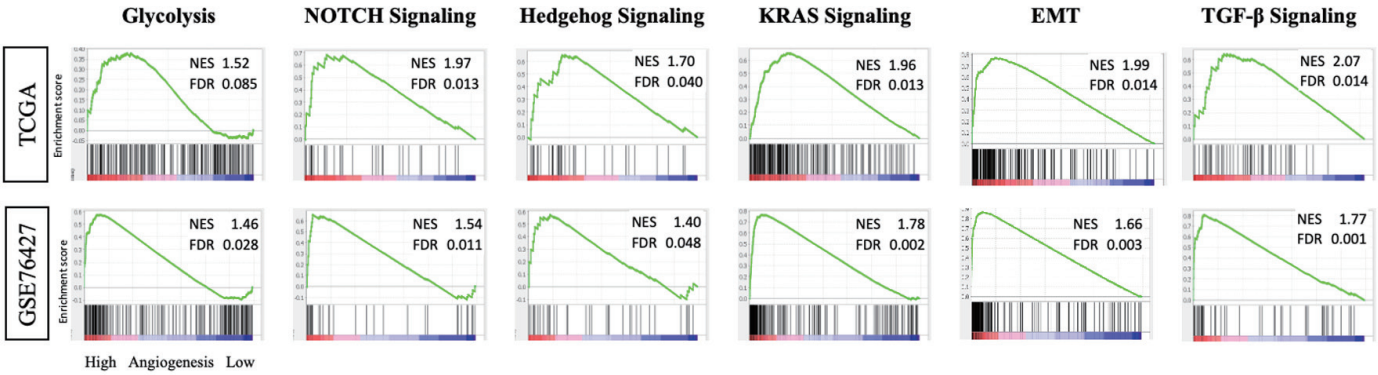

Figure 4. Tumors with high intra-tumoral angiogenesis score demonstrate enrichment of pro-tumor gene sets in the TCGA and GSE76427 cohorts. GSEA with NES and FDR for glycolysis, Notch signaling, Hedgehog signaling, KRAS signaling, EMT, and TGF-β signaling. EMT: epithelial mesenchymal transition; FDR: false discovery rate; GSEA: Gene Set Enrichment Analysis; NES: normalized enrichment score; TGF-β: transforming growth factor-beta.

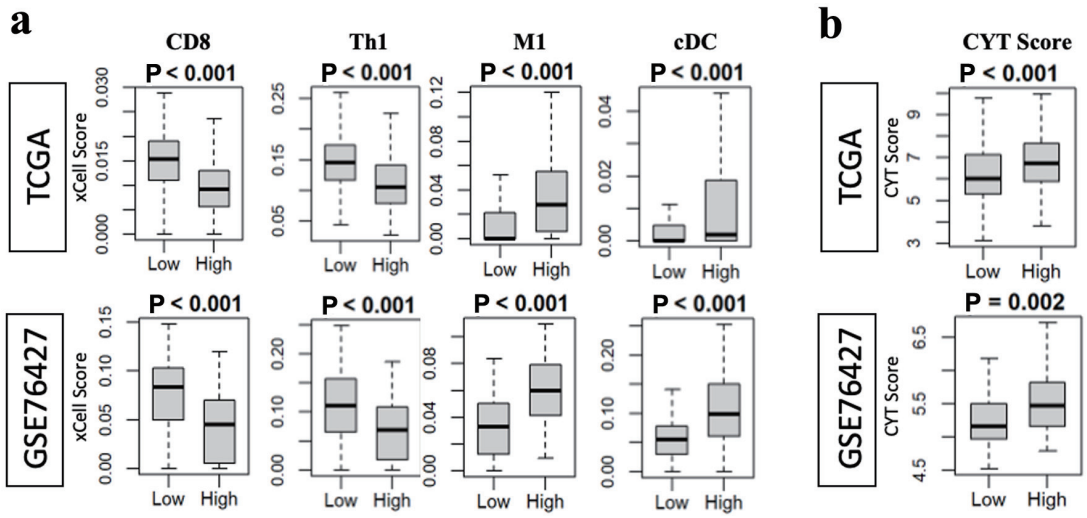

Figure 5. High angiogenesis score is associated with increased CYT in both the TCGA and GSE76427 cohorts, while pro-and anti-tumor infiltrating immune cells showed variable presence. (a) CD8 and Th1 infiltration was associated with low angiogenesis score while M1 macrophages and cDC infiltration was associated with high angiogenesis score. (b) CYT score was correlated with tumors which had high angiogenesis score. cDC: conventional dendritic cell; CYT: cytolytic activity.

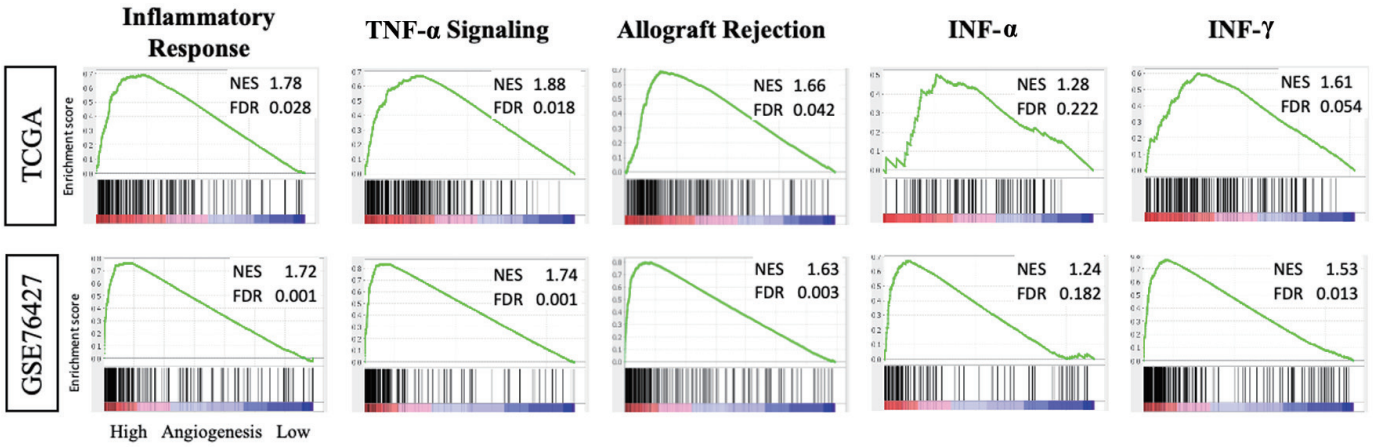

Figure 6. Tumors with high intra-tumoral angiogenesis score demonstrate enrichment of inflammatory response gene sets in the TCGA and GSE76427 cohorts. GSEA with NES and FDR for inflammatory response, TNF-α, allograft rejection, INF-α and γ. FDR: false discovery rate; GSEA: Gene Set Enrichment Analysis; INF-α and γ: interferon-alpha and gamma; NES: normalized enrichment score; TNF-α: tumor necrosis factor-alpha.

AS. In order to elucidate the cumulative effect of this cellular infiltration pattern, we calculated the CYT score based on the quantitative measure of transcript levels of GZMA and PRF1. Tumors with high AS had a higher CYT score in both cohorts (Fig. 5b).

High angiogenesis tumors enrich immune response-related gene sets

Since the CYT score was elevated in tumors with high AS, then the adjunct cellular pathways associated with increased reactionary inflammation should also show a similar pattern. GSEA was applied to tumors with high AS and inflammatory response gene sets were examined (Fig. 6). Signaling pathways associated with tumor necrosis factor-alpha (TNF-α) and allograft rejection were enriched in both cohorts. Additionally, the interferon-alpha/gamma (INF-α/γ) and inflammatory response pathways also demonstrated significant enrichment in high AS tumors.

High angiogenesis HCC shows increased immune checkpoint marker expression

Amongst patients whose tumors demonstrated high AS in the first 3 years, a transient survival benefit appears to develop and subsequently dissipate. This may indicate that there is a period of time during which intra-tumoral angiogenesis primes the host immune system for a transient anti-tumor response after which tumor escape leads to patient demise. To investigate this mechanism further, we sought to characterize the expression of checkpoint markers (Fig. 7). A high AS correlated with increased expression of programmed cell death protein 1 (PD1), programmed death ligand 1 (PD-L1), and programmed death ligand 2 (PD-L2) in the TCGA cohort. In both the TCGA and GSE cohorts, high tumor AS correlated with higher levels of cytotoxic T lymphocyte-associated protein 4 (CTLA-4).

Discussion

The results of this study indicate that angiogenesis is a cornerstone in the immunogenic potential of HCC. The AS derived from the MSigDB hallmark annotated gene set was used to investigate two large patient cohorts. Tumors with high AS in both cohorts demonstrated higher expression of VEGF (VEGFC), endothelial cells markers (CD31, VWF), and vascular stability markers (TIE1 and 2, ANGPT1, VE-cadherin, CLDN5 and JAM2) leading credence to the score serving as a reasonable metric for tumor angiogenesis. In these cohorts, AS was not associated with clinical outcomes including DFS and OS. Additionally, tumors with high AS showed enrichment of classic pro-tumor gene sets including Notch, Hedgehog, KRAS, TGF-β, glycolysis and EMT. The TME within these patient cohorts were variable, showing both pro- and anti-tumor signatures; however, the aggregate effect as measured by CYT was elevated in tumors with a high AS. High AS tumors also showed enrichment of the pro-inflammatory gene sets IFN-α/γ, TNF-α, allograft rejection and inflammatory response. Finally, both cohorts showed that tumors with higher AS also had increased immune checkpoint marker expression including PD1, PD-L1, PD-L2, and CTLA-4.

The current paradigm for the treatment of HCC hinges on anatomic resectability [35]. However, up to 70% of patients worldwide may not be candidates for surgery or transplantation at diagnosis; thus, the treatment of most patients relies on a combination of local therapies (i.e., ablation, transarterial chemoembolization, transarterial radioembolization, etc.) and systemic therapy [36, 37]. Unfortunately, traditional chemotherapeutics have shown suboptimal efficacy for HCC. In the contrary, metronomical delivery of anti-angiogenic chemotherapy, such as capecitabine, demonstrated significant anti-

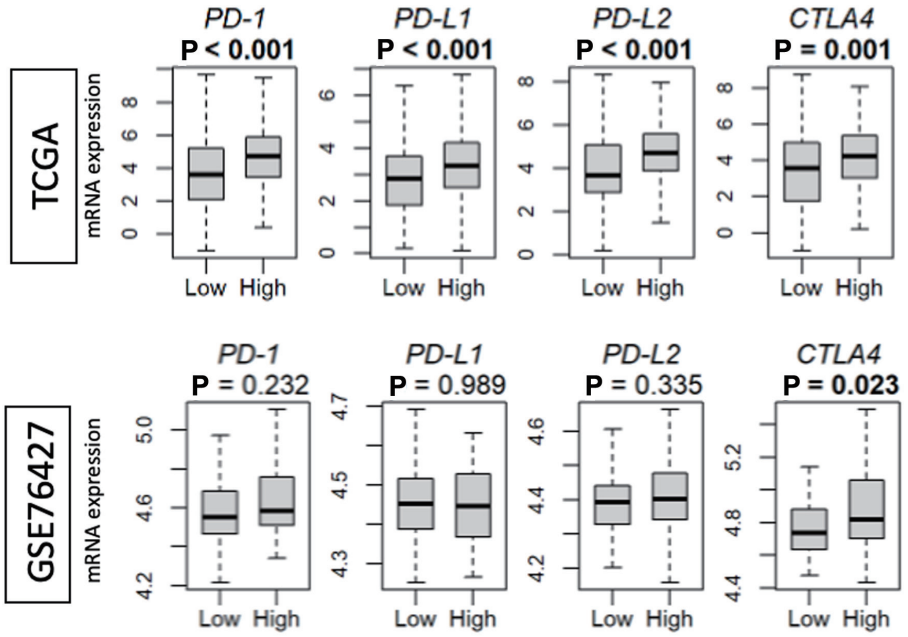

Figure 7. Intra-tumoral angiogenesis score correlates with checkpoint marker expression in the TCGA and GSE76427 cohorts. High versus low, median angiogenesis pathway score. PD1: programmed cell death protein 1; PD-L1 and 2: programmed death ligand 1 and 2; CTLA-4/CD152: cytotoxic T lymphocyte-associated protein 4.

tumor efficacy with a good safety profile and significant radiological tumor response in cases of HCC that failed sorafenib [38-42]. Thus, current management guidelines are based on targeted anti-angiogenic agents and, more recently, immunotherapeutics [43-45]. However, immunotherapy has associated complications; therefore, a metric to better select patients is needed to reduce those complications [46].

Although overall HCC tends to be a malignancy characterized by arterialization, this study identifies a subset of patients whose tumors may reside at the higher end of this spectrum, and in turn exhibit a more immunogenic signature [47]. Other investigations have shown similarly that HCC may be an immunologically heterogenous disease, with some tumors being at least theoretically more susceptible to checkpoint therapy [48]. Current studies indicate that immunotherapy may be effective in up to 30% of patients, but there is no distinct maker to identify immunotherapy candidacy [49]. Here, we suggest that the AS may provide a way to identify tumors whose transcriptomic signature and aggregate cellular infiltration pattern may lead to increased susceptibility to immune modulation. *In-vivo* investigation could readily be supported by the framework established by AS in this investigation. The results of this study further support the potential therapeutic impact of enhanced anti-tumor effects by combination strategy of tyrosine kinase inhibitors as antiangiogenic agents and immune checkpoint inhibitors. This dual synergistic effect has been recently reviewed, addressing the rationale of ongoing clinical trials [50].

There are some limitations to the interpretation of our results. This was a retrospective analysis performed on banked and cataloged tissue and transcriptomic data. As demonstrated by other investigations, the intra-tumoral and peri-tumoral tissue can be highly heterogeneous, and thus, tissue sampling error may play a role. The cohorts examined only had limited clinical annotation, and thus, extrapolation to highly selected patients is difficult. It has been reported that there is variability in HCC-related angiogenesis and microenvironment by underlying liver disease. It would be ideal to document the underlying liver disease in TCGA and GSE7642733 cohorts; however, we did not have access to such data at this time. Additionally, whether the single or multiple nodules of HCC have a clinical impact on this study is of interest. However, we do not have access to number of nodules of HCC on the cohorts we used, and we were unable to conduct such analyses. Finally, the AS is a derivative score that would require prospective validation before clinical application.

In conclusion, the angiogenesis score in HCC appears to be a reasonable metric for intra-tumoral angiogenesis. HCC, which has high AS, may be pro-immunogenic, and thus, this score may help stratify patients in which a more selective application of immunotherapy could be achieved.

Acknowledgments

None to declare.

Financial Disclosure

This research was supported by National Institutes of Health, USA (grant number R01CA160688, R37CA248018, R01CA250412, R01CA251545, and R01EB029596), as well as US Department of Defense BCRP (grant number W81XWH-19-1-0674 and

W81XWH-19-1-0111) to K. Takabe. National Cancer Institute, cancer center support grant P30CA016056 to Roswell Park Comprehensive Cancer Center.

Conflict of Interest

The authors have no potential conflict of interest to disclose.

Informed Consent

Not applicable.

Author Contributions

Conceptualization: R. Vaghjiani, R. Wu, and K. Takabe. Data analyses: R. Vaghjiani, R. Wu, and K. Takabe. Writing - original draft preparation: R. Vaghjiani. Writing - review and editing: K. Tung, T. Ishikawa, and K. Takabe. Supervision: T. Ishikawa and K. Takabe. Funding acquisition: K. Takabe. All authors have read and agreed to the published version of the manuscript.

Data Availability

The data that support the findings of this study are available from the corresponding author, KT, upon reasonable request.

Abbreviations

ANGPT1: angiopoietin 1; CD31/PECAM-1: platelet endothelial cell adhesion molecule; cDC: conventional dendritic cell; CD144/VE-cadherin: vascular endothelial cadherin; CD152/CTLA-4: cytotoxic T lymphocyte-associated protein 4; CLDN5: Claudin5; CYT: cytolytic activity; DFS: disease-free survival; EMT: epithelial mesenchymal transition; FDR: false discovery rate; GSEA: Gene Set Enrichment Analysis; HCC: hepatocellular carcinoma; INF-α and γ: interferon-alpha and gamma; JAM2: junction adhesion molecule 2; LECs: lymphatic endothelial cells; MECs: microvascular endothelial cells; NES: normalized enrichment score; OS: overall survival; PD1: programmed cell death protein 1; PD-L1 and 2: programmed death ligand 1 and 2; TIE1 and 2: tyrosine kinase with immunoglobulin-like and epidermal growth factor-like domains 1 and 2; TNF-α: tumor necrosis factor-alpha; VEGF: vascular endothelial growth factor; VWF: von Willebrand factor

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