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Brief Communication

Gastric cancer with enhanced myogenesis is associated with less cell proliferation, enriched epithelial-to-mesenchymal transition and angiogenesis, and poor clinical outcomes

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Abstract: Gastric cancer (GC) remains a lethal disease, with over 26,000 new cases and more than 11,000 deaths annually in the US. Thus, a deeper understanding of GC biology is critical to improve survival. Myogenesis is the formation of muscle fibers, which is a mesodermal tissue. In cancer, epithelial-to-mesenchymal transition (EMT) is a known phenomenon that promotes metastasis and poor survival. Given that myogenesis produces mesenchymal cells, we hypothesized that GC with increased myogenesis is linked to aggressive tumor behaviors and less favorable outcomes. In this study, three GC patient cohorts: TCGA (*n*=375), GSE26253 (*n*=432), and GSE84437 (*n*=482), were analyzed. The "MYOGENESIS" set in the Hallmark collection which comprises 200 myogenesis-related genes was analyzed to perform gene set variation analysis to create a score to quantify the myogenesis activity. Our results showed that T category of AJCC cancer staging that reflects the tumor invasion to stomach wall consistently correlated with myogenesis activity in two GC cohorts. High myogenesis GC was associated with lower cell proliferation, evidenced by reduced proliferation scores, decreased Ki67 gene expression, and less enrichment of E2F Targets, G2M checkpoint, MYC Targets V1, and V2 gene sets. High myogenesis tumors showed increased stromal cells (fibroblasts and adipocytes) infiltration within the tumor microenvironment, as well as less silent and non-silent mutation rates and copy number alterations. Higher lymphocyte infiltration, leukocyte fraction, T-cell receptor richness, and B-cell receptor richness were associated with high myogenesis GC. However, infiltration of CD4 cells, T helper type 1 and 2 cells, Natural Killer cells, regulatory T cells, and plasma cells was lower, with increased infiltration of dendritic cells in high myogenesis GC. High myogenesis GC enriched EMT, Hedgehog, TGF-β, and KRAS gene sets. Furthermore, it was associated with enhanced angiogenesis, evidenced by enrichment of Angiogenesis, Coagulation, and Hypoxia gene sets, and increased infiltration of microvascular and lymphatic endothelial cells and pericytes. High myogenesis GC consistently correlated with worse overall survival in all three cohorts, and worse disease-specific and progression-free survival in the TCGA cohort. Hence, our findings suggest that GC with enhanced myogenesis is associated with decreased cell proliferation, increased EMT and angiogenesis, and worse prognosis.

Keywords: Gastric cancer, myogenesis, gene expression, GSEA, GSVA, signaling, prognosis

Introduction

Gastric cancer (GC) continues to be a formidable disease in the United States, resulting in over 26,000 new cases and more than 11,000 deaths annually [1]. Despite an increase in 5-year survival rates from 15% in the 1970s to 33% in the 2010s, these rates remain relatively

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low compared to other cancer types [2]. Gastric cancer is often marked by non-specific symptoms, and it often progresses unnoticed due to the delay in onset of clinically detectable symptoms [3]. When the disease is diagnosed at the metastatic stage, the prognosis is generally poor and radical surgery is not indicated [4]. Hence, elucidating the mechanisms associated with GC progression is a crucial first step to treat this deadly condition.

Myogenesis is the formation of muscle fibers that originate from the mesoderm [5], which also gives rise to blood vessels, lymphatic tissue and connective tissues. This embryological concept is extremely important in oncology. Epithelial-to-mesenchymal transition (EMT) is a process in which cancer epithelial cells transform into mesoderm-derived mesenchymal cells that enable malignant cancer cells to acquire the ability to invade vessels, resist apoptosis, and disseminate to generate distant metastasis [6], which is one of the hallmarks of cancer and is associated with poor survival outcomes [7]. Hence, cancer cells exhibiting a mesenchymal phenotype are believed to be involved in the EMT. In this context, we speculate that quantifying the pathways generating mesenchymal cells, such as muscle cells derived from mesoderm would reflect the activity of EMT. Here, we hypothesized that GC with enhanced myogenesis is associated with EMT and unfavorable outcomes.

Advances in technology have significantly facilitated data acquisition, such as obtaining a comprehensive transcriptome through RNA-sequence. The use of bioinformatic analyses helps reveal the details of complex biological processes [8]. However, interpreting the clinical significance of individual gene expressions without considering their biological context can be challenging and prone to errors [9]. Understanding the profile of multiple gene expressions organized according to the gene functions provides a more comprehensive and relevant perspective [10]. For this reason, we utilized the Gene Set Variant Analysis (GSVA), a sophisticated computational tool that assesses the biological activity of a specific signaling pathway. This tool has been employed to derive scores from these pathways [11-15]. In our study, we used GSVA and integrated multiple genes to enhance the model's explanatory power and to obtain a global perspective on myogenesis activity in gastric cancer. Based on our current understanding, this is the initial study exploring myogenesis activity in GC across multiple independent large patient cohorts.

Methods

Clinical data acquisition for GC patients

We accessed transcriptomic and clinical details of GC patients from the TCGA database related to stomach adenocarcinoma (*n*=375) [16] through the Genomic Data Commons Data Portal (GDC). Mutation information was obtained via cBioportal [17]. Additionally, we analyzed two comprehensive gastric cancer datasets, GSE26253 [18] and GSE84437 [19], each containing transcriptomic data for 432 patients and 482 patients respectively. While the TCGA dataset included overall survival (OS), progression free survival (PFS), and disease-specific survival (DSS), both GSE26253 and GSE84437 provided only OS. The normalized genomic and clinical datasets were obtained from the GEO database of the US National Institutes of Health. Our analytical approach utilized log2-transformed gene expression values. Since TCGA and GEO data are public and do not contain any identifiable information, there was no need for an Institutional Review Board (IRB) approval for our study.

Gene set enrichment analysis (GSEA)

We employed GSVA [20] to generate a score from the gene expression data similar to our previous studies [21-24]. We utilized the "HALLMARK_MYOGENESIS" gene set from the Molecular Signatures Database (MSigDb) Hallmark collection (http://www.gsea-msigdb.org) [25] for our analysis and performed gene set enrichment analysis (GSEA) to differentiate between tumors with low and high myogenesis levels. In alignment with the Broad Institute's guidelines, gene sets achieving a false discovery rate (FDR) of < 0.25 were considered to have achieved statistically significant enrichment in the GSEA. The primary outcome of this study focuses on the clinical relevance, particularly survival, while the secondary outcome examines aspects of cancer biology related to this phenomenon.

Cell composition of the tumor microenvironment (TME)

As previously detailed, we used the xCell algorithm to assess the correlation between myogenesis high and low groups and the infiltration of stromal and immune cells in the tumor microenvironment (TME) [26-30]. In their evaluation of the TCGA cohort, Thorsson et al. [31] offered supplementary scores, including indel and single-nucleotide variant (SNV) neoantigens, copy number alteration (CNA), silent and non-silent mutations, homologous recombination defects (HRD), intratumor heterogeneity, and proliferation score. They also provided immune-related score, including tumor infiltrating lymphocyte (TIL) regional fraction, interferon (IFN)-γ response, leukocyte fraction, T cell receptor (TCR) and B cell receptor (BCR) richness and lymphocyte infiltration signature. In previous discussions, we outlined the process of computing the cytolytic activity score (CYT), which entailed analyzing gene expression levels for *perforin* and *granzyme A* [32].

Statistical analysis

All statistical evaluations were conducted using R software (version 4.1.3, www.r-project.org). The comparisons between groups were made using Mann-Whitney U test, and the Kruskal-Wallis test. Turkey's boxplots were employed to represent interquartile ranges. The relationship between myogenesis level and survival outcomes, including OS, PFS, and DSS, was analyzed through the Cox-proportional hazards regression model and visualized through the Kaplan-Meier survival curve. We deemed *p*-value below 0.05 as statistically significant in all the tests conducted.

Results

Myogenesis enhanced GC was associated with higher T-category of AJCC staging

Initially, we explored the clinical significance of myogenesis level among GC patients. Our primary testing cohort comprised GC patients from The Cancer Genome Atlas (TCGA) and we utilized GSE84437 as a validation cohort. Supplementary Figure 1 showcases histograms representing myogenesis levels across two distinct cohorts: TCGA (n=375) and GSE84437 (n=482). The expression levels in these cohorts demonstrate an approximate bell-curve distribution. Within each group, the top two tertiles were defined as the myogenesis high group, while the remainder constituted the low myogenesis group, as separated by the green lines (Supplementary Figure 1). Myogenesis high GC consistently correlated with a significantly higher T-category in both cohorts (both $P < 0.001$). However, the relationship with N-category of AJCC staging showed significance in one cohort but lacked validation in the other (**Figure 1A**). GC with high myogenesis predominantly manifested as mucinous, diffuse, papillary, signet, and tubular histological types, in descending order (**Figure 1A**). These findings suggest that myogenesis enhanced GC is associated more with a higher T category of AJCC staging.

Myogenesis enhanced GC had decreased enrichment of cell proliferation-related gene sets, lower proliferation score and decreased expression of Ki-67 gene (MKI67)

Next, we investigated if there was a correlation between the myogenesis level and cancer cell proliferation. Our analysis revealed that in the TCGA cohort, myogenesis high GC exhibited a reduced proliferation score (**Figure 1B**, $P < 0.001$). Consistently, in the TCGA and GSE84437 cohorts, Ki-67 (MKI67) gene expression, a cell proliferation marker was found to be lower in myogenesis high GC (**Figure 1C**; both $P < 0.001$). Additionally, myogenesis high GC in the GSE84437 cohort demonstrated decreased enrichment in several gene sets linked to cell proliferation, such as MYC Targets v1 and v2, G2M checkpoint, and E2F Targets, which also indicate that myogenesis high GC is less proliferative. However, this pattern was not seen in the TCGA cohort (**Figure 1D**). These findings suggest an association between myogenesis high GC and decreased cellular proliferation.

GC with enhanced myogenesis had higher infiltration of stromal cells in tumor microenvironment (TME) and was associated with lower silent and non-silent mutation rate and copy number alteration (CNA)

Previous studies have consistently demonstrated that cancers with high proliferation rates tend to have reduced stromal cell infiltration in the TME [27, 33, 34]. Considering this, we explored the relationship between myogenesis

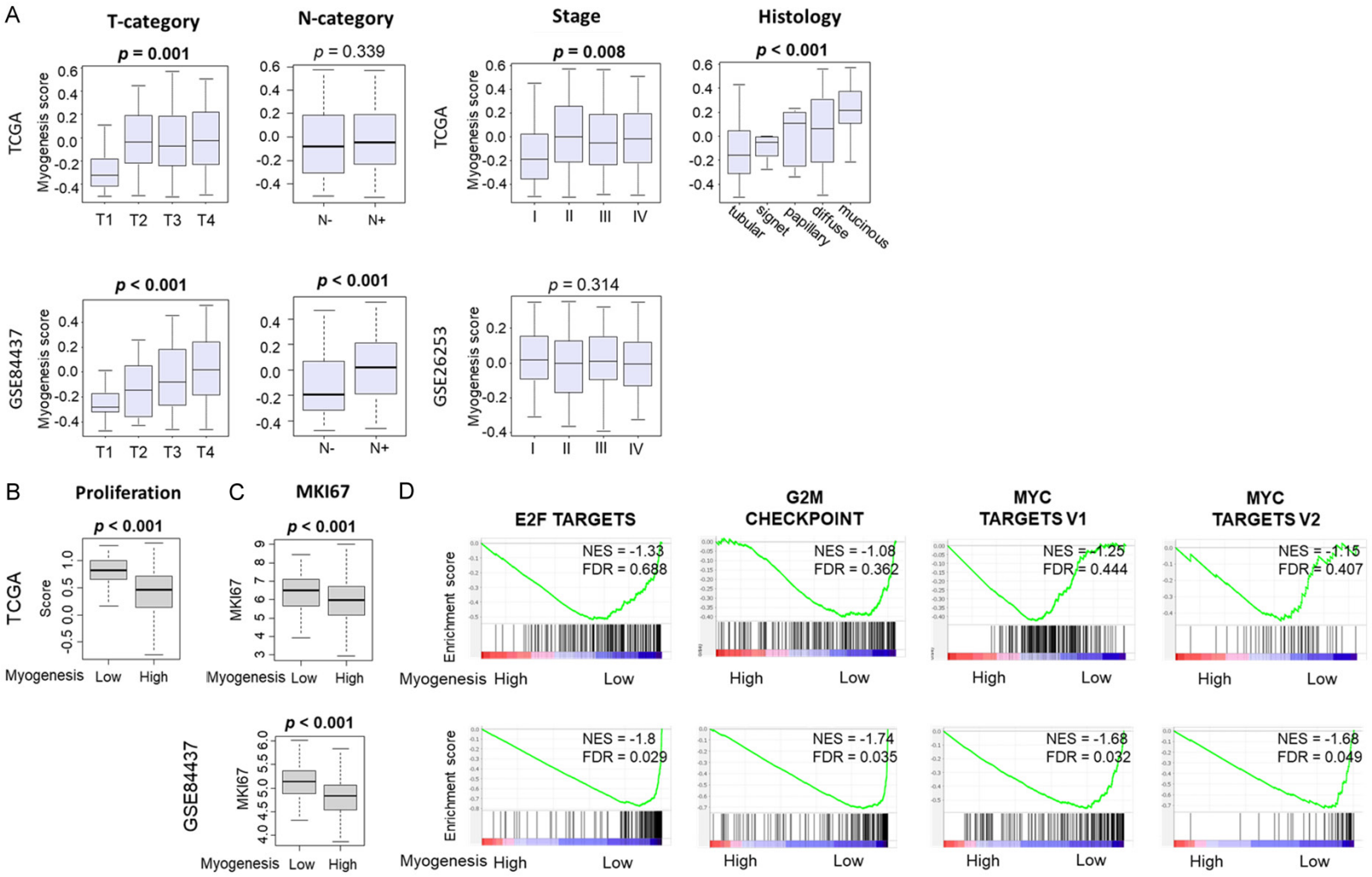

Figure 1. Association of myogenesis level with clinical parameters and proliferation of cancer cells. A. Association of high myogenesis GC with tumor T-category, N-category, stage, and histological types in GC patients from both cohorts. The analysis of variance (ANOVA) test was utilized to obtain the *p*-values. B. Proliferation score based on myogenesis level in the TCGA cohort. C. Expression of Ki-67 proliferation marker (MKI67) in TCGA and GSE84437 cohorts by myogenesis high and low GC groups. D. Cell proliferation-related gene sets enhancement in GC stratified based on high and low myogenesis scores from the TCGA and GSE84437 cohorts.

level and the presence of stromal cells, specifically adipocytes and fibroblasts, in the TME across both the TCGA and GSE84437 cohorts. In alignment with the previous observations, GC characterized by high myogenesis activity was associated with increased infiltration of fibroblasts and adipocytes in the TME, which is in agreement with diminished cancer cell proliferation in both datasets (Supplementary Figure 2A). Furthermore, genomic instability driven by mutations is a recognized catalyst for the initiation and aggressiveness of cancer cells. Utilizing the previously calculated scores by Thorsson and colleagues [28, 35, 36], our examination disclosed a noteworthy link between a myogenesis high GC and diminished silent and non-silent mutation frequencies, as well as copy number alteration (CNA) within the TCGA cohort (Supplementary Figure 2B). Nevertheless, there were no notable correlations with single nucleotide variant (SNV) neoantigens, intratumor heterogeneity, nor homologous recombination deficiency (HRD). These findings imply that GC with an increased myogenesis activity tend to have lower mutation frequencies and genomic instability, reflecting their less proliferative characteristics.

Immune cell infiltration in GC by the myogenesis activity did not show any definitive trend

Immune cells play a pivotal role in influencing cancer cell behavior within the TME [37]. Hence, we explored any potential correlation between the myogenesis activity and the infiltration of immune cells. In TCGA cohort, lymphocyte infiltration signature, leukocyte fraction, and T-cell receptor (TCR) richness and B-cell receptor (BCR) richness were higher in myogenesis high GC (**Figure 2A**). Further, consistently in both cohorts, we found significant infiltrations of dendric cells (DCs) in myogenesis high GC (**Figure 2B**, P=0.019 and P=0.002 respectively). The CD8 cells displayed a decrease in TCGA cohort and increase in infiltration in GSE84437 cohort (**Figure 2B**; both $P < 0.05$). Interestingly, the infiltration of CD4 cells, T helper type 1 (Th1) cells, and NK cells (favorable immune cells) and T helper type 2 (Th2) cells, regulatory T cells (Tregs), and plasma cells (unfavorable immune cells) exhibited lower levels in both the TCGA and GSE84437 cohorts (**Figure 2B**). A significant decrease in infiltration of M2 macrophages was observed in the GSE84437 cohort; however, this finding was not validated in the TCGA cohort (**Figure 2B**, $P < 0.001$ in GSE84437). Furthermore, a noteworthy decrease was observed in the cytolytic activity (CYT), which indicates immune cell-mediated destruction within the TME, among myogenesis high GC only in GSE84437 but in the TCGA cohort, this was not observed to be significant (**Figure 2C**).

Myogenesis high GC enriched multiple gene sets that aggravate cancer including Epithelial Mesenchymal Transition (EMT), Hedgehog, TGF-β, and KRAS signaling gene sets

Given that myogenesis is the process of generating muscle fibers, which are mesoderm-derived tissues, we investigated whether it was related to cancer aggravating gene sets including EMT. As expected, our findings revealed that myogenesis high GC enriched several gene sets that exacerbate cancer, such as EMT, Hedgehog, and upregulated KRAS signaling consistently in both cohorts (**Figure 3A**, all $FDR < 0.25$).

Myogenesis high GC was associated with enhanced angiogenesis, evidenced with enrichment of angiogenesis, coagulation, and hypoxia gene sets, and infiltration of microvascular and lymphatic endothelial cells and pericytes

Considering that myogenesis high GC was linked to an enrichment of several cancer-aggravating gene sets, we sought to understand its association with other hallmarks of cancer. Interestingly, gene sets associated with angiogenesis, coagulation, and hypoxia were enriched in myogenesis high GC, suggesting increased angiogenesis (**Figure 3B**). In addition, both microvascular and lymphatic endothelial cells, as well as pericytes, were more abundant in myogenesis high GC, indicating a higher presence of mature blood vessels and lymphatic ducts (**Figure 3C**). These findings suggest that myogenesis high GC is linked to increased angiogenesis and the presence of mature vessels.

Myogenesis enhanced GC was associated with worse survival outcomes

Considering that myogenesis high GC was linked to EMT and angiogenesis, we sought to

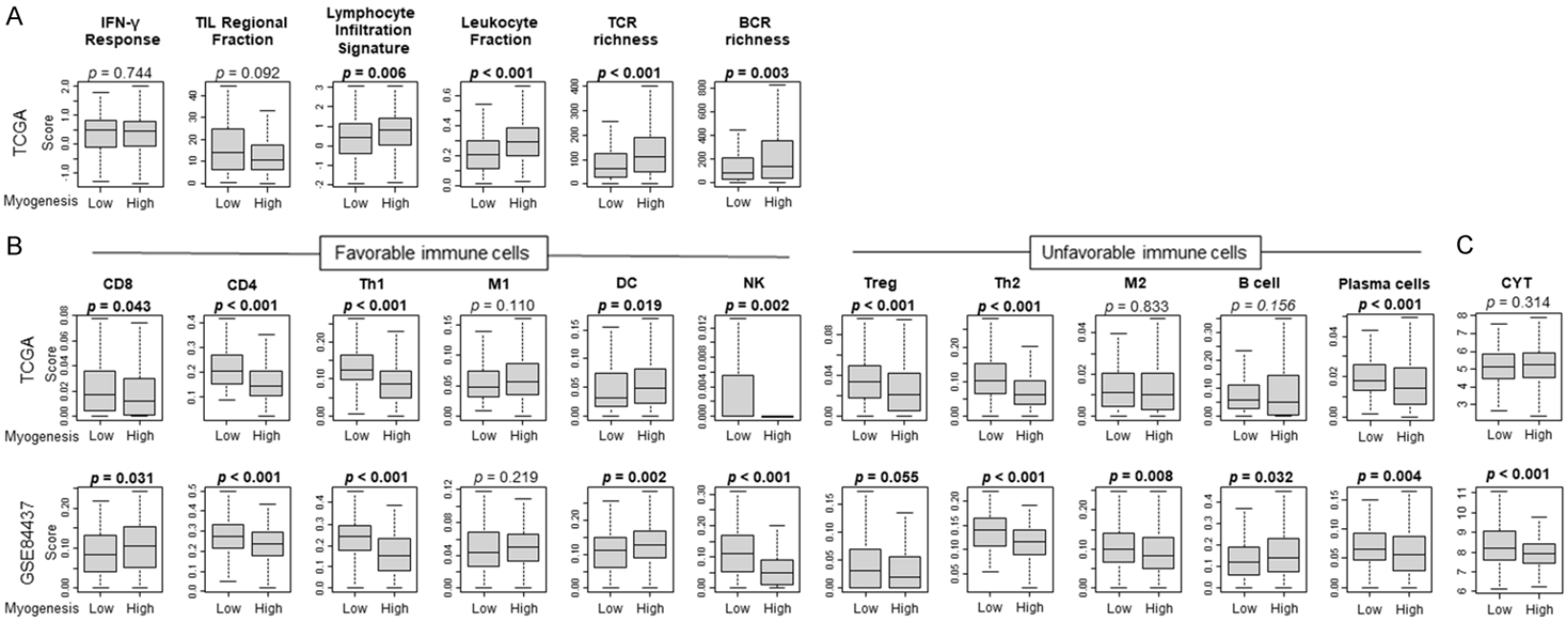

Figure 2. Immune cell infiltration in GC tumor microenvironment (TME) in relation to myogenesis level. A. Lymphocyte infiltration signature, leukocyte fraction, and T cell and B cell receptor richness by myogenesis high and low groups in TCGA cohort. B. Various immune cell infiltrations in TCGA and GSE84437 cohorts by myogenesis high and low GC groups. C. Cytolytic activity (CYT) score in GC cases from GSE84437 and TCGA cohorts based on myogenesis level.

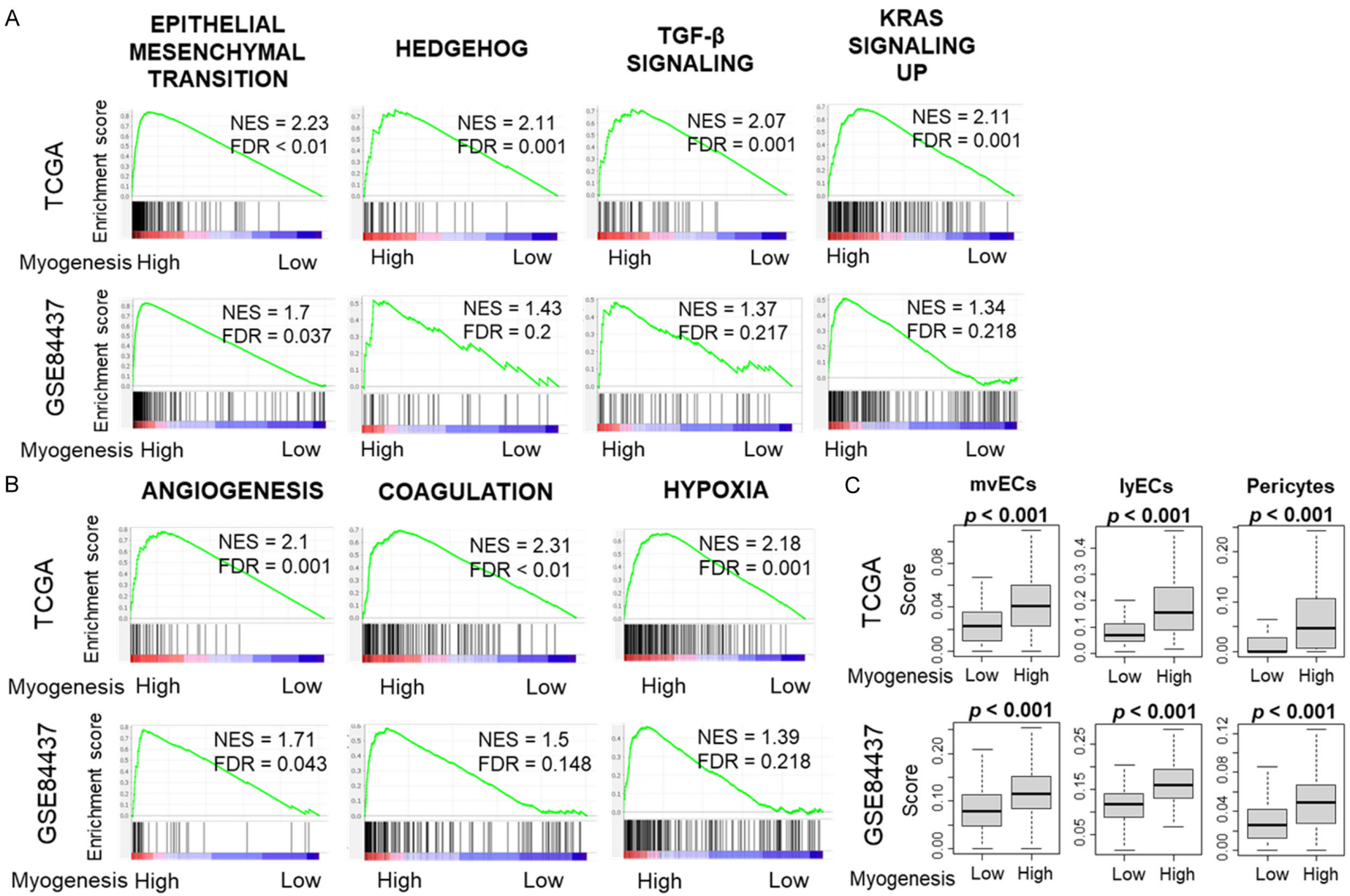

Figure 3. Association of myogenesis high and low GC with Epithelial Mesenchymal Transition, Hedgehog, TGF-β signaling, and KRAS signaling gene sets as well as angiogenesis related gene sets, microvascular (mv) and lymphatic (ly) endothelial cells (EC), and pericytes. A. Enrichment of cancer aggravating gene sets in GC by myogenesis high and low groups from TCGA and GSE84437 cohorts. B. Enrichment of angiogenesis related gene sets in GC from TCGA and GSE84437 cohorts by myogenesis high and low groups. C. Infiltration contrast of mvECs, lyECs, and pericytes in the high and low myogenesis GC.

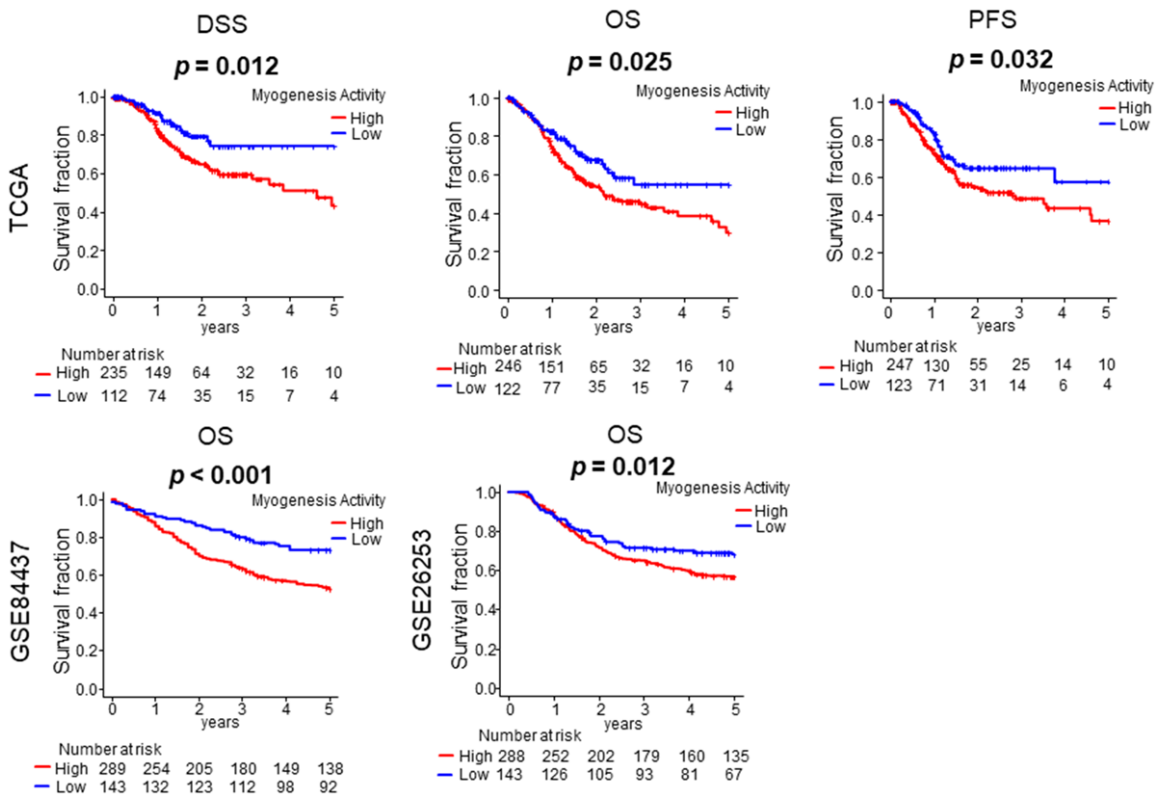

Figure 4. Survival outcomes in GC patients by myogenesis high and low groups. The Kaplan-Meier survival plots illustrate a comparison between tumors demonstrating high (red lines) and low (blue lines) myogenesis level, accompanied by the log-rank test. Disease-specific survival (DSS), and progression-free survival (PFS) outcomes of myogenesis high and low GC patients from the TCGA cohort and overall survival (OS) of these patients from TCGA, GSE84437, and GSE26253 cohorts.

understand its association with survival outcomes in GC patients. In the TCGA dataset, it was observed that GC patients with high myogenesis activity had notably poorer DSS, OS, and PFS outcomes (**Figure 4**, with *p* values of 0.012, 0.025, and 0.032, respectively). This observation for OS was validated in two other datasets, GSE84437 and GSE26253 (**Figure 4**; $P < 0.001$ and 0.012, respectively).

Discussion

The present investigation delves into the clinical significance of myogenesis activity in GC. Analyses using both the TCGA and GSE84437 cohorts indicated that high myogenesis GC was consistently linked to a higher tumor T category of AJCC cancer staging and displayed lower cell proliferation rates, marked by decreased levels of the proliferation score, expression of Ki67 gene and less enhancement of gene sets related to cell proliferation. Note that the T category of AJCC cancer staging in GC is defined by the depth of cancer invasion to the stomach wall and not by the size of the tumor. Additionally, these tumors exhibited a higher infiltration of stromal cells within the TME while displaying reduced mutation rates, all consistent with less cancer cell proliferation. While immune cell infiltration did not show a definitive pattern, myogenesis high GC did show significant enrichment in gene sets known to exacerbate cancer progression including EMT, TGF-β, Hedgehog, and KRAS signaling gene sets. Furthermore, myogenesis high GC was associated with enhanced angiogenesis, evidenced by the enrichment of Angiogenesis, Coagulation, and Hypoxia gene sets, and infiltration of microvascular and lymphatic endothelial cells and pericytes. GC with enhanced myogenesis was also linked with poorer survival outcomes, including DSS, OS and PFS.

Each of the myogenesis-related genes is known to participate in multiple biological activities

beside myogenesis. Thus, analyzing a single gene may not accurately reflect the myriad pathways involved in myogenesis. To address this, we adopted the myogenesis score, consisting of 200 myogenesis-related genes, to aggregate the overall activity of these genes. This approach diminishes the effects of extraneous bioactivities not related to myogenesis. By employing the computational algorithm Gene Set Variation Analysis (GSVA), we can explore gene expression within a pathway instead of concentrating on an individual gene [20]. Enhancing the accuracy of gene coordination, simplifying the model, and improving the applicability of predictive models, this pathway-centric approach has proven its clinical relevance in various cancer types. For example, we previously employed similar pathway scores, such as E2F Targets and G2M Checkpoint pathway scores, utilizing the MSigDb Hallmark gene set of the GSVA [12-14, 24]. Furthermore, we conducted GSEA on the complete Hallmark gene set to assess whether additional signaling pathways are involved and found that the myogenesis score enriches pathways linked to metastasis, such as EMT and angiogenesis.

Comprising proliferating cancer cells, stromal cells, vascular endothelial cells, infiltrating inflammatory cells, and various associated cells, the TME is a distinctive milieu that arises during tumor progression through interactions with the host [38]. We have previously reported that cells within the TME other than the cancer cells such as fibroblasts [39], immune cells [29, 35], and adipocytes [27, 40, 41], and microvessel endothelial cells [33, 42] are associated with cancer biology and prognosis. However, to date, myocytes has never been studied. To our knowledge, this study is the first to quantify the activity of myogenesis and its clinical relevance.

Dysregulation of cellular proliferation, extensively examined as one of the hallmarks of cancer [43], is crucial for predicting tumor behavior [44]. Aggressive tumors typically exhibit higher proliferative rates [45]; nonetheless, other reports suggest an inverse relationship between cancer cell proliferation and tumor aggressiveness [41, 46]. The association between low cellular proliferation and poor prognosis may be linked to hypoxic regions within the tumor. Hypoxic conditions have been demonstrated to decelerate tumor growth while facilitating the onset of EMT leading to invasion [44, 47], which was also seen in this study results.

In this study, we hypothesized that GC with enhanced myogenesis may exhibit aggressive behavior and unfavorable outcomes due to their association with the transformation into mesenchymal cells, which are implicated in EMT. Indeed, our results show that myogenesis high GC were less proliferative and enriched gene sets related with initiation of metastasis, including EMT and TGF-β. Notably, in many advanced tumors, TGF-β signaling shifts from suppressing cell proliferation to activating EMT, thereby endowing cancer cells with traits associated with high-grade malignancy [7, 43]. Furthermore, in gastric cancer, the activation of the Hedgehog signaling pathway is associated with tissue invasion and increased metastatic potential [48].

Additionally, the role of angiogenesis in tumor growth and metastasis is well recognized in multiple cancer types [21, 33, 42]. In agreement, our study showed that myogenesis high GC was linked with enhanced angiogenesis and infiltration of microvascular and lymphatic endothelial cells and pericytes, which may contribute to poorer survival. We previously found that GC with a high angiogenesis score exhibited enrichment in coagulation, hypoxia, EMT, and TGF-β signaling pathways [42]. This indicate that high angiogenesis GC is linked with enhanced TGF-β signaling and EMT, both of which are known to exacerbate cancer progression and metastasis [49]. Weidner et al. [50] observed that blood vessel number and density correlate with metastatic frequency in invasive breast cancer, and similar observations have been made in gastrointestinal tumors [51, 52]. In another study, we reported that increased intra-tumoral expression of pro-coagulation genes predicts angiogenesis, EMT, and worse patient survival in GC [53]. Intriguingly, this report also found an association between high pro-coagulation gene expression and myogenesis activity [53]. While the causal relationships remain unclear, angiogenesis, EMT, myogenesis, and coagulation appear to be interconnected in GC. Interestingly, our data indicated that myogenesis high GC had reduced proliferation capacity, consistent with our prior work on GC [53]. We speculate that myogenesis activity reflects initiation of metastasis by pro-

moting EMT and angiogenesis. Furthermore, GC with enhanced myogenesis may exhibit cancer biology that is less inclined to proliferate locally but more prone to acquire EMT and metastasize, leading to worse survival outcomes. Whether myogenesis is inherently challenging or elevated in conjunction with other pathways remains an open question, pointing to future research avenues.

This study emphasizes the clinical relevance of myogenesis activity in GC, presenting a novel perspective. However, it is important to acknowledge its limitations. The study's retrospective design, relying on pre-existing cohorts, is susceptible to selection bias. The database had incomplete clinical data, leading to the assumption that all patients received standard treatment. Although the TCGA dataset included OS, PFS, and DSS, both GSE26253 and GSE84437 provided only OS. Furthermore, gene expression was analyzed at a solitary time point - during surgical removal, without follow-up data on potential change in expressions. While our bioinformatics results do not establish a definitive mechanism, they offer insights into the relationship between myogenesis activity in GC and patient outcomes. We are aware that some argue the necessity of experimental verification, however, we respectfully disagree with that point of view because it is well known that neither in vitro or in vivo systems completely replicate human cancer and its TME. Therefore, even if additional experiments yield results inconsistent with ours, which does not necessarily mean that our results are untrue. We believe the only valid verification of our study will be to conduct prospective clinical trial on patients, however, that was not practically feasible at this point.

Conclusion

High myogenesis GC was found to be associated with decreased cell proliferation, increased EMT and angiogenesis, and poorer patient outcomes. The results highlight the myogenesis activity's significance as a prognostic biomarker for GC. However, further prospective experimental studies are necessary to gain a deeper understand of the role of myogenesis within the TME of GC and other cancers.

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Disclosure of conflict of interest

None.

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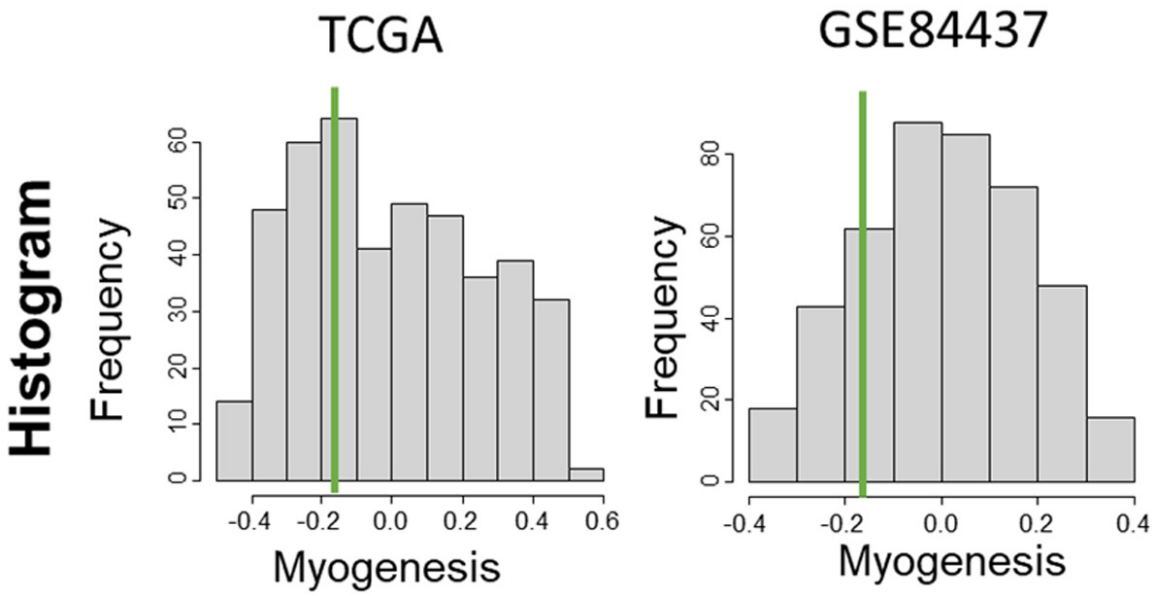
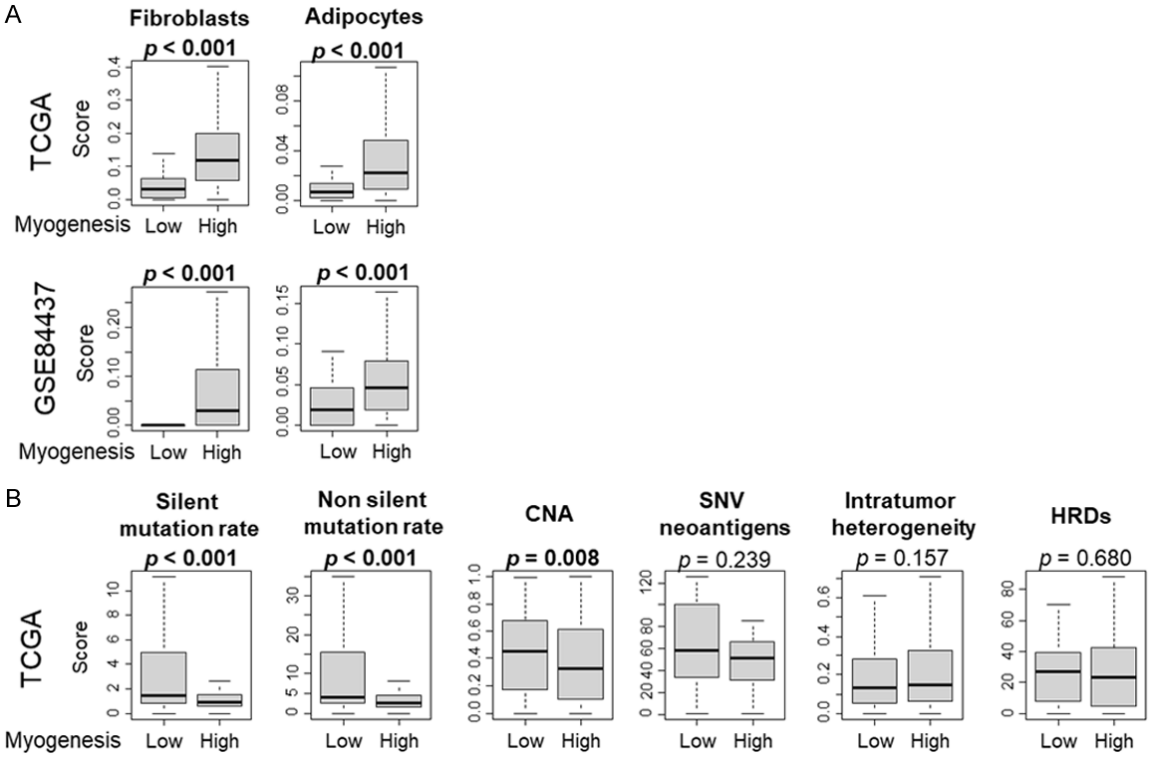


Supplementary Figure 1. Histograms of TCGA and GSE84437 cohorts by myogenesis level. Distribution of myogenesis level in TCGA (n = 375) and GSE84437 (n = 432) cohorts, highlighting the division between high and low myogenesis groups (green line, bottom 33%).



Supplementary Figure 2. Stromal cell infiltration in the tumor microenvironment (TME) and genomic characteristics in GC patients based on myogenesis level. A. Analysis from TCGA and GSE84437 cohorts showing infiltration levels of various stromal cell types such as fibroblasts and adipocytes. B. Comparison of copy number alteration (CNA), single-nucleotide variant (SNV) neoantigens, silent and non-silent mutation rates, intratumor heterogeneity, and homologous recombination defects (HRDs) between high and low myogenesis GC.