### Original Article Higher intra-tumoral expression of pro-coagulation genes is a predictor of angiogenesis, epithelial mesenchymal transition and worse patient survival in gastric cancer

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Abstract: Coagulation regulates angiogenesis in cancer, and is associated with tumor development and metastasis. To date, there have been no studies quantifying the state of intra-tumoral coagulation. We measured intra-tumoral coagulation gene expression using the "Hallmark-COAGULATION" gene set in the MSigDB, performing gene set variation analysis and then assigning a "coagulation score" to quantify gene expression. Clinical, histologic, and genetic data were analyzed in 807 gastric cancer patients from the TCGA\_STAD and GSE84437 databases. Tumors with increased expression of pro-coagulation genes were consistently associated with higher AJCC T-categories (p = 0.018), lymph node metastasis (p = 0.036), and stage (p = 0.006) in both cohorts. Patients with high coagulation scores were found to have worse disease-specific survival and overall survival (OS) (p = 0.019 and 0.011, respectively) in TCGA, and worse OS in GSE84437 cohort (p = 0.012). Higher expression of pro-coagulation genes correlated with increased intra-tumoral angiogenesis, as well as increased proportions of lymphatic and microvascular endothelial cells, endothelial cells, and pericytes, calculated by xCell algorithm. High coagulation scores were significantly associated with low tumor mutation burden, but not with intratumor heterogeneity and homologous recombination deficiency. Gastric cancers with high coagulation scores contained higher amounts of M1 macrophages and dendritic cells, and low numbers of Th1 cells (all P<0.001). Genes for epithelial mesenchymal transition (EMT), myogenesis, apical junction, transforming growth factor (TGF)-β signaling, and angiogenesis were enriched in high coagulation score-gastric cancers (all false discovery rate <0.25). In conclusion, gastric cancers expressing higher levels of pro-coagulation genes demonstrate increased angiogenesis, EMT, TGF-β signaling and worse patient prognosis.

Keywords: Coagulation, gastric cancer, prognosis, signaling, angiogenesis, EMT

### Introduction

Hypercoagulable state is the second leading cause of death among patients with advanced cancer, only after organ failure by metastatic disease [1]. It is also known as cancer-associated thrombosis and may manifest as Trousseau's syndrome (thrombophlebitis migrans) or thromboembolic events such as venous thromboembolism (VTE) or stroke. The mechanism of cancer-associated thrombosis is not fully understood, but there are several known interactions between tumor cells and host cells that influence the host coagulation cascade [2].

Interestingly, several recent studies have demonstrated that the reverse of this relationship is also true, and that coagulation factors also promote tumor metastasis and cancer progression via various proposed pathways [3, 4]. For example, platelets contribute to metastasis by forming a physical shield around tumor cells which protects them from host natural killer (NK) cells. Platelets have also been shown to promote an epithelial to mesenchymal transition (EMT) in tumor cells, which is associated with an invasive phenotype [5, 6]. Further, thrombin has been shown to increase cancer proliferation, migration and angiogenesis in preclinical models [7, 8]. Cancer cells promote the secretion of von Willebrand Factor from blood endothelial cells [9, 10], which also has been reported to contribute to the process of EMT [11]. With that said, these findings were made in in vitro and in vivo systems, and their clinical relevance remains unclear.

Gene set variation analysis (GSVA) is a well-validated method of characterizing gene expression by organizing genes into sets according to functional pathways, rather than measuring activity of individual genes [12]. This method provides a relevant clinical context for interpreting gene expression data, and our group has extensive experience using the GSVA algorithm to predict clinical outcomes using large transcriptome databases. For example, we found that increased expression of the G2M checkpoint pathway genes in pancreatic cancer is predictive of treatment response and overall survival [13-15]. In patients with hepatocellular carcinoma we found that tumor growth and patient survival correlated with unfolded protein response signaling, which has been implicated in tumorigenesis [16]. Currently, GSVA scoring is widely used to estimate the activity of several pathways in cancer research [17-20].

Here, we hypothesize that higher intra-tumoral expression of pro-coagulation genes predicts increased angiogenesis, cancer cell proliferation, EMT, and poorer survival in gastric cancer patients.

### Materials and methods

### Patients

Transcriptomic as well as clinical demographic data of The Cancer Genome Atlas Stomach

Adenocarcinoma project (TCGA-STAD, n = 375) was obtained through The Genomic Data Commons portal [21, 22]. The Pan-Cancer Clinical Data Resource, which is a standardized dataset of clinical outcomes of patients included in TCGA, was used to obtain information on survival endpoints [23]. A second cohort, GSE-84437 (n = 432, Yoon et al.) [24] was obtained from the Gene Expression Omnibus (GEO) repository and used to validate our findings. All analyses were performed using  $\log_2$ -transformed gene expression.

### Coagulation score

We utilized the gene set "HALLMARK\_COA-GULATION" from the Molecular Signatures Database (MSigDB) hallmark collection [25], which is comprised of genes encoding components of blood coagulation, and calculated a "coagulation score" using GSVA as published previously [15, 19, 20, 26, 27]. A high score represents increased expression of pro-coagulation genes. The median of the score was used as a cut-off to split high vs. low score groups.

Hallmark pathway gene set enrichment analysis

The Gene Set Enrichment Analysis (GSEA) Java application (vers. 4.0) was used to analyze Hallmark collection gene sets in the Molecular Signatures Database (MSigDB). GSEA [28] was used to identify differences in gene expression between the high- and low-score groups as we previously described [29-34]. A false discovery rate (FDR) <0.25 was chosen as the cutoff indicative of statistical significance, as recommended for the GSEA method.

### Tumor immune microenvironment analysis

xCell software was used to quantify the relative abundance of stromal and immune cells in each sample from their transcriptome profile [35], as we previously reported [36-41].

### Other statistical analyses

R software (version 4.0.2) was used for all analyses and figures generated in this study. Each boxplot illustrates the median and interquartile range (IQR). Kruskal-Wallis and Mann Whitney U tests were used for group comparisons, and Spearman's correlation coefficient was used



**Figure 1.** Relationship between the coagulation score and clinical cancer aggressiveness. A. Boxplots of the coagulation score by AJCC T- and N-category, in the TCGA and GSE84437, and AJCC stage and Nottingham histological grade in the TCGA cohort. Kruskal-Wallis and Mann Whitney U tests were used to determine *p* values. B. Kaplan-Meier curves of disease specific (DSS) and overall survival (OS) in the TCGA (n = 368) and OS in the GSE84437 (n = 432) cohorts of Low (blue line) and High (red line) in coagulation score. Log-rank test was used to determine *p* values.

for two factor comparisons. Groups were separated into "high" vs. "low" scores relative to the median score. Survival curves were plotted using the Kaplan-Meier method with log-rank test. *P*-values <0.05 were regarded as statistically significant.

### Results

### Increased expression of pro-coagulation genes predicts advanced gastric cancer stage and poorer prognosis

Transcriptomic data from two large gastric cancer cohorts, TCGA and GSE84437, were analyzed using GSVA, as we have previously de-

scribed [22]. First, we studied the relationship between the intra-tumoral expression of procoagulation genes and clinical aggressiveness, using American Joint Committee on Cancer (AJCC) T-category/N-category/stage, pathological grade, and patient survival in two independent cohorts, TCGA and GSE84437. In TCGA, tumors with high coagulation scores had advanced AJCC T-category and increased likelihood of lymph node metastasis (Figure 1A, P = 0.018 and 0.036, respectively). This association was validated by GSE84437 (P = 0.006and P<0.001, respectively). High scores consistently predicted advanced stage in TCGA (P = 0.006) as well, but did not correlate with pathological grade (P = 0.753).

Consequently, TCGA patients with high coagulation scores also had poorer disease specific survival (DSS) and overall survival (OS) (**Figure 1B**, P = 0.019 and 0.011, respectively). This association between coagulation score and OS was validated by GSE84437 (P = 0.012). These results indicate that intra-tumoral coagulation was significantly associated with larger, more aggressive gastric cancers and with poor patient prognosis. Next, we further investigated whether previously proposed mechanisms have clinical relevance in large gastric cancer patient cohorts.

### A high coagulation score correlated with angiogenesis in gastric tumors

Multiple previous studies have demonstrated an association between pro-coagulation factors and angiogenesis in various tumors [2, 42]. To this end, we investigated the relationship between the coagulation and angiogenesis scores, which were analyzed by the same methodology, GSVA. We found that coagulation strongly correlated with angiogenesis, but not with tissue factor (TF) expression (Figure 2A; r = 0.810 and 0.838, respectively, both P< 0.01 for angiogenesis). Gastric cancers with high coagulation scores demonstrated significant enrichment of angiogenesis gene sets in both the TCGA and GSE84437 cohorts (Figure 2B; normalized enrichment score (NES) = 2.24 and 1.71, respectively). Congruently, high-coagulation gastric tumors contained significantly higher proportions of angiogenesis-related vascular cells, including endothelial cells, microvascular endothelial cells, pericytes, and lymphatic endothelial cells across both cohorts (Figure 2C; all P<0.001). Tumors with higher coagulation scores also demonstrated increased expression of genes related to angiogenesis (von Willebrand Factor (vWF) and CD31), endothelial cells, and vascular stability (VEcadherin, ANGPT1, TIE2, TIE1, JAM2 and Claudin 5) (Figure 2D and 2E; all P<0.001). In summary, the expression of coagulation and angiogenesis gene sets were closely linked in malignant gastric tumors.

# High coagulation score did not correlate with immune cell infiltration or host immune response

Given that coagulopathy often occurs with vascular injury in clinical practice [43], we next investigated the relationship of coagulation and immune response in the tumor microenvironment. We found that increased expression of pro-coagulation genes was not predictive of either tumor infiltrating lymphocyte (TIL) regional fraction score nor interferon (IFN)-y activity in the TCGA cohort (Figure 3A). Gastric cancers with high coagulation scores did not enrich IFN-α nor IFN-y signaling by GSEA in the GSE84437 cohort (Figure 3B). Next, we investigated the relationship between coagulation and immune cell infiltration using xCell algorithm. Although high coagulation gastric cancers contained higher proportions of dendritic cells (DC) and M1 macrophages (all p<0.001) and relatively lower proportions of T helper type 1 (Th1) cells, no other immune cells consistently correlated with coagulation score across both TCGA and GSE96058 cohorts (Figure 3C). Therefore, we conclude that expression of procoagulation genes does not correlate with the host immune response nor immune cell infiltration in the gastric cancer tumor microenvironment.

## Higher expression of pro-coagulation genes correlated with low mutation load in gastric tumors

Since mutation load is known to correlate with aggressiveness of cancer [41, 44-47], we examined the association between the coagulation score and tumor mutation load. Although low- and high-coagulation score groups did not show significant differences with regard to intratumor heterogeneity nor homologous recombination deficiency (**Figure 4A**; P = 0.134 and 0.243, respectively), gastric cancers with high coagulation scores demonstrated high levels of SNV neoantigens, fraction of genome altered, and both silent and non-silent mutation rate in TCGA (**Figure 4B**; P<0.001, P<0.001, P = 0.022, and P = 0.011, respectively).

### Coagulation score did not correlate with cell proliferation signaling in gastric cancer

Given that highly proliferative cancer results in worse survival [13, 48, 49], we next queried whether a high coagulation score predicts increased expression of cell proliferation pathways. The score did not correlate with *MKI*67 expression, a cell proliferation marker, in two cohorts. None of the cell proliferation-related gene sets in Hallmark collection, including



### Coagulation worsens gastric cancer



**Figure 2.** Relationship between the coagulation score and angiogenesis-related genes in the GSE84437 and TCGA cohorts. (A) Scatter plots of the Spearman's rank correlation test (r) between coagulation score and angiogenesis score or *TF* (tissue factor) gene expression. (B) Angiogenesis gene set enrichment plots with false discovery rate (FDR) and normalized enrichment score (NES). Classical gene set enrichment analysis (GSEA) method was used to determine FDR and NES. Boxplots of the comparison between low and high coagulation score in (C) angiogenesis-related cells; endothelial cells, microvascular endothelial cells (mvE), lymphatic endothelial cells (lyE), and pericytes. (D) Endothelial cell markers; CD31, von-Willebrand factor (*VWF*), (E) vascular stability-related genes; *TIE1*, *TIE2*, *ANGPT1*, *E*-cadherin, *Claudin5 and JAM2*. P value was analyzed with Mann Whitney U test.



**Figure 3.** Association between the coagulation score and immune function and cell fractions in gastric cancer. A. Box plots of tumor infiltrating lymphocytes (TIL) regional fraction score and interferon (IFN)- $\gamma$  response in the TCGA cohort. B. GSEA plots with FDR and NES for the IFN- $\alpha$  and IFN- $\gamma$  signaling gene sets in the GSE84437 cohort. The classical GSEA method was used to determine FDR and NES. C. Boxplots of infiltrating immune cells including CD8<sup>+</sup> T cells, CD4<sup>+</sup> memory T cells, T helper type 1 (Th1), dendritic cells (DCs), M1 macrophages, regulatory T cells (Tregs), and Th2 cells, and M2 macrophages, by low and high coagulation score in the TCGA and GSE84437 cohorts. *P* value was analyzed with Mann Whitney U test.



Figure 4. Association of the coagulation score with intratumor heterogeneity, homologous recombinant deficient (HRD), and mutation-related score in the TCGA cohort. (A) Box plots of intratumor heterogeneity, HRD, and (B) mutation-related score, including silent and non-silent mutation rate, fraction altered, and single nucleotide variant (SNV) neoantigens by low and high coagulation score groups. *P* value was analyzed with Mann Whitney U test.



**Figure 5.** Correlation of the coagulation score with *MKi67* expression and cell proliferation-related gene sets score in the TCGA and GSE84437 cohorts. A. Scatter plots between the coagulation score and *MKi67* gene expression. B. Correlation plots between coagulation score and cell proliferation-related gene sets score, including MITOTIC spindle E2F targets, G2M checkpoint, MYC targets v1, and MYC targets v2. A Spearman's rank correlation test (r) was used where blue indicates positive and red indicates negative correlation.

those for mitotic spindle, MYC Targets v1 and v2, G2M Checkpoint, and E2F Targets, were highly expressed in high-coagulation gastric cancers (Figure 5A and 5B).

Genes for transforming growth factor (TGF)-β signaling epithelial mesenchymal transition (EMT), and myogenesis, apical junction, were highly expressed in high-coagulation gastric cancers

Finally, we examined the correlation between coagulation and pathways related to transition and transformation of malignant cells, an important factor in tumor progression. The high-coagulation score group significantly enriched EMT, as evidenced by the highest NES across both cohorts (**Figure 5**; NES = 2.25 and 1.75, respectively). Furthermore, they also demonstrated higher expression of genes en-

coding myogenesis, apical junction, and  $TGF-\beta$  signaling across both cohorts (**Figure 6**).

### Discussion

Bleeding tendency and thrombotic events are very common in patients with advanced cancer [43, 50]. Most cancer cells constitutively express tissue factor (TF) on the cell surface that generates thrombin by combining with factor VIIa to activate factor IX to IXa and X to Xa on the activated platelet surface, resulting in the conversion of prothrombin to thrombin. Thus, malignancy initiates a vicious cycle in which greater tumor burden supplies greater thrombin and platelet-tumor interaction. However, it has not been fully elucidated whether tumor expression of these coagulation cascade genes augments the malignant biology of cancer.



**Figure 6.** Gene Set Enrichment Analysis (GSEA) demonstrating enrichment gene sets in high coagulation score gastric cancer in the GSE84437 and TCGA cohorts. Epithelial mesenchymal transition (EMT), myogenesis, apical junction, and *TGF*-β signaling gene sets demonstrated significant enrichment consistently in both cohorts. NES and FDR were determined with the classical gene set enrichment analysis (GSEA) method.

In recent years, the use of large transcriptome databases and gene set analysis has facilitated massive growth in the field of translational research [51]. Additionally, the ability to digitally dissect tumor samples and determine their cellular composition allows researchers to gain a broader picture of the tumor microenvironment [35, 52, 53]. Here, we demonstrated the clinical relevance of intra-tumoral pro-coagulation gene expression in gastric cancer. Using multiple large cohorts of patients with gastric cancer, we found that intratumoral expression of pro-coagulation genes was associated with angiogenesis and EMT, but not consistently with cell proliferation.

Moreover, we discovered that gastric cancers with higher expression of coagulation pathways did not demonstrate enrichment of cell proliferation- nor immune-related gene sets, which have previously been reported to be important for tumor aggressiveness. However, our results indicate that gastric cancers with high coagulation scores did demonstrate EMT, angiogenesis, apical junction, and *TGF*-β signaling, which play key roles in the transformation and migration of malignant cells [54]. The requirement of angiogenesis for tumor growth and metastasis is well recognized [55]. Mary et al. reported that thrombin, one of the major players in coagulation signaling, activates tumor cell adhesion to platelets, endothelial cells, and subendothelial matrix proteins, which enhances tumor cell growth, and stimulates tumor cell angiogenesis [56]. Our results show that although TF gene expression did not correlate with angiogenesis, coagulation score strongly correlated with angiogenesis score.

Interpreting the clinical relevance of any single gene out of its biological context is challenging and prone to error [57]. Assays which measure gene expression within sets organized according to their function provide a more comprehensive and relevant analysis [58-61]. Thus, the GSVA method is utilized in this study to provide a global picture of the coagulation pathway within gastric cancers. It is difficult to elucidate these pathways in *in vitro* or *in vivo* studies because the complex interactions within the tumor microenvironment are not easily replicated in *in vitro* experiments, and difficult to isolate in *in vivo* studies. Our results indicate that previously reported relationships between coagulation signaling and tumor growth also occur within tumors. The nature of this type of study does not lend itself to determining causality, but supports previously made observations that factors within the coagulation cascade subsequently promote angiogenesis, EMT and TGF-beta signaling.

By using transcriptomic expression across multiple cohorts, we showed that intratumoral coagulation is linked to outcomes in gastric cancer. Although prognostic biomarkers have been developed in gastric cancer, none have yet been clinically applied. Although further investigation is required, the coagulation score may prove to be a useful biomarker for gastric cancer patients. Additional studies are needed to determine the clinical utility of the intratumoral coagulation score.

There are several limitations to interpreting this study. This is a retrospective study that does not allow us to account for confounding factors that may influence clinical outcomes. Patient outcome is naturally affected by multiple clinical factors, such as comorbidities, functional status, surgical and systemic treatment, for which limited information is available in a transcriptomic database. Additionally, this study does not provide original data on the mechanisms that underlie the associations of tumor coagulation with outcomes and various tumor features of gastric cancer.

In summary, the coagulation score provides a method to quantify the intra-tumoral expression of coagulative factors, which is associated with angiogenesis, EMT and TNF-beta signaling and worse outcomes of gastric cancer patients.

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### Disclosure of conflict of interest

None.

### Abbreviations

AJCC, American Joint Committee on Cancer; FDR, false discovery rate; GSEA, gene set enrichment analysis; GSVA, gene set variation analysis; NES, normalized enrichment score; TCGA, The Cancer Genome Atlas.

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