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Prognostic and predictive value of angiogenesis-associated serum proteins for immunotherapy in esophageal cancer

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ABSTRACT

Background Immune checkpoint inhibitors (ICIs) have significantly improved patient survival in multiple cancers. However, therapy response in esophageal cancer is limited to subgroups of patients and clinically useful predictive biomarkers are lacking.

Methods We collected a series of plasma samples from 91 patients with esophageal cancer before and after ICI treatment. The Olink Immuno-Oncology panel (92 proteins) with proximity extension assays was used to detect the dynamic changes in plasma and potential biomarkers associated with treatment outcomes. We screened all survival-related proteins and established a risk score model to better predict the prognosis and treatment response in patients with esophageal cancer immunotherapy.

Results We found that 47 out of 92 quantified proteins had significant changes in plasma levels during ICI treatment ($p<0.050$), and these changed proteins were involved in immune-related reactions, such as intercellular adhesion and T-cell activation. Notably, the baseline levels of three angiogenesis-related proteins (IL-8, TIE2, and HGF) were significantly associated with the survival outcomes of patients treated with ICIs ($p<0.050$). According to these prognostic proteins, we established an angiogenesis-related risk score, which could be a superior biomarker for ICI response prediction. In addition, antiangiogenic therapy combined with ICIs significantly improved overall survival compared with ICI monotherapy ($p=0.044$).

Conclusions An angiogenesis-related risk score based on three proteins (IL-8, TIE2, and HGF) could predict ICI response and prognosis in patients with esophageal cancer, which warrants verification in the future. Our study highlights the potential application of combining ICIs and antiangiogenic therapy and supports Olink plasma protein sequencing as a liquid biopsy method for biomarker exploration.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The immune checkpoint therapy has revolutionized the treatment of esophageal cancer. However, additional efforts should be made to investigate effective biomarkers for optimal patient selection.

WHAT THIS STUDY ADDS

⇒ We identified angiogenesis-associated serum proteins, IL-8, TIE2, and HGF, which were associated with the prognosis of esophageal cancer patients treated by immunotherapy. Therefore, we established an angiogenesis-associated risk score containing these three proteins, which represent the immunosuppressive status and better predict the immune checkpoint inhibitor response in patients with esophageal cancer.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The angiogenesis-associated risk score exhibited superior predictive value in predicting the efficacy and prognosis of patients with esophageal cancer to immunotherapy, which may facilitate the patient selection and outcome prediction of immunotherapy in esophageal cancer. Our results support the application of plasma proteome sequencing for biomarker exploration and provide important insight for combining immunotherapy and antiangiogenic therapy in the future.

INTRODUCTION

Esophageal cancer is a high-incidence and idiopathic tumor in China and is the seventh most common cancer and the sixth most common cancer-related cause of death in the world.[1] Most patients are diagnosed at an advanced stage, with a 5-year survival rate of less than 5%.[2] Immune checkpoint inhibitors (ICIs) targeting anti-CTLA-4 and anti-programmed cell death protein 1 (anti-PD-1) have become one of the most promising approaches in cancer immunotherapy.[3] Several immunotherapeutic clinical studies have focused on esophageal cancer, revealing that ICIs have dramatically improved the survival of patients with esophageal cancer.[4 5] However, the objective response rate (ORR) of ICI monotherapy in esophageal cancer was

only 10%–20%,[6] and only a subset of patients could derive benefits from ICI therapy, underscoring the urgent need for effective biomarkers for optimal patient selection. Several immunotherapeutic biomarkers have been investigated in esophageal cancer, such as tumor mutational burden (TMB),[7] PD-L1 expression,[8] and gene expression profile (GEP).[9] However, most of them have poor prediction effects. These approaches have several limitations, including limited accessibility and temporal and spatial heterogeneity, emphasizing the urgent need to explore new optimal predictors.

Growing evidence has shown that peripheral blood biomarkers, such as exosomes,[10] circulating tumor DNA,[11] cell-free DNA,[12] cell-free RNA,[13] and the detection of proteins in peripheral blood, that play an 'executive' role in cell physiological function, may provide a large amount of information to represent the tumor immune microenvironment. Plasma-based protein biomarker tests are used to predict prognosis and monitor the efficacy of treatment. Based on the latest Olink proteome technology, a large-scale plasma proteome analysis was recently conducted on a cohort of patients with cancer before receiving ICI treatment, and leukemia inhibitory factor LIF was screened as a new peripheral blood protein marker for predicting the efficacy of ICIs.[14] Compared with traditional biomarkers, such as PD-L1 expression status, circulating biomarkers offer a promising alternative to address the pitfalls associated with the analysis of tumor tissue, such as temporal and spatial tumor heterogeneity. This method provides a unique and previously unexplored view of the dynamic changes in the proteins present in plasma.

To study the dynamic changes in plasma protein during ICIs and surrogate the predicted biomarkers for esophageal cancer, we used Olink to detect the plasma protein landscape before and after ICIs in patients with esophageal cancer. We found that cytokine–cytokine receptor interaction, T-cell activation, and other immune-related pathways were significantly upregulated after immunotherapy. Therefore, we constructed an angiogenesis-associated signature based on survival-related plasma proteins for predicting ICIs, implying the potential utility of antiangiogenic therapy plus ICIs in the future.

METHODS

The study design is shown in figure 1. A brief description is given below.

Study population and data collection

This retrospective study recruited 91 patients with esophageal cancer who failed standard therapy and received immunotherapy or immunotherapy combined with antiangiogenic targeted therapy at the Department of Gastroenterology, Peking University Cancer Hospital from January 2016 to March 2021. The patients who received treatment with any anticancer chemotherapy within 8 weeks before the first ICI dose were excluded. We analyzed a series of pretreatment and post-treatment plasma samples with a median of 35 days between samples (range: 14–134, SD: 22). We used proximity extension assays (PEAs) to analyze 158 plasma samples (67 matches) from 91 patients with esophageal cancer. All blood samples were collected in EDTA tubes. Blood samples were centrifuged at 3000×g for 10 min, and plasma samples were separated at 1800×g for 8 min. Plasma samples were stored at −80°C until further analysis.

Patient clinical data included age at treatment initiation, sex, Eastern Cooperative Oncology Group (ECOG) stage, pathological type, pathological stage, the best response to treatment, date of disease progression (PD), and/or date of death. Tumor efficacy was measured by imaging or physical examination according to RECIST V.1.1 and iRECIST. Response was defined as complete

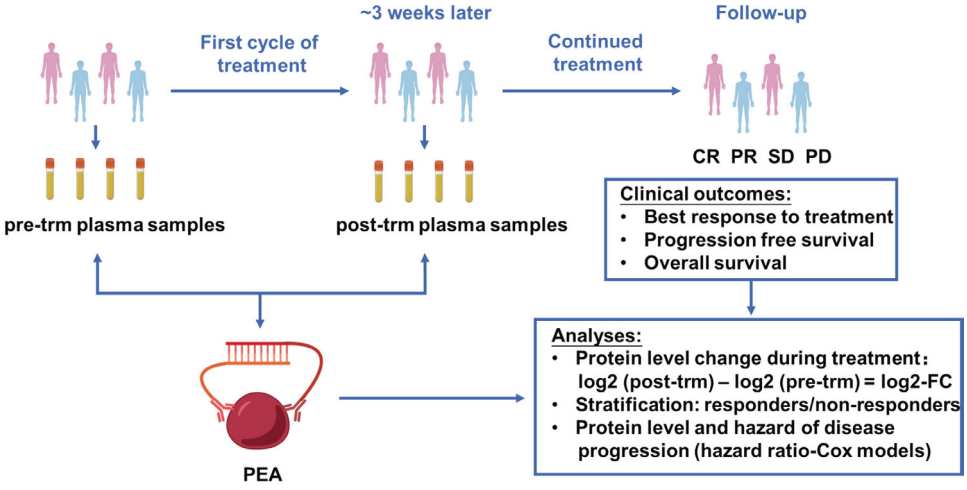

Figure 1 Study workflow. Plasma samples were collected pretreatment at baseline and during treatment with ICIs in the study cohort. Treatment outcomes were followed prospectively. CR, complete response; ICIs, immune checkpoint inhibitors; log2-FC, log2-fold-change; PD, progressive disease; PEA, proximity extension assays; PR, partial response; post-trm, after the first treatment cycle; pre-trm, pretreatment; SD, stable disease.

response (CR), partial response (PR), or stable disease (SD) ≥24 weeks. Non-responders (NR) were defined as patients with PD or SD<24 weeks. The date of progression was recorded at the time of diagnostic imaging. Progression-free survival (PFS) was defined as the time from the start of treatment until the date of PD or death. Overall survival (OS) was defined as the time from the start of treatment to the date of death.

Serum protein expression assay

The plasma samples were additionally analyzed using PEAs at the clinical biomarkers' facility at SciLifeLab, Uppsala, Sweden. In total, 92 human protein biomarkers (online supplemental table S0, additional file 1) were measured using the Olink Immuno-Oncology I panel (www.Olink.com). The final assay readout is presented in normalized protein expression values, which is an arbitrary unit on a log2-scale where a higher value corresponds to a higher protein expression.

Measurements were performed using 1 µL of each sample. In PEAs, a pair of oligonucleotide-labeled antibodies bind to their targeted protein in the samples. If the probes are in close proximity, the oligonucleotides will hybridize in a pairwise manner. The addition of a DNA polymerase leads to a proximity-dependent DNA polymerization event, generating a unique PCR target sequence. The target sequence was detected and quantified using a microfluidic real-time PCR instrument (Biomark HD, Fluidigm). Data are then quality controlled and normalized using an internal extension control and an interplate control to adjust for intrarun and inter-run variation.

Functional enrichment analyses

The differential serum protein expression before and after treatment was tested using the Wilcoxon rank-sum test. Proteins with a p value less than 0.05 were selected as subjects for enrichment analyses. The enrichment analysis was performed on the Kyoto Encyclopedia of Genes and Genomes (KEGG) as well as Gene Ontology Biological Process (GO) to categorize the biological functions of genes found in these three modules using the "cluster Profiler" package in R.

Construction of the angiogenesis-associated prognostic model

A risk score was calculated for each sample based on the expression level of 3 angiogenesis-associated serum proteins (IL-8, TIE2, and HGF) in the Cox model. The regression terms for the Cox model were fitted by the 'predict' function (type="risk") from the "survival" package in R. The risk scores of patients according to the expression of the model proteins and the formula is risk score=(0.50713)×IL-8+(0.38762)×TIE2+(−0.09996)×HGF. All samples were divided into high-risk and low-risk groups by the median risk score. Time-dependent receiver operating characteristic (ROC) curves were plotted using the 'survival ROC' package to calculate the area under the ROC curve (AUC) value at 6, 12, and 18 months of the angiogenesis-associated prognostic model.

Prognostic analysis

The expression profiles of 92 serum proteins from patients in our cohort with survival information were analyzed via univariate Cox regression analysis. Variables with both log-rank and likelihood p<0.05 on univariate analysis were entered into the multivariate Cox model. Next, the "step" function in the survival "package" with the option direction="both" works by comparing Akaike's information criterion (AIC) improvements from dropping each candidate variable and adding each candidate variable between the upper and lower bound regressor sets supplied from the current model and by dropping or adding the one variable that leads to the best AIC improvement (smallest AIC).

Statistical analysis

Quantitative variables are presented as the median±IQR, and categorical variables are presented as proportions. The Wilcoxon test was used for non-normally distributed variables. Fisher's exact test was used for comparisons between two categorical variables. Survival analysis was performed using a Kaplan-Meier survival plot, and the log-rank test p value was calculated. All statistical analyses were performed with GraphPad Prism statistical software V.8.0 (GraphPad Software) and R statistical software V.3.6.2 (R Project for Statistical Computing). A p<0.05 was considered statistically significant, and tests were two tailed.

RESULTS

Patient cohorts and plasma samples

We enrolled 91 patients with esophageal cancer treated with ICIs at our center, of whom 9 received anti-PD-1/PD-L1 plus anti-CTL-4 ICIs, 64 received ICI monotherapy, and 18 received ICI plus antiangiogenic therapy (figure 2A, table 1). Eighty-four (92.3%) patients were diagnosed with esophageal squamous cell carcinoma cancer. According to RECIST V.1.1, 24 patients were identified as responders (R), and 65 patients were identified as NR. The other clinical characteristics are listed in table 1. Responders and NRs did not have differences in clinical characteristics at baseline (table 1).

Therefore, we collected the baseline peripheral blood of these 91 patients and post-treatment blood from 67 patients to perform paired plasma proteomic assays before and after treatment.

Changes in plasma protein levels before and after immunotherapy for esophageal cancer

We first intended to explore the influence of ICIs on the plasma proteome, regardless of response to treatment. We found that the plasma levels of 47 of 92 analyzed proteins significantly changed in response to anti-PD-1 treatment. Specifically, 2 were downregulated after

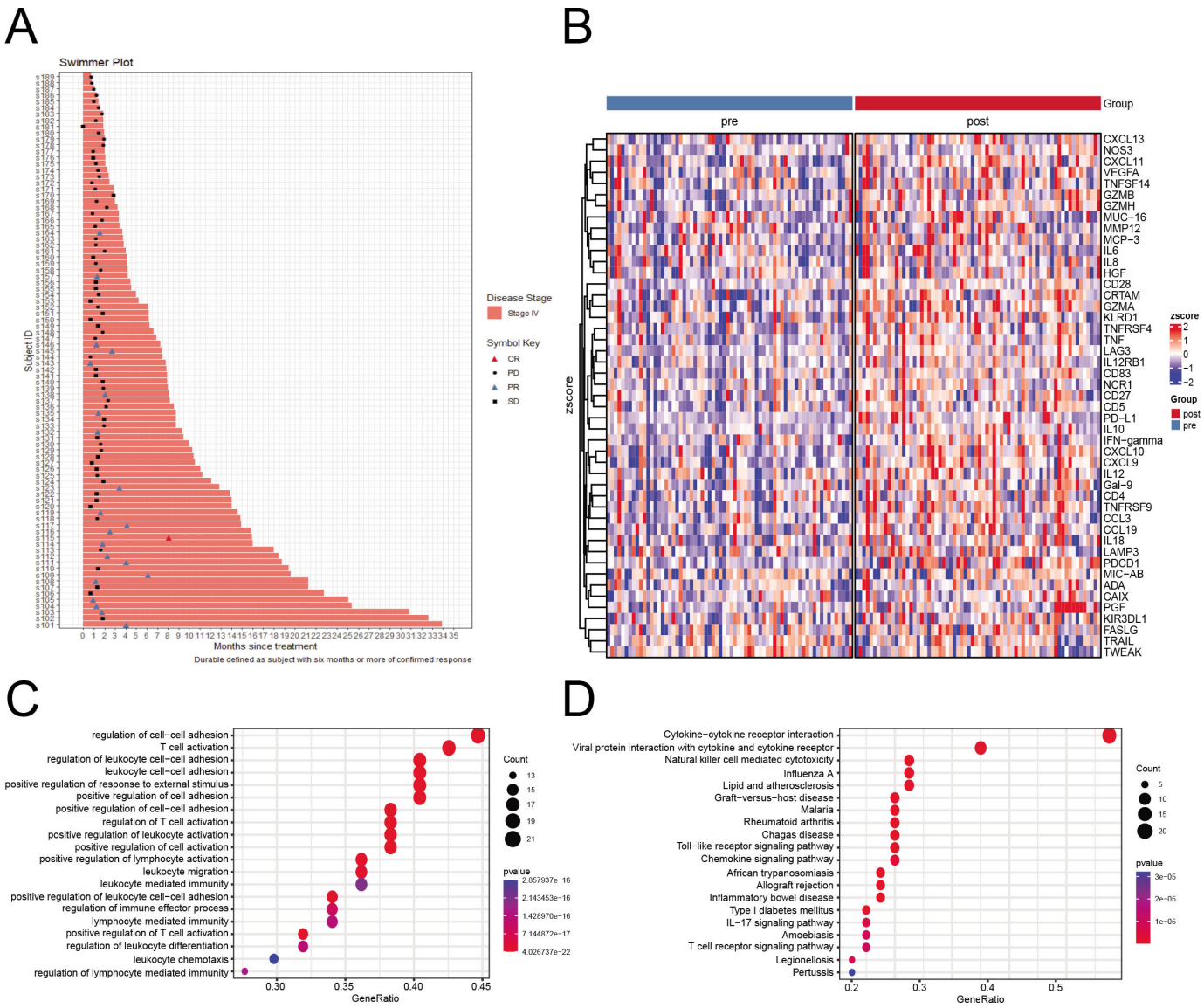

Figure 2 Treatment outcomes and changes in serum proteome of all patients included in the study. (A) Swimmer's plot showing clinical course and outcome events of each patient in the study cohort. (B) Heatmap depicting the relative expression levels of plasma proteins before (pre) and after (post) treatment in esophageal cancer. Each column of the heatmap shows a patient, while the rows represent different molecules. Color scale in the heatmap represents scores standardized across rows. (C) GO analysis of change in protein plasma levels during treatment. (D) KEGG analysis of change in protein plasma levels during treatment. CR, complete response; GO, gene ontology; KEGG, the Kyoto Encyclopedia of Genes and Genomes; PD, progressive disease; PR, partial response; SD, stable disease.

treatment (TRAIL and TWEAK), and the remaining 45 were upregulated after treatment ($p<0.05$, figure 2B, online supplemental table S1, additional file 1). Functions enrichment and signaling pathway analyses were performed based on the GO and KEGG databases to further explore the biological functions of the alternating proteins (figure 2C,D). Some signaling pathways from the GO set were significantly upregulated after ICI therapy, including the regulation of cell–cell adhesion, T-cell activation, and the regulation of leucocyte cell–cell adhesion (figure 2C). Similarly, according to KEGG terms, the changed proteins during ICI treatment were enriched in cytokine–cytokine receptor interactions, viral protein interaction with cytokine and cytokine receptors, and natural killer cell-mediated cytotoxicity (figure 2D).

Stratified analysis of changed plasma proteins during ICI treatment based on patient response

We further explored the changed plasma proteins during ICI treatment in responders (R) and NR. For responders (R), 22 of the 92 analyzed plasma proteins were upregulated after ICI treatment ($p<0.05$, online supplemental figure S1A and table S2, additional file 1). For NR, three plasma proteins (TRAIL, TWEAK, and ICOSLG (Inducible T cell costimulatory ligand) were decreased and the remaining 47 proteins were increased after ICI treatment

Table 1 Baseline clinical characteristics of patients with esophageal cancer

(A) Patients and clinical characteristics	Overall
n	91
Age (median (IQR))	58.52 (54.23–64.29)
Gender (%)	
Male	75 (82.4)
Female	16 (17.6)
ECOG (%)	
0	19 (20.9)
1	69 (75.8)
2	3 (3.3)
Treatment (%)	
Anti-PD-1 and anti-CTL4 ICIs	9 (9.9)
ICI monotherapy	64 (70.3)
ICI plus antiangiogenic treatment	18 (19.8)
Pathological type (%)	
Squamous carcinoma	84 (92.3)
Other	7 (7.7)
Stage (%)	
IV	91 (100.0)
Best response to treatment (%)	
Response=R	24 (26.4)
Non-response=NR	65 (71.4)
NA	2 (2.2)

(B) Patients and clinical characteristics	NR	R	P value	
n	65	24		test
Age (median (IQR))	57.86 (53.11–63.33)	61.81 (57.25–65.06)	0.067	Nonnorm
Gender (%)			0.542	Fisher's exact
Male	52 (80.0)	21 (87.5)		
Female	13 (20.0)	3 (12.5)		
ECOG (%)			0.376	Fisher's exact
0	11 (16.9)	7 (29.2)		
1	51 (78.5)	17 (70.8)		
2	3 (4.6)	0 (0.0)		
Treatment (%)			0.739	Fisher's exact
Anti-PD-1 and anti-CTL4 ICIs	7 (10.8)	2 (8.3)		
ICI monotherapy	47 (72.3)	16 (66.7)		
ICI plus antiangiogenic treatment	11 (16.9)	6 (25.0)		
Pathological type (%)			0.669	Fisher's exact
Squamous carcinoma	59 (90.8)	23 (95.8)		
Other	6 (9.2)	1 (4.2)		
Stage (%)			NA	
IV	65 (100.0)	24 (100.0)		
Best treatment response (%)			NA	
Complete response	0 (0.0)	1 (4.2)		
Partial response	0 (0.0)	21 (87.5)		
Stable disease	24 (36.9)	2 (8.3)		

Continued

Table 1 Continued

(B) Patients and clinical characteristics	NR	R	P value	
n	65	24		test
Progression disease	41 (63.1)	0 (0.0)		
Progression-free survival, median (months)	1.8 (95% CI 1.5 to 2.1)	11.4 (95% CI 3.8 to 19.1)	<0.001	Log-rank
Overall survival, median (months)	6.6 (95% CI 3.4 to 9.9)	18.4 (95% CI 13.5 to 23.3)	<0.001	Log-rank

(A) Overall patients table. (B) Stratificational patients table.
P values are obtained with non-norm, t, Wilcoxon, Fisher, or log-rank test.
ECOG, Eastern Cooperative Oncology Group Performance Status score; ICIs, immune checkpoint inhibitors; ICOSLG, Inducible T cell costimulatory ligand; NA, not applicable; NR, non-responders; R, responders.

(p<0.05, online supplemental figure S1D and table S3, additional file 1). These significantly changed proteins were mainly enriched in immune-related biological pathways, such as intercellular adhesion, T-cell activation, and cytokine-cytokine receptor interaction, regardless of patient's response (online supplemental figure S1B,C and E,F). Specially, we found that CD5 and IL-15 were elevated after treatment only in responders, while 30 plasma proteins were detected to be altered in NRs (online supplemental figure S2).

Protein plasma levels can predict OS and PFS

To explore the utility of plasma for ICI prediction, we analyzed the association between pretreatment plasma protein levels and survival outcomes. Univariate Cox analysis showed that 7 protein plasma levels (TNFRSF12A, CD83, ICOSLG, CD5, TRAIL, TNFRSF21, and DCN) were associated with superior OS in patients receiving treatment, whereas 17 proteins (CSF-1, TIE2, HGF, ANGPT2, ADA, VEGFA, IL-8, PDGF-subunit-B, CXCL11, MCP-3, TNFSF14, ANGPT1, IL-7, IL-6, MMP12, EGF, and CD40-L) were associated with unfavorable OS (p<0.05; online supplemental table S4, additional file 1).

When analyzing the PFS endpoint, we detected six plasma proteins related to shortened PFS (p<0.05; online supplemental table S5, additional file 1): interleukin-8 (IL-8), angiopoietin-1 receptor (TIE2), monocyte chemotactic protein 1 (MCP-1), hepatocyte growth factor (HGF), C-C motif chemokine 20 (CCL20), and tumor necrosis factor (TNF). Interestingly, three proteins, IL-8, TIE2, and HGF, were significantly associated with both shorter OS (IL-8: HR=1.920, 95% CI: 1.438 to 2.565, p<0.001; TIE2: HR=3.212, 95% CI: 1.259 to 8.198, p=0.015; HGF: HR=2.318, 95% CI: 1.144 to 4.698, p=0.022; online supplemental table S4, additional file 1) and PFS (IL-8: HR=1.761, 95% CI: 1.354 to 2.292, p<0.001; TIE2: HR=2.326, 95% CI: 1.051 to 5.150, p=0.039; HGF: HR=2.010, 95% CI: 1.083 to 3.732, p=0.028; online supplemental table S5, additional file 1). These proteins have been revealed to promote tumor angiogenesis functions,[15–17] suggesting that angiogenesis-related factors may be a possible cause of poor prognosis in patients with esophageal cancer.

Establishment of a risk score related to angiogenesis-associated proteins

We next performed time-dependent ROC curve analysis according to these three plasma proteins and found that single factors could obtain a modest predictive effect for OS (figure 3A–C). We sought to integrate these three factors into a prediction model and named them "angiogenesis-associated risk scores" for their functions involved in tumor angiogenesis. Interestingly, the angiogenesis-associated risk score achieved better prediction efficacy, with a 6-month AUC of 0.863, and an 18-month AUC of 0.716 (figure 3D).

Importantly, this model also has a good predictive effect on the treatment response of patients with esophageal cancer. The AUC of this risk score for predicting ICI response reached 0.822 (figure 3H), which is superior to the predictive efficacy of single angiogenesis-associated proteins for treatment response (figure 3E–G). In addition, we also analyzed the association between PD-L1 expression and ICI treatment outcomes and found that PD-L1 expression was not associated with the ICI treatment outcomes of esophageal cancer (online supplemental figure S3). These results confirmed that the angiogenesis-associated risk score was a good predictor of both prognosis and treatment response in patients with esophageal cancer immunotherapy.

The angiogenesis-associated risk score is associated with immunosuppression

We investigated the association of the angiogenesis-associated risk score and other plasma proteins. We divided the patients into two groups according to the angiogenesis-associated risk score. We performed a correlation analysis of the risk score and all plasma protein levels (figure 4, online supplemental table S6, additional file 1). IL-6, IL-8, MMP12, CSF-1, HGF, ANGPT1, and LAP-TGF-beta-1 were strong risk score correlation proteins, and only TRAIL was a negatively correlated factor. Notably, among the strongly associated factors, MMP12 and CSF-1, are both associated with the chemotaxis of macrophage macrophages, especially M2-type macrophages.[18–20] IL-8 and IL-6 are considered immunosuppressive factors secreted by myeloid-derived suppressor cells (MDSCs) and

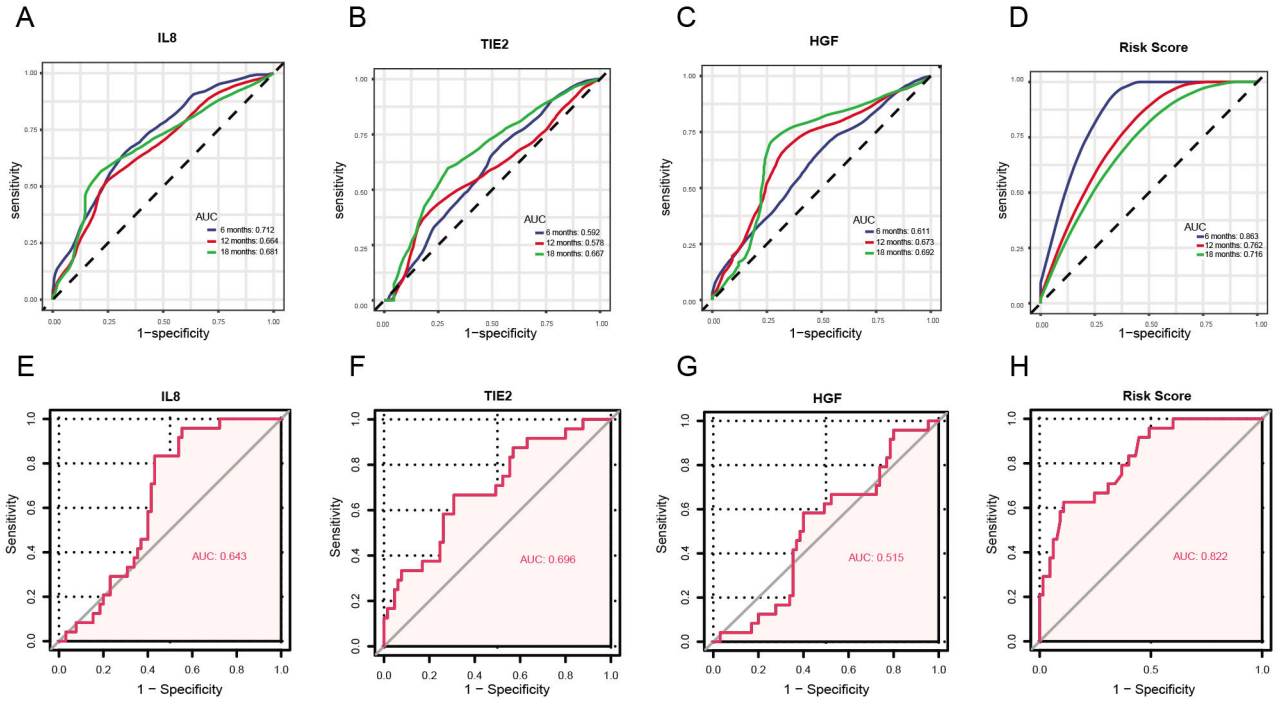

Figure 3 Analysis of the prognostic and predictive value of the angiogenesis associated proteins. The three angiogenesis-associated proteins (IL-8, TIE2, HGF) were simultaneously included in the Cox proportional hazard model to calculate the risk score, which showed that the angiogenesis-associated risk score was a good predictor of prognosis (D) and treatment response (H) in patients with esophageal cancer immunotherapy, but the single factor effect was not significant (A–C, E–G).

macrophages and are negative prognostic factors of ICIs in melanoma, non-small cell lung cancer, colorectal cancer, gastric cancer, and hepatocellular carcinoma.[21–23] These results suggest that a correlation between the angiogenesis-associated risk score and immunosuppression, partially explaining the predictive value of the risk score.

The influence of antiangiogenic treatment schemes on prognosis

Because several angiogenesis-related serum proteins predict poor immunotherapeutic outcomes, we hypothesize, that targeting antiangiogenic treatment could be a potential combination partner for ICIs. We further grouped the patients into monoICI and ICI plus anti-angiogenesis groups to investigate the impact of combination ICIs on the prognosis of patients with esophageal cancer. Interestingly, we found that ICIs combined with antiangiogenic treatment significantly improved OS compared with ICI monotherapy ($p<0.05$, online supplemental figure S4C,D). In addition, anti-CTLA4 plus anti-PD1/PD-L1 did not considerably improve the OS and PFS of patients with esophageal cancer compared with anti-PD1/PD-L1 monotherapy (online supplemental figure S4A,B). These results further support that the combination of ICIs and antiangiogenic treatment may provide improved clinical benefits for patients with advanced esophageal cancer.

DISCUSSION

In this study, we used the Olink Immuno-Oncology panel to identify plasma biomarkers of response to ICIs in patients with esophageal cancer. By analyzing 92 protein biomarkers in this panel, we identified angiogenesis-associated serum proteins, IL-8, TIE2, and HGF, which were associated with the prognosis of immunotherapy in esophageal cancer. Therefore, we established an angiogenesis-associated risk score containing these three proteins. This risk score could represent the immunosuppressive status and better predict the ICI response.

Currently, multiple biomarkers have been explored to predict ICI response in esophageal cancer, such as TMB,[7] PD-L1 expression,[8] and GEP.[9] However, most of these existing biomarkers do not have good sensitivity or specificity and require biopsy samples, which may be unavailable for some patients with esophageal cancer and may not represent the entire tumor immune microenvironment. To overcome these disadvantages of current biomarkers, liquid biopsy has emerged as an ideal technology for exploring optimal biomarkers.[24] At present, many studies have observed that various peripheral biomarkers, including circulating tumor DNA, exosomes, cell-free DNA, and cell-free RNA have the potential to predict the response and survival of ICIs across multiple cancer types.[25] Beyond these approaches, circulating proteins are attractive factors for detecting the peripheral tumor immune microenvironment, providing important insight for biomarker research. The recently developed methodology, PEA, integrated the specificity of antibody-linked

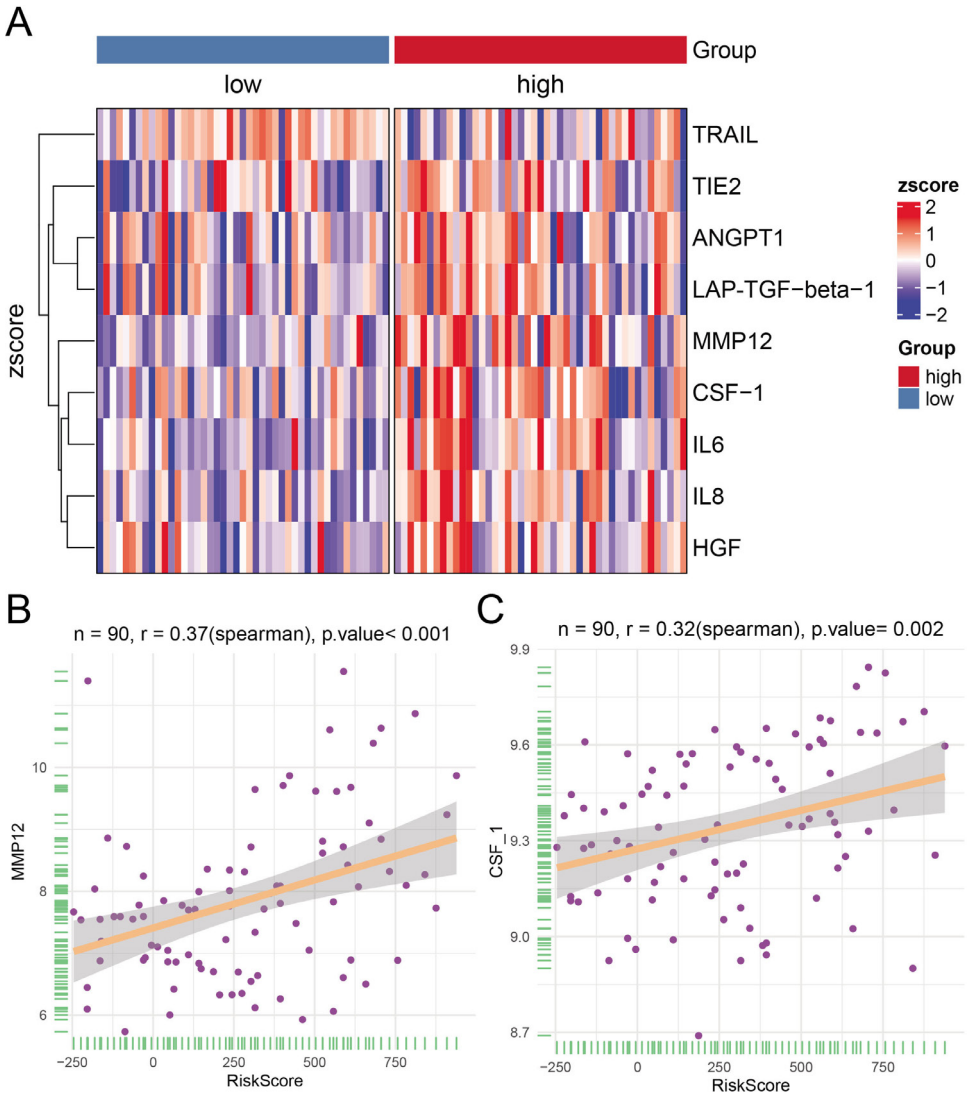

Figure 4 Correlation analysis between risk score and serum proteins. (A) Heatmap of highly correlated proteins by risk group. (B) Correlation analysis between risk score and MMP12; (C) Correlation analysis between risk score and CSF-1.

detection and the sensitivity of the PCR, enabling the precise multiplex detection of low-abundance proteins using only microliter quantities of sera.[26] High-throughput, low-volume PEA technology from Olink has been used in protein marker discovery, drug research and development, translational medicine, etc.[14 27 28] We chose the 92 proteins using the Olink immuno-oncology panel for their involvement in tumor immunity, chemotaxis, vascular and tissue remodeling, apoptosis, metabolism, and autophagy.[29] To our knowledge, this study is the first to describe the plasma proteome landscape of immunotherapy in esophageal cancer, allowing us to better pinpoint the molecular changes that were associated with ICI treatment efficacy from the peripheral perspective.

We observed that several protein baseline levels were associated with OS or PFS in treated patients. Some of these makers have also been reported to harbor predictive value, mainly discovered by transcriptomic sequencing on tumor tissues, such as the ICOSLG[30] and IL-6.[31] However, our findings were based on the perspective of peripheral proteins, indicating the intriguing interactions of the tumor microenvironment and peripheral environment, which deserves further investigation. In addition, it was of interest to see that some of the classical markers of response to PD-1 blockade in melanoma (eg, CXCL9 and CXCL10) are not suitable for use as biomarkers in our group of patients, suggesting that these markers may be useful for target engagement but not response prediction in multiple indications (online supplemental figure S5 and tables S4,S5, additional file 1).

Of particular interest, we focused on three plasma proteins, IL-8, TIE2, and HGF, due to their significant association with both OS and PFS ($p<0.05$, HR>1; online supplemental tables S4,S5, additional file 1). Previous studies have confirmed that these three plasma proteins are related to the state of angiogenesis or immune suppression.[15–17] IL-8 (CXCL8) is a proinflammatory chemokine and a chemoattractant for myeloid leucocytes and induces neutrophil degranulation.[32] IL-8 may also enhance tumor cell growth and metastasis through multiple

mechanisms.[33] Previous reports have shown that patients with high IL-8 have a high level of circulating immunosuppressive monocytic and granulocytic MDSCs, which are associated with poor outcome to ICIs.[34] The second protein, Tie2, is expressed by vascular and lymphatic endothelial cells (ECs), endothelial progenitor cells, and several cancer cells[35] and regulates tumor angiogenesis, growth, and metastasis to distant organs.[36] Recent studies have revealed that the Ang-Tie2 system functions as an angiogenic switch during tumor progression and metastasis.[37] More importantly, HGF is a mesenchymal cytokine and acts as a potent mitogen and pro-motility agent for epithelial cells by activating mitogenesis, morphogenesis, and angiogenesis.[38] C-Met is the HGF receptor on epithelial cells and belongs to the tyrosine kinase family of receptors. Aberrant HGF/C-Met activation is involved in various types of carcinomas including human esophageal squamous cell carcinomas.[39] Serum HGF levels have been evaluated and reported to be related to poor prognosis in pancreatic cancers,[40] cervical cancer,[41] lung cancers,[42] and esophageal squamous cell carcinomas.[43] Based on our findings and previous studies, we introduced an angiogenesis-associated risk score model containing IL-8, TIE2, and HGF as a novel biomarker to predict the efficacy of ICIs. This risk score's predictive value outperformed that of a single angiogenesis-associated protein.

According to the angiogenesis-associated risk score model, patients with esophageal cancer could be divided into two groups: a high-risk score group and a low-risk score group. Interestingly, IL-6, IL-8, MMP12, CSF-1, HGF, ANGPT1, LAP-TGF-beta-1, TIE2, and TRAIL were enriched in the low-risk score group, and most of these factors are related to immunosuppression. For example, CSF1 is a central cytokine that regulates monocyte/macrophage differentiation, survival, and proliferation and promotes their recruitment and polarization.[19] Recent studies have confirmed that ZFP64 transcriptionally regulates CSF1 expression by directly binding to its promoter region, and that secreted CSF1 strongly induces M2 polarization of recruited macrophages, thereby inducing a suppressive tumor immune microenvironment and promoting tumor progression.[44] IL-6,[45] MMP12[46] and TGF-beta[47] are all related to the chemotaxis and functions of M2-type macrophages. These results further confirmed that the angiogenesis-associated risk score could be considered a potential parameter to evaluate the immunosuppressive status of esophageal cancer.

In recent years, multiple efforts have been paid to testing combination ICI regimens, some of them have shown encouraging clinical results in the treatment of esophageal cancer.[48] Our study suggests that aberrant angiogenesis is the most important factor driving the suppressive tumor microenvironment in esophageal cancer, suggesting that ICIs plus antiangiogenic therapy may be an optimal combination strategy.[48] Meng *et al* have reported that camrelizumab plus apatinib achieved a promising ORR of 34.6%,[49] which is numerically higher compared with the results of anti-PD-1 monotherapy and other dual ICI combinations.[50] Our study results also found that ICIs plus antiangiogenic therapy improved the survival of patients with esophageal cancer more than ICI monotherapy, highlighting the effect of antiangiogenic therapy that may favor remodeling of the tumor immune microenvironment and maximize immunotherapy. These results provide clinical evidence supporting immunotherapy combined with antiangiogenic therapy and merit prospective clinical studies for validation in the future.

Our study also has some limitations. First, the study was a retrospective analysis involving a limited sample size of only 91 patients with advanced esophageal cancer. The angiogenesis-associated risk score model could be more convincing if validated in a prospective study with a large sample size in the future. Second, we only detected 92 plasma proteins in the Immuno-Oncology panel. Although these proteins could represent the tumor peripheral microenvironment, some other proteins may also be involved in shaping the microenvironment, which could be detected in the peripheral blood. Additionally, more research is still needed to explore the specific mechanisms by which these plasma proteins regulate tumor angiogenesis and the tumor microenvironment. Third, although we found that patients treated with ICIs plus antiangiogenic agents could derive more clinical benefit, the number of patients treated in combination in this article was not big enough and a prospective randomized study containing a larger sample size should be conducted to verify the superiority of this combination. Moreover, in our combination group, antiangiogenic agents included apatinib, anlotinib, and surufatinib. Other specific angiogenesis-related proteins, such as TIE-2, may also aid in remodeling the angiogenesis status, providing clinical insight for future clinical trial design.

Conclusions

In summary, we identified three angiogenesis-related proteins IL-8, TIE2, and HGF based on Olink proteomics. The angiogenesis-associated risk score exhibited superior predictive value in predicting the efficacy and prognosis of patients with esophageal cancer to immunotherapy, which may help facilitate the individualized management of immunotherapy in esophageal cancer. Our results support the application of plasma proteome sequencing and provide a clinical implication for combinations of ICIs with antiangiogenic therapy in the future.

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