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High TSTA3 Expression as a Candidate Biomarker for Poor Prognosis of Patients With ESCC

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Abstract

Esophageal squamous cell carcinoma is the sixth most lethal cancer worldwide and the fourth most lethal cancer in China. Tissue-specific transplantation antigen P35B codifies the enzyme GDP-D-mannose-4,6-dehydratase, which participates in the biosynthesis of GDP-L-fucose. GDP-L-fucose is an important substrate involved in the biosynthesis of many glycoproteins. Cancer cells are often accompanied by the changes in glycoprotein structure, which affects the adhesion, invasion, and metastasis of cells. It is not clear whether tissue-specific transplantation antigen P35B has any effect on the development of esophageal squamous cell carcinoma. We used an immunohistochemical method to assess the expression of tissue-specific transplantation antigen P35B in 104 esophageal squamous cell carcinoma samples. The results showed tissue-specific transplantation antigen P35B expression was associated with some clinical features in patients, such as age ($P = .017$), clinical stage ($P = .010$), and lymph node metastasis ($P = .043$). Kaplan-Meier analysis and log-rank test showed that patients with esophageal squamous cell carcinoma having high tissue-specific transplantation antigen P35B expression had a worse prognosis compared to the patients with low expression ($P = .048$). Multivariate Cox proportional hazards regression model showed that high expression of tissue-specific transplantation antigen P35B could predict poor prognosis for patients with esophageal squamous cell carcinoma independently. In conclusion, abnormal fucosylation might participate in the progress of esophageal squamous cell carcinoma and tissue-specific transplantation antigen P35B may serve as a novel biomarker for prognosis of patients with esophageal squamous cell carcinoma.

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Keywords
ESCC, glycosylation, TSTA3, Prognosis, biomarker

Abbreviations
AFP, α-fetoprotein; CI, confidence interval; ESCC, esophageal squamous cell carcinoma; FUTs, fucosyltransferases; GMD, GDP-D-mannose-4,6-dehydratase; H-Score, Histoscore; IHC, immunohistochemical; LN, lymph node; OS, overall survival; PH, proportional hazards; ROC, receiver operating characteristic; TSTA3, tissue-specific transplantation antigen P35B

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Introduction

Esophageal carcinoma (EC) remains one of the major cancers worldwide and ranks fourth in terms of cancer incidence in China,[1-3] where the major histologic type is esophageal squamous cell carcinoma (ESCC).[2] Although there is a steady development in surgical treatment and chemotherapy, the 5-year survival rate of patients with ESCC is still low because most patients with ESCC are diagnosed in the mid-late stage of the disease.[2] Therefore, biomarkers for early diagnosis and detection are much required for ESCC.

The formation of cancer cells is often accompanied by changes in the cytomembrane glycoprotein structure.[4] These glycoproteins or glycosylated structures are often secreted or broken down from the cell membrane; some of them have been used as tumor markers for cancer detection and evaluation of treatment efficacy, such as sialyl Lewis X-I antigen, α-fetoprotein (AFP), and carbohydrate antigen-19-9.[5,6] These studies highlight a requirement for GDP-L-fucose by cancer cells. There are 2 major pathways to synthesize GDP-L-fucose: *de novo* and salvage, with the *de novo* one providing 90% of the GDP-L-fucose in the human body.[7] Tissue-specific transplantation antigen P35B (TSTA3), also known as GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-reductase, and GDP-D-mannose-4,6-dehydratase (GMD) participate in the *de novo* way and convert cellular GDP-D-mannose into GDP-L-fucose.[7] GDP-L-fucose is the substrate of several fucosyltransferases (FUTs). Altered expressions of GDP-L-fucose play an important role in tumorigenesis, invasion, and metastasis of various cancers.[6,8,9] In the process of malignant transformation, increased FUTs accelerates the fucosylation of different sialylated precursors. This leads to enhancement of cell adhesion and migration.[10,11] Enhanced expression of TSTA3 has a significant correlation with FUTs expression in colorectal cancer,[12] suggesting that TSTA3 plays critical role in tumor progression.

Previously, we showed missense mutation frequency of *TSTA3* gene was 2% in ESCC.[13] *TSTA3* gene is located in a chromosomal locus (8q24.3) that frequently amplifies in ESCC.[14] These findings suggested that *TSTA3* gene is closely related to the progression of ESCC. However, the expression and mechanism of TSTA3 in ESCC is unclear. In the present study, we have revealed that increased TSTA3 protein expression was associated with clinical stage and lymph node (LN) metastasis in patients with ESCC. More importantly, increased TSTA3 expression could independently predict poor prognosis for patients with ESCC. Our results indicated that abnormal expression of TSTA3 may contribute toward ESCC progression, and TSTA3 may act as a potential novel biomarker for prognosis of patients with ESCC.

Materials and Methods

Patients and Tissue Specimen

All the 104 ESCC tissues used in the current study were collected during our previous study[13] from patients untreated with neoadjuvant therapy at least for 3 years prior to surgery. The tissues were embedded in paraffin to generate tissue chips for large-scale immunohistochemical (IHC) analysis. Overall survival (OS) was defined as the time interval from primary surgery until death due to any cause or terminal time of follow-up without any death events. Clinical staging of ESCC was determined according to the American Joint Commission on Cancer/International Union Against Cancer TNM-staging system, 7th Edition (2010). Details of clinicopathological features such as histological grade, LN involvement, clinical stage, and TSTA3 of IHC-defined classifications are summarized in Supplementary Table S1.The study was approved by the Shanxi Medical University (approval no 2009029). The informed consent was obtained from all participants.

IHC Analysis

The expression of TSTA3 in tumor tissues was examined by IHC staining. The IHC staining was performed using the Envision Labelled Peroxidase System (Maixin, Fuzhou, China). Briefly, sections were incubated with 1:400 diluted anti-TSTA3 (rabbit polyclonal antibody, ab190002; Abcam, Cambridge, UK) for 14 hours at 4°C. After washing with phosphate-buffered saline, the thrombotic microangiopathies were incubated with the 1:20 diluted secondary antibody (MaxVision HRP-Polymer anti-rabbit IHC kit, KIT-5920; Maixin, Fuzhou, China) at 37°C for 20 minutes, followed by detection using the DAB detection kit (Maixin, Fuzhou, China). After being counterstained with hematoxylin and bluing reagent, all section images were scanned at 100×. Cytoplasmic expression of the TSTA3 protein was

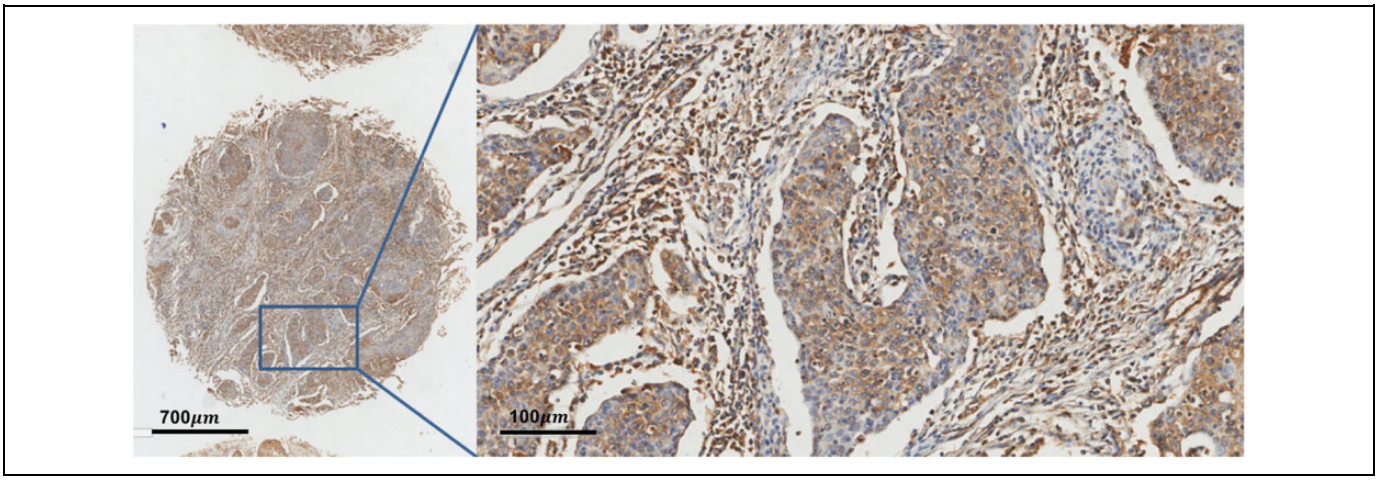

Figure 1. Expression of TSTA3 in ESCC tumor tissues. TSTA3 indicates tissue-specific transplantation antigen P35B; ESCC, esophageal squamous cell carcinoma.

determined using fully automatic digital pathological scanning apparatus (Aperio, Vista, CA, USA) and analyzed using Image Scope software v12.0 (Aperio, Vista, CA, USA). Histoscore (H-Score) was calculated by a semiquantitative assessment of both the staining intensity and the percentage of positive cells.

Statistical Analysis

The receiver–operating characteristic (ROC) curve was used to get the optimized cutoff value of TSTA3 protein, which divided the patients into 2 groups: the $TSTA3_{high}$ group and $TSTA3_{low}$ group, respectively. Correlation analysis of TSTA3 expression and clinical pathologic factors was performed with a χ^2 test. Survival analysis was carried out using Kaplan-Meier analysis and log-rank test. Univariate and multivariate survival analyses were carried out by a Cox proportional hazards (PHs) regression model after PH-assumption tests. All calculations were performed with SPSS 18.0 for Windows statistical software package (SPSS Inc, Chicago, Illinois). P values <.05 were considered statistically significant.

Results

Expression of TSTA3 Protein in ESCC Tumor Tissues

First, TSTA3 protein levels were measured in 104 primary tumor tissues. The results of IHC showed that TSTA3 was expressed in the cytoplasm of ESCC tissues (Figure 1). The H-Score of TSTA3 protein ranged from 66.3158 to 297.3680 in ESCC tumor tissues, and the median was 184.926.

Relationships Between TSTA3 Expression and Clinicopathological Characteristics in Patients With ESCC

According to the ROC curve analyses (area under curve = 0.709, P = .00037, 95% confidence interval (CI), 0.600-0.818; Figure 2A), all cancer samples were divided into 2 groups: $TSTA3_{low}$ (H-Score <195.2735) and $TSTA3_{high}$ (H-Score ≥195.2735).

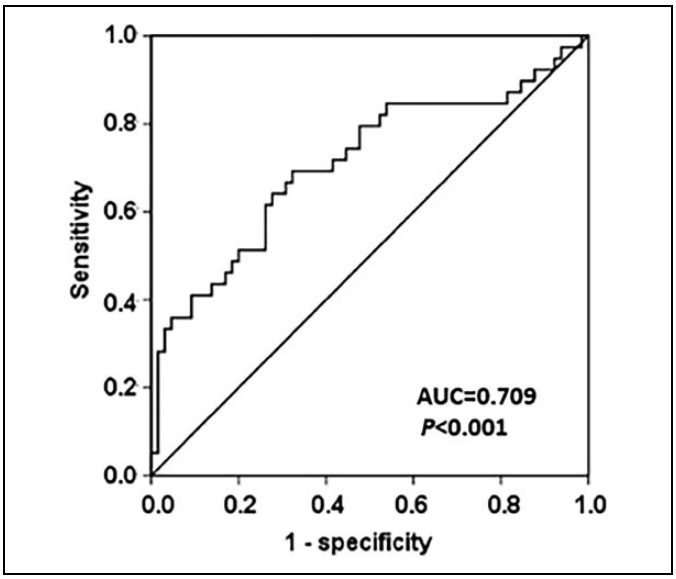

Figure 2. ROC curve analyses of TSTA3 expression in ESCC. The ROC curve analyses showed that the optimum cutoff value was 195.2735 (AUC = 0.703, P = .00037, 95% CI, 0.600-0.818). ROC indicates receiver operating characteristic; TSTA3, tissue-specific transplantation antigen P35B; ESCC, esophageal squamous cell carcinoma; AUC, area under curve; CI, confidence interval.

The TSTA3 protein expression level was associated with age (P = .017, χ^2 = 5.983), alcohol history (P = .007, χ^2 = 9.817), clinical stage (P = .010, χ^2 = 6.996), and LN metastasis (P = .043, χ^2 = 4.876; Table 1). No obvious correlations were seen in patients of different gender, histological grade, smoking history, or esophageal site (Table 1). Although the correlation was not statistically significant, TSTA3 protein expression levels tended to have a correlation with invasion depth (T1+T2 vs T3, P = .055, χ^2 = 4.131; Table 1).

Table 1. Association of *TSTA3* Levels With Clinicopathological Factors in Patients With ESCC.

Variables	Cases	*TSTA3* Level		χ^2 Test	
		Low (%)	High (%)	χ^2	*P*
Age (years)					
<60	63	40	23	5.983	.017
≥60	41	16	25		
Gender					
Female	32	16	16	0.275	.672
Male	72	40	32		
Grade					
1-2	70	35	35	1.275	.299
3	34	21	13		
Smoking history					
Current	48	24	24	4.542	.103
Never	31	14	17		
Missing	25	18	7		
Alcohol history					
Current	36	26	10	9.817	.007
Never	43	22	21		.056 (current vs never)
Missing	25	8	17		
Site					
Upper	4	1	3	2.590	.274
Middle	70	41	29		
Below	30	14	16		
Depth of invasion					
T1	8	6	2	4.299	.117
T2	24	16	8	4.131	.055 (T1+T2 vs T3)
T3	72	34	38		
Clinical stage					
I+II	47	32	15	6.996	.010
III+IV	57	24	33		
LN					
N (−)	40	27	13	4.876	.043
N (+)	64	29	35		

Abbreviations: TSTA3, tissue-specific transplantation antigen P35B; ESCC, esophageal squamous cell carcinoma; LN, lymph node.

Prediction of OS Based on the TSTA3 Protein Levels

The OS of $TSTA3_{low}$ patients ranged from 34 to 55 months, and the median was 45 months. The OS of $TSTA3_{high}$ ranged from 26 to 41 months, and the median was 33 months. Kaplan-Meier analysis showed the patients in the $TSTA3_{low}$ group had a longer survival time than those in the $TSTA3_{high}$ group ($P = .048$; Figure 3A). The same trend was observed in the patients with ESCC without alcohol history ($P = .048$; Figure 3B), with grade 3 ($P = .020$; Figure 3D), and with the lower EC ($P = .038$; Figure 3E). Such trend was more remarkable only in male patients ($P = .017$; Figure 3C).

Cumulative OS of patients in some groups such as old patients (≥60 years, $P = .059$; Figure 4A), with LN metastasis ($P = .064$; Figure 4B), or with esophageal full thickness invaded ($P = .068$, Figure 4C) tended to be affected by the expression of TSTA3. However, there was no difference between the patients with high TSTA3 and the ones with low TSTA3 in clinical stage I+II or III+IV ($P = .374$ and $P = .133$, respectively; Figure 4D). Although these trends were not statistically significant, and further study with larger cohort is needed to confirm the findings.

Univariate and Multivariate Analysis to Determine the Prognostic Value of TSTA3 Protein Levels

To evaluate the predictive value of the TSTA3 expression for OS status in patients with ESCC, univariate and multivariate Cox PH regression models were performed (Table 2) after PH-assumption tests (Supplementary Table S2). The univariate analysis results showed the association between OS and TSTA3 protein levels in patients with ESCC, and the PH of $TSTA3_{high}$ was not statistically significant (hazard ratio [HR] = 1.967; 95% CI, 0.987-3.923; $P = .055$). In multivariate analysis with an entered stepwise Cox PH regression model, interferences of other significant factors in Kaplan-Meier survival analysis were eliminated, and the PH of $TSTA3_{high}$ was statistically significant ($P = .012$). The results showed that $TSTA3_{high}$ was a significant risk prognostic factor for patients with ESCC. The risk of death in $TSTA3_{high}$ patients was HR = 2.816-fold (95% CI, 1.253-6.329) and higher than the $TSTA3_{low}$ patients. According to the study of Lo *et al* [15] on the difference between univariate and multivariate analysis, TSTA3 protein levels may independently predict poor prognosis for patients with ESCC.

Discussion

Esophageal squamous cell carcinoma is one of the major malignancies with poor prognosis because it lacks specific early diagnostic and prognostic markers. Our present study found that the TSTA3 protein expression level was associated with the clinical stage and LN metastasis status. The $TSTA3_{low}$ patients also had a longer survival time than those with a high level of TSTA3, and TSTA3 maybe serve as a potential prognosis marker of ESCC.

The TSTA3, also known as FX, is an NADPH-binding protein[16] and plays a key role in the course of glycosylation.[7] Glycosylation is necessary for regulation of the function of cells and proteins and plays important roles in a variety of biological functions, including cell–cell and cell–substrate interactions, adhesion, cell immunogenicity, and cell signaling. Aberrations of glycosylation are involved in a number of diseases, such as tumorigenesis[17] and chronic inflammation.[18] Fucosylation is one of the most common glycosylation modifications on glycoproteins and glycolipids, and abnormal fucosylation is closely related to the tumor.[19,20] In head and neck, breast, and colorectal cancers, as one of the core-fucosylated-proteins, annexin I can modulate adhesion functions of cancer cells by controlling intracellular calcium release,[21] annexin II can interact with matrix proteins and specific protease to regulate prothrombin activation, cell movement, and adhesion.[22-24] Expression change in fucosylated glycoproteins has been observed in liver cancer cell lines with discrepant migration abilities.[25] Fucosylation associated with tumor

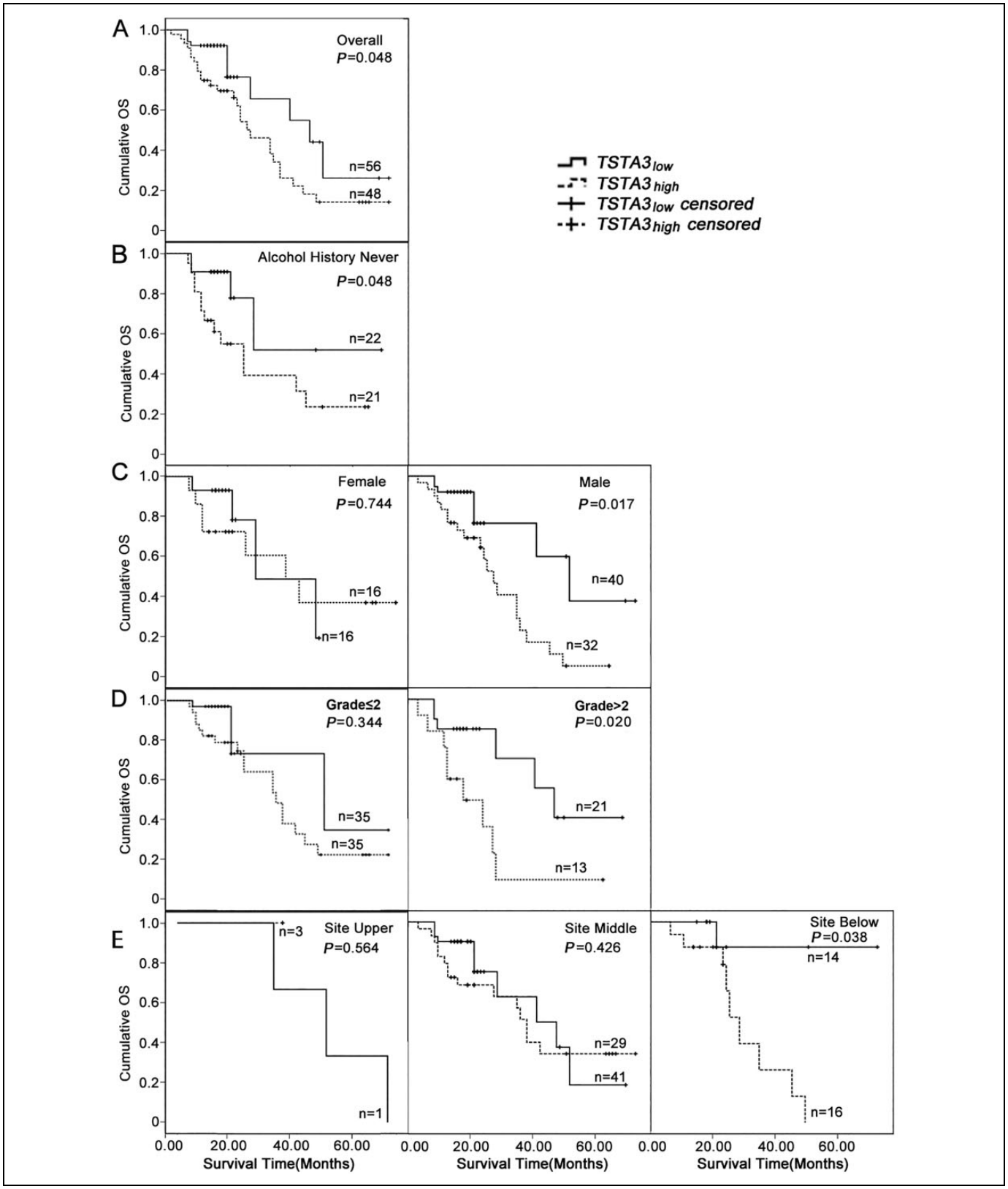

Figure 3. Kaplan-Meier survival curves of patients with ESCC with different TSTA3 protein expression. A, Cumulative OS in the overall study population. B, Cumulative OS in patients without alcohol history. C, Cumulative OS of patients in the female and male groups. D, Cumulative OS in patients with ESCC with grades 1-2 and 3. E, Cumulative OS in patients with ESCC with carcinoma in the upper, middle, and below sites of the esophagus. ESCC indicates esophageal squamous cell carcinoma; TSTA3, tissue-specific transplantation antigen P35B; OS, overall survival.

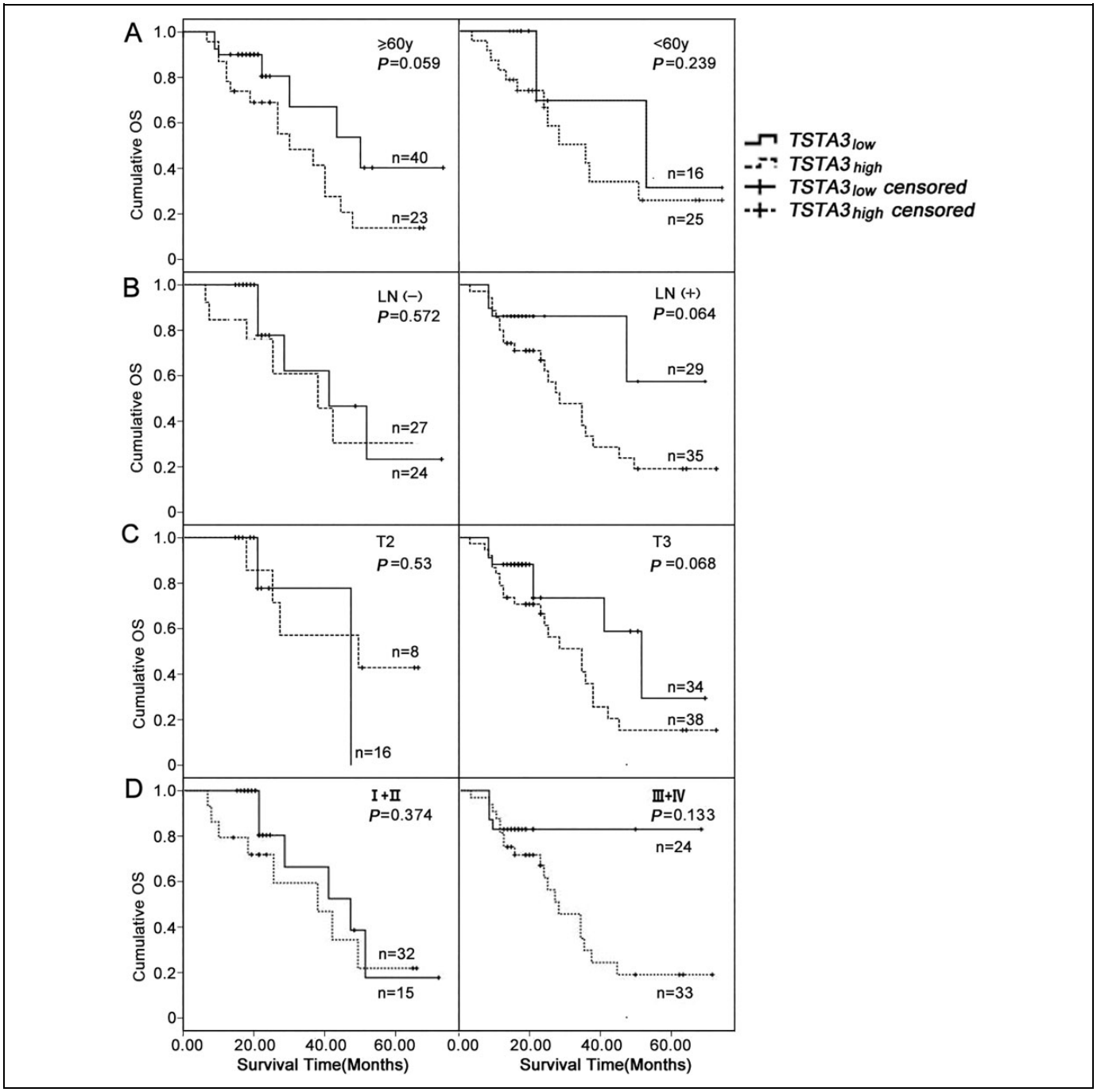

Figure 4. Kaplan-Meier survival curves of patients with ESCC with different TSTA3 protein expression in the different age, LN status, and invasive depth groups. A, Cumulative OS of patients in the age <60 years group and in the age ≥60 years group. B, Cumulative OS in patients with ESCC with or without LN metastasis. C, Cumulative OS in patients with ESCC with T2 and T3 of tumor invasive depth. D, Cumulative OS in patients with ESCC with different clinical stages. ESCC indicates esophageal squamous cell carcinoma; TSTA3, tissue-specific transplantation antigen P35B; LN, lymph node; OS, overall survival.

immune escape and deficiency in GMDs can lead colon cancer cells to escape from natural killer cell-mediated tumor surveillance through downregulation of fucosylation.[26,27]

As one of the key enzymes in fucosylation, TSTA3 participates in the metabolism of mannose and directly produces metabolites—GDP-L-fucose. Increased expression of TSTA3 can synchronously induce an elevation in both core-fucosylated and fucosylated glycoproteins,[28]which play important roles in the progression of cancer. In hepatocellular carcinoma, the expression of TSTA3 was increased, followed by increases in GDP-L-fucose and α1-6-fucosyltransferases expression.[29] In head/neck tumors, SialylLewisX, a fucosylated glycoprotein that mediates cell-to-cell recognition processes, can be induced by high-

Table 2. Univariate and Multivariate Cox Models for the Association Between Survival and Clinicopathological Factors in Patients With ESCC.

Variables		Univariate Analysis			Multivariate Analysis		
		HR	95% CI	*P* Value	HR	95% CI	*P* Value
Age	<60 vs ≥60	1.161	0.606-2.224	.652	1.042	0.492-2.206	.914
Gender	Female vs male	0.723	0.358-1.459	.365	0.528	0.240-1.162	.112
Site	Upper-middle vs below	1.151	0.559-2.371	.702	1.490	0.673-3.298	.326
Grade	1+2 vs 3	0.728	0.381-1.391	.337	0.518	0.245-1.095	.085
N	N (−) vs N (+)	0.712	0.365-1.389	.319	0.622	0.149-2.599	.515
T	T1+T2 vs T3	0.567	0.269-1.198	.137	0.568	0.222-1.453	.238
Clinical stage	I+II vs III+IV	0.706	0.372-1.340	.287	2.085	0.475-9.159	.330
TSTA3 level	High vs Low	1.967	0.987-3.923	.055	2.816	1.253-6.329	.012

Abbreviations: ESCC, esophageal squamous cell carcinoma; HR, hazard ratio; CI, confidence interval; TSTA3, tissue-specific transplantation antigen P35B.

level TSTA3.[30] As our results showed in ESCC, $TSTA3_{high}$ patients tended to have a deeper invasion depth and a higher LN metastasis rate, which indicated that high TSTA3 may promote the invasion and metastasis of ESCC cells, more experiments are required for making clear whether such function is through aberrant fucosylation of glycoproteins. Additionally, our results also showed TSTA3 was associated with alcohol history. In fact, alcohol consumption is one of the major risk factors for esophageal cancer.[31] In consideration of a large amount of missing data of drink (nearly 25%) in our study, further study is needed to be done in a larger cohort.

Since most focus glycoproteins are secreted proteins or cell membrane protein on the surface, abnormal glycoproteins on the tumor cells which break away from the primary tumor site or release to blood could be used as a tumor marker. SialylLewisX, with high expression in breast cancer, ovarian cancer, liver cancer, and colorectal cancer, has been proved to be a tumor-associated antigen.[32-35] Engineering anti-Lewis-Y hu3S193 and IGN311 antibodies have been tested as passive immunotherapy approaches of epithelial cancers and may be applied clinically.[36,37] Core-fucosylated AFP (AFP - L3) compared to AFP has a more specific value of the diagnosis of liver cancer.[20,38] Serum-fucosylated haptoglobin with high levels is considered as a novel prognostic biomarker for malignant tumors, such as ovarian cancer, lung cancer, breast cancer, and pancreatic cancer.[39-41] Our study also showed $TSTA3_{low}$ patients had a longer survival time than those with a high level of TSTA3, and TSTA3 might be a potential molecular predictor of prognosis in patients with ESCC.

In conclusion, TSTA3 protein levels were correlated with clinical stage and LN metastasis in ESCC and could potentially predict poor prognosis for patients with ESCC independently. As a promising candidate for predicting poor prognosis in ESCC, further research is needed to clarify the mechanism of carcinogenesis and identify the potential clinic applications of TSTA3.

Authors' Note

Jie Yang, Pengzhou Kong, Jian Yang, Zhiwu Jia, Xiaoling Hu, and Zianyi Wang contributed equally to this work.

Declaration of Conflicting Interests

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Supplemental Material

Supplementary material for this article is available online.

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