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ORIGINAL RESEARCH

High expression of uPA related to p38MAPK in esophageal cancer indicates poor prognosis

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Background: The aim of the study was to investigate the relationship between urokinase-type plasminogen activator (uPA) and mitogen-activated protein kinase 38 (p38MAPK), and preliminarily analyze their relationship with clinical characteristics of esophageal cancer.

Materials and methods: Immunohistochemistry and Western blot were used to detect the expressions of uPA and p38MAPK in patients with esophageal cancer. The relationship between them and clinicopathological features was analyzed by chi-squared test and Spearman correlation. Prognosis was performed using Kaplan–Meier and Cox proportional hazard models analysis.

Results: The expressions of uPA and p38MAPK proteins were significantly higher in esophageal squamous cell carcinoma or adenocarcinoma than in normal esophageal mucosa tissue (both $P<0.0001$). The expression of uPA was significantly correlated with the depth of invasion of esophageal cancer ($P=0.0067$), tumor size ($P=0.0364$), and pathological stage ($P<0.0001$); p38MAPK expression vs esophageal cancer tissue type ($P=0.0043$), esophageal cancer infiltration depth ($P=0.0097$), tumor size ($P=0.0015$), and pathological stage ($P<0.0001$). Both were not significantly associated with lymph node staging, gender, age, and esophageal cancer histological type. There was a positive correlation between uPA and p38MAPK expressions ($r=0.7301$, $P=0.0104$). Kaplan–Meier analysis showed that the overall survival time of patients with positive expression of uPA or p38MAPK protein was significantly shorter, and the time of recurrence or metastasis of esophageal cancer was significantly earlier in patients with uPA-positive expression. Multivariate analysis of Cox model showed that uPA, p38MAPK, and pathological staging were independent factors influencing survival.

Conclusion: The expressions of uPA and p38MAPK may play an important role in the progression of esophageal cancer, and there is a close relationship between the two proteins, which may be one of the prognostic indicators.

Keywords: esophageal cancer, urokinase-type plasminogen activator, mitogen-activated protein kinase 38, prognosis

Introduction

Esophageal cancer is one of the most common malignant tumors of the digestive system in the world. There are about 240,000 new cases of esophageal cancer in China every year.[1–3] According to statistics, its 5-year survival rate is 3.5%–13.9% in Xinjiang. The cancer mortality rate is 13.05/100,000, of which the Kazakh (Ha) mortality rate is 68.88/100,000, which seriously threatens people's health.[4–6]

Since the study in 1976 that urokinase-type plasminogen activator (uPA) is present in human ovarian tumor cells, the relationship between uPA and tumor invasion and metastasis has been paid more and more attention. It is widely distributed in human tumor cells. The expression level in malignant tumor tissues is higher than that in their corresponding normal tissues.[7,8] The uPA system may regulate angiogenesis, invasion,

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and metastasis of digestive tract tumors in a variety of ways, and is related to monocyte activation antigen and matrix metalloproteinase production.[9] Studies have shown that[10] uPA mainly participates in the inhibition of excessive degradation of extracellular matrix (ECM) through the urokinase-type plasminogen activator receptor (uPAR)/plasminogen activator inhibitor type 1 (PAI-1) complex, thereby inhibiting tumor metastasis. Recently, more studies have shown[11–14] that uPA may be associated with the mitogen-activated protein kinase 38 (p38MAPK) signaling pathway in a variety of tumor progression, and is found to be highly expressed in both tumors and associated with clinical stage and lymph node metastasis, such as in breast cancer, ovarian cancer, and lung cancer. It was also found that blocking the p38MAPK signaling pathway in ovarian cancer, can inhibit uPA expression. The p38MAPK signaling pathway can inhibit uPA expression. Although some progress has been made on this research, there are few more detailed studies on them, such as whether p38MAPK participates in the formation of uPA/uPAR/PAI-1 and affects the degradation of the outer matrix (ECM), or their relationship in the esophagus carcinoma progression.

P38 is an important member of the mitogen-activated protein kinase (MAPK), which has six isoforms, p38α1, p38α2, p38β1, p38β2, p38γ, and p38δ. Studies have found that extracellular stimuli such as cytokines and bacterial pathogens can activate the phosphorylation of the Thr site of p38MAPK, which transduces signals to the nucleus to initiate related target genes to promote transcription.[15–17] p38MAPK is known to be involved in a variety of different biological processes, including promoting apoptosis, participating in inflammatory reactions, malignant tumor development, and regulating transcription and translation of many different proteins, but its research in esophageal cancer is relatively rare. In our previous study, we found that uPA and p38MAPK may be closely related to esophageal cancer. The purpose of this study was to investigate the relationship between the two in esophageal cancer and its relationship with clinicopathological features and prognosis of esophageal cancer.

Materials and methods

Materials

One hundred eighty-four cases of esophageal cancer specimens confirmed by pathology in the First Affiliated Hospital of Sun Yat-sen University from 2007 to 2011 and 62 cases of adjacent normal tissues (>5 cm from the edge of esophageal cancer, pathologically confirmed normal mucosal tissue) were selected. All cases were not given any radiotherapy or chemotherapy before surgery. According to the TNM standard staging of the eighth edition of esophageal cancer in 2017, gender, age, degree of cell differentiation, tissue type, depth of invasion, and pathological staging were selected as clinical characteristic parameters. Follow-up was by telephone and mail; overall survival was calculated from the date of diagnosis to death or August 2, 2017. Progression-free survival begins with treatment of esophageal cancer patients, until observation progression or death or until August 2, 2017. There were no cases of loss of follow-up. The uPA mouse antihuman monoclonal antibody (sc-59727, concentrated) was purchased from Santa Cruz Biotechnology Inc., Dallas, TX, USA mouse antihuman p38MAPK (AN1020) was from Abgent, San Diego, CA, USA. The ready-to-use Elivision mainly includes the following: avidin (Reagent A), biotin-horseradish peroxidase (anti-mouse/rabbit IgG, Reagent B), which is purchased from Fuzhou Maixin Biotechnology Development Co., Ltd. with DAB staining solution (Fuzhou Maixin Biotechnology Development Co., Ltd). B-actin antibody was purchased from Beijing Zhongshan Biotechnology Company. Immunoblot chemiluminescence reagent (enhanced chemiluminescence [ECL] reagent) was purchased from Jiangsu Biyuntian Biotechnology Research Institute. Polyvinylidene fluoride or polyvinylidene difluoride (PVDF) membranes were purchased from Millipore, Burlington, MA, USA.

Immunochemistry

Specimens were fixed in 10% formalin, embedded in paraffin, and made into 3–4-μm-thick paraffin sections. According to Elivision method, paraffin sections were placed in an oven at 67°C for 2 hours, dewaxed and hydrated, rinsed with 7.4 pH PBS for 3 times, 3 minutes each time, then antigen repair. One drop of 3% H_2O_2 was added to each section and incubated for 10 minutes at room temperature to block endogenous peroxidase activity, and rinsed with PBS for 3 times, 3 minutes each time. Then, PBS was removed and primary antibody – anti-uPA (1:200) and anti-p38MAPK (1:150) – was added dropwise and incubated for 2 hours at room temperature. Washed with PBS for 3 times, 5 minutes each time. PBS was removed, reagent A added, incubated at room temperature for 20 minutes, rinsed with PBS for 3 times, 3 minutes each time. PBS was removed , reagent B added, incubated at room temperature for 30 minutes, then rinsed with PBS for 3 times, 5 minutes each time. Counterstained with hematoxylin, 0.1% HCL differentiation, rinsed with distilled water, dehydrated with gradient alcohol, transparent with xylene, sealed with neutral gum, observed after drying.

Result evaluation

The results were independently read by three pathologists and scored according to the intensity of staining and the percentage of positive cells. Ten high-power fields of each slice were selected, and the percentage of positive cells was counted. The scoring criteria were as follows: no positive cells, 0; ≤25%, 1; 25%–50%, 2; 50%–75%, 3; >75%, 4. Staining intensity score criteria: no coloring, 0; light yellow, 1; brownish yellow, 2; tan, 3. The final staining results were multiplied by the percentage of positive cells and staining intensity, with 0–2 being negative and ≥3 being positive.

Western blotting

One hundred microliters of RIPA lysate was added to 20 mg of tissue, homogenized on ice, centrifuged at 12,000 rev/minute for 10 minutes at 4°C, and the supernatant was separated to determine the protein concentration. The protein was separated by 10% SDS-PAGE electrophoresis with a loading of 40 μg in a volume of 20 μL. After the electrophoresis, a "sandwich" was prepared, and the protein was electrotransferred to the PVDF membrane. After blocking with 5% nonfat dry milk for 2 hours, added anti-uPA (1:600), anti-p38MAPK (1:500), and mouse anti-β-actin (clone AC-15; 1:10,000, Sigma-Aldrich). After washing the membrane, added the secondary antibody, incubated for 50 minutes at room temperature on the shaker. At the end, the membrane was washed five times with TBST for 5 minutes each time, and the ECL was exposed. Gelpro 32 analysis was performed for gel image analysis.

Statistical methods

Data were analyzed using Graphpad Prism 6.0 software. Count data were expressed as rates and chi-squared tests were used for comparison between groups. Spearman test was used for correlation analysis and Kaplan–Meier and Cox proportional hazards regression were used for univariate and multivariate analyses. $P<0.05$ was considered statistically significant. "*" stands for "$P<0.01$," "**" stands for "$P<0.001$," "***" stands for "$P<0.0001$."

Results

Expressions of uPA and p38MAPK in esophageal cancer and adjacent tissues

After immunohistochemistry test, according to the above methods, the expressions of uPA and p38MAPK in esophageal squamous cell carcinoma and adenocarcinoma were found to be higher than those in normal esophageal tissues (both $P<0.0001$): uPA (squamous cell carcinoma: 89/112, adenocarcinoma: 61/72, and normal tissue: 15/62) and p38MAPK (squamous cell carcinoma: 92/112, adenocarcinoma: 61/72, normal tissue: 20/62). Further detection by Western blot found similar results, compared with normal esophageal tissue; the expression of uPA or p38MAPK protein in esophageal squamous cell carcinoma or adenocarcinoma was higher than that of normal esophageal tissue, and all P-value < 0.0001 (Table 1 and Figure 1).

Relationship between uPA and p38MAPK and their relationship with clinicopathological parameters

After further analysis, it was found that there was a close correlation between the expressions of uPA and p38MAPK in esophageal cancer tissues ($r=0.7301$, $P=0.0104$, Table 1). At the same time, uPA expression was significantly different between esophageal cancer infiltration depth (T stage, $P=0.0067$), tumor length ($P=0.0364$), and pTNM stage ($P<0.0001$), but there was no significant relationship between tissue type, morphologic type, lymph node staging, gender, and age. p38MAPK expression was correlated with esophageal cancer infiltration depth (T stage, $P=0.0097$), tumor length ($P=0.0015$), pTNM stage ($P<0.0001$), and tissue type ($P=0.0043$), but it was also not significantly associated with morphologic type, age, gender, and lymph node staging (Table 2).

Relationship between uPA, p38MAPK, and prognosis in patients with esophageal cancer

After Kaplan–Meier analysis, the survival time of uPA- and p38MAPK-positive expression patients was found to be significantly shorter than that of negative patients (former, median survival: 42 vs 96 months, $P<0.0001$; latter, median survival: 45 vs 78 months, $P=0.0121$). At the same time, it was found that patients with uPA-positive expression had a shorter progression-free time than negative patients (Median time:16 vs 70 months, $P=0.0032$), whereas there was no significant difference between p38MAPK-positive and p38MAPK-negative patients (Figure 2 and Table 3).

Cox multivariate regression analysis was performed based on patient age, gender, depth of tumor invasion, lymph node metastasis, lesion length, pTNM, uPA, and p38MAPK protein expressions. The results showed that uPA, p38MAPK protein expressions, and pTNM staging were independent prognostic factors (Table 3).

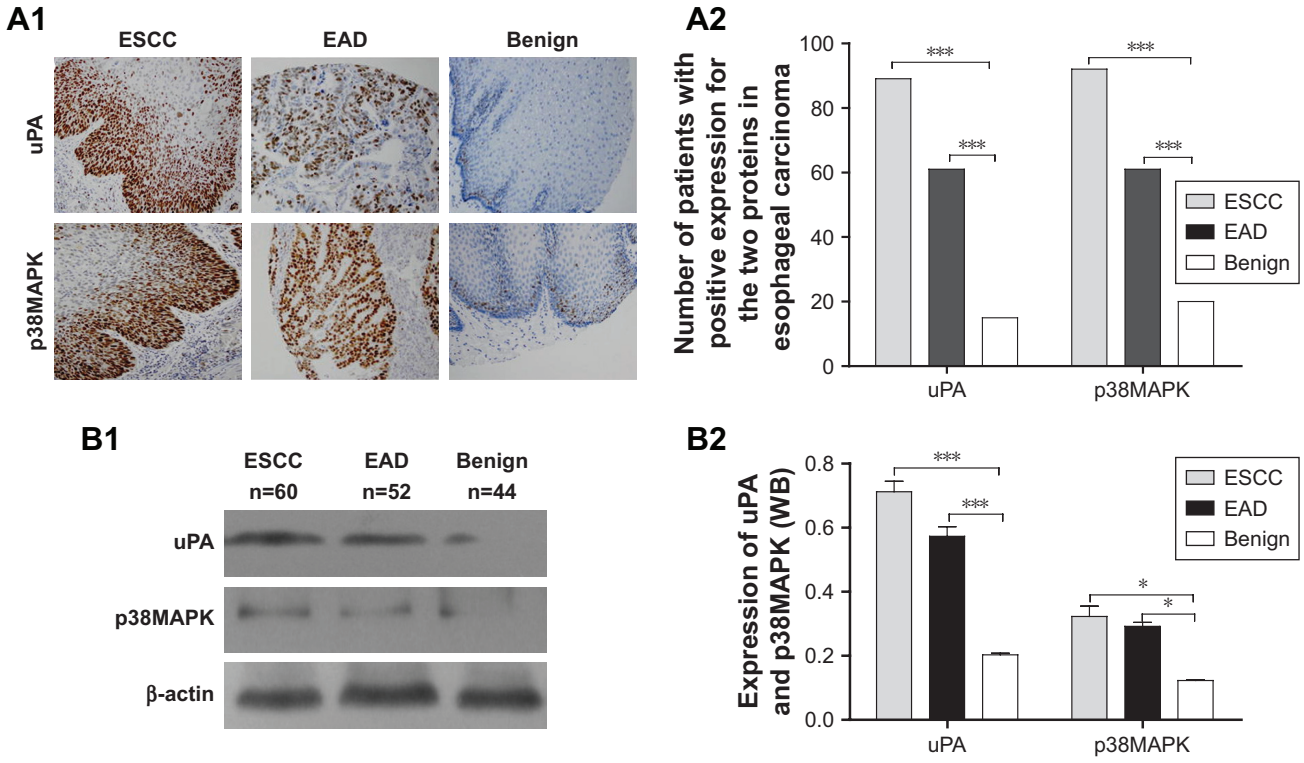

Figure 1 uPA and p38MAPK expressions in esophageal cancer via IHC and WB.

Notes: According to the method, the expressions of the two proteins in esophageal cancer and adjacent normal tissues were analyzed. (**A1**, **A2**) The expressions of uPA and p38MAPK in ESCC, adenocarcinoma, and adjacent normal tissues (×200) were observed. The analysis found that the expressions of both proteins in squamous cell carcinoma and adenocarcinoma were much higher than those in adjacent normal tissues. (**B1**, **B2**) The expressions of the two proteins in esophageal cancer were analyzed by Western blot, and similar results were obtained. ***$P<0.0001$.

Abbreviations: EAD, esophageal adenocarcinoma; ESCC, esophageal squamous cell carcinoma; IHC, immunohistochemistry; p38MAPK, mitogen-activated protein kinase 38; uPA, urokinase-type plasminogen activator; WB, Western blot.

Discussion

Esophageal cancer is one of the most common malignant tumors in the world and is one of the important threats to human life.[18,19] At present, after extensive research, the diagnosis and treatment of esophageal cancer has been greatly expanded, mainly including chemotherapy and surgical treatment. However, the recurrence rate and metastasis rate are still high, which is an important limitation that restricts the therapeutic effect.[20–22] This study explored the relationship between uPA, p38MAPK, and esophageal cancer, explored its possible mechanism, and provided a preliminary scientific basis for expanding the new diagnosis and treatment of esophageal cancer.

A number of studies have confirmed that uPA plays an important role in tumor progression in a variety of tissues, such as breast cancer and ovarian cancer,[23–25] which is often

Table 1 Both proteins' expression in ESCC, EAD, and benign tissue

Variable factors	uPA				p38MAPK			
	ESCC		EAD		ESCC		EAD	
	+	–	+	–	+	–	+	–
Cancer	89	23	61	11	86	26	67	5
Benign	15	47	15	47	20	42	20	42
P-value	<0.0001		<0.0001		<0.0001		<0.0001	
Spearman correlation S(r) between expressions of uPA and p38MAPK								
Patients (n)					***R***		***P*-value**	
uPA vs p38MAPK	184				0.7301		0.0104	

Abbreviations: EAD, esophageal adenocarcinoma; ESCC, esophageal squamous cell carcinoma; p38MAPK, mitogen-activated protein kinase 38; uPA, urokinase-type plasminogen activator.

Table 2 Correlation of uPA and p38MAPK with clinicopathologic parameters

Groups	uPA		*P*-value	p38MAPK		*P*-value
	−	+		−	+	
Gender						
Male	16	78	0.7047	17	77	0.6967
Female	18	72		14	76	
Age (years)						
<60	14	81	0.1890	16	79	1.0000
≥60	20	69		15	74	
Histological type						
SCC	23	89	0.4388	26	86	0.0043
AD	11	61		5	67	
Morphologic type						
Ulcer	6	41	0.5338	5	42	0.2723
Fungoid	9	36		10	35	
Medullary	12	38		11	39	
Constriction	7	35		5	37	
T stage						
T1+T2	22	57	0.0067	20	59	0.0097
T3+T4	12	93		11	94	
N stage						
N0	20	68	0.1848	18	70	0.2402
N1–3	14	82		13	83	
Tumor size						
<3 cm	22	66	0.0364	23	65	0.0015
≥3 cm	12	84		8	88	
pTNM						
I	15	23	<0.0001	13	25	<0.0001
II	13	34		14	33	
III	3	56		3	56	
IV	3	37		1	39	

Abbreviations: AD, adenocarcinoma; p38MAPK, mitogen-activated protein kinase 38; SCC, squamous cell carcinoma; uPA, urokinase-type plasminogen activator.

higher in cancer tissues than in normal tissues. For example, Goscinski et al[26] found that the positive expression of uPA was significantly higher in patients with distant metastasis of esophageal squamous cell carcinoma than in patients without distant metastasis. The higher the tumorigenicity of esophageal squamous cell carcinoma with higher uPA expression, the stronger is the tumor invasiveness and the tumor growth, suggesting that uPA may be involved in the occurrence and development of esophageal squamous cell carcinoma, participating in tumor tissue angiogenesis, and facilitating tumor cell metastasis. These data are consistent with our findings. In this study, the expression of uPA in esophageal squamous cell carcinoma and adenocarcinoma was much higher than that in normal esophageal tissue, suggesting that it may bear an important role in esophageal cancer progression.

MAPK is an important key molecule in the cell signaling pathway. p38 is one of its family members. When activated by upstream signals, p38 transmits cytoplasmic signals to the nucleus, activates specific nuclear transcription factors, regulates target genes, and participates in regulating cell growth and differentiation. p38 has six different subtypes, and its biological effects may differ under different cells and stimuli. Kim et al[27] found that inhibition of p38MAPK activity can reduce the ability of cancer cells to exercise and reduce invasion and metastasis. Studies on human choriocarcinoma have shown that activated p38 enhances the invasive behavior of human trophoblast cells and plays an important role in the formation of choriocarcinoma.[15,16,28] This study showed that the expressions of uPA and p38MAPK in esophageal cancer tissues were much higher than those in adjacent normal esophageal tissues, suggesting that both proteins may play an important role in the progression of esophageal cancer. Further analysis revealed that there is a close positive correlation between the expressions of both proteins. These data suggest an important possibility: do not rule out the role of uPA and p38MAPK in promoting the progression of esophageal cancer in the same pathway. Considering p38MAPK as a key molecule for several important signaling pathways,[29,30] we believe that uPA may be involved either upstream or downstream of it. The conclusion of this study has not been reported yet. Although this study only analyzes semiquantitative immunohistochemistry and there is no further observation and determination of the relationship between the two proteins, it provides an important direction for revealing the mechanism of esophageal cancer progression.

In further analysis of its relationship with clinical pathological parameters of patients with esophageal cancer, the close relationship between them is further indicated. We found that the positive expressions of uPA and p38MAPK are closely related to the cancer size, depth of invasion, and pathological stage of esophageal cancer. This confirms that both proteins are involved in the progression of esophageal cancer, which is consistent with some conclusions about the relationship among uPA, p38MAPK, and esophageal cancer.[31–33] In these studies, uPA or p38MAPK was also observed to be closely related to esophageal cancer size and pTNM staging. In this study, we also found that uPA has no significant relationship with esophageal cancer tissue type, and p38MAPK is relatively high in adenocarcinoma. This phenomenon has attracted our attention, which may suggest

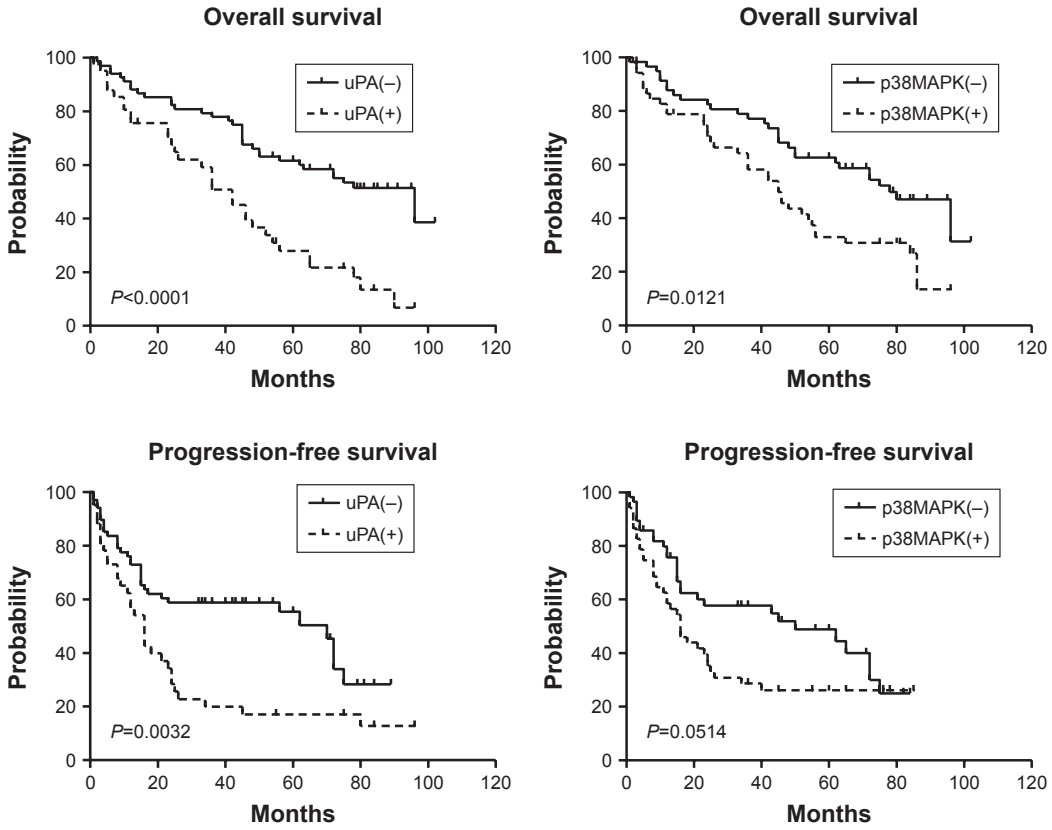

Figure 2 Analysis of the relationship between the two proteins and prognosis of esophageal cancer by Kaplan–Meier analysis.
Notes: The median survival time of patients with positive expression of both proteins was significantly shorter than that of patients with negative expression. Meanwhile, the time to progression of uPA-positive patients was shortened, but there was no significant difference between the time of progression of p38MAPK-positive patients and p38MAPK-negative patients.
Abbreviation: p38MAPK, mitogen-activated protein kinase 38; uPA, urokinase-type plasminogen activator.

Table 3 Kaplan–Meier and Cox multivariate proportional hazard analyses for overall survival

Factors	Univariate analysis		Multivariate analysis	
	Log-rank	*P*-value	Hazard ratio (95% CI)	*P*-value
Gender				
Male			0.8469 (0.5161–1.390)	0.5111
Female				
Age (years)				
<60			0.6673 (0.4041–1.102)	0.1140
≥60				
Tumor size				
<3 cm			0.7383 (0.4431–1.230)	0.2442
≥3 cm				
uPA				
Negative	15.44	<0.0001	0.3294 (0.1893–0.5732)	0.0302
Positive				
p38MAPK				
Negative	6.300	0.0121	0.5253 (0.3177–0.8684)	0.0122
Positive				
pTNM				
I–II	8.425	0.0214	0.8315 (0.5021–0.9501)	0.0413
III–IV				

Abbreviations: p38MAPK, mitogen-activated protein kinase 38; uPA, urokinase-type plasminogen activator.

that the role or mechanism of p38MAPK is subtly different in different tissue types of esophageal cancer tissues. There is no research on the mechanism of p38MAPK in different tissue types of esophageal cancer, which is worthy of further research.

In the analysis, we performed a complete prognosis of overall survival and progression-free prognosis for 184 patients. The similarity in characteristics and functions of the two were observed again. We found that the median survival time of patients with positive protein expression was significantly shortened, which is consistent with the above data and also with most of research on their relationship between single uPA or p38MAPK and esophageal cancer.[31,34,35] At the same time, we found that only the uPA-positive patients had a significant reduction in the progression-free time, and the p38MAPK-positive patients did not significantly shorten the progression time. For the cause of this phenomenon, we consider not to rule out the possible errors caused by follow-up data collection (accurate time for progression-free progress has an error). In further Cox multivariate analysis, this study found that both proteins and pTNM staging can be used as independent prognostic factors for esophageal cancer.

In this study, we found that uPA and p38MAPK proteins may play important roles in the progression of esophageal cancer, and there may be synergistic effects in this process, which provide a new perspective for us to further explore the mechanism of esophageal cancer progression. At present, the diagnosis and treatment of esophageal cancer mainly depends on surgery and chemotherapy, which has a great impact on the quality of life of patients and has limitations. Therefore, these results are of great value to explore novel treatment methods. The role of uPA and p38MAPK in the progression of esophageal cancer may provide a scientific basis for exploring the feasibility of biologically targeted therapy for esophageal cancer.

Ethical approval and written informed consent

All procedures performed in the studies involving human participants were approved by the Ethics Committee of the First Affiliated Hospital, Sun Yat-sen University and conducted in accordance with the 1964 Helsinki declaration. Written informed consent was obtained from all participants included in the study.

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Disclosure

The authors report no conflicts of interest in this work.

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