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Circulating uPA as a potential prognostic biomarker for resectable esophageal squamous cell carcinoma

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Abstract

Previous research showed that the 4 genes of matrix metallopeptidase 9 (MMP9), cyto-keratin 20 (CK20), cyto-keratin 19 (CK19) and urokinase type plasminogen activator (uPA) are detectable in the peripheral blood. All the 4 genes are related to tumor invasion and metastasis. However, whether their expression is associated with clinicopathologic factors and the prognosis of patients with esophageal squamous cell carcinoma (ESCC) is still confused. Expression levels of MMP9, CK20, CK19, and uPA were evaluated by quantificational real-time polymerase chain reaction (qRT-PCR) in peripheral blood of 205 ESCC patients who received radical resection. The cut-off value was 1000 copy numbers. Their impacts on clinicopathologic factors and survival were investigated. The uPA expression positively correlated with gender ($P = .046$) and tumor size ($P = .046$). Meanwhile, CK19 expression positively correlated with tumor size ($P = .029$), vascular invasion ($P = .024$), and CK20 expression positively correlated with tumor size ($P = .035$) and degrees of differentiation ($P = .032$). Moreover, the overexpression of MMP9 has a correlation with postoperative radiotherapy ($P = .041$) and chemotherapy ($P = .012$). Among the 4 genes, only uPA is a prognostic indicator for disease-free survival and overall survival both in univariate analysis and multivariate analysis ($P = .015$). This study suggests that circulating uPA mRNA in peripheral blood can serve as a potential unfavorable prognosis biomarker in ESCC. Further perspective, multi-center and large-scale study is still needed.

Abbreviations: CI = confidence interval, CK-19 = cyto-keratin 19, CK-20 = cyto-keratin 20, DFS = disease-free survival, EC = esophageal cancer, ESCC = esophageal squamous cell carcinoma, HR = hazard ratio, MMP9 = matrix metallopeptidase 9, OS = overall survival, uPA = urokinase type plasminogen activator.

Keywords: cyto-keratin 19 (CK-19), cyto-keratin 20 (CK-20), esophageal squamous cell carcinoma (ESCC), matrix metallopeptidase 9 (MMP9), urokinase type plasminogen activator (uPA)

1. Introduction

Esophageal cancer (EC) is one of the most common cancers in Asia and the eighth cause of cancer related-death.[1] Esophageal squamous cell carcinoma (ESCC) is the majority pathological subtype of EC in China.[1] Surgery is one of the main treatments for EC patients and the 5-year survival rate in operable ESCC patients is only from 20% to 36%.[2] The most responsible reasons for the failure of treatment and the majority of cancer-related deaths are regional recurrence and distant metastases. Thus, it is important to detect sensitive and specific biomarkers to identify potential postoperative EC patients who are tend to have early invasion and metastasis.

As we know, fibrin degradation and vascular formation effect can result in degradation of extracellular matrix and basement membrane which are the critical processes of tumor invasion and metastasis. The expression of matrix metallopeptidase 9 (MMP9), a member of the MMP family, can directly degrade the extracellular matrix. Thus, it serves a crucial role in cell migration. It has been reported to be involved in esophageal carcinoma metastasis,[3] and high level of MMP9 expression is associated with lymph node metastasis in esophageal carcinoma.[4] Cytokeratin 19 (CK19) and cytokeratin 20 (CK20) are the principal structural elements of the cytoskeleton of epithelial cells. They expressed during the process of transformation of the tissue from normal to tumor tissue.[5] Therefore, CK19 and CK20 can serve as epithelial tumors metastasis molecular markers.[6] ESCC is originated from the squamous epithelium cell. Thus, CK19 and CK20 may be the potential prognostic biomarker for ESCC. Urokinasetype plasminogen activator (uPA), as a proteolytic factor, can activate a variety of fibrinolytic enzymes. It is not only as a predictive biomarker, but also as a new therapeutic target because it can promote tumorous infiltration and metastasis by degrading extracellular matrix and basement membrane.[7]

Although, many studies have proved that MMP9, CK20, CK19, and uPA expression which detected by IHC and RT-PCR in tissue could serve as potential prognosis biomarker for various

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tumor patients.[8–12] However, tissue biopsy is invasive and associated with a higher risk of complications for the patient. Up to date, only a few small-size studies[13–15] have investigated the correlation between the expression of MMP9, CK19, CK20, and uPA in peripheral blood and the risk of EC . Therefore, we conducted this large-scale study to further determine whether MMP9, CK20, CK19, and uPA, detected in peripheral blood, can be a useful biomarker for patients with ESCC.

2. Methods

2.1. Patients and blood samples

Two hundred five patients with resectable ESCC diagnosed and treated at the Department of Thoracic Surgery of Zhejiang Cancer Hospital in China between September 2009 and March 2012 were included in the retrospective database for analysis. All patients were pathologically diagnosed by surgical specimens. This study was approved by the medical ethics committee of Zhejiang Cancer Hospital.

The following criteria should be satisfied:

1. patients who didn't receive any preoperative treatment (chemotherapy and/or radiotherapy);
2. all patients should have R0 esophagectomy;
3. records with sufficient information including the follow-up information for analysis.

The exclusion criteria were as follows:

1. patients who presented with other malignant disease within 5 years;
2. patients with a noncurative resection;
3. patients who died within 30 days after operation.

Preoperative EDTA anticoagulated whole blood samples (2–3 ml) were collected from all patients with ESCC before surgery. Immediately after collection, blood samples were stored at −80°C until further processing.

2.2. RNA extraction and PCR

Total RNA was extracted using the Trizol reagent (Invitrogen Biotech, Shanghai, China). All RNA preparation and processing steps were conducted under RNAse-free conditions. Total RNA was diluted into 10 ng/μl and dissolved in diethylpyrocaronate-treated water (DEPC water), then stored at –80°C until use. RNA concentration was accessed by a spectrophotometer and the purity and integrity were detected using A260/A280 ratio. Electrophoresis on denaturated 1% agarose gel was used to further confirm the quality and concentration of the RNA samples.

The quality of the total RNA was checked by ethidium-stained 2% agarose gels. The reverse transcription reaction was carried out with a PrimeScriptTM RT reagent Kit (TaKaRa, Dalian, China) in 10 μl solution containing 2 μl of RNA extract, 2 μl 5×PrimeScript Buffer, 0.5 μl PrimeScript RT Enzyme Mix I, 0.5 μl Oligo dT Primer, 0.5 μl Random 6 mers and 4.5 μl RNase Free dH_2O. For the synthesis of cDNA, reaction mixtures were incubated at 37°C for 15 minute, at 85°C for 5 second, and then held at 4°C.

SYBR green-based real-time quantitative polymerase chain reaction assays were used for the gene expression analysis of MMP9, CK20, CK19, and uPA according to the manufacturer's protocol of the Doagnostic Kit for MMP9/CK-19/CK-20/UPA-mRNA Kit (BioPerfectus technologies, Taizhou, China). The amounts of mRNAs were calculated by a standard curve constructed with the use of 5 μl quantitative standard reference which provided in the Kit. Next, 20 μl reaction mixtures consisted of 2 μl of cDNA solution, 17.8 μl gene-specific reaction mixtures and 0.2 Taq DNA polymerase was used to perform quantitative PCR, which was run on a 7500 Real-time PCR system (Applied Biosystems). Quantiative PCR was conducted with following cycle parameters: enzyme activation at 95°C for 5 minute, 40 cycles at 95°C for 30 second and 60°C for 30 second.

3. Statistical analysis

The association between MMP9, CK20, CK19, and uPA expression level and clinicopathological factors, such as gender, age, differentiation, tumor size, venous/lymphatic invasion, lymph node metastasis, and postoperative treatment, was analyzed using the x^2 test or Fisher exact test. The disease-free survival (DFS) and overall survival (OS) of the patients was calculated by the Kaplan Meier method, and the survival curves were tested by the log-rank method. Multivariate Cox regression analysis was performed to include the parameters that were found to be significant by the univariate analysis. All results were analyzed using SPSS 18.00 statistical software (SPSS 18.0 Inc., Chicago, IL). A P value $< .05$ was considered to be statistically significant.

4. Results

4.1. Patient characteristics

Relationship between clinicopathological factors and MMP9, CK20, CK19, and uPA expression in the peripheral blood were presented in Table 1. This study examined a total of 205 ESCC patients (182 men and 23 women) with a mean age of 61 years (range, 39–81). To evaluate the correlation between the MMP9, CK20, CK19, and uPA mRNA levels and the clinicopathological characteristics, patients were divided into 2 groups according to copy numbers. The cut-off levels for MMP9, CK20, CK19, and uPA were set at 1000 copies, which were defined according to product manual suggestions. As showed in Table 1, a statistically significant association was observed between MMP-9 expression and postoperative radiotherapy ($P=.041$) or chemotherapy ($P=.012$). CK-20 high expression was associated with poor differentiation ($P=.032$) and larger tumor size ($P=.035$). A correlation between CK-19 high expression and larger tumor size ($P=.022$) and venous/lymphatic invasion ($P=.024$) were observed.

4.2. Univariate and multivariate Cox analysis of prognostic factors in OS and DFS

Kaplan–Meier DFS and OS curves of the ESCC cancer patients according to the status of MMP9, CK20, CK19, and uPA levels were examined (Fig. 1). For DFS analysis, all 205 patients with underwent curative surgery were included for DFS analysis. The DFS of patients in the high uPA expression group showed significantly worse survival rates than those who were in the low uPA expression group ($P=.048$) (Fig. 1D). OS analysis revealed that the patients in the high uPA expression group had significantly worse survival rates than those who were in the low uPA expression group ($P=.016$) (Fig. 2D). These results

Table 1

Expression levels of the 4 genes and associations with clinicopathological characteristics.

Characteristics	All (number of patients)	MMP-9 low expression	MMP-9 high expression	*P* value	CK-20 low expression	CK-20 high expression	*P* value	CK-19 low expression	CK-19 high expression	*P* value	uPA low expression	uPA high expression	*P* value
Gender													
Female	23	19 (11.4%)	4 (10.3%)	.832	20 (12.3%)	3 (7.0%)	.321	21 (13.1%)	2 (4.4%)	.103	21 (13.8%)	2 (3.8%)	**.046**
Male	182	147 (88.6%)	35 (89.7%)		142 (87.7%)	40 (93.0%)		139 (86.9%)	43 (95.6%)		131 (86.2%)	51 (96.2%)	
Age													
<60 years old	91	73 (44.0%)	18 (46.2%)	.805	72 (44.4%)	19 (44.2%)	.976	68 (42.5%)	23 (51.1%)	.304	66 (43.4%)	25 (47.2%)	.636
≥60 years old	114	93 (56.0%)	21 (53.8%)		90 (55.6%)	24 (55.8%)		92 (57.5%)	22 (48.9%)		86 (56.6%)	28 (52.8%)	
Smoking													
Never	45	34 (20.7%)	11 (28.2%)	.313	37 (23.1%)	8 (18.6%)	.526	36 (22.8%)	9 (20.0%)	.692	38 (25.2%)	7 (13.6%)	.086
Ever	158	130 (79.3%)	28 (71.8%)		123 (76.9%)	35 (81.4%)		122 (77.2%)	36 (80.0%)		113 (74.8%)	45 (86.5%)	
Alcohol consumption													
Never	48	42 (25.3%)	6 (15.4%)	.188	40 (24.7%)	8 (18.6%)	.402	37 (23.1%)	11 (24.4%)	.853	40 (26.3%)	8 (15.1%)	.097
Ever	157	124 (74.7%)	33 (84.6%)		122 (75.3%)	35 (81.4%)		123 (76.9%)	34 (75.6%)		112 (73.7%)	45 (84.9%)	
Family history of cancer													
No	152	123 (74.1%)	29 (74.4%)	.973	118 (72.8%)	34 (79.1%)	.407	118 (73.8%)	34 (75.6%)	.807	113 (74.3%)	39 (73.6%)	.914
Yes	53	43 (25.9%)	10 (25.6%)		44 (27.2%)	9 (20.9%)		42 (26.2%)	11 (24.4%)		39 (25.7%)	14 (26.4%)	
Differentiation													
Well	13	9 (5.6%)	4 (10.5%)	.384	8 (5.1%)	5 (11.9%)	**.032**	10 (6.5%)	3 (6.8%)	.523	8 (5.4%)	5 (10.0%)	.508
I ntermediate	72	61 (37.9%)	11 (28.9%)		52 (33.1%)	20 (47.6%)		53 (34.2%	19 (43.2%)		54 (36.2%)	18 (36.0%)	
Poor	114	91 (56.5%)	23 (60.5%)		97 (61.8%)	17 (40.5%)		92 (59.4%)	22 (50.0%)		87 (58.4%)	27 (54.0%)	
Tumor size													
<3cm	60	52 (31.3%)	8 (20.5%)	.182	53 (32.7%)	7 (16.3%)	**.035**	53 (33.1%)	7 (15.6%)	**.022**	50 (32.9%)	10 (16.7%)	.053
≥3cm	145	114 (68.7%)	31 (79.5%)		109 (67.3%)	36 (83.7%)		107 (66.9%)	38 (84.4%)		102 (67.1%)	43 (81.1%)	
T stage													
T1–2	66	52 (31.5%)	14 (35.9%)	.599	57 (35.2%)	9 (21.4%)	.089	55 (34.4%)	11 (25.0%)	.239	55 (36.2%)	11 (21.2%)	**.046**
T3	138	113 (68.5%)	25 (64.1%)		105 (64.8%)	33 (78.6%)		105 (65.6%)	33 (75.0%)		97 (63.8%)	41 (78.8%)	
Venous/lymphatic invasion													
No	144	115 (69.7%)	29 (74.4%)	.566	117 (72.2%)	27 (13.2%)	.314	119 (74.4%)	25 (56.8%)	**.024**	110 (72.4%)	34 (65.4%)	0.340
Yes	60	50 (30.3%)	10 (25.6%)		45 (27.8%)	15 (35.7%)		41 (25.6%)	19 (43.2%)		42 (27.6%)	18 (34.6%)	
Perineural invasion													
No	137	112 (67.9%)	25 (64.1%)	.652	112 (69.1%)	25 (59.5%)	.237	112 (70.0%)	25 (56.8%)	.099	105 (69.1%)	32 (61.5%)	0.318
Yes	67	53 (32.1%)	14 (35.9%)		50 (30.9%)	17 (40.5%)		48 (30.0%)	19 (43.2%)		47 (30.9%)	20 (38.5%)	
Lymph node metastasis													
N0–1	145	118 (71.1%)	27 (69.2%)	.819	115 (71.0%)	30 (69.8%)	.876	114 (71.2%)	31 (68.9%)	.758	111 (73.0%)	34 (64.2%)	.221
N2–3	60	48 (28.9%)	12 (30.8%)		47 (29.0%)	13 (30.2%)		46 (28.8%)	14 (31.1%)		41 (27.0%)	19 (35.8%)	
Clinical stage													
I+II	96	77 (46.4%)	19 (48.7%)	.793	76 (46.9%)	20 (46.5%)	.963	76 (47.5%)	20 (44.4%)	.717	74 (48.7%)	22 (41.5%)	.367
III+IV	109	89 (53.6%)	20 (51.3%)		86 (53.1%)	23 (53.5%)		84 (52.5%)	25 (55.6%)		78 (51.3%)	31 (58.5%)	
Postoperative radiotherapy													
No	157	132 (79.5%)	25 (64.1%)	**.041**	123 (75.9%)	34 (79.1%)	.665	122 (76.2%)	35 (77.8%)	.831	120 (78.9%)	37 (69.8%)	.176
Yes	48	34 (20.5%)	14 (35.9%)		39 (24.1%)	9 (20.9%)		38 (23.8%)	10 (22.2%)		32 (21.1%)	16 (30.2%)	
Postoperative chemotherapy													
No	135	116 (69.9%)	19 (48.7%)	**.012**	105 (64.8%)	30 (69.8%)	.543	104 (65.0%)	31 (68.9%)	.627	103 (67.8%)	32 (60.4%)	.329
Yes	70	50 (30.1%)	20 (51.3%)		57 (35.2%)	13 (30.2%		56 (35.0%)	14 (31.1%)		49 (32.2%)	21 (39.6%)	

Bold values indicate statistical significance ($P < .05$).

CK-20 = cyto-keratin 20, MMP9 = matrix metallopeptidase 9.

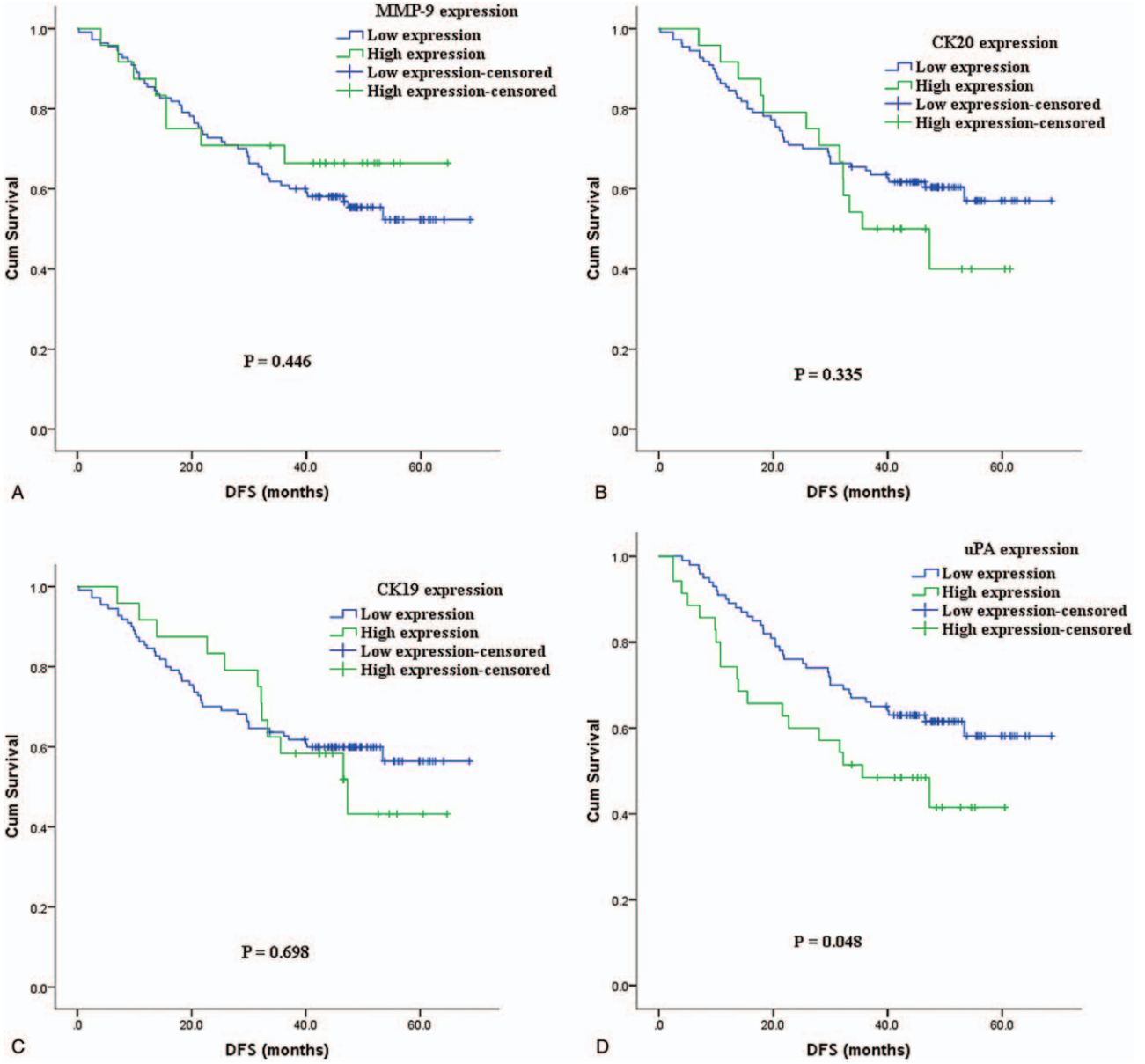

Figure 1. Kaplan–Meier disease-free survival estimates for patients with resectable esophageal squamous cell carcinoma according to circulating MMP9, CK20, CK19, and uPA expression levels. CK-19 = cyto-keratin 19, CK-20 = cyto-keratin 20, MMP9 = matrix metallopeptidase 9, uPA = urokinase type plasminogen activator.

suggest that high expression uPA is associated with poor prognosis in ESCC patients.

The results of univariate and multivariate Cox proportional hazard regression analysis for OS and DFS are shown in Tables 3. In the univariate analysis, tumor size, lymph node metastasis, T stage, and clinical stage showed significance for DFS and OS (Table 2). In the multivariate analysis, gender, smoking history, and T stage showed significance for both DFS and OS. These results suggest that uPA has independent prognostic value for both OS and DFS (Table 3).

5. Discussion

ESCC is an invasive malignant tumor with a high mortality rate (109.5 per 100,000).[16] Obviously, the efficacy of simple surgical treatment is unsatisfactory.[2] It is thus crucial to correctly identify those patients who have an increased risk of cancer recurrence and may benefit from adjuvant treatment. The aim of this study is to examine the expression level of 4 tumor-promoting biomarkers including MMP9, CK20, CK19, and uPA in the peripheral blood and the prognosis of patients with ESCC. As a result, none of the expression level of MMP9, CK20, or CK19, except uPA, was related to the DFS or OS of resectable ESCC.

uPA, a serine protease with multiple function, acts as risk assessment and a possible treatment target in many cancers,[10,17,18] such as breast cancer,[9,19,20] pancreatic cancer,[21,22] prostate cancer,[23,24] and ovarian cancer.[25–27] In particular, uPA can accelerate tumor metastasis and promote tumor angiogenesis by degrading extracellular matrix (ECM) and

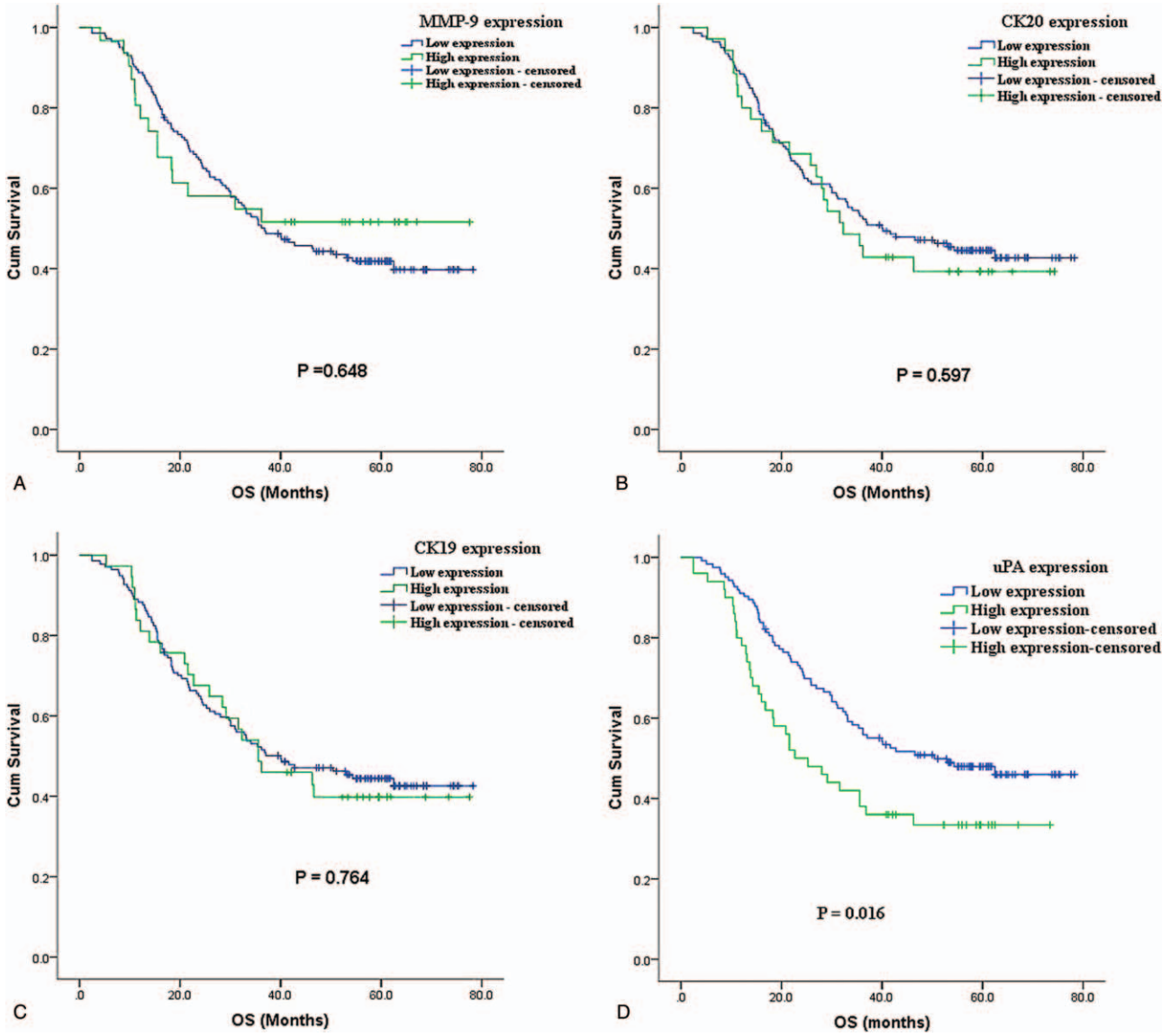

Figure 2. Kaplan–Meier overall survival estimates for patients with resectable esophageal squamous cell carcinoma according to circulating MMP9, CK20, CK19, and uPA expression levels. CK-19 = cyto-keratin 19, CK-20 = cyto-keratin 20, MMP9 = matrix metallopeptidase 9, uPA = urokinase type plasminogen activator.

basement membranes, such as vimentin and fibronectin, involving in epithelial-mesenchymal transition (EMT).[27,28] Moreover, international guidelines (AGO, St. Gallen, ASCO) recommend the use of uPA and plasminogen activator inhibitor-1 (PAI-1) expression to better assess potential clinical benefit from adjuvant systemic treatment of breast cancer.[29–31]

The role of uPA overexpression in EC was also investigated. uPA overexpression was in tissue which accessed by immunohistochemistry related to clinical stage, differentiation and lymph node metastasis.[32] However, we found no correlation between uPA overexpression and lymph node metastasis. Torzewski et al[33] have proved that the intensity of uPA expression detected by immunohistochemistry was an independent risk prognostic factor in 150 potentially curatively resected ESCC patients. In our study, circulating uPA mRNA was found to be an independent predictor of a poor outcome in univariate and multivariate analysis. In addition, uPA expression was related to T stage, and uPA expression of the group with T3 stage were significantly higher than those of the group with T1–2 stage ($P = .046$).

Because a blood sample is more easily to be obtained, circulating uPA mRNA overexpression maybe a clinically useful, non-invasive screening strategy for ESCC patients. Furthermore, we usually access circulating protein using immunofluorescence, mass spectrometry, protein microarray, or Elisa. Obviously, qPCR which use to test mRNA expression is more economically and widespread use in most hospital. To our best knowledge, this is the first and large-scale study to demonstrate that uPA mRNA overexpression in the peripheral blood can be used as a reliable surrogate method to predict poor survival of resectable ESCC. However, the optimal cut-off value of circulating uPA mRNA expression is needed to be further validated. In addition, no healthy control group was evaluated in this study.

Based on our findings, we clearly demonstrated that circulating uPA mRNA in peripheral blood may be a promising prognostic predictor in ESCC patients postoperatively. It might be a

Table 2

Univariate analysis of factors that influence the progression-free survival and overall survival.

Factors	Patients (n)	*P* value of DFS	*P* value of OS
Gender			
Female	23	.453	.961
Male	182		
Age			
<60 years old	91	.166	.133
≥60 years old	114		
Smoking			
Never	45	.361	.128
Ever	158		
Alcohol consumption			
Never	48	.165	.108
Ever	157		
Family history of cancer			
No	152	.293	.885
Yes	53		
Differentiation			
Well	13	.459	.820
Intermediate	72		
Poor	114		
Tumor size			
<3cm	60	**.050**	**.030**
≥3cm	145		
T stage			
T1–2	66	**.002**	**<.001**
T3	138		
Venous/lymphatic invasion			
No	144	.386	.958
Yes	60		
Perineural invasion			
No	137	.347	.183
Yes	67		
Lymph node metastasis			
N0–1	145	**.048**	**<.001**
N2–3	60		
Clinical stage			
I+II	96	**.008**	**<.001**
III+IV	109		
MMP-9			
Low expression	166	.446	.648
High expression	39		
CK-20			
Low expression	144	.335	.597
High expression	43		
CK-19			
Low expression	160	.698	.764
High expression	45		
uPA			
Low expression	152	**.048**	**.016**
High expression	53		
Postoperative radiotherapy			
No	157	.328	.162
Yes	48		
Postoperative chemotherapy			
No	135	.879	.524
Yes	70		

DFS = disease-free survival; OS = overall survival, uPA = urokinase type plasminogen activator.
Bold values indicate statistical significance ($P < .05$).

potential new and interesting way in screening and monitoring of ESCC patients.

Author contributions

Conceptualization: Xiao He.
Data curation: Xiaoling Xu.
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Investigation: Xiao He.
Project administration: Guanxia Zhu.
Validation: Hong Ye.
Writing – original draft: Xiao He.
Writing – review & editing: Hong Ye.

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Table 3

Multivariate analysis of progression-free survival and overall survival in ESCC.

	Characteristics		Progression-free survival			Overall survival		
Factors	Unfavorable	Favorable	Hazard ratio	95% CI	*P* value	Hazard ratio	95% CI	*P* value
Gender	Male	Female	0.335	0.142–0.791	**.013**	0.494	0.245–1.000	**.050**
Age	≥ 60 years old	< 60 years old				1.503	0.974–2.319	.066
Smoking history	Yes	No	2.206	0.996–4.885	**.051**	2.326	1.239–4.364	**.009**
T stage	T3	T1+T2	2.888	1.464–5.698	**.002**	2.487	1.445–4.281	**.001**
Lymph node metastasis	N3	N0–2	1.678	0.959–2.936	.070	2.119	1.378–3.256	**.001**
uPA	(+)	(-)	1.972	1.113–3.492	**.020**	1.819	1.161–2.851	**.009**

Bold values indicate statistical significance ($P < 0.05$).
ESCC = esophageal squamous cell carcinoma, uPA = urokinase type plasminogen activator.

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