MEDICAL SCIENCE MONITOR

Prognostic Significance of Periostin and Mammalian Target of Rapamycin (mTOR) in Locally Advanced Esophageal Squamous Cell Carcinoma

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Periostin and the mammalian target of rapamycin (mTOR) are involved in several cancers. This study aimed to evaluate the expression level of periostin and mTOR in locally advanced esophageal squamous cell carcinoma (ESCC) and to analyze their correlations with prognostic value.

Material/Methods: Expression levels of periostin and mTOR were examined by immunohistochemistry in locally advanced ESCC and corresponding adjacent normal tissue of 71 patients. The expression of periostin and mTOR were correlated with clinicopathologic characteristics by χ^2 test or Kruskal-Wallis analysis. The prognostic factors of periostin and mTOR on overall survival (OS) and disease-free survival (DFS) were assessed using Kaplan-Meier and Cox regression methods, respectively.

Results: The high expression of periostin was significantly correlated to tumor stage (P=0.000), vascular invasion (P=0.027), differentiation (P=0.002), invasion depth (P=0.023), and lymph node metastasis (P=0.017). The high expression of mTOR was associated with tumor stage (P=0.001), lymphatic metastasis (P=0.014), and differentiation (P=0.036). Expression levels of periostin and mTOR was positively correlated (r=0.416, P=0.000). The OS and DFS in patients in the high-periostin group were significantly shorter than those in the low-periostin group, (both P<0.001). Similar results were found in mTOR analysis. Moreover, Cox regression analysis showed that the expressions of periostin and mTOR, along with tumor stage, were the independent factors affecting the survival time of ESCC patients.

Conclusions: Expressions of periostin and mTOR are related to multiple clinicopathologic features. High expression of periostin and mTOR were independent risk factors of ESCC patients, which might offer a potential target strategy for ESCC treatment in the future.

MeSH Keywords: Esophageal Neoplasms • Immunohistochemistry • Prognosis • TOR Serine-Threonine Kinases

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Background

Esophageal cancer (EC) is the eighth most common malignant tumor globally, while cancer-associated mortality ranks sixth among all carcinomas [1,2]. Esophageal squamous cell carcinoma (ESCC) in developing countries accounts for 80% of esophageal cancer [2,3]. The early symptoms of esophageal cancer have no specificity, and early screening and diagnosis methods are shortage in clinical practice of esophageal cancer. Once the disease is detected, the course of disease has entered the advanced stage [4]. Besides with surgery and chemoradiotherapy, targeted therapy is currently the effective treatment strategies for patients with locally advanced ESCC. However, the 5-year survival rate of EC in the relevant literature was about 20% [1,5]. Therefore, it is necessary to screen out more biomarkers for monitoring the progression of ESCC.

Periostin is an extracellular matrix and secreted protein, which is highly expressed in inflammation in adults and embryos [6,7]. Currently, periostin is also upregulated in some malignant cancers, such as pancreatic, ovarian, colorectal, breast, lung, head and neck, thyroid, gastric, neuroblastoma, hepatic, and esophageal cancer [8–16]. An in vitro study of ESCC found the expression of periostin protein was decreased by inhibiting EGFR and by restoring wild-type p53 signaling [17]. An in vivo experiment showed that periostin in ESCC xenograft tumors of mice might play an important role in detection of preneoplastic lesions [18]. Additionally, periostin binding with some integrins ($\alpha v \beta 3$, $\alpha 6 \beta 4$, and $\alpha v \beta 5$) can interact with some cell surface receptors and then activate downstream proteins to promote tumor invasion and metastasis by PI3K-Akt and/or other signaling pathways [14,15]. The mammalian target of rapamycin (mTOR) is a downstream target protein of the PI3K/Akt. The correlation between periostin and mTOR and their prognostic significance is currently unclear. Therefore, we observed periostin and mTOR expression in ESCC tissues using immunohistochemistry (IHC) method, and investigated not only the association between periostin and mTOR, but also their prognostic significance in locally advanced ESCC patients.

Material and Methods

Tissue samples

In this retrospective study, 71 cases of locally advanced ESCC and paired adjacent normal esophageal epithelium were collected from patients who underwent radical esophageal resection from 8 April 2009 to 23 June 2011 at Anhui Provincial Hospital. All patients had no other malignant tumor and were not treated with any anti-tumor therapy before the operation. All cases had a complete clinical data, including overall survival (OS) and disease-free survival (DFS) time. The pathological diagnosis was confirmed as squamous cell carcinoma by the double-blind method. The duration of follow-up was from the time of surgery to death or study deadline (September 19th, 2015). All patients were simultaneously detected by periostin and mTOR. This study was approved by the Research Ethics Committee of Anhui Provincial Hospital, and all patients signed informed consent.

Immunohistochemistry

The Envision (DAKO) method of immunohistochemistry staining was used to determine the expression of periostin and mTOR protein. Primary rabbit polyclonal anti-periostin and anti-mTOR antibody were purchased from Abcam, Ltd, Shanghai, China and used at a working concentration of 1: 150. Briefly, all specimens were cut into sections 4 micrometers thick, deparaffinized, and rinsed. The specimen slices incubated in citrate buffer (pH=6.0) were heated to retrieve antigen using a microwave oven. The sections were then incubated with primary antibody at room temperature following blocking of endogenous peroxidases and washing 3 times in buffer. The incubation time was 2 h. After incubating with secondary antibody at room temperature for 30 min, the sections were stained. The secondary antibody was purchased from Zhongshan Jinqiao Co., Beijing, China. A known positive tissue slice was used as a positive control and slides processed with PBS were the negative control.

Result evaluation

The stained sections were examined independently and blindly by 2 experienced pathologists. The standard for judging the expression level of proteins by IHC was partly based on previous research [9]. Grading was performed according to the staining intensity and distribution of positive tumor cells. The degree of staining was divided into 4 grades: negative, light yellow, brownish-yellow, and brown. Moreover, the scores of staining intensity above were 0, 1, 2, and 3 points. The degree of stain-positive tumor cells distribution was also divided into 4 grades. We randomly selected 10 fields under 400×magnification to observe positive cells and carefully calculated the proportion of positive cells by counting 200 cells per field. When the percentage of stain-positive cells was less than 5%, the score was zero point. On the contrary, when percentage of stain-positive cells was more than 50%, the score was 3 points. The scores of stain-positive cells which ranked 5–25% or 25–50% were 1 and 2 points, respectively. The final scores=score of stain intensity×score of stain-positive cell distribution. Scores of more than 4 points were defined as high expression, and the low-expression group contained a small number of expressive slices or no expressive slices.
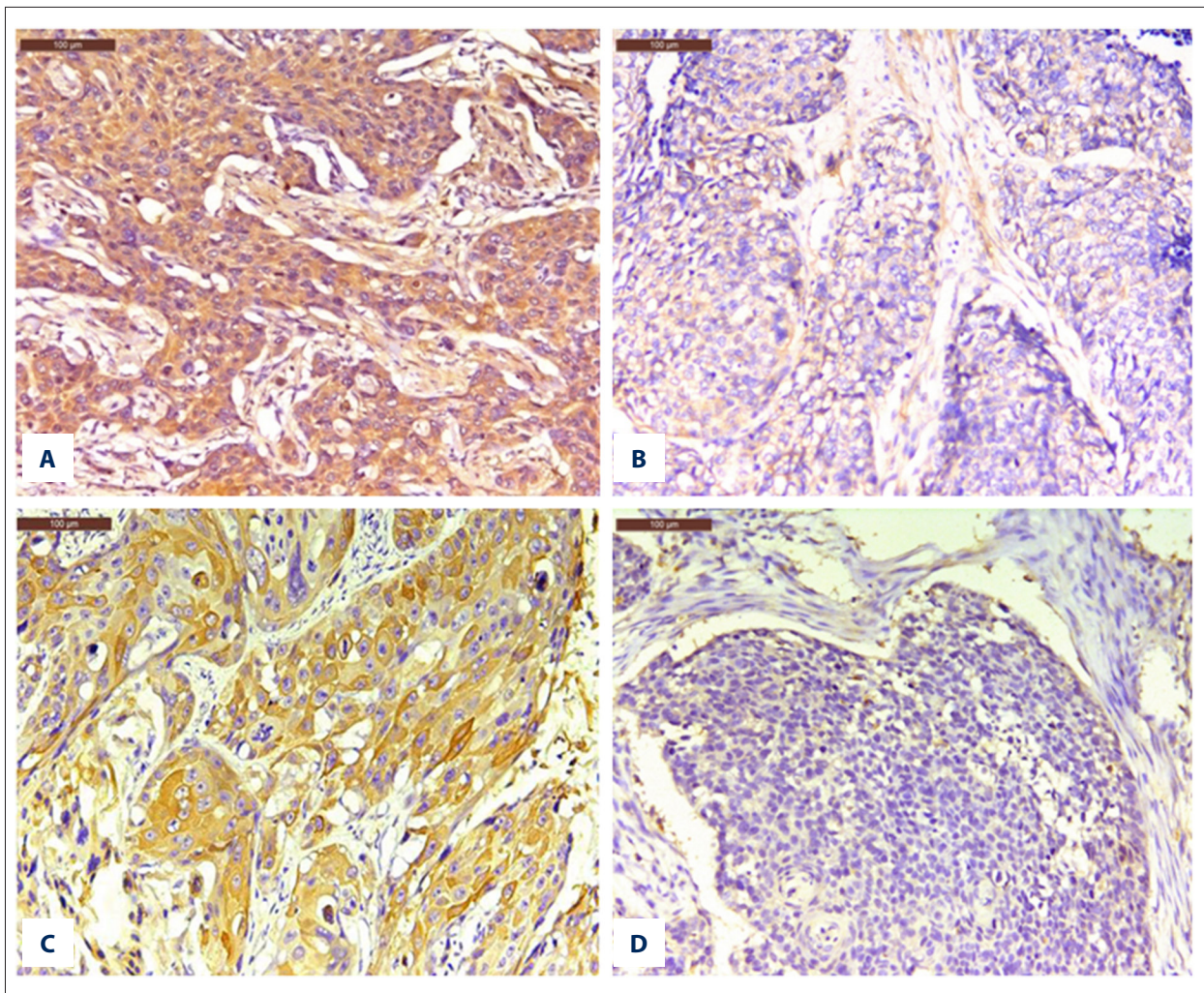
Figure 1. IHC staining for periostin and mTOR in ESCC tissues (200×). (**A**) IHC high for periostin in ESCC tissues; (**B**) IHC low for periostin in ESCC tissues; (**C**) IHC high for mTOR in ESCC tissues; (**D**) IHC low for mTOR in ESCC tissues.

Statistical analysis

Data were analyzed using SPSS software (vision 13.0). The chi-square test and Kruskal-Wallis analysis were used to assess the correlation between the expression of these 2 proteins and clinicopathologic characteristics. The expression of periostin was correlated with mTOR expression by Spearman rank correlation tests. Kaplan-Meier curve and log-rank test were conducted to compare the correlation between survival time (DFS and OS) and these 2 proteins. Multivariate analysis was assessed by using the Cox regression model. All *P* values <0.05 were regarded as statistically significant.

Results

Periostin expression in ESCC and its relationship with clinicopathological features

In our study, periostin was expressed in tumor cytoplasm and also extracellular fibroblast cells (Figure 1). Periostin was highly expressed in the cytoplasm of neoplastic cells (61.97%, 44/71) and adjacent normal esophageal squamous epithelium (32.39%, 23/71). Moreover, according to its positive expression in cytoplasm, the IHC scores in ESCC tumor and control normal squamous epithelium showed significant discrimination (*P*<0.001, Figure 2). In the present study, patients were divided into high and low expression groups according to periostin levels. Periostin in the high group was significantly associated with vascular invasion (*P*=0.027), tumor stage (*P*=0.000), differentiation (*P*=0.002), depth of invasion (*P*=0.023), and lymph node metastasis (*P*=0.017), whereas the relationship
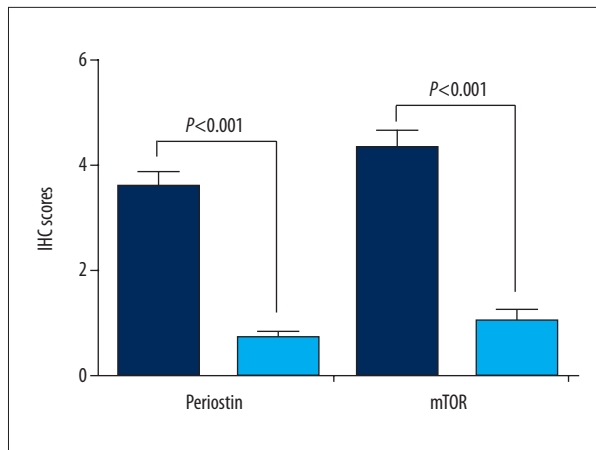
Figure 2. IHC scores of periostin and mTOR expression in the cancer group was higher than that in the normal control group (*P*<0.001).

between periostin and other clinicopathological parameters, such as tumor location, sex, and age, showed no significance (all *P*>0.05, Table 1).

mTOR expression in ESCC and its relationship with clinicopathological features

Based on analysis results of IHC, mTOR is mainly expressed in the cytoplasm of tumor cells (Figure 1). The high expression rate of mTOR in tumor and control normal esophageal squamous epithelium was 69.01% (49/71) and 35.21% (25/71), respectively. Compared with normal tissues, mTOR was expressed at higher rates in ESCC (*P*<0.001, Figure 2). mTOR in the high expression group was not significantly associated with sex, age, depth of invasion, vascular invasion, tumor size, or location (all *P*>0.05), whereas its high expression was only significantly associated with differentiation (*P*=0.036), lymph node metastasis (*P*=0.014), and tumor stage (*P*=0.001, Table 1).

Correlation analysis of periostin and mTOR

The expression of periostin was correlated with mTOR expression, which was calculated by Spearman analysis. Periostin and mTOR in the high group were found in 37 of 71 (52.11%). On the contrary, periostin and mTOR in the low group were found in 15 of 71 (21.13%). Spearman correlation analysis showed that periostin in the high group was positively associated with high mTOR expression (r=0.416; *P*=0.000; Table 2).

Survival analysis of periostin and mTOR

Kaplan-Meier analysis showed that OS in locally advanced ESCC patients with high expression of periostin (27.96±2.12 months) was worse than in those with low expression of periostin (45.84±2.82 months, Figure 3A), showing a significant

difference (χ^2=18.890, *P*=0.000, Table 3). The OS of patients with high mTOR levels was significantly shorter than in those with low mTOR levels, with OS time of 29.27±1.94 months and 46.98±3.52 months, respectively (χ^2=18.826, *P*=0.000; Figure 3B). Similarly, DFS in the high-periostin and high-mTOR groups was significantly worse than in the low-periostin and low-mTOR group, respectively (16.68±1.62 months *vs.* 37.78±3.52 months; 18.51±1.56 months *vs.* 38.50±4.48 months; both *P*<0.001; Figure 3C, 3D).

Results of univariate analysis show that prognosis in patients with ESCC is significantly associated with the following features: expression of periostin and mTOR, invasion depth, differentiation grade, lymphatic metastasis, and tumor stage (all *P*<0.05, Table 3). Multivariate Cox regression analysis showed that periostin and mTOR were independent prognostic factors, along with tumor stage (Table 4).

Discussion

Esophageal carcinoma, a common malignant digestive tract tumor, has a high degree of invasion and a low survival rate of 5 years [1,4]. Currently, accumulating data in the literature demonstrate that periostin is upregulated in a variety of carcinomas, and is involved in tumor invasion, angiogenesis, and metastasis, as well as cell survival [7,14]. However, few studies have focused on the combined expression of periostin and mTOR in ESCC and their prognostic value. The correlation between periostin and mTOR remains unknown.

In the present study, periostin was expressed at significantly higher levels in cancer tissue than in tumor-adjacent normal squamous epithelium, consistent with previous studies [15,16,19]. Michaylira et al. [17] found that periostin was overexpressed in cells with both high EGFR expression and mutant p53 status. Periostin was reported to be highly expressed in ESCC xenograft tumors of mice [18]. The results of these studies suggest that upregulated expression of periostin is frequent in ESCC, and high expression of periostin might be involved in tumorigenesis of ESCC. Our research indicates that periostin in locally advanced ESCC is associated with tumor invasion depth, differentiation, vascular invasion, lymph node metastasis, and tumor stage, consistent with the results of Wang et al. [15]. Paradoxically, Luo et al. [16] reported that periostin was not associated with the degree of differentiation. These conflicting results were probably caused by differences in sample selection and evaluation criteria for IHC methods. Survival analysis also showed that patients in the high-periostin group had significantly shorter OS and DFS than in the low periostin group, suggesting that periostin as an extracellular matrix protein might affect invasion and the process of ESCC [14–17].

Table 1. Relationship between periostin, mTOR, and clinicopathological characteristics in 71 locally advanced ESCC patients.

Characteristics	n	Periostin			mTOR		
		High	Low	P	High	Low	P
Sex							
Male	41	25	16	0.804	26	15	0.233
Female	30	19	11		23	7	
Age (years)							
<60	23	14	9	0.895	15	8	0.632
≥60	48	30	18		34	14	
Tumor length (cm)							
<5	57	33	24	0.153	39	18	0.827
≥5	14	11	3		10	4	
Tumor location							
Upper	6	2	4	0.624	2	4	0.536
Middle	36	24	12		27	9	
Low	29	18	11		20	9	
Invasion depth							
pT1	4	0	4	0.023	2	2	0.154
pT2	20	11	9		12	8	
pT3	47	33	14		35	12	
Differentiation grade							
Well	15	4	11	0.002	8	7	0.036
Moderate	44	30	14		30	14	
Poor	12	10	2		11	1	
Vascular invasion							
Yes	15	13	2	0.027	11	4	0.926
No	56	31	25		38	18	
Lymphatic metastasis							
Yes	44	32	12	0.017	35	9	0.014
No	27	12	15		14	13	
TNM stage							
I	7	1	6	0.000	1	6	0.001
II	31	16	15		20	11	
III	33	27	6		28	5	

Table 2. The expression correlation between periostin and mTOR (cases).

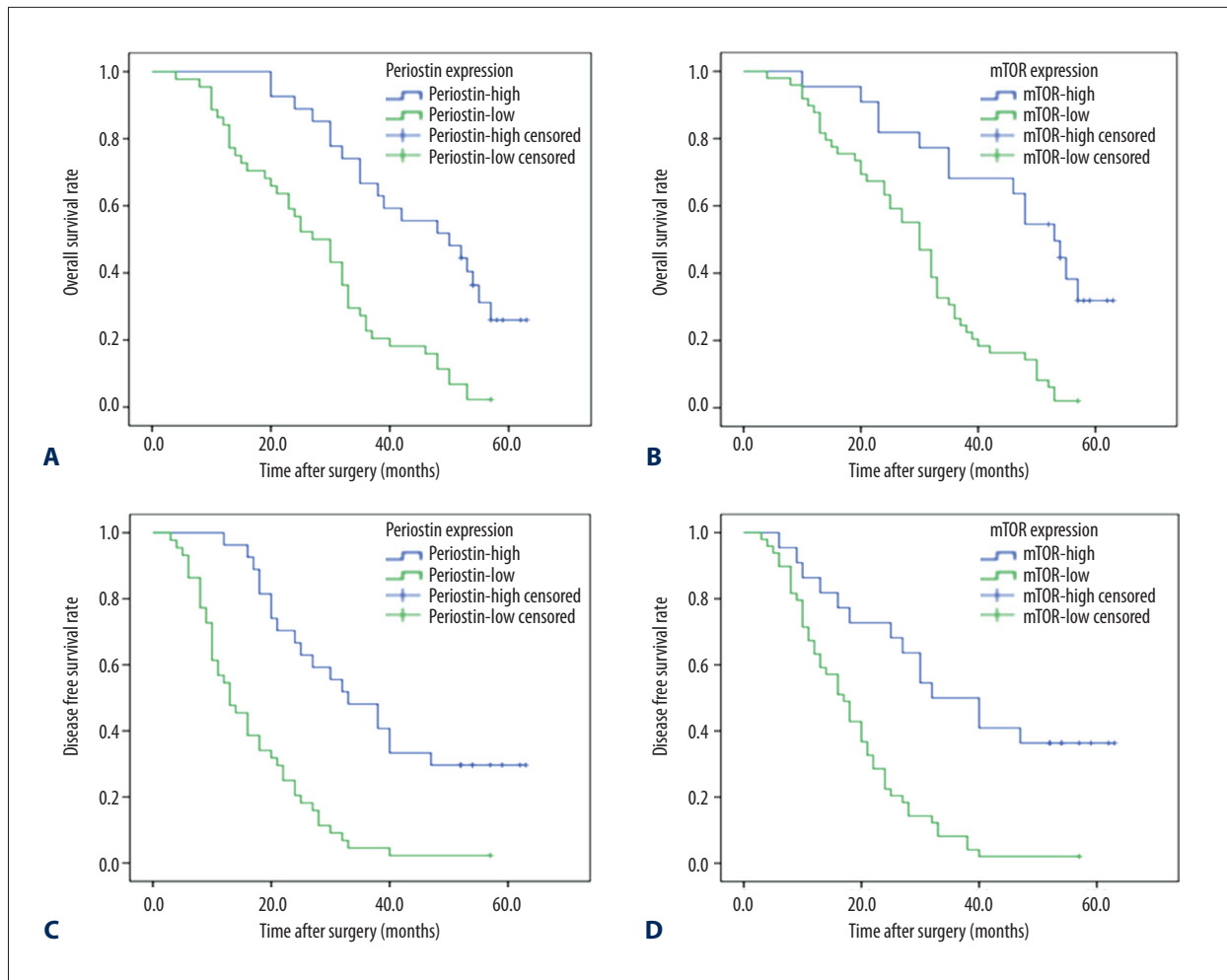|		Periostin		r	P value
		High (n=44)	Low (n=27)		
mTOR	High (n=49)	37	12	0.416	0.000
	Low (n=22)	7	15		

Figure 3. Kaplan-Meier survival curves of the OS and DFS in ESCC patients. (**A**) OS curve of ESCC patients based on periostin expression; (**B**) OS curve of ESCC patients based on mTOR expression; (**C**) DFS curve of ESCC patients based on periostin expression; (**D**) DFS curve of ESCC patients based on mTOR expression.

mTOR, as an atypical serine/threonine acid protein kinase, can be activated by PI3K/Akt and control phosphorylation of multiple downstream target protein, which regulates the cell cycle, proliferation, and invasion [20–23]. The high expression rate of mTOR in our samples was consistent with the results of Lu et al. [4]. Hou et al. [24] indicated that the sensitivity to cisplatin in EC9706 was increased after silencing mTOR expression by RNA interference cells, suggesting that the high expression of mTOR in ESCC was a common phenomenon *in vivo* and *in vitro*. Some scholars found that p-mTOR protein had no significant association with differentiation, lymph node metastasis, or tumor staging between positive and negative group [25,26], but Chuang et al. [27] and Boone et al. [28] found p-mTOR expression level was associated with tumor grade and differentiation, respectively. The role of mTOR in ESCC is presently unclear. However, Lu et al. [4] found that high expression of mTOR was significantly associated with differentiation, invasion depth, lymph node metastasis, and tumor

staging. The present study found that mTOR was only associated with differentiation, lymph node metastasis, and tumor staging, which was similar to the findings of Lu et al. [4] in 2015. Larger studies are needed to determine the reasons for the discrepancy between these studies. Survival analysis showed that the OS and DFS in the high-mTOR group were significantly worse than in the low-mTOR group, consistent with previous research [4,25]. Moreover, multivariate analyses showed that high expression of periostin and mTOR were independent risk factors determining the survival of patients with locally advanced ESCC, and might be prognostic factors in targeted therapy of ESCC.

Research showed that periostin binding with integrin (αvβ3, αvβ5, α6β4) was closely related to a variety of cell surface receptors and signaling pathways [14]. Periostin can activate target proteins of FAK and Akt/PKB pathway [14,15,29,30], and mTOR is one of the downstream targets of Akt. Until present,

Table 3. Univariate analysis of clinicopathologic features associated with survival time.

Characteristics	DFS		OS	
	95%CI	*P*	95%CI	*P*
Periostin				
Low	30.877–44.679	0.000	40.303–51.374	0.000
High	13.506–19.857		23.805–32.104	
mTOR				
Low	29.728–47.272	0.000	40.084–53.883	0.000
High	15.463–21.557		25.454–33.076	
Sex				
Male	18.686–29.558	0.709	28.874–39.230	0.690
Female	19.467–21.667		29.875–41.642	
Age (years)				
<60	15.061–28.244	0.534	24.693–38.293	0.425
≥60	20.912–30.546		31.585–40.832	
Tumor length (cm)				
<5	21.003–30.646	0.267	31.214–40.028	0.288
≥5	14.049–27.094		23.020–40.408	
Tumor location				
Upper, middle	18.717–28.950	0.684	29.592–39.555	0.761
Low	19.409–32.591		28.795–41.205	
Invasion depth				
pT1, pT2	25.776–43.474	0.001	35.333–49.834	0.002
pT3	16.327–22.737		26.647–35.072	
Differentiation grade				
Well, moderate	21.879–31.205	0.030	32.627–41.015	0.027
Poor	10.228–22.105		15.999–34.334	
Venous invasion				
Yes	12.260–30.406	0.358	23.730–40.404	0.568
No	21.104–30.075		31.118–39.870	
Lymphatic metastasis				
Yes	14.692–23.354	0.001	24.870–33.857	0.002
No	27.374–40.774		37.857–49.460	
TNM stage				
I, II	28.261–40.054	0.000	39.090–49.092	0.000
III	11.483–16.517		20.495–27.869	

Table 4. Multivariate analysis of characteristics associated with OS and DFS.

Factor	OS			DFS		
	HR	95%CI	*P*	HR	95%CI	*P*
Periostin (high *vs.* low)	2.260	1.143–4.468	0.019	2.265	1.181–4.342	0.014
mTOR (high *vs.* low)	2.117	1.008–4.447	0.048	2.309	1.080–4.938	0.031
Sex (Male *vs.* Female)	1.397	0.745–2.618	0.297	1.243	0.668–2.311	0.493
Age (<60 *vs.* ≥60)	0.694	0.381–1.261	0.230	0.670	0.374–1.201	0.179
Tumor length (<5 *vs.* ≥5)	1.065	0.546–2.079	0.853	1.288	0.657–2.525	0.461
Tumor location (Upper, middle *vs.* Low)	1.045	0.591–1.848	0.880	0.916	0.515–1.628	0.764
Invasion depth (pT3 *vs.* pT1, pT2)	1.632	0.761–3.502	0.209	1.923	0.838–4.411	0.123
Differentiation grade (poor *vs.* well/moderate)	1.514	0.744–3.080	0.253	1.550	0.787–3.055	0.205
Venous invasion (Yes *vs.* No)	0.805	0.402–1.610	0.539	0.898	0.451–1.790	0.760
Lymphatic metastasis (Yes *vs.* No)	0.851	0.310–2.337	0.754	1.232	0.423–3.588	0.702
Stage (III *vs.* I, II)	3.983	1.383–11.472	0.010	3.180	1.086–9.313	0.035

the relationship between periostin and mTOR has been unclear in ESCC. To better understand their value in ESCC, we performed Spearman correlation analysis, which indicated that periostin and mTOR had a positive correlation (*r*=0.416), suggesting that high expression of periostin might be associated with abnormal activation of mTOR. However, the specific mechanism is still unclear and we plan to perform related experiments to demonstrate the underlying mechanisms in future.

There were some limitations in our study. First, our sample size of patients was not large. Secondly, we only examined the protein level of periostin and mTOR by IHC without exploring the detailed molecular mechanisms. Larger studies are required to explore these hypotheses.

Conclusions

Collectively, the results of our study suggest that abnormality of periostin and mTOR in locally advanced ESCC may be closely linked to multiple clinicopathologic features. We found a positive correlation between periostin and mTOR. High expression of periostin and mTOR were independent risk factors determining the DFS and OS of patients with locally advanced ESCC, and might offer a potential target strategy for ESCC treatment in the future.

Conflict of interest

None.

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