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FOXA1 and CK7 expression in esophageal squamous cell carcinoma and its prognostic significance

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Esophageal Squamous Cell Carcinoma (ESCC) is one of the most common malignant tumors in human. Some ESCC cells express adenocarcinoma cell markers, such as Cytokeratin 7 (CK7), but the clinical significance of these cells in ESCC is unknown. Immunohistochemical analysis of CK7 and Fork head box protein A1 (FOXA1, an upstream regulator of CK7) was performed on 610 ESCC specimens using tissue microarray. In total, positive staining of CK7 was 59/594 (10%). CK7 expression was correlated with ESCC differentiation (p=0.006). The expression of CK7 is associated with poor overall survival (OS) of ESCC patients (p=0.0498). FOXA1 positive staining was 180/586 (31%). FOXA1 expression correlates with differentiation (p<0.0001) and vascular invasion status (p=0.016) of ESCC. FOXA1 expression was non-independently correlated with poor prognosis of OS in ESCC patients (p=0.1198), but correlated with the prognosis of ESCC patients in some specific pathological characteristics, such as age less than 61 years (p=0.0066), tumor located in the middle segment of esophagus (p=0.0046), and non-lymph node metastasis (p=0.0377). Correlation analysis between the CK7 and FOXA1 expression was positive (p<0.0001). In conclusion, FOXA1 expression was positively correlated with CK7 expression. CK7 expression is an independent prognostic factor for ESCC, and FOXA1 is a non-independent prognostic factor. Both, CK7 and its upstream factor FOXA1 can be used as potential targets for ESCC therapy.

Key words: FOXA1, CK7, esophageal squamous cell carcinoma, prognosis

Esophageal cancer is the fifth common cancer in the world, and due to the poor survival rate it confers, ranks fourth among all cancers in mortality [1, 2]. Overall incidence rates are two-fold higher in less-developed countries compared with more-developed geographic regions, with the highest rates occurring in Asia [1]. Incidence and mortality rates are two to three fold higher in males than females [1]. ESCC is the predominant histologic type of esophageal cancer worldwide[2]. Because of the lack of early clinical symptoms of ESCC patients and the slow development of symptoms, ESCC invasion ability is very strong and results in a high degree of malignancy of ESCC and poor prognosis. Epidemiological investigation showed that the 5-year survival rate of patients with ESCC was only about 35% [3–6]. In order to reduce the morbidity and mortality of patients with ESCC, it is urgent to select suitable tumor markers. Therefore, the study of tumor markers is of great significance in the development, diagnosis and treatment of cancer [7–10].

The expression of cytokeratin in the majority of epithelial tissues is stable. It is involved in the formation of cytoskeleton in the form of intermediate filament proteins. In the case of epithelial tumor and tumor metastasis, cytokeratin still maintains its expression. CK7 is a type II cytokeratin, it is often used as a marker of epithelial tumor, lung adenocarcinoma, breast invasive ductal carcinoma, endometrial cancer, ovarian cancer, renal cell carcinoma or transitional cell carcinoma. Although CK7 is rarely expressed in squamous cell carcinoma, the studies have reported the expression of CK7 in a number of ESCC part of the squamous epithelium, and found that there is a relationship between CK7 and prognosis of different clinical stages in patients with ESCC [11, 12].

FOXA1 is a member of the transcription factors family, which is associated with the pathogenesis of lung cancer, esophageal cancer and prostate cancer [13]. FOXA1 recruits different transcription factors to perform different functions. FOXA1 is a decisive factor to affect ER binding to chromatin.

FOXA1 binding to ER can control both the activity and the expression levels of ER alpha in breast cancer, which affects the growth and migration of mammary gland cancer cells [14]. Research suggests that FOXA1 is the upstream gene of CK7 and can regulate the expression of CK7 [14–17].

In this study, CK7 and FOXA1 expression were detected in 610 ESCC samples. We characterized CK7 and FOXA1 expression in ESCC tissues for association with clinicopathological data and prognosis.

Patients and methods

ESCC organization source. A cohort of 610 subjects with ulcerative ESCC was recruited between 2008 and 2014 from the Department of thoracic surgery, Affiliated Hospital of Jining Medical University (Shandong, PR China). We collected relevant clinical data and prognostic information of patients. Among them, 470 cases were male and 140 cases were female (3.4:1). Age ranges from 34 to 83 years old (mean age, 61 years). 318 patients have long-term follow-up results and the mean survival time was 29 months (1–95.2 months).

All biopsies were immediately fixed in 4% buffered paraformaldehyde, routinely processed, and embedded in paraffin. Tumors were classified according to the standard TNM staging guidelines of UICC [18]. The study protocol had been reviewed and approved by the local ethics committee. All patients gave written consent for the tissue samples. The study was approved by the ethics committee of Jining Medical University. Each patient signed an informed consent form.

Tissue microarray. Representative areas of the ESCC were marked on each hematoxylin-eosin (H&E) slide and tissue paraffin block, and the marked areas of tissue paraffin blocks were sampled for the TMAs. The TMAs were assembled with a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD) as described by Kallioniemi et al[19].

Immunohistochemical staining of the CK5/6, CK7, and FOXA1 proteins were performed using the streptavidin-peroxidase (S-P) method as previously described with minor modifications [20]. Briefly, each TMA section was deparaffinized and rehydrated, antigen retrieval was done at 95 °C in 1x EDTA (Ethylenediaminetetraacetic acid) buffer (pH 9.0) for 15 min. Inactivation of endogenous peroxidase was performed by using 0.3% H_2O_2-methanol for 30 min. Nonspecific binding was prevented by incubation with normal serum for 20 min at room temperature (RT), followed by incubation with the primary antibody CK7 and CK5/6 (Fuzhou Maixin Biotech.Co., Ltd., China) or FOXA1 (ab173287, Abcam Inc. Cambridge, MA, USA), at 4 °C overnight. Antibody binding was detected using Envision reagents (Dako REAL EnVision Detection System; peroxidase/DAB1, Dako Cytomati on, Denmark). The immune reaction was visualized by an incubation with 3,3'-diaminobenzidine chromogen substrate (DAB 1 Chromogen, DAKOVR, Carpinteria, CA) for 10 min at RT. Finally, slides were counterstained with haematoxylin-eosin, dehydrated and cover-slipped with amounting automat (Sakura GLC 550, Tissue-TekVR, Alphen aanden Rijn, The Netherlands). CK7 and FOXA1 immunoreactivity was scored by two independent pathologists without prior knowledge of patients' clinicopathological characteristics according to the intensity and extent of staining. Briefly, in the tumor cells, low: no staining, weak staining or strong stains in less than 25% of tumor cells; high: moderate staining or strong stains in more than 25% of tumor cells [21, 22].

Data analysis. Differences between groups were estimated using Pearson's c^2 to analyze the association of CK7 and FOXA1 expression with clinicopathological characteristics by using the SPSS 13.0 software package (SPSS, Chicago, IL). The Kaplan-Meier method was used to determine the probability of survival and data was analyzed with the logrank test, GraphPad Prism software (version 6, La Jolla, CA) was used for the analysis. In the analyses, a p-value of <0.05 was considered significant.

Results

Glandular epithelium-like ESCC cells express high level of CK7. In our study, a subset of cancer cells, inner blue line region cells (Figure 1), were found to have simultaneously high expression of both the biomarkers for squamous epithelium (CK5/6) and glandular epithelium (CK7) by immunohistochemical staining in ESCC samples at low-magnification (Figure 1B, C) and high-power magnification (Figure S1B, C). We define these subset cells as Glandular Epithelium Cell-like ESCC Cells (GECEC). Normal squamous epithelial cells, inner black line region cells, only express CK5/6 protein, but not CK7 (Figure 1B, C). Typical ESCC cells, inner red line region cells also highly expressed CK5/6 protein, but CK7 had low or no expression (Figure 1B, C). FOXA1, the upstream regulator of CK7, was highly expressed in normal squamous epithelial cells and GECEC (Figure 1D).

CK7, FOXA1 expression and its relationship with clinicopathological characteristics and prognosis of ESCC patients. In total, CK7 strong staining was found in 59 cases (Table 1). The statistical results reveal that CK7 expression was only associated with ESCC differentiation (p=0.006), but uncorrelated with other clinicopathological characteristics (Table 1). Kaplan-Meier analysis was performed in 312 ESCC patients based on the follow up information. The data showed that the CK7 expression was associated with poor OS of ESCC patients (p=0.0498) (Figure 3A).

FOXA1 positive staining was found in 180 cases (30%). FOXA1 expression was associated with tumor vascular invasion (p=0.016) and the degree of differentiation ($p<0.001$) of ESCC patients (Table 1). There was no relationship between the expression of FOXA1 and the prognosis of OS in ESCC patients (p=0.1198) (Figure 3B).

CK7 and FOXA1 expression was independently associated with the prognosis of ESCC patients in some specific pathological characteristics. The ESCC patients were

grouped according to the characteristics of esophagus. The data showed that CK7 expression was also independently associated with the poor prognosis of ESCC patients, such as male (p=0.0104, Figure 4A), the tumor diameter more than 4 cm (p=0.0037, Figure 4B), clinical stage I+II (p=0.0464, Figure 4C), non-lymph node metastasis (p=0.0217, Figure 4D), and non-neurological invasion (p=0.0158, Figure 4E).

Although FOXA1 expression was non-independently correlated with poor prognosis of OS in ESCC patients, our data showed that the FOXA1 expression was independently associated with the poor prognosis of ESCC patients in some specific pathological characteristics, such as patients' age <61 years (p=0.0066, Figure 5A), the middle esophageal location (p=0.0046, Figure 5B), and non-lymph node metastasis (p=0.0377, Figure 5C).

Expression relationship between CK7 and FOXA1. In total, CK7 expression was positively correlated with FOXA1 expression (p<0.0001, Figure 6).

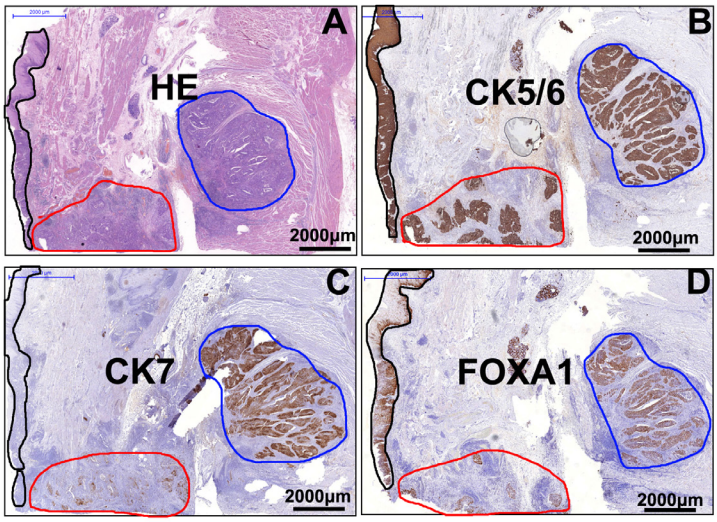


Figure 1. Glandular epithelium and squamous epithelium biomarkers were co-expressed in GEEC. A, Hematoxylin & Eosin staining. Immunohistochemistry staining for B, squamous epithelium biomarkers, CK5/6, C, glandular epithelium biomarkers, CK7, and D, CK7 up stream regulator, FOXA1. Scale bar, 2000 μm.

Discussion

Our study found that CK7 expression was associated with ESCC differentiation. This is consistent with the study of Yamada A et al. Our data also revealed that high expression of CK7 was an independent poor prognosis marker for ESCC patients. Yamada et al. [23] noted that CK7 expression was a useful biomarker for predicting the outcome of stage I/IIA/IIB ESCC patients [23]. Oue et al. [24] pointed out that ESCC patients with CK7-positive, adjuvant chemotherapy tended to be beneficial for patients with stage II/III.

Immunostaining markers such as CK5/6 can be used to distinguish squamous epithelium, whereas CK7 is used for glandular epithelium [25]. In our study, we found a subpopulation of ESCC cells that positively co-express CK5/6 and CK7. A study has shown that almost all ESCC highly expressed CK5/6 protein, and only one of the 64 samples showed an immunohistochemical score of CK5/6 less than 10% [26], so we only detected the CK7 expression by ESCC TMA. The functions and meanings of the GECEC are not clear. Adenosquamous esophageal carcinoma (ACS) is another esophageal cancer subtype, a rare histological variant comprising 1% of esophageal adenocarcinoma (ACA) in the Unites States [27]. Patients with ASC had worse OS compared to ACA, but not ESCC in both univariate and multivariate analyses (OR=0.76;

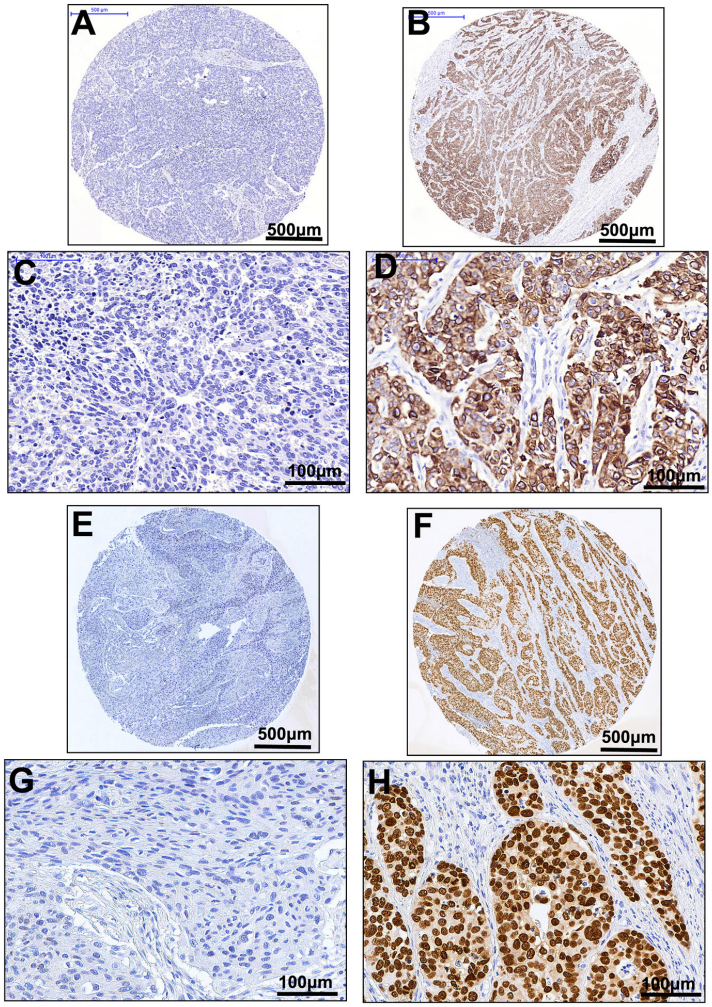


Figure 2. Immunohistochemistry staining for CK7 and FOXA1 proteins in ESCC samples. A&C, CK7 staining negative, B&D, CK7 staining positive, E&G, FOXA1 staining negative, F&H, FOXA1 staining positive. Scale bar, A, B, E, F, 500 μm, C, D, G, H, 100 μm.

Table 1. CK7 and FOXA1 expression in ESCC patients and its clinicopathological significance.

	CK7			FOXA1		
	High (n=59) N (%)	Low (n=535) N	p-value	High (n=180) N (%)	Low (n=406) N	p-value
Age (years)			0.36			0.302
≥61	33(56)	281		98(54)	210	
<61	26(44)	254		82(46)	196	
Gender			0.379			0.532
Male	47(80)	411		139(77)	313	
Female	12(20)	124		41(23)	93	
Tumor size (cm)			0.368			0.53
>4	23(39)	192		65(36)	147	
≤4	36(61)	343		115(64)	259	
Stage			0.519			0.348
I+II	17(29)	151		48(27)	117	
III+IV	42(71)	384		131(73)	289	
LNM			0.311			0.298
Negative	29(49)	286		93(52)	221	
Positive	30(51)	249		87(48)	185	
Nerve invasion			0.203			0.084
Negative	54(92)	464		162(90)	347	
Positive	5(8)	54		18(10)	59	
Vascular invasion			0.593			0.016
Negative	55(93)	497		161(90)	385	
Positive	4(7)	38		19(10)	21	
Differentiation			0.006			<0.001
High	6(10)	87		13(7)	80	
Middle	10(34)	175		66(37)	117	
Low	43(56)	273		101(56)	209	
Tumor location			0.731			0.286
Up	0(0)	5		3(2)	2	
Middle	31(78)	304		94(74)	234	
Down	9(22)	102		30(24)	80	
Invasion depth			0.88			0.934
Mucous layer	3(5)	20		7(4)	16	
Muscle layer	15(25)	137		41(23)	108	
Whole layer	41(70)	377		131(73)	282	

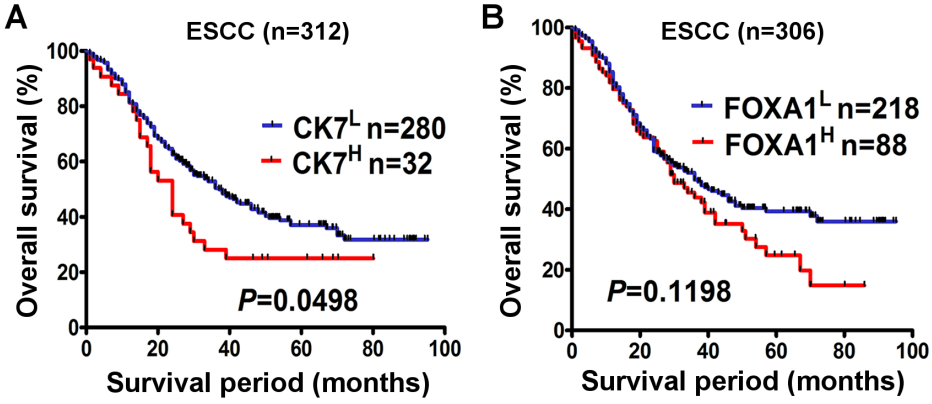


Figure 3. Relationship between ESCC CK7 and FOXA1 status and patients' overall survival. Kaplan-Meier survival curves for A, CK7, B, FOXA1.

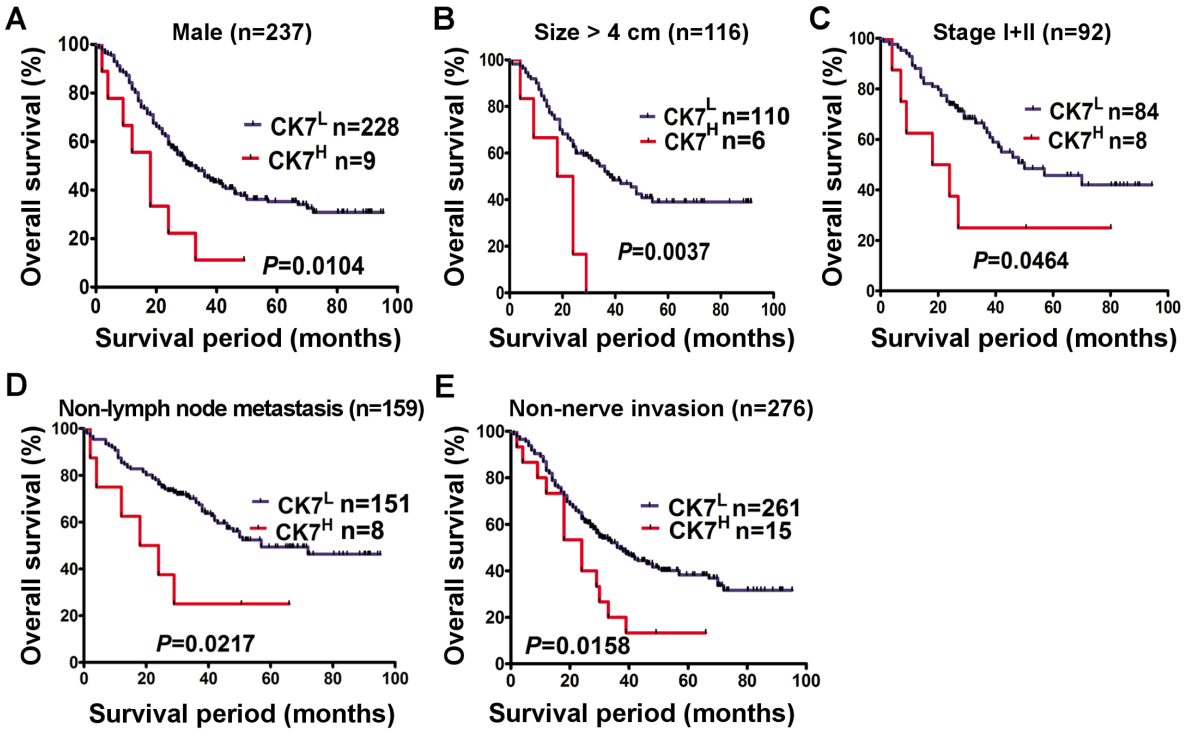


Figure 4. Relationship of CK7 expression and patients' overall survival. For A, male, B, stage I+II, C, tumor size more than 4 cm, D, non-lymph node metastasis, E, non-nerve invasion.

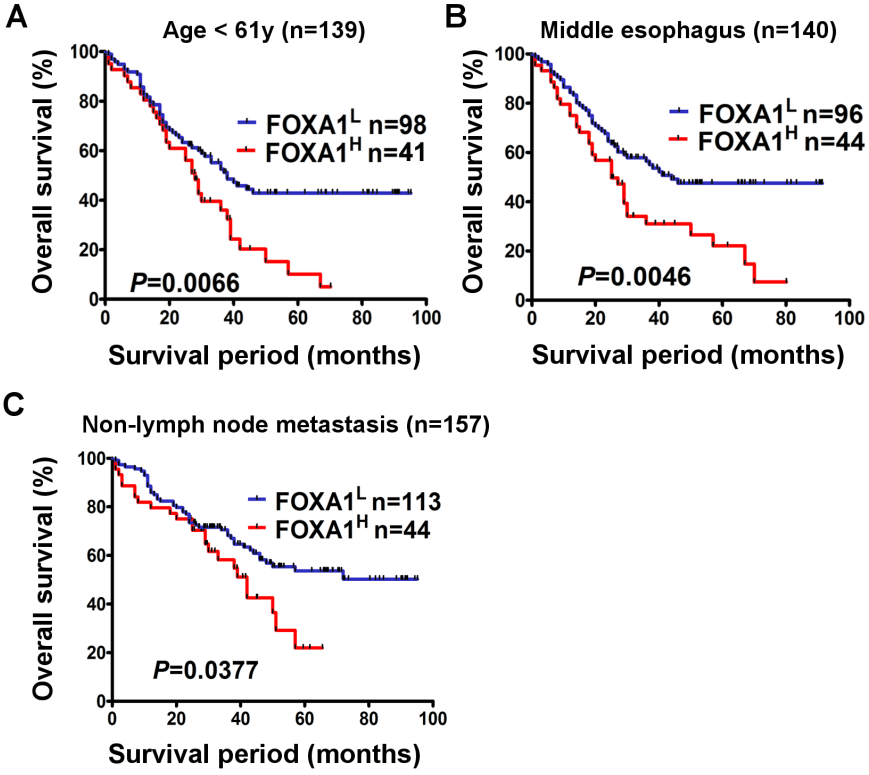
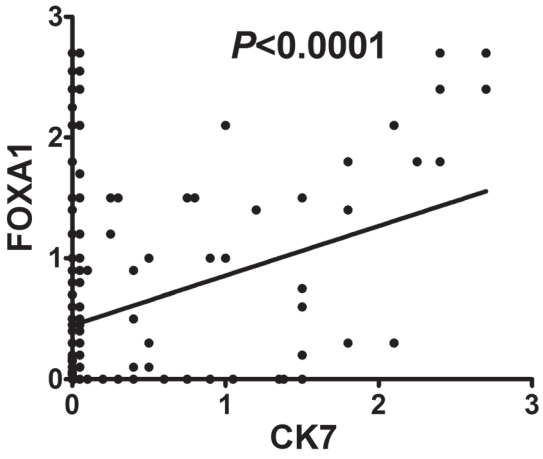


Figure 5. Relationship of FOXA1 expression and patients' overall survival. For A, age<61 years old, B, middle esophagus, C, non-lymph node metastasis.



Pearson r	0.2125
95% confidence interval	0.1338 to 0.2885
P value (two-tailed)	< 0.0001
P value summary	***

Figure 6 Correlation between FOXA1 and CK7 expression. Dots represent CK7 and FOXA1 staining of each ESCC sample, n=584. Formula = protein intensity * staining area/100, intensity from 0–3, and staining area from 0–100%.

p<0.05 and OR=0.86; p<0.05 respectively) [27]. GECEC had no glands compared to ASC, but had worse OS compared to ESCC patients, suggesting that GECEC may be a subtype of esophageal cancer with poor prognosis.

High-risk human papillomavirus (HPV), especially HPV16, correlates with cancerogenesis of human ESCC and HPV16 related to a poor prognosis of ESCC patients in China [28, 29]. Transcriptionally active HPV infection was associated with CK7 [30]. CK7 related to a viral episomal replication and CK19 to viral integration, contributing to viral replication and malignant transformation in high risk human papillomavirus (HR HPV) infected cells in cervical tumor [31]. Therefore, CK7 associated with a poor prognosis in patients may promote HPV infection and activation.

FOXA1 is the upstream gene of CK7 and can regulate the expression of CK7 [14–17]. Our data show that FOXA1 expression positively correlated with CK7 expression. However, the prognostic relationship between FOXA1 expression and ESCC patients has not been reported. Our data show that FOXA1 is an independent poor prognosis marker for ESCC patients and it is a poor prognosis marker for ESCC patients' age less than 61 years old. Single-nucleotide polymorphisms (SNPs) play an important role in ESCC carcinogenesis. A study revealed that there was significantly decreased ESCC risk associated with the FOXA1 rs12894364 C>T and rs2145146 C>A polymorphisms among older patients [32]. This may explain why younger patients have poor prognosis. Furthermore, there was significantly increased ESCC risk associated with the FOXA1 rs7144658 T>C polymorphism among male patients [32], this may be the cause of poor prognosis in male patients with CK7 expression.

Our data also show that FOXA1 is a poor prognosis marker for ESCC patients without lymph node metastasis, suggesting that lymph node metastasis plays an important role in the prognosis of ESCC patients. Although our data reveal that FOXA1 is a poor prognosis marker for ESCC patients' tumor located in the middle of esophagus, suggesting that the prognosis of ESCC patients may be associated with the tumor location. When the tumor occurs in the upper esophagus or lower segment, due to the special parts which connect throat or stomach, and because the site of lymph node and blood vessels is very rich, it is prone to lymph node metastasis and blood metastasis. It is possible to explain why FOXA1-expression ESCC patients with low lymph node metastasis are associated with poor prognosis. Furthermore, most of the esophageal cancers occur in the middle third (>50%) [33–35], the detection of FOXA1 expression has important significance in the treatment and prognosis. Additional larger studies are required to confirm our findings.

We also found that CK7 expression was associated with poor prognosis in male ESCC patients, but not FOXA1. Studies have shown that FOXA1 can regulate the androgen receptor (AR) genome group [36], suggesting that the poor prognosis of CK7 positive male patients may be related to androgen or AR.

In conclusion, FOXA1 expression was positively correlated with CK7 expression. CK7 expression was independent, while FOXA1 expression was non-independent, and correlated with poor prognosis in ESCC patients, and therefore, we believe that CK7 and FOXA1 expression can be used as molecular markers to predict the prognosis of patients with ESCC, and may be potential molecular targets for ESCC therapy. In addition, we will continue to study the relationship between the expression of FOXA1 and ESCC patients, and the specific regulator pathways between FOXA1 and CK7, then provide more powerful theoretical basis for molecular therapy of ESCC patients.

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