Decreased mRNA expression of transcription factor forkhead box F2 is an indicator of poor prognosis in patients with resected esophageal squamous cell carcinoma

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Abstract. The transcription factor forkhead box F2 (FOXF2) is an evolutionarily conserved DNA-binding protein involved in embryogenesis and metabolism. Although recent studies prove that FOXF2 is a tumor suppressor in various human cancers, the role of FOXF2 in esophageal squamous cell carcinoma (ESCC) remains unknown. Therefore, samples were collected from 188 ESCC patients, including 33 pairs of tumor and non-tumor tissues, and *FOXF2* mRNA expression was investigated by quantitative polymerase chain reaction. The results demonstrated that *FOXF2* mRNA is downregulated in tumor tissues compared to paired non-tumor tissues (P=0.048). The receiver operating characteristic curve analysis indicated 1.2 as a cut-off point and, thus, 125 and 63 tumors were classified as low- and high-level *FOXF2* mRNA expression, respectively. We observed that low-level *FOXF2* mRNA expression in the tumors was associated with a higher frequency of lymph node metastasis (P=0.044), an effect further suggested by the multivariate logistic regression analysis (P=0.060). According to the univariate Cox analysis, patients harboring tumors with low-level *FOXF2* mRNA expression had a significantly increased mortality risk compared to those with high-level expression (hazard ratio=1.700, 95% confidence interval, 1.077-2.681), with 5-year survival rates of 41.1 and 61.9%, respectively. This negative prognostic effect of low-level *FOXF2* mRNA expression was further validated in the multivariate Cox analysis (P=0.021). The subgroup analysis demonstrated that the effect of *FOX2* mRNA expression was limited to male patients and those with advanced-stage disease. Taken together, these findings suggest that *FOXF2* may be an anti-oncogene for ESCC and decreased *FOXF2* mRNA expression is associated with a poor prognosis in patients with ESCC.

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Introduction

Esophageal squamous cell carcinoma (ESCC) is a common type of cancer worldwide. Despite the significant improvements in diagnostic methods, surgical resection and multidisciplinary therapy, the outcome remains dismal, with a 5-year survival rate of <40% (1,2). Although the tumor-node-metastasis staging system is well established (3), patients with the same stage commonly present with different survival rates. Therefore, it is crucial to evaluate the molecular and genetic characteristics of ESCC.

The forkhead box family of transcription factors comprises evolutionarily conserved DNA-binding proteins that are present in several organisms (4,5). As a member of this family, transcription factor forkhead box F2 (FOXF2) has been well described as an essential signaling molecule for embryogenesis and metabolism (6-12). Experiments *in vivo* have revealed cleft palate and gastrointestinal defects in *FOXF2* knockout mice (6,7). Studies in humans also demonstrated associations between *FOXF2* mutations and congenital diseases (8,9). In addition, a recent study proved that FOXF2 is involved in the process of glycose metabolism (10).

Recently, decreased FOXF2 expression was shown to promote tumor development (13) and display several functions critical for cancer initiation and progression. Moreover, reduced *FOXF2* mRNA expression was found to be associated with early-onset metastasis and poor prognosis in breast cancer patients (14). It was also reported that FOXF2 may be

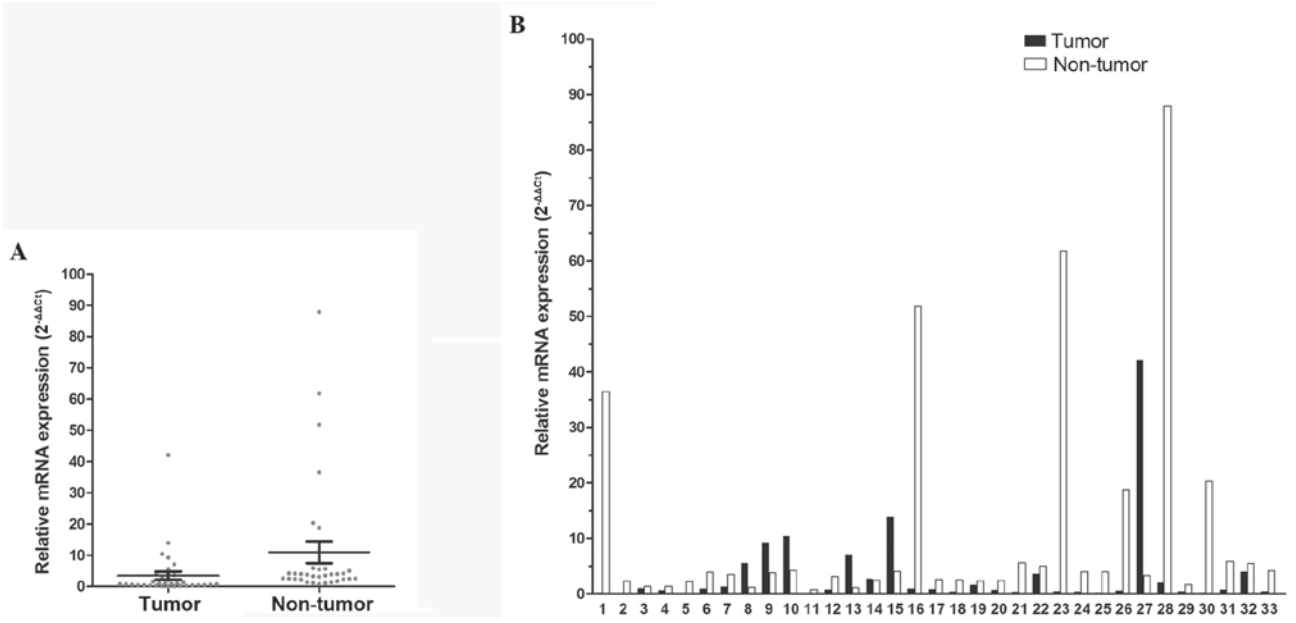

Figure 1. Analysis of *FOXF2* mRNA expression in esophageal tissues. (A) *FOXF2* mRNA expression was significantly downregulated in tumor tissue (P=0.048). (B) *FOXF2* mRNA levels in 33 paired non-tumor and tumor tissues were compared individually. The gene expression results were normalized to the internal control glyceraldehyde-3-phosphate dehydrogenase. *FOXF2*, forkhead box F2.

downregulated by microRNA-182 (15), which has been proved to accelerate metastasis and promote cell invasion (16,17). However, the clinicopathological and prognostic significance of FOXF2 in human ESCC remains unknown.

To elucidate the clinicopathological and prognostic value of FOXF2 in ESCC, we determined *FOXF2* mRNA expression by quantitative polymerase chain reaction (qPCR) and evaluated its feasibility as a biomarker for ESCC patients.

Materials and methods

Patient selection. Following approval by the local Institutional Ethics Committee, a series of 188 consecutive patients with ESCC who underwent esophagectomy with extended two-field lymphadenectomy at the Department of Thoracic Oncology of Sun Yat-sen University Cancer Center between January, 2002 and December, 2008, were enrolled in this study. Written informed consent was provided by the participants for their clinical records to be used in this study. All patient data were anonymized and de-identified in a confidential manner.

The inclusion criteria were as follows: i) Pathologically diagnosed ESCC; ii) complete surgical resection; iii) no distant metastasis; iv) no preexisting/concurrent malignant disease or a second primary tumor; v) no perioperative mortality; and vi) availability of fresh samples. Patients receiving neoadjuvant or adjuvant treatment were excluded. The pretreatment evaluation included a complete history and physical examination, complete blood cell count, serum biochemistry, chest radiography, esophageal barium meal, computed tomography scan of the cervical region, chest and abdomen, endoscopy and ultrasonography scan of the abdomen. The pathological staging was reverified based on the 7th American Joint Committee on Cancer (AJCC) staging system (3).

Following primary treatment, the majority of the patients were followed up in the outpatient clinic every 3 months during the first 2 years, every 6 months during years 3-5 and every 12 months thereafter. The survival status was reverified using the best available method in June, 2014. The median time from surgery to the last censoring date for the entire cohort was 68.5 months.

qPCR assays. Fresh tumor and non-tumor samples were collected from regions that were macroscopically assessed as neoplastic and normal, respectively. The samples were immediately stored on dry ice after resection and then frozen at -80˚C. Total RNA was extracted from the specimens using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Each cDNA was synthesized from 1 μg of total RNA using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) and stored at -80˚C. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), a housekeeping gene, was selected as an internal standard to control for amplification variability. The following primers were used: *FOXF2*, forward 5'-CACTACTGGACCATCGACCC-3'; and reverse 5'-CTCACCACGCGGTGGTACAT-3' (NCBI: NM_001452.1); and *GAPDH*, forward 5'-ACTTCAACAGCGACACCCACTC-3' and reverse 5'-TACCAGGAAATGAGCTTGACAAAG-3' (NCBI: NM_001256799.1). As the reverse transcription (RT)-qPCR assays were not performed at the same time, we utilized *FOXF2* mRNA expression of the EC109 ESCC cell line as an internal control (calibrator) to adjust variation. The PCR mixture of each PCR analysis included 0.12 μl of cDNA, 5 μl of 2X Power SYBR-Green PCR Master mix (Applied Biosystems, Life Technologies, Grand Island, NY, USA), 0.25 μl of 20 mmol/l forward primer, 0.25 μl of reverse primer and 4.38 μl of distilled water. RT-qPCR was performed using LightCycler 480 (Roche Applied Science, Penzberg, Germany) with the following thermal cycling profile: 95˚C for 10 min, followed by

Table I. Correlations between *FOXF2* expression and clinicopathological characteristics.

Characteristics	Total (n=188)	*FOXF2* mRNA expression		P-value[a]
		High (n=63)	Low (n=125)	
Age, years				0.198
≤59[b]	98	37 (58.7)	61 (48.8)	
>59	90	26 (41.3)	64 (51.2)	
Gender				0.468
Female	51	15 (23.8)	36 (28.8)	
Male	137	48 (76.2)	89 (71.2)	
Tumor location				0.374
Upper	36	15 (23.8)	21 (16.8)	
Middle	104	35 (55.6)	69 (55.2)	
Lower	48	13 (20.6)	35 (28.0)	
Tumor length, cm				0.380
≤4.2[b]	90	33 (52.4)	57 (45.6)	
>4.2	98	30 (47.6)	68 (54.4)	
Tumor cell differentiation				0.888
High	45	14 (22.2)	31 (24.8)	
Moderate	97	34 (54.0)	63 (50.4)	
Poor	46	15 (23.8)	31 (24.8)	
pT stage				0.353
T1b-T2	46	18 (28.6)	28 (22.4)	
T3-T4a	142	45 (71.4)	97 (77.6)	
pN stage				0.044
N0	100	40 (63.5)	60 (48.0)	
N1/N2/N3	88	23 (36.5)	65 (52.0)	
AJCC stage				0.088
I-II	106	41 (65.1)	65 (52.0)	
III	82	22 (34.9)	60 (48.0)	
Resected lymph nodes, no. ± SD	24.0±11.8	23.8±11.9	24.6±11.9	0.656

[a]χ^2 test; [b]Median. *FOXF2,* forkhead box F2; AJCC, American Joint Committee on Cancer; SD, standard deviation.

40 cycles of amplification (95˚C for 10 sec and 60˚C for 20 sec) and then 72˚C for 30 sec. Each assay was performed at least three times. Any samples with a coefficient of variance >10% were retested. The relative expression level of *FOXF2* mRNA for each sample was calculated as $2^{-\Delta\Delta Ct_{sample}}$ as follows: $\Delta\Delta Ct_{sample} = \Delta Ct_{calibrator} - \Delta Ct_{sample}$, where $\Delta Ct_{calibrator}$ of *FOXF2* mRNA = $\Delta Ct_{calibrator}$ of *FOXF2* - $Ct_{calibrator}$ of *GAPDH*; $\Delta Ct_{sample} = Ct_{sample}$ of *FOXF2* - Ct_{sample} of *GAPDH*. The value of the Ct difference is equal to a 2^n-fold difference.

Statistical analysis. The statistical analysis was performed using SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). A paired two-tailed t-test was employed to analyze the difference in *FOXF2* mRNA expression between the paired tumor and non-tumor tissues. A receiver operating characteristic (ROC) curve was generated to select the optimal cut-off value and, therefore, divided the *FOXF2* mRNA expression into two groups, namely low- and high-expression groups. The χ^2 test was used to determine the associations between *FOXF2* mRNA expression and categorized clinicopathological parameters. To determine the factors associated with an increased risk of lymph node metastasis (LNM), crude and adjusted analyses were performed using univariate and multivariate logistic regression.

The cancer-specific survival (CSS) was calculated from the date of surgery to either the date of death from ESCC or the last follow-up. The survival analysis was performed using the Kaplan-Meier method and the differences between curves were assessed by the log-rank test. Univariate and multivariate Cox regression analyses were used to identify the factors associated with prognosis. For selecting variables for the multivariate logistic/Cox regression model, a cut-off value of P=0.10 was used. P<0.05 was considered to indicate a statistically significant difference.

Table II. Univariate and multivariate logistic regression analyses of factors associated with lymph node metastasis.

	Univariate analysis		Multivariate analysis	
Factors	HR (95% CI)	P-value	HR (95% CI)	P-value
Age, years (≤59/>59)	1.078 (0.607-1.912)	0.799	-	NA[a]
Gender (F/M)	1.915 (0.985-3.724)	0.055	1.834 (0.921-3.655)	0.085
Tumor location (U/Md/L)	1.178 (0.765-1.814)	0.458	-	NA[a]
Tumor differentiation (high/moderate/poor)	1.337 (0.882-2.027)	0.171	-	NA[a]
Tumor length, cm (≤4.2/>4.2)	1.425 (0.801-2.546)	0.228	-	NA[a]
pT stage (T1b-T2/T3-T4a)	2.186 (1.087-4.398)	0.028	2.186 (1.087-4.398)	0.028
Resected lymph nodes, no. (≤21/>21)	0.984 (0.554-1.747)	0.955	-	NA[a]
FOXF2 mRNA expression (high/low)	1.884 (1.012-3.507)	0.046	1.828 (0.975-3.430)	0.060

[a]Not assessed due to an insignificant result in the univariate analysis (P>0.1). F, female; M, male; U, upper; Md, middle; L, lower; HR, hazard ratio; CI, confidence interval; *FOXF2*, forkhead box F2.

Results

Human FOXF2 mRNA expression in tumor and non-tumor tissues. In total, 33 patients with paired tumor and non-tumor tissues were enrolled in this study. *FOXF2* was found to be significantly downregulated in tumor compared to non-tumor tissues from the same patient (P=0.048, Fig. 1A). The median value of the normal/tumor (N/T) ratio of *FOXF2* mRNA expression was 4.1; the N/T ratio was >two-fold in 66.7% of the patients (22/33) and the highest ratio was ≤165.91-fold (Fig. 1B).

Association between FOXF2 mRNA expression level and clinicopathological parameters. A total of 188 patients with available tumor tissue samples were enrolled in this study, with a median age of 59 years (range, 34-88 years). The patient characteristics are summarized in Table I. The median value of *FOXF2* mRNA expression was 0.70 in the tumor tissue samples (range, 0.02-42.10). According to the ROC curve (Fig. 2), the optimal cut-off value of *FOXF2* mRNA with the best discriminatory power was 1.2. Using this cut-off value, the entire cohort was classified into two groups, namely high-level (>1.2; n=125) and low-level (≤1.2; n=63) *FOXF2* mRNA expression. There was no significant association between *FOXF2* mRNA expression and age, gender, tumor location, tumor length, tumor cell differentiation, pathological T stage, AJCC stage and the number of resected lymph nodes. However, we observed that the rate of LNM was significantly associated with a low level of *FOXF2* mRNA expression (P=0.044) (Table I).

Evaluation of FOXF2 mRNA expression as a risk factor for LNM. In the univariate logistic regression analysis, patients with a low level of *FOXF2* mRNA expression exhibited a significantly higher risk of LNM compared to those with a high level of expression, with a hazard ratio (HR) of 1.884 [95% confidence interval (CI): 1.012-3.507]. This effect was further observed in the multivariate logistic analysis, with a marginal significance (P=0.060) and an adjusted HR of 1.828 (95% CI: 0.975-3.430). The independent risk factor for LNM was advanced pathological T stage (P=0.028) (Table II).

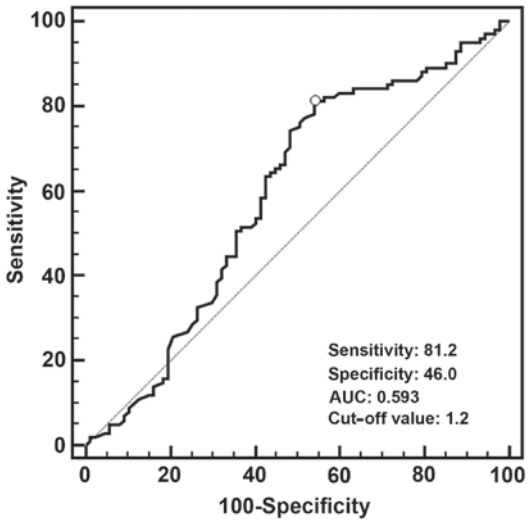

Figure 2. Receiver operating characteristic curve using *FOXF2* mRNA expression levels. The optimal cut-off value was 1.20, with a sensitivity of 81.2% and a specificity of 46.0%. AUC, area under the curve; *FOXF2*, forkhead box F2.

Association between FOXF2 mRNA expression level and CSS. In this study, the median survival time was 54.0 months, with an estimated 5-year CSS of 48.0%. In the univariate Cox analysis, patients with low-level *FOXF2* mRNA expression exhibited a significantly enhanced mortality risk compared to those with high-level expression (HR=1.700, 95% CI: 1.077-2.681), with a 5-year CSS of 41.1 and 61.9%, respectively (Fig. 3A). This effect was further verified in the multivariate Cox analysis, with an adjusted HR of 1.714 (95% CI: 1.085-2.708). Other negative prognostic factors with independent significance included advanced AJCC stage (P<0.001) and a resected lymph node number of ≤21 (P=0.016). The details of the univariate and multivariate analyses are shown in Table III.

Prognostic significance of FOXF2 mRNA expression level in the subgroup analysis. The association between *FOXF2* mRNA expression and CSS across strata of other potential predictors of patient outcome were assessed. As shown in Table IV, following

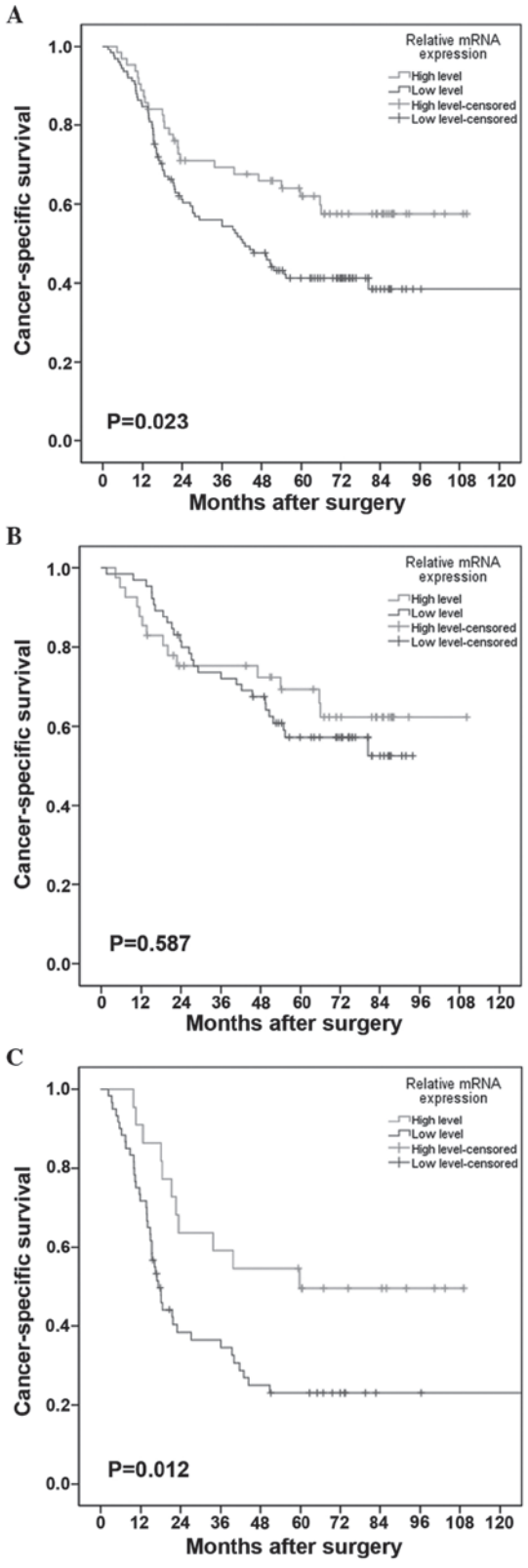

Figure 3. Cancer-specific survival curves stratified by *FOXF2* mRNA expression levels in (A) the entire cohort, (B) patients with stage I/II disease and (C) patients with stage III disease. *FOXF2*, forkhead box F2.

adjustment for known prognostic factors, the increased risk of cancer-related mortality conferred by low-level *FOXF2* mRNA expression was unchanged in male patients (adjusted HR=1.790, 95% CI: 1.044-3.072) and patients with advanced-stage disease (adjusted HR=2.924, 95% CI: 1.466-5.833). However, this association was insignificant in other subgroup analyses. The CSS curves stratified by *FOXF2* mRNA expression level in patients with stage I/II and patients with stage III disease are shown in Fig. 3B and C, respectively.

Discussion

It was previously demonstrated that FOXF2 acts as a tumor suppressor in breast and prostate cancer (14,15). In this study, we observed that *FOXF2* mRNA expression was significantly higher in normal esophageal tissue compared to that in tumor specimens. Thus, FOXF2 may act as a tumor suppressor in ESCC. We hypothesized that FOXF2 maintains a normal expression level in normal esophageal epithelial cells and plays an important role in balancing cell behavior, such as proliferation, differentiation, mitotic cycle and apoptosis, which was also supported by previous studies (7,11-13). However, FOXF2 expression may be compromised in ESCC during the course of tumorigenesis, similar to prostate cancer (18,19). Although the precise mechanism is not known, one possible explanation involves FOXF2 downregulation by microRNA-182 (15), the overexpression of which has been observed in various human cancers, such as colorectal (20), cervical (21) and non-small-cell lung cancer (22). In addition, we observed that a low level of *FOXF2* mRNA expression was associated with a high rate of LNM; the same result was obtained in patients with breast cancer (14).

When our analyses focused exclusively on patient survival, the level of *FOXF2* mRNA expression was found to be an independent and significant predictive variable. The 5-year CSS was significantly higher in patients with high-level *FOXF2* mRNA expression compared to that in patients with low-level expression. The effect of *FOXF2* on prognosis may be attributed to its intrinsic nature as an anti-oncogene.

Several studies have been conducted to elucidate how FOXF2 exerts its tumor suppressor effect. It was reported that FOXF2 may downregulate matrix metalloproteinases (MMPs) and upregulate tissue inhibitor of metalloproteinase-3, a known inhibitor of MMPs (15,19). Due to their ability to degrade the extracellular matrix, activated MMPs accelerate metastasis and decrease survival in patients with ESCC (23), colorectal (24), breast (25) and gastric cancer (26). In addition, FOXF2 was reported to inhibit the Wnt pathway (7,13), the activation of which has been associated with a high risk of metastasis and a poor outcome in patients with pancreatic (27), breast (28) and prostate cancer (29). However, the potential mechanisms of FOXF2 as a tumor suppressor in ESCC remain unknown and require further investigation.

In our subgroup analysis, we observed that the increased risk conferred by low-level *FOXF2* mRNA expression was limited to patients with stage III disease. A possible explanation may be associated with the fact that the survival rate is generally relatively low in patients with advanced ESCC; therefore, there may be a tendency to observe significant survival differences according to a certain factor. Second, the superior outcomes in the high *FOXF2* mRNA expression group may be partially associated with the role of FOXF2 in suppressing metastasis (7,13,15,19); this effect is expected to be more prominent in patients with a high predisposition for metastasis,

Table III. Univariate and multivariate Cox analyses of factors associated with cancer-specific survival.

	Univariate Cox analysis		Multivariate Cox analysis	
Factors	HR (95% CI)	P-value	HR (95% CI)	P-value
Age, years (≤59/>59)	1.300 (0.872-1.936)	0.198	-	NA[a]
Gender (F/M)	1.119 (0.717-1.745)	0.621	-	NA[a]
Tumor location (U/Md/L)	1.131 (0.837-1.527)	0.422	-	NA[a]
Tumor differentiation (high/moderate/poor)	1.352 (1.009-1.812)	0.043	1.276 (0.939-1.735)	0.119
Tumor length, cm (≤4.2/>4.2)	0.913 (0.613-1.360)	0.654	-	NA[a]
AJCC stage (I/II/III)	2.384 (1.660-3.424)	<0.001	2.482 (1.728-3.566)	<0.001
Resected lymph nodes, no. (≤21/>21)	0.646 (0.431-0.971)	0.035	0.606 (0.403-0.910)	0.016
FOXF2 mRNA expression (high/low)	1.700 (1.077-2.681)	0.023	1.714 (1.085-2.708)	0.021

[a]Not assessed due to an insignificant result in the univariate analysis (P>0.1). F, female; M, male; U, upper; Md, middle; L, lower; HR, hazard ratio; CI, confidence interval; *FOXF2*, forkhead box F2; AJCC, American Joint Committee on Cancer.

Table IV. Subgroup analysis for *FOXF2* mRNA expression.

		FOXF2 mRNA expression (no. of events/no. at risk)		Multivariate Cox analysis		
Factors	5-year OS (%)	High	Low	HR	95% CI	P-value
Age, years						
≤59[a]	53.1	13/37	34/61	1.862	0.980-3.539	0.058
>59	42.4	12/26	38/64	-	-	NA[b]
Gender						
Female	46.6	7/15	20/36	-	-	NA[b]
Male	49.9	18/48	52/89	1.790	1.044-3.072	0.034
Tumor location						
Upper	56.4	5/15	11/21	-	-	NA[b]
Middle	46.0	15/35	41/69	1.667	0.921-3.017	0.091
Lower	47.1	5/13	20/35	-	-	NA[b]
Tumor length, cm						
≤4.2[a]	47.4	14/33	35/57	-	-	NA[b]
>4.2	49.8	11/30	37/68	1.777	0.945-3.340	0.074
AJCC stage						
I-II	61.3	14/41	28/65	-	-	NA[b]
III	20.7	11/22	44/60	2.924	1.466-5.833	0.002
Resected lymph nodes, no.						
≤21[a]	41.7	15/32	43/68	-	-	NA[b]
>21	55.6	10/31	29/57	2.038	0.987-4.205	0.054

[a]Median. [b]Not assessed due to an insignificant result in the univariate analysis (P>0.1). *FOXF2*, forkhead box F2; OS, overall survival; HR, hazard ratio; CI, confidence interval; AJCC, American Joint Committee on Cancer.

which was also indicated by Kong *et al* (14); in that study, the poor effect of decreased *FOXF2* mRNA expression on survival in breast cancer was limited to patients with a triple-negative profile, a well-known subtype characterized by early-onset metastasis and a dismal outcome (30). Additionally, we found *FOXF2* mRNA expression to be associated with survival in male patients. Since the interaction between gender and FOXF2 remains unknown, we hypothesized that this phenomenon may be partially attributed to the high risk of LNM in male patients (adjusted HR=1.834, 95% CI: 0.921-3.655) in this study.

To date, advances in molecular biology have led to the rapid development of individualized management in various human cancers. Based on a cohort of patients treated by surgery alone, we observed that patients with low-level *FOXF2* mRNA expression had a significantly lower CSS compared to those with high-level expression. Furthermore, this effect was independent of the aggressiveness of lymphadenectomy and patient characteristics. In this sense, surgery alone may be insufficient for patients with a low level of *FOXF2* mRNA expression and multidisciplinary therapy should be recommended. Future studies should focus on the interaction between chemotherapy/chemoradiotherapy and FOXF2 expression to verify our hypotheses.

In conclusion, *FOXF2* may be an anti-oncogene in ESCC. Decreased *FOXF2* mRNA expression was found to be associated with poor prognosis in patients with completely resected ESCC. However, the clinical value of the changes in *FOXF2* mRNA levels in ESCC require further validation by large multicenter studies.

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