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Cytoplasmic Forkhead Box M1 (FoxM1) in Esophageal Squamous Cell Carcinoma Significantly Correlates with Pathological Disease Stage

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Abstract Esophageal cancer is a deadly cancer with esophageal squamous cell carcinoma (ESCC) as the major type. Until now there has been a lack of reliable prognostic markers for this malignancy. This study aims to investigate the clinical correlation between Forkhead box M1 (FoxM1) and patients' parameters in ESCC.

Methods Immunohistochemistry was performed to investigate the expression and localization of FoxM1 in 64 ESCC tissues and 10 nontumor esophageal tissues randomly selected from 64 patients before these data were used for clinical correlations.

Results Cytoplasmic and nuclear expressions of FoxM1 were found in 63 and 16 of the 64 ESCC tissues, respectively. Low cytoplasmic expression of FoxM1 was correlated with early pathological stage in ESCC ($P = 0.018$), while patients with nuclear FoxM1 were younger in age than those without nuclear expression ($P < 0.001$). Upregulation of *FoxM1* mRNA was found in five ESCC cell lines (HKESC-1, HKESC-2, HKESC-3, HKESC-4, and SLMT-1) when compared to non-neoplastic esophageal squamous cell line NE-1 using quantitative polymerase chain reaction (qPCR). Except for HKESC-3, all studied ESCC cell lines demonstrated a high expression of FoxM1 protein using immunoblot. A high mRNA level of *FoxM1* was observed in all of the ESCC tissues examined when compared to their adjacent nontumor tissues using qPCR.

Conclusion Cytoplasmic FoxM1 was correlated with pathological stage and might be a biomarker for advanced ESCC.

Introduction

Forkhead box M1 (FoxM1) is a member of the Forkhead transcription factor family, which is evolutionarily conserved and is defined by having a common DNA-binding domain called Forkhead or winged-helix domain [1]. The *FOXM1* gene is located on chromosome 12 12p13.3 [2]. It is involved in an array of biological processes, including metabolism and development, cell proliferation, apoptosis and invasion, longevity, and cancer [3]. *FoxM1* has high expression in fetal tissues with active cell proliferation and its expression is negligible in differentiated cells [4]. It regulates the cell cycle by modulating the transcription of various cell cycle-related genes essential for G1-S and G2-M progression, chromosome stability, and segregation, including $p27^{kip1}$, cyclin B1, and cyclin D [5–8]. Recently, *FoxM1* has been linked to tumorigenesis and progression of certain cancers. Recurrent DNA copy number alterations (CNA) were reported in an array-based comparative

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genome hybridization study of malignant peripheral nerve sheath tumor (MPNST). This gain/loss in copy number of *FOXM1* is associated with survival in MPNST [2]. Upregulation of *FoxM1* expression is found in basal cell carcinomas [9], glioblastomas [10], non-small-cell lung cancers [11], and breast cancers [12, 13]. Mice with *FoxM1* conditionally deleted from hepatocytes were highly resistant to liver tumor induction by phenobarbital and diethylnitrosamine [14]. Moreover, expression of the *FoxM1* transgene in mice increased with the size of the colorectal tumors while conditional *FoxM1* knockout mice showed reduced growth of colorectal tumors [6]. These results suggest a role for *FoxM1* in tumor initiation and progression [4]. To our knowledge, no study has investigated the clinical significance of FoxM1 in esophageal squamous cell carcinoma (ESCC), which is a major type of esophageal cancer [15]. The aim of this present study was to investigate the prognostic significance of FoxM1 in ESCC.

Materials and methods

Human esophageal cell lines

Five human ESCC cell lines, HKESC-1, HKESC-2, HKESC-3, HKESC-4 and SLMT-1, and one human non-neoplastic esophageal squamous cell line, NE-1, were used in this study. These cell lines were previously established by our team [16–20]. NE-1 cells were cultured in keratinocyte-SFM medium supplemented with bovine pituitary extract and human recombinant epidermal growth factor (Invitrogen, Carlsbad, CA). HKESC-1 and HKESC-4 cells were maintained in Minimum Essential Medium (MEM) medium (Invitrogen) with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen) and 1% penicillin and streptomycin (P/S) (Invitrogen). HKESC-2, HKESC-3, and SLMT-1 cells were cultured in MEM medium with 20% FBS and 1% P/S. All cell lines were maintained in a humidified atmosphere at 37°C containing 5% CO_2.

Patients and clinical specimens

We recruited 64 ESCC patients who had undergone esophagectomy between 1997 and 2005 at the Department of Surgery, Queen Mary Hospital, Hong Kong. Consent to use clinical specimens for this study was obtained from the Institutional Review Board of The University of Hong Kong/Hospital Authority Hong Kong West Cluster (HKU/HA HKW IRB). There were 47 men and 17 women and the mean age was 64.6 years (range = 40–87 years). None of the patients had received neoadjuvant chemotherapy or radiotherapy before esophagectomy. Data of patient demographics, histopathology, and long-term follow-up were captured in a prospectively collected database. Specimens of primary tumor and nontumor tissues obtained from near the proximal resection margins were stored at –80°C and retained for laboratory studies and subsequent analyses. The site and size of the tumors were recorded. Standard tissue blocks were prepared by fixing the specimens in 10% formalin before embedding in paraffin wax. Five-micron sections were prepared and stained with hematoxylin and eosin for light microscopy. Stained sections were reviewed and tumors were graded according to the criteria of the World Health Organization (WHO). Cancers were staged according to TNM classification.

Reverse transcription (RT) and quantitative polymerase chain reaction (qPCR)

The expression of *FoxM1* mRNA in esophageal cell lines and clinical specimens was determined using qPCR. RNA was extracted using TRIzol reagent (Invitrogen), according to manufacturer's instructions. RT and qPCR were performed as described [21, 22]. Five hundred nanograms of RNA was reverse-transcribed into cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen), following the manufacturer's protocol. cDNA (0.3 and 0.6 μg) from cultured cells and tissues was used for qPCR using Platinum Quantitative PCR SuperMix-UDG w/ROX (Invitrogen) and *FoxM1*-specific primers (forward: 5′-ACC CAA ACC AGC TAT GAT GC-3′ and reverse: 5′-GAA GCC ACT GGA TGT TGG AT-3′). *β-Actin* was used as an internal control in a parallel experiment, using its specific primers (forward: 5′-CCA TCA TGA AGT GTG ACG TG-3′ and reverse: 5′-ATC CAC ATC TGC TGG AAG GT-3′). qPCR was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Carlsbad, CA). Each sample was performed in at least duplicates.

Immunoblot

The protein level of FoxM1 in esophageal cell lines was detected using immunoblot as described [23, 24]. Briefly, cultured cells were washed twice with ice-cold 1 × phosphate-buffered saline (PBS, pH 7.4). Ice-cold cell lysis buffer (Cell Signaling Technology, Danvers, MA) was added to cell monolayers to extract proteins by scraping. Cell lysates were left on ice for 15 min before being centrifuged at 13,200 rpm for 15 min at 4°C. Protein concentration was estimated using Bradford Protein Assay solution (Bio-Rad, Hercules, CA). Following the addition of 6 × sample buffer to the cell lysates, 25 μg of protein was resolved onto an 8% sodium dodecyl sulfate (SDS) polyacrylamide gel at room temperature before the transfer of proteins onto the polyvinylidene fluoride (PVDF) membrane for 2.5 h at 4°C. After

blocking with 5% skimmed milk in Tris-buffered saline with Tween-20 (TBST) at room temperature for 2 h, membranes were probed with polyclonal rabbit anti-FoxM1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1,500 overnight at 4°C. Monoclonal mouse anti-β-actin antibody (Sigma-Aldrich, St Louis, MO) at 1:10,000 was used as a loading control. After washing with TBST, membranes were incubated with either goat anti-rabbit (Zymed, Carlsbad, CA) (1:7,000 for detecting anti-FoxM1 antibody) or goat anti-mouse (Zymed) (1:20,000 for detecting anti-β-actin antibody) secondary antibodies conjugated with horseradish peroxidase (HRP) for 1 h at room temperature. Following washing with TBST, signals were visualized using ECL Plus Western blotting reagent pack (GE Healthcare Biosciences, Piscataway, NJ). The apparent molecular weight of FoxM1 and β-actin on gels was 100 and 42 kDa, respectively.

Immunohistochemistry

Five-micron sections from tumors and nontumor tissues were prepared and mounted on glass slides for immunohistochemistry as previously described [23, 25, 26]. Sections were deparaffinized and rehydrated. Antigen retrieval was performed by heating sections in 0.1 M citrus buffer (pH 6.0) in a microwave for 10 min. Endogenous peroxidase activities were blocked by incubating sections with 3% hydrogen peroxide at room temperature for 20 min. After washing with TBS (pH 7.6), nonspecific binding sites were blocked with 10% normal goat serum (Dako, Glostrup, Denmark) in TBST at room temperature for 1 h. Sections were incubated with anti-FoxM1 antibody at 1:600 overnight at 4°C. After washing with TBS, sections were incubated with anti-rabbit-labeled polymer-HRP provided in EnVision + System-HRP (Dako) at room temperature for 45 min to detect the primary antibody. Signals were visualized by incubating sections with liquid DAB + (diaminobenzidine) (Dako) before counterstaining with hematoxylin. A colorectal carcinoma section from the tissue microarray was used as a positive control (Fig. 1a), while normal goat serum as a substitution for anti-FoxM1 antibody was used as a negative control (Fig. 1b). Stained sections were examined by Dr. Chan under light microscope. The expression of cytoplasmic FoxM1 was evaluated according to the methodology of Liu et al. [10] with modifications. The expression of FoxM1 was categorized according to the percentage of cancer cells stained positive with anti-FoxM1 antibody (score from 0 to 3, where $0 \leq 5\%$, $1 = 6$–25%, $2 = 26$–50%, and $3 \geq 51\%$) and the intensities of the immunostain (1 +, 2 +, 3 +). A weighted index score (0–9) was calculated for each section by multiplying the values of these two categories. For example, the weighted index score should be 6 if 30% of cancer cells were stained (score = 2) with strong signals (score = 3). For cytoplasmic FoxM1 expression, tumors were further grouped into a low-expression group (weighted index score = 0–4) and a high-expression group (weighted index score = 5–9). For nuclear FoxM1, its expression segregated the tumors into an "absence" group or a "presence" group.

Statistical analysis

Data are expressed as mean ± SEM. Pearson's χ^2 test was used to assess the correlations between immunohistochemical data and categorical clinicopathological characteristics. The Kaplan-Meier method was used for survival analysis, and the differences in survival were estimated using the log-rank test. A P value of less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 12.0 for Windows (SPSS, Inc., Chicago, IL).

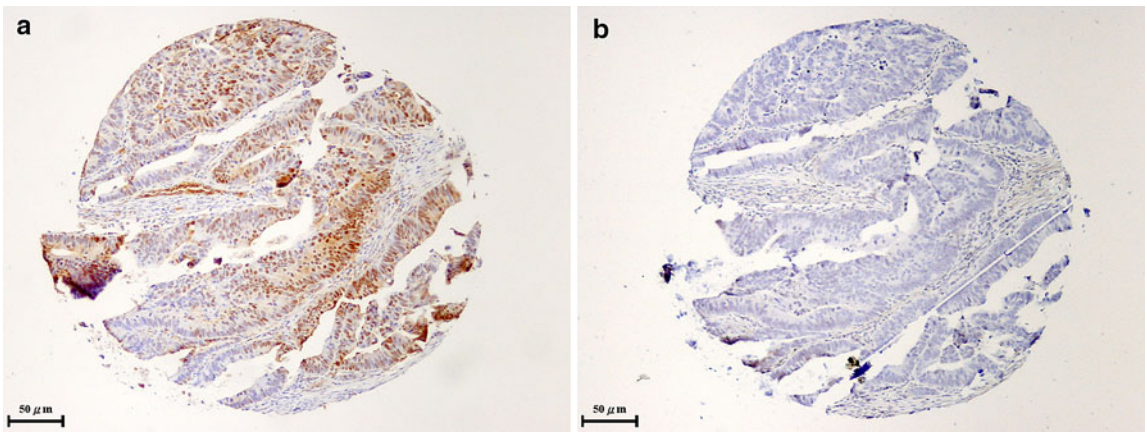

Fig. 1 Immunohistochemical staining of FoxM1 in tissue microarray of colorectal cancers. Anti-FoxM1 antibody was used to stain FoxM1 in colorectal cancer tissues from tissue microarray (**a**). A negative control was included in a parallel experiment (**b**). Original magnification, 200×

Results

High mRNA level of *FoxM1* in ESCC cell lines and tissues

A high level of *FoxM1* mRNA was found in ESCC cell lines compared to that in the non-neoplastic squamous cell line NE-1 using qPCR. An increase of 24.34-, 57.45-, 103.72-, 25.81-, and 13.92-fold in the mRNA level of *FoxM1* was observed in HKESC-1, HKESC-2, HKESC-3, HKESC-4, and SLMT-1 cells, respectively, compared to that in NE-1 cells (Fig. 2). All of ten ESCC tissues studied showed an increase in *FoxM1* mRNA when compared to their corresponding nontumor counterparts. The upregulation of *FoxM1* ranged from about 3- (patient 9) to 500-fold (patient 6) (Fig. 3).

High protein level of FoxM1 in ESCC cell lines

The protein level of FoxM1 in cultured cells was analyzed using immunoblot. Non-neoplastic NE-1 cells expressed an undetectable protein level of FoxM1. Except HKESC-3 cells, ESCC cells, including HKESC-1, HKESC-2, HKESC-4, and SLMT-1, showed an elevated protein level of FoxM1 compared to NE-1 cells (Fig. 4).

Low expression of cytoplasmic FoxM1 associated with early-stage ESCC

Immunohistochemistry was performed to investigate the expression and localization of FoxM1 in 64 ESCC tissues and 10 randomly selected nontumor tissues from the same patient cohort. Cytoplasmic expression of FoxM1 was found in 98.44% (63/64) of ESCC tissues (Fig. 5), while its nuclear expression was observed in 25% (16/64) of ESCC

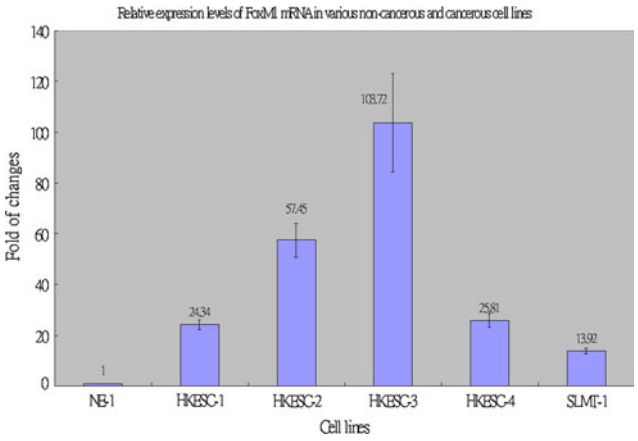

Fig. 2 High mRNA level of *FoxM1* in ESCC cell lines. qPCR was performed to investigate the mRNA level of *FoxM1* in ESCC cell lines (HKESC-1, HKESC-2, HKESC-3, HKESC-4, and SLMT-1) and the non-neoplastic esophageal cell line NE-1. High mRNA level of *FoxM1* was associated with ESCC cells. Each sample was repeated at least in duplicate

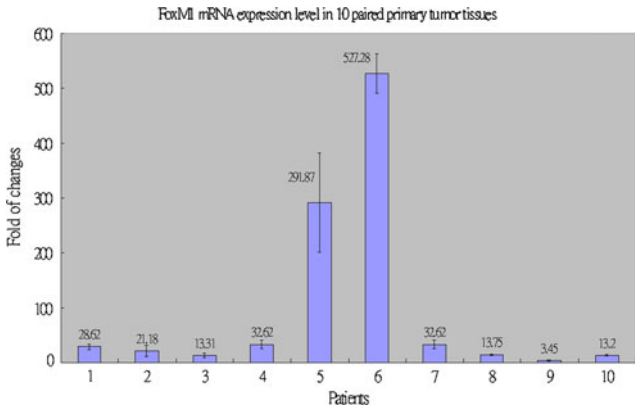

Fig. 3 High mRNA level of *FoxM1* in ESCC tissues. qPCR was performed to investigate the mRNA level of *FoxM1* in ten pairs of ESCC tissues and their adjacent nontumor tissues. High expression of *FoxM1* mRNA was found in all of the ESCC cases. The expression of *FoxM1* in nontumor tissues was arbitrarily set to 1. Each sample was repeated at least in duplicate

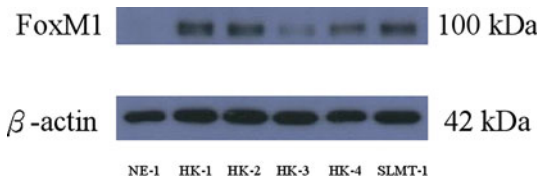

Fig. 4 High protein level of FoxM1 in ESCC cell lines. Immunoblot was performed to examine the protein level of FoxM1 in ESCC cell lines (HKESC-1/HK-1, HKESC-2/HK-2, HKESC-3/HK-3, HKESC-4/HK-4, and SLMT-1) and non-neoplastic esophageal cell line NE-1. High protein level of FoxM1 was associated with most ESCC cell lines, except HK-3

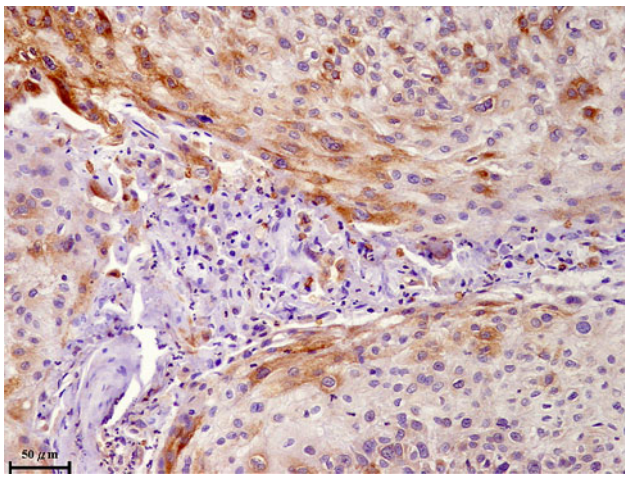

Fig. 5 Cytoplasmic localization of FoxM1 in ESCC tissues. Immunohistochemistry was used to study the expression and localization of FoxM1 in ESCC tissues and most cases of ESCC tissues had cytoplasmic staining of FoxM1. Original magnification, 200×

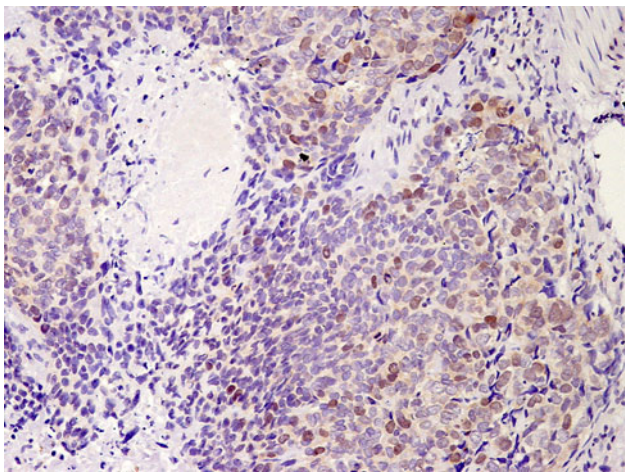

Fig. 6 Nuclear localization of FoxM1 in ESCC tissues. Immunohistochemistry was used to reveal the expression and localization of FoxM1 in ESCC tissues and about 25% of ESCC cases had tumors with nuclear FoxM1. Original magnification, 200×

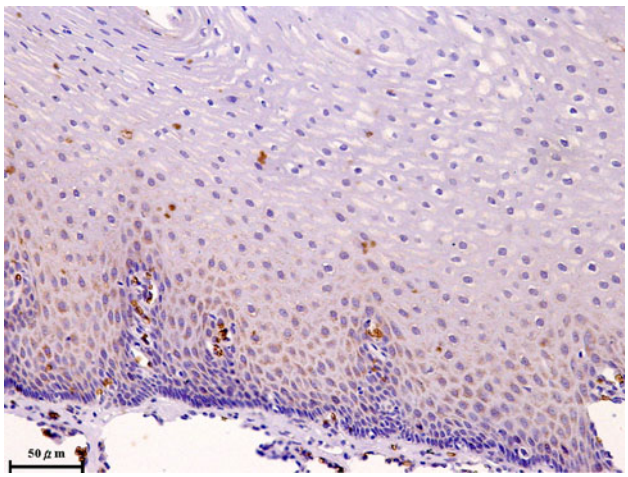

Fig. 7 Localization of FoxM1 in nontumor tissues of ESCC patients. Immunohistochemistry was used to examine the expression and localization of FoxM1 in nontumor tissues from ESCC patients, which showed cytoplasmic FoxM1 expression in the proliferating layer. Original magnification, 200×

tissues (Fig. 6). All nontumor tissues were positive for cytoplasmic FoxM1, whereas 20% (2/10) of them expressed nuclear FoxM1. Specifically, FoxM1 expression was found mainly in the proliferating layer of the non-neoplastic epithelium and the expression was relatively low in the more differentiated layer (Fig. 7).

Patients with lower expression of cytoplasmic FoxM1 were more likely in the early stage of ESCC ($P = 0.018$) than those with higher expression (19/37, 51.4% vs. 6/27, 22.2%, respectively) (Table 1). No significant difference in survival after surgery could be found between patients with low expression of cytoplasmic FoxM1 and those with high

Table 1 Correlations between cytoplasmic FoxMl expression and various clinicopathological parameters in ESCC

Clinicopathological parameters	Subgroups	Low expression	High expression	*P*-value
Age (mean in years)		64.3	65	0.797
Gender	Female	11	6	0.502
	Male	26	21	
Smoking	Non smoker	21	10	0.119
	Smoker	16	17	
Level of Tumor	Upper	5	5	0.895
	Middle	21	16	
	Lower	9	5	
	Double	2	1	
Tumor differentiation	Poor	10	4	0.481
	Moderate	21	17	
	Well	6	6	
R catergory	R0	29	16	0.098
	R1/R2	8	11	
T-stage	Early (T1/T2)	9	4	0.35
	Advanced (T3/T4)	28	23	
N-stage	N0	18	8	0.126
	N1	19	19	
M-stage	M0	33	25	0.645
	Mla/Mlb	4	2	
Overall pathological stage	Early (Stage I/II)	19	6	0.018
	Advanced (Stage III/IV)	18	21	

expression (14.75 vs. 23.28 months, respectively, $P = 0.977$) (Fig. 8). Patients with nuclear expression of FoxM1 were younger than those without nuclear FoxM1 (57 vs. 67.13 years, $P < 0.001$) (Table 2). The median postoperative survival for patients with nuclear FoxM1 was similar to those without (14.75 vs. 25.64 months, $P = 0.697$) (Fig. 9).

Discussion

This study used immunohistochemistry to study the correlations between FoxM1 and various clinicopathological parameters in ESCC patients. Our data show that cytoplasmic FoxM1 is associated with pathological disease stage. This is consistent with the report by Liu et al. [10], which demonstrated that the expression of *FoxM1* was higher in patients with high-grade glioma. Another study conducted by Chan et al. [27] also reported that a higher *FoxM1* level was significantly associated with

Table 2 Correlations between nuclear FoxMl expression and various clinicopathological parameters in ESCC

Clinicopathological parameters		Absence	Presence	*P*-value
	Subgroups			
Age (mean in years)		67.13	57	<0.001
Gender	Female	13	4	0.87
	Male	35	12	
Smoking	Non smoker	20	11	0.06
	Smoker	28	5	
Level of Tumor	Upper	9	1	0.371
	Middle	27	10	
	Lower	9	5	
	Double	3	0	
Tumor differentiation	Poor	11	3	0.937
	Moderate	28	10	
	Well	9	3	
R catergory	R0	34	11	0.874
	R1/R2	14	5	
T-stage	Early (T1/T2)	9	4	0.59
	Advanced (T3/T4)	39	12	
N-stage	N0	19	7	0.769
	Nl	29	9	
M-stage	M0	44	14	0.62
	Mla/Mlb	4	2	
Overall pathological stage	Early (Stage I/II)	18	7	0.657
	Advanced (Stage IMV)	30	9	

advanced-stage cervical cancer. All of the above findings supported a hypothesis that cytoplasmic FoxM1 might play a role in the progression of ESCC.

Expression of nuclear FoxM1 significantly correlates with the age of ESCC patients, i.e., patients with nuclear FoxM1 are younger than those without nuclear FoxM1. Ly et al. [28] previously compared the expression levels of various genes in fibroblasts from healthy individuals of different ages (young, middle age, and old age) and from patients with Hutchinson-Gilford progeria, which is a rare genetic disease accompanied by accelerated aging. Among the different genes, *FoxM1* was always downregulated in middle-aged, old-aged, and diseased when compared to young individuals [28]. Nuclear FoxM1 is an active form and regulates the expression of proliferation-related genes like cyclins [6, 7, 29, 30], which are crucial to the cell cycle. It is thus reasonable to find the expression of nuclear FoxM1 in younger ESCC patients since they are likely to have a higher rate of cell proliferation compared to the older patients.

We have found a tendency for patients with a smoking habit to have no expression of nuclear FoxM1. Previous studies have demonstrated that the salivary level of epidermal growth factor (EGF) was 32% lower in smokers than in nonsmokers [31, 32]. In addition, phosphorylation of EGF receptor in the buccal cavity was impaired in smokers as well [32]. Since the EGF receptor is involved in the mitogen-activated protein kinase (MAPK) pathway [33], this in turn activates *FoxM1* [34]. Therefore, smoking-induced reduced salivary EGF level and deficiency in EGF receptor phosphorylation likely inhibit the activation of *FoxM1* in esophageal epithelial cells of ESCC patients

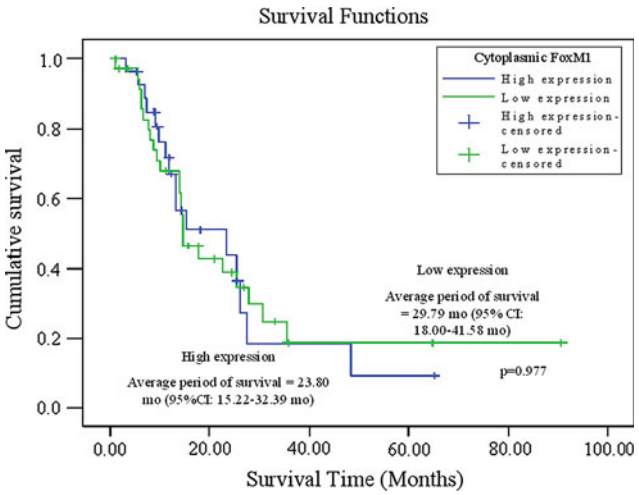

Fig. 8 Kaplan-Meier survival curves for cytoplasmic FoxM1. Kaplan-Meier survival curves were constructed for patients having high ($n = 27$) or low ($n = 37$) expression of cytoplasmic FoxM1. Log-rank test, $P = 0.977$

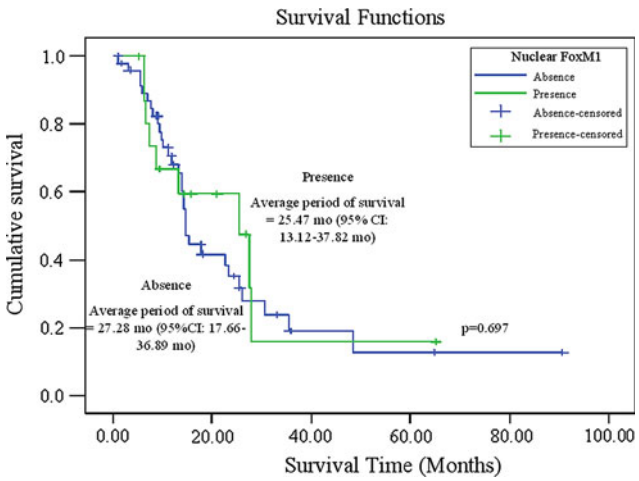

Fig. 9 Kaplan-Meier survival curves for nuclear FoxM1. Kaplan-Meier survival curves were generated for patients based on the presence ($n = 16$) or absence ($n = 48$) of nuclear FoxM1. Log-rank test, $P = 0.697$

who smoke. This might finally translate into a significant association between smoking and the absence of nuclear FoxM1 in ESCC patients.

HKESC-3 cells have the highest mRNA level but lowest protein level of FoxM1 when compared to the other studied ESCC cell lines. This could be the result of the presence of post-translation modifications in the *FoxM1* gene in ESCC. A review from Calnan and Brunet [35] stated the presence of a wide range of post-translation modifications such as phosphorylation, acetylation, and ubiquitination in the *FoxO* gene, which is also a member of the Forkhead transcription factor as is *FoxM1*. Thus, it is possible that a similar post-translation modification would occur in other members of the Forkhead transcription factor family like *FoxM1*.

In summary, we have shown that a low cytoplasmic FoxM1 level is correlated with early-stage ESCC and that nuclear FoxM1 expression was found in young ESCC patients. Therefore, FoxM1 is a potential biomarker for late-stage ESCC.

Disclosure The authors have no conflict of interest to declare.

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