

# Cyclin A1 is associated with poor prognosis in oesophageal squamous cell carcinoma

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Received April 23, 2018; Accepted April 2, 2019

DOI: 10.3892/ol.2019.10377

**Abstract.** Dysregulation of cyclin A1 (CCNA1) is implicated in the carcinogenesis, progression and metastasis of many types of solid tumours. In the present study, an mRNA single-channel expression profile chip experiment revealed that the CCNA1 mRNA levels in oesophageal squamous cell carcinoma (ESCC) were increased >10-fold compared with those in the adjacent non-cancer tissues. Reverse transcription-quantitative polymerase chain reaction and immunohistochemistry analyses were performed to additionally investigate the role of CCNA1 in the development and progression of ESCC in patients treated by radical resection of the oesophagus. The association between CCNA1 mRNA expression and the clinicopathological parameters of patients with ESCC was statistically analysed. The results indicated that upregulation of CCNA1 occurred in ~70% of patients with ESCC, and increased CCNA1 mRNA expression was significantly associated with advanced clinical stage, lymph node metastasis, invasiveness and poor clinical outcome, including disease-free survival and overall survival rates. Taken together, the data suggested that CCNA1 had an important function in ESCC

development and progression, and may serve as a prognostic biomarker and therapeutic target in ESCC.

## Introduction

Oesophageal cancer is one of the leading causes of cancer mortality worldwide (1). Oesophageal squamous cell carcinoma (ESCC) is the major histological type of oesophageal cancer in Eastern Asia (>90%). Epidemiological evidence suggests that carcinogen exposure and nutritional deficiency may be major risk factors for oesophageal cancer development (2). Genetic susceptibility may be an even more significant risk factor in high incidence areas of Northern China, such as variants in the phospholipase C epsilon 1 gene at 10q23. The 5-year survival rate of patients with ESCC is <20%, due to the lack of effective tools for early diagnosis and treatment (3). Therefore, it is crucial to understand the pathogenesis of ESCC. At present, the molecular basis for the development and progression of ESCC remains unclear.

Cyclin A1 (CCNA1) is an A-type cyclin characterised by a typical periodicity in protein abundance throughout the cell division cycle. CCNA1 binds cyclin-dependent kinase 2 (CDK2) and cell division cycle 2 (CDC2) kinases in the S and G2 phases, respectively, which results in 2 distinct kinase activities, and thereby regulates separate functions in the cell cycle (4,5). This cyclin has also been demonstrated to bind to important cell cycle regulators, including retinoblastoma (Rb) family proteins, transcription factor E2F-1, and the p21 family proteins (5-8). CCNA1 was initially identified as a protein essential for spermatogenesis and leukaemogenesis (9,10); however, evidence indicates that dysregulation of CCNA1, including its methylation or hypermethylation (11-14), is closely associated with carcinogenesis, cancer progression and metastasis in several solid tumour types (5,7,15,16). Furthermore, hypermethylation of the CCNA1 promoter is a potential marker for distinguishing the histopathological stage during multistep carcinogenesis (13,17), which indicates

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**Key words:** cyclin A1, oesophageal squamous cell carcinoma, clinicopathological parameters, prognosis, survival

its important role in early diagnosis. Hypermethylation of CCNA1 may be used as a tool for the identification of head and neck squamous cell carcinoma (HNSCC) subjects at risk of developing second primary carcinomas (18,19), suggesting its potential effect on patient prognosis. In addition, CCNA1 methylation is a potential predictive marker for chemotherapy sensitivity (20-22), which may aid in the development of effective therapy regimens.

Although the evidence of CCNA1 carcinogenicity in humans is compelling, its involvement in ESCC has not yet been reported. The present study demonstrated that CCNA1 mRNA was significantly upregulated in ESCC, and that its expression was associated with poor cell differentiation, advanced clinical stage, lymph node metastasis and invasiveness. Patients with high levels of CCNA1 had decreased disease-free survival (DFS) and overall survival (OS) rates, compared with patients with decreased levels of CCNA1. These data suggested that CCNA1 may serve a crucial role in the tumorigenesis and progression of ESCC and identified CCNA1 as a potential prognostic biomarker and therapeutic target in ESCC.

## Materials and methods

**Ethics statement.** The present study was approved by the Ethics Committee of Nanjing Medical University Cancer Centre (Nanjing, China). All patients provided written informed consent. All specimens were handled and stored anonymously according to ethical and legal standards.

**Study population.** Tumour samples were obtained from December 2007 to January 2012, from patients with pathologically confirmed ESCC (n=78) during radical surgery of the oesophagus at the Affiliated Nanjing Hospital of Nanjing Medical University (Nanjing, China). No patients had received antitumour therapy, including chemotherapy, radiotherapy or chemoradiation prior or subsequent to the surgery. The adjacent non-cancerous tissue was defined as tissue  $\geq 2$  cm from the edge of the tumour. The clinicopathological parameters of the 78 patients with ESCC were obtained from medical records and pathology reports. The ESCC specimens were staged in accordance with the 7th edition of the American Joint Committee on Cancer/Union for International Cancer Control tumor, node, metastasis (TNM) staging system (23). The grading and histopathological subtyping of the ESCC specimens was based on WHO Classification of Tumors of the Digestive System (24). Patient consent was obtained prior to use of the clinical materials for research purposes. The patients regularly attended follow-up visits. OS was defined as the interval between the dates of surgery and mortality or last observation. DFS was defined as the interval from the date of surgery to that of recurrence, the last follow-up or mortality. Data were censored at the last follow-up visit for surviving patients with no disease recurrence.

**RNA isolation and Agilent mRNA single-channel expression profile chip experiment.** Total RNA was extracted from 5 representative paraffin blocks of cancerous and paired adjacent non-cancerous tissues using an EasyPure RNA kit (Beijing Transgen Biotech Co., Ltd.), then purified and fluorescently

labelled using a Crystal core<sup>®</sup> cRNA amplification marker kit (Beijing CapitalBio Technology Co., Ltd.), according to the manufacturer's protocol. The chip was scanned using Agilent G2565CA Microarray Scanner (Agilent Technologies, Inc.) to obtain hybrid images. The Feature Extraction image analysis software was used to analyse the chip image and convert the image signals into digital signals. The raw data were inputted into GeneSpring GX 10 software (Agilent Technologies Inc.), and the signal was normalized by the percentile shift method. Then, the differential genes were confirmed by a standard of absolute fold change  $\geq 2$ .

**Reverse transcription quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted by Trizol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and reverse transcribed to cDNA by the PrimeScript<sup>™</sup> RT reagent kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the supplier's protocol. The following primers were used: Cyclin A1 forward, 5'-CTCCTCTCCCAGTCTGAAGA-3'; cyclin A1 reverse, 5'-CAGGAAGTTGACAGCCAGAT-3'; GAPDH forward, 5'-AACAGCGACACCCACTCCTC-3'; GAPDH reverse, 5'-GGAGGGGAGATTCAGTGTGGT-3'. The two-step PCR protocol was used as follows: Initial denaturation at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 1 min and annealing at 60°C for 34 sec, followed by an extension at 95°C for 15 sec, 60°C for 1 min and 95°C for 5 sec. qPCR was performed using SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II (Takara) and an ABI 7500 Real-time PCR system and the StepOnePlus<sup>™</sup> 2.0 software (Bio-Rad Laboratories, Inc.). The real-time values were averaged for each sample and compared using the Ct method. The relative expression level of the target gene, defined as a fold change, was calculated using the  $2^{-\Delta\Delta Cq}$  (25) method, where  $\Delta\Delta Cq = \text{mean } Cq_{\text{CCNA1}} - \text{mean } Cq_{\text{GAPDH}}$ . A total of 2 independent experiments were performed to analyse the relative gene expression, and each sample was examined in triplicate.

**Immunohistochemistry (IHC).** For IHC analysis of CCNA1 protein expression, 20 paraffin-embedded ESCC tumour specimens (n=10) and the corresponding normal epithelial samples (n=10) were selected. The reagents used in immunohistochemistry were purchased from Fuzhou Maxim Biotechnology Development Co., Ltd. Briefly, tissue sections were cut from the paraffin blocks (5  $\mu\text{m}$ ) and mounted on microscope slides. Slides were placed in 60°C, baked for 20 min before being deparaffinised with xylene, then orderly soaked in anhydrous ethanol, 95% ethanol and 75% ethanol, and blocked with 10% normal goat serum at room temperature for 30 min. The slides were subsequently incubated with a rabbit polyclonal antibody against CCNA1 (cat. no., ab118897, Abcam) at a dilution of 1:100 at 4°C overnight, followed by incubation with biotinylated goat anti-rabbit immunoglobulin (cat. no., KIT-5004) at a dilution of 1:100 for 60 min at 37°C. CCNA1 expression was evaluated as the percentage of tumour cells with positive staining at each high-power field with a light microscope (OLYMPUS BX46; Olympus Corporation; magnification, x100), and at least 5 HPFs were assessed. The H-score method (26) was used to analyse each sample on a continuous scale of 0-300. The percentage of cells at different staining intensities [no staining, weak (yellow), moderate (brown), or strong (dark brown)] was

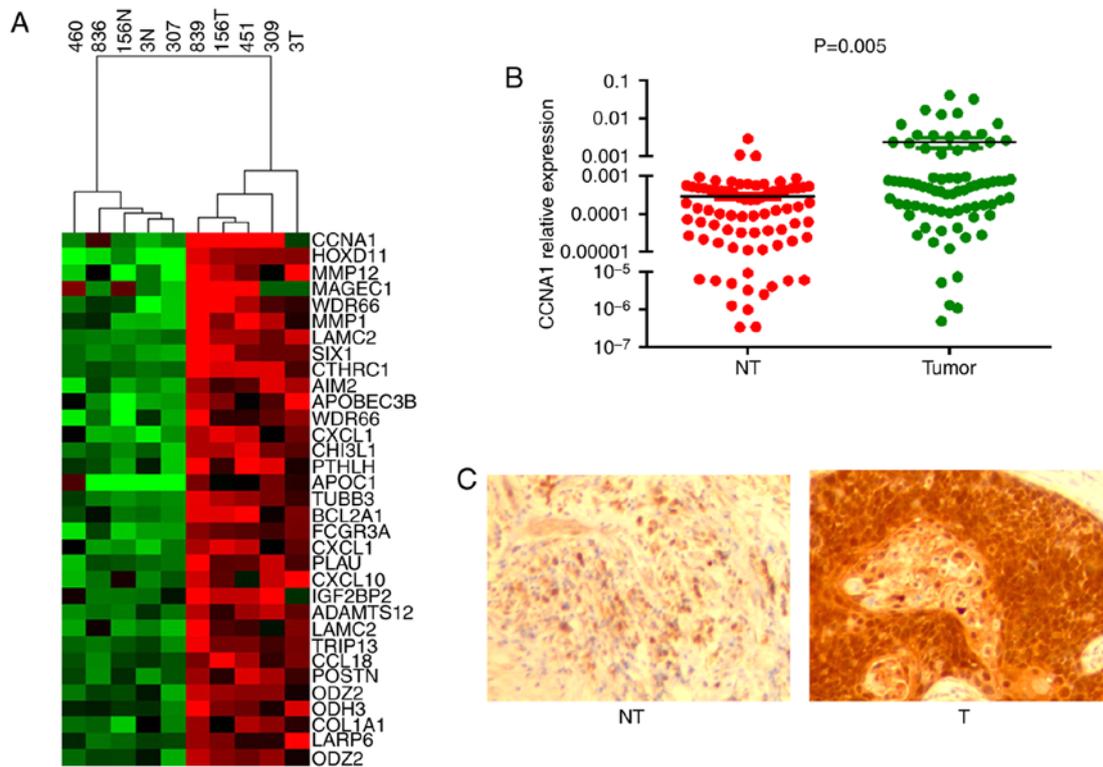


Figure 1. CCNA1 upregulation in ESCC. (A) A total of 5 mRNA single-channel expression profile chips all revealed that CCNA1 levels in ESCC (5 right-hand columns) were increased >10-fold compared with those in NTs (5 left-hand columns). (B) Cyclin A1 mRNA expression was significantly increased in the ESCC tissues compared with the matched adjacent NTs ( $P=0.005$ ) using reverse transcription quantitative polymerase chain reaction. (C) Representative CCNA1 protein expression in a pair of ESCC (right) and adjacent NT (left) samples from 1 patient, detected by immunostaining with anti-CCNA1 antibody. Positive staining is indicated by brown colouring. Magnification,  $\times 100$ . CCNA1, cyclin A1; ESCC, oesophageal squamous cell carcinoma; NT, non-cancer tissues.

determined by visual assessment, with the score calculated using the formula: (Percentage of weakly stained tumour cells) + (2x percentage of moderately stained tumour cells) + (3x percentage of strongly stained tumour cells). Samples were subsequently classified as low (H-score <200; IHC negative) or high ( $\geq 200$ ; IHC positive) for CCNA1 protein expression.

**Statistical analysis.** All statistical analyses were performed using SPSS version 19.0 (IBM Corp.). Paired Student's t-test was used in the comparison of the relative expression levels of CCNA1 mRNA between tumour tissues and the adjacent non-cancer tissues (NTs). Pearson's  $\chi^2$  test or Fisher's exact test and logistic regression analysis were used to examine the association between CCNA1 expression and the clinicopathological parameters. DFS and OS were estimated using the Kaplan-Meier method, with a 95% confidence interval. Differences between survival curves were calculated using the log-rank test. Prognostic factors were examined by multivariate analyses using the Cox proportional hazards model.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**CCNA1 is upregulated in ESCC tissues.** All 5 mRNA chips revealed that CCNA1 mRNA levels in ESCC were increased >10-fold compared with those in the adjacent NTs ( $P=0.007$ ; Fig. 1A). RT-qPCR was performed for 78 pairs of primary ESCC tumours and their corresponding adjacent NTs. It was revealed that upregulation of CCNA1 mRNA was present in

55/78 (70.51%) ESCC tumours, whereas upregulation was present in 23/78 of the adjacent NTs (29.49%). The relative expression levels of CCNA1 mRNA were significantly increased in tumour tissues compared with the adjacent NTs ( $P=0.005$ ; Fig. 1B). IHC analysis indicated that cyclin A1-positive staining was present in 7/10 ESCC specimens (70%) and 2/10 adjacent NTs (20%), although there was no statistical difference ( $P=0.07$ ). CCNA1 protein was primarily located in the nucleus of tumour cells, as indicated by the brown staining pattern (Fig. 1C).

**CCNA1 upregulation is associated with ESCC progression.** The association between CCNA1 mRNA expression levels and the clinicopathological features of ESCC were subsequently evaluated (Table I). The results revealed that CCNA1 upregulation was correlated with the tumour invasiveness ( $P=0.04$ ), advanced clinical stage ( $P=0.001$ ) and lymphatic metastasis ( $P=0.029$ ). However, univariate analysis revealed that CCNA1 upregulation was not significantly associated with sex, age or cell differentiation. Binary logistic regression analysis indicated that the expression of CCNA1 was increased in cases with poor cell differentiation ( $P=0.01$ ) and advanced clinical stage ( $P < 0.001$ ; Table II). No distant metastasis cases were presented in the present study.

**CCNA1 upregulation in tumour cells predicts poor prognosis.** The 5-year survival rate of the ESCC patients enrolled in the present study was 24.36% (19/78). During the follow-up period, 53 patients (67.95%) were diagnosed with tumour recurrence

Table I. Association between CCNA1 expression and oesophageal squamous cell carcinoma clinicopathological characteristics.

Characteristics	Number	CCNA1 expression		P-value
		High (%)	Low (%)	
Age, years				0.777
<60	29	21 (38.18)	8 (34.78)	
≥60	49	34 (61.82)	15 (65.22)	
Sex				0.482
Male	52	38 (69.09)	14 (60.87)	
Female	26	17 (30.91)	9 (39.13)	
Cell differentiation				0.073
Well	12	7 (12.73)	5 (21.74)	
Moderate	56	38 (69.09)	18 (78.26)	
Poor	10	10 (18.18)	0 (0)	
Tumour invasion depth				0.04
T1	7	3 (5.45)	4 (17.39)	
T2	18	10 (18.18)	8 (34.78)	
T3	53	42 (76.36)	11(47.83)	
T4	0	0 (0)	0 (0)	
Lymph node metastasis				0.029
N0	51	31 (56.36)	20 (36.36)	
N1	22	19 (34.55)	3 (13.04)	
N2	5	5 (9.09)	0 (0)	
Distant metastasis				-
M0	78	55 (100)	23 (100)	
M1-x	0	0 (0)	0 (0)	
Clinical stage				0.001
I	19	8 (14.55)	11 (47.83)	
II	33	23 (41.82)	10 (43.48)	
III	26	24 (43.64)	2 (8.70)	
IV	0	0 (0)	0 (0)	

CCNA1, cyclin A1. P-values were calculated using Pearson's  $\chi^2$  test or Fisher's exact test, as appropriate.

Table II. Binary logistic regression analysis between cyclin A1 expression and oesophageal squamous cell carcinoma clinicopathological characteristics.

Variable	Hazard ratio (95% confidence interval)	P-value
Cell differentiation, well/moderate/poor	6.195 (1.555-24.670)	0.010
Clinical stage, I/II/III	5.114 (2.063-12.676)	<0.001

Variables were analysed by Pearson  $\chi^2$  test or Fisher's exact test.

and progression. In the overexpression group, the median OS and DFS were 20 and 14 months, respectively. Univariate analysis demonstrated that high CCNA1 expression, advanced clinical stage, and higher T and N classifications predicted a shorter OS in ESCC, although multivariate analysis demonstrated that only overexpression of CCNA1 and advanced clinical stage were independent prognostic factors for ESCC (Table III). Kaplan-Meier analyses and log-rank tests revealed

that patients with high CCNA1 expression had significantly shorter DFS (P=0.006) and OS (P=0.003) rates, compared with the low CCNA1 expression group (Fig. 2).

### Discussion

CCNA1 primarily functions in the meiotic cell cycle, but also contributes to the cell cycle progression in somatic cells (5).

Table III. Analyses of prognostic parameters in patients with oesophageal squamous cell carcinoma.

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Clinical stage, I		<0.001	7.535 (2.656-21.371)	<0.001
II	0.007 (0.001-0.054)			
III	0.158 (0.083-0.300)			
Cell differentiation, well/moderate/poor	-	0.905	-	-
Lymph node metastasis, N0/N1-N2	0.101 (0.053-0.192)	<0.001	-	-
Tumour invasion depth, T1-T2/T3	0.164 (0.069-0.387)	<0.001	-	-
Sex, male/female	2.168 (1.136-4.137)	0.013	-	-
Age, y, <60/≥60	-	0.697	-	-
CCNA1, low/high expression	0.387 (0.198-0.756)	0.003	0.456 (0.220-0.943)	0.034

HR, hazard ratio; CI, confidence interval; CCNA1, cyclin A1. P-values were obtained by univariable analysis, Kaplan-Meier analysis and the log-rank test.

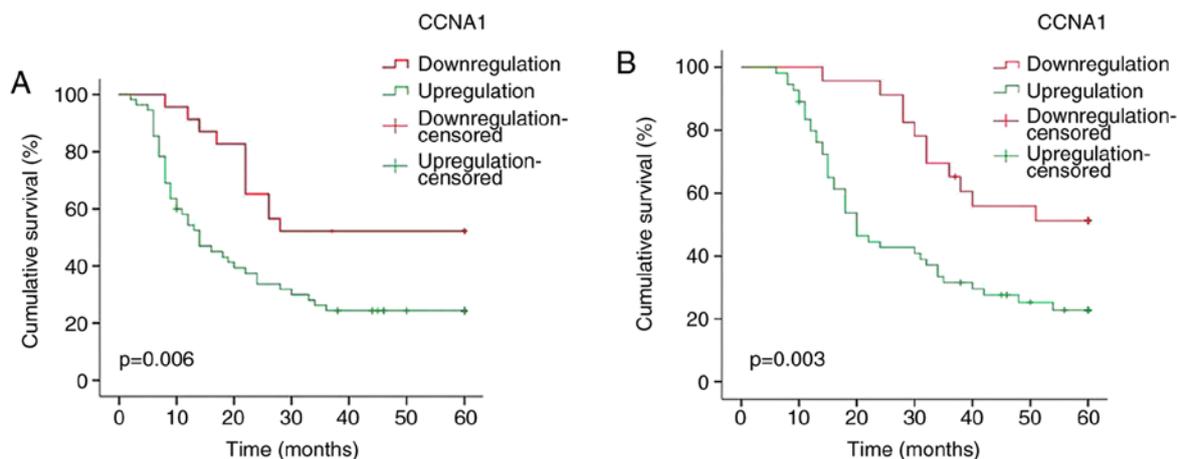


Figure 2. CCNA1 upregulation is associated with poor prognosis in patients with ESCC. Patients with increased levels (green line) of CCNA1 exhibited poorer (A) disease-free survival (log-rank  $P=0.006$ ) and (B) overall survival (log-rank  $P=0.003$ ) compared with those with decreased CCNA1 expression (red line). CCNA1, cyclin A1.

In addition, CCNA1 has been demonstrated to serve as an oncogene and tumour suppressor (7,27), with crucial roles in the carcinogenesis, progression and metastasis of several solid tumour types (11-16). In addition, CCNA1 is considered to be a promising tumour antigen for anticancer T-cell therapies (28,29), and increased CCNA1 expression results in chemoresistance to certain cytotoxic drugs, including paclitaxel, doxorubicin and 5-fluorouracil (20,22).

However, the role of CCNA1 in ESCC remains largely unknown; to the best of our knowledge, the present study is the first to describe the involvement of CCNA1 in ESCC. It was revealed that CCNA1 mRNA expression was significantly increased in the ESCC tissues compared with paired non-cancerous tissue using mRNA chips and RT-qPCR. Increased expression of CCNA1 protein was observed in ESCC tissues (70% positive) compared with paired non-cancerous tissues (20% positive) by IHC, which was consistent with the CCNA1 mRNA expression results. However, no statistical significance was observed; this discrepancy may be due to the

small sample size. CCNA1 protein was predominantly located in the nucleus of tumour cells, where it may bind CDK2 or CDC2 kinases to control the cell cycle at the G1/S or G2/M transitions (5,7). This suggests that CCNA1 may have an important role in the development and progression of ESCC.

A tissue microarray study of 149 patients with bladder cancer revealed that CCNA1 expression predicts progression, and recent clinical evidence has demonstrated that CCNA1 contributes to cancer invasion via multiple cellular mechanisms (30,31). Consistent with these data, the present study revealed that ESCC tumours with high CCNA1 expression were more invasive, prone to lymph node metastasis and in advanced clinical stages, suggesting a prognostic role of CCNA1 for ESCC progression. Furthermore, the data confirmed that increased levels of CCNA1 were associated with poorer cell differentiation. Therefore, CCNA1 expression has the potential to be used as a biomarker to determine whether adjuvant therapy following surgery is required. Survival analysis revealed that the poorer outcomes were associated with numerous factors, including

high CCNA1 expression, advanced clinical stage, T and N classification and sex. However, only CCNA1 overexpression and advanced clinical stage were independent prognostic factors for ESCC survival. In agreement with previous data (29,31,32), the data from the present study clearly demonstrated that a subset of patients with upregulated CCNA1 mRNA expression exhibited demonstrated DFS and OS rates. The present study suggested that CCNA1 may be an important oncogene in ESCC development and progression.

CCNA1 is an important cell-cycle regulator, and previous molecular studies have revealed that the oncogenic function of CCNA1 was closely associated with its effect on cellular proliferation (33-37). In addition, CCNA1 was functionally associated with vascular endothelial growth factor, matrix metalloproteinase (MMP)2, MMP9 and oestrogen receptor  $\alpha$  to promote cancer progression (30,38-40). However, the particular molecular mechanism of CCNA1 in ESCC requires additional study.

Although the results of the present study require additional validation due to certain limitations, they elucidated the clinical importance of CCNA1 in ESCC progression. In particular, the effect of CCNA1 expression on survival was highlighted. The data presented suggested that CCNA1 was an important prognostic factor and a diagnostic molecular marker in ESCC. Furthermore, CCNA1 has potential as a novel therapeutic target in patients with ESCC.

### Acknowledgements

The authors would like to thank Dr. Jian Zou and Ying Yin (Central Laboratory, Wuxi People's Hospital) for their advice and input. The authors acknowledge colleagues in the Department of Pathology (Wuxi People's Hospital) for providing the facilities.

### Funding

The present study is supported by National Natural Science Foundation of China (grant no. 81672423), Jiangsu Natural Science Foundation (grant no. BK20151292), Jiangsu Provincal Medical Innovation Team of the Project of Invigorating Health Care through Science, Technology and Education, and the 333 Project Foundation of Jiangsu Province (grant no. BRA2014355).

### Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

### Authors' contributions

XH, XC and SL were responsible for the conception and design of the study. XH, TF and SL developed the methodology. XH, QL and XC were responsible for the acquisition of data, including recruitment and management of patients and provision of facilities. XH, TF, JM, QL, WS and YL analysed and interpreted the data, including statistical analysis, biostatistics and computational analysis. XH and TF were responsible for writing and revision of the manuscript. XH and XC provided administrative, technical and

material support, including reporting or organizing data, and construction of databases.

### Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Nanjing Medical University Cancer Centre (Nanjing, China). All patients provided written informed consent. All specimens were handled and stored anonymously according to ethical and legal standards.

### Patient consent for publication

All patients provided written informed consent.

### Competing interests

The authors declare that they have no competing interests.

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