RESEARCH ARTICLE

Stathmin is a Marker of Progression and Poor Prognosis in Esophageal Carcinoma

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Abstract

Stathmin, also called oncoprotein 18, is a founding member of the family of microtubule-destabilizing proteins that play a critical role in the regulation of mitosis. At the same time stathmin has been recognized as one of responsible factors in cancer cells. The aim of this study was to assess stathmin status, its correlations with clinicopathological parameters and its role as a progosnostic marker in EC patients. The protein and mRNA levels of stathmin were examined byimmunohistochemistry (IHC) and in situ hybridization in 100EC tissues and adjacent noncancerous tissues. mRNA and protein expression of stathmin in three EC cell lines(EC9706, ECa109, EC1 commonly used in research) were also analyzed using immunocytochemistry, western blot and in situ hybridization. The prognostic value of Stathmin expression within the tumor tissues were assessed by Cox regression and Kaplan-Meier analysis. We showed that stathmin expression was significantly higher in EC tissues than in adjacent noncancerous tissues. High stathmin immunostaining score in the EC was positively correlated with tumor differentiation, Tumor invasion, Lymph node metastases, and TNM stage. In addition, we demonstrated that three EC cell lines examined, were constitutively expressing a high level of stathmin. Of those, EC-1 showed the strongest mRNA and protein expression for the stathmin analyzed. Kaplan-Meier analysis showed that significantly longer 5-year survival rate was seen in EC patients with high Stathmin expression, compared to those with low expression of Stathmin expression. Furthermore, multivariate Cox proportional hazard analyses revealed that Stathmin was an independent factors affecting the overall survival probability. In conclusion, our data provide a basis for the concept that stathmin might be associated with EC development and progression.. High levels of Stathmin expression in the tumor tissues may be a good prognostic marker for patients with EC.

Keywords: Esophageal carcinoma - stathmin - biomarker

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Introduction

Esophageal carcinoma (EC) is one of the most aggressive carcinomas of the gastrointestinal tract worldwide (Hu et al., 2013). More than half of all EC cases in the world occurred in China, while Linzhou City, a region in northwest China, has the highest EC rates (Song et al., 2011). Until now, EC has low survival rate because of its aggressive nature, distant metastasis, and largely unknown molecular mechanism of progression (He et al., 2012; Wang et al., 2013;). To determine a specific biomarker at the gene level and establish effective prevention, obtaining an early diagnosis method has become one of the research focuses in the field of tumor and EC prevention (Qi et al., 2013).

Stathmin, also called oncoprotein 18, is a microtubule-destabilizing phosphorprotein, microtubules are essential for many cellular processes, including intracellular transport, mitosis, sustain of cell shape and cell motility (Tsvetkov et al., 2013; Li et al., 2012; Tian et al., 2012). At molecular level, stathmin depolymerizes microtubules by either sequestering free tubulin dimers or directly inducing microtubule-catastrophe. Studies have showed that stathmin, which is frequently overexpressed in a variety of human cancers, has a close correlation with cancer cell differentiation, TNM classification and Lymph node metastases (Liu et al., 2013). It is necessary for overexpression of stathmin to maintain proliferation of cancer cell. It suggests that stathmin participates in the tumorigenesis and tumor development and provides an attractive oncobiological marker and molecular target for cancer therapy (Karst et al., 2011; Jeon et al., 2010).

However, there are fewer reports about stathmin expression and biological characteristic in EC and prognosis of EC patients (Liu et al., 2013). In the current study, the expression of stathmin in EC and EC cells lines were investigated and the correlations between stathmin parameters of expression and clinicopathological parameters were analyzed. Furthermore, the prognostic significance of Stathmin in human EC was also explored.

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Materials and Methods

Ethics statement

This study was performed under a protocol approved by the Institutional Review Boards of The First Affiliated Hospital of Zhengzhou Medical University and all examinations were performed after obtaining written informed consents.

Patients and tissue specimens

100 patients with histologically confirmed esophageal carcinoma underwent esophagectomy between June 2009 and June 2011 at The First Affiliated Hospital of Zhengzhou University entered the study. The group of patients consisted of 68 males and 32 females with ages ranging between 43 and 78 years old (mean age: 64 years old). Tumor was classified and staged with World Health Organization criteria. Of 100 cases, 71 and 29 were well, moderately or poorly differentiated EC respectively. 52 cases were accompanied with lymph node metastasis and 48 cases had no lymph node metastasis. The invasion depth was divided into two groups consisting of 34 cases with invasion of the superficial layer and 66 with invasion of the deep layer. The TNM classification included grade I, II (39 cases) and grade III, IV (61 cases). None of the patients received any type of therapy prior to surgery. Informed consent was obtained from all of the patients, and the study was approved by the Research Ethics Committee of Zhengzhou University. In each case, hematoxylin- and eosin-stained preparations were subjected to histopathological evaluation by two pathologists.

Cell lines and cell culture

The human esophageal cancer cell line Eca109 were saved in the Tumor Center Laboratory of Zhengzhou University, human esophageal cancer cell line EC-1 were obtained from the Biological Engineering Laboratory of Molecular Biology of Zhengzhou University and human esophageal cancer cell line EC9706 were obtained from State Key Laboratory of Molecular Oncology of Cancer Institute and Hospital, Chinese Academy of Medical Sciences. All Cells were cultured in 1640 medium containing 10% fetal bovine serum (FBS), 100 mg/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Immunohistochemistry and immunocytochemistry of stathmin

Every tissue specimen was taken from the tumor focus, as well as the distal normal mucosa, and was fixed in 10% buffered formalin, embedded in paraffin and sectioned into 4-μm slices. They were deparaffinized in xylene and then rehydrated through a graded alcohol series. 10 mM Citric acid buffer, pH 6.0 was used as standard microwave-based antigen retrieval methods (70°C for 15 minutes). The endogenous peroxidase activity of specimens was blocked by immersing the slides in a 3% hydrogen peroxide (H_2O_2) solution in methanol for 25 minutes at room temperature. Briefly, sections were blocked in bovine serum for 20 min to reduce non-specific background staining. The blocked sections were incubated with primary antibody stathmin (Santa Cruz Biotechnology, Santa Cruz, CA; sc-48362, 1:100), diluted in PBS at 4°C for overnight, followed by staining with a streptavidin-biotin peroxidase kit (Zhongshan Goldenbridge Biotechnology, Beijing, China). Between each immunostaining step, slides were washed briefly in PBS buffer. Sections known to stain positively were included in each batch and negative controls were prepared by replacing the primary antibody with PBS buffer. For stathmin, cytoplasmic immunoreactivity was scored by its extent and intensity. Staining intensity was graded as follows19: negative (0), weak (1), moderate (2) and strong (3). Staining extent was rated according to the percentage of positive cells. Samples with no stained tumor cells were rated as 0, those with <10% of stained tumor cells were rated as 1, those 11%-50% of stained tumor cells were rated as 2, those with 50-75% of stained tumor cells were rated as 3 and those with >75% of stained tumor cells were rated as 4.19 The score of staining intensity multiplied by the score of extent equals an overall staining score. An overall staining score of 0-3 and >3 were regarded as negative (-) and positive (+) protein expression, respectively.

For stathmin immunocytochemical analysis of glioma cell lines, the cells were plated on glass coverslips placed into 12-well plates, and allowed to adhere overnight. Then, the cells were fixed in paraformaldehyde at 4% for 15 minutes, followed by permeabilization with 0.05% Triton X-100 for 4 minutes at room temperature. The immunocytochemistry procedure was performed using the method as described above.

In Situ Hybridization

4-6μm thickness tissue sections of each case were placed sequentially on each positive charged slide. The sections were deparaffinized, digested by pepsin (DAKO, 1.3 mg/ml) for 20 minutes, washed in sterile water, then 100% ethanol, and air dried.

The biotin-labeled stathmin cDNA probes (5'-AGC TTC ATG GGA CTT GCG TCT-3') were diluted to a concentration of 6-12 ng/20 μl using in situ hybridization buffer. The probes and tissue RNA were co-denatured at 42°C for 12h-16h minutes. This was followed by a wash in a solution of 0.1×SSC with 2% BSA for 5 min. Followed by incubation with streptavdin alkaline phosphates (SA-AP) in a heater at 37°C for 30 min, the blue color was developed by incubation of the slide with nitroblue tetrazolium and bromchloroindolyl phosphate (NBT/BCIP) at 37°C. The colorimetric reaction was monitored visually and stopped by placing the slides in water when background coloring started to appear on the negative control (scrambled probe), varying from 15-30 min. The slides were counterstained with nuclear fast red (Enzo Diagnostics, NY) to visualize the nuclei, before cover glass mounting. Representative and viable tissue sections were scored manually semiquantitatively for cytoplasmic staining. The dominant staining intensity in tumor cells was scored as: 0=negative; 1=weak; 2=intermediate; 3=strong. All samples were anonymized and independently scored by one experienced pathologist and one technician.

Western blot

EC-9706, Eca-109 and EC-1 cells were harvested and lysed in modified radioimmune precipitation assay lysis buffer with 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 0.5% deoxycholic acid, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 1 mM sodium orthovanadate, and 10 μg/ml soybean trypsin inhibitor in 50 mM Tris buffer, pH 7.4. The proteins were separated by SDS-PAGE gel electrophoresis and then transferred onto a PVDF membrane (Stathmin: 20V, 25min; β-actin: 20V, 60min). After blocking with 0.1% Tween 20 and 5% nonfat dry milk in Tris-buffered saline at 4°C overnight, then the membrane was incubated with primary antibody (Stathmin: 1:1000) at room temperature for 2h, and then incubated with horseradish peroxidase-conjugated secondary antibody (1:4000) for 1h. Protein bands were detected (the colored membranes) with the enhanced chemiluminescence (ECL) system and exposed to X-ray film. All analyses were performed in triplicate.

Statistical analysis

Statistical analyses were performed using the SPSS 17.0. Chi2 and Spearman rank correlation were used to analyze associations between the parameters of stathmin expression and clinicopathological features. Associations between age and categorical variables were determined using the unpaired student's t-test or one-way analysis of variance. The survival rates after tumor removal were calculated by the Kaplan-Meier method, and differences in survival curves were analyzed by the Log-rank tests. Multivariate survival analysis was performed on all the significant characteristics measured by univariate survival analysis through the Cox proportional hazard regression model. All reported *p*-values were two sided and reported at a significance level of 5%.

Results

Stathmin protein expression in esophageal carcinoma

Immunohistochemistry was performed to determine the expression levels of stathmin proteins in EC tissues in comparison with the corresponding normal esophageal mucosa tissues. Stathmin positive signals showed brown-yellow granules in the cytoplasm (Figure1A, B). The positive rates of stathmin were73.0% in EC tissues and 30.0% in the corresponding normal esophageal mucosa tissues (Table 1). Moreover, the expression of stathmin protein in EC tissues were significantly higher than that in the corresponding normal esophageal mucosa tissues (p=0.000).

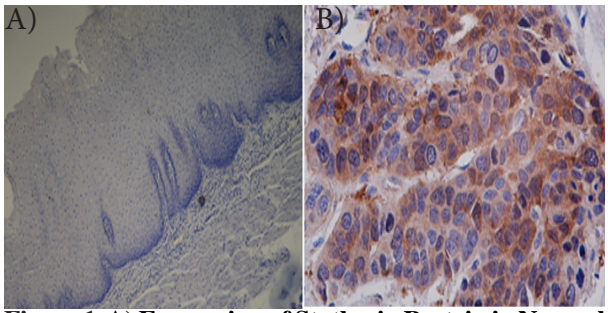

Figure 1. A) Expression of Stathmin Protein in Normal Esophageal Mucosa (×200), B) Expression of Stathmin in Esophageal Squamous Cell Cancer (ESCC) (×400)

Table 1. Frequency of Stathmin Protein Expression in Benign and Malignant Esophageal Tissues

	Number of cases	ESCC	Normal	*p* values
stathmin	100	73 (73.0)%	30 (30.0)%	0

*ESCC: esophageal squamous cell carcinoma; Normal: normal esophageal mucosa.

Association between stathmin protein expression and clinicopathological parameters

The over-expression of stathmin was associated with poorly differentiated tumors, and this correlation was statistically significant (p=0.017). Additionally, stathmin protein overexpression was significantly correlated with the lymph node metastasis of esophageal carcinoma. The positive rates of stathmin were 50.0% and 94.2% in non-metastatic and metastasis EC, respectively (p=0.000). For the TNM clinical classification, stathmin positive rate was only 62.5% in early clinical stage of esophageal carcinoma, however significantly higher in late stage cases (80.3%), and the difference was statistically significant (p=0.008). Also the positive rate of stathmin protein expression was significantly higher in superficial invasion depth than it in

Table 2. Stathmin Protein Expression in Relation to Clinical Pathological Characteristics in EC

Clinicopathologic features	n	stathmin -	+	* (+%)	*p*
Age(years)					
≤60	63	17	46	73	0.996
>60	37	10	27	72.8	
Sex					
male	54	14	40	74.1	0.793
female	46	13	33	71.7	
Tumor differentiation					
Well, Moderate	71	24	47	66.2	0.017
Poor	29	3	26	89.7	
Tumor invasion					
Superficial layer	34	20	14	41.2	0
Deep layer	66	7	59	89.4	
Lymph node metastases					
Yes	52	3	49	94.2	0
No	48	24	24	50	
TNM					
I+II	39	15	24	62.5	0.008
III+IV	61	12	49	80.3	

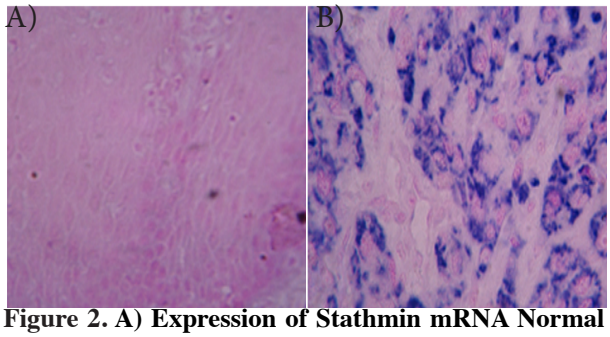

Figure 2. A) Expression of Stathmin mRNA Normal Esophageal Mucosa(×200), B) Expression of Stathmin in Esophageal Squamous Cell Cancer (ESCC) (×400)

muscularis invasion depth (p=0.000). However, stathmin protein expression level was not correlated with the patient age (p=0.996), gender (p=0.793). (Table 2)

Stathmin mRNA Expression in Esophageal Carcinoma

Furthermore, the mRNA level of stathmin was detected in esophageal carcinoma tissues by in situ hybridization (Figure 2A, B). The expression of stathmin was positively detected in 75.0 % esophageal carcinoma tissues, while the positive stathmin expression was found only in 25.0 % normal esophageal mucosa tissues. The difference of stathmin expression was statistically significant between esophageal carcinoma and normal esophageal mucosa tissues (p=0.000) (Table 3). This finding matches with the IHC result reported in this study, i.e, stathmin was found over-expressed in esophageal carcinoma.

Association between stathmin mRNA expression and clinicopathological parameters

To understand the clinical significance of stathmin mRNA expression in EC tissues, the correlation between the expression levels of stathmin mRNA and clinicopathological parameters was analyzed. We also found that there were no differences in the expression levels of stathmin mRNA in EC between male and female patients groups (p=0.996), or between different age group (p=0.487). However, the expression levels of stathmin mRNA in EC were positively correlate with degree of tumor differentiation (p=0.008), invasion depth (p=0.000), lymph node metastasis (p=0.000) and TNM classification (p=0.044) (Table 4).

Table 3. Frequency of Stathmin mRNA Expression in Benign and Malignant Esophageal Tissues

	Number of cases	ESCC	Normal	*p* values
stathmin	100	75	25	0

*ESCC, esophageal squamous cell carcinoma; Normal, normal esophageal mucosa

Table 4. Stathmin mRNA Expression in Relation to Clinical Pathological Characteristics in EC

Clinicopathologic features	n	stathmin			
		-	+	* (%)	*p*
Age					
≤60	63	16	47	74.6	0.996
>60	37	9	28	75.7	
Gender					
male	54	12	42	77.8	0.487
female	46	13	33	71.7	
Tumor differentiation					
Well, Moderate	71	23	48	67.6	0.008
Poor	29	2	27	93.1	
Tumor invasion					
Superficial layer	34	18	16	47.1	0
Deep layer	66	7	59	89.4	
Lymph node metastases					
Yes	52	0	52	100	0
No	48	25	23	47.9	
TNM					
I+II	39	14	25	64.1	0.044
III+IV	61	11	50	81.9	

*Frequency

Stathmin mRNA and protein expression in esophageal carcinoma cell lines

To further study the role of stathmin in EC, we screened for stathmin expression in human EC cell lines, EC9706, Eca109 and EC-1 by immunocytochemistry, western blot and in situ hybridization. Through immunocytochemistry analysis with mAb stathmin, we found that stathmin which localized in intracellular sites (blue color) was expressed at high levels in all these three cell lines (Figure 3A). While no specific signals could be detected in cells hybridized with scramble probe. The three cell lines expressed stathmin at varying intensities: EC9706 contained 84.6%±5.2% positive cells, ECa109 contained 86.6%±6.2% positive cells, EC-1 contained 87.1%±6.9%

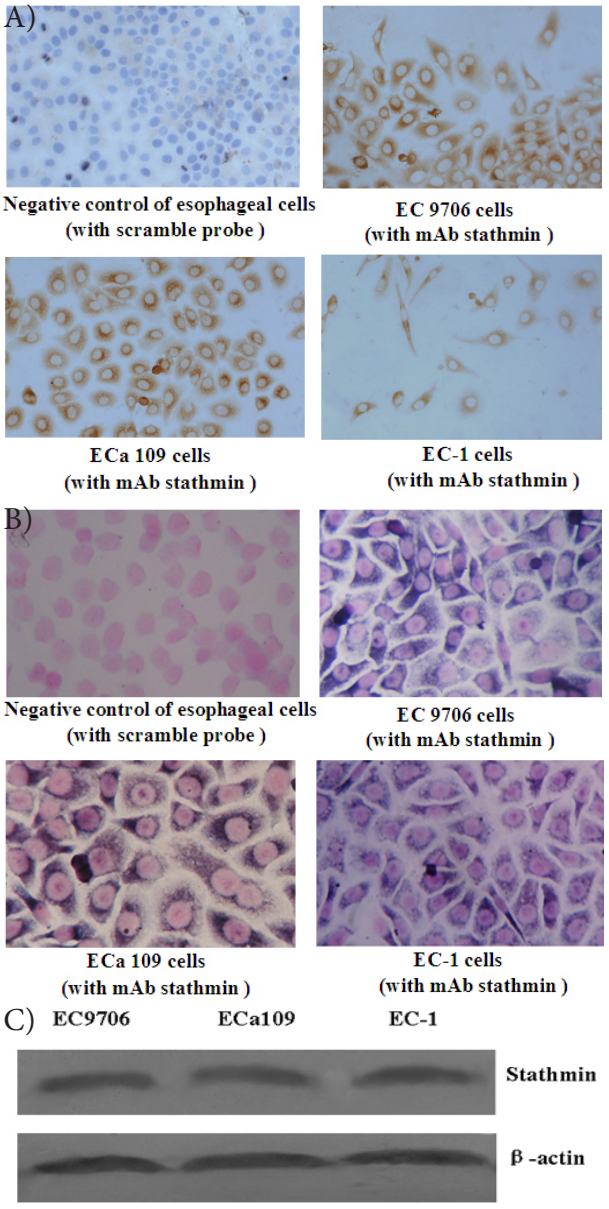

Figure 3. Expression of Stathmin in Esophageal Cancer Cell Lines. A) The expression of Stathmin protein in esophageal cancer cell lines: EC9706, ECa109 and EC-1 was detected by using immunocytochemistry method. **B)** The expression of Stathmin mRNA in esophageal cancer cell lines: EC9706, ECa109 and EC-1 was detected by using in situ hybridization method. **C)** The expression of Stathmin protein in esophageal cancer cell lines: EC9706, ECa109 and EC-1 was detected by using western blot method

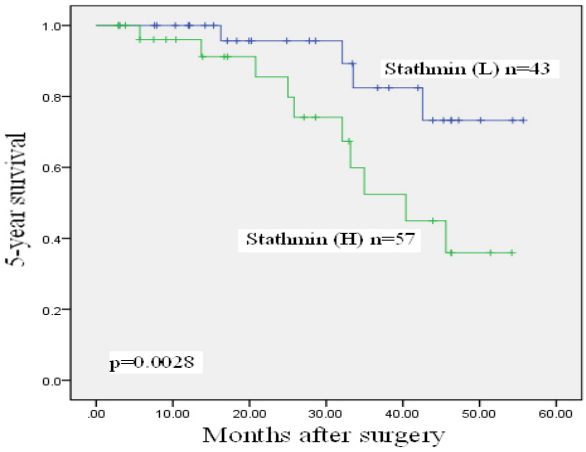

Figure 4. Kaplan-Meier Analyses of 5-year Survival Rates in 100 ESCC Patients in Relation to Stathmin Protein Overexpression. Patients with ESCC with high Stathmin expression had lower 5-year (p<0.001) survival rates than those with low Stathmin expression as determined using the Kaplan-Meier method.(H: high Stathmin expression, L: low Stathmin expression)

positive cells, but these differences were not statistically significant (p=0.924). Then, in situ hybridization assays were performed on these three kinds of EC cell lines in order to validate the expression of stathmin. We found that in EC cell lines, the signals of stathmin probe (blue color) were dispersed uniformly throughout the cytoplasm of all three kinds of tumor cells (Figure 3B). No specific signals could be detected in cells hybridized with scramble probe. The three cell lines expressed stathmin at just about the same levels: EC9706 87%.5±7.2%, ECa109 88.2%±7.6%, EC-1 88.1%±6.5% (p=0.833). Furthermore, these results were further confirmed by Western blot (Figure 3C).

Correlation between Stathmin expression and patient survival in human ESCC

At the time of data analysis (April, 2014), patients with EC with high Stathmin expression had lower disease-free and 5-year survival rates than those with low Stathmin expression as determined using the Kaplan-Meier method. (Figure 4)

Univariate Cox regression analysis also identified that clinical variables including age, sex, tumor differentiation, invasion depth, TNM stage and lymph node metastasis and Stathmin expression were significantly associated with overall survival (p>0.05). Furthermore, to evaluate the potential of Stathmin expression as an independent predictor for overall survival of EC, multivariate Cox regression analyses were performed. While the others failed to demonstrate independence, tumor differentiation (HR:1.053, 95%CI: 0.925-1.989,P=0.0025), invasion depth (HR: 1.213, 95%CI: 0.684-1.593,p<0.001) and lymph node metastasis (HR: 1.276,95%CI: 0.824-2.014, p<0.001) and Stathmin expression (HR: 1.867, 95%CI: .076-3.561, p<0.001) may play a role in predicting the overall survival in EC (p<0.05).

Discussion

Esophageal carcinoma (EC) is lethal malignancy with very high mortality rate (Wang et al., 2013; Wu et al., 2013). Despite decades of research, the prognosis for EC remains dismal and absence of sensitive and specific marker (s) is one of the major factors for poor prognosis of EC patients (Yoon et al., 2011; Yuen et al., 2011). Hence, there has been a growing emphasis on the identification of molecular markers that can identify EC at an early and potentially resectable stage.

Stathmin is a microtubule-regulating protein that has an important role in the assembly and disassembly of the mitotic spindle (Wang et al., 2007; Holmfeldt et al., 2001; Iancu-Rubin et al., 2011). Stathmin is frequently overexpressed in many human cancers including lung (Han et al., 2013), bladder (Wosnitzer et al., 2011), endometrial (Werner et al., 2014), and oral cancer (Kouzu et al., 2006) and Yuan et al reported that the high level of stathmin expression is required for the maintenance of high proliferation rate of tumor cells (Yuan et al., 2012), however information of stathmin expression in esophageal carcinoma is limited thus far. In order to clarify the relative status of stathmin in EC, we detected the mRNA and protein expression of stathmin in human esophageal carcinoma tissues using immunocytochemistry, and in situ hybridization. In this study, it has reported that stathmin expression was increased in human esophageal carcinoma compared with normal esophageal esophageal mucosa tissues and stathmin protein level increased with tumor differentiation degree. Furthermore, stathmin levels were shown to significantly differ among patients with lymph node metastasis. This may be because stathmin is identified to interfere with microtubule dynamics, by adjusting the balance of microtubule dynamics, stathmin could take part in the proliferation, differentiation and metastasis of cancers (Miceli et al., 2013; Yuan et al., 2012). Our findings also showed that high stathmin expression were significantly associated with tumor invasion and TNM clinical classification. These findings suggested that stathmin may be an important factor for the formation and progression of esophageal carcinoma. The same is observed for stathmin mRNA expression. In the past reports, stathmin was shown as an oncogene in many kinds of tumors, which promoted proliferation, invasion and metastasis in a variety of tumors (D'Andrea et al., 2012; Ying et al., 2013). All the above results suggest stathmin acts as a pivotal factor contributing to progression of EC and Stathmin could be considered as a potentially valuable prognostic indicator in patients with esophageal carcinoma.

Since there is obvious evidence about the influence of stathmin on the progression and development of EC, we studied its expression pattern in three different EC cell lines. In the present study, we found a cytoplasmatic staining for stathmin and we also positively detected stathmin expression in three human EC cell lines EC-9706, ECa-109 and EC-1 by immunocytochemistry and western blot analysis. We further investigated the mRNA level of stathmin expression using in situ hybridization and data were matched to protein expression levels studied by immunocytochemistry and western blot analysis.

In this study, the prognosis of EC patients with a high expression of Stathmin was poor, and Cox regression

analysis indicated that high expression level of Stathmin was a significant prognostic factor for a poor survival rate of EC patients. These findings raised the possibility that Stathmin not only facilitated tumor differentiation, invasion depth, lymph node metastasis, but also aggressive cancer behavior, resulting in poor prognosis for EC patients.

Taken together, our data indicate that stathmin may contribute in EC progression and development. Stathmin may be used as a biomarker in the prognosis of EC and development of methods aiming at selective blockade of stathmin thus may constitute a new approach for cancer therapy and prevention.

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