

ORIGINAL ARTICLE

Expression of Stathmin and vascular endothelial growth factor C in esophageal cancer and their combined diagnostic value

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Summary

Purpose: To investigate the expression of Stathmin and vascular endothelial growth factor C (VEGF-C) in the serum of patients with esophageal cancer (EC) and the diagnostic value of the combined two factors.

Methods: 88 EC patients (the EC Group) treated in Shaanxi Provincial People's Hospital and 50 healthy people (the Control Group) were selected as subjects of this study. After detecting the levels of Stathmin mRNA and VEGF-C mRNA using real-time quantitative PCR (qRT-PCR) and the expression levels of Stathmin protein and VEGF-C protein using enzyme-linked immunosorbent assay (ELISA), analysis of the relationship between the expression levels of Stathmin mRNA and VEGF-C mRNA in the serum and the clinicopathological features of EC were analyzed.

Results: The levels of Stathmin mRNA and VEGF-C mRNA, Stathmin protein and VEGF-C protein in the serum of the Control Group were lower than those in the EC group ($p < 0.001$). The mRNA and protein expressions of Stathmin

and VEGF-C in the serum of EC group were related to lymph node metastasis, clinical stage, grade of differentiation and degree of infiltration ($p < 0.05$). Compared with the combined detection of mRNA, the sensitivity of separate detection of Stathmin mRNA and VEGF-C mRNA was lower, but the specificity of the separate detection of Stathmin mRNA was higher ($p < 0.05$). Compared with the sensitivity of the combined detection of protein, the sensitivity of the separate test of Stathmin protein and VEGF-C protein was lower ($p < 0.05$).

Conclusion: Stathmin and VEGF-C could be used as markers for early diagnosis of EC, and the combined detection of the two factors could improve the sensitivity of diagnosis since the expression levels of mRNAs and proteins of Stathmin and VEGFC in the serum of EC patients were proved to be higher than those of healthy volunteers.

Key words: combined detection, esophageal cancer, growth factor C, Stathmin, vascular endothelial growth factor

Introduction

Esophageal cancer (EC) is a malignant tumor of the digestive tract with high invasiveness. Its mortality rate ranks sixth in the world, and over 450,000 people pass away because of EC every year [1,2] yet the general public is not very aware of it. Despite the rapid development of medical technology, the incidence of EC may continue to rise [3]. As far as clinical practice is concerned, the most important treatment for EC is still surgical resection with unfortunately moderate-poor results achieving a median survival time of less

than 18 months, and a 5-year survival rate of less than 20% [4]. However, the latest studies have shown that endoscopic submucosal dissection for early EC patients can achieve a higher R0 resection rate, and very little recurrence rate after surgery [5]. Therefore, considering the key importance of early diagnosis and timely symptomatic treatment to improve the survival rate of EC, to find a simple, efficient and non-invasive method to diagnose EC is of great significance for the screening examination of EC.

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New serum biomarkers are urgently needed for the early diagnosis of EC since the serum biomarkers used at present to diagnose EC, such as carcinoembryonic antigen, squamous cell carcinoma antigen and p53, have unsatisfactory sensitivity and specificity in the early diagnosis of EC [6]. As a cytosolic phosphoprotein that inhibits tubulin polymerization and promotes microtubule depolymerization, Stathmin is involved in the carcinogenesis and invasion of various epithelial malignant tumors and its high expression level is proved to be an independent factor for poor prognosis of EC, correlated with the clinical stage, nodal metastasis, and grade of infiltration of EC [7,8]. At the same time, higher levels of Stathmin are also independent prognostic factors after Ivor-Lewis esophagectomy. Estimating Stathmin expression levels can improve the prognostic stratification of patients with stage IIA EC as Stathmin with high expression is an independent prognostic factor after Ivor-Lewis esophagectomy [9]. In addition, vascular endothelial growth factor (VEGF) is a major promoter of cell proliferation and migration and angiogenesis, with high expression levels in body fluids and various malignancies [10]. Being an important member of the VEGF family, VEGF-C has been proved not to be favorable for the prognosis of EC patients when the expression level is excessively high [11]. Given the fact that the diagnostic value of Stathmin and VEGF-C to EC was seldom studied despite their promotion in the tumor progression and their relationship with poor prognosis in EC patients, this study detected the expression of Stathmin mRNA, VEGF-C mRNA, Stathmin protein and VEGF-C protein in the serum of EC patients and explored the diagnostic value of Stathmin, VEGF-C and their combination.

Methods

Experimental subjects

This study was a retrospective analysis of 88 EC patients from the EC Group (60 men and 28 women, aged from 23 to 71 with an average age of 43.68±7.84 years) from January 2015 to June 2017, treated in Shaanxi Pro-

vincial People’s Hospital and 50 healthy people from the Control Group during the same period (34 males and 16 females, aged from 21 to 70 with an average age of 42.74±7.31 years).

Inclusion criteria: 1) No radiotherapy, chemotherapy and surgery were performed 6 months before the study entry on the patients of EC Group diagnosed by histopathological examination; 2) no subject was younger than 18 years and all were in good health; 3) all subjects signed a complete informed consent form.

Exclusion criteria: 1) people with heart, brain, liver and kidney dysfunction or severe coagulopathy; 2) people with secondary EC; 3) people with severe mental illness, confusion or communication disorders.

Experimental reagents and instruments

TRIzol (Chongqing Pulike Biotechnology Co., Ltd., 15596026); Reverse Transcription Kit (Beijing Protein Innovation Co., Ltd, BPI01030); ABI PCR Instrument (Shanghai Oulu Biotechnology Co., Ltd., 9700); TransScript Green TwoStep qRT-PCR SuperMix (TransGen Biotech Co., Ltd, AQ201); ddH₂O (Beijing Dingguo Changsheng Biotechnology Co., Ltd., PER 018-1); Stathmin ELISA Kit (Nanjing Saihongrui Biotechnology Co., Ltd., SEC892Mi02); VEGF-C ELISA Kit (Shanghai Fanke Biotechnology Co., Ltd., FK-KJ0113); FLUOstar Omega Automatic Multi-mode Microplate Reader (Bio-Gene Technology Ltd., FLUOstar Omega); Micro-ultraviolet visible Spectrophotometer (Thmorgan Biotechnology Co., Ltd, MD2000); High speed and large capacity refrigerating centrifuge (Eppendorf China Ltd., China).

Experimental primers

The experimental primers were designed by Primer Premier 5.0 (Premier, Palo Alto CA, USA) primer design software, and generated by Shanghai Boya Co., Ltd.. The sequences of experimental reference genes and Stathmin and VEGF-C primer sequences are shown in Table 1.

Experimental methods

Collection of serum samples

3 ml of blood sample were taken from each subject’s elbow vein using a vacuum blood collection needle after 8 h of fasting which were kept at room temperature for 2 h, then were centrifuged at 10,000 rpm for 10 min at 4°C. The serum was separated and dispensed. The samples required for the experiment were taken for subsequent studies, and the excess serum samples were stored at -80 °C for future use.

Table 1. Sequences of primers

| | | Sequence (5’→3’) | Product length (bp) |
|----------|----------------|--------------------------------------|---------------------|
| Stathmin | Forward Primer | 5’-CCCCTTCCCTCCAAAGAA-3’ | 267 |
| | Reverse Primer | 5’-TCGCAAACGTTCCAGTTTGG-3’ | |
| VEGF-C | Forward Primer | 5’-ACCAAACAAGGAGCTGGATG-3’ | 435 |
| | Reverse Primer | 5’-TGGTGGTGGAACTTCTTTCC-3’ | |
| β-actin | Forward Primer | 5’-TGACGGGGTCACCCACACTGTGCCCATCTA-3’ | 610 |
| | Reverse Primer | 5’-CTAGAAGCATTTCGGGTGGACGATGGAGGG-3’ | |

Real-time fluorescence quantitative RT-PCR (qRT-PCR) was used to determine the expression levels of Stathmin mRNA and VEGF-C mRNA in the serum of patients from two groups

Total RNA was extracted using Trizol reagent, and then the protein purity and concentration of the extracted total RNA were measured by spectrophotometer (The absorbance ratio at the wavelength of 260 nm and 280 nm is 1.8-2.0, which is considered to be qualified in purity), the integrity of the extracted RNA was tested by agarose gel electrophoresis. Then, reverse transcription of the extracted total RNA into cDNA using a reverse transcription kit was done in strict accordance with the instructions. After amplification of the target gene using the ABI PCR amplification instrument, the PCR reaction system consisted of 0.4 μ L of the forward primers and 0.4 μ L of reverse primers, 1 μ L of cDNA, 0.4 μ L of Passive Reference Dye (50x) (optional), 10 μ L of 2 \times TransScript[®] Tip Green qRT-PCR SuperMix. The system was added to 20 μ L with dd H₂O. The reaction condition was 30 s for denaturation at 94°C; 5 s for annealing at 94°C; 30 s for extension at 60°C; 40 circulations with β -actin as an internal reference. Three independent experiments were

performed, and the Ct value was recorded, then the data analysis was performed using the formula $RQ=2^{-\Delta\Delta Ct}$.

Detection of the expression level of Stathmin protein and VEGF-C protein in the serum by enzyme-linked immunosorbent assay (ELISA)

The double antibody sandwich method was used to determine the concentration of Stathmin protein and VEGF-C in the serum sample to be tested. Standard holes were set on coated wells and loaded with 50 μ L of sample (the concentration was 0.15-1.8 μ g/L) and the blank holes and sample holes were also set separately. Forty μ L of the dilution were added to the sample hole, then 10 μ L of the sample were added to dilute the sample 5 times, and mixed gently. Each reaction hole was sealed and incubated for 30 min at 37°C. After diluting the concentrated washing solution with distilled water for 20 times, the sealing film was peeled off, and the liquid in the reaction hole was discarded. The diluted washing solution was filled in each hole and then was dried after 30 s (herein after referred to as washing). The washing was repeated 5 times, and each reaction hole was sealed

Table 2. Comparison of general clinical data

| Clinical factors | EC Group (n=88) | Control Group (n=50) | t/x ² | p |
|---|------------------|----------------------|------------------|-------|
| Age (years), mean \pm SD | 43.68 \pm 7.84 | 42.74 \pm 7.31 | 0.694 | 0.489 |
| Body mass index (kg/m ²), mean \pm SD | 23.38 \pm 1.35 | 23.41 \pm 1.24 | 0.129 | 0.897 |
| Gender, n (%) | | | 0.000 | 0.982 |
| Male | 60 (68.18) | 34 (68.00) | | |
| Female | 28 (31.82) | 16 (32.00) | | |
| Smoking history, n (%) | | | 0.016 | 0.900 |
| Yes | 52 (59.09) | 29 (58.00) | | |
| No | 36 (40.91) | 21 (42.00) | | |
| Poor diet history, n (%) | | | 2.063 | 0.151 |
| Yes | 74 (84.09) | 37 (74.00) | | |
| No | 14 (15.91) | 13 (26.00) | | |
| Serum creatinine, n (%) | | | 0.029 | 0.865 |
| <133 μ mol/L | 68 (77.27) | 38 (76.00) | | |
| \geq 133 μ mol/L | 20 (22.73) | 12 (24.00) | | |
| Blood urea nitrogen, n (%) | | | 0.001 | 0.979 |
| <7.14mmol/L | 72 (81.82) | 41 (82.00) | | |
| \geq 7.14mmol/L | 16 (18.18) | 9 (18.00) | | |
| Lymphatic metastasis, n (%) | | | | |
| No | 23 (26.14) | - | | |
| Yes | 65 (73.86) | - | | |
| Clinical stage, n (%) | | | | |
| I/II | 31 (35.23) | - | | |
| III/IV | 57 (64.77) | - | | |
| Grade of differentiation, n (%) | | | | |
| Low differentiation | 48 (54.55) | - | | |
| High/medium differentiation | 40 (45.45) | - | | |
| Infiltration degree, n (%) | | | | |
| Serosa not infiltrated | 16 (18.18) | - | | |
| Serosa infiltrated | 72 (81.82) | - | | |

with a sealing film again, and incubated at 37°C for 30 min. After washing 5 times, 50 µL of the developer A and 50 µL of the developer B were sequentially added in each hole and gently shaken, then they were kept for reaction at 37°C for 15 min in the dark. Finally, 50 µL of the stop solution were added to terminate the reaction, and the yellow color appeared in the reaction hole. The blank hole was used as the zero reference value and the optical density (OD value) in each reaction hole was measured using a wavelength of 450 nm of a spectrophotometer. Then the Stathmin protein and VEGF-C protein concentration in the sample were calculated by a standard curve.

Statistics

The experimental data were statistically analyzed using SPSS19.0 statistical software (SPSS Inc., Chicago, IL, USA). Comparison of the measurement data expressed as mean±standard deviation (SD) between the two groups was performed using the t-test to detect the two independent samples; comparison of the count data expressed by percents (%) between the two groups was performed using the chi-square test. Finally, the

receiver operating characteristic curve (ROC curve) was performed. Statistical significance was set at p<0.05.

Results

Comparison of general clinical data

As shown in Table 2, no statistical difference was seen between the EC Group and the Control Group in terms of general clinical data such as gender, age, body mass index (BMI), smoking history, and poor diet history (p>0.05).

Comparison of relative expression levels of Stathmin mRNA and VEGF-C mRNA in the serum

The relative expression levels of Stathmin mRNA and VEGF-C mRNA in the serum of the EC Group were 1.53±0.44 and 1.42±0.21, while the relative expression levels of Stathmin mRNA and VEGF-C mRNA in the serum of the Control Group were 1.02±0.13 and 1.05±0.22, lower than those in the EC Group (p<0.001; Table 3).

Table 3. Comparison of relative expression levels of Stathmin mRNA and VEGFC mRNA in the serum

| | Stathmin | VEGF-C |
|----------------------|-----------|-----------|
| EC Group (n=88) | 1.53±0.44 | 1.42±0.21 |
| Control Group (n=50) | 1.02±0.13 | 1.05±0.22 |
| t | 7.990 | 9.778 |
| p | <0.001 | <0.001 |

Table 4. Comparison of expression levels of Stathmin protein and VEGF-C protein in serum (mean±SD)

| | Stathmin protein (ng/mL) | VEGF-C protein (pg/mL) |
|----------------------|--------------------------|------------------------|
| EC Group (n=88) | 5.27±2.62 | 241.27±32.74 |
| Control Group (n=50) | 2.43±1.21 | 176.43±46.75 |
| t | 8.671 | 8.675 |
| p | <0.001 | <0.001 |

Table 5. Relationship between Stathmin mRNA, VEGF-C mRNA expression and clinical pathological features of EC (mean±SD)

| General clinical data | n | Stathmin mRNA | t | p | VEGF-C mRNA | t | p |
|-----------------------------|----|---------------|-------|--------|-------------|-------|--------|
| Gender | | | 0.857 | 0.394 | | 1.874 | 0.064 |
| Male | 60 | 1.56±0.42 | | | 1.37±0.18 | | |
| Female | 28 | 1.48±0.38 | | | 1.45±0.20 | | |
| Age (year) | | | 1.538 | 0.130 | | 1.554 | 0.124 |
| ≤50 | 41 | 1.61±0.31 | | | 1.38±0.23 | | |
| >50 | 47 | 1.47±0.51 | | | 1.46±0.25 | | |
| Lymph node metastasis | | | 3.039 | 0.003 | | 3.540 | 0.001 |
| No | 23 | 1.32±0.18 | | | 1.28±0.16 | | |
| Yes | 65 | 1.72±0.62 | | | 1.52±0.31 | | |
| Clinical stage | | | 3.184 | 0.002 | | 3.130 | 0.002 |
| I/II | 31 | 1.23±0.35 | | | 1.32±0.34 | | |
| III/IV | 57 | 1.62±0.63 | | | 1.65±0.53 | | |
| Grade of differentiation | | | 4.430 | <0.001 | | 4.151 | <0.001 |
| Low differentiation | 48 | 1.72±0.69 | | | 1.23±0.08 | | |
| High/medium differentiation | 40 | 1.27±0.13 | | | 1.67±0.73 | | |
| Infiltration degree | | | 3.097 | 0.003 | | 3.397 | 0.001 |
| Serosa not infiltrated | 16 | 1.25±0.13 | | | 1.17±0.12 | | |
| Serosa infiltrated | 72 | 1.75±0.64 | | | 1.65±0.56 | | |

Comparison of the expression levels of Stathmin protein and VEGF-C protein in the serum

The expression levels of Stathmin protein and VEGF-C protein in the serum of the EC Group were 5.27 ± 2.62 ng/mL and 241.27 ± 32.74 pg/mL, while the expression levels of Stathmin protein and VEGF-C protein in the serum of the Control Group were 2.43 ± 1.21 ng/mL and 176.43 ± 46.75 pg/mL, lower than those of the EC Group ($p < 0.001$; Table 4).

The relationship between mRNA and protein expression of Stathmin and VEGF-C and clinicopathological features of EC

As shown in Table 5, the expression of Stathmin mRNA and VEGF-C mRNA in the serum of

the EC Group was not related to the gender and age of patients ($p > 0.05$), but it was related with lymph node metastasis, clinical stage, differentiation grade and the degree of infiltration ($p < 0.05$). As shown in Table 6, the expression of Stathmin protein and VEGF-C protein in the EC Group was also not related to the gender and age of patients ($p > 0.05$), but it was related with lymphatic metastasis, clinical stage, grade of differentiation and degree of infiltration ($p < 0.05$).

Diagnostic value of Stathmin mRNA, VEGF-C mRNA, Stathmin protein and VEGF-C protein in the serum

According to the expression levels of Stathmin mRNA, VEGF-C mRNA, Stathmin protein, VEGF-C

Table 6. Relationship between Stathmin protein, VEGF-C protein expression and clinical pathological features of EC (mean \pm SD)

| General clinical data | n | Stathmin protein (ng/mL) | t | p | VEGF-C protein (pg/mL) | t | p |
|-----------------------------|----|--------------------------|-------|-----------|------------------------|-------|-------|
| Gender | | | 0.329 | 0.743 | | 0.439 | 0.662 |
| Male | 60 | 243.45 \pm 31.43 | | | 173.73 \pm 45.58 | | |
| Female | 28 | 241.12 \pm 29.93 | | | 178.38 \pm 47.95 | | |
| Age (years) | | | 0.730 | 0.467 | | 0.376 | 0.708 |
| ≤ 50 | 41 | 244.74 \pm 34.63 | | | 174.54 \pm 43.64 | | |
| > 50 | 47 | 239.53 \pm 32.25 | | | 178.26 \pm 48.57 | | |
| Lymph node metastasis | | | 2.078 | 0.041 | | 2.084 | 0.040 |
| No | 23 | 235.62 \pm 31.47 | | | 162.53 \pm 43.46 | | |
| Yes | 65 | 253.47 \pm 36.65 | | | 186.36 \pm 48.32 | | |
| Clinical stage | | | 2.446 | 0.017 | | 2.025 | 0.046 |
| I/II | 31 | 236.72 \pm 27.36 | | | 163.47 \pm 43.98 | | |
| III/IV | 57 | 255.54 \pm 37.74 | | | 184.75 \pm 48.69 | | |
| Grade of differentiation | | | 4.122 | < 0.001 | | 2.506 | 0.014 |
| Low differentiation | 48 | 263.64 \pm 35.38 | | | 189.74 \pm 58.64 | | |
| High/medium differentiation | 40 | 235.58 \pm 26.85 | | | 163.44 \pm 39.25 | | |
| Infiltration degree | | | 2.212 | 0.036 | | 2.210 | 0.036 |
| Serosa not infiltrated | 16 | 229.53 \pm 31.56 | | | 160.23 \pm 41.12 | | |
| Serosa infiltrated | 72 | 249.54 \pm 37.53 | | | 186.23 \pm 48.53 | | |

Table 7. Comparison of the diagnostic value of Stathmin mRNA, VEGF-C mRNA, Stathmin protein, VEGF-C protein and combined detection of mRNA and protein in the serum

| | Diagnostic critical value | AUC (95%CI) | Youden index | p | Sensitivity % | Specificity % |
|-------------------------------|---------------------------|---------------------|--------------|-----------|---------------|---------------|
| Stathmin mRNA | 1.158 | 0.831 (0.659-0.846) | 0.681 | < 0.001 | 76.14* | 92.00# |
| VEGF-C mRNA | 1.164 | 0.861 (0.843-0.967) | 0.561 | < 0.001 | 82.95* | 82.00 |
| Combined detection of mRNA | - | 0.921 (0.748-0.910) | 0.761 | < 0.001 | 95.45 | 74.00 |
| Stathmin protein (ng/mL) | 3.750 | 0.847 (0.646-0.836) | 0.650 | < 0.001 | 75.00‡ | 90.00 |
| VEGF-C protein (pg/mL) | 220.6 | 0.898 (0.659-0.846) | 0.681 | < 0.001 | 76.14‡ | 92.00 |
| Combined detection of protein | - | 0.904 (0.671-0.855) | 0.693 | < 0.001 | 94.32 | 82.00 |

*The sensitivity of Stathmin mRNA was lower than that of the combined detection of two mRNAs ($\chi^2=11.550$, $p=0.001$), so was the sensitivity of VEGF-C mRNA ($\chi^2=5.641$, $p=0.018$); #the specificity of Stathmin mRNA was higher compared with that of the combined detection of two mRNAs ($\chi^2=5.741$, $p=0.017$); ‡the sensitivity of Stathmin protein was lower than that of the combined detection of two proteins ($\chi^2=10.490$, $p=0.001$), so was the sensitivity of VEGF-C protein ($\chi^2=11.550$, $p=0.001$).

protein, combined detection of Stathmin and VEGF-C mRNA, and combined detection of Stathmin and VEGF-C protein in the serum of patients from the EC Group and people from the Control Group, the ROC curve was drawn. The area under the ROC curve of Stathmin mRNA was 0.831 (0.659-0.846), with a diagnostic value of 1.158 ($p < 0.001$); the area under the ROC curve of VEGF-C mRNA was 0.861 (0.843-0.967), with a diagnostic critical value of 1.164 ($p < 0.001$); the area under the ROC curve of mRNA combined detection was 0.921 (0.748-0.910) ($p < 0.001$); the area under the ROC curve of Stathmin protein was 0.847 (0.646-0.836), with a diagnostic critical value of 3.750 ng/mL ($p < 0.001$); the area under the ROC curve of VEGF-C protein was 0.898 (0.659-0.846), with a diagnostic critical value 220.6 pg/mL ($p < 0.001$); the area under the ROC curve of protein combined detection was 0.904 (0.671- 0.855) ($p < 0.001$). Compared with the combined detection of mRNA, the sensitivity of separate detection of Stathmin mRNA and VEGF-C mRNA was lower, but the specificity of the separate detection of Stathmin mRNA was higher ($p < 0.05$). Compared with the sensitivity of the combined detection of protein, the sensitivity of separate test of Stathmin protein and VEGF-C protein was lower ($p < 0.05$; Table 7 and and Figures 1,2).

Discussion

EC is a malignant tumor with a high invasion potential. Its yearly incidence is ever increasing in developed countries [12], severely threatening human life. At present, the common clinical diagnosis and treatment method is by gastroscopy, which is invasive and has low patient compliance. Most patients have entered the advanced stage of EC at the time of diagnosis because early symptoms of most EC patients are not obvious, contributing to poor prognosis [13,14]. Compared with gastroscopy, blood tests have a lower threshold, less invasiveness, higher patient acceptance, and greater economic benefits [15]. Therefore, this study aimed to seek new EC serum biomarkers to provide a reference value for the diagnosis of early EC, thereby further improving clinical efficacy.

Some authors already proved that Stathmin protein and VEGF-C protein have higher expression levels in the serum or cancer tissues of EC patients [16,17], but the experimental conditions were different. In these previous studies, the paraffin-embedded specimens were subjected to organic solvent treatment in the experimental process, which easily caused certain errors in the staining results, and no discussions about the Stathmin and mRNA were

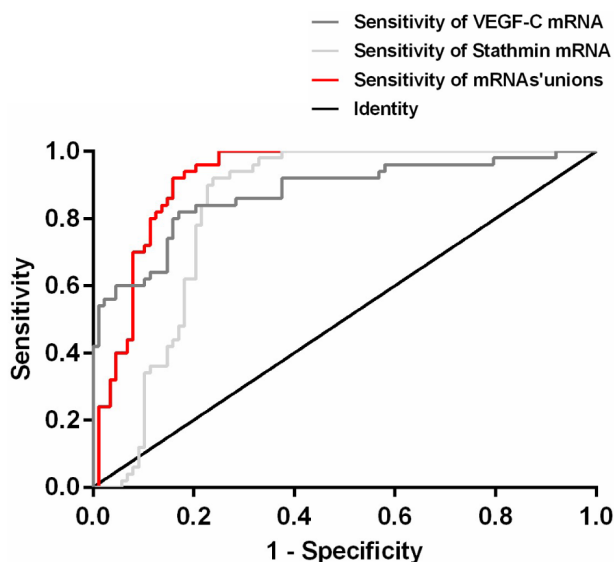


Figure 1. The ROC curve of Stathmin mRNA, VEGF-C mRNA and combined detection of the two mRNAs. According to the ROC curve of the single detection of the expression levels of Stathmin mRNA, VEGF-C mRNA and the combined detection of the expression level of the two mRNAs in the serum of the EC Group and the Control Group, the area of Stathmin mRNA under the ROC curve was 0.831 (0.659-0.846), with a diagnostic critical value of 1.158 ($p < 0.001$); the area of VEGF-C mRNA under the ROC curve was 0.861 (0.843-0.967), with a diagnostic critical value of 1.164 ($p < 0.001$); the area of the combined two mRNAs under the ROC curve was 0.921 (0.748-0.910) ($p < 0.001$).

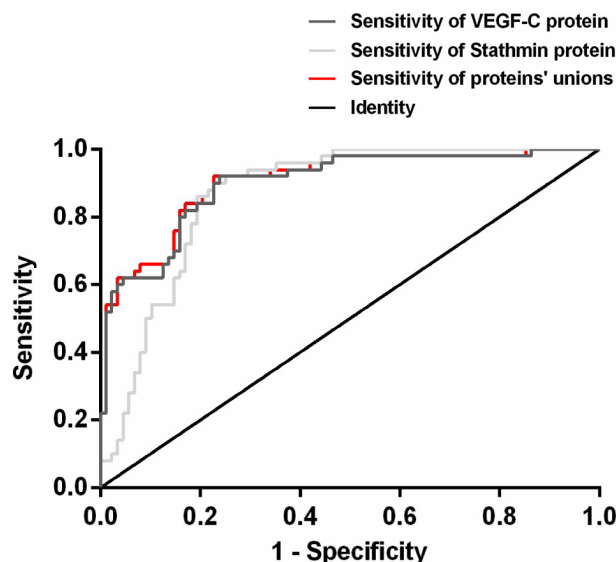


Figure 2. The ROC curve of Stathmin protein, VEGF-C protein and combined detection of the two proteins. According to the ROC curve of the single detection of the expression levels of Stathmin protein, VEGF-C protein and the combined detection of the expression level of the two proteins in the serum of the EC Group and the Control Group, the area of Stathmin protein under the ROC curve was 0.847 (0.646-0.836), with a diagnostic critical value of 3.750 ng/mL ($p < 0.001$); the area of VEGF-C protein under the ROC curve was 0.898 (0.659-0.846), with a diagnostic critical value of 220.6 pg/mL ($p < 0.001$); the area of the combined two proteins under the ROC curve was 0.904 (0.671-0.855) ($p < 0.001$).

made together. Therefore, in this experiment, we simultaneously detected the expression levels of Stathmin mRNA, VEGF-C mRNA, Stathmin protein and VEGF-C protein in the serum, which was more comprehensive than the design of previous experiments.

The results of this study showed that no difference existed between the EC Group and the Control Group in terms of general clinical data such as gender, age and diet; the expression levels of Stathmin mRNA, VEGF-C mRNA, Stathmin protein and VEGF-C protein in the serum of the Control Group were lower than those in the EC Group; the expressions of Stathmin mRNA, VEGF-C mRNA, Stathmin protein and VEGF-C protein were not related to the gender and age of patients, but were related to the nodal metastasis, clinical stage, grade of differentiation and degree of infiltration; both the areas of combined detection of two proteins and combined detection of two mRNAs under the ROC curve were larger than that of the single detection; the sensitivity of the combined detection of Stathmin mRNA and VEGF-C mRNA (95.45%) was higher than that of the single tests (76.14%, 82.95%), and the sensitivity of the combined detection of Stathmin protein and VEGF-C protein (94.32%) was higher than the single tests (75.00%, 76.14%); the specificity of the combined detection of Stathmin mRNA and VEGF-C mRNA (74.00%) was lower than that of single detection of Stathmin mRNA (92.00%); the specificity of Stathmin protein, VEGF-C protein and combined detection were 90.00%, 92.00%, and 82.00%, respectively. These results suggested that the mRNA and protein of Stathmin and VEGF-C were higher in the serum of EC patients than in the people from the Control Group, and the combined detection could improve the sensitivity of detection. The specificity of the combined detection of Stathmin protein and VEGF-C protein was not statistically different from single detection, showing both combined detection and single detection had high specificity in the diagnosis of EC. Stathmin was found to be overexpressed in a variety of malignant tumors and could promote the development of tumors which explained why the decreased expression level of Stathmin could lower the ability of cell proliferation and metastasis, thus inducing apoptosis of malignant cells [18]. Kang et al [19] and other authors also discovered obvious decrease in the proliferation, invasion and migration of tumor cell and the blocking of the G1 phase after downregulating the expression of Stathmin protein. Some other studies found that Stathmin was also involved in the development of many malignant tumors such as breast cancer [20], nasopharyngeal carcinoma [21], and non-small cell lung cancer [22]. The study made

by Jiang et al [23] and other researchers indicated that as an indicator of the prognosis of esophageal squamous cell carcinoma, Stathmin with low expression could significantly inhibit the proliferation, invasion and metastasis of EC tumors. These aforementioned studies showed that Stathmin was closely related to the occurrence and development of EC tumor cells, and might become a more sensitive biomarker in the early diagnosis of EC. VEGF-C was found to be able to promote the development of tumors by activating the specific receptor VEGFR-3, and to increase the migration and invasion of solid tumors such as breast cancer and skin cancer [24,25]. Zhang et al [26] and other researchers found that the positive rate of VEGF-C in the serum of EC patients is three times as high than that of non-EC patients, capable of being a potential biomarker for EC diagnosis, which is consistent with the findings of this study. Another study [27] found the importance of VEGF-C for the development of lymphatic vessels in EC patients, and its potential to be a biological indicator of the prognosis of esophageal squamous cell carcinoma, which also supports the results of this study from the aspect that VEGF-C was of great significance for the early diagnosis of EC. No research on the use of Stathmin combined with VEGF-C in the diagnosis of EC patients has been carried out so far. The results of this study showed that Stathmin and VEGF-C had good diagnostic value for EC patients, and the value of the combined use of Stathmin and VEGF-C was higher.

The limitations of experimental conditions and the not large sample size have a certain impact on the experimental results and need the supplement of the sample capacity in subsequent studies. In addition, this study did not further explore the specific mechanism of Stathmin and VEGF-C in EC using animal models or further studies by other researchers to support the results of the present study.

In summary, this study found that the expression of Stathmin mRNA, VEGF-C mRNA, Stathmin protein and VEGF-C protein in the serum of EC patients were higher than in healthy volunteers, and that the expression levels of Stathmin mRNA, VEGF-C mRNA, Stathmin protein and VEGF-C protein were related to nodal metastasis, clinical stage, grade of differentiation and degree of infiltration of the patients, suggesting that Stathmin and mRNA could be used as biomarkers for early diagnosis of EC, with higher diagnostic performance when combined together.

Conflict of interests

The authors declare no conflict of interests.

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