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Original Article

Characterization of tissue chromogranin A (CgA) immunostaining and clinicohistopathological changes for the 125 Chinese patients with primary small cell carcinoma of the esophagus

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SUMMARY. **The rarity of primary small cell carcinoma of the esophagus (PSCE) has limited the clinical feature and survival analysis with large sample size. Tissue chromogranin A (CgA) protein expression has been reported to be a useful biomarker for diagnosing PSCE. Interestingly, recent studies have indicated tissue CgA as a significant prognostic marker in multiple human cancers, but without PSCE. The present study, thus, was undertaken to characterize the clinicopathological changes and to evaluate the associations of tissue CgA expression with clinical response on Chinese PSCE patients. All the 125 PSCE patients were enrolled from our 500,000 esophageal and gastric cardia carcinoma databases (1973–2015), constructed by the cooperative team from more than 700 hospitals in China and established by Henan Key Laboratory for Esophageal Cancer Research in Henan, China. Immunostaining for CgA showed that CgA was mainly located in cytoplasm of tumor cells with a positive detection rate of 44.6%. The CgA positive expression rate in PSCE at lower segment of the esophagus (72.2%) was higher than that at middle segment (41.5%) ($P = 0.001$). However, CgA protein expression did not correlated with lymph node metastasis ($P = 0.767$), TNM staging ($P = 0.740$), tumor invasion ($P = 0.253$), gender ($P = 0.262$), and age ($P = 0.250$). Multivariate survival analysis showed that the patients with higher CgA protein expression had a superior long survival than those without CgA expression ($P = 0.037$). The clinicopathological analysis showed that PSCE occurred predominantly in male (M:F = 1.9:1) at the middle segment (68%) of the esophagus. Histologically, 89.6% were pure PSCE and 10.4% were mixed type with either squamous cell carcinoma (8%) or adenocarcinoma (2.4%). It was noteworthy that, with the in-depth invasion from T1 to T2 and T3, the positive lymph node metastasis rate increased dramatically from 38%, 56% to 74%, respectively. The survival rates of 1-, 2-, 3-, and 5-year were 64%, 35%, 18%, and 7%, respectively. The Kaplan–Meier survival analysis showed that the young patients (≤60 years) had longer survival than the elderly ($P = 0.011$). Interestingly, multivariate survival analysis revealed that the patients with mixed PSCE had a significantly better survival than those with pure PSCE ($P = 0.015$). Furthermore, the median survival time for the patients with and without lymph node metastasis was 1.16 and 2.03 years, respectively. But, the difference was not significant ($P = 0.143$). Univariate analysis did not show any survival influence by gender, tumor location, tumor invasion depth, and TNM staging. It was noteworthy that, of the 13 early PSCE patients (T1N0M0), only one patient had more than 5 year survival, the others died with less than one or two year (65%). The present study indicates that the PSCE is of badly worsen prognosis, even in the pathological early stage. Tissue CgA protein expression is a promising maker not only for diagnosis and also for prognosis. Further assessment is needed to establish specific PSCE pathological staging system and to clarify the mechanisms of CgA protein in PSCE progression and prognosis.**

KEY WORDS: **chromogranin A, immunohistochemistry, prognosis, small cell carcinoma.**

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INTRODUCTION

Primary small cell carcinoma of the esophagus (PSCE), a subtype of neuroendocrine neoplasm (G3) by 2010 World Health Organization (WHO) classification,[1] is an extremely rare aggressive malignant disease. Histopathologically, PSCE is characterized by chief component of neuroendocrine differentiation tumor cells. Some PSCE cases may include other carcinomatous components, such as squamous cell carcinoma or adenocarcinoma.[2] Since the first report by McKeown in 1952,[3] most publications concerning PSCE have been case reports. Because of its rarity, overall data regarding PSCE are scarce, and its clinicopathological characteristics are far from being well established.

The diagnosis criteria for PSCE includes histopathological changes and couple of immunostaining biomarkers including tissue chromogranin A (CgA), neuron-specific enolase (NSE), synaptophysin (Syn), neuronal cell adhesion molecules 56 (CD56), and Ki67, which have been routinely used as a diagnostic biomarker for PSCE.[4–6] Furthermore, Ki67 index has been used to characterize the G3 subtype with an index of >20%. It is noteworthy that the main prognostic indicators for PSCE are, until recently, the disease stage.[7] Though neuroendocrine differentiation is considered to influence the clinical behavior of PSCE, the neuroendocrine markers, including CgA, have not yet been well characterized as prognostic indicators in PSCE clinical practice. CgA is an acidic glycoprotein belonging to a family of regulated secretory proteins stored in the dense core granules of the adrenal medulla and of many other neuroendocrine cells and neurons.[8] Interestingly, the accumulated evidences have suggested that CgA is more than a diagnostic marker for small cell carcinoma, but as a significant prognostic marker in multiple human cancers, including small cell carcinoma of the uterine cervix[9] and adenocarcinoma of the prostate,[10] but without PSCE. Recently, it has been reported that CgA serum level as a marker of progression in hepatocellular carcinoma.[11] The patients with higher serum CgA level have a poor survival, compared to those with lower CgA level, suggesting that the CgA is useful in monitoring progression of disease and may assist as a prognostic indicator.[11] Similar results have been observed in gastroenteropancreatic neuroendocrine tumors. However, the alterations of CgA in tissue expression have not been determined for influencing on PSCE survival. The present study, thus, was undertaken to characterize the clinicopathological changes and to evaluate the associations of CgA tissue expression with clinical response on 125 Chinese patients with PSCE, which were diagnosed strictly based on the PSCE criteria by 2010 WHO.[1]

MATERIALS AND METHODS

Patients

All the 125 patients histopathologically confirmed with PSCE were enrolled from our 500,000 esophageal and gastric cardia carcinoma databases (1973–2015), constructed by the cooperative team from more than 700 hospitals in China and established by Henan Key Laboratory for Esophageal Cancer Research of the First Affiliated Hospital of Zhengzhou University in Henan, China.[12] All the patients were performed surgical treatment. Of the patients, there were 82 males (65.6%) with an average age of 61.7 ± 8.9 (range: 45–85 years) and 43 females with an average age of 65.1 ± 7.4 (range: 49–88 years). All the patients had not received any preoperative chemoradiotherapy. The written informed consent was given to each patient. The study protocol was approved by the Medical Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

Diagnosis criteria, tumor staging, follow-up, and surgical specimen preparation

Based on the PSCE diagnosis criteria by 2010 WHO,[1] all the PSCE patients in this study had been performed the immunostaining of CgA, NSE, Syn, CD56, and Ki67. The diagnosis was made based on the histopathological changes and these immunostaining biomarkers. In brief, the special morphological features of solid or clustered growth patterns, the different cell proliferation of ki67 index >20% and the positive immunostaining of biomarkers contribute to the final diagnosis of PSCE. Due to lacking of special pathological staging for PSCE, the PSCE patients were staged according to 2009 American Joint Committee on Cancer (AJCC) TNM staging system for esophageal squamous cell carcinoma.[13] Follow-up was performed either through telephone or home interview to each patient yearly until death. The last follow-up was June 2016, with a successful rate of 100%. All the surgical specimens from each patient were formalin fixed, paraffin-embedded. 5 μm serial tissue sections were collected for H&E staining and immunohistochemistry.

Protein immunostaining

Immunohistochemical analysis was performed on 5 μm tissue sections according to standardized procedure. The primary antibodies of CgA monoclonal antibody (1:200), Syn monoclonal antibody (1:400), CD56 monoclonal antibody (1:200), NSE monoclonal antibody (1:1000), and Ki67 monoclonal antibody (1:500) were obtained from Abcam (USA). After the immunocomplex was detected by avidin-biotin complex kit, the sections were counterstained,

dehydrated, and mounted. PBS and positive lung cancer slides were used for negative and positive controls, respectively. Immunoreactivity results were assessed by two pathologists independently. The final immunostaining results were denoted as positive (≥25% of positive cells) or negative (<25% of the positive tumor cells). The evaluation of Ki67 expression was performed in at least 2000 tumor cells.[1]

Statistical analysis

All statistical analyses were conducted using IBM SPSS ver. 23.0 (IBM Inc., Armonk, NY, USA). Chi-square and binary logistic regression were used to analyze the correlations of CgA expression with clinicopathological features. The Kaplan–Meier survival analysis and log-rank test were used to calculate cumulative survival rates. Cox proportional hazards regression models were used to compute odds ratio (OR) and 95% confidence interval (CI) for multivariate survival analysis in a forward stepwise manner. Receiver operating characteristic (ROC) curve was used to determine the predictive value for the probability of PSCE. Any result with a *p*-value of less than 0.05 was considered as statistically significant.

Table 1 Patient information and clinical characteristics *n* (%)

Variables	Number of patients
Gender	
Male	82 (65.6)
Female	43 (34.4)
Age (years)	
≤60	50 (40.0)
>60	75 (60.0)
Histology	
Pure PSCE	112 (89.6)
Mixed PSCE	13 (10.4)
Tumor gross type	
Ulcerative	63 (50.4)
Plaque	30 (24)
Medullary	25 (20)
Mushroom	7 (5.6)
Tumor invasion depth	
T1	13 (10.4)
T2	50 (40)
T3	62 (49.6)
Lymph node metastasis	
Yes	76 (60.8)
No	49 (39.2)
Tumor location	
Upper	12 (9.6)
Middle	85 (68.0)
Lower	28 (22.4)
TNM grading	
I	13 (10.4)
IIA+IIB	53 (42.4)
III	56 (44.8)
IV	3 (2.4)

PSCE, primary small cell carcinoma of the esophagus.

RESULTS

Clinicopathological characteristics

The clinical feature of our patients was similar to that reported in literature with a male-to-female ratio of 1.9:1 and a mean age of 64 years. Table 1 summarized the patient clinicopathological characteristics. Interestingly, of the 125 cases with radical esophagectomy, 112 patients (89.6%) were pure PSCE, 13 patients (10.4%) had mixed histological type, including 10 patients (8%) with foci of frank squamous cell carcinoma and 3 (2.4%) with foci of frank esophageal adenocarcinoma. Furthermore, 60% of the PSCE patients had lymph node metastasis, which was apparently higher than esophageal squamous cell carcinoma. Apparently, similar with esophageal squamous cell carcinoma, PSCE occurred predominantly in the middle segment of the esophagus (68%), followed by lower segment, and with a majority of ulcerative gross type. Again, based on TNM staging, 90% of the patients had been in middle (stage IIA, IIB, III) and late stage (stage IV) at diagnosed. Early PSCE (stage I, T1N0M0) accounted only 10%, which was a little higher than esophageal squamous cell carcinoma. It was noteworthy that, with the in-depth invasion from T1 to T2 and T3, the positive lymph node metastasis rate increased dramatically from 38% to 56% and 74%, respectively. Moreover, of the 125 patients with radical esophagectomy, there were 24 patients with age of more than 70 years and three patients with age of more than 80 years, only one patient in these elderly patients was in early stage (T1N0M0) at diagnosed.

Immunostaining results

Because of that some paraffin blocks were not large enough to perform immunostaining, or, the section containing very few tumor cells, only 92 cases were finally performed immunostaining for in-depth analysis. A nested or organoid growth pattern and peripheral palisading of tumor cell nests were observed in most cases (Fig. 1A). Immunostaining results showed that none of the patients had completely negative expressions of the five immunostaining biomarkers (CD56, NSE, Syn, CgA, and Ki67) in this study. The immunoreactivity of CgA was mainly located in the cytoplasm of tumor cells (Fig. 1B) and there was 55.4% of the PSCE patients were negative for CgA expression. However, in the patients with negative CgA expression, the other four immunostaining biomarkers were alternatively positive. Even though only half of the patients were positive for CgA, the other four biomarkers were positive alternatively in these patients. The positive expression rates were 76% for two biomarkers, 28% for three biomarkers, and 4% for four biomarkers. In order to further evaluate the diagnostic significance of CgA, CD56, NSE, Syn, and Ki67 in PSCE, we preformed the ROC curve analysis of the predictive value for the sensitivity and

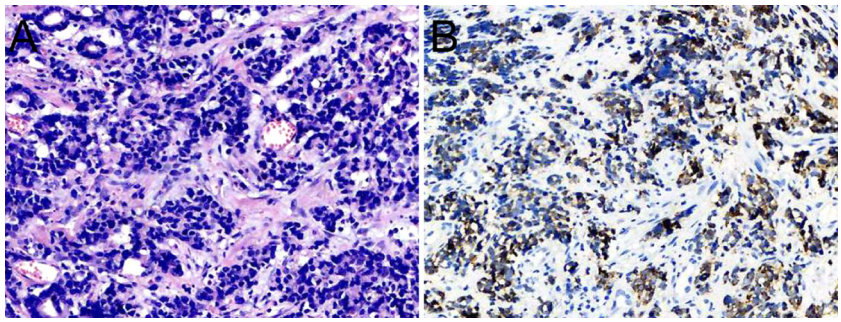

Fig. 1 Hematoxylin and eosin stained section and chromogranin A (CgA) immunostaining in primary small cell carcinoma of the esophagus (PSCE). (A) Small, round-shaped carcinoma cells with a high nucleus-to-cytoplasm ratio in a nested growth pattern. (B) The positive expression for CgA was observed in the PSCE area (Mag. × 200).

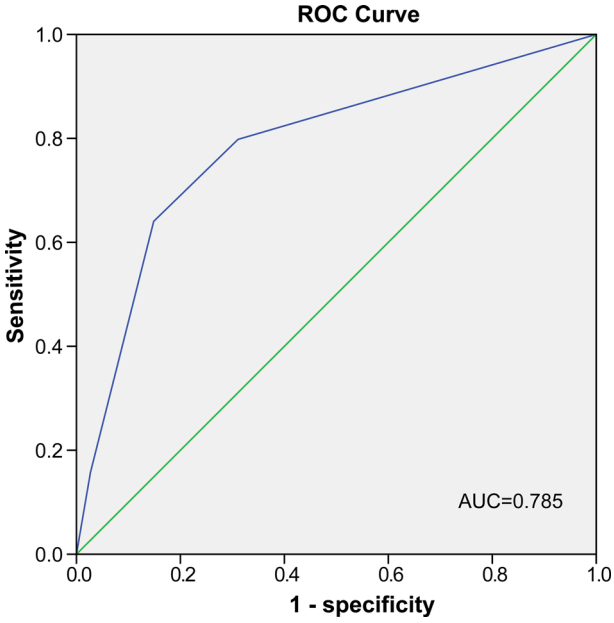

Fig. 2 Receiver operating characteristic (ROC) curve analysis of the predictive value for the probability of PSCE with successive addition of five immunostaining biomarkers (CD56, Syn, NSE, CgA, and Ki67). The area under the ROC curve with five markers was 0.79 (95% CI 0.73–0.85) with the optimal cut off value of 2.5, the sensitivity of 64% and the specificity of 85%, $P < 0.001$.

specificity of these five immunostaining biomarkers in PSCE diagnosis with successive addition of CgA, CD56, NSE, Syn, and Ki67. The area under the ROC curve of cumulative five biomarkers for the probability of PSCE was 0.79 (95% CI 0.73–0.85) with the optimal cut off value of 2.5, the sensitivity and specificity were 64% and 85%, respectively, $P < 0.001$ (Fig. 2). The average Ki67 index was >35%, which was consistent with the G3 NET/NEC subtype classification by 2010 WHO.

Surprisingly, CgA protein expression did not correlate with lymph node metastasis ($P = 0.767$), TNM staging ($P = 0.740$), tumor invasion ($P = 0.253$), gender ($P = 0.262$), and age ($P = 0.250$) (Table 2). However, the significant associations were observed between the CgA expression and tumor location ($P = 0.001$). The CgA positive expression rate in PSCE at lower segment of the esophagus was higher than that at middle segment (72.2% vs. 41.5%).

Survival analysis for various potential prognostic factors

Up to June 2016, of the 125 patients, only 36 patients (30%) remained alive. The survival rates of 1-, 2-, 3- and 5-year were 64%, 35%, 18%, and 7%, respectively. The medium survival time of patients was 14.4 months, ranging from 4 to 196.8 months.

The Kaplan–Meier survival analysis showed that the young patients with age below or equal to 60 years had a longer survival than the elderly patients above 60 years (2.01 vs. 1.18 years, $P = 0.011$, Fig. 3A). Interestingly, the patients with mixed histological type of small cell carcinoma together with squamous cell carcinoma or adenocarcinoma had a better survival than those with pure PSCE (5.36 vs. 3.47 years, $P = 0.015$, Fig. 3B). The median survival time of the patients with and without lymph node metastasis was 1.16 and 2.03 years, respectively, but, no survival difference was observed for these two groups ($P = 0.143$). Univariate analysis did not show any survival influence by gender ($P = 0.409$), tumor location ($P = 0.575$), tumor invasion ($P = 0.713$), and TNM staging ($P = 0.476$). Multivariate survival analysis revealed that the patients with mixed PSCE had a significantly better survival than those with pure PSCE ($P = 0.015$) and that the patients with higher CgA protein expression had a superior long survival than those without CgA expression ($P = 0.037$) (Table 3).

It was noteworthy that, of the 13 early PSCE patients (T1N0M0) identified mostly in younger patients, eight patients had died with a worsen survival: three patients with less than one year survival (37.5%), another three patients with one and half year survival (37.5%), one patient with two year survival (12.5%), and only one patient with more than 5 year survival (12.5%). The early PSCE patient seems to

Table 2 Relationship between tissue CgA expression and clinicopathological characteristics

Variables	Total	CgA *n* (%)		*p*
		Positive	Negative	
Gender				
Male	57	28 (49.1)	29 (50.9)	0.262
Female	35	13 (37.1)	22 (62.9)	
Age (years)				
≤60	41	21 (51.2)	20 (48.8)	0.250
>60	51	20 (39.2)	31 (60.8)	
Histology				
Pure PSCE	79	35 (44.3)	44 (55.7)	0.729
Mixed PSCE	13	5 (38.5)	8 (61.5)	
Tumor invasion depth				
T1	13	5 (38.5)	8 (61.5)	0.253
T2	36	13 (36.1)	23 (63.9)	
T3	43	23 (53.5)	20 (46.5)	
Lymph node metastasis				
Yes	54	24 (44.4)	30 (55.6)	0.767
No	38	16 (42.1)	22 (57.9)	
Tumor location				
Upper	9	0 (0)	9 (100)	0.001
Middle	65	27 (41.5)	38 (58.5)	
Lower	18	13 (72.2)	5 (27.8)	
TNM staging				
I	11	3 (27.3)	8 (72.7)	0.740
II	78	36 (46.2)	42 (53.8)	
III	3	1 (33.3)	2 (66.7)	

CgA, chromogranin A; PSCE, primary small cell carcinoma of the esophagus.

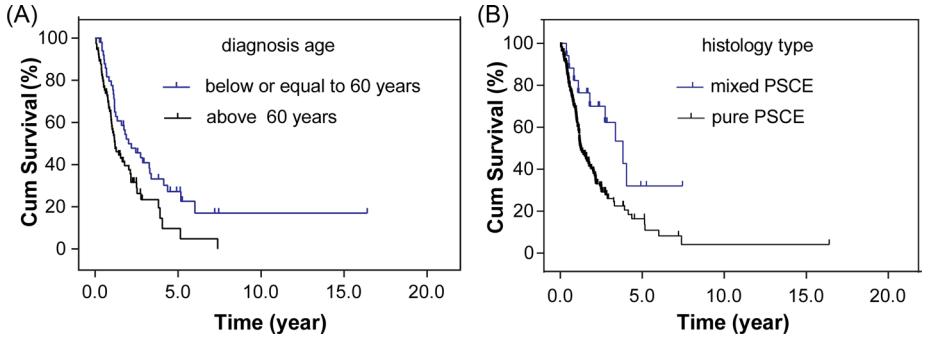

Fig. 3 Kaplan–Meier curves of overall survival for PSCE patients. Young patients with age below or equal to 60 years had a better survival than the elderly patients above 60 years ($P = 0.011$, A). The mixed PSCE patients had a better survival than those with pure PSCE ($P = 0.015$, B).

have a badly worsen survival than the early esophageal squamous cell carcinoma. Furthermore, of the three elderly patients (more than 80 years), one patient with age of 85 years died one month after radical esophagectomy, another elderly patient with age of 88 years died eight months late, and the third one with age of 80 years died forty-six months late. Moreover, in the group of 24 patients with 70–78 years old, 15 patients had died with a worsen survival: nine patients with less than one year survival (60%), another three patients with one and half year survival (20%), three patients with two year survival (20%). Of these 24 elderly patients, only one patient was identified as early PSCE at diagnosis, and unfortunately, this early PSCE patient also died 14 months late after radical surgery.

DISCUSSION

As we know, this is the largest sample size report on CgA protein expression in tissue level with PSCE prognosis. Our study demonstrates that the patients with CgA overexpression in tissue level had a superior long survival than those without CgA expression after excluding from all of potentially confounding factors by multivariate analysis, indicating that CgA is more than a diagnostic marker for PSCE, but as a significant prognostic marker. Interestingly, recent studies indicate that tissue CgA overexpression is correlated with the prognosis for small cell carcinoma in cervix[9] and prostate.[10] Furthermore, serum CgA has been demonstrated as a prognostic biomarker for small cell carcinoma in lung,[14] prostate,[15] and even

Table 3 Survival analysis for prognosis of 125 PSCE patients

Variables	Univariate			Multivariate		
	n	χ^2	*P*	*n*	OR (95% CI)	*P*
Gender						
Male	82	0.683	0.409	51	ref	
Female	43			30	1.34 (0.71–2.53)	0.369
Age(years)						
≤60	50	6.392	0.011	36	ref	
>60	75			45	0.72 (0.42–1.24)	0.235
Histology						
Pure PSCE	112	5.909	0.015	68	ref	
Mixed PSCE	13			13	2.81 (1.22–6.47)	0.015
Tumor invasion depth						
T1	13	0.676	0.713	13	ref	
T2	50			28	1.24 (0.56–2.77)	0.604
T3	62			40	0.93 (0.49–1.72)	0.811
Lymph node metastasis						
Yes	76	2.147	0.143	51	ref	
No	49			30	0.91 (0.46–1.78)	0.781
Tumor location						
Upper	12	1.109	0.575	9	ref	
Middle	85			54	0.45 (0.15–1.41)	0.255
Lower	28			18	0.84 (0.41–1.69)	0.639
TNM staging						
I	13	1.485	0.476	11	ref	
II	109			67	0.93 (0.15–5.78)	0.936
III	3			3	1.05 (0.23–4.85)	0.955
CgA expression						
Negative	51	1.237	0.266	45	ref	
Positive	41			36	1.87 (1.04–3.37)	0.037

CgA, chromogranin A; CI, confidence interval; OR, odds ratio; PSCE, primary small cell carcinoma of the esophagus.

in hepatocellular carcinoma of elderly patients.[11] The mechanism for CgA in small cell carcinoma prognosis is not clear. Recently, it has been reported that CgA serum level is a useful marker in monitoring progression of hepatocellular carcinoma. The hepatocellular carcinoma patients with higher CgA level have poor survival, compared to those with lower CgA level, the CgA may assist as a prognostic indicator in hepatocellular carcinoma.[11] Further study is necessary to assess the role of this protein in tumor progression and to clarify the mechanisms on tumor prognosis.

The present study also demonstrates that CgA expression is associated with location of PSCE, i.e., CgA overexpression occurs predominantly in PSCE at the lower segment of the esophagus. One possibility is that PSCE may be originated from the amine precursor uptake and decarboxylase cells of the esophagus, which mainly exist in the distal esophagus.[16,17]

Another interesting finding in this study is that the mixed PSCE patients with squamous cell carcinoma or adenocarcinoma have a longer survival than those with pure PSCE by either univariate or multivariate survival analysis. Previous studies have displayed that almost one third of the PSCE coexists with either squamous cell carcinoma and/or adenocarcinoma,[18,19] which is higher than in our study (10%). The mixed PSCE might originate from multipotential stem cells of esophageal mucosa.[20] Obviously, pure PSCE has a poorer differentiation than mixed PSCE, which may contribute to the observed different prognosis.

In contrast to the better survival in early squamous cell carcinoma (with a 5-year survival of more than 80%),[21] the early PSCE patients (T1N0M0) in this study have been identified with a badly worsen survival, which is less than 10%. The reason for this poor prognosis is not clear. Nishimaki *et al.*[22] has reported that three PSCE patients with tumors limited to the submucosal layer die within 11 months while two patients with advanced lesions have long-term survivals. Other authors have also reported long-term survivors with advanced PSCE lesions.[23–25] These results indicate that the outcome of treatment on PSCE appears to depend largely on behavior of individual tumors and therefore is unpredictable[19] and moreover, the TNM staging system for esophageal squamous cell carcinoma may not well suitable for PSCE. Further study is needed to assess the pathological staging for PSCE.

In the present study, 60% of PSCE cases have occurred lymph node metastasis at the time of diagnosis, which is obviously higher than esophageal squamous cell carcinoma.[26] It has been reported that lymph node metastasis significantly influences PSCE survival.[7] Surprisingly, our result does not show any effect of lymph node metastasis on PSCE survival. The median survival time of patients with and without lymph node metastasis is 1.16 and 2.03 years, respectively, in this study. The overall too short survival time

may be one of the reasons for negative influence of lymph node metastasis on PSCE prognosis.

It is noteworthy that the elderly patients with more than 70 years at diagnosis account for 21% in this study. Even though all these elderly patients are performed radical esophagectomy, however, the survival is badly worsen, 66% (12/18) of the elderly patients died within one month after radical surgery. The literature for operative reports on the elderly cancer patients is very limited.[27] Because of the worsen survival for the elderly PSCE patients after radical surgery, the selection of operative method should be critical with precise overall evaluation on the elderly PSCE patients.

In summary, the PSCE is of badly worsen prognosis, even in the pathological early stage. Tissue CgA protein expression is a promising maker not only for diagnosis, but also for prognosis. Further assessment is needed to clarify the role and the mechanisms of CgA protein in PSCE progression and prognosis and to establish specific pathological staging system for PSCE.

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