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High Expression of S100A6 Predicts Unfavorable Prognosis of Lung Squamous Cell Cancer

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Data Interpretation D
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Background: S100 family of proteins is mainly involved in regulation of intracellular calcium homeostasis. Aberrant expression of S100 family members has been reported in many types of cancers. However, as a member of S100 family, the prognostic value of S100A6 for lung squamous cell carcinoma (SCC) has not been well-studied.

Material/Methods: Using immunohistochemistry, we investigated the expression of S100A6 in 177 patients with SCC and further divided the cohort into a high S100A6 expression group and a low S100A6 expression group. The chi-square test was applied to analyze the correlation between S100A6 expression and clinicopathological factors. Univariate analysis using the Kaplan-Meier method was performed to compare the difference in survival rates between the high S100A6 expression group and the low S100A6 expression group; multivariate analysis with Cox regression model was used to identify independent prognostic risk factors.

Results: In our experiment, we demonstrated that the expression of S100A6 was significantly associated with patient age and tumor differentiation. High-expression of S100A6 was shown to be substantially related to the unfavorable prognosis of SCC. Moreover, our results confirmed that S100A6 was an independent risk factor for SCC prognosis, and could predict unfavorable prognosis.

Conclusions: High-expression of S100A6 was identified as an independent unfavorable prognostic factor for SCC, suggesting that targeting S100A6 may result in the development of potential targeted drug for SCC.

MeSH Keywords: Lung Neoplasms • Neoplasms, Squamous Cell • Prognosis • S100 Proteins

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Background

Lung cancer is the most common malignancy in humans, and results in the most cancer-related deaths worldwide [1]. Lung cancer continues to be a medical and financial burden, as well as a threat to people's health, especially in air polluted countries such as India and China [2]. Lung cancer is classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which have different treatment strategies and prognosis [3]. NSCLC makes up about 85% of all lung cancers, and is divided into subtypes, including adenocarcinoma (AC) and squamous cell carcinoma (SCC) which account for approximately 40% and 35% of all lung cancers, respectively [2,4,5]. NSCLC tumors have remarkable molecular heterogeneity, requiring customized therapy [1,6]. New prognostic biomarkers of NSCLC are urgently needed and could help more precisely stratify patients and identify high-risk patients.

The S100 family consists of at least 20 members, differentiated by calcium-binding EF-hand motifs [7]. The S100 family is involved in several biological processes, including immune response and differentiation and growth, through regulating cytoskeleton dynamics and enzyme activity. Most members of the S100 family are believed to function via binding to distinct intracellular compounds, but the precise function is not well-defined. Most S100 genes are confined on the human chromosome 1q21, which is repeatedly rearranged in human tumors [4,8]. Many S100 family members are reported to be aberrantly expressed in different kinds of tumors and tumor stages, including lung cancer [9,10]. S100A6, also known as calcyclin, can regulate the dynamics of cytoskeleton constituents, cell growth and differentiation, and calcium homeostasis [11–13]. Although S100A6 has been shown to be overexpressed in several types of human tumors, its function and clinical role in lung cancer is still unknown.

In our experiment, we investigated the expression of S100A6 in 177 SCC patients, using immunohistochemistry (IHC), and divided the patients into a high S100A6 expression group and a low S100A6 expression group according to our study IHC-based cutoff values. Furthermore, we evaluated the clinical significance of S100A6 in SCC, including the correlation between clinicopathological factors and survival rates.

Material and Methods

Patients and follow-ups

We enrolled patients who underwent radical surgery for SCC from 2004 to 2015 at Linyi People's Hospital. In all, 177 patients were included in the study based to the following criteria: 1) available paraffin-embedded tissues; 2) no adjuvant chemotherapy or radiation therapy before or after surgery; and 3) underwent radical resection of tumor. All the resected samples were obtained from the Department of Pathology after the consent of the patients. The study was approved and supervised by the Ethics Board of Linyi People's Hospital. The stage of SCC was identified by the 7th American Joint Committee on Cancer/International Union against Cancer (AJCC/UICC) pathological tumor node metastasis (pTNM) stage system (2009).

Immunohistochemistry

The diagnosis of SCC was double confirmed and the area for IHC was selected by two independent pathologists. The protocol for IHC staining was performed as described in previous studies [14,15]. In brief, the slides were deparaffinized first and then rehydrated with graded ethanol to deionized water. Sodium citrate buffer at pH 6 was used to incubate the slides to get the optimal antigen retrieval. The blockage of endogenous peroxidase enzyme was achieved by incubation in 3% H_2O_2 for 20 minutes. After rinsing with phosphate buffer saline (PBS) three times the slides were incubated in 1% bovine serum albumin for 20 minutes; then the samples were incubated in diluted primary antibody of S100A6 (1: 100) overnight at 4°C. The primary antibodies of S100A6 were purchased from the Santa Cruz Biotechnology (sc-50409, CA, USA). Samples were then rinsed with PBS, and the corresponding secondary antibody was applied for incubation for 20 minutes, and the streptavidin-peroxidase complex was used to react with the samples for 30 minutes after rinsing. Then 3,3'-diaminobenzidine solution was used for antigen visualization.

Evaluation of IHC staining and scoring system

Two senior pathologists evaluated the results of IHC with no prior awareness of clinical data. The final IHC results for S100A6 were defined as the product of the score of staining intensity multiplied by the score of positive cell percentage as described in previous studies [16,17]. The score of positive cell percentage was graded as: 0 (10% positive cells); 1 (10–30% positive cells); 2 (30–50% positive cells); 3 (50% or more positive cells). The score of staining intensity was described as follows: score 0 for negative staining; score 1 for weak staining; score 2 for moderate staining; and score 3 for strong staining. The cutoff was generated by receiver operating characteristic (ROC) curve and defined as the point with the highest specificity and sensitivity [18]. Based on the cutoff values, the cohort was divided into two groups: the group with high expression of S100A6 and the group with low expression of S100A6.

Statistical analysis

All the data was analyzed by the software SPSS 17.0 (Chicago, IL, USA). Chi-square test was used to analyze the correlation

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Table 1. Characters of patients.

Characters	Number	Percentage
Gender		
Male	114	65.52%
Female	63	36.21%
Age		
<60	56	32.18%
≥60	121	69.54%
Tumor diameter (cm)		
≤3	68	39.08%
>3	109	62.64%
Differentiation		
Well	92	52.87%
Moderately+Poorly	85	48.85%
Lymph node metastasis		
No (N0)	111	63.79%
Yes (N1–3)	66	37.93%
TNM stage		
I	111	63.79%
II	40	22.99%
III	26	14.94%
Smoking		
No	81	46.55%
Yes	96	55.17%
S100A6		
Low	119	68.39%
High	58	33.33%

between the expression of S100A6 and clinicopathological factors. The overall survival curve was generated by the Kaplan-Meier method; the log-rank test was used to compare the statistical difference between the group with high-expression of S100A6 and the group with low-expression of S100A6. The Cox regression hazard model was performed to identify the independent prognostic factors. Statistically significant was defined as $p<0.05$ in our study.

Results

Basic information of patients

A total of 177 patients were included in our cohort, which consisted of 114 males and 63 females (Table 1). Consistent with previous research, male and older patients were dominant in our cohort. Male patients accounted for 65.52%, and

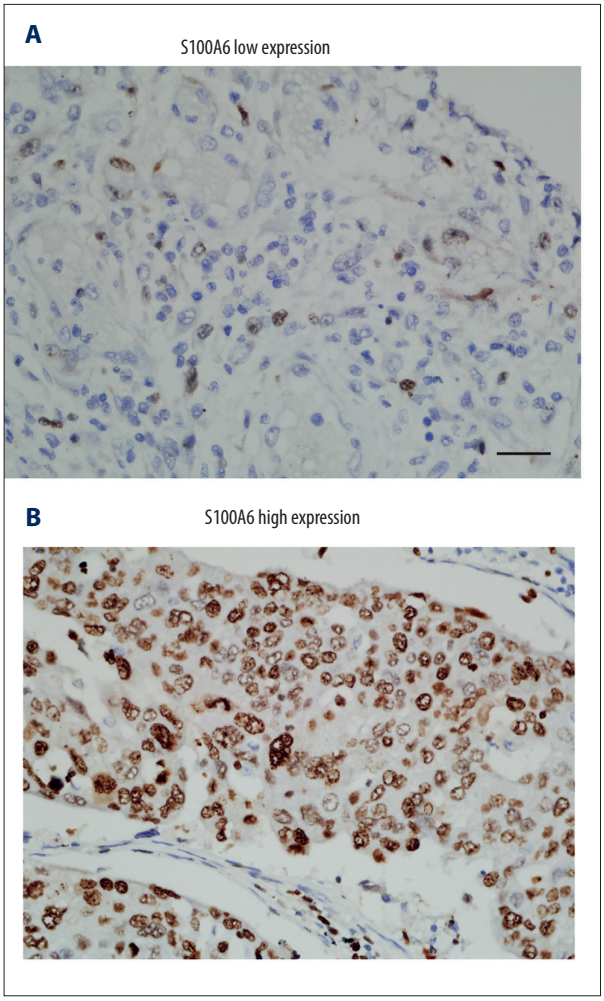

Figure 1. Representative immunohistochemical images of high-expression and low-expression of S100A6. (**A**) Representative image of S100A6 low-expression. Scale bar: 50 μm. (**B**) Representative image of S100A6 high-expression.

elderly patients (≥60 years of age) accounted for 69.54%. The cohort was divided into a group with S100A6 low-expression and a group with S100A6 high-expression which accounted for 68.39% (119/177) and 33.33% (58/177), respectively. Representative images are displayed in Figure 1A and 1B. In most cases, S100A6 could be observed in the nucleus and cytoplasm of SCC cells (Figure 1).

The associations of S100A6 and clinicopathological factors

The chi-square test was performed to screen potential risks affected by S100A6 (Table 2). The clinicopathological factors included patient age, sex, smoking status, tumor diameter, tumor differentiation, lymph node metastasis and TNM stage. In our cohort, S100A6 high-expression was more frequent in cases with poorer differentiation ($p=0.011$), indicating that

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Table 2. Correlation between S100A6 and clinicopathologic parameters.

Characters	S100A6		P
	Low	High	
Gender			
Male	72	42	0.135
Female	47	16	
Age			
<60	44	12	**0.038**
≥60	75	46	
Tumor diameter (cm)			
≤3	47	21	0.743
>3	72	37	
Differentiation			
Well	70	22	**0.011**
Moderately+Poorly	49	36	
Lymph node metastasis			
No	76	35	0.741
Yes	43	23	
TNM stage			
I	76	35	0.794
II	27	13	
III	16	10	
Smoking			
No	56	25	0.634
Yes	63	33	

* Chi-square test.

S100A6 may influence the differentiation-related cellular processes. Interestingly, older patients also had a higher S100A6 expression, suggesting S100A6 may be involved in cell senescence, which may be possible because S100A6 could regulate intracellular calcium homeostasis.

The associations of S100A6 and the overall survival rate

The correlations between the overall survival rate and clinicopathological factors were analyzed by the log-rank test; the survival curve was determined using the Kaplan-Meier method (Table 3). S100A6 high-expression was demonstrated to be significantly correlated to a lower overall survival rate (p=0.006) (Figure 2), suggesting S100A6 is a valid prognostic biomarker. Moreover, the TNM stage (p<0.001) and lymphatic invasion status (p<0.001) were also identified to be related to the prognosis. Patients with positive lymph nodes and advanced TNM stage had a more unfavorable prognosis. No other clinicopathological factors were shown to be significantly associated with survival rates.

Multivariate analysis

Multivariate analysis was applied for the identification of independent prognostic factors with Cox regression hazard model (Table 4). All the factors in the univariate analysis were used in the Cox regression hazard model except the TNM stage because of its important interaction with lymph node status. In multivariate analysis, S100A6 was confirmed as an independent prognostic factor of SCC in our study (p=0.016, 95%

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Table 3. Univariate analysis.

Characters	5-year survival rate %	P*
Gender		
Male	24.4	0.823
Female	27.6	
Age		
<60	32.9	0.205
≥60	11.0	
Tumor diameter (cm)		
≤3	17.4	0.691
>3	32.3	
Differentiation		
Well	29.4	0.804
Moderately+Poorly	31.9	
Lymph node metastasis		
No	32.9	P<0.001
Yes	11.0	
TNM stage		
I	32.9	P<0.001
II	23.1	
III	0	
Smoking		
No	26.7	0.245
Yes	23.8	
S100A6		
Low	27	0.006
High	19	

* Log-rank test.

CI=1.101–2.517, HR=1.66), which means S100A6 itself could predict poorer prognosis of SCC. Additionally, lymph node metastasis was also defined as a risk factor, predicting unfavorable prognosis (p<0.001, 95% CI=1.440–3.429, HR=2.16).

Discussion

The S100 family consists of many members with various heterogeneities. The oncogenic role of the S100 family has been suggested in several types of cancers, but different S100 members have been reported to be differently expressed in different tumors, indicating that S100 family members have

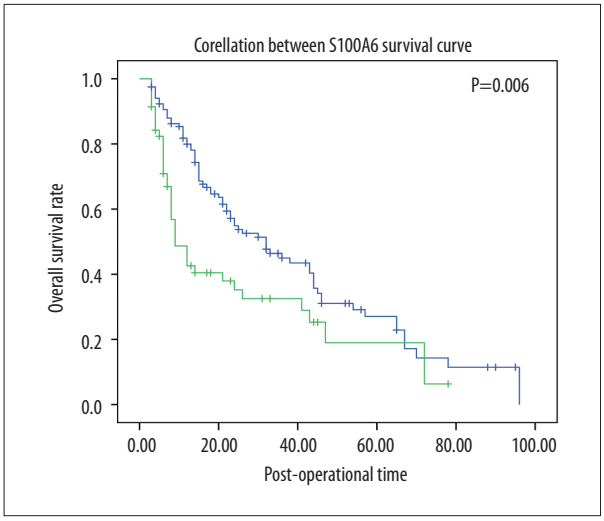

Figure 2. Prognostic significance of S100A6. The overall survival curve was drawn with Kaplan-Meier method and stratified with S100A6 expression. The group with S100A6 high-expression had poorer prognosis than those with S100A6 low-expression (p=0.006).

distinct tissue specificities. For example, S100A4 was shown to be linked with metastasis in breast cancer, but its expression had no relation with the melanoma metastasis [19,20]. The correlation between the S100 family and NSCLC has been reported sporadically. Diederichs at al. compared the mRNA level between primary tumors and metastasis, and demonstrated that S100P overexpression was more frequent in the metastasis of lung cancer and was associated with poorer prognosis [4]. Additionally, S100A4 was also identified as a prognostic marker of lung SCC [21]. Wang et al. showed that overexpression of S100A2 could indicate poorer prognosis of patients with stage I NSCLC [9]. However, the role of S100A2 in lung cancer remains controversial. Some evidence has been found to suggest that S100A2 is a suppressor of NSCLC, while other evidence has considered S100A2 as a predictor of poorer prognosis of NSCLC [4,8,10,22,23].

In the S100 family, S100A6 has been shown to be overexpressed in several types of human tumors, including osteosarcoma, hepatocellular carcinoma, gastric cancer, colorectal cancer, cholangiocarcinoma, and pancreatic cancer [12,24–31]. In lung cancer, emerging evidence has indicated an oncogenic role for S100A6. The level of S100A2 and S100A6 in serum is significantly elevated in early stage NSCLC [32], showing the potential oncogenic role of S100A2 and S100A6 in early stage NSCLC. Moreover, S100A6 has been reported to be correlated with survival of patients with stage I NSCLC [33], but this finding was generated from a small sample size and AC and SCC were not researched separately. Our finding that S100A6 could predict unfavorable prognosis of SCC is valuable for the potential screening of drug targets for lung cancer, which is essential

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Table 4. Multivariate analysis.

Characters	HR	95% CI	P*
Gender			
Male	1		
Female	0.95	0.623–1.457	0.830
Age			
<60	1		
≥60	1.14	0.731–1.793	0.556
Tumor diameter (cm)			
≤3	1		
>3	0.99	0.661–1.487	0.967
Differentiation			
Well	1		
Moderately+Poorly	1.14	0.754–1.724	0.535
Lymph node metastasis			
No	1		
Yes	2.16	1.440–3.429	**<0.001**
Smoking			
No	1		
Yes	0.76	0.513–1.130	0.176
S100A6			
Low	1		
High	1.66	1.101–2.517	0.016

* Cox-regression model; HR – means hazard ratio; 95% CI – means 95% confidential interval.

for the development of new targeted drugs. Certainly, experiments *in vitro* and research on underlying molecular mechanisms are also necessary to explain why S100A6 leads to poorer prognosis of lung cancer. S100A6 could play a role in the regulation of many cellular processes, and not be limited to influencing the calcium homeostasis. The intracellular molecular target of S100A6 has not been well identified; it was not the primary focus of our study to reveal the underlying mechanisms. However, we hope our study can trigger more interest in this area and further the understanding of the molecular mechanisms involved.

Conclusions

We investigated the expression of S100A6 in 177 patients with SCC and evaluated its clinical significance. We found that S100A6 was significantly associated with patient age and tumor differentiation. Our results identified S100A6 as an independent prognostic risk factor in SCC; and it could predict unfavorable prognosis. This suggests that targeting S100A6 may lead to the future development of potential targeted drugs for SCC.

Conflicts of interest

The authors have no conflicts of interest.

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