ORIGINAL ARTICLE

Overexpression of S100A13 protein is associated with tumor angiogenesis and poor survival in patients with early-stage non-small cell lung cancer

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Abstract

Background: S100A13 plays a key role in tumor growth and metastasis. The purpose of this study was to investigate the prognostic significance of S100A13 expression, microvessel density (MVD), and survival in early stage non-small cell lung cancer (NSCLC).

Methods: In silico analysis was performed to determine the associations between S100A13 and NSCLC. The data of 82 patients with early-stage NSCLC who underwent radical resection were evaluated. Paraffin-embedded tumor specimens were stained with S100A13 and CD31 (a specific endothelial marker) using immunohistochemical methods. Prognostic significance was assessed by univariate and multivariate analyses.

Results: S100A13 messenger RNA was overexpressed in NSCLC, especially in advanced stage. Of the 82 NSCLC specimens examined, 37 (45.1%) cases exhibited S100A13 overexpression and 31 (37.8%) showed high MVD. Univariate analysis indicated that gender, age, smoking status, histology type, tumor differentiation, and T stage were not significantly associated with prognosis. However, the overall and disease-free survival rates of patients with S100A13 overexpression and high MVD were significantly lower than in the remaining cases. Multivariate analysis demonstrated that only S100A13 overexpression was an independent factor for poor prognosis in early-stage NSCLC. Statistical analysis demonstrated that the MVD was significantly higher in tumors with high (67.6%, 25/37) compared to low S100A13 expression (13.3%, 6/45) ($P < 0.01$).

Conclusions: High S100A13 expression is closely associated with high intratumoral angiogenesis and poor prognosis in patients with stage I NSCLC. Immunohistochemical evaluation of S100A13 expression, along with an examination of the perioperative extent of angiolymphatic invasion, has value for predicting prognosis.

Introduction

Primary lung cancer is the leading cause of cancer-related death worldwide.[1,2] With advances in many aspects of the classification, diagnosis, and treatment of non-small cell lung cancer (NSCLC), the overall survival (OS) of patients with early-stage NSCLC has improved after complete resection; however, postoperative recurrence and metastasis are also major obstacles to prolonged survival in early-stage NSCLC.[3–6] The detection of tumor molecular markers may help to predict local recurrence and distant metastases and thus more aggressive treatment regimens can be administered.[7–10]

The S100 family is the largest subfamily of calcium binding proteins of the EF-hand type and includes at least 25 distinct members.[11,12] The S100 protein family has been implicated in multiple stages of tumorigenesis and progression by various mechanisms including proliferation,

apoptosis, angiogenesis, metastasis, tumor microenvironment, and cancer stem cells.[11,13–16] S100A13 is a small calcium (Ca2+) binding protein belonging to the S100 family and the C-terminus of the S100A13 protein ends with a special motif, which has also been observed in metastasis-associated proteins S100A4 and S100A10.[17,18] S100A13 is upregulated in human astrocytic gliomas and melanoma, where it correlates with VEGF-A and FGF-1 expression and angiogenesis, and is also associated with a more aggressive, invasive phenotype in lung cancer-derived cell lines.[19–23] To our knowledge no previous studies have investigated a correlation between S100A13 and angiogenesis and prognosis in surgically resected early-stage NSCLC.

The aim of the present study was to investigate S100A13 protein expression in a sample of 82 stage I NSCLC patients, and to determine a correlation between S100A13 protein expression and clinical pathological features, intratumoral angiogenesis, and survival.

Methods

In silico analysis

The S100A13 expression profile was analyzed using The Cancer Genome Atlas (TCGA, https://cancergenome.nih.gov/) and Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geoprofiles/) databases. Kaplan–Meier plotter (http://kmplot.com) and Oncolnc data sets (http://www.oncolnc.org, data gathered from TCGA) were used to analyze the association between S100A13 expression and patient prognosis. Microarrays (GSE19804) were searched from GEO profiles and analyzed with R language, and differentially expressed RNAs (logFC>2) were adopted. A STRING data set (https://string-db.org/) was used to analyze protein interactions (PI). Pearson's correlation analysis was performed between S100A13 and other molecules via Oncomine. Gene Ontology (GO) enrichment was analyzed using the Database for Annotation, Visualization and Integrated Discovery (https://david.ncifcrf.gov). GO enrichment was classed with adjusted *P* values, and the top five were accepted. The Kyoto Encyclopedia of Genes and Genomes (KEGG) PATHWAY database (http://www.kegg.jp) was used to analyze the associated pathways of the key molecules.

Patients

This retrospective study included a total of 82 sequential patients who underwent radical lobectomy for NSCLC between January 2007 and December 2008 at the Affiliated Hospital of Qingdao University and Peking University First Hospital. Chest and upper abdomen computed tomography (CT) scans and bronchoscopy were routinely performed before surgery. Whole-body bone scans and CT scans of the brain were used to exclude possible metastasis. All patients underwent complete tumor resection with systematic lymph node dissection. The International Union Against Cancer Tumor Node Metastasis (TNM) classification was used to

Table 1 Correlation between S100A13 protein and intratumoral neoangiogenesis and clinicopathologic features in NSCLC specimens (*n* = 82)

Variables	No. of patients (%)	S100A13 expression			Intratumoral neoangiogenesis (MVD)		
		High	Low	*P*	High	Low	*P*
Gender				0.934			0.335
Male	24	11	13		11	13	
Female	58	26	32		20	38	
Age				0.118			0.148
< 65	32	11	21		9	23	
≥ 65	50	26	24		22	28	
Smoking				0.204			0.928
Heavy (≥ 20cps)	17	10	7		7	10	
Light (< 20cps)	20	6	14		7	13	
Never	45	21	24		17	28	
Tumor type				0.727			0.933
Adeno	56	26	30		21	35	
SCC	26	11	15		10	16	
Differentiation				0.643			0.536
Well	26	12	14		10	16	
Moderately	32	16	16		14	18	
Poorly	24	9	15		7	17	
T Stage				0.007			0.001
T1	40	12	28		8	32	
T2	42	25	17		23	19	

Adeno, adenocarcinoma; cps, cigarettes per day; MVD, microvessel density; NSCLC, non-small cell lung cancer; SCC, squamous cell carcinoma.
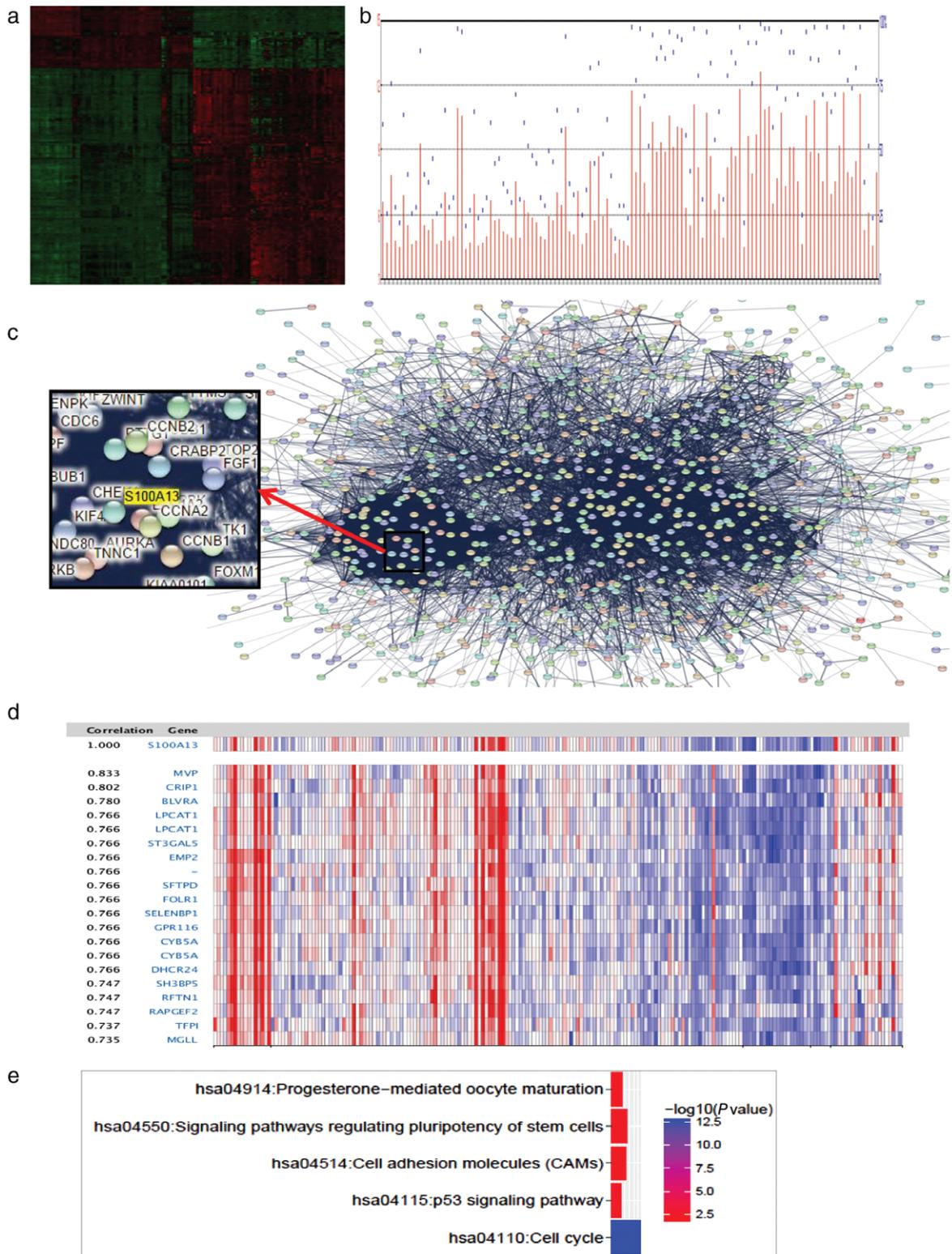
Figure 1 S100A13 is overexpressed and functions as a core molecule in non-small cell lung cancer (NSCLC). (a) Different expression between tumor tissues (right, n = 60) and adjacent normal tissues (left, n = 60) in NSCLC patients. (b) S100A13 expression profile in GSE19804 (left: normal; right: cancer). (c) Protein interaction network of the different expressed molecules (logFC>2). The left picture shows the location of S100A13. (d) Pearson's correlation analysis of S100A13 and other molecules in NSCLC. (e) Gene Ontology function enrichment of the molecules correlated with S100A13.

determine disease stage. None of the patients had received preoperative or postoperative adjuvant therapy before their tumor relapse. Written informed consent was obtained from all patients and the ethical committee of our hospital approved the study.

All patients were followed up in the outpatient department every three months for the first two years and semi-annually thereafter. Follow-up assessments included the evaluation of clinical information such as tumor recurrence, progression, OS, disease-free survival (DFS), and cause of death, with collaboration between thoracic surgeons. The clinicopathologic characteristics of the patients are listed in Table 1.

Immunohistochemical staining for S100A13 and CD31

Formalin-fixed paraffin-embedded surgical specimens of tissue blocks were collected. Serial tissue sections were cut from each tissue block at 4 μm intervals, stained with hematoxylin and eosin, and reviewed to confirm the diagnosis and presence of cancer cells. After dewaxing in xylene and rehydrating in a graded series of alcohols, endogenous peroxidase was blocked with 3% H_2O_2. Immunohistochemical staining for S100A13 and CD31 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was carried out using a Histostain-SP kit (Zymed Laboratories Inc., San Francisco, CA, USA).

Two pathologists blinded to clinicopathologic factors and clinical outcomes evaluated S100A13 expression. For each sample, S100A13 expression was evaluated as percentage of positive cells in a total of at least 1000 tumor cells. A value of 1% positive cells was used as the cutoff to distinguish negative from positive tumors. Following previous literature, cytoplasmic staining intensity was scored semiquantitatively into different grades on an arbitrary four-tiered scale from 0 to 3. The following scoring criteria were applied: grade 0, no detectable immunostaining of tumor cells; 1+, weak staining of the majority of tumor cells; and 2+ and 3+, moderate and strong staining intensity of tumor cells, respectively. Weak, moderate, and strong staining patterns were defined as positive in subsequent statistical analysis.

The microvessel density (MVD) was determined by counting CD31-labeled microvessel endothelial cells. Highly vascular areas were identified by scanning tumor sections at low power. Subsequently, counts of the stained microvessels were performed on three consecutive high-power (200× magnification) fields. MVD was defined as the number of manually counted vessels per mm^2 and presented as the mean of three hot spots. The median MVD served as the cutoff; expression was classified as low if less than the median, and high otherwise.

Statistical analysis

The relationship between S100A13 and CD31 expression and clinical-pathological characteristics was analyzed using the chi-square test. Survival curves were obtained using the Kaplan–Meier method. Multivariate analyses were performed using a Cox regression model to identify independent prognostic factors. All statistical analyses were carried out using SPSS version 13.0 (SSPS Inc., Chicago, IL, USA). Statistical differences of $P < 0.05$ were considered statistically significant.

Results

Bioinformatics analysis reveals the important role of S100A13 in non-small cell lung cancer

Using in silico analysis with a GEO microassay (GSE19804) (Fig 1a), we found that S100A13 is significantly upregulated in lung cancer tissues (left, $n = 60$) compared to adjacent normal tissues (right, $n = 60$) (3.1-fold change, $P < 0.01$) (Fig 1b). We analyzed all differentially expressed molecules between lung cancer and normal tissues (logFC>2) using the STRING dataset, and obtained a large PI network containing 1376 molecules (Fig 1c, right). From this network, two main clusters were found, and may indicate the underlying angiogenesis mechanisms in NSCLC. The location of S100A13 in the left cluster demonstrated its important role in NSCLC (Fig 1c, left).

Pearson's correlation analysis was performed to tentatively explore the function of S100A13 in NSCLC cells. Molecules associated with S100A13 were determined, and the top 20 molecules according to correlation coefficient were listed (Fig 1d). We then analyzed the main functions of these molecules. GO function enrichment analysis indicated that S100A13 mainly regulated the cell cycle in NSCLC cells (Fig 1e).

Using another dataset from TCGA, we determined that S100A13 is upregulated in tumor tissues in early-stage NSCLC patients compared to normal tissues ($P < 0.01$) (Fig 2a). Furthermore, T2 patients showed a higher level of S100A13 than T1 patients ($P < 0.01$) (Fig 2b). We analyzed the relationship between S100A13 and survival in NSCLC patients using the Kaplan–Meier plotter, and the results showed that a higher level of S100A13 was associated with poor prognosis in lung adenocarcinoma (Fig 2d), lung squamous cell carcinoma (Fig 2e), and NSCLC (Fig 2c) patients ($P < 0.01$).

The results of in silico analysis demonstrated that S100A13 is overexpressed in NSCLC and is associated with shorter survival. More experiments are necessary to verify these results.
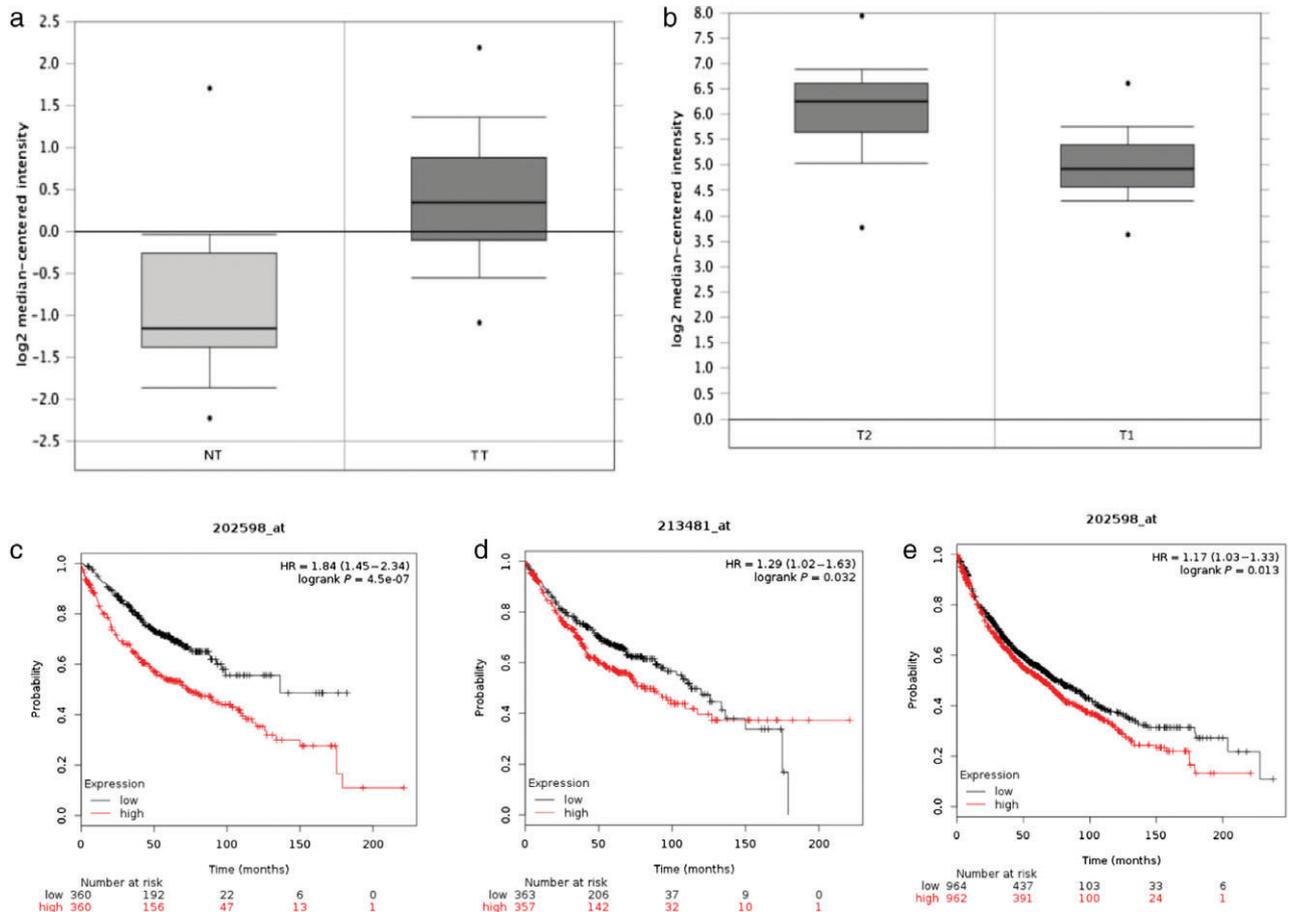
Figure 2 S100A13 is upregulated in early-stage non-small cell lung cancer (NSCLC) patients and is correlated with poor prognosis. (**a**) Relative expression of normal tissues (NT, left) versus tumor tissues (TT, right) and (**b**) T2 (left) versus T1 (right). Survival curves according to S100A13 expression in (**c**) NSCLC, (**d**) lung adenocarcinoma, and (**e**) lung squamous cell carcinoma. HR, hazard ratio.

Correlation between S100A13 protein and intratumoral neoangiogenesis and clinicopathologic features

S100A13 expression was assessed using a four-point scoring system to determine the number of positively stained tumor cells. As shown in Figure 3, S100A13 was positively immunostained in the cytoplasm and nuclei of lung cancer cells. Of the 82 NSCLC specimens examined in this study, 37 (45.1%) cases exhibited S100A13 protein overexpression (Fig 3). We next analyzed the correlation between positive S100A13 expression and various clinicopathological factors. Our data showed that S100A13 protein overexpression was significantly higher in T2 than in T1 patients (60.0% vs. 30.0%; $P = 0.007$). However, there was no statistically significant correlation between S100A13 protein expression and other clinicopathologic features in lung cancer patients ($P > 0.05$) (Table 1).

Microvessel density was our preferred parameter to quantify the extent of tumor vascularization, and intratumoral MVD was quantified by counting CD31-positive endothelial cells in the lung cancer tissues (Fig 3). The MVD staining intensity ranged broadly from 7 to 86 microvessels/200× magnification fields. Based on the cutoff value of 35 microvessels/200× magnification fields, 31(37.8%) cases showed high MVD. MVD was significantly higher in T2 than in T1 patients (54.8% vs. 20.0%; $P = 0.001$), while no significant correlations were observed between MVD and other clinicopathologic factors of NSCLC ($P > 0.05$) (Table 1).

Correlation between S100A13 expression and intratumoral neoangiogenesis and patient survival

Follow-up information was available for 82 NSCLC patients for periods ranging from 19 to 96 months (median 61.5).

Figure 3 S100A13 expression and CD31 were stained by immunohisto-chemical method in non-small cell lung cancer (NSCLC) tissues. (**a**) High (strong positive) and (**b**) low (negative) S100A13 expression. (**c**) Tumor area with high and (**d**) low microvessel density by CD31 staining.
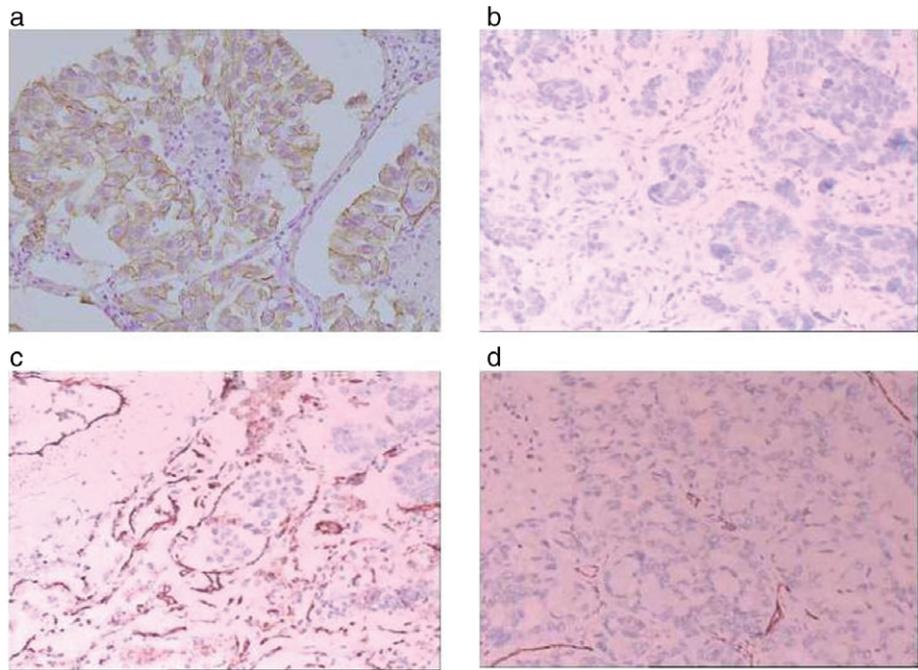
Univariate analysis using the Kaplan–Meier method indicated that gender, age, smoking status, histology type, tumor differentiation, and T stage were not significantly associated with prognosis. However, the OS rate of patients with S100A13 protein overexpression (43.2% vs. 75.6%; *P* = 0.001) (Fig 4a) and high MVD (45.2% vs. 70.6%;
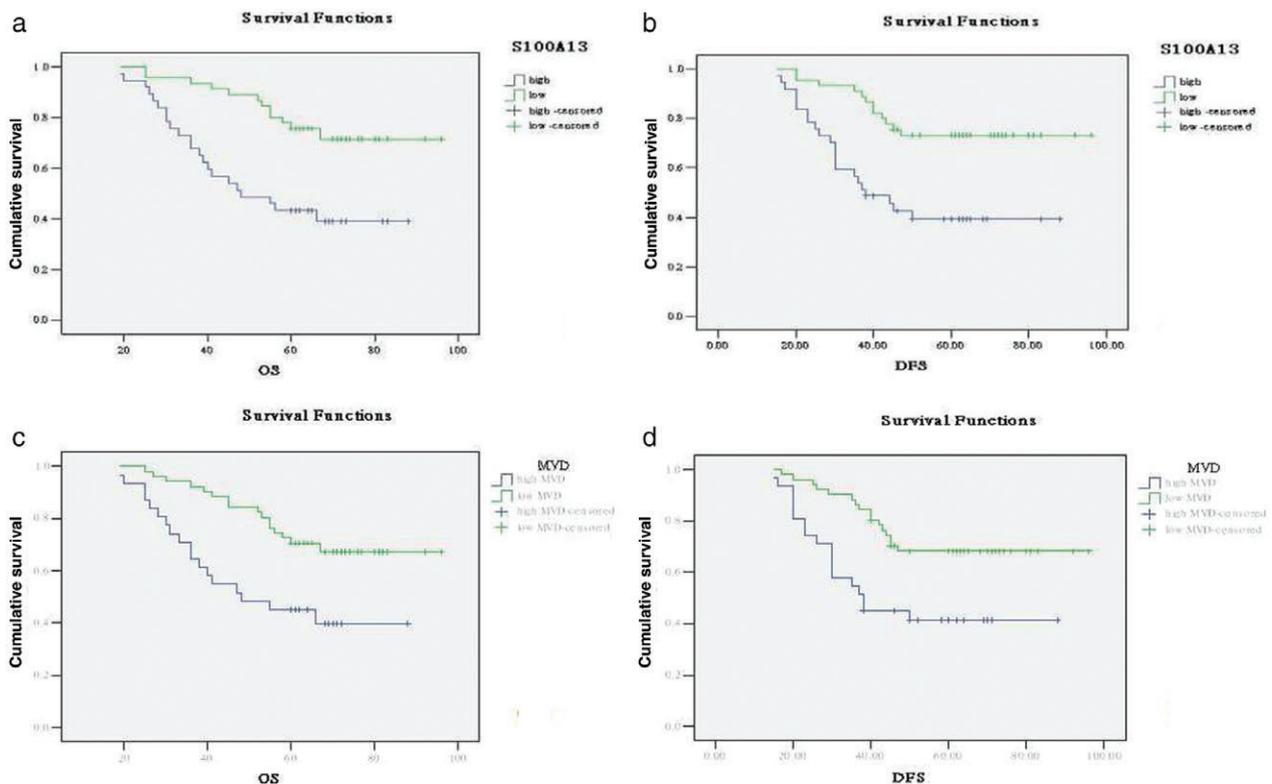

Figure 4 Kaplan–Meier curves of overall survival (OS) (**a**) and disease-free survival (DFS) (**b**) in patients with stage I non-small cell lung cancer according to high (**c**) and low S100A13 expression and microvessel density (MVD) (**d**).

$P = 0.004$) (Fig 4c) was significantly lower than in the remaining cases. Kaplan–Meier analysis of DFS also demonstrated poor five-year survival in patients with S100A13 protein overexpression (39.3% vs. 73.2%; $P = 0.001$) (Fig 4b) and high MVD (41.4% vs. 68.2%; $P = 0.004$) (Fig 4d).

Multivariate analysis using a Cox proportional hazard model was conducted to determine the independent prognostic effects of the various clinicopathological parameters. Our results demonstrated that only S100A13 protein overexpression was an independent factor for poor prognosis in early-stage NSCLC patients ($P < 0.005$) (Table 2).

Correlation between S100A13 overexpression and microvessel density

We first analyzed the correlation between MVD and S100A13 protein expression in human NSCLC samples. Statistical analysis demonstrated that the MVD was significantly higher in tumors with high S100A13 protein expression (67.6%, 25/37) than in those with low expression (13.3%, 6/45) ($P < 0.01$).

To explore the possibility of crosstalk between S100A13 and MVD, we examined the survival differences of patients stratified with low and high MVD according to S100A13 protein expression status. Patients without S100A13 protein overexpression and high MVD had low OS ($P = 0.034$), while those without S100A13 protein overexpression and low MVD had high DFS ($P = 0.026$) (Table 3). However, in patients with S100A13 protein overexpression, there were no significant differences in survival between the low and high MVD groups ($P = 0.256$ for OS and $P = 0.287$ for DFS) (Table 3).

Discussion

Tumor microvascular density is used to determine the extent of neovascularization and is a quantitative indicator of the degree of tumor neovascularization, suggesting tumor recurrence, metastatic potential, and long-term survival. Neovascularization is important for the rapid growth and metastasis of solid tumors.[24,25] Increased MVD is regarded as a significant and independent prognostic indicator in a number of human cancers.[26–31] Several studies have shown that S100A13 is overexpressed in several solid tumors and is involved in angiogenesis and metastatic development.[21–23]

In the present study, we examined S100A13 protein expression and MVD in 82 surgical cases with early-stage pulmonary adenocarcinoma by immunohistochemical staining. S100A13 overexpression in tumor tissues was common in stage I NSCLC patients (37/82, 45.1%) and was significantly associated with increased intratumoral MVD ($P < 0.05$), as assessed by CD31 staining. This result suggests that the S100A13 protein might promote tumor progression and development by inducing tumor angiogenesis. Pierce *et al.* reported that an increase in S100A13 transcript is associated with a more aggressive invasive phenotype and clustered with several other well-documented cancer/metastasis-related genes involved in processes such as angiogenesis.[22]

There were more female than male patients included in our study; however, this was unintentional as specimens were randomly collected. Subsequent testing indicated no statistical difference between the different cohorts; however, we should not exclude the probability that our conclusion is based on gender distinction.

Our subsequent analyses further suggested that S100A13 protein overexpression is significantly correlated with decreased OS and DFS rates in patients with stage I NSCLC, compared to patients without S100A13 overexpression ($P < 0.05$). Our study also demonstrated that increased MVD combined with higher S100A13 expression could indicate a poorer prognosis in early-stage pulmonary adenocarcinoma patients and is a useful prognostic marker in cancer. However, high MVD is not an independent

Table 2 Multivariate analysis of pathological factors and molecular markers in stage I NSCLC patients ($n = 82$)

	RR		*P*		95% CI	
Variables	OS	DFS	OS	DFS	OS	DFS
CD31	0.682	0.694	0.347	0.371	0.307–1.515	0.311–1.545
High S100A13 expression	1.244	1.429	0.036	0.027	1.193–3.021	1.186–3.991

Table 3 Survival differences stratified by low and high MVD in the S100A13 overexpressed and non-overexpressed subgroups (log-rank test)

	S100A13 overexpression			S100A13 non-overexpression		
Five-year survival	Low MVD (%)	High MVD (%)	P	Low MVD (%)	High MVD (%)	P
Overall	50.0	40.0	0.256	76.9	66.7	0.034
Disease-free	47.6	35.0	0.287	74.2	64.7	0.026

prognostic factor for poor survival, because high MVD may result from high S100A13 expression. Multivariate analysis based on the current data supports the notion that overexpression of S100A13, but not MVD, is an independent factor predicting a poor outcome for stage I NSCLC patients.

The correlation observed between S100A13 and MVD indicates that S100A13 exerts important functions in lung cancer progress, such as invasion and metastasis, or cell proliferation and apoptosis. Our next study (titled "Expression of osteopontin in non-small cell lung cancer and correlative relation with microvascular density") will focus on these further functions of S100A13 in lung adenocarcinoma. Although the prognostic role of MVD in NSCLC has been comprehensively investigated, its accuracy remains controversial. In our study, we verified a close correlation between MVD and prognosis in lung adenocarcinoma patients. Furthermore, S100A13 expression promotes MVD and prognosis. More experiments will be performed to further assess the potential role of S100A13 as a clinical biomarker.

In summary, we conclude that higher S100A13 expression is closely associated with high intratumoral angiogenesis and poor prognosis in patients with stage I NSCLC. Immunohistochemical evaluation of S100A13 expression, along with an examination of the perioperative extent of angiolymphatic invasion, has value for predicting prognosis.

Acknowledgments

Disclosure

No authors report any conflict of interest.

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