



## Tumor expression of S100A6 correlates with survival of patients with stage I non-small-cell lung cancer

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### ABSTRACT

**Background:** In a previously published *in vitro* study based on top-down proteomics we found that the calcium-binding proteins S100A6 and S100A4 were affected by exposure to ionizing radiation in a p53-dependent fashion. Both proteins showed post-translational modification changes, and S100A6 also showed increased expression and translocation in response to irradiation. Aim of the present study was to evaluate the expression of S100A6 and S100A4 in non-small-cell lung cancer (NSCLC).

**Methods:** S100A6 expression on archival tumor cell lysates from 39 patients with radically resected NSCLC was assessed with SELDI-TOF-MS. S100A6 identity was confirmed using a SELDI-based antibody-capture method on lysates from the A549 lung cancer cell line, cell lysates from two freshly prepared NSCLC samples, four plasma samples and one pleural effusion sample. Immunostainings for S100A6, S100A4 and p53 were performed on tissue microarrays containing 103 stage I surgically resected NSCLC cases and 14 normal lung parenchyma specimens.

**Results:** The presence of post-translationally modified S100A6 forms was confirmed with SELDI-MS on enriched tumor cell lysates, as well as in plasma and pleural effusion samples. In addition, high S100A6 peak intensity was associated with longer median survival (35 months vs. 18 months for high and low peak intensity, respectively;  $p = n.s.$ ). The immunohistochemical analysis showed that 25% of tumors were S100A6 positive. S100A6 expression correlated directly with non-squamous histology ( $p < 0.0001$ ) and S100A4 expression ( $p = 0.005$ ), and inversely with p53 expression ( $p = 0.01$ ). S100A6-positive cases showed a trend of longer survival compared with S100A6-negative cases ( $p = 0.07$ ). This difference became significant when the analysis was restricted to p53-negative cases ( $n = 72$ ). In this subgroup of patients, whose tumors likely exhibit a functional p53, S100A6 was an independent prognostic factor of improved survival at multivariate analysis (HR 0.49, 95% CI 0.27–0.81,  $p = 0.017$ ).

**Conclusions:** In this study we have validated on clinical material our previous findings on cell lines in terms of S100A6 expression and post-translational modifications pattern in NSCLC. Moreover, the survival results obtained in p53-negative stage I NSCLC cases support the proposed pro-apoptotic function of S100A6 and suggest the hypothesis of a cross regulation between these two proteins.

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### 1. Introduction

Non-small-cell lung cancer (NSCLC) is the leading cause of cancer-related deaths in the Western Countries [1]. Despite all progresses made in the last decades to define the biology of this disease, there is still an urgent need to discover and validate biomarkers

that could be included in the elaboration of treatment algorithms and assist in therapeutic decisions. The choice to administer further treatment after radical surgical resection in stage I NSCLC is one of those clinical situations where the availability of useful biomarkers for patient selection would be of high value. In this setting, in fact, the administration of adjuvant chemotherapy to unselected population has lead to conflicting results [2].

S100A6 and S100A4, members of the S100 protein family, are small calcium-binding proteins that have previously been related with prognosis and risk of metastasis in several tumor types [3]. Although the function of S100 proteins is still poorly understood, the lack of a proper catalytic domain suggests that their activity is

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mainly mediated via protein–protein interaction. There are more than 90 target proteins reported in the literature connecting S100 proteins to biological roles such as cytoskeleton rearrangement, cellular mobility and metastasis, chemotaxis, cell cycle regulation, protein degradation control and transcription factor regulation [4].

We have recently shown that, *in vitro*, S100A6 and S100A4 are up-regulated or altered by post-translational modifications (PTMs) in connection with genotoxic stress caused by irradiation [5]. The novel PTM changes of S100A6 and S100A4 (cysteinylation and glutathionylation), the subcellular relocalization of S100A6 after irradiation and the dependency of these events upon p53 status, directed our interest to study the potential clinical impact of these proteins in lung cancer. Although several studies have been published about the expression of S100A4 in lung cancer, to our knowledge an extensive report on S100A6 in NSCLC is still missing.

The aims of the present study were first to determine whether the post-translational modifications pattern of S100A4 and S100A6 detected on lung cancer cell lines could be assessed in clinical material. Secondly, we aimed to study the p53 relation and possible prognostic value of S100A6 and S100A4 in stage I NSCLC.

## 2. Materials and methods

### 2.1. Cell culturing and protein extraction

The lung cancer cell line A549 was cultured in Dulbecco's modified Eagle medium with 10% calf serum and 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA). Twenty-four hours after seeding, cells were harvested and then lysed using repeated freeze–thaw cycles and soluble proteins were extracted in a pH 7.5 lysis buffer including 0.1% Triton X-100, 1% CHAPS and Complete mini EDTA free protease inhibitors (Roche Diagnostics, Mannheim, Germany).

### 2.2. Patients characteristics

In the present study two separate patient cohorts with early stage NSCLC were included. No patients in either cohort received

peri-operative chemotherapy or radiotherapy. Follow-up data were obtained from the Swedish Cancer registry and from single patient files. Patients were scored as events at the date of death for any cause, or censored at the date of last follow-up.

Cohort I consisted of 39 patients (54% males, 46% females) with NSCLC who received curative surgery at Karolinska Hospital Solna between 1980 and 1992. Tumor cell suspensions were available from patients in this cohort. Cases were selected from a biobank of 75 samples on the basis of a diagnosis of lung adenocarcinoma or squamous-cell carcinoma, and on a protein concentration in tumor cell lysates >1 mg/ml (see below). Most of patients ( $n=31$ ) had stage I disease. Median age at surgery was 65 years (range 39–84 years). There were 33 events and 6 censored cases. Follow-up in all censored cases was longer than 11 years. Unfortunately, from this patient cohort it was not possible to retrieve further tumor material for confirmatory tests, in the form of formalin-fixed and paraffin-embedded or fresh frozen specimens. Tumor cell suspensions remained the only available source material.

Cohort II consisted of 103 patients with pathological stage I NSCLC who received curative surgery defined as lobectomy, bilobectomy or pneumonectomy. Patients were selected on the basis of the availability of formalin-fixed and paraffin-embedded tumor tissue. Primary tumors were resected between 1987 and 1992. Patient characteristics are listed in Table 1. Median age at surgery was 68 years (range 41–82 years). For correlation analyses and survival analyses, patients were grouped according to age at surgery in adults (<70 years, 60 patients) and elderly (>70 years, 43 patients). This cohort included 85 cases scored as events, and 18 cases scored as censored. Median follow-up of censored cases was 90.5 months (range 1–206 months). Two patients were excluded from survival analysis, but included in the correlation analyses for markers expression: one patient for post-operative mortality, one patient for intra-lobar metastasis (T4).

The study was approved by the Institutional Review Boards at Karolinska Institutet and at Stockholm's County Council. The collection of this patient material was carried out before the law of informed consent referred to biological human material (law number 1995:831) was implemented in Sweden on 1 July 1996.

**Table 1**

Immunohistochemical expression of p53, S100A4 and S100A6 according to clinicopathologic characteristics of cases in cohort II

	Number of patients	p53 expression			S100A4 expression <sup>a</sup>			S100A6 expression		
		Negative	Positive	<i>p</i>	Negative	Positive	<i>p</i>	Negative	Positive	<i>p</i>
All cases	103	72	31		80	22		77	26	
Age at surgery										
Adults <70 years	60	41	19	0.7	46	13	0.9	46	14	0.5
Elderly >70 years	43	31	12		34	9		31	12	
Gender										
Male	63	41	22	0.1	50	12	0.5	52	11	0.02
Female	40	31	9		30	10		25	15	
Surgery										
Lobectomy	84	60	24	0.4	62	21	0.06	60	24	0.1
Pulmonectomy–bilobectomy	12–7	12	7		18	1		17	2	
Differentiation										
Well	22	19	3	0.01	14	8	0.08	11	11	0.004
Moderate	39	30	9		30	9		29	10	
Low	42	23	19		36	5		37	5	
Histology										
Adenocarcinoma–BAC	33–18	42	9	0.006	31	20	<0.0001	26	25	<0.0001
Squamous	52	30	22		49	2		51	1	
T-stage										
T1	40	32	8	0.09	31	9	0.9	28	12	0.3
T2	62	40	22		48	13		49	13	

<sup>a</sup> S100A4 expression not evaluable in one case.

### 2.3. Preparation of surgical specimens for tumor proteome profiling

Tumor cell suspensions from patients in cohort I were obtained directly after surgical resection. Macroscopically distinguishable tumor tissue, without necrosis, was excised and minced in the presence of collagenase IV and DNase I (Boehringer Mannheim, Mannheim, Germany). Tumor material was gently passed through a stainless steel mesh into balanced salt solution (BSS) medium. A tumor cell-enriched suspension was obtained by centrifugation (60 × g, 5 min) and further fractionated on Percoll (Pharmacia, Milton Keynes, UK) gradients (25 g, 5 min). Tumor cell-enriched pellets were frozen in liquid nitrogen until analysis.

The results obtained on this material were confirmed on two NSCLC samples freshly prepared after surgical resection with a method modified from Forsberg et al. [6]. Briefly, fresh samples from a region pathologically confirmed to be highly representative of tumor content were minced and filtered through a 70- $\mu$ m cell strainer (BD Biosciences, Bedford, MA, USA). Red blood cells were hemolyzed by incubating samples in hemolysis buffer (0.1 mM EDTA, 10 mM KHCO<sub>3</sub>, 154 mM NH<sub>4</sub>Cl) for 10 min. Samples were centrifuged (200 × g, 10 min) and finally washed in phosphate buffer solution and centrifuged (300 × g, 10 min) to obtain a tumor cell-enriched pellet that was then frozen at –80 °C until analysis.

### 2.4. Preparation of plasma and pleural effusion

Pleural effusion and peripheral venous blood were collected in EDTA-coated tubes and stored at +4 °C. Within 2 h of collection, samples were centrifuged twice (1500 × g for 10 min and 3000 × g for 10 min) to obtain filament- and cell-free plasma and pleural effusion. Samples were frozen at –80 °C until analysis.

### 2.5. SELDI-TOF-MS analysis

Surface enhanced laser desorption/ionization (SELDI) mass spectrometry analysis was used to determine S100A6 levels in cell lysates from cultured cells and surgical specimens (cohort I) and on plasma and pleural effusion. Prior to analysis, cells were lysed as described above and protein concentration was normalized to 1.0 mg/ml. Mean protein concentration in cell lysates from surgical specimens was 3.2 mg/ml (range 1.1–11.9 mg/ml). Samples were profiled using the ProteinChip® SELDI-TOF-MS system Protein Biology System IIC (PBSIIC) (Bio-Rad, Hercules, CA, USA). Each sample was analyzed in duplicates on a reverse phase chip surface (H50, 15% acetonitrile). After incubation, washing and application of matrix solution (sinapinic acid), the ProteinChip® arrays were analyzed and spectra collected in the 3–35 kDa range. Each spot was analyzed twice with separate settings of laser intensity, detector sensitivity and time lag focusing to allow optimal detection of spectral regions between 3–10 kDa and 10–35 kDa. All spectra were baseline corrected (using a peak width value of 8) and peak detection was performed using signal/noise value of 4 and valley depth value of 2 (first pass). The data analysis was performed using the Ciphergen Express package software (Bio-Rad, Hercules, CA, USA). The technical reproducibility of the quantitative analysis of proteins level obtained with SELDI-TOF-MS was tested applying two samples in six replicates on ProteinChip® array surfaces. The average coefficient of variation (CV) of peptide and protein peak intensity was 15% (range 11–19%). Identification of S100A6 was confirmed using antibody capture, as described in [5]. Anti-S100A6 antibody (Sigma, Saint Louis, MO, USA) coupled to RS100 ProteinChip® (Bio-Rad, Hercules, CA, USA) was used to capture S100A6 protein from tumor cell lysates and body fluids. Anti-p21 antibody (St. Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used as a negative

control. The S100A6 post-translational modification pattern seen on MS-profile was confirmed using reduction of the cysteinylated and glutathionylated forms as described in [5].

### 2.6. Tissue microarray construction

Tissue microarray (TMA) was constructed using a robotized arrayer (ATA-27 automated arrayer, Beecher Instruments, Sun Prairie, WI, USA) as previously described [7]. Formalin-fixed paraffin-embedded tumors from patients in cohort II were used as donor blocks. Core samples (0.6-mm diameter) were obtained from two different tumor-rich areas in donor blocks, selected from the corresponding section stained with hematoxylin and eosin, and were arrayed in duplicates in the host block. From TMA, 4- $\mu$ m sections were cut and adhered to the slide according to manufacturer instructions. The upper and lower sections obtained from the TMA were stained with hematoxylin and eosin to check the quality of sample cores in terms of tumor and stromal representation.

A TMA with 14 samples of normal lung parenchyma was constructed separately. Cores (1-mm diameter) from donor blocks were arrayed in duplicates in a host block using a manual arrayer (MTA-I, Beecher Instruments, Sun Prairie, WI, USA). Sections from this TMA were stained to assess marker expression in normal lung tissue.

### 2.7. Immunohistochemistry (IHC)

TMA slides were deparaffinized, rehydrated through graded ethanol to deionized water and pretreated with sodium citrate buffer at pH 6 in microwave for 20 min for optimal antigen retrieval. After rinsing, the endogenous peroxidase activity was blocked by hydrogen peroxide, 0.5% for 30 min. Sections were then rinsed and incubated with blocking serum (1% bovine serum albumin) for 20 min at room temperature and later incubated with a primary antibody overnight in humidity chamber at +8 °C. The following primary antibodies were used: a mouse monoclonal Ab anti-p53 (DO-1) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:50, a rabbit monoclonal Ab anti-S100A4 (NeoMarkers, Fremont, CA, USA) diluted 1:100 and a mouse monoclonal Ab anti-S100A6 (Sigma, Saint Louis, MO, USA) diluted 1:2000. As secondary antibodies, biotinylated horse anti-mouse or goat anti-rabbit IgG were used, diluted 1:200 (Vector Laboratories, CA, USA), for incubation for 30 min. After rinsing, sections were allowed to react for another 30 min with avidin–biotin–peroxidase complex. The peroxidase reaction was developed using 3,3-diaminobenzidine for 6 min. Nuclear counterstaining was performed with Mayers hematoxylin. Tris buffered saline pH 7.4 was used for rinsing between the steps. Negative controls were performed omitting the primary antibody. As positive controls, colon cancer and melanoma were used for p53 and S100A4, respectively. For S100A6, one lung cancer specimen known to express this protein was used.

### 2.8. Evaluation of IHC staining and scoring system

Scoring of the TMA was performed by an experienced pathologist, who was blind to clinical correlates and survival outcomes. Each case was scored by combining the evaluation of four cores from two different TMA slides. Cases that were not successfully evaluated with TMA analysis were re-stained on an entire section and scored using the same scoring system. On the basis of previous reports, immunoreactivity was scored as either positive or negative using a cut-off of 10% of nuclei for p53 [8] and a cut-off of 30% of cells for S100A4 [9], independently on staining intensity. For S100A6 we applied a scoring system that has been developed and validated in pancreatic cancer, the disease where this protein has been more

extensively studied [10]. The intensity of staining was graded as follows: grade 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The immunoreactivity of the sample was determined by the percentage of positive cells: one point for <20%, two points for 20–50%, and three points for >50% of cells. The total score for each case was obtained as the product of intensity of staining and percentage of cells stained. Negative or weakly positive cases had a score of 0–3, moderately positive cases had a score of 4–6, and strongly positive cases had a score of >6. S100A6 expression was then finally divided into two groups: negative cases (with a score  $\leq 3$ ) and positive cases (with a score  $\geq 4$ ).

### 2.9. Statistical analyses

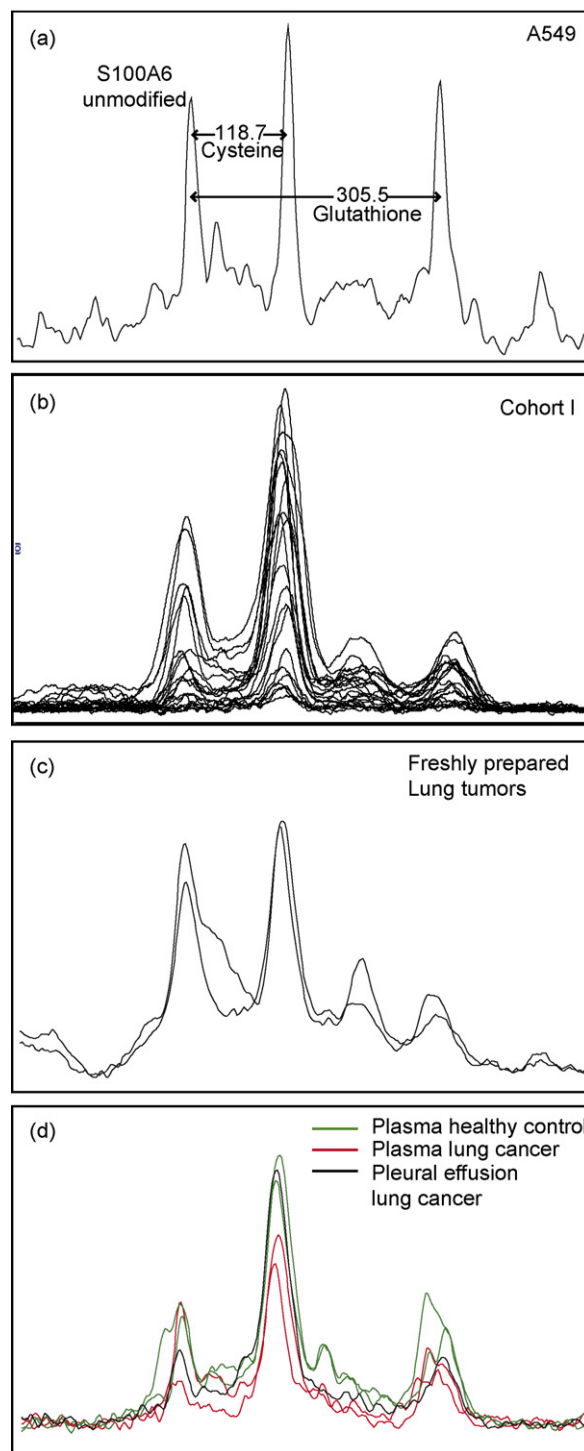
Statistical analyses were performed using the JMP software 4.0.4 and the StatView software 5.0.1 (SAS Institute, Cary, NC, USA). To determine the associations between diverse clinicopathologic variables and markers expression, the Fischer's exact test and the chi-square test were used. Univariate survival analyses were calculated using the Kaplan–Meier method, and curves were examined using the log-rank test. To determine which factors had an independent impact on survival multivariate analysis was performed using the Cox-s proportional hazard method. A  $p$  value < 0.05 was considered statistically significant; all tests were two-sided.

## 3. Results

Based on our study on S100A6 levels and kinetics in lung cancer cell lines [5], we pursued to determine the S100A6 expression in NSCLC using retrospective tumor cell material. We used mass spectrometry-based proteomics to assess S100A6 level and PTM pattern on tumor cell lysates from cohort I ( $n = 39$ ). Additionally, we determined the immunohistochemical expression of S100A6 on paraffin-embedded sections from cohort II ( $n = 103$ ).

### 3.1. S100A6 levels in cohort I

Cohort I included 39 early stage NSCLC patients with over 10 years of follow up. In all cases an  $m/z$  peak at 10.2 kDa was detected in the mass spectra. This peak corresponds to S100A6 (Fig. 1). The post-translational modification pattern of S100A6 (cysteinylated, glutathionylated and non-modified S100A6), that we previously reported in lung and colon cancer cell lines [5] could also be confirmed in this surgical material (Fig. 1a and b). The most represented peak in the cluster, i.e., the peak with the highest intensity, corresponded to the cysteinylated form of S100A6, and in the present cohort had a median relative intensity of 51.4. Based on this value, S100A6 peak intensity was scored as low, if below or equal to the median, or high, if above the median. Patients with a high S100A6 peak intensity had a survival advantage over patients with a low S100A6 peak intensity, with median survival times (MST) of 35 months vs. 18 months, respectively ( $p = n.s.$ ). Median survival time in the entire cohort was 27 months. Tumor cells from this patient cohort had been collected during several years and stored over a long period of time in liquid nitrogen. To verify the findings obtained in cohort I, we profiled a NSCLC cell line (A549) and two freshly collected lung cancer samples using an Ab-capture based SELDI-TOF-MS method. As shown in Fig. 1a–c, the post-translational modifications pattern of S100A6 was similar between the cell line, the samples in cohort I and the fresh lung cancer specimens. Moreover, with the same method, we analyzed one sample of malignant pleural effusion and plasma samples from two patients with advanced NSCLC and two healthy controls. Results are shown



**Fig. 1.** SELDI-TOF-MS overlay spectra of S100A6 expression and post-translational modifications pattern. A549 cell lysate (a). Archival enriched tumor cell lysates from representative cases in cohort I (b). Enriched tumor cell lysates from freshly prepared NSCLC samples (c). Plasma and pleural effusion samples (d).

in Fig. 1d. To our knowledge, this is the first time that the presence of S100A6 is detected in plasma and pleural effusion.

### 3.2. S100A6 expression in cohort II

The mass spectrometry analysis of samples from cohort I displayed a variable expression of S100A6, and suggested a potential

**Table 2**  
Correlation between markers expression

	P53 expression			S100A4 expression		
	Negative	Positive	<i>p</i>	Negative	Positive	<i>p</i>
<b>S100A6 expression</b>						
Negative	49	28	0.01	65	11	0.005
Positive	23	3		15	11	
<b>S100A4 expression</b>						
Negative	54	26	0.2			
Positive	18	4				

clinical implication of this finding in correlation with patients' survival.

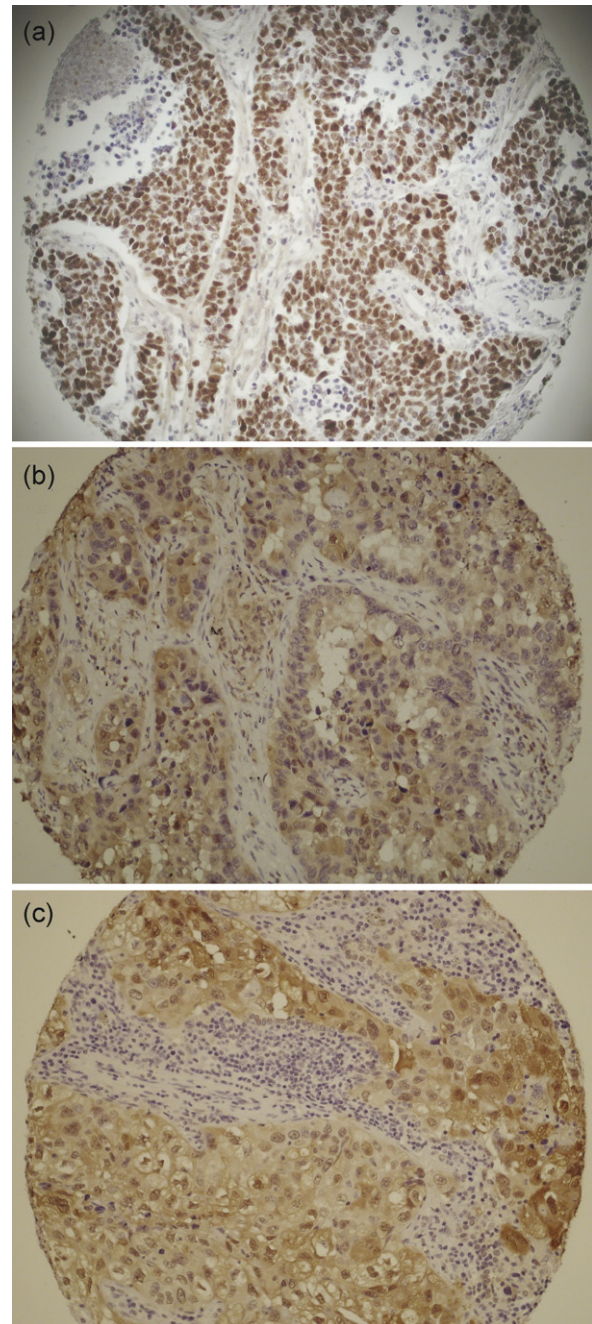
To validate the role of S100A6 as a prognostic marker in lung cancer and to detect possible correlations with p53 and S100A4 expression we used IHC on TMA sections. This analysis was performed on a separate well-characterized patient cohort of 103 stage I NSCLC cases (cohort II).

Immunostaining of TMA sections with primary antibodies was successful in the great majority of cases. p53, S100A4 and S100A6 were not evaluable on TMA sections in 2, 11 and 5 cases, respectively, that were instead evaluated and scored on entire histological sections. In one case, due to lack of material, S100A4 expression was not evaluable. As expected, p53 was expressed only in tumor cells nuclei, and was positive in 31/103 cases. S100A4 and S100A6 expression was negative in all components of normal lung and bronchial tissues i.e., type I and II alveolar cells, ciliated bronchial epithelium, peribronchial glands. In tumor tissues, S100A4 and S100A6 expression was mainly cytoplasmatic. Though nuclear expression could not be excluded, no cases with exclusive nuclear expression were observed. Cases scored as positive were 22/102 for S100A4 and 26/103 for S100A6.

The correlation between marker expression and diverse clinicopathologic characteristics of patients is also shown in Table 1.

No statistically significant correlations were observed between the expression of p53, S100A4 or S100A6 and either age at surgery, kind of surgery or T-stage. As expected, p53 was significantly more expressed in squamous-cell carcinomas and in tumors with poor differentiation. On the contrary, both S100A4 and S100A6 were more expressed in non-squamous tumors (adenocarcinomas and BACs) (Fig. 2). S100A6 was significantly more expressed in well differentiated tumors, and this, together with the higher expression in non-squamous cases might explain the relative higher prevalence of S100A6 positive cases in women (37.5%) compared to men (17.4%). More interesting was the relationship observed between the immunostaining results of the diverse markers (Table 2). S100A6 expression correlated with both p53 expression and S100A4 expression ( $p=0.01$  and  $p=0.005$ , respectively). S100A6 was significantly more expressed in p53-negative cases (32%) compared with p53-positive cases (9%), but, on the contrary, it was found to be more expressed in S100A4-positive cases (50%) than in S100A4-negative cases (13.7%). Not confirming what has been reported elsewhere [11], no correlation between S100A4 and p53 expression was observed.

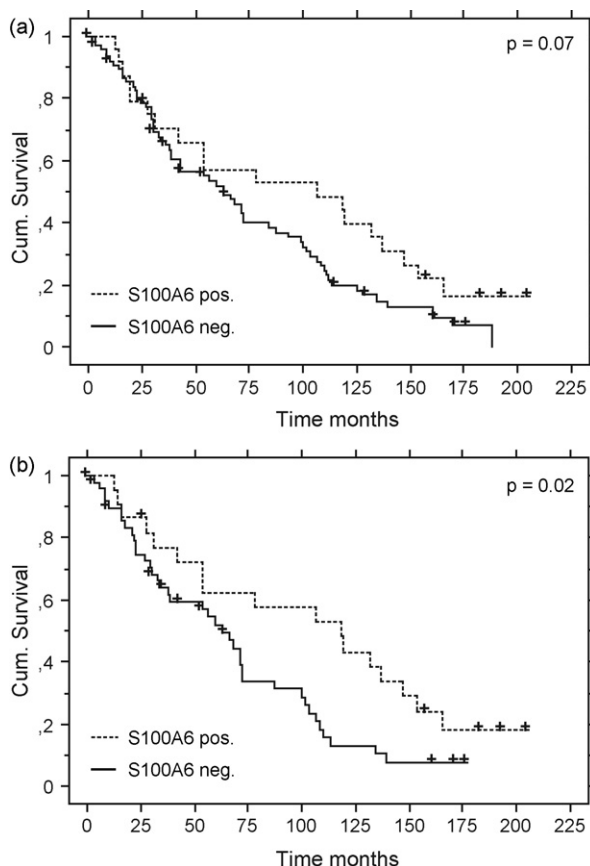
Survival analyses are shown in Fig. 3. MST in the overall patient cohort was 65.5 months, with a 5-year survival rate of 53.2%. No prognostic impact was shown for both p53 and S100A4 expression. Patients with a positive expression of S100A6 had a MST of 92.5 months, while patients with a negative expression of S100A6 had a MST of 61.5 months. This difference did not reach statistical significance ( $p=0.07$ ) (Fig. 3a). When selecting only p53-negative cases (72 patients), patients with a positive S100A6 expression had a significant longer MST compared to patients with a negative S100A6 expression (112 months vs. 61.5 months, respectively; HR



**Fig. 2.** Representative positive immunostainings for p53 (a), S100A4 (b) and S100A6 (c).

0.52, 95% CI 0.29–0.94;  $p=0.02$ ) (Fig. 3b). At multivariate analysis, after adjusting by T-stage, histology, grade of differentiation and kind of surgery, age at surgery and S100A6 expression were significant independent prognostic factors in p53-negative cases (HR 0.47, 95% CI 0.27–0.81,  $p=0.007$  for age at surgery <70 years; HR 0.49, 95% CI 0.27–0.81,  $p=0.017$  for S100A6 positive expression).

To further explore the possible prognostic impact of the co-expression of the two S100 proteins, the subset of patients with a positive S100A4 expression was further divided in two groups according to S100A6 expression. MST in 11 patients whose tumors expressed both S100A4 and S100A6 was 132 months, compared to 63 months in 11 patients whose tumors were S100A4 positive but S100A6 negative (log-rank  $p=0.01$ ).



**Fig. 3.** Univariate survival analysis of stage I NSCLC patients from cohort II based on positive (dotted line) or negative (straight line) S100A6 expression. Whole patient population (101 patients) (a), p53-negative cases (72 patients) (b).

#### 4. Discussion

In the present study we used a mass spectrometry-based method to validate on clinical material our previous *in vitro* findings obtained on lung cancer cell lines. We demonstrated that the PTM pattern of S100A6 was consistent even in surgically resected NSCLC. We also found that the relative intensity of the peak corresponding to the cysteinylated form of S100A6 correlated with survival. However, we could not determine what would be the possible clinical implications of this PTM pattern, in terms of lung cancer specificity or possible modifications after genotoxic stress, such as neoadjuvant treatments.

Moreover, S100A6 was detectable in plasma from NSCLC patients and healthy controls, as well as in pleural effusion from one patient with NSCLC. This latter finding, that to date has not been previously reported, might support the potential extracellular function of S100A6 in the induction of apoptosis through the activation of the extrinsic pathway, as proposed in a recently published *in vitro* study [12].

To study further the implications of the survival results obtained in cohort I, we determined the immunohistochemical expression of S100A6 in a distinct group of patients composed of 103 stage I NSCLC cases. S100A6 was significantly more expressed in non-squamous tumors, and showed a significant direct and inverse correlation with the expression of S100A4 and p53, respectively. *In vitro* we found that the increase in S100A6 levels and the PTM pattern changes after irradiation were dependent upon TP53 status, both expression and PTM changes being enhanced only in TP53 wt cells and not in TP53-/- cells. The results from the present study

support this preclinical evidence, since the immunohistochemical expression of p53 likely reflects the presence of a missense gene mutation that leads to the nuclear accumulation of p53 [13].

Finally, increased S100A6 expression indicated a beneficial trend for survival in our patient population. This benefit turned independently significant when analyzing only p53-negative cases. These results support the hypothesis of a possible role of p53 in regulating S100A6 cellular level and function in apoptosis [14] and cytoskeleton rearrangement [15]. It is worth to be noted that in the present patient cohort the immunohistochemical expression of p53 was not of prognostic importance. Although meta-analyses of retrospective data have found an overall negative prognostic impact of p53 expression in resected NSCLC [16], results from single investigations are conflicting. For instance, if considering studies that included mainly stage I NSCLC cases, a positive correlation between p53 protein expression and worse overall survival was detected in some reports [17,18], but not confirmed in others [19,20]. Moreover, the immunohistochemical detection of the nuclear presence of p53 alone might not provide full information about the functional status of the TP53-dependent pathways. In the present study, the lack of correlation between p53 expression and patients' survival may well depend upon sample size limitations, but it is also likely that other p53-independent pathways might have driven the biological behavior of these tumors.

This is the first time that the expression and prognostic role of S100A6 have been specifically assessed in NSCLC. In relation to studies performed on other tumor forms, the evaluation of S100A6 yielded conflicting results. In pancreas [10,21,22], thyroid [23,24] and colorectal [25] cancers, S100A6 expression has been directly correlated with the neoplastic phenotype, being more expressed in cancer than in normal tissues. Additionally, S100A6 expression was reported to be a negative prognostic factor in pancreatic tumors [10], and in colorectal cancer it was more expressed in liver metastasis than in primary tumors [25]. Completely opposite results have been obtained in osteosarcoma [15] and cholesteatoma [26], where increased S100A6 levels in tumor cells have been coupled with improved survival. Moreover, loss of S100A6 in tumors compared to benign proliferative lesions has been observed for prostate cancer [27] and melanoma [28]. Particularly interesting is how epigenetic changes, namely promoter hypermethylation, would down-regulate S100A6 during cancer progression [29]. Although tissue specific function may well vary from different tumor types, in our study all normal lung specimens were negative for S100A6 staining, and only 25% of tumors were positive. An evaluation of S100A6 expression on premalignant lung lesions could help to clarify whether this protein might in some way be involved in lung carcinogenesis and cellular response to smoking-related damage.

In terms of S100A4, it has been studied as a mediator of invasion and metastasis, and more consistently correlated with poor prognosis in various tumor types [30]. We failed to demonstrate a negative prognostic role of S100A4 expression in NSCLC, as well as possible correlations with p53 status. This might depend on sample size limitations. In previous reports the expression of S100A4 in NSCLC, assessed with IHC [9,11] or real-time PCR [31], was correlated with poor survival. However, these studies included mainly, [9] if not exclusively, [11,31] adenocarcinomas, and a subgroup survival analysis on stage I node-negative cases was lacking. In our stage I specimens, S100A4 was expressed in 39% of non-squamous tumors, although without any prognostic meaning (data not shown). This might implicate a role of S100A4 in the metastatic phenotype of NSCLC at later stages, or histology specificity.

The survival differences that we observed in S100A4 positive cases with different S100A6 expression suggest that in NSCLC S100A6 could act antagonistically to S100A4 and modulate its

metastatic potential. Such functional regulation is further supported by the fact that several S100 proteins form heterodimers. One example of functional regulation through formation of S100-heterodimers is S100A1 reduction of S100A4-induced motility and growth in soft agar and metastasis *in vivo* [32]. S100A1 by itself did not reduce cell motility, invasion and formation of metastases but only reduced the effects of S100A4. It is tempting to speculate a similar function of S100A6 in interfering with S100A4 activities. Furthermore, since both S100A6 and S100A4 have shown to bind tropomyosin [33,34] this binding could be competitive and have effects on S100A4-induced cell migration. However this needs to be studied further.

Another member of the S100 family that has been previously studied in lung cancer is S100A2. The expression of this putative tumor suppressor gene, at either the mRNA or protein level, has been assessed in several papers, with conflicting results [11,35–40]. The largest of these reports, which is a study with a similar design to the present paper, evaluated the immunohistochemical expression of S100A2 on tumor samples from 113 patients with stage I NSCLC [39]. Although no correlation was observed between S100A2 expression and any tumor or patient characteristics, S100A2-positive cases (70%) showed a worse prognosis compared with S100A2 negative cases (5-year survival rate of 38% vs. 72%, respectively,  $p < 0.001$ ). An evaluation of S100A2 expression was beyond the scope of this paper, since we lacked specific pre-clinical evidence supporting possible interactions between S100A2 and S100A6.

The main limitations of the present study are the small sample size and the lack of clinical information about progression-free survival and cancer specific survival. This sensibly decreases the statistical power to perform correlation analyses on multiple biomarker expression. Moreover, although the decision to assess the expression of S100A4 and S100A6 was based on our *in vitro* study on lung cancer cell lines [5], other S100 proteins should also be evaluated. Finally, we included in our analysis only p-stage I patients due to the possible clinical implications of our findings in the management of node-negative NSCLC. However, to speculate on the potential role of S100A4 and S100A6 in the invasion process, these proteins should be assessed also in tumors at later stages, including nodal and distant metastases.

In conclusion, our study for the first time provides information about the PTM pattern of S100A6 in NSCLC tissue, the presence of S100A6 in plasma and pleural effusion, and the possible prognostic implications of S100A6 in stage I NSCLC in correlation with p53 expression. Altogether, this study further contributes to the understanding of S100 proteins significance in lung tumors.

### Conflict of interest statement

No conflict of interest to declare.

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