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ORIGINAL RESEARCH

Prognostic significance of different immunohistochemical S100A2 protein expression patterns in patients with operable nonsmall cell lung carcinoma

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Abstract: S100 proteins are involved in carcinogenesis, metastasis, and survival. S100A2 is a member of the S100 family, and its expression and precise role in patients with non-small cell lung carcinoma (NSCLC) has been debated. Therefore, we examined the immunohistochemical expression patterns of S100A2 in NSCLC in relation to clinicopathological parameters, important molecular biomarkers, and patient outcome. Microarray data for 74 paraffin-embedded specimens from patients with NSCLC were immunostained for S100A2 and p53 proteins. Immunohistochemical staining patterns of S100A2 in the NSCLC tissue samples examined were either nuclear (nS100A2), cytoplasmic (cS100A2), or both. A significant association between nS100A2 positivity and better disease-free interval was observed (hazards ratio 0.47; 95% confidence interval 0.23–0.99; $P = 0.047$). Similarly, cS100A2 negativity was marginally associated with shorter overall survival ($P = 0.07$). Patients without lymphatic infiltration and an earlier disease stage had significantly better overall survival and disease-free interval. The S100A2 expression pattern in operable NSCLC varies widely, and this differential expression (nuclear, cytoplasmic or both) seems to correlate with prognosis. Intensity of expression was highest in the early and advanced stages, and equally distributed in the middle stages. This observation may be indicative of a dual role for this protein both during earlier and advanced disease stages, and may also explain the differential immunoexpression of S100A2. Analysis of the disease-free interval showed that nS100A2-negative and p53-positive expression was associated with a statistically significant ($P = 0.003$) shorter disease-free interval in comparison with nS100A2-positive and p53-negative expression (12 versus 30 months, respectively). Further studies are required to establish whether S100A2 protein may have a substantial role as a prognostic or predictive indicator in this unfavorable group of patients.

Keywords: S100A2, expression, lung cancer, thoracic surgery

Introduction

Lung carcinoma is the leading cause of cancer death worldwide, and accounted for 18% of cases in the global statistics for 2008.[1] Metastasis and its complications are the main factors contributing to mortality in patients with lung cancer. Invasion and migration are typically considered essential steps for expression of the metastatic potential of a tumor. Therefore, reliable prognostic biomarkers that may help in optimal treatment planning are urgently needed.[2,3]

The S100 protein family is a multigenic group of cytoplasmic EF-hand Ca^{2+}-binding proteins that have been proposed as potential biomarkers. Their expression

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has been involved in both intracellular and extracellular functioning. Regulation of protein phosphorylation, enzyme activity, calcium homeostasis, cytoskeletal components, and transcriptional activity are examples of such actions. S100 proteins exert a range of effects on p53 activity. S100A4 and S100B inhibit p53 transcriptional activity and S100A2 promotes it, while S100A4 enhances p53-dependent apoptosis. Many members of this family have a role in modulating cytoskeletal dynamics. Their action in actin, myosin, and tropomyosin, and direct interaction with tubulins and intermediate filaments are implicated in metastasis. Some S100 members, including S100A1 and S100A11, play a role in modulating cell proliferation. Some members act extracellularly as tumor promoters and others as tumor suppressors.[4]

S100A2 is a member of this family of proteins, with conflicting published results in cancer patients. Feng et al[5] suggested S100A2 to be a putative tumor suppression factor in the early stages of carcinogenesis in the human lung. Downregulation of S100A2 in lung cancer, melanoma,[6] and breast cancer has been reported to be associated with tumor progression.[7] Similarly, S100A2 overexpression has also been significantly associated with poor survival and a high risk of metastasis in surgically resected nonsmall cell lung cancer (NSCLC).[3]

Heighway et al[8] and Wang et al[9] have proposed that S100A2 is frequently overexpressed in lung cancer. Although most normal lung cells do not express S100A2, strong expression was found in a line of basilar epithelial pulmonary cells that cover the large airways.[10] This finding further supports the hypothesis that these normal cells may actually represent the type of pulmonary cell that is most exposed to oncogenic factors and overexpression of S100A2, so represents an early event in carcinogenesis in the human lung. We hypothesized that the role of S100A2 is not clearly defined, having noticed an imbalance in the intensity of expression of the protein between the early, late, and middle stages of cancer, and undertook this study to investigate whether at least a dual role of the protein may exist.

Materials and methods

Paraffin-embedded specimens from 74 patients with NSCLC were examined. All patients underwent radical excision of their primary tumor (lobectomy or pneumonectomy), together with regional lymphadenectomy between January 2002 and December 2005. Histology reports were issued in accordance with World Health Organization criteria.[11] Staging was performed according to the Seventh Edition of TNM in Lung Cancer.[12] Institutional review board approval from Evangelismos General Hospital, Athens, Greece, was obtained to use archive material for research purposes. Patient survival was calculated from the day of surgery until death in months.

Tissue microarrays

Representative areas from each tumor in hematoxylin and eosin-stained sections were chosen and subsequently punched out from donor paraffin blocks in order to construct tissue microarrays with a manual model ATA-100 tissue arrayer (Chemicon International, Temecula, CA). One to five 2 mm wide tissue cores were chosen from each tumor. All cases were distributed over eight tissue microarray recipient paraffin blocks, which were incubated at 56°C for 5 minutes in order for recipient and donor paraffin to adhere to each other.

Immunohistochemistry

Immunostaining of the tissue microarrays was performed on 3 μm thick sections deparaffinized in xylene and hydrated in a series of graded alcohol dilutions. The slides were boiled in a microwave at 650 W for 20 minutes, immersed in a high pH target retrieval solution (K8004, DAKO, Carpinteria, CA) and subsequently cooled at room temperature for 20 minutes. Endogenous peroxidase activity was blocked using the S2001 peroxidase-blocking reagent (K5007, DAKO). Immunohistochemistry was performed using the DAKO Autostainer Plus device. Sections were incubated with primary antibodies against S100A2 (mouse monoclonal, clone DAK-S100A2/1, DAKO, 1/100 dilution), Ki67

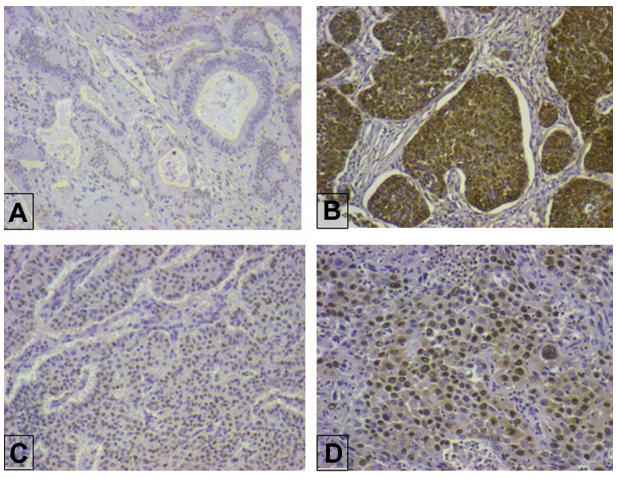

Figure 1 Representative nonsmall cell lung cancer cases with different S100A2 immunohistochemical staining patterns. (**A**) Negative, (**B**) cytoplasmic and nuclear, (**C**) nuclear score 2, and (**D**) nuclear score 3.

Table 1 Patient characteristics

Variable	Number of patients	Percentage (%)
Age (years)		
Mean ± SD	65.51 ± 7.74	
Gender		
Male	59	79.7
Female	15	20.3
Smoking (p/years)		
Mean ± SD	59.66 ± 31.47	
Histology		
Adenocarcinoma	29	39.2
Squamous	28	37.8
Adenosquamus	5	6.8
Large cell/neuroendocrine	9	12.2
Other	3	4.1
Grade		
Low	17	23
Medium-low	19	25.7
Medium	38	51.4
Stage		
IA	7	9.5
IB	24	32.4
IIB	17	23.0
IIIA	22	29.7
IIIB	4	5.4
FEV_1		
<70	12	16.2
>70	62	83.8
Operation		
Lobectomy	36	48.6
Pneumonectomy	22	29.7
Wedge	11	14.9
Bilobectomy	3	4.1
Other	2	2.7
Pleural invasion		
No	41	55.4
Yes	33	44.6
Infiltration of vascular branches		
No	39	52.7
Yes	35	47.3
Infiltration of lymph vessels		
No	57	77.0
Yes	17	23.0
Preoperative chemotherapy		
No	72	97.3
Yes	2	2.7
Postoperative chemotherapy		
No	17	23.0
Yes	57	77.0
Preoperative radiotherapy		
No	72	97.3
Yes	2	2.7
Postoperative radiotherapy		
No	40	54.1
Yes	34	45.9
Site of metastasis		
None	19	25.7
Brain	7	9.5
Liver	10	13.5
Thoracic wall	3	4.1
Lymph nodes	3	4.1
Lung	18	24.3
Pleura	2	2.7
Bones	10	13.5
Mediastinum	1	1.4
Kidneys	1	1.4

(mouse monoclonal, clone MIB-1, DAKO, 1:100 dilution), cleaved Caspase-3 (Asp175) (rabbit monoclonal, clone 5A1, Cell Signaling, Beverly, BA, 1:100 dilution), and p53 (mouse monoclonal, clone DO-7, DAKO, 1/50 dilution). The slides were then incubated with Envision-horseradish peroxidase for 30 minutes, following which the antigen-antibody complex was visualized using DAB chromogen for 10 minutes. All sections were lightly counterstained with hematoxylin prior to mounting. All series included both positive (ie, tissues known to express the relevant antigen) and negative (ie, duplicate sections processed as above, apart from omitting incubation with the primary antibody solution) controls.[10]

Evaluation of the immunostained slides consisted of estimating the percentage of positive cells per tissue microarray punch for all antibodies. For Ki67 and p53, only nuclear staining was considered as positive, whereas both nuclear and cytoplasmic staining were considered as positive for the remaining antibodies, ie, S100A2 and Caspase 3. If more than one punch was used from a single case, the average percentage was calculated. For evaluation of immunoreactivity in tumor cells, a dichotomized scoring system was used as follows: p53 positivity if > 20% of tumor cells demonstrated nuclear staining,[13] a high Ki-67 labeling index if > 30% tumor cells stained, and Caspase-3 positivity if nuclear or cytoplasmic staining was present in >3.22% of tumor cells, according to the 75th percentile.

Immunostained slides for S100A2 were evaluated by estimating the percentage of positive cells per case. Both nuclear and cytoplasmic staining were observed. Immunostaining was registered as predominantly nuclear, predominantly cytoplasmic, or both. The intensity of immunostaining was semiquantitatively assessed as follows: 0, no staining; 1, weak staining; 2, moderate staining; or 3, intense staining. For data grouping, a case was considered to be positive for nuclear, cytoplasmic, or both nuclear and cytoplasmic

Table 2 Pathologic characteristics

Variable	Number of patients	Percentage (%)
Nuclear S100A2		
Negative	22	29.7
Positive	52	70.3
Cytoplasmic S100A2		
Negative	27	36.5
Positive	47	63.5
Intensity of S100A2		
0	12	16.2
1	11	14.9
2	36	48.6
3	15	20.3
p53		
Negative	28	37.8
Positive	39	52.7
Ki67		
Negative	39	52.7
Positive	27	36.5
Caspase 3		
Mean value ± SD	1.93 ± 1.50	

staining if > 10% of the cells were scored with 1, 2, or 3 intensity.[10] Staining patterns are shown in Figure 1.

Statistical analysis

Quantitative variables were expressed as the mean, standard deviation, median, and interquartile range. Categorical variables were expressed as frequencies and percentages. We used the Kolmogorov-Smirnov test and normal probability plots to test the normal distribution of the calculations. The protein categories were compared using the Chi-squared test for qualitative data. However, if a cell in the table had few expected cases (ie, less than five), Fisher's Exact test was used. All continuous variables were compared using the Student's *t*-test, and the Mann-Whitney U test was used when their distributions were not normal. Kaplan-Meier estimates and log rank tests were used to estimate and compare survival functions between the two protein categories (ie, negative versus positive). Survival analysis

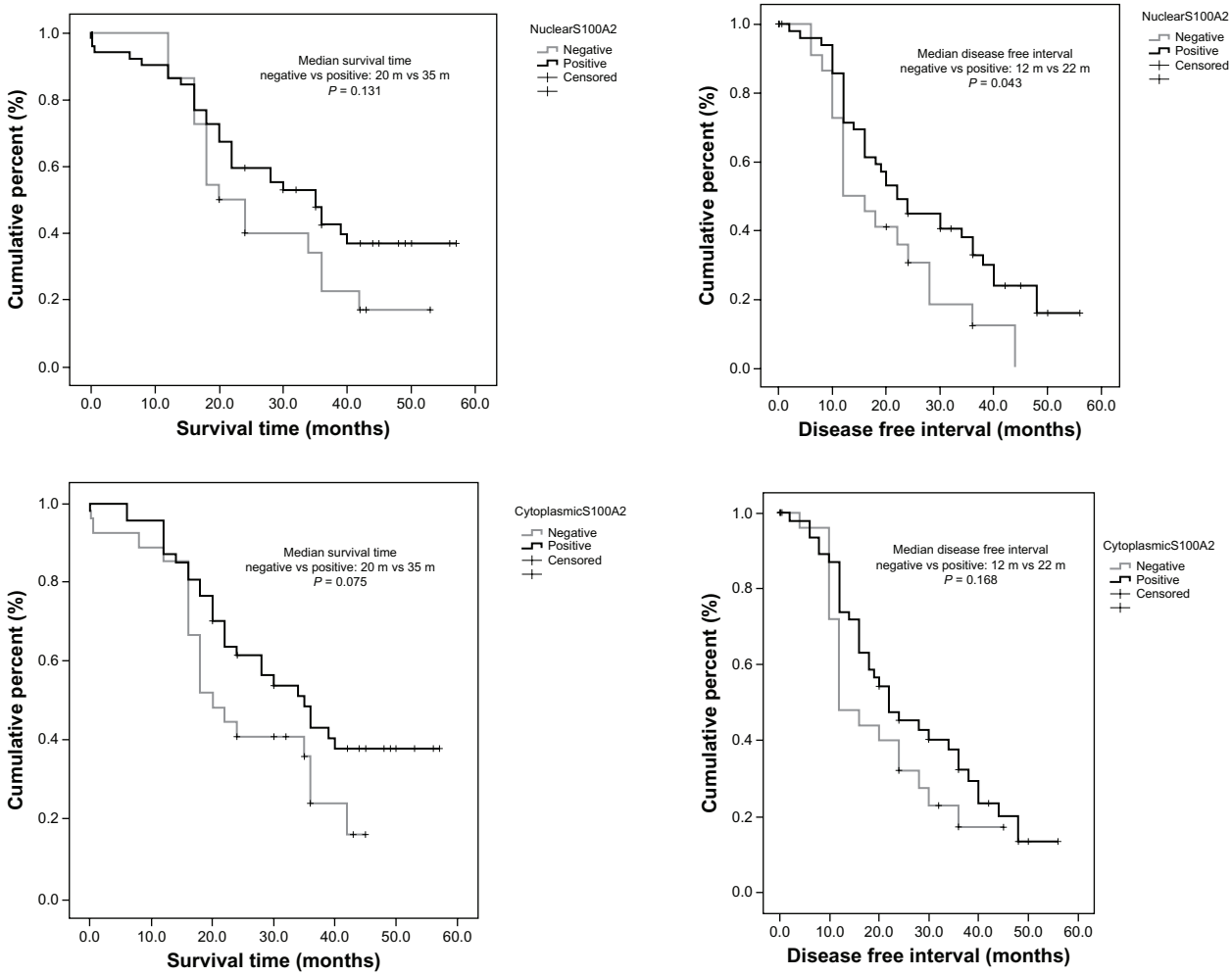

Figure 2 Mantel Cox survival and disease-free interval analysis for nuclear S100A2 and cytoplasmic S100A2.

included Kaplan-Meier estimates and Cox proportional hazards models, using disease-free interval and mortality as the outcomes of interest. Estimated time to an event was calculated as time from day of surgery to death or to a diagnosed metastasis. Each outcome was analyzed using multivariate Cox proportional hazards model [adjusted hazard ratio (95% confidence interval)] for clinical and demographic variables. All multivariate regressions were adjusted for variables that were significantly different between protein categories from univariate analysis or believed to be potential confounders. All tests were two-tailed, and statistical significance was considered for P values < 0.05. We also took into account all the statistical differences between $0.05 < P < 0.1$ given that they could highlight a possible tendency. Statistical analysis of the data was performed using the Statistical Package for the Social Sciences version 16.0 (SPSS Inc, Chicago, IL).

Results

Seventy-four patients (59 male, 79.7%) were enrolled in the study. Their age ranged from 36 to 80 years, with a median age of 66 years. In this cohort, 57 (77.02%) patients were smokers, with 43 (75.43%) being current smokers and 14 (24.57%) being former smokers, and 12 (16.2%) having a forced expiratory volume in one second < 70%. Regarding histology, 29 (39.2%) were diagnosed with adenocarcinoma, 28 (37.8%) with squamous cell carcinoma, five (6.8%) with adenosquamous carcinoma, nine (12.2%) with large cell/neuroendocrine neoplasms, and three (4.1%) with other types of NSCLC. Clinical data for the patients are given in Table 1.

Table 3 Univariate analysis of qualitative variables for nuclear S100A2 and cytoplasmic S100A2

Variables	Nuclear S100A2		*P*-value	Cytoplasmic S100A2		*P*-value
	Negative	Positive		Negative	Positive	
Gender						
Male	19 (86.4%)	40 (76.9%)	0.529	20 (74.1%)	39 (83.0%)	0.382
Female	3 (13.6%)	12 (23.1%)		7 (25.9%)	8 (17%)	
Stage						
I	6 (27.3%)	25 (48.1%)	0.125	8 (29.6%)	23 (48.9%)	0.143
II–III	16 (72.7%)	27 (51.9%)		19 (70.4%)	24 (51.1%)	
Histology						
Squamus	6 (27.3%)	22 (42.3%)	0.334	7 (25.9%)	21 (44.7%)	0.031
Adenocarcinoma	12 (54.5%)	17 (32.7%)		10 (37%)	19 (40.4%)	
Adenosquamus	2 (9.1%)	3 (5.8%)		5 (18.5%)	0 (0%)	
Large cell/neuroendocrine	2 (9.1%)	7 (13.5%)		4 (14.8%)	5 (10.6%)	
Other	0 (0%)	3 (5.8%)		1 (3.7%)	2 (4.3%)	
Grade						
Low	6 (27.3%)	11 (21.2%)	0.831	7 (25.9%)	10 (21.3%)	0.835
Low/middle	5 (22.7%)	14 (26.9%)		6 (22.2%)	13 (27.7%)	
Middle	11 (50.0%)	27 (51.9%)		14 (51.9%)	24 (51.1%)	
Pleural infiltration						
Yes	11 (50%)	22 (42.3%)	0.613	13 (48.1%)	20 (42.6%)	0.808
No	11 (50%)	30 (57.7%)		14 (51.9%)	27 (57.4%)	
Vascular infiltration						
Yes	10 (45.5%)	25 (48.1%)	1.000	13 (48.1%)	22 (46.8%)	1.000
No	12 (54.5%)	27 (51.9%)		14 (51.9%)	25 (53.2%)	
Lymph vessels infiltration						
Yes	2 (9.1%)	15 (28.8%)	0.087	6 (22.2%)	11 (23.4%)	1.000
No	20 (90.9%)	37 (71.2%)		21 (77.8%)	36 (76.6%)	
p53						
Positive	9 (50%)	30 (61.2%)	0.420	12 (52.2%)	27 (61.4%)	0.603
Negative	9 (50%)	19 (38.8%)		11 (47.8%)	17 (38.6%)	
FEV_1 (%)						
>70%	20 (90.9%)	42 (80.8%)	0.491	24 (88.9%)	38 (80.9%)	0.517
<70%	2 (9.1%)	10 (19.2%)		3 (11.1%)	9 (19.1%)	
Ki67						
Positive	5 (27.8%)	22 (45.8%)	0.263	10 (43.5%)	17 (39.5%)	0.797
Negative	13 (72.2%)	26 (54.2%)		13 (56.5%)	26 (60.5%)	

Table 4 Univariate analysis of quantitative variables for nuclear S100A2 and cytoplasmic S100A2

Variables	N	Mean ± SD	*P*-value
Nuclear S100A2			
Age			
Negative	22	65.27 ± 5.48	0.863
Positive	52	65.61 ± 8.57	
Smoking (p/years)			
Negative	22	65.23 ± 30.61	0.326
Positive	52	57.31 ± 31.83	
Maximum tumor size			
Negative	22	5.62 ± 4.02	0.614
Positive	51	5.14 ± 3.57	
Apoptosis (Caspase 3)			
Negative	19	2.13 ± 2.15	0.557
Positive	52	1.85 ± 1.60	
Cytoplasmic S100A2			
Age			
Negative	27	63.70 ± 8.26	0.291
Positive	20	66.25 ± 7.81	
Smoking (p/years)			
Negative	27	52.22 ± 34.98	0.138
Positive	20	66.75 ± 29.12	
Maximum tumor size			
Negative	27	5.66 ± 4.35	0.812
Positive	20	5.36 ± 4.06	
Apoptosis (Caspase 3)			
Negative	25	1.77 ± 1.34	0.890
Positive	20	1.71 ± 1.58	

Positive nuclear expression of S100A2 was demonstrated in 52 (70.3%) cases and positive cytoplasmic expression in 47 (63.5%) cases. p53 positivity was present in 39 (52.7%) cases and high Ki67 in 27 (36.5%). The mean Caspase 3 value was 1.93 ± 1.50. There was an unequal distribution of S100A2 intensity between the cohorts. An intensity of 0 was noted in 12 (16.2%), 1 in 11 (14.9%), 2 in 36 (48.6%), and 3 in 15 (20.3%) cases (Table 2).

Follow-up was available for all patients, with a median follow-up of 24 months and a range of 0.2–57 months. Forty-seven patients (63.5%) died during the follow-up period. The median survival time was 30.3 months (95% confidence interval 28.5–35.8 months). Using the multivariate Cox proportional hazards model we investigated the effect of different immunostaining patterns (nS100A2, cS100A2, and iS100A2) on overall survival and disease-free interval when modified for possible important confounders (Figure 2).

Relationship between different tumor variables and S100A2

A significant association between cytoplasmic positivity and squamous cell carcinoma histology was noted in the univariate analysis ($P = 0.031$). Nuclear S100A2 positivity showed a trend toward significance ($P = 0.087$), with positive lymph vessel infiltration (Table 3). Intensity of S100A2 revealed that there was a statistically significant relationship with disease stage ($P = 0.023$). There was an unequal distribution of the highest intensity of S100A2 that was noticed in the early and advanced stages. No significant associations between S100A2 and other tumor parameters examined were observed (Tables 4–6).

Nuclear S100A2 immunoreactivity, disease-free interval, and overall survival

There was no statistically significant relationship between nS100A2-positive immunostaining and overall survival ($P = 0.114$), lymphatic infiltration ($P = 0.099$), or disease stage ($P = 0.269$). There was a statistically significant relationship between nS100A2 positivity and disease-free interval (hazards ratio 0.47, 95% confidence interval 0.23–0.99; $P = 0.047$). Positive patients had a 53% lower risk of relapse versus negative patients (Table 7).

Cytoplasmic S100A2 immunoreactivity, disease-free interval, and overall survival

There was no statistically significant relationship between cS100A2 positivity and overall survival. Regarding the relationship between S100A2 intensity versus disease-free interval and overall survival, no statistically significant correlations were found ($P = 0.077$ and $P = 0.097$, respectively). However, patients with intensity 2 had a 68% lower probability of death

Table 5 Univariate analysis of quantitative variables for S100A2 intensity

Variable	S100A2 intensity	N	Mean ± SD	*P*-value
Age	0	12	64.25 ± 4.35	0.936
	1	11	66.18 ± 9.13	
	2	36	65.58 ± 8.59	
	3	15	65.87 ± 7.22	
Smoking (p/years)	0	12	55.00 ± 35.48	0.337
	1	11	69.09 ± 22.56	
	2	36	54.58 ± 35.48	
	3	15	68.67 ± 20.57	
Maximum tumor size	0	12	6.25 ± 4.76	0.197
	1	11	6.58 ± 4.57	
	2	35	4.17 ± 2.33	
	3	15	6.18 ± 4.24	
Caspase 3	0	10	1.60 ± 1.40	0.752
	1	11	1.54 ± 1.43	
	2	35	2.07 ± 2.04	
	3	15	2.09 ± 1.52	

Table 6 Univariate analysis for S100A2 intensity in relation to clinicopathologic parameters

	S100A2 intensity				*P*-value
	0	1	2	3	
Gender					
Male	9 (75%)	9 (81.8%)	26 (72.2%)	15 (100%)	0.153
Female	3 (25%)	2 (18.2%)	10 (27.8%)	0 (0%)	
Stage					
I	2 (16.7%)	6 (54.5%)	20 (55.6%)	3 (20%)	0.023
II–III	10 (83.3%)	5 (45.5%)	16 (44.4%)	12 (80%)	
Histology					
Squamus	2 (16.7%)	5 (45.5%)	13 (36.1%)	8 (53.3%)	0.117
Adenocarcinoma	7 (58.3%)	2 (18.2%)	14 (38.9%)	6 (40%)	
Adenosquamus	2 (16.7%)	0 (0%)	2 (5.6%)	1 (6.7%)	
Neuroendocrine	1 (8.3%)	4 (36.4%)	4 (11.1%)	0 (0%)	
Other	0 (0%)	0 (0%)	3 (8.3%)	0 (0%)	
Grade					
Low	3 (25%)	4 (36.4%)	6 (16.7%)	4 (26.7%)	0.008
Low/middle	3 (25%)	2 (18.2%)	5 (13.9%)	9 (60%)	
Middle	6 (50%)	5 (45.5%)	25 (69.4%)	2 (13.3%)	
Pleural infiltration					
Yes	6 (50%)	5 (45.5%)	16 (44.4%)	6 (40%)	0.965
No	6 (50%)	6 (54.5%)	20 (55.6%)	9 (60%)	
Vascular infiltration					
Yes	6 (50%)	5 (45.5%)	14 (38.9%)	10 (66.7%)	0.344
No	6 (50%)	6 (54.5%)	22 (61.1%)	5 (33.3%)	
Lymph vessels infiltration					
Yes	2 (16.7%)	3 (27.3%)	9 (25%)	3 (20%)	0.909
No	10 (83.3%)	8 (72.7%)	27 (75%)	12 (80%)	
p53					
Positive	5 (55.6%)	7 (63.6%)	16 (50%)	11 (73.3%)	0.483
Negative	4 (44.4%)	4 (36.4%)	16 (50%)	4 (26.7%)	
FEV_1 (%)					
>70%	12 (100%)	8 (72.7%)	29 (80.6%)	13 (86.7%)	0.298
<70%	0 (0%)	3 (27.3%)	7 (19.4%)	2 (13.3%)	
Ki67					
Positive	2 (22.2%)	5 (50%)	12 (37.5%)	8 (53.3%)	0.431
Negative	7 (77.8%)	5 (50%)	20 (62.5%)	7 (46.7%)	

than those having intensity 3 ($P = 0.014$). Similarly, patients in stage II had a 2.4-fold higher probability of death versus patients in stage I ($P = 0.028$, Table 7).

Combined S100A2 and p53 analysis

We further investigated whether p53 expression in combination with different S100A2 expression patterns had any major impact on outcome overall. This was done separately for nuclear and cytoplasmic S100A2 expression (Tables 8 and 9).

In analysis of variance and multiple comparisons between groups, Caspase 3 expression showed a trend towards significance ($P = 0.083$) with nS100A2-positive and p53-negative expression in comparison with nS100A2-negative and p53-positive expression. Caspase 3 expression showed a trend toward significance ($P = 0.094$) in relation to cS100A2 positivity and p53 negativity in comparison with cS100A2-positivity and p53-positivity. Disease-free interval analysis between the categories showed that nS100A2-negative and p53-positive expression was associated with a statistically significant ($P = 0.003$) shorter disease-free interval in comparison with the nS100A2-positive and p53-negative group (12 versus 30 months, respectively).

Discussion

Metastasis is a complex process that involves several as yet undefined steps. In general, invasion into the surrounding tissues, entrance into lymphatics or the bloodstream, and migration are considered essential for metastasis. Biomarkers are typically expressed by neoplastic lung tissue and are used to define its metastatic potential and likely patient outcome. Potentially important biomarkers include the S100 protein family members. The S100 protein family is a multigenic group of cytoplasmic EF-hand Ca^{2+}-binding

Table 7 Multivariate survival and disease free interval analysis for nuclear S100A2, cytoplasmic S100A2 and intensity of S100A2

	Reference category	AHR	95.0% CI		*P*-value
Survival analysis					
Nuclear S100A2	Negative	0.54	0.25	1.16	0.114
Infiltr lymph vessels	No	1.83	0.89	3.75	0.099
p53	Negative	1.49	0.74	3.00	0.267
Ki67	Negative	1.08	0.55	2.12	0.821
Caspase 3	–	1.05	0.88	1.25	0.596
Disease stage	I	1.50	0.73	3.07	0.269
Disease free interval					
Nuclear S100A2	Negative	0.47	0.23	0.99	0.047
Infiltr lymph vessels	No	1.91	0.96	3.80	0.065
p53	Negative	1.26	0.66	2.43	0.484
Ki67	Negative	1.29	0.69	2.40	0.429
Caspase 3	–	1.12	0.96	1.30	0.158
Disease stage	I	1.49	0.78	2.85	0.227
Survival analysis					
Cytoplasmic S100A2	Negative	0.576	0.289	1.150	0.118
Infiltr lymph vessels	No	1.790	0.884	3.626	0.106
p53	Negative	1.531	0.760	3.085	0.234
Ki67	Negative	0.953	0.497	1.826	0.884
Caspase 3	–	1.075	0.901	1.283	0.421
Disease stage	I	1.543	0.755	3.151	0.234
Disease free interval					
Cytoplasmic S100A2	Negative	0.612	0.315	1.187	0.146
Infiltr lymph vessels	No	1.822	0.926	3.582	0.082
p53	Negative	1.279	0.665	2.461	0.461
Ki67	Negative	1.082	0.591	1.981	0.797
Caspase 3	–	1.158	0.992	1.352	0.063
Disease stage	I	1.627	0.852	3.109	0.141
Survival analysis					
Intensity S100A2					0.097
0		0.62	0.25	1.55	0.304
1	3	0.52	0.18	1.53	0.237
2		0.32	0.13	0.80	0.014
Grade					0.260
Low middle	Low	0.51	0.21	1.22	0.132
Middle		0.97	0.46	2.07	0.943
Disease stage	I	1.03	0.48	2.23	0.939
Disease free interval					
Intensity S100A2					0.077
0		0.83	0.34	2.02	0.683
1	3	0.25	0.07	0.86	0.028
2		0.54	0.23	1.22	0.139
Grade					0.798
Low middle	Low	0.77	0.35	1.68	0.509
Middle		0.91	0.43	1.91	0.796
Disease stage	I	0.97	0.47	2.00	0.941

proteins comprising 21 known human members. This protein is expressed in different ways in different cell types, and its actions involve regulation of the inflammatory response,[14] cell-cycle progression, and differentiation.[15]

These proteins are localized in specific cellular compartments. Some of them relocate and transduce Ca^{2+} signaling after Ca^{2+} activation. By this method they interact with different targets specific for S100 proteins. Some members of this protein family are even secreted from cells exerting extracellular cytokine-like activities. S100A2 expression in most cancers is predominantly cytoplasmic, and is explained by the continuous nature of cytoplasmic accumulation throughout the cell cycle in malignant cells. Nuclear positivity of the protein is considered to be transient. Nuclear localization predominates in normal epithelial squamous cells. This observation is supported by immunolocalization

Table 8 Combined nuclear and cytoplasmic S100A2 and p53 expression in relation to FEV_1, lymph vessel invasion, Ki 67 and Caspase 3 expression

	$FEV_1 > 70$	$FEV_1 < 70$	Lymph invasion		Ki67–	Ki67+	Caspase 3
			Negative	Positive			
Nuclear– and p53–	0 (0%)	9 (100%)	8 (88.9%)	1 (11.1%)	6 (66.7%)	3 (33.3%)	9
Nuclear– and p53+	1 (11.1%)	8 (88.9%)	8 (88.9%)	1 (11.1%)	7 (77.8%)	2 (22.2%)	8
Nuclear+ and p53–	4 (21.1%)	15 (78.9%)	13 (68.4%)	6 (31.6%)	13 (72.2%)	5 (27.8%)	19
Nuclear+ and p53+	6 (20%)	24 (80%)	22 (73.3%)	8 (26.7%)	13 (43.3%)	17 (56.7%)	30
P-value	0.470		0.501		0.118		0.054
Cytoplasmic– and p53–	0 (0%)	11 (100%)	7 (63.6%)	4 (36.4%)	7 (63.6%)	4 (36.4%)	11
Cytoplasmic– and p53+	3 (25%)	9 (75%)	10 (83.3%)	2 (16.7%)	6 (50%)	6 (50%)	11
Cytoplasmic+ and p53–	4 (23.5%)	13 (76.5%)	14 (82.4%)	3 (17.6%)	12 (75%)	4 (25%)	17
Cytoplasmic+ and p53+	4 (14.8%)	23 (85.2%)	20 (74.1%)	7 (25.9%)	14 (51.9%)	13 (48.1%)	27
P-value	0.323		0.634		0.429		0.094

studies showing that the S100A2 protein is preferentially located in the nucleus in normal tissues.[16–18] The same relocation of S100A2 from the nucleus to the cytoplasm has been observed in normal cultured human keratinocytes.[19] In our study, we noticed an equal distribution of S100A2 positivity between the nucleus and cytoplasm. It seems that there was a balanced expression of the protein between nucleus and cytoplasm for the specific cohort of stage and grade of patients in our study.

Table 9 Multivariate survival and disease free interval analysis of combined nuclear and cytoplasmic S100A2 and p53 expression

	Number of events	AHR	95.0% CI	
Survival analysis				
Nuclear S100A2– and p53–	3	4.749	26.025	44.642
Nuclear S100A2– and p53+	1	0.000	43.000	43.000
Nuclear S100A2+ and p53–	10	3.073	35.323	47.369
Nuclear S100A2+ and p53+	10	2.796	37.338	48.300
P-value = 0.434				
Disease free interval				
Nuclear S100A2– and p53–	6	3.872	12.856	28.033
Nuclear S100A2– and p53+	9	1.809	10.233	17.323
Nuclear S100A2+ and p53–	12	4.320	22.903	39.836
Nuclear S100A2+ and p53+	23	2.898	19.928	31.290
P-value = 0.031				
Survival analysis				
Cytopl S100A2– and p53–	3	4.095	31.391	47.442
Cytopl S100A2– and p53+	3	3.756	28.971	43.696
Cytopl S100A2+ and p53–	10	3.463	33.080	46.655
Cytopl S100A2+ and p53+	8	2.842	39.230	50.370
P-value = 0.173				
Disease free interval				
Cytopl S100A2– and p53–	7	4.585	14.813	32.787
Cytopl S100A2– and p53+	10	2.600	10.176	20.369
Cytopl S100A2+ and p53–	11	4.529	22.162	39.916
Cytopl S100A2+ and p53+	22	2.897	19.711	31.067
P-value = 0.151				

Overexpression of S100A2 has been found in ovarian cancer, melanoma, lymphoma, gastric tumors, and epithelial tumors of the skin.[20–22] S100A2 is overexpressed in various stages of gastric cancer, and is considered to be an early tumorigenic event rather than a tumor progression marker. Heighway et al[8] showed that S100A2 was strongly expressed in most lung cancers, and a study by Wang et al[9] concluded that the S100A2 protein is strongly expressed in NSCLC. These findings are in disagreement with those reported by Feng et al.[5]

Regarding intensity of S100A2 expression, we found this to be highest in the early and advanced disease stages, and evenly distributed in the middle stages. We do not have an obvious explanation for this finding. However, this observation may be indicative of a dual role for this protein, mostly in the earlier and advanced stages of the disease. This may also explain the differential location of expression of S100A2 in both the nucleus and cytoplasm.

S100A2 has been described as a potential tumor suppressor in human cancer. Its expression has been associated with tumor progression, especially in early lung cancer,[5] melanoma,[23] breast,[7] and esophageal cancer.[24] These findings are in keeping with those in our study. In contrast data reported from Roy Castle group[10] did not provide data on survival, and data from Munster University[25] explored a patient cohort containing a higher proportion of adenocarcinoma cases. This group reported that patients who overexpressed S100A2 has a worse prognosis than controls with low S100A2 expression. In their cohort, two of every three patients were diagnosed with adenocarcinoma, and stage IIIA was considered as "early". In our study, we included approximately equal numbers of adenocarcinomas and squamous carcinomas, and we considered early-stage disease to be stage I only. We included all other stages into a category called stage II for statistical analysis.

Although we found an association between nuclear and/or cytoplasmic S100A2 positivity and a better outcome, our results were not always statistically significant in this regard. However, a statistical association was found between nS100A2 positivity and longer disease-free interval. We attempted to support these findings further by combining p53 and S100A2 expression. The p53 tumor suppressor gene is a key regulator of the cell cycle and triggers apoptosis in response to DNA damage or stress. It has been suggested that patients with p53 positivity (indicative of absence of wild-type p53 protein) have a worse prognosis than those without p53 overexpression, but derive more benefit from adjuvant chemotherapy. In the present study, we also evaluated the relationship between combined p53/S100A2 expression, disease-free interval, and overall survival. Although the number of patients in our study was small, we observed that nuclear S100A2 negativity along with p53 positivity was significantly associated with a shorter disease-free interval. This observation is in keeping with the roles previously proposed for both S100A2 and p53, and should be studied more extensively because they may serve as an important prognostic tool.

In conclusion, we observed S100A2 protein to be evenly distributed in the nucleus and cytoplasm in patients with operable NSCLC. It seems that overexpression of the protein either in the nucleus or in the cytoplasm is associated with a better outcome in operable NSCLC patients. Additional studies in larger cohorts of patients are warranted to explore further the precise role and function of S100A2 in NSCLC.

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Disclosure

The authors report no conflicts of interest in this work.

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