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S100A2–S100P expression profile and diagnosis of non-small cell lung carcinoma: Impairment by advanced tumour stages and neoadjuvant chemotherapy

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ABSTRACT

Early and correct diagnosis of non-small cell lung carcinoma (NSCLC) is essential for the choice of an appropriate anti-cancer therapy. Besides the histopathological diagnosis, molecular profiling by detection of the tumour-associated gene expression might play an upcoming role. As proteins of the S100 gene family show a distinct cell type-specific expression profile, our study focused on the relevance of the S100 family for identification and classification of NSCLCs. Among the S100 members, we identified the expression of S100A1, S100A2, S100A4, S100A6, S100A9 and S100P in human lung carcinoma cells ($H358^{p53-}$, $A549^{p53+}$) or NSCLC tissues. Distinct S100 members are increased in NSCLCs compared with control lung specimens depending on the histopathological subtype. In particular, S100A2 was upregulated in squamous cell carcinomas, whereas S100P was mainly increased in adenocarcinomas. The upregulation of either S100A2 or S100P was detected in early but less in advanced tumour stages and not at all in NSCLC patients who had received neoadjuvant chemotherapy. In conclusion, our study indicates an important role of the S100A2–S100P expression profile for molecular diagnosis of NSCLCs at early and, therefore, prognostically more favourable tumour stage. As the S100A2–S100P profile also allows the histopathological classification, it might significantly support the conventional tumour diagnostics.

1. Introduction

Lung cancer is associated with an extremely poor diagnosis and patient's survival highly depends on histology and cancer stage.[1] The early detection and correct diagnosis of lung carcinomas are therefore essential for the choice of an appropriate anti-cancer therapy. Although the cancer phenotype includes a broad selection of characteristic features,[2] the lung cancer classification is still based on clinicopathological features. Presently, a number of microarray studies suggest the identification of malignant tissues by detection of the whole expression pattern of genes.[3–6] However, the detection of the molecular profile is not yet introduced in cancer diagnostics. This can be partially explained by the extensive costs, an ongoing development of the microarray technologies with missing standardization and the large number of redundant data. Finally, a small number of significant genes might be sufficient for diagnosis of malignant tissues, identification

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of the respective histopathological subtype as well as tumour staging. Our study focussed on the family of S100 proteins because the majority of S100 genes is localised on human chromosome 1q21, a region that is frequently rearranged in tumours.[7] Moreover, S100 members are differentially expressed in various malignancies depending on the type[8–14] and stage of cancer[15–18] including lung cancer.[16,17,19].

The multigene family of S100 proteins consists of at least 20 members, which are commonly characterised by the calcium-binding EF-hand motifs.[20] Members of the S100 family are low-molecular weight proteins forming homo- or heterodimers. As S100 proteins contribute to the regulation of cytoskeleton dynamics and enzyme activity, they are involved in several biological processes including immune response, differentiation and growth.[21] Although their precise function is not well understood, most S100 proteins are believed to mediate regulatory functions via binding to and modulating the biological activity of distinct intracellular compounds. Moreover, defined S100 proteins exert their biological effect through binding to cell surface receptors after release from the cell.[20]

As S100 proteins are not ubiquitously expressed they seem to mediate specific functions in different types of cells.[20] Although it is still under discussion as to what extent members of the S100 family contribute to cancer development,[22] their cell type-specific expression suggests a significant role of S100 proteins in the histopathological classification of tumours. Therefore, our study focused on the importance of the S100 gene family for identification of non-small cell lung carcinomas (NSCLCs) and the impact of multiple alterations that occur in advanced tumour stages and in response to neoadjuvant chemotherapy.

2. Materials and methods

2.1. Patients of study

We studied the tumour and corresponding normal (control) lung tissue of 48 patients with non-small cell lung carcinoma (NSCLC) of either adenocarcinoma histology or squamous cell carcinoma histology (Table 2). They were grouped depending on size of the primary tumour (T), nodal involvement (N) and distant metastasis (M) according to the WHO guidelines.[23] Fourteen NSCLC patients received neoadjuvant chemotherapy (Table 2), which was approximately finished one month prior to surgical tumour resection. The therapy-induced tumour regression was estimated according to the *Bochum* regression grading.[24] The local ethics committee approved this study.

2.2. Isolated lung cells of study

Human bronchiolo-alveolar cell lines A549 and H358 were used. Tumour fibroblasts were isolated from the resected lung tumour after enzymatic digestion with 0.05% trypsin and 15U collagenase P (Roche; Mannheim, Germany). WI-38 cells correspond to primary human fibroblasts from foetal lung (ATCC; Manassas, VA, USA). All cells were cultured in DMEM containing 10% FCS. NHBECs were isolated from resected bronchi and then cultured onto fibronectin/collagen-coated dishes in epith-o-ser medium (C·C·Pro; Neustadt/W., Germany).[25] Monocytes were isolated from human buffy coat by standard centrifugation through a Ficoll density gradient (Biochrom; Berlin, Germany).

2.3. Expression analysis

RNA of cultured cells was extracted by use of the RNeasy Kit (Qiagen, Hilden, Germany). RNA of the tissues was isolated by standard guanidinium isothiocyanate/caesium chloride centrifugation and quantified by UV/VIS spectrophotometry. In a reverse transcription reaction (RT), cDNA was synthesised with Superscript II™ reverse transcriptase (Invitrogen; Carlsbad, CA, USA). Thereafter, cDNA was amplified by real-time PCR containing 5 pmol of each gene-specific primer (Table 1) and SYBR green-PCR mix (Bio-Rad; Munich, Germany). External cDNA standards with identical primer-binding sites were established for amplification of a concentration series of standard molecules. Real-time PCR was performed using the iCycler iQ™ system (Bio-Rad; Hercules, CA, USA). Calculating the amplified signal of the sample cDNA in relation to the standard cDNA curve assessed the gene expression. Standard-calibrated PCR of *survivin* was described earlier.[26]

For immunoblot analysis, proteins were extracted from tissues in Tris-buffered SDS solution containing protease inhibitors, separated by SDS polyacrylamide gel-electrophoresis and blotted onto nitro-cellulose membrane.[27] Cytosolic fractions were prepared after permeabilizing the cells in HEPES buffer containing 50 μg/ml digitonin (Sigma). The remaining nuclei-enriched fraction was spun down at 200*g* for 10 min and lysed in Tris-buffered SDS. Mouse anti-S100A2 antibody was used for S100A2 detection (Sigma). GAPDH immunoblotting with a rabbit polyclonal antibody (Abcam, Cambridge, UK) indicates protein loading. Primary antibodies were visualised by HRP-conjugated secondary antibodies and ECL^{plus} detection (Amersham; Buckinghamshire, UK). The intensities of visualised signals were analysed by use of the LAS 3000 computer-based imaging system (FUJIFilm; Tokyo, Japan) equipped with AIDA 3.5. software (Raytest; Straubenhardt, Germany).

2.4. Immunocyto- and immunohistochemistry

Lung samples were fixed in PBS-buffered 4% formalin and embedded in paraffin. Sections of 2–3 μm were cut, dewaxed, and rehydrated. Blocking, detection, and staining was performed using the ZytochemPlus HRP detection kit (Zytomed Systems; Berlin, Germany). Prior to specific antibody staining, slides were incubated for 10 min in 0.05% Pronase E Solution. Mouse anti-S100A2 (Sigma; 1:100 in PBS) or rabbit anti-S100P (BD Bioscience; 1:400 in PBS) were applied in a humidified chamber at 37 °C for 30 min. After co-staining of the nuclei with haematoxylin standard solution, slides were embedded in glycerol–gelatin mounting medium and examined independently by two pathologists.

For S100A2 immunocytochemistry, cells were fixed in 4% PBS-buffered formaldehyde, permeabilized in 0.5% Nonidet NP-40, blocked with 10% goat serum albumin (DAKO) and stained overnight with the mouse anti-S100A2 antibody (1:100 in PBS) at 4 °C. S100A2 detection was performed with

Table 1 – RT-PCR primers

mRNA	Accession number	Primer	5′–3′ sequence	Annealing (°C)
S100A1	NM_006271	Sense	TGCACTGCTCATATGGGCTCTG	55
		Antisense	ATGTGGCTGGATCCTCAACTGT	
S100A2	NM_005978	Sense	AAGAGGGCGACAAGTTCAAG	58
		Antisense	ATCCATGGCAGGAAGTCAAG	
S100A4	NM_002961	Sense	CTGACTGCTCATATGGCGTGCC	52
		Antisense	CCAACCGGATCCGAGGAGTTTT	
S100A6	NM_014624	Sense	GCCCTCACATATGGCATGCCC	52
		Antisense	TATGGATCCTCAGCCCTTGAGG	
S100A8	NM_002964	Sense	ACAAGTACTCCCTGATAAAGGGG	50
		Antisense	TGCCAG(G)CCCATCTTTATCACCAG	
S100A9	NM_002965	Sense	ATGGAGGACCTGGACACAAATGC	58
		Antisense	TCGTCACCCTCGTGCATCTTCTC	
S100A12	NM_005621	Sense	TAGGCTGGGCATATGACAAAAC	58
		Antisense	TAAAAAGGATCCAGAGAGCTAC	
S100B	NM_006272	Sense	GAGCCCCTCATATGTCTGAGC	55
		Antisense	TGGCTGGATCCTAATCTCACT	
S100P	NM_005980	Sense	ATCTAGCACCATGACGGAAC	55
		Antisense	AGCCTAGGGGAATAATTGCC	
S100Z	AF_437876	Sense	ATGCCCACCCAGCTCGAGATGGCCATGGAC	69
		Antisense	CTCAAGCAGCTTTCTCCAAAGCCCCCAAGC	
18S rRNA	M_10098	Sense	GTTGGTGGAGCGATTTGTCTGG	60
		Antisense	AGGGCAGGGACTTAATCAACGC	

Table 2 – Clinical data of NSCLC patients

	Squamous cell carcinoma		Adenocarcinoma	
Without neoadjuvant chemotherapy				
TNM	IA–IB	IIA–IV	IA–IB	IIA–IV
Number	$n = 6$	$n = 9$	$n = 7$	$n = 8$
Median (range) *survivin* mRNA (a.U.)	117 (22–742)		210 (3–1480)	
With neoadjuvant chemotherapy				
TNM	IA–IB	IIA–IV	IA–IB	IIA–IV
Number (*n*)	4	3	2	4
Median (range) *survivin* mRNA (a.U.)	171 (100–660)		175 (22–742)	
Chemotherapy drug (*n*)	Carboplatin/Paclitaxel (5) Carboplatin/Gemcitabine (1) Doxorubicin/Vincristin (1)		Carboplatin/Paclitaxel (3) Carboplatin/Etoposide/ Vincristin (2) Carboplatin/ Paclitaxel/Etoposide (1)	

a.U., *arbitrary units*.

Alexa Fluor®488-conjugated secondary antibodies (Molecular Probes Europe). Slides were embedded in Mowiol mounting medium (Merck; Bad Soden, Germany) and analysed by fluorescence microscopy.

2.5. Data analysis

Patient's data are given as median. Box and Whisker plots were constructed for illustration: the line within the box marks the 50th percentile (median), boundaries of the box indicate the 25th and 75th percentiles, and Whiskers above and below the box represent the 5th and 95th percentiles. Student's paired *t*-test determined statistical significance of patient's expression data followed by Wilcoxon Signed Rank test or by the ANOVA on Ranks procedure followed by Dunn's method if suggested (SigmaStat and SigmaPlot softwares; Jandel Corp.; San Rafael, CA, USA). The correlation coefficient *R* of the linear regression analysis for significance was tested using the two-sided test. *P* values less than 0.05 indicate a significant difference of data.

3. Results

3.1. Members of the S100 family in lung cells and tissues

Human lung tissues and tumour specimens (representative mix of six NSCLC patients each) as well as lung-associated cells had been analysed to get an overview as to which members of the S100family are expressed in the lung. In lung and tumour specimens, we identified the mRNA expression of several S100 members: S100A1, S100A2, S100A4, S100A9 and S100P (Fig. 1). S100A8, S100A12 and S100Z mRNAs were also detected in lung and tumour tissues but primarily in isolated

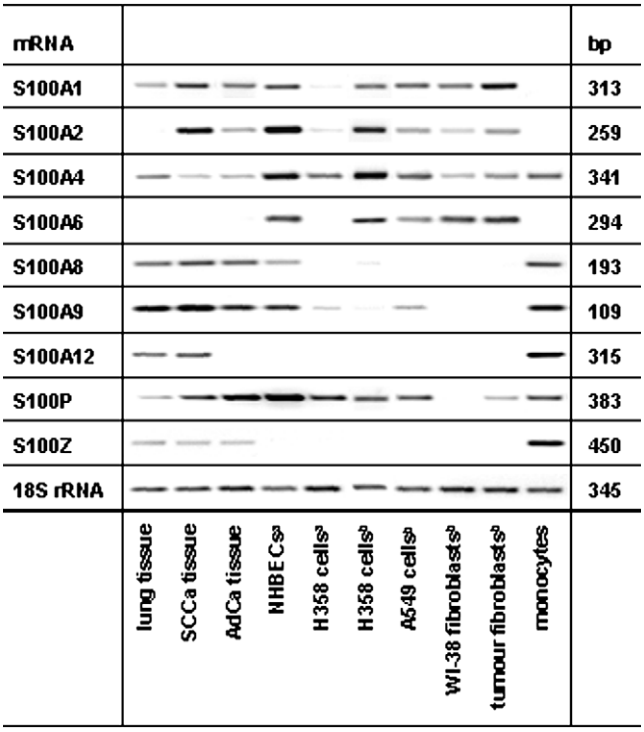

Fig. 1 – mRNA expression of several members of the S100 family is shown after PCR amplification for human lung tissue (sample mix containing RNAs from *n* = 6 NSCLC patients), lung-related cells (cultivation in [a]epith-o-ser medium without FCS or [b]DMEM containing 10% FCS) and freshly prepared monocytes. Amplification of 18S rRNA indicates RNA loading per RT-PCR.

monocytes (Fig. 1). Several S100 proteins were correspondingly transcribed in lung epithelial cell lines (H358, A549) and in primary human bronchial epithelial cells (NHBECs). However, *in vitro* levels of the S100 mRNAs are affected by medium conditions as indicated for H358 cells (Fig. 1a and b). Lung fibroblasts particularly expressed S100A1 and S100A6 (Fig. 1). S100B was transcribed in none of these cells (not shown).

3.2. *S100 expression and localisation in NSCLC*

As the expression of S100A1, S100A2 and S100P was highly detectable in the tumour specimens (Fig. 1), we selected these S100 members for detailed analyses of patients with NSCLC. Moreover, S100A1, S100A2 and S100P were less expressed in monocytes indicating a moderate influence of the tissue-associated immune cells on the total S100 expression in lung specimens. Subsequent quantitative mRNA analyses revealed S100A2 and S100P to be upregulated in NSCLCs, whereas the expression of S100A1 remained unchanged (Fig. 2). However, the upregulation of S100A2 and S100P strongly depended on the histological subtype. While S100A2 mRNA was primarily elevated in squamous cell lung carcinomas, the increased mRNA level of S100P was prominent in lung adenocarcinomas (Fig. 2).

Detailed investigations indicated that the upregulation of S100A2 mRNA mainly corresponds to patients with early tumour stages (TNM IA–IB) but less to patients with advanced tumour stages (TNM IIA–IV) (Fig. 3). NSCLC patients receiving neoadjuvant chemotherapy did not show higher mRNA levels of S100A2 (Fig. 3). Subsequent protein analyses by immunoblot technique (Fig. 4a and b) and immunohistochemistry (Fig. 5a) confirmed the differential expression of S100A2, which was associated with a positive correlation of S100A2 mRNA and protein (Fig. 4a).

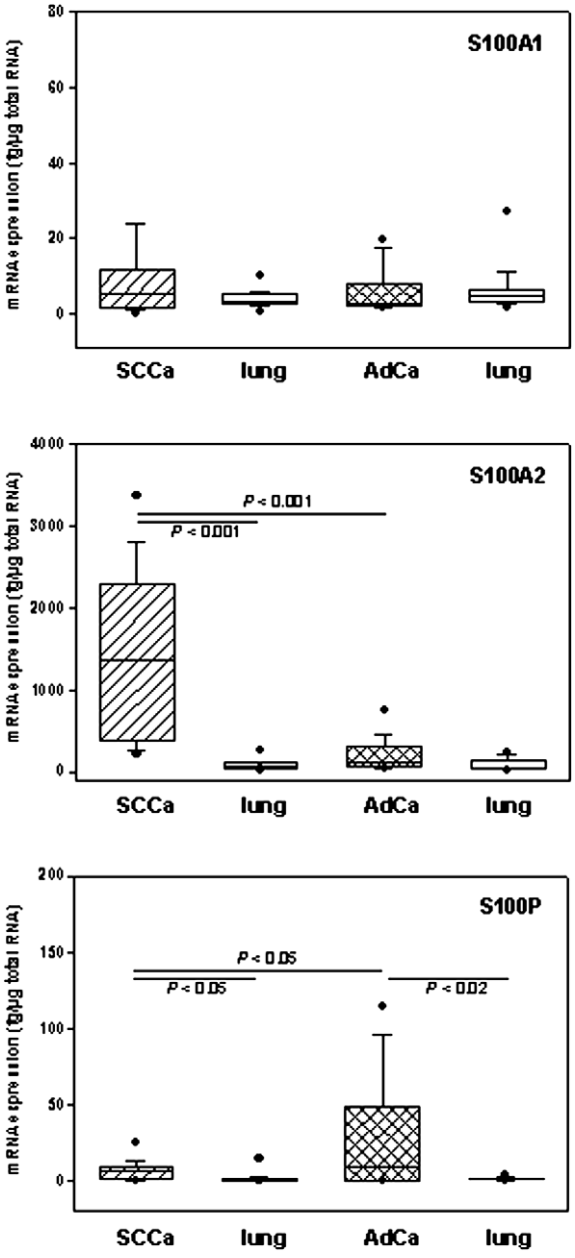

Fig. 2 – Box and Whisker plots indicate the mRNA expression of S100A1, S100A2 and S100P in squamous cell lung carcinoma (SCCa; *n* = 15) and lung adenocarcinoma (AdCa; *n* = 15) compared with the corresponding control lung tissues as analysed by real-time PCR.

Although S100A2 has been originally described as a nuclear protein,[28] our immunohistochemical analysis suggests a mostly cytosolic staining of S100A2 in NSCLC cells (Fig. 5a). This cytosolic localisation of S100A2 has been confirmed for

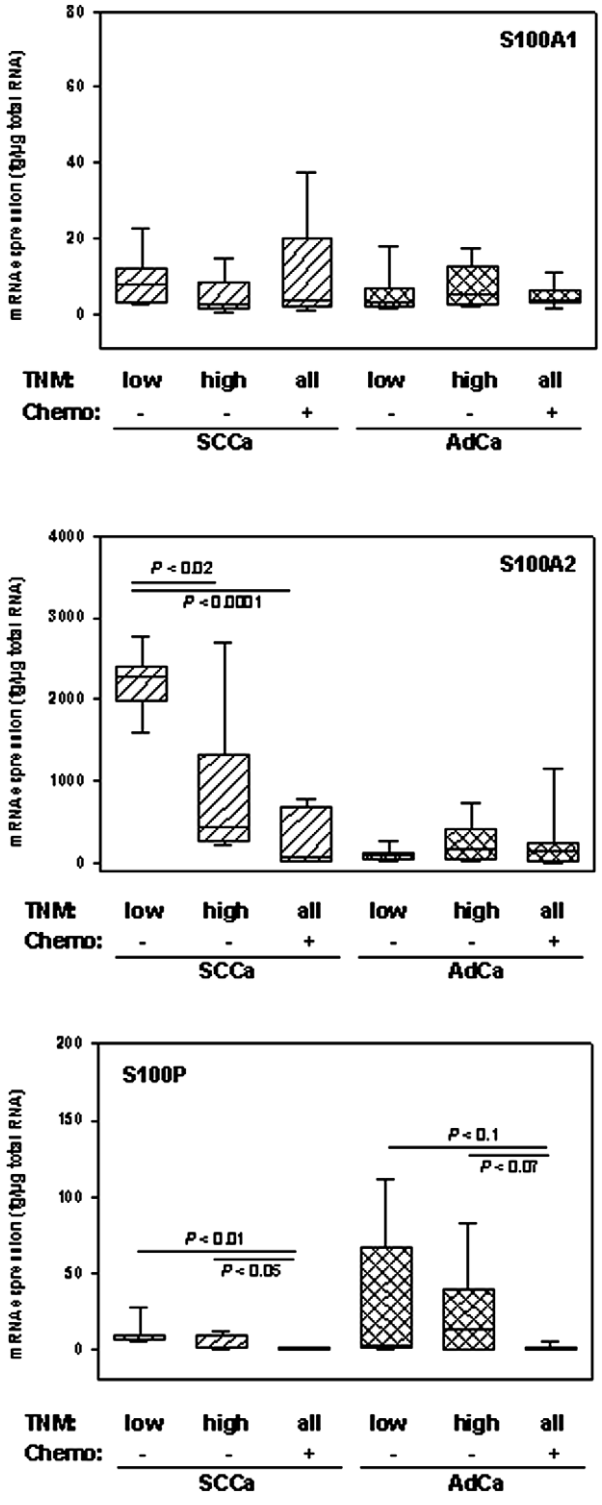

Fig. 3 – Median mRNA expression of S100A1, S100A2 and S100P in squamous cell lung carcinoma (SCCa) and lung adenocarcinoma (AdCa) depending on TNM staging of cancer patients without chemotherapy compared with patients who received neoadjuvant chemotherapy (Chemo) (Number of patients per subgroup, see Table 2).

H358 and A549 lung cancer cells, whereas NHBECs frequently showed S100A2 staining at the plasma membrane (Fig. 5b). Only a sub-population of the transformed or primary lung epithelial cells revealed nuclear S100A2, which can be detected for p53-expressing A549 cells and NHBECs as well as for *p*53-deleted H358 cells (Fig. 5b). Analysing protein fractions of H358 cells confirmed less amount of nuclear S100A2 compared with the strong detection of S100A2 in the cytosol (Fig. 5c).

While S100A2 was upregulated in squamous cell lung carcinomas, the mRNA level of S100P was predominately induced in lung adenocarcinomas (Fig. 2) but not in those patients who had received neoadjuvant chemotherapy (Fig. 3). Similarly to S100A2, the differential expression of S100P in NSCLC tended to increase in early tumour stages compared with advanced stages (Fig. 3). The mRNA data have been confirmed by immunohistochemistry of selected NSCLC specimens that indicates a scattered distribution of cells expressing S100P in adenocarcinomas as compared to S100A2 in squamous cell carcinomas (Fig. 5a).

3.3. *Differential expression of S100A2–S100P in NSCLC diagnostics*

Additionally, we plotted the mRNA levels of S100A2 and S100P for data formation in a graphical cluster. Low-stage tumours revealed a well-defined cluster of the S100A2–S100P expression data allowing the precise differentiation of NSCLC patients with an adenocarcinoma from those with a squamous cell carcinoma (Fig. 6). This distinct S100A2–S100P expression cluster was impaired by advanced tumour stages and even more by chemotherapy (Fig. 6). However, neoadjuvant chemotherapy does not generally affect the expression of cancer-associated genes as demonstrated for *survivin* mRNA (Table 2). A direct correlation between the S100 expressions and the histologically estimated stage of tumour regression according to the *Bochum* regression grading has not been found (data not shown).

4. Discussion

Gene expression profiling of tumours is supposed to improve the correct diagnosis of non-small cell lung carcinomas (NSCLCs). In this context, members of the S100 gene family might have diagnostic significance as they are characterised by a distinct expression profile depending on the type of primary or transformed cell.[20] Thus, we initially studied which S100 proteins are expressed in human lung tissue and lung-related cells.

Among the members of the S100 family, we identified the expression of S100A1, S100A2, S100A4, S100A6, S100A9 and S100P in lung carcinoma cells (H358, A549), primary epithelial cells from human bronchus (NHBEC) and whole lung tissue. However, several S100 members are exclusively expressed in immune cells as demonstrated for S100A8, S100A12 and S100Z in isolated monocytes. This might also explain why the expression of these S100 proteins has not been identified in primary or transformed lung epithelial cells but only in lung tissue, which is normally infiltrated with blood cells.

Distinct S100 members showed an elevated expression in NSCLCs compared with control lung tissues. This has been mainly observed for the mRNA and protein expression of S100A2 and S100P, respectively, depending on the histological

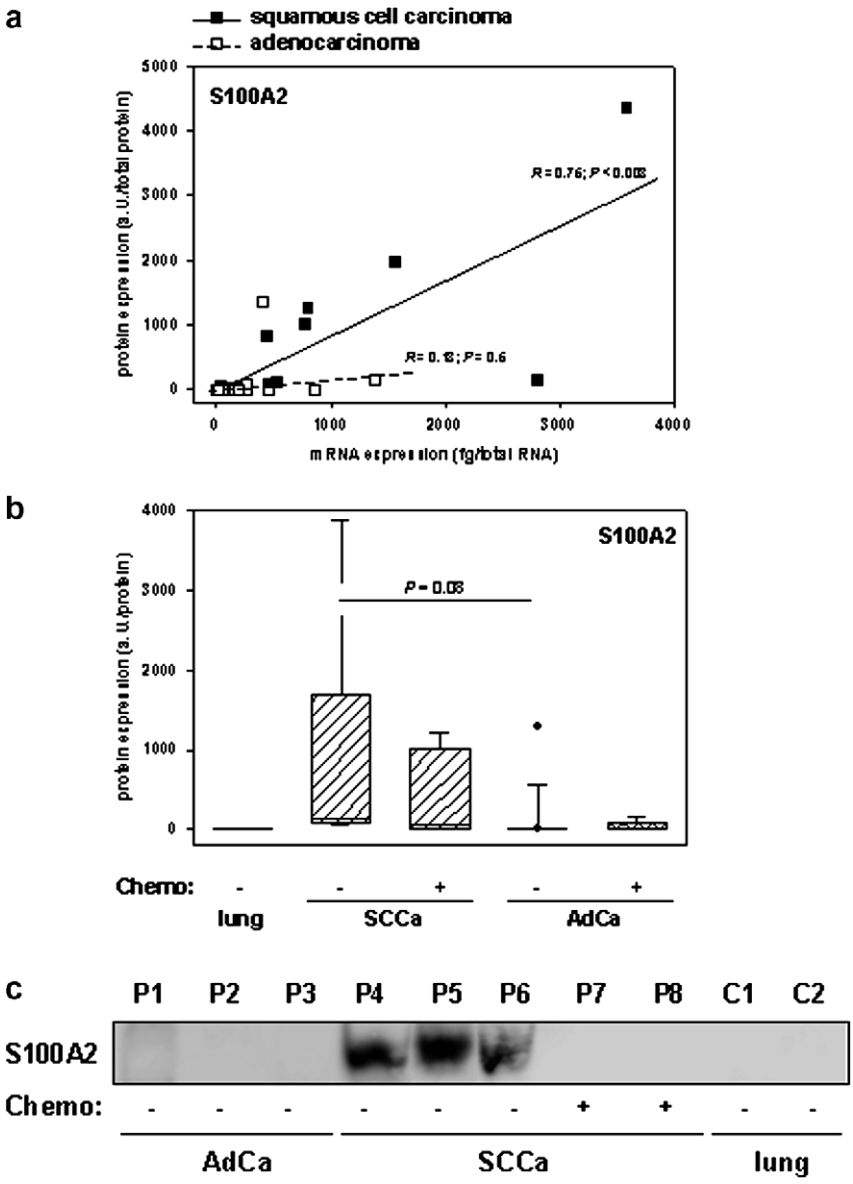

Fig. 4 – (a) S100A2 immunoblot analyses of NSCLC samples indicate the correlation of the mRNA and protein level in lung tumours. (b) Differential expression of S100A2 in squamous cell lung carcinomas (SCCa) and lung adenocarcinomas (AdCa) is graphed for NSCLC patients without and with neoadjuvant chemotherapy (Chemo) and control lung tissue ($n = 4$). (c) Representative S100A2 immunoblots of tumour samples from selected patients (P) and control lung tissue (C).

subtype and tumour stage. While the expression level of S100A2 was strongly upregulated in squamous cell lung carcinomas but not in lung adenocarcinomas, S100P was more expressed in adenocarcinomas compared with squamous cell lung carcinomas. This histology-specific expression of S100A2 and S100P might be an additional tool for the identification and classification of NSCLCs. However, the histopathological potential of the S100A2–S100P expression profile is strongly influenced by the tumour stage, as lung tumours of advanced stages did not show a differential expression of S100A2 and S100P, respectively. The lower level of S100A2 and S100P, respectively, in high-stage compared with low-stage tumours can result not only from indirect but also from direct gene-regulatory mechanisms. In this regard, the hypermethylation of promoter regions is a characteristic feature of gene silencing during the tumour progression.[2] As the gene expression of S100A2 and S100P highly depends on the methylation state,[29,30] DNA hypermethylations might be one reason explaining the low expression of those S100 proteins in advanced tumours.

The application of the S100A2–S100P expression cluster for identification and histopathological classification was also not suitable for NSCLC specimens of patients who had received neoadjuvant chemotherapy. They commonly showed a low level of the S100A2 and S100P in lung tumours, which can be partially explained by the mostly advanced tumour stages of those patients receiving neoadjuvant chemotherapy. Nevertheless, it might also reflect one of the multiple alterations of the tumour tissue that occurs in response to chemotherapy[24] because the histology-related S100A2–S100P

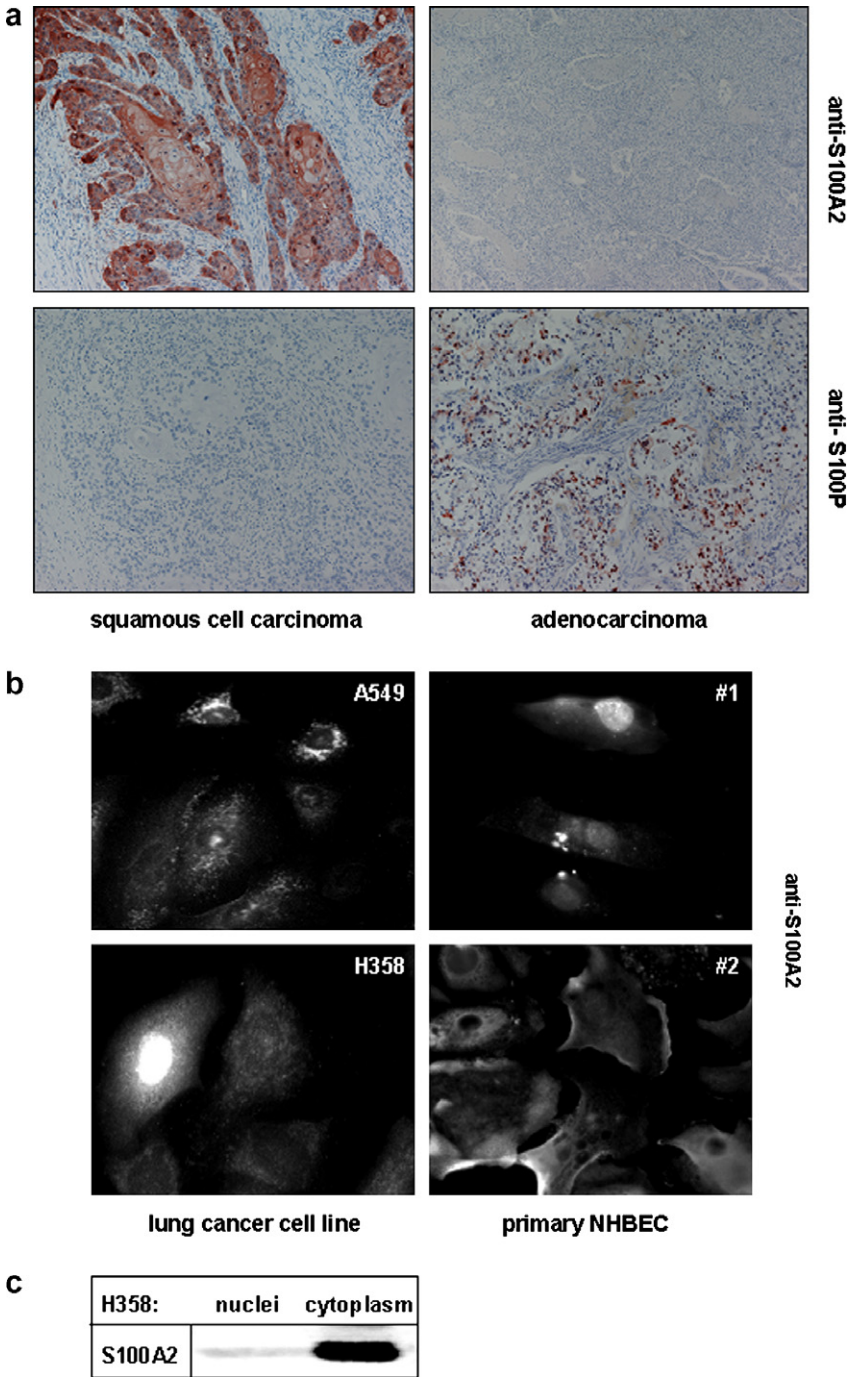

Fig. 5 – (a) Immunohistochemistry of S100A2 and S100P, respectively, for selected squamous cell lung carcinoma and lung adenocarcinoma specimens. (b) S100A2 immunofluorescence of lung cancer cell lines (A549, H358) and two preparations of NHBECs (#1 and #2). (c) S100A2 immunoblot of nuclear and cytoplasmic protein fraction isolated from H358 cells.

expression cluster was even more impaired in patients with neoadjuvant chemotherapy than in patients with high-stage tumours without chemotherapy.

As patients' survival depends on the early identification of the tumour, the increased expression of either S100A2 or S100P in low-stage tumours might be helpful. The upregulation of either S100A2 or S100P in lung carcinomas also confirms recent data of gene array analyses, which revealed S100A2 and S100P as significant targets for early-stage and, therefore, prognostically favourable NSCLC.[16,17] These and our data also contradict earlier reports describing a reduced expression of S100A2 in lung carcinomas[19] and breast cancer.[29] However, some controversy about the expression of S100A2 might result from the fact that previous studies did not analyse S100A2 depending on the histopathological subtype of the malignant carcinoma.

S100A2 is frequently described as a nuclear protein that interacts with p53 thereby enhancing the transcriptional

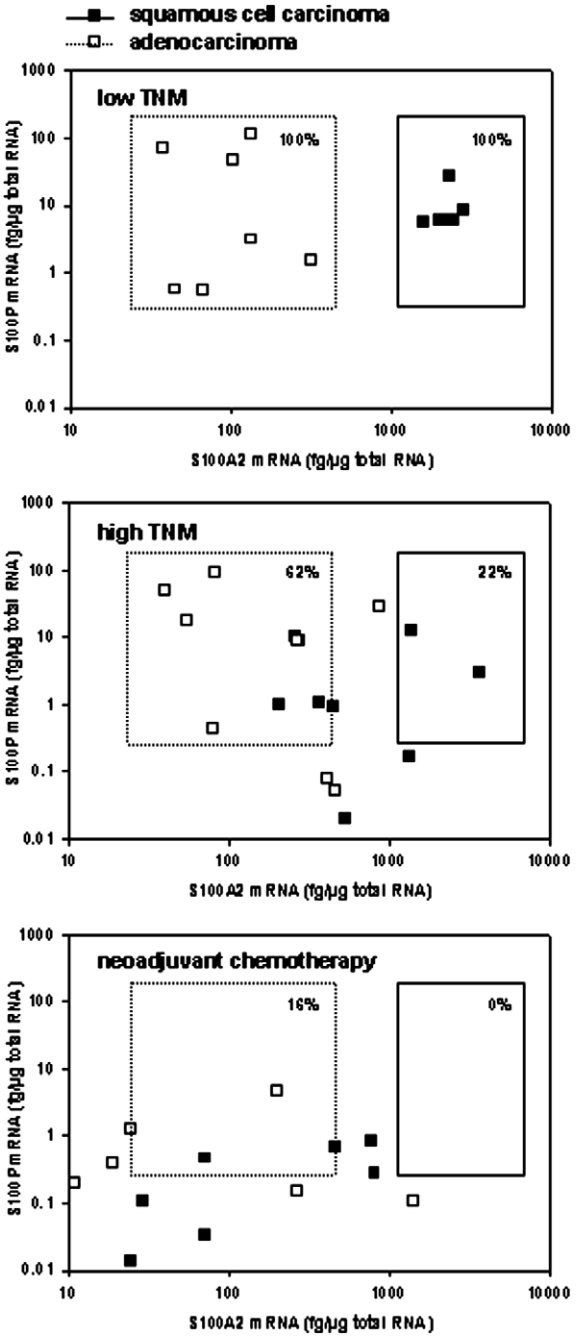

Fig. 6 – Graphical plotting of the individual mRNA levels for S100A2 and S100P in squamous cell lung carcinomas and lung adenocarcinomas depending on TNM stage and neoadjuvant chemotherapy. Percentage number of tumour specimens is given, which can be correctly assigned to the S100A2–S100P expression cluster (box) indicating either squamous cell carcinoma or adenocarcinoma histology.

activity of p53.[28] In contrast, we found a preferred cytosolic staining of S100A2 in NSCLC sections and cells as recently observed for hepatocellular carcinoma cells.[31] One might think that this divergent observation relates to the cellular p53 status because H358 cells carry a homozygous *p*53 deletion.[32] However, A549 cells and NHBECs having a normal *p*53 status showed cytosolic S100A2, too.

Despite of considerable advantages in the specificity and quantification, the cell type-independent detection of the expression profile is the main disadvantage of all analyses using total tissue samples. Besides the important function of non-malignant cells of the tumour stroma in tumour development and resistance,[33] tumour stage- and therapy-associated alterations in the cellular composition of the malignant tissue affect the expression data. Therefore, the application of gene expression clusters might have significant supportive function in tumour diagnosis but it will not completely replace the clinico- and histopathological investigations.

Taken together, the induced expression of S100A2 in NSCLC considerably indicates an early-stage squamous cell lung carcinoma tissue, whereas the expression of S100P identifies an early-stage lung adenocarcinoma. Although the application of whole-genome microarrays provides a larger scale of expression data, our study demonstrated that the detection of only few but defined genes might be sufficient to support the conventional tumour diagnostics.

Conflict of interest statement

None declared. All authors of this work disclose any financial relationships and personal relationships, respectively, with other people or organisations.

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