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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.

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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

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