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Intracellular ATP-Binding Cassette Transporter A3 is Expressed in Lung Cancer Cells and Modulates Susceptibility to Cisplatin and Paclitaxel

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Key Words
ABCA3 · ATP-binding cassette transporter · Cisplatin · Drug resistance · Lung cancer · Paclitaxel

Abstract
Patients with advanced-stage bronchial cancer benefit from systemic cytostatic therapy, in particular from regimens integrating cisplatin and taxanes. However, eventual disease progression leads to a fatal outcome in most cases, originating from tumor cells resisting chemotherapy. We here show that the intracellular ATP-binding cassette transporter A3 (ABCA3), previously recognized as critical for the secretion of surfactant components from type 2 pneumocytes, is expressed in non-small-cell lung cancer (NSCLC) cells. With some heterogeneity in a given specimen, expression levels detected immunohistochemically in primary cancer tissue were highest in adenocarcinomas and lowest in small cell lung cancers. Genetic silencing of ABCA3 in the NSCLC cell line models A549, NCI-H1650 and NCI-H1975 significantly increased tumor cell susceptibility to the cytostatic effects of both cisplatin (in all cell lines) and paclitaxel (in two of three cell lines). Taken together, ABCA3 emerges as a modulator of NSCLC cell susceptibility to cytostatic therapy.
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Introduction

Despite improvements in diagnostics and therapy, 5-year overall survival rates of patients with lung cancer remain bleak (15–18%) [1]. In patients without metastatic involvement, multidisciplinary approaches including surgery, radiotherapy and chemotherapy can achieve cure for some patients. For patients with metastatic disease, representing 40% of all patients at primary diagnosis [2], transient control of cancer growth can only be provided by systemic cytostatic treatment regimens. Within such regimens, combinations of vinca alkaloids, anthracyclines, topoisomerase inhibitors, antimetabolites and DNA alkylating drugs have shown activity against lung cancer, with limited promising results obtained with combination protocols integrating platinum derivatives and taxanes for patients with non-small-cell lung cancer (NSCLC) and platinum combined with etoposide in small-cell lung cancer (SCLC) patients [1]. Thus, as current standard of care, platinum-based combination chemotherapy has generated a plateau in the overall response rate of about 25–35% in the first-line treatment of stage-IV NSCLC patients [3]. In patients who experience primary progress or relapse of disease, tumor regrowth must

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originate from cells resisting chemotherapy, and several mechanisms protecting lung cancer cells from cytostatic effects have been described, ranging from impaired apoptosis induction and tumor dormancy to classical multidrug resistance and cellular drug extrusion by drug-transporting proteins such as ATP-binding cassette (ABC) transporters and lung resistance protein [4–12].

Analyzing the intrinsic drug efflux capacity pertinent to leukemic progenitor cells with the side population phenotype, we previously detected a specific role for the ABC transporter A3 (ABCA3) in drug resistance of myeloid leukemia as well as neuroblastoma stem cells [13–15]. Genetic manipulation studies clearly established an association of ABCA3 expression with drug resistance, and the intracellular localization of ABCA3 in the membranes of lysosome-related organelles led to the discovery of ABCA3-mediated lysosomal drug sequestration as a contributing mechanism [16, 17]. Intriguingly, ABCA3 has previously been described as a key regulating transport protein in the process of surfactant extrusion into the pulmonary alveolae [18, 19]. Children with functional relevant mutations in the ABCA3 gene experience severe respiratory distress syndrome after birth, and experimental study of surfactant biogenesis revealed a critical role of ABCA3 function for the amount and composition of phospholipids in the vesicular transport of cytoplasmic multilamellar bodies of type 2 pneumocytes to the multitrabecular bodies carrying the surfactant into the alveolae [20–22]. With type 2 pneumocytes as the major cells of physiological ABCA3 expression as well as the transporter function in vesicular transport and secretion, we here addressed ABCA3 expression in transformed bronchoepithelial cells and its role in the secretion of the cytostatic drugs most commonly used in lung cancer therapy.

Materials and Methods

Cell Culture, Lentiviral Transfection and ABCA3 Detection

The human lung cancer cell lines A549 [23], NCI-H1650 [24] and NCI-H1975 [24] as well as the SCLC cell line NCI-H69 [25] were propagated in RPMI 1640. HEK293T cells were grown in DMEM. The stable ABCA3-eGFP and eGFP transfectants HEK293/ABCA3-eGFP and HEK293-eGFP described previously [17] were routinely cultured in DMEM supplemented with 300 μg/ml G418 (Carl Roth, Karlsruhe, Germany). All media were supplemented with 10% heat-inactivated fetal calf serum (Gibco-BRL, Karlsruhe, Germany), penicillin/streptomycin (Sigma-Aldrich Chemie, Steinheim, Germany/Biochrom, Berlin, Germany) and GlutaMAX I (Gibco-BRL). For shRNA-mediated silencing of ABCA3 in the lung cancer cell lines, two validated specific shRNA sequences [the RNAi Consortium, www.broadinstitute.org/rnai/trc: TRC clone ID TRCN0000059338, here referred to as shABCA3.38: forward 5′-CCGG(GCCCAGCTCATTGGGAAATTT)CTCGAG(AAATTTCCCAATGAGCTGGGC)TTTTTG-3′ and reverse 5′-AATTCAAAAA(AAATTTCCCAATGAGCTGGGC)CTCGAG(GCCCAGCTCATTGGGAAATTT)-3′, and TRC clone ID TRCN0000059339, here referred to as shABCA3.39: forward 5′-CCGGGCCCAGCTCATTGGGAAATTTCTCGAGAAATTTCCCAATGAGCTGGGCTTTTTG-3′ and reverse 5′-AATTCAAAAAGCCCAGCTCATTGGGAAATTTCTCGAGAAATTTCCCAATGAGCTGGGC-3′] were cloned into pLKO.1-eGFP (Addgene, Cambridge, Mass., USA) and lentiviral particles produced in the HEK293T producer cell line with the plasmids pCMV-ΔR8.91 (containing gag, pol and rev genes) and pMD.G (VSV-G-expressing plasmid), as previously reported [26]. Selection was performed using puromycin. Cells were cultured without puromycin 7 days before viability assays. Stable ABCA3 knockdown cell lines, i.e. A549^{shABCA3}, NCI-H1650^{shABCA3} and NCI-H1975^{shABCA3} were propagated like the corresponding wild-type cell lines as described above. Expression of the lentiviral construct was determined by flow-cytometric evaluation of eGFP, and only samples exceeding 90% marker expression were used in further experiments. For ABCA3 detection in in vitro propagated cell populations, we performed indirect immunocytology (dilution 1:200) following cytocentrifugation as well as Western blot (dilution 1:500) of whole-cell lysates as described before, either using a primary polyclonal rabbit antibody against human ABCA3 or a commercially available monoclonal rabbit antibody to ABCA3 (HPA007884; Sigma-Aldrich Chemie) [16, 17, 19, 27].

Immunohistochemistry

The human lung cancer tissue microarray (TMA) LC2001 was obtained from a commercial tissue bank (US Biomax Inc., Rockville, Md., USA). Clinical information on the 187 patients with lung cancer (TMA) is summarized in table 1. All samples were achieved by surgery of the primary tumor before any treatment. Outcome data were not available in this cohort. The mean age of the patients was 56.4 ± 8.2 years (range 30–78). Histological subtypes of lung cancer were adenocarcinoma, squamous cell carcinoma, SCLC, carcinoid and mucoepidermoid carcinoma at the following percentages: 33.2, 42.2, 12.8, 7.5 and 4.3%, respectively. Pathological staging of these patients was performed according to the 6th edition of the International System for Staging Lung Cancer [28]. Immunohistochemistry was performed using the primary antibody to ABCA3 (HPA007884; Sigma-Aldrich Chemie) diluted 1:150 in AK diluent (Dako, Hamburg, Germany) and stained following standard protocols [17]. Secondary antibody (AP anti-rabbit polymer; Zytomed Systems, Berlin, Germany) was visualized using liquid permanent red chromogen in liquid permanent red chromogen substrate buffer (Dako). ABCA3 cytoplasmic intensity staining levels were analyzed by panel agreement of two independent reviewers as negative, weak, intermediate or strongly positive. The proportion of positive tumor in relation to the whole tumor was determined in percent. Normal epithelial lung tissue adjacent to the tumor area as well as 8 tissue samples of normal lung tissue (LC2001) were used as positive (type 2 pneumocytes) and negative controls.

Viability Assays and Statistical Evaluation

Cell sensitivity to cytostatic drugs was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described [17]. Cells were seeded in triplicate in 96-well culture plates at a density of 1×10^5 cells/well

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Table 1. Characteristics and staging of 187 patients (TMA LC2001)

Mean age ± standard deviation, years	56.4±8.2	
Range	30–78	
All patients	187	100.0%
Gender		
Female	57	30.5%
Male	130	69.5%
Histological subtype		
Adenocarcinoma	62	33.2%
Squamous cell carcinoma	79	42.2%
SCLC	24	12.8%
Carcinoid	14	7.5%
Mucoepidermoid carcinoma	8	4.3%
Grading		
I	11	5.9%
II	77	41.2%
III	72	38.5%
Unknown	27	14.4%
pT status		
T1	33	17.6%
T2	120	64.2%
T3	29	15.5%
T4	5	2.7%
pN status		
N0	131	70.1%
N1	38	20.3%
N2	17	9.1%
N3	1	0.5%
Pathological stage (UICC, 6th edition)		
I	119	63.6%
II	37	19.8%
IIIa	25	13.4%
IIIb	5	2.7%
IV	1	0.5%

Numbers (%) of patients are shown except for age.

and were treated with the indicated concentrations of cisplatin (Teva, Ulm, Germany), vinorelbine (Pierre Fabre Pharma, Freiburg, Germany) and paclitaxel (Teva) for 3 h each, washed and then incubated for a period of 24 h. After 24 h at 37°C, the culture volume of 100 µl was supplemented with MTT in phosphate-buffered saline to achieve a final concentration of 0.5 mg/ml. After a 4-hour incubation, the supernatant was discarded, and adherent cells resuspended in 30% (v/v) dimethyl sulfoxide, 5% (v/v) formic acid and 1% (w/v) Triton X-100 (all from Sigma) dissolved in isopropanol. Light absorbance from formazan was measured at 540 nm on a Tecan SLT photometer (Tecan SLT Spectra). We expressed the effect on viability as the ratio of values from treated samples with wild-type ABCA3 versus the knockdown variant. EC_{50} was defined as the concentration of drug causing a 50% inhibition of cell growth in wild-type cells compared with knockdown variants.

To estimate differences between cohorts of samples with and without experimental intervention, two-way ANOVA with a Bonferroni post hoc test was applied, with differences of $p < 0.05$ considered significant.

Results

Expression of ABCA3 in Lung Cancer Cells

The physiological expression of ABCA3 in specialized alveolar cells had raised our interest for the prevalence and intratumoral expression pattern of the ABC transporter in transformed pulmonary cells. First, we analyzed 187 cases of primary lung cancer for ABCA3 protein expression in a TMA representing all major types of epithelial lung cancer tumors. Whereas we found no or only very low levels of ABCA3 in small fractions of cells in SCLC tissues, the majority of cancers with non-small cell differentiation showed significant expression of the ABCA3 transporter protein (fig. 1; table 2). Within the NSCLC, expression patterns varied, ranging from >75% strongly positive cells in some cases of adenocarcinoma to low-level expression in cases of predominant squamous cell differentiation (fig. 1; table 3). Of note, intratumoral expression patterns were heterogeneous in all histological subtypes, with subfractions of cells displaying higher amounts of the ABCA3 transporter (table 3).

With respect to subgroups as gender, grading, pT/N status or pathological stage, there were no major differences in the two groups of staining intensity (negative/weak and intermediate/strong) except for the histological subtype (table 2).

In NSCLC, we found cytoplasmic expression of the transporter in all three cell lines tested, which was comparable to the level obtained by ectopically enforced expression in HEK293 cell lines (fig. 2a). In contrast to the NSCLC cell lines, the SCLC cell line NCI-H69 showed no expression of ABCA3 (fig. 2a).

The intracellular staining pattern, with ABCA3 being organized in cytoplasmic vesicular structures, was reminiscent of the staining profile of ABCA3 previously found in other malignancies [15, 17]. Thus ABCA3 is expressed in the majority of NSCLC samples with remarkable inter- and intratumoral heterogeneity.

Role of ABCA3 in the Susceptibility of Lung Cancer Cell Lines to Cisplatin and Paclitaxel

Our previous findings associated ABCA3 expression with leukemia cell resistance against anthracyclines and vinca alkaloids. Therefore, we assessed to which extent ABCA3 protects lung cancer cells against the cytostatic effects of drugs typically applied to control disease in lung cancer patients, i.e. cisplatin and paclitaxel. Thus, we exposed the lung cancer cell lines A549, NCI-H1650, NCI-H1975 and their knockdown variants to cisplatin, pacli-

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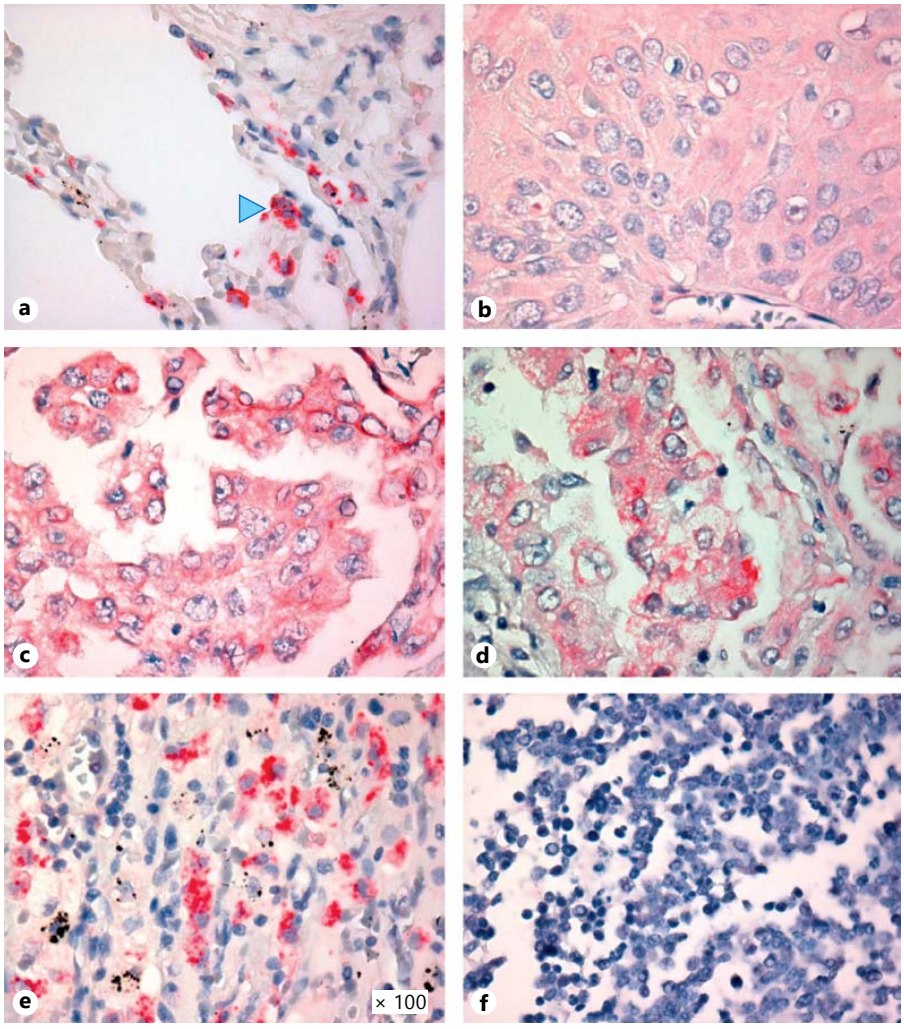

Fig. 1. Expression patterns of ABCA3 in histological subtypes of bronchial carcinoma. Tissue sections were stained by indirect immunoperoxidase reaction with a polyclonal rabbit anti-ABCA3 antibody as primary antibody, and characteristic examples for histological subtypes of bronchial carcinoma are shown. In normal adult lung tissue, ABCA3 expression was restricted to type 2 pneumocytes (**a**; arrowhead). In samples with squamous cell differentiation, the cytoplasm of the tumor cells displayed a homogeneously diffuse, faint reactivity (**b**), whereas in samples with adenocarcinoma morphology, ABCA3 expression levels were generally higher with a heterogeneous expression pattern in the tumor histology (**c**, **d**). Cells with strong ABCA3 expression with a heterogeneous pattern were found in cases of bronchoalveolar differentiation (**e**). SCLC cases did not show reactivity with the anti-ABCA3 antibody (**f**).

taxel and vinorelbine in a dose-escalating schedule and compared the susceptibility of the variants to the parental cells. Across NSCLC cell lines, we observed a significant increase in the cytostatic efficacy of cisplatin (all cell lines) and paclitaxel (A549 and NCI-H1650) in cell lines with stably silenced ABCA3 expression compared to their parental controls (fig. 3). With respect to vinorelbine, we found a tendency to increased susceptibility associated with ABCA3 suppression, albeit with lower consistency across the cell lines (fig. 3, far right column).

Discussion

We report the first data on the expression pattern and potential functional significance of ABCA3 in lung cancer, particularly in patients with predominant adenocarcinoma differentiation. These findings may have several implications for the understanding of lung cancer disease.

First, the differences in ABCA3 expression observed between NSCLC and SCLC samples add to the knowledge about the fundamental biological differences between these two disease groups. ABCA3 expression in lung cancer appears to be closely associated with adenoid differentiation, an observation recently also made in breast cancer. In mammary cancer tissue, the level of ABCA3 expression correlated with the degree of glandular differentiation, and lower levels of ABCA3 expression were associated with a more aggressive phenotype of the tumor cells [29]. This appears similar to the situation in lung cancer with low expression levels of ABCA3 in SCLC, the most aggressive variant of the disease. Clinically, SCLCs are characterized by enhanced susceptibil-

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Table 2. Expression of ABCA3 in lung cancer subtypes

Characteristics		Staining intensity			
		negative/weak		intermediate/strong	
		n	%	n	%
Age, years		54.2±10.0		56.1±9.6	
Range		32–78		30–77	
Patients	Total	108	57.8	79	42.2
	Female	33	57.9	24	42.1
	Male	75	57.7	55	42.3
Histological subtype	Adenocarcinoma	22	35.5	40	64.5
	Squamous cell carcinoma	53	67.1	26	32.9
	SCLC	19	79.2	5	20.8
	Carcinoid	8	57.1	6	42.9
	Mucoepidermoid carcinoma	6	75.0	2	25.0
Grading	I	6	54.5	5	45.5
	II	41	53.2	36	46.8
	III	45	62.5	27	37.5
	Unknown	16	59.3	11	40.7
pT status	T1	19	57.6	14	42.4
	T2	67	55.8	53	44.2
	T3	19	65.5	10	34.5
	T4	3	60.0	2	40.0
pN status	N0	70	53.4	61	46.6
	N1	24	63.2	14	36.8
	N2	13	76.5	4	23.5
	N3	1	100.0	0	0.0
Pathological stage (UICC, 6th edition)	I	67	56.3	52	43.7
	II	19	51.4	18	48.6
	IIIa	18	72.0	7	28.0
	IIIb	3	60.0	2	40.0
	IV	1	100.0	0	0.0

Numbers and percentages of negative and weakly positive versus intermediate and strongly positive tumor cases are listed. Note the inverse relationship between ABCA3 positivity of high ABCA3 expression in NSCLC/adenocarcinoma tissue and low ABCA3 expression in the SCLC samples. Furthermore, major differences in gender, grading, pT/N status or pathological stage were not detected.

Table 3. Intratumoral heterogeneity of ABCA3 expression in lung cancer tissue

Histological subtype	Intermediate/strong positivity for ABCA3 cluster (positivity/tumor sample)					
	0%	≤1%	>1–≤25%	>25–≤50%	>50–≤75%	>75%
Adenocarcinoma	22 (35.5)	14 (22.6)	7 (11.3)	6 (9.7)	2 (3.2)	11 (17.7)
Squamous cell carcinoma	53 (67.1)	11 (13.9)	11 (13.9)	2 (2.5)	0	2 (2.5)
SCLC	19 (79.2)	2 (8.3)	3 (12.5)	0	0	0
Carcinoid	8 (57.1)	3 (21.4)	0	1 (7.1)	1 (7.1)	1 (7.1)
Mucoepidermoid carcinoma	6 (75.0)	1 (12.5)	1 (12.5)	0	0	0
Total	108 (57.8)	31 (16.6)	22 (11.8)	9 (4.8)	3 (1.6)	14 (7.5)

The numbers and percent of cases of intermediate and strongly positive cells per tumor sample are listed for all samples and the histological subtypes. Highest proportions of positive cells were observed in samples with adenocarcinoma differentiation.

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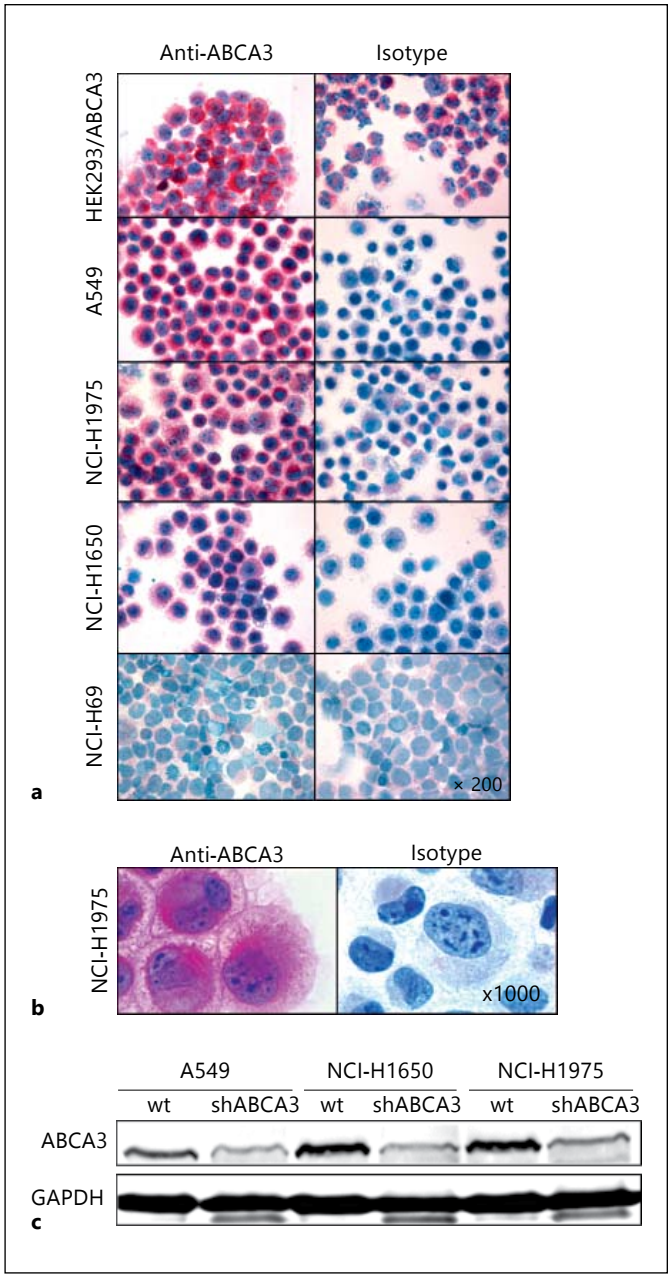

Fig. 2. Expression of ABCA3 in NSCLC and SCLC cell lines. Cell line samples were spun onto slides by cytocentrifugation and stained with anti-ABCA3 polyclonal antibody by indirect immunocytology (**a**). The expression levels in the three NSCLC cell lines A549, NCI-H1975 and NCI-H1650 were comparable to the levels achieved by enforced ectopic expression in HEK293/ABCA3 cells (**a**; upper lane). The SCLC cell line NCI-H69 showed no or very weak expression of ABCA3. As shown for NCI-H1975 at higher magnification, ABCA3 was found in a punctate pattern in the cytoplasm, with some perinuclear accumulation (**b**). ABCA3 transporter expression was also detected by Western blot in the three NSCLC cell lines (A549, NCI-H1975 and NCI-H1650), documenting significant silencing of gene expression by lentiviral shRNA transduction (**c**).

ity to cytostatic agents compared to NSCLC. In accordance with our data, the association of ABCA3 expression levels with chemotherapy resistance has also been noted in other studies. Assessing ABC transporter expression in 60 cancer cell lines (NCI-60), profiling of mRNA revealed an association of lung cancer histology and ABCA3 expression, and levels were highest in the adenocarcinoma cell lines NCI-H522, EKVX and A549 [30].

Secondly, we observed considerable intratumoral heterogeneity of ABCA3 in the primary lung cancer tissue, ranging from strong to virtually absent transporter expression in the cells of the individual cell clone (table 3). This finding is reminiscent of recent observations made on the expression of ABCA3 in leukemia and lymphoma [16, 17, 31, 32], in which transporter expression was clearly associated with a drug-resistant leukemia cell phenotype [13, 16, 17]. As for lung cancer tissue, it remains to be shown whether the strongly ABCA3-positive cells in a given tumor represent the cell fraction surviving chemotherapy, e.g. by comparing ABCA3 in tissues from primary and relapsed/refractory cases of adenocarcinoma. Different levels of heterogeneity in ABCA3 expression may reflect the wide range of response to chemotherapy. Recent observations in chronic myeloid leukemia, as well as in solid neoplasia, employing a subtractive comparative genomic hybridization-based approach to the comparison of chemotherapy-naïve and -resistant cell lines support the concept that ABCA3 expression levels increase with acquired drug resistance in cancers [33].

Third, the role of ABCA3 in the secretory function of type 2 pneumocytes, specifically its role in the formation of intracellular multilamellar bodies and consecutively extracellular multitrabecular bodies, raises the question to which extent such vesicles are secreted by cancer cells and how such vesicles might interfere with lung cancer biology and treatment. For aggressive B-cell lymphomas, we recently reported that ABCA3 is involved in tumor cell secretion of exosomes and that such tumor-derived exosomes protect cancer cells against complement-dependent cytotoxicity of therapeutic antibodies [26]. These findings were mirrored by observations in epithelial cancer cells, for which exosomal shedding of the epidermal growth factor receptor was described to allow tumor cells to evade antibody-dependent cellular cytotoxicity [34]. It is currently open to question to which extent and to which morphological subtype of extracellular vesicles the expression of ABCA3 in lung cancer cells contribute, and whether

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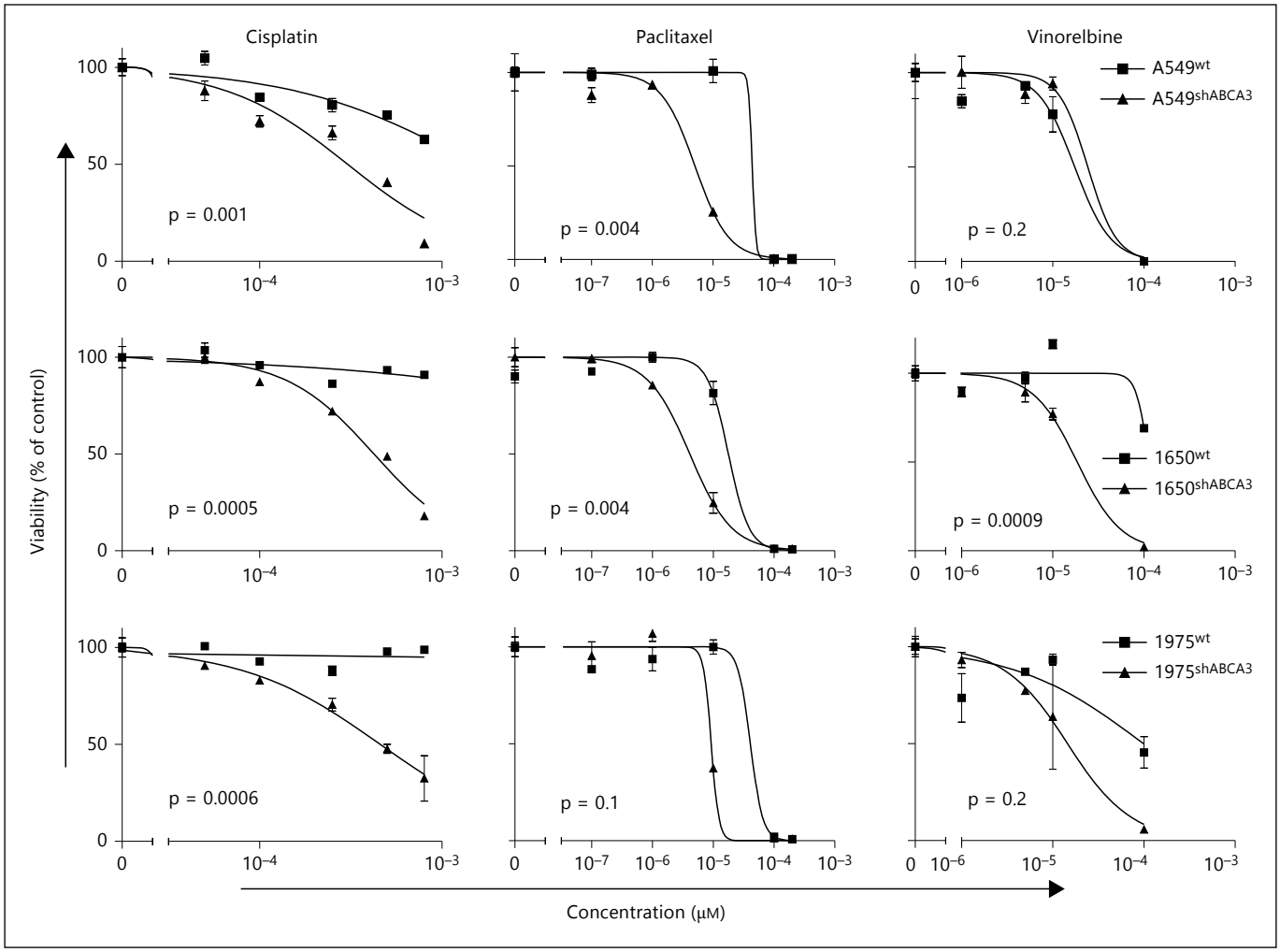

Fig. 3. Silencing of ABCA3 sensitizes lung cancer cell lines to the cytostatic effects of cisplatin and paclitaxel. Silencing of ABCA3 was achieved in the cell lines A549, NCI-H1650 and NCI-H1975 by lentiviral shABCA3 constructs and stable variant cell populations exposed to cisplatin, paclitaxel and vinorelbine for 3 h each at increasing concentrations as indicated. Viability of cells was measured by MTT test after 24 h, and values are reported as percent of untreated controls, with error bars representing standard deviations of triplicates. Differences between experimental groups were evaluated by two-way ANOVA with a Bonferroni post hoc test; p values were integrated into the graphs.

such vesicles traffic or bind chemotherapy or therapeutic antibodies.

Fourth, several further ABC transporters have been recognized to interfere with cytostatic drugs in lung cancer [35, 36]. In particular the transporter ABCB1 (MDR1, P-glycoprotein) and ABCC1 (multidrug resistance protein, MRP1) at the plasma membrane, as well as lung resistance protein at the nuclear membrane, were expressed in lung cancer cells and associated with drug resistance against classical ABC transporter substrates [37, 38]. Noteworthy, the resistance mechanisms supported by classical ABC transporter function do not alter cellular accumulation of cisplatin and do not protect cancer cells from cisplatin-mediated cytotoxicity. Thus MDR1 and MRP1 expression levels were not correlated with chemosensitivity or intracellular/intranuclear accumulation of cisplatin in several cell lines [9]. It remains to be investigated whether cisplatin is actually sequestered and exported in exocytic vesicles, as discussed above, or whether the protective effect of increased ABCA3 function is based on its effects on cellular phospholipid composition and susceptibility to proapoptotic stimuli.

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Based on these data, we are now focusing on NSCLC patients treated with cisplatin-based chemotherapy in a neoadjuvant setting, and tumor samples were examined before and after systemic therapy. The aims of the following study are to detect possible changes in ABCA3 expression, particularly in chemoresistant lung cancer tumors, and to investigate the effect of different ABCA3 levels in lung cancer on the prediction of response to chemotherapy and prognosis.

Inhibitors of ABCA3 are already established and further preclinical studies in lung cancer are planned in order to elucidate potential effects in combination with systemic therapy.

Taken together, intracellular ABCA3 is differentially expressed in histological subtypes of human lung cancer and modulates susceptibility of NSCLC cell lines to cisplatin and paclitaxel in vitro. Further analysis of the subcellular mechanisms of cancer cells and the evaluation of effects from specific interference with ABCA3 function may eventually contribute to refinements in the cytostatic treatment of lung cancer.

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Disclosure Statement

We declare that none of the authors has any financial interest related to this work.

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