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Screening for circulating nucleic acids and caspase activity in the peripheral blood as potential diagnostic tools in lung cancer

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ABSTRACT

The focus of the current investigational study was to examine whether circulating nucleic acids (i.e., DNA and microRNAs) have the potential to become suitable blood-based markers for diagnosis and progression of lung cancer. The concentrations of cell-free DNA and four circulating microRNAs (miR10b, miR34a, miR141 and miR155) as well as the caspase activity were measured in serum of 35 lung cancer patients (19 non-small-cell lung cancer, 8 small cell lung cancer patients and 8 patients with indefinite cancer type), 7 patients with benign lung tumors and 28 healthy individuals by PicoGreen, TaqMan MicroRNA, and Caspase-Glo®3/7 assay, respectively. The data were correlated with the established risk factors for lung cancer progression. The concentrations of cell-free DNA ($p = 0.0001$), serum microRNAs ($p = 0.0001$) and caspase activities ($p = 0.0001$) significantly discriminated cancer patients from healthy individuals. Serum DNA, caspase activities and RNA levels could not distinguish between patients with benign lung disease and cancer patients. However, the levels of miR10b ($p = 0.002$), miR141 ($p = 0.0001$) and miR155 ($p = 0.007$) were significantly higher in lung cancer patients than those in patients with benign disease. As determined by the Spearman–Rho test, high levels of cell-free DNA significantly correlated with elevated circulating caspase activities ($p = 0.0001$). In lung cancer patients high serum miR10b values associated with lymph node metastasis ($p < 0.03$) and elevated levels of TPA (tissue polypeptide antigen, $p = 0.01$), whereas high serum miR141 values associated with elevated levels of uPA (urokinase plasminogen activator, $p = 0.02$).

The findings of our pilot study suggest that the assays for circulating DNA, microRNAs and caspase activities in blood might become novel minimally invasive diagnostic tools for detection and risk assessment of lung cancer, provided that their clinical utility can be confirmed in larger prospective trials.

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Abbreviations: EMT, epithelial mesenchymal transition; miRs, microRNAs; NSCLC, non-small-cell lung cancer; SCLC, small cell lung cancer; TPA, tissue polypeptide antigen; uPA, urokinase plasminogen activator.

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1. Introduction

Lung cancer is classified in different cancer types, namely non-small-cell lung cancer (NSCLC) including squamous cell carcinoma, adenocarcinoma, large cell carcinoma, and small cell lung cancer (SCLC). The majority (70–80%) of the lung cancer patients have NSCLC (Alberg and Samet, 2003). Previous studies have shown that the pandemic of lung cancer parallels with trends of cigarette smoking, and environmental exposures, such as asbestos and air pollution. Approximately 85% of the patients who develop lung cancer die from this disease (Alberg and Samet, 2003). This high rate of mortality is caused by the difficulty to diagnose early lung cancer and by the rapid progression of the disease in advanced stages (Balch et al., 2009). The current therapeutic strategies for lung cancer include combinations of cytotoxic chemotherapy and targeted biological therapies, such as bevacizumab and erlotinib. However, adjuvant chemotherapy only results in a modest extension of survival and is significantly toxic for the patients. Therefore, the development of clinically validated biomarkers is a crucial challenge to advance the field of molecular biology in lung cancer.

Elevated levels of nucleic acids which display genetic and epigenetic alterations of the primary tumor circulate in blood of tumor patients, including lung cancer patients (Fleischhacker and Schmidt, 2007; Holdenrieder et al., 2008, 2004; Pathak et al., 2006; Chen et al., 1996). So far, it is unknown whether there is a relationship between an increased quantity of extracellular nucleic acids circulating in the plasma or serum of tumor patients and the possibility to detect genetic or epigenetic alterations in these samples. The mechanism of the release of nucleic acids into the blood circulation occurs either by proliferating or dying tumor cells, such as apoptotic and necrotic cells (Stroun et al., 2001). Caspases which belong to the cysteine proteases are involved in the apoptotic cascade. Their deregulation is a further essential aspect for cancer development and progression (Lopez-Otin and Matrisian, 2007). During apoptosis caspase-activated endonucleases induce fragmentation of DNA into oligo- and mononucleosomes (Enari et al., 1998). This DNA is then packed in apoptotic blebs that are subsequently engulfed by neighboring macrophages and released into the blood circulation.

Although most of the literature has focused on the quantification of circulating DNA (Fleischhacker and Schmidt, 2007; Pathak et al., 2006; Chun et al., 2006; Schwarzenbach et al., 2004, 2008), only some studies have analyzed the clinical relevance of circulating microRNAs (miRs) in peripheral blood for diagnosis and prognosis (Lawrie et al., 2008; Mitchell et al., 2008; Zhu et al., 2009). As far as we know, there is only one study on circulating cell-free miRs in blood of lung cancer patients (Hu et al., 2010). Protected from the degradation by endogenous RNase activity, miRs circulate in a remarkably stable form in blood (Esquela-Kerscher and Slack, 2006).

MiRs are small regulatory, non-coding RNA molecules and consist of approximately 22 nucleotides. They modulate the activity of specific mRNA molecules, e.g. the transcripts of tumor suppressor genes, by binding sequence-specifically to their target mRNA, and inhibit their translation into polypeptides. Moreover, miRs seem to be involved in the regulation of

different cellular processes, e.g. apoptosis, hematopoietic cell differentiation, metabolism, neural development and metastasis. Since half of human miRs are located in fragile chromosomal regions, which can exhibit amplifications, deletions or translocations, their expression is frequently deregulated during tumor development. In tumors the change in their expression causes that they may function as oncogenes (Kloosterman and Plasterk, 2006; Stefani and Slack, 2008). Among the miRNAs identified, miR10b, miR34a, miR141 and miR155 have been associated with cancer progression (Ma et al., 2007; Baffa et al., 2009). In NSCLC patients, aberrant miR34a expression may be an independent prognostic marker for metastatic relapse and a useful tool to help guide treatment decisions (Gallardo et al., 2009). In univariate analysis expression of miR155 correlated with poor survival of lung cancer patients (Yanaihara et al., 2006).

Since there is an urgent need for a minimally invasive test to identify lung cancer and to discriminate malignant from benign tumors, we assessed the clinical relevance of circulating nucleic acids and their relationship to caspase activities in blood serum of lung cancer patients in comparison to patients with benign tumors and healthy individuals.

2. Materials and methods

2.1. Patient design

Between October 1997 and December 2000, blood serum was collected from 35 patients with lung cancer before any treatment. All patients analyzed had histologically proven epithelial cancer and gave their informed consent. Patient characteristics at the time of diagnosis are shown in Table 1.

The treatment protocol was as followed:

Chemotherapy: As induction chemotherapy cisplatin and etoposide were applied as previously described (Eberhardt et al., 1998). In brief, 60 mg/m² cisplatin were administered intravenously as a 1 h infusion on days 1 and 7 of each cycle. 150 mg/m² etoposide were given as a 1 h infusion on days 3–5. Three chemotherapy cycles were given at a three-weeks interval. Supportive treatment consisted of 5-HT₃ antagonists (ondansetron) and dexamethasone as antiemetic therapy, hydration with 0.9% normal saline and furosemide plus magnesium infusion before cisplatin therapy.

Chemoradiotherapy: Hyperfractionated accelerated radiotherapy was daily performed with a single dose of 1.5 Gy and a minimum interval of 6 h between the two doses. The total dose for patients undergoing surgery was 45 Gy. The total dose for inoperable patients was 60 Gy. Most patients received prophylactic cranial irradiation with a total dose of 30 Gy. Concomitant chemotherapy started on day 2 of the radiotherapy using cisplatin 50 mg/m² on days 2 and 8 and etoposide 100 mg/m² on days 4–6.

Surgery: After repeated mediastinoscopy patients with negative mediastinal lymph nodes and a few with only one positive lymph node underwent definitive surgery 3–5 weeks after the end of radiation therapy. Operative procedures

Table 1 – Patient characteristics at the time of primary diagnosis of lung cancer and correlations of the serum levels of DNA, caspases, total RNA and miRs with these parameters.

Parameters	Patients (%)	DNA Levels	Caspase Activities	Total RNA	miR10b	miR34a	miR141	miR155
Mean ± Standard Deviation (relative values)								
Lung cancer patients								
Total	35							
Age	55 years (range 32–71 years)							
Gender								
Female	12 (34.3)	15223.2 ± 1807.4	84178 ± 103349.1	4.6 ± 2.7	1.5 ± 1.1	1.5 ± 0.4	0.2 ± 0.1	2.9 ± 1.8
Male	23 (65.7)	12458.5 ± 6063.9	12101.3 ± 5444.9	5.0 ± 2.2	2.1 ± 1.4	2.2 ± 1.4	0.2 ± 0.2	2.9 ± 1.5
Histology								
NSCLC	19 (70.4)	12414.4 ± 3583.3	44883.6 ± 75506.1	4.7 ± 2.4	2.0 ± 1.4	2.0 ± 1.3	0.2 ± 0.2	2.9 ± 1.7
SCLC	8 (29.6)	19558 ± 8913.8	11990 ± 8584.3	6.0 ± 0.8	1.8 ± 0.8	1.9 ± 0.7	0.1 ± 0.1	2.8 ± 0.9
Tumor stage								
pT1-2	12 (44.4)	14215.3 ± 1834.6	61045 ± 97491.4	4.1 ± 2.1	1.4 ± 0.7	1.4 ± 0.4	0.1 ± 0.02	1.6 ± 0.7
pT3-4	15 (55.6)	13213.7 ± 5927.7	30391.1 ± 58949.8	5.1 ± 2.3	2.1 ± 1.4	2.1 ± 1.3	0.2 ± 0.2	3.3 ± 1.5
Diagnosis subgroup								
M0	17 (63)	13191 ± 5591.4	28506 ± 55918.9	4.8 ± 2.4	1.9 ± 1.3	2.0 ± 1.3	0.2 ± 0.2	2.8 ± 1.6
M1	10 (37)	14624.3 ± 1983.5	77546.7 ± 112303.3	5.5 ± 1.5	2.2 ± 1.3	1.6 ± 0.3	0.3 ± 0.2	3.4 ± 1.8
Lymph node metastasis								
N0	4 (15.4)	11608.3 ± 4174.5	9243.3 ± 4026.8	4.6 ± 3.3	1.2 ± 0.4^a	2.0 ± 0.8	0.1 ± 0.1	1.8 ± 0.9
N1-3	22 (84.6)	14095.9 ± 5215.8	48997 ± 78341.9	5.0 ± 2.0	2.2 ± 1.4^a	1.9 ± 1.3	0.2 ± 0.2	3.2 ± 1.6
Grading								
II	7 (33.3)	12035.8 ± 3030.1	11906 ± 5226.6	5.3 ± 2.8	1.4 ± 1.2	1.8 ± 1.2	0.2 ± 0.2	1.9 ± 1.6
III	14 (66.7)	14450.6 ± 5847	57271.3 ± 86581.8	4.6 ± 2.0	2.3 ± 1.2	2.1 ± 1.2	0.2 ± 0.2	3.5 ± 1.2
TPA								
≤ 70	11 (37.9)	13794.7 ± 3383.5	74580 ± 95200.9	4.8 ± 3.1	0.9 ± 0.6^b	1.3 ± 0.8	0.2 ± 0.2	2.1 ± 1.8
> 70	18 (62.1)	13288 ± 6270.4	10031.4 ± 4818.4	4.9 ± 1.6	2.6 ± 1.1^b	2.4 ± 1.2	0.2 ± 0.2	3.4 ± 1.2
uPA								
≤ 850	8 (42.1)	15108.4 ± 6910.8	50956 ± 87541.4	6.8 ± 1.1	2.1 ± 2.0	2.1 ± 1.9	0.1 ± 0.04^c	2.6 ± 1.9
> 850	11 (57.9)	12530.3 ± 3432.8	32865 ± 62399	3.9 ± 2.0	1.8 ± 0.9	1.9 ± 0.8	0.3 ± 0.2^c	3.0 ± 1.4
Patients with benign lung disease								
Total	7							
Age	69 years (range 63–75 years)							
		13029 ± 9396	32217 ± 26452	7.0 ± 4.4	0.5 ± 0.5	1.5 ± 0.9	0.0 ± 0.0	1.3 ± 0.8
Healthy controls								
Total	28							
Age	42 years (range 21–67)							
		7532 ± 838	5725 ± 2351	2.8 ± 1.3	0.3 ± 0.2	2.1 ± 4.6	0.7 ± 2.8	1.0 ± 0.5

Abbreviations: NSCLC, non-small-cell lung cancer; SCLC, small cell lung cancer; M0, patients with localized lung cancer; M1, patients with metastatic lung cancer; TPA, tissue polypeptide antigen (U/L); uPA, urokinase plasminogen activator (pg/ml).

^a $p < 0.03$ (p values as determined by Chi-square and Fischer's exact test).

^b $p = 0.01/p = 0.03$ (p values as determined by Mann–Whitney-U test).

^c $p < 0.02$ (p values as determined by Chi-square and Fischer's exact test).

included lobectomies, bilobectomies, sleeve resections, chest wall resections, or pneumonectomies were performed as clinically indicated.

Serum samples of 7 patients with suspicion of lung cancer, that shaped up as non-cancerous diseases were collected between November 2005 and February 2010 as control cohort of patients with benign lung tumors. The small size of the cohort is because of the rare cases in clinical daily routine (Table 1).

Additionally, 11 healthy female and 17 male controls aged between 21 and 67 (mean age 42) with no history of cancer and in good health based on self-report were recruited. The examination of the samples was approved by the local ethics review

board. Table 1 summarizes the clinical data and the laboratory values of the cancer patients.

2.2. Determination of serum markers TPA and uPA

TPA (tissue polypeptide antigen) was measured using the Liaison TPA-M kit (Byk Sangtec, Dietzenbach, Germany), a two-site immunoluminometric assay (sandwich principle) based on paramagnetic particles coated with three Mabs and a polyclonal tracer antibody labeled with an isoluminol derivative. The Mabs used in the assay are directed against various epitopes on CK 8, 18 and 19.

UPA (urokinase plasminogen activator) was determined using the uPA sandwich enzyme immunoassay (Oncogene Science, Cambridge, MA) that utilizes two Mabs to human uPA as the capture reagents, immobilized on the interior surface of microplate wells.

2.3. Cell culture

For investigations of miR expression in cell lines, the lung cancer cell line H1395 was cultured in DMEM (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (fetal calf serum; PAA Laboratories, Cölbe, Germany), 2 mM L-glutamin (Invitrogen) and 200 U/mL antibiotic-antimycotic solution (PAA). Micrometastatic lung cell line LC-M1 was cultured in RPMI (Invitrogen) with 2 mM L-glutamin (Invitrogen), 200 U/mL antibiotic-antimycotic solution (PAA), 1x Insulin-Transferrin-Selenium-A (Gibco, Eggenstein, Germany), 50 ng/mL human EGF (epidermal growth factor; Macs Miltenyi Biotec, Bergisch Gladbach, Germany) and 10 ng/mL human bFGF (basic fibroblast growth factor; Macs Miltenyi Biotec). To analyze the methylation status of the miR promoters, each cell line was stimulated by 1 μ M 5-aza-2'-deoxycytidine (5-aza-CdR, Sigma-Aldrich, Taufkirchen, Germany) for 72 h 5-aza-CdR-treated cells and mock controls were stimulated by 500 nmol/L trichostatin A (TSA, Sigma-Aldrich) for 24 h following the 48-h 5-aza-CdR or mock incubation.

2.4. Quantification of circulating cell-free DNA in blood serum

Serum was processed immediately after blood withdrawal and repeatedly centrifuged to avoid cell lyses and to remove any potential contaminating cells. For quantification of circulating cell-free DNA in serum, the Quant-iT PicoGreen dsDNA Kit (Invitrogen) was performed according to the manufacturer's instructions. Briefly, to compare the single assays, a five-point standard curve of 1000, 100, 10, 1 and 0 ng/mL Lambda DNA was prepared. The experiments were done in duplicates. Following 5 min incubation at room temperature, the intensity of fluorescence was measured at 520 nm on a Mastercycler realplex (Eppendorf, Hamburg, Germany).

2.5. Quantification of the caspase 3 and 7 activity in blood serum

For measurements of the activity of caspase 3 and 7, the Caspase-Glo[®]3/7 Assay (Promega, Mannheim, Germany) was carried out according to the manufacturer's instructions. The Caspase-Glo[®]3/7 Assay is based on the cleavage of the DEVD sequence of a luminogenic substrate by the caspases 3 and 7 which results in a luminescent signal. The fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 527 nm on a Mikrowin 2000 Plate Reader (Berthold Technologies, Barsinghausen, Germany).

2.6. Extraction of total RNA and reverse transcription

Isolation of total RNA from blood serum and cultured cell lines, and reverse transcription were performed by the mirVana PARIS kit (Ambion, Darmstadt, Germany) and the

TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions, respectively. The extracted RNA from 400 μ L serum samples and 1×10^6 lysed cells was quantified on a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, USA). One μ L RNA derived from serum or cultured cells was reverse transcribed on an MJ Research PTC-200 Peltier Thermal Cycler (Global Medical Instrumentation, Minnesota, USA).

2.7. Preamplification of miR141 and miR16 cDNA

Based on the low expression of miR141 detected in serum, a preamplification of its cDNA was performed. To properly normalize the expression data of miR141, cDNA of the reference miR16 was also preamplified. Both cDNAs were preamplified in 7.5 μ L Taq PCR Mastermix and 0.75 μ L 20x TaqMan MiRNA Assay mix by using the Taq PCR Mastermix Kit (Qiagen, Hilden, Germany). The PCR was run on an MJ Research PTC-200 Peltier Thermal Cycler (Global Medical Instrumentation): 1 cycle at 95 °C for 5 min, 15 cycles at 95 °C for 20 s, 60 °C for 20 s and 72 °C for 20 s and a terminal cycle at 72 °C for 5 min.

2.8. Quantitative real-time PCR of miR10b, miR34a, miR141 and miR155

For quantitative real-time PCR the miR-specific TaqMan MicroRNA Assays (Applied Biosystems) for miR16 (reference miR), miR10b, miR34a, miR141 and miR155 were used. In a 10 μ L-reaction, 1 μ L of cDNA or 3 μ L preamplified cDNA was mixed with 5 μ L TaqMan 2xUniversal PCR Master Mix No AmpErase UNG and 0.5 μ L of its miR-specific TaqMan MicroRNA Assay Mix on a twin-tec real-time PCR plate (Eppendorf). The quantitative real-time PCR reaction was performed on a Mastercycler Realplex (Eppendorf) at 95 °C for 10 min and 40 cycles at 95 °C for 15 s and 60 °C for 60 s.

The obtained data of the miR expression levels were calculated and evaluated by the ΔC_t method as follows: $\Delta C_t = \text{mean value } C_t (\text{reference miR16}) - \text{mean value } C_t (\text{miR of interest})$. The relative expression of miR of interest corresponded to the $2^{-(\Delta C_t)}$ value.

As recommended by the manufacturer (Applied Biosystems), miR16 has been chosen as reference to normalize of the expression levels of our miR panel. To effectively normalize the expression data of miR10b, miR34a, miR141 and miR155, we analyzed the miR16 expression levels and found that the levels remained relatively constant across the serum samples. We calculated a mean value of 25.33, 24.33 and 21.17, with a standard deviation of 1.24, 2.39 and 0.98 for the subgroups of M0 patients, M1 patients and healthy individuals, respectively. So, the mean C_t values of miR16 are somewhat higher in lung cancer patients than in healthy individuals. Standardized RNA volumes were used for real-time PCR, leading to similar mean values of miR16 in the patient cohorts and healthy controls.

2.9. Statistical analysis

The statistical analyses were performed using the SPSS software package, version 18.0 (SPSS Inc. Chicago, IL). The chi-

Square or two-tailed Fischer's exact test was used to identify possible associations between DNA and miR concentrations in blood and the clinical and histopathological risk factors of the lung cancer patients. For non parametric comparisons, univariate analyses of the Mann Whitney-U test of two independent variables and bivariate analyses of the Spearman–Rho test were used. Kaplan–Meier plots were drawn on to estimate overall survival and recurrence, and the Log rank test was used for statistical analyses. Diagnostic power of the single markers was analyzed by receiver operating characteristic (ROC) curves. Areas under the curve (AUC) were calculated. A p value ≤ 0.05 was considered as statistically significant. All p values are two-sided.

3. Results

3.1. Quantification of circulating DNA and caspase activities in blood serum

We compared the levels of cell-free DNA in blood serum of 35 lung cancer patients including 19 NSCLC, 8 SCLC patients and 8 patients with indefinite cancer type with those of 7 patients with benign lung disease and 28 healthy individuals. As shown in the box plots of [Figure 1A](#), patients with benign lung disease ($p = 0.001$, Mann–Whitney-U test) and cancer patients ($p = 0.0001$) had significantly higher DNA levels in their blood than healthy individuals. The median DNA values in blood serum of the cancer patients and patients with benign lung tumors were approximately 4-fold and 1.5-fold higher than in healthy individuals, respectively. However, the DNA concentrations in serum could not discriminate between benign and malignant lung disease ($p = 0.099$). In order to determine the sensitivity and specificity of the cell-free DNA in distinguishing between lung cancer and benign lung disease or healthy controls, we performed ROC analysis and used area under the curve (AUC) to test its performance. The AUC value was 0.943, demonstrating the significant difference of the DNA levels between lung cancer patients and healthy individuals ([Figure 1B](#)) and 0.702, demonstrating the significant difference of the DNA levels between lung cancer patients and patients with benign lung disease ([Figure 1C](#)). Moreover, in serum of cancer patients with metastatic disease (M1) the mean DNA yield was somewhat higher than that of cancer patients with primary lung cancer (M0) (data not shown).

During apoptosis caspases are activated and cause DNA fragmentation resulting in the release of DNA into the blood circulation. To determine whether the activities of caspases 3 and 7 associate with the levels of circulating DNA in serum of patients, we quantified them in blood serum by the Caspase-Glo[®]3/7 Assay. As shown in the box plots of [Figure 1D](#), the relative caspase activities were significantly higher in patients with lung cancer ($p = 0.0001$, Mann–Whitney-U test) and benign tumors ($p = 0.003$) than those in blood of healthy individuals. The median values of the caspase activities in blood serum of the cancer patients and patients with benign disease were approximately 2.5-fold and 5-fold higher than in healthy individuals, respectively. The AUC value of ROC analysis was 0.896, demonstrating the

significant difference of the activities between lung cancer patients and healthy controls ([Figure 1E](#)) and 0.335, demonstrating the difference between lung cancer patients and patients with benign lung tumors ([Figure 1F](#)). The inconsistent AUC values reflect the results from [Figure 1D](#), since the caspase activities in serum had no significant diagnostic power to distinguish between benign and malignant disease ($p = 0.181$). As shown in a scatter plot, we found significant correlations of the elevated levels of circulating DNA with the increasing caspase activities using the Spearman–Rho test ($p = 0.0001$, $r = 0.529$; [Figure 1G](#)).

3.2. Expression pattern of miR10b, miR34a, miR141 and miR155 in basal and stimulated lung cancer cell lines

Before examining the signature of the four miRs (miR10b, miR34a, miR141 and miR155) in human blood, we quantified their relative expression patterns in the lung cancer cell line H1395 and the micrometastatic lung cancer cell line LC-M1 by determining the low cycle threshold (Ct) values. The micrometastatic cell line LC-M1 was established from disseminated tumor cells of bone marrow derived from a lung cancer patient ([Pantel et al., 1995](#)). Its invasive phenotype has been supported by the constitutive expression of several adhesion molecules characteristic for metastasis ([Putz et al., 1999](#)). As shown in [Figure 2A](#), the relative level of miR141 was much higher in H1395 cells than in LC-M1 cells, whereas the levels of miR10b, miR34a, and miR155 in both cell lines did not differ in such a extent. In order to clarify whether both DNA methylation and histone deacetylation are involved in activation of the miR promoters, the cell lines H1395 and LC-M1 were incubated with the DNA demethylating agent 5-aza-CdR and/or the histone deacetylase inhibitor TSA. 5-aza-CdR could only upregulate the basal expression of miR141 in LC-M1 cells, but had no effect on miR141 levels in H1395 cells. TSA had neither alone nor in concert with 5-aza-CdR a stimulatory effect on this promoter activity ([Figure 2B](#)). These data suggest that in LC-M1 cells the low expression level of miR141 may be caused by hypermethylation of its promoter, whereas the chromatin structure seems to have no implication in activation. Moreover, 5-aza-CdR and/or TSA could not stimulate the basal transcript levels of the other miRs (miR10b, miR34a and miR155) in both cell lines (data not shown).

3.3. Signature of miR10b, miR34a, miR141 and miR155 in blood serum

Following quantification of total RNA, miR10b, miR34a, miR141 and miR155 in the cell lines, we compared their concentrations in serum of the lung cancer patients with those of the patients with benign lung disease and healthy individuals. As shown in the box plots of [Figure 3A](#) and determined by the normalized Ct values, the lung cancer patients had significantly higher circulating RNA ($p = 0.009$) and miR (miR10b, miR34a, miR141 and miR155) levels ($p = 0.0001$) in their blood than healthy individuals (Mann Whitney-U test). The median values of total RNA, miR10b, miR34a, miR141 and miR155 in blood serum of the tumor patients were approximately 2-, 11-, 5-, 10- and 4-fold higher than in healthy individuals, respectively. In order to determine the sensitivity and specificity

Figure 1 – Elevated DNA and caspase levels in blood of lung cancer patients The box plots show the comparison of circulating DNA concentrations (A) and caspase activities (D) in blood of healthy individuals ($n = 28$), patients with benign lung disease ($n = 7$) and lung cancer patients ($n = 35$). The ROC analyses show the profiles of sensitivity and specificity of DNA concentrations to discriminate lung cancer patients from healthy controls (B) and from patients with benign lung disease (C), as well as the profiles of sensitivity and specificity of caspase activities to discriminate lung cancer patients from healthy controls (E) and from patients with benign lung disease (F). The scatter plot (G) shows the relative DNA concentrations versus the caspase activities. The significant p values are indicated as determined by Mann Whitney-U test and Spearman-Rho test.

of total cell-free RNA and the miRs in distinguishing between lung cancer and healthy controls, we performed ROC analysis to test their performance. Overall, the AUC values exceed 0.704 for total RNA and 0.899 for each miR demonstrating the significant differences of the RNA levels between lung cancer patients and healthy individuals (Figure 3B).

Surprisingly, the RNA concentration was higher in serum of patients with benign lung disease than in lung cancer patients, but these different yields were not significant ($p = 0.084$). However, the serum RNA levels in patients with benign tumors were significantly higher than those in healthy individuals

($p = 0.001$). As the serum DNA amounts, the serum RNA amounts do not differentiate between malignant and benign tumor patients. With the exception of serum miR34a, the levels of miR10b ($p = 0.002$), miR141 ($p = 0.0001$) and miR155 ($p = 0.007$) in patients with benign disease were significantly lower than the levels in cancer patients. The miR concentrations were similar between healthy individuals and patient with benign disease (Figure 3A). Overall, the AUC values exceed 0.286 for total RNA and 0.963 for each miR demonstrating the significant differences of the miR levels between lung cancer patients and patients with benign lung tumors (Figure 3C).

Figure 2 – MiR expression in basal and stimulated cell lines as determined by quantitative real-time PCR Basal expression levels of miR10b, miR34a, miR141 and miR155 in the lung cancer cell line H1395 and the micrometastatic lung cell line LC-M1 (A). LC-M1 cells were treated with 1 μ mol/L 5-aza-CdR for 72 h and/or 0.5 mmol/L TSA for the last 24 h. The transcript levels of miR141 were determined prior and after stimulation (B).

3.4. Relationship of circulating DNA and microRNA levels with the clinical data

We compared the concentrations of circulating serum DNA, total RNA, miR10b, miR34a, miR141, and miR155 with the clinical and histopathological data of the lung cancer patients. For these statistical evaluations the chi-Square or two-tailed Fischer's exact test, and the Mann Whitney-U test of the non parametric comparison of two independent variables were used. As summarized in Table 1, the increased serum levels of miR10b correlated significantly with positive lymph node status. Approximately 85% of the patients with lymph node metastasis had high serum values of miR10b in their blood ($p < 0.03$). It has been reported that elevated levels of the tumor markers TPA and uPA associate with tumor progression and poor outcome of lung cancer patients (Chen et al., 2009; Mazar, 2008). Our statistical analyses show that high serum values of miR10b and miR141 correlated with the elevated serum levels of TPA and uPA, respectively. Alike, 85% of the patients who had high expression levels of TPA and uPA had also significantly high serum values of miR10b ($p = 0.01$) and miR141 ($p = 0.02$), respectively.

4. Discussion

In the present study, we demonstrated that the concentrations of cell-free nucleic acids (DNA, RNA and miRs) were significantly higher in blood of lung cancer patients than in normal controls, and thus, may discriminate cancer patients from healthy individuals. Considering the different values in the both patient cohorts, the DNA and RNA levels could not discriminate between benign and malignant lung tumors. Nevertheless, there was a rise in DNA leading from healthy individuals over patients with benign tumors to lung cancer patients. Surprisingly, serum levels of total RNA were higher in

patients with benign lung tumors than in lung cancer patients. In several studies increased presence of circulating DNA in blood of lung cancer patients has been reported (Pathak et al., 2006). After chemotherapy of SCLC cancer patients increasing plasma DNA concentrations significantly associated with tumor progression. Their quantification is an accurate technique amenable to standardization, which was also shown to complement current methods for the prediction of patient survival (Gautschi et al., 2004). As far as we know, only two studies investigated the DNA levels in plasma of patients with benign lung disease (Xie et al., 2004; Schmidt et al., 2008). Whereas a study showed that the significantly increased DNA levels could discriminate between benign and malignant lung disease (Xie et al., 2004), the other study found no significant differences of these populations (Schmidt et al., 2008).

To determine the mechanism underlying the DNA release into the blood circulation, we investigated its relationship with the apoptotic pathway. We detected a significant correlation with an increase in the circulating activities of caspases 3 and 7 in blood of all patients. This suggests that the induction of DNA release may occur particularly by activating the caspases 3 and 7 and reflects their active role in tumorigenesis. High serum levels of caspase activities could only discriminate between healthy individuals and tumor patients, but not between benign and malignant tumors. As the total RNA levels, the caspase activities were higher in patients with benign lung tumors than in patients with lung cancer. Why the total RNA levels and caspase activities were so high in our cohort of patients with benign disease is unknown, but may reflect the high rates of cell death during inflammation processes.

To date, mainly in tumor cells and tissues of lung cancer patients, the expression of miRs have previously been examined (Baffa et al., 2009; Hayashita et al., 2005; Bommer et al., 2007; Wang et al., 2009). As far as we know, there is only one study on circulating miRs in blood of these cancer patients (Hu et al., 2010). Our analyses show significantly elevated

Figure 3 – Elevated levels of RNA and miRs in blood of lung cancer patients The box plot shows the significantly different amounts of RNA, miR10b, miR34a, miR141 and miR155 which circulate in blood of healthy individuals ($n = 28$), patients with benign lung disease ($n = 7$) and patients with lung cancer ($n = 35$). The small integrated diagram shows a magnification of the low serum concentrations of miR141. The significant p values are indicated as determined by Mann Whitney-U test (A). The ROC analyses show the profiles of sensitivity and specificity of each miR to discriminate lung cancer patients from healthy controls (B) and from patients with benign lung disease (C).

transcript levels of our miRs panel in serum of lung cancer patients in comparison to healthy individuals and patients with benign lung disease. To date, no studies on circulating miRs in blood of patients with benign lung disease have been published. Our study shows for the first time that with the exception of miR34a, the miRs miR10b, miR141 and miR155 could discriminate between benign and malign lung disease. Although miR34a was reported to be downregulated in NSCLC tissues and other tumor entities (Bommer et al., 2007; Braun et al., 2008), our data clearly refer to an increased transcript level in lung cancer (NSCLC and SCLC) patients, which may be caused by an upregulation of the gene expression and/or a pronounced stability of this molecule. Admittedly, miRs have numerous target mRNA molecules and can, therefore, repress the transcription of several genes. In discharge of this interplay of multi-regulatory functions, the effective impact of miR34a is hard to interpret. Our observation on its upregulation is in line with Dutta et al. who described a high incidence of miR34a overexpression in various tumor types and undetectable expression in only poorly differentiated adenocarcinomas of stomach and renal cell carcinomas. Their data showed that miR34a overexpression supported cell proliferation in the majority of carcinomas (Dutta et al., 2007).

In addition, we detected that the increase in serum levels of miR141 in lung cancer patients paralleled with increasing concentrations of the serine protease uPA which is involved in extracellular matrix degradation. The secretion of high amounts of uPA has been described to associate with the invasion of cancer cells (Mazar, 2008). It was reported that miR141 activates the epithelial differentiation of diverse tumor cells and is involved in the epithelial mesenchymal transition (EMT) which may be an important step in tumor progression. This process is thought to contribute to the invasiveness and dissemination of epithelial tumor cells (Burk et al., 2008). Downregulation of miR141 in tumor patients associated with tumor progression and might be directly involved in cancer metastasis (Baffa et al., 2009). Its elevated transcript level in blood of our lung cancer patient cohort which associated with the serum uPA level may deliver a further indication of its role in aggressive diseases.

Besides miR141, miR10b may also play an essential role in EMT process, because its expression is induced by the binding of the EMT-inducing transcription factor TWIST at its putative promoter leading to an increased expression of the prometastatic gene RhoC (Ma et al., 2007). Overexpression of miR10b in non-metastatic breast cancer cells enables them to acquire invasive and metastatic behavior. Conversely, gene silencing of miR10b inhibits Twist-mediated cell migration and invasion (Ma et al., 2007). Its potential as a target for anti-metastatic therapy was showed in mice bearing highly metastatic cells. Administration of miR10b antagomirs, a class of chemically modified anti-miRNA oligonucleotides, to these mice markedly suppressed formation of lung metastases in a sequence-specific manner. The miR10b antagomir, which was well tolerated by normal animals, appears to be a promising candidate for the development of new anti-metastasis agents (Ma et al., 2010). Our analyses in blood serum complement this recent observation, and show that elevated levels of circulating miR10b may represent a novel minimally invasive diagnostic tool in the

characterization of lymph node-positive lung cancer gene targets. Although we detected a significant relationship of miR10b with lymph node metastasis, we detected no such association with distant metastasis suggesting that the up regulation of miR10b is acquired in the course of lung tumor progression and, in particular, during the acquisition of metastatic potential. Also, we detected an association between the miR10b yields and serum levels of TPA, which is a degradation product of the intermediate filament cytoskeleton formed by the cytokeratins 8, 18 and 19. These cytokeratins are expressed in simple epithelium, including benign and malignant lung epithelia. TPA serves as a tumor marker for lung cancer patients, since its serum levels are elevated in cancer patients and correlated with an unfavorably clinical outcome (Chen et al., 2009; Kasimir-Bauer et al., 2003). Our findings endorse previous studies on tumor cells and tissues showing that miR10b correlate with tumor progression and metastatic disease of breast cancer (Ma et al., 2007; Baffa et al., 2009).

To investigate the regulatory mechanism underlying the gene repression of miRs, we performed expression analyses in micrometastatic tumor cells and a tumor cell line. Gene silencing has been described to be reversed by epigenetic drugs, such as DNA demethylating agents and histone deacetylase inhibitors, leading to promoter demethylation and activation (Lujambio and Esteller, 2007). We observed that DNA hypomethylation may also be the mechanism responsible for the aberrant expression of miR141 in the micrometastatic lung cancer cell line LC-M1, because the demethylating treatment of these cells by 5-aza-CdR resulted in activation of miR141. In contrast, 5-aza-CdR had no stimulatory effect on the other miRs in both cell lines. Moreover, our administration of the cells by histone deacetylase inhibitor TSA had no effect on the expression levels, indicating that histone acetylation is not involved in the transcriptional activation of this miR.

The main goal for treating cancer patients in the future is “targeted therapy”. Thus, patients will be treated according to their personal expression profile not only on the primary tumor but also on metastases. Lung cancer biopsies only give a restricted information about the phenotype as well as the genotype of the tumor. In addition, the re-evaluation of an expression profile, e.g. receptor status, for selecting the appropriate therapy by a tissue biopsy of the metastatic lesion is not feasible due to location of the metastatic site. Furthermore, the expression profiles may vary between different metastatic sites and also change during treatment. Therefore, the screening of circulating nucleic acids might be a strategy with potential clinical application since serum samples can easily be taken during follow-up of the patients.

5. Conclusion

In conclusion, our quantitative analyses show that the aberrantly high levels of circulating DNA, caspase activities and total RNA detected in blood serum discriminate between cancer patients and healthy individuals. The apoptotic pathway may be involved in the DNA release into the blood circulation. High levels of circulating miRs were found to be specific for lung cancer patients, whereas the miR levels were similar in

healthy individuals and patients with benign lung tumors. Screening for serum miRs could provide additional diagnostic information on this cancer entity, and become a valuable non-invasive test to monitor disease progression and to distinguish between benign and malignant tumors. The main caveat of our study is the small size of the patient cohorts, particularly the subgroup of the patients with benign lung tumors. However, in the clinical practice patients with benign lung disease are rare cases.

Apart from the significant relationship of serum miR10b levels with lymph node metastasis, no further associations of the miRs were detected with the pTNM stage. This lack of correlation implicates the small size of our patient collective and the arbitrarily selected miR panel. Analyses of additional miRs may improve our molecular approach, and the miRs may be revealed by more systematic profiling, using a microRNA expression microarray. For an advanced detection and monitoring of the disease, we plan to perform microarray chip analyses to select the most clinically relevant miRs. The subsequent quantification of these miRs in single real-time PCR analyses and in larger cohorts of lung cancer patients with follow-up studies might improve the identification of potential targets. Moreover, it is necessary to enroll a larger cohort of patients with benign lung tumors to determine whether the analyzed markers are cancer-specific and provide new insights into the complex pathogenesis of lung cancer.

Conflict of interest

All authors disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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