Dissertation

Differential expression of miRNAs in A549 cells and plasma samples from lung adenocarcinoma patients and healthy donors

submitted by

Dr. Irina ROBINSON

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Department of Internal Medicine, Division of Pulmonology

under the Supervision of

Assoz. Prof. Priv.-Doz. Dr.rer.nat. Andelko HRZENJAK

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Statutory Declaration

I hereby declare that this dissertation is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this dissertation. Due acknowledgement has been made in the text to all other material used. Throughout this dissertation and in all related publications I followed the "*Guidelines of the Medical University of Graz on Good Scientific Practice*".

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Disclosures

This dissertation project resulted in the publication of the following scientific paper:

Robinson I, Bertsch A, Leithner K, Stiegler P, Olschewski H, Hrzenjak A. Circulating microRNAs as molecular biomarkers for lung adenocarcinoma. Cancer Biomark. 2022;34(4):591-606. doi: 10.3233/CBM-210205.

The data contained in the publication will also be discussed and presented in this thesis.

Following co-authors actively contributed to the results of the thesis and the aforementioned publication:

Alexandra BERTSCH - Department of Internal Medicine, Division of Pulmonology, MUG

Katharina LEITHNER - Department of Internal Medicine, Division of Pulmonology, MUG

Philipp STIEGLER - Department of Transplant Surgery, MUG

Horst OLSCHEWSKI - Department of Internal Medicine, Division of Pulmonology, MUG

Andelko HRZENJAK - Department of Internal Medicine, Division of Pulmonology, MUG

All co-authors have explicitly agreed to the use of their published data in this thesis, and permission to reproduce illustrations and figures from own or third-party publications has been granted.

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Circulating microRNAs as molecular biomarkers for lung adenocarcinoma

Irina Robinson [1], Alexandra Bertsch [1], Katharina Leithner [1 2], Philipp Stiegler [3], Horst Olschewski [1], Andelko Hrzenjak [1 4]

doi: 10.3233/CBM-210205.

[1] Department of Internal Medicine, Division of Pulmonology, MUG.

[2] BioTechMed-Graz, Graz, Austria.

[3] Department of Transplant Surgery, MUG.

[4] Ludwig Boltzmann Institute for Lung Vascular Research, Medical University of Graz, Graz, Austria.

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Abstract

MicroRNAs (miRNAs) are small non-coding RNA molecules, which take part in gene regulation on post-transcriptional level. They are involved in different biological processes, but also in tumorigenesis. In this thesis, we wanted to extend our knowledge about miRNA expression and they role in lung cancer, to find out a potential circulating miRNA that could be promising as a diagnostic biomarker for early lung cancer detection. In the first part of the thesis, we aimed to analyze the global expression of 752 miRNAs *in vitro* in A549 lung adenocarcinoma (LUAD) cells and in primary, non-malignant bronchial epithelial (BE) cells from healthy donors, and to identify the most prominently deregulated miRNAs. Expression of 752 miRNAs in LUAD and BE cells was assessed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) with mean-centering restricted (MCR) normalization. Out of 752 miRNAs, 37 miRNAs were significantly deregulated in A549 cells compared to BE cells. After setting $\Delta\Delta Ct \geq 2.5$, eighteen significantly deregulated miRNAs were chosen, 8 were up- and 10 down-regulated in A549 cells compared to BEC cells. Based on those *in vitro* data, the aim of the second part of this thesis was to analyze miRNAs expression in plasma samples from patients with histologically diagnosed lung adenocarcinoma and healthy donors (n = 18 for each group, totally n = 36). We also aimed to identify stabile expressed miRNAs in plasma. We found four significant deregulation miRNAs in plasma samples (miR-15b-3p, miR-148a-3p, miR-193b-3p, and miR-195-5p) in LUAD patients compared to donors' samples. One miRNA (miR-195-5p) was up-regulated and three miRNAs (miR-15b-3p, miR148a-3p, and miR-193b-3p) were down-regulated in plasma sample of LUAD patients compared to healthy donors. Two miRNAs were used for normalization (miR-16-5p and miR-191-5p), because of their known and proved stability. Finally, by different *in silico* tools, we identified the target genes influenced by deregulated circulating miRNAs and determine their assignments to specific pathways relevant for biology of lung adenocarcinoma. Based on literature, 107 validated genes are regulated by these four miRNAs. Out of 107 validated target genes, five (PTEN, CXCR4, IGF1R, FGF2, and PD-L1) were described to be regulated with more than one of four deregulated miRNAs. Concerning their molecular function, 107 direct target genes were annotated to five main functional groups: binding, catalytic activity, molecular function regulator, molecular transducer activity, and transporter activity. In conclusion, we could demonstrate the relevant differences between cancer and control miRNA expression *in vitro* and in plasma samples of LUAD patients compared to healthy donors. These four dysregulated miRNAs are promising as a diagnostic biomarkers for lung adenocarcinoma.

Abstrakt

MicroRNAs (miRNAs) sind kleine nicht kodierende RNA-Moleküle, die an der Genregulation auf posttranskriptioneller Ebene beteiligt sind. Sie haben eine wichtige Rolle in verschiedenen biologischen Prozessen, aber auch in der Tumorentstehung. Mit dieser Dissertation erweitern wir das bestehende Wissen über miRNAs und ihre Rolle bei Lungenkrebs, mit dem Ziel zirkulierende miRNA zu finden, die als diagnostische Biomarker für die Früherkennung von Lungenkrebs dienen können. Im ersten Teil dieser Arbeit analysierten wir die globale Expression von 752 miRNAs *in vitro* in A549-Lungenadenokarzinom (LUAD)-Zellen und in primären, nicht-malignen Bronchialepithel (BE)-Zellen von gesunden Spendern und identifizierten die deregulierten miRNAs. Die Expression von 752 miRNAs in LUAD- und BE-Zellen wurde mittels reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysiert und mittels mean-centering restricted (MCR) Normalisierung ausgewertet. Von 752 miRNAs, waren 37 miRNAs in A549-Zellen im Vergleich zu BE-Zellen signifikant dereguliert. Nach der Einstellung von $\Delta\Delta Ct \geq 2,5$ wurden achtzehn signifikant deregulierte miRNAs ausgewählt; 8 waren hoch- und-10 herunterreguliert. Basierend auf diesen *in vitro* Daten, untersuchten wir die miRNAs Expression in Plasmaproben von Patienten mit Lungenadenokarzinom und gesunden Spendern (n = 18 pro Gruppe). Wir fanden vier signifikant deregulierte miRNAs (miR-15b-3p, miR-148a-3p, miR-193b-3p und miR-195-5p) bei LUAD-Patienten im Vergleich zu Spenderproben. Eine miRNA (miR-195-5p) war hochreguliert und drei miRNAs (miR-15b-3p, miR148a-3p und miR-193b-3p) waren herunterreguliert. Zwei miRNAs wurden für die Normalisierung verwendet (miR-16-5p und miR-191-5p), aufgrund ihrer bekannten und nachgewiesenen Stabilität. Schließlich identifizierten wir mit verschiedenen *in silico* Tools die Zielgene, die von deregulierten zirkulierenden miRNAs beeinflusst werden und für die Biologie des Lungenadenokarzinoms relevant sind. Basierend auf der Literatur wurden 107 validierte Gene gefunden. Fünf davon (PTEN, CXCR4, IGF1R, FGF2 und PD-L1) wurden von mehr als einer von vier deregulierten miRNAs als reguliert beschrieben. Hundertsieben direkte Zielgene wurden der fünf funktionellen Gruppen zugeordnet: Bindung, katalytische Aktivität, molekularer Funktionsregulation, molekulare Transducer-Aktivität und Transportaktivität. Zusammenfassend konnten wir die relevanten Unterschiede zwischen Lungenkrebs- und Kontroll-miRNA-Expression *in vitro* und in Plasmaproben von LUAD-Patienten im Vergleich zu gesunden Spendern aufzeigen. Vier deregulierte miRNAs sind vielversprechend als diagnostischer Biomarker für das Lungenadenokarzinom.

Abbreviations and Definitions

ACO	asthma-COPD overlap
AIS	adenocarcinoma *in situ*
ALK	anaplastic lymphoma kinase
APA	acinar predominant adenocarcinoma
ARG	amphiregulin
BE	bronchial epithelial
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BTC	betacellulin
COPD	chronic obstructive pulmonary disease
CT	computer tomography
Ct	Cycle of threshold
CTCs	circulating tumor cells
ctDNA	circulating tumor DNA
CTECs	circulating tumor vascular endothelial cells
CTLA-4	cytotoxic T-lymphocyte antigen 4
CXCR4	C-X-C chemokine receptor 4
DNMTA	a central DNA methyl transferase in mammalian cells
Drosha	double-stranded RNA-specific endoribonuclease
DYNLT1	dynein light chain Tctex-type 1
EBUS	endobronchial ultrasound
ECOG	Eastern Cooperative Oncology Group
EDTA	ethylenediaminetetraacetic acid

EGFR	epidermal growth factor receptor
EGN	epigen
EPR	epiregulin
ERBB2	erythroblastic oncogene B
ESCC	esophageal squamous cell carcinoma
ESMO	European society for Medical Oncology
Exp5	Exportin 5
FCS	fetal calf serum
FGF2	fibroblast growth factor 2
FOXO	forkhead box O transcription factors
GC	gastric cancer
HB-EGF	heparin-binding EGF-like growth factor
HCC	Hepatocellular carcinoma
HER	human epidermal growth factor receptor
IASLC	international association for the study of lung cancer
IGF	insulin-like growth factor
IMA	invasive mucinous adenocarcinoma
KRAS	Kirsten rat sarcoma viral oncogene
LDCT	low dose computed tomography
LUAD	lung adenocarcinoma
mAbs	monoclonal antibodies
MAPK	mitogen-activated protein kinase
MC	mean-centering
MCR	mean-centering restricted
MET	mesenchymal to epithelial transition

MIA	minimally invasive adenocarcinoma
miRNAs	microRNAs
NGS	next generation sequencing
NLST	National Lung Screen Trial
NSCLC	non-small cell lung cancer
OS	overall survival
PBS	phosphate buffered saline
PD-1	programmed cell death 1
PDL-1	programmed death ligand 1
PET	positron emission computer tomography
PI3K	phosphoinositide 3-kinase
PPA	papillary predominant adenocarcinoma
pre-miRNAs	precursor miRNA
pri-miRNAs	Primary miRNAs
PTEN	phosphatase and tensin homolog
qPCR	real-time quantitative polymerase chain reaction
RBPs	RNA-binding proteins
RG	reference genes
RIN	RNA integrity number
ROS1	proto-oncogene C-Ros-1
RT	room temperature
RTKs	receptor tyrosine kinases
RT-qPCR	reverse transcription-quantitative polymerase chain reaction
SABR	stereotactic ablative body radiotherapy
T790M	threonine 790 with methionine

TEPs	tumor-educated blood platelets
TGF-a	transforming growth factor a
TKI	tyrosine kinase inhibitors
TNM	Tumour, Node, Metastasis
TP53	tumor protein p53
TTF-1	thyroid transcription factor 1
UTR	untranslated region
VATS	video-assisted thoracoscopic surgery
YBX1	Y-box binding protein 1

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1. Introduction

1.1. Lung cancer

1.1.1. Lung cancer statistics

Lung cancer is quite common cancer in both sexes. In year 2020, more than 2,2 million new cases of lung cancer were diagnosed worldwide (1). By the incidence rate, lung cancer takes the leading position together with breast cancer. The number of deaths caused by lung cancer in 2020 in both sexes and all ages was almost 1.800.000 cases. It is more than 18% among all cancer types, and it takes the leading position in the world. Lung cancer is still the main cause among cancer mortality worldwide in men, and it is the second most frequent type after breast cancer in women. (1). The 5-year survival of patients with lung cancer is about 19% (2). In comparison to other cancer types, lung cancer survival rate has remained almost the same as 50 years ago. Of course, the survival rate depends on the stage at which the disease had been detected. The survival rate in other malignant diseases is increasing. For example, Sasaki at al. showed that the 5-year survival rate of patients with tumors of the hematopoietic and lymphoid tissues has recently increased and it is approximately 90% (3). This trend is connected with the new therapy and diagnostic options for blood cancer. The decreased survival rate is associated with the late stage of the disease and this unfavorable factor has influence on the overall survival for patients with lung cancer and therefore the tools for early lung cancer detection are needed.

1.1.2. Lung cancer causes

There are many causes of lung cancer development, one of which is tobacco smoking. Smoking increases the risk of lung cancer by approximately 30 times in comparison to non-smokers. Cigarette smoke leads to DNA damages and to the gene mutation, thereby working as a carcinogen. The consequence of this is a change in expression of tumor suppressor genes, such as tumor protein p53 (TP53), and in oncogenes, such as Kirsten rat sarcoma viral oncogene (KRAS), leading to the uncontrolled cellular growth and cancer development (4). Every cigarette contains a mixture of different chemical components, such as 1,3-butadiene, cyanide, arsenic, polycyclic aromatic hydrocarbons and others, that are toxic not only for the respiratory tract, but for the whole body (5). Secondhand and passive smoking are also proven to be the causes of the development of respiratory diseases, including lung cancer (6). E-cigarettes contain a lot of liquids and metals that have negative side effects to the health with serious (or different) consequences, such as lung cancer (7). Many studies showed that the exposure to

asbestos leads to the high risk of lung cancer occurrence (8), (9). In lung cancer patients, Turner at al showed positive association between the mortality of never-smokers and air pollution. This 26-year long prospective study with a large cohort of 188,699 individuals demonstrated, that prolonged explosion of ambient fine particulate caused by air pollution is correlated with lung cancer mortality in lifelong never-smokers (10). The chronic obstructive pulmonary disease (COPD) can also increase the risk of lung cancer development (11).

1.1.3. Lung cancer screening

The aim of the screening is to detect lung cancer in the early stage when cancer is still curable and, therefore, to decrease the lung cancer incidence and mortality worldwide. Early cancer means that the lesion is ≤ 1 cm in a computer tomography (CT) scan (12). Already in the 1980-s there were many attempts to find out the screening technique for the reduction of lung cancer death cases. The data showed no advantage of using the chest radiography and sputum cytology in comparison to usual medical control as a screening routine (13), (14). A study analyzed the efficiency of CT screening, where about 85% of participants were diagnosed with lung cancer in stage I with a 10-year survival rate of 88%, but those patients who underwent surgical treatment had a 92% survival rate. The screening with CT is beneficial for lung cancer detection in people with high risk, like age and cigarette consumption (15). In many medical guidelines the low-dose CT scan is recommended for screening, based on potential benefits: the reduced lung cancer mortality and the discovery of potential significant health disorders (16), (17), (18). The problems of the screening are possible complications caused by the invasive procedures and the following false positive results and overdiagnosis, but also additional radiation exposure and high costs. Currently there is no strong evidence of minimal invasive biomarkers for early lung cancer detection, but different biomarkers are under investigation, including microRNAs.

1.1.4. Lung cancer diagnostics

At the moment of the first lung cancer diagnosis, most patients have different symptoms, such as dyspnea, hemoptysis, chest pain, cough, weight loss or hoarseness. However, some patients have no claims and are presented with suspicious lesion in chest imaging. For the diagnosis, tissue biopsies are used to differentiate the histology of cancer and to plan the future strategy for therapy. Not only clinical symptoms, but also medical history, physical examinations, laboratory tests and imaging are needed for every patient with a suspicion of lung cancer. Additional imaging, like positron emission tomography scan, brain magnet resonance tomography and ultrasound, is recommended to reveal metastases and for stage detection.
There are many invasive methods used in daily routine to reach the tumor for further determination of the diagnosis. The established technique for the diagnostics is bronchoscopy. Endobronchial ultrasound-guided transbronchial fine needle aspiration is used for the determination of the lymph nodes stage. In some cases, computerized tomography or ultrasound guided transthoracic fine needle aspiration can be useful, especially when the tumor is located in the periphery of lungs. The video-assisted thoracoscopic surgery (VATS) can be also applied for tumor tissue extraction. Patients with malignant pleural effusion should undergo thoracocentesis for additional diagnostics and therapy. With help of these methods, it is possible to receive the tumor tissue for histological, immunochemical and molecular testing. All these invasive methods are associated with possible serious complications, such as bleeding, pneumothorax or hypoxemic respiratory failure and death. Unfortunately, there are not any exact minimal invasive diagnostic tools for lung cancer determination, which would be precise enough and without a high risk of complications.

1.1.5. Liquid biopsy as diagnostics tool

Liquid biopsy is a diagnostic method by which cancer biomarkers such circulating tumor DNA (ctDNA), miRNA, circulating tumor cells (CTCs), tumor-educated blood platelets (TEPs), circulating tumor vascular endothelial cells (CTECs) can be detect. Due to minimal invasive technique, it can be analyzed from collected blood and urine. Peripheral venous blood is the most frequent liquid biopsy test in the daily praxis. It was shown that there is correlation between ctDNA level and cancer mass, suggesting that measurement of ctDNA may be evaluating factor of efficacy after therapy (19). However, the lung cancer diagnosis is still based

on tissue biopsy, and it is recommended to be used as the gold standard for primary lung cancer detection. In comparison with standard invasive tumor detection, ctDNA collection can be repeated many times. CtDNA is commonly used in the clinical practice, for example for detection of epidermal growth factor receptor (EGFR) resistance mutation - threonine 790 with methionine (T790M) mutation. In some clinical cases when the patient is insufficient fit for invasive diagnostics or there is not enough tissue materials for molecular testing, ctDNA examination can be alternative for determination of lung cancer progression under therapy (20). This tool significantly improved the therapeutic setting for non-small cell lung cancer (NSCLC) patients to determine the drug response and if it is necessary to start the third-generation tyrosine kinase inhibitors (TKI) therapy - osimertinib (21). It was shown that patients, who were treated with this substance, had significantly longer progression free survival than patients who received standard EGFR-TKI therapy (gefitinib or erlotinib) (22). CtDNA analysis has high specificity with low false positive rate, but the sensitivity rate is approximately 60-70 %, that means, that the lack of T790M mutation does not exclude the presence of mutation (20). The recommended method for liquid biopsy analysis is the next generation sequencing (NGS) method. This method is based on detection of large genes regions in one run and focused on genes of interest for clinical praxis (23). Additionally, due to NGS method, tumor suppressor genes and previously undetected genes can be discovered, that makes this method in comparison to qPCR-based method, more useful (23). The international association for the study of lung cancer (IASLC) recommends using liquid biopsies for all patients with NSCLC in advanced stage and patients with clinical characteristics, like never smoker or young individuals, that may indicate molecular mutations (23).

Liquid biopsy gives an opportunity for the early cancer detection, especially when tissue biopsy cannot be received, and for monitoring of therapy efficiency and evaluation of the treatment response. However, development of the highly sensitive methods for lung cancer diagnostics are needed. There are many preanalytical steps of ctDNA testing, which are recommended by European Medicines Agency and International Organization for Standardization (Table 1) (24).

European society for medical oncology demonstrated four scenarios, when ctDNA analysis is used in the clinical practice. In the first case, when molecular testing helps to identify clinically actionable targets for possible target therapy like EGFR mutation, v-raf murine sarcoma viral oncogene homolog B1 (BRAF) mutation, anaplastic lymphoma kinase (ALK) mutation, proto-oncogene c-ros-1 (ROS1) mutation and other. In the second case, it is applied

after curative surgery to evaluate possibility for adjuvant treatment. In the third case, liquid biopsy can be also used for prediction of tumor recurrence after therapy and in the fourth case for detection of therapy resistance and tumor progress (24).

Table 1. Preanalytical conditions for ctDNA mutation testing in NSCLC.

Blood collection and plasma preparation	■ IASLC recommends the maximum time from blood collection to plasma preparation should be 2 h for ethylenediaminetetraacetic acid (EDTA) tubes ■ there is no standardized volume announced, but 2x 10 ml is recommended ■ blood should be collected in suitable tubes to prevent coagulation ■ there were good results, if blood was collected with EDTA tubes and stored for a maximum of 4 h at room temperature or for 24 h at 4˚C
Storage and preparation of plasma	■ Cell-free plasma should be stored at 20˚C or 80˚C, but for long long-term it should be storage at 80˚C ■ A 'double spin' plasma isolation procedure is recommended

1.1.6. Lung adenocarcinoma

Lung adenocarcinoma is a malignant epithelial tumor and is the most common histological type of lung cancer. Lung adenocarcinoma is the most frequent lung cancer type in never-smoking patients. Women develop more often non-smoking-associated lung cancer than men (25). Lung adenocarcinoma is very rare among young people under the age of 35. A study demonstrated that almost one third of patients under the age of 35 were male, 90% of those patients had never smoked and 70% were diagnosed with Tumour, Node, Metastasis (TNM) stage I and 16% with stage IV (26). In almost 70% of patients under the age of 35 different genetic mutations were detected. The most prevalent mutations were erythroblastic oncogene B (ERBB2) with 22%, EGFR with 14%, and ALK with 16%. Mutation status can have correlation with TNM stage, clinocopatological characteristics and overall survival. For example, ALK expression was frequently found in the late stage of lung adenocarcinoma and in poorly differentiated tumours, but ERBB2 mutation is more frequent in well differentiated tumours. The long follow-up data showed that ALK expression was associated with a poor prognosis in comparison to ERBB2 mutation (26). The overall survival prognosis is dependent on many factors, one of them being cancer histology. The poorest prognosis is associated with micropapillary, solid and invasive mucinous adenocarcinoma (27). Patients after lung resection with adenocarcinoma and EGFR mutation have a better survival prognosis (28). Patients with adenocarcinoma with lepidic components also have a better prognosis (28). Yanagawa et al. showed that patients with adenocarcinoma in situ (AIS) and minimally invasive adenocarcinoma (MIA) had a 100% 5-year survival rate, whereas patients with adenocarcinoma and lipid components had a 94.9% 5-year survival rate. Patients with solid predominant adenocarcinoma had the poorest prognosis with 54% (29). With the immunohistochemical marker thyroid transcription factor 1 (TTF-1), it is possible to distinguish between lung adenocarcinoma and other kinds of lung cancer, for example, mesothelioma. TTF-1 expression has a high occurrence for lung adenocarcinoma and, therefore, it is possible to differentiate between original lung adenocarcinoma and metastatic adenocarcinoma from other organs, excluding thyroid cancer (30).

1.1.7. Staging for non-small cell lung cancer

The International Association for the Study of Lung Cancer updated the lung cancer staging using the database of more than 95.000 patients. The last 8th edition was published in 2017 (31). The general aim of the TNM classification is to enable the common understanding between clinical specialists worldwide about anatomical extensiveness of lung cancer, to guide treatment recommendations and prognosis of the disease. Based on the TNM classification, lung cancer is divided into stages. There are three categories that describe how expanded the tumor is: T for the size of the primary tumor in cm and the invasion into anatomical structures, N for the lymph nodes, and M for the single or multiple distant metastasis. T, N and M categories are divided into numerous subgroups and typify the exact tumor characteristics (31). Positron emission computer tomography (PET), CT and bronchoscopy with endobronchial ultrasound (EBUS) are essential for the diagnostics and staging of lung cancer.

1.1.8. Molecular testing of non-small cell lung cancer

Molecular analysis of every tissue sample of lung cancer is becoming a standard of care. The discovery of the mutations in specific genes in lung cancer led to the development of targeted therapy and a new management of the treatment. Some mutations are well investigated and used in the daily oncological praxis. EGFR was one of the first receptor tyrosine kinases (RTKs) which was studied. EGFR belongs to other RTK family members, which consists of three other RTKs: ErbB2/ HER2, ErbB3/HER3, and ErbB4/HER4 (32). Seven different ligands can regulate EGFR: EGF, transforming growth factor a (TGF-a), heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), amphiregulin (ARG), epigen (EGN) and epiregulin (EPR) (32). Results of one study showed, that there was no significant association between the presence of EGFR gene mutations and sex or smoking status (33). These data are not in agreement with another study, where frequent expression of EGFR mutations was detected in nonsmoking lung cancer patients (34). Kosaka et al showed, that EGFR mutations were found almost only in female patients with lung adenocarcinomas with nonsmoking status (35). Analyzed data of another work demonstrated that tumor stage did not correlate with the existence of EGFR mutations (34), suggesting that EGFR mutations do not have connection with disease progression. Some randomized studies have shown that expression of EGFR mutation acts as the predictive factor of the therapy response to first-generation TKIs in cases of advanced lung adenocarcinoma (36), (37). Efficacy of the therapy with TKIs in the same

cases is restricted, because of drug resistance occurrence. One of the most investigated mutations is T790M. The T790M mutation appears approximately in 50 percentage of cases, which leads to the resistance development to TKIs drugs like gefitinib and erlotinib (38).

ALK is a tyrosine kinase transmembrane protein that belongs to the insulin receptor superfamily and contains 1620 amino acids (39). During embryogenesis ALK takes part in the development of brain and neurons (39). ALK expression was identified in different histological lung cancer types, but the most prevalent was adenocarcinoma (40), (41). It has been shown that ALK positive patients tend to have advanced cancer stage (42). Patients with ALK expression and EGFR-mutation displayed similar sensitivity to the platinum-based chemotherapy (43).

The human homolog ROS1 is a gene, which is located at chromosome 6q22 (44). ROS1 is a receptor tyrosine kinase that occurs in approximately 1%-2% of lung carcinoma cases, but it appears also in other type of tumors, like cholangiocarcinoma, gastric carcinoma and colorectal carcinoma (45). One of the known ROS1 function is regulation of cellular proliferation, differentiation and cancer development, including lung cancer (46). Detection of ROS1 can be done by using fluorescence *in situ* hybridization, real-time PCR, and immunohistochemistry. Microarray analysis displayed increased ROS1 expressions in lung adenocarcinoma subtype (47). Published results show that presence of ROS1 fusion was prevalent detected in female NSCLC patients without smoking history in advanced tumor stages (48).

The BRAF mutation was observed in different cancer types, but prevalent in melanoma patients and in lung cancer patients (49). BRAF mutation had the correlation between increased mutation expression and clinicopathological features, like sex and smoking status. Male with positive smoking history had enlarged existence of BRAF mutation (50). There were no strong associations between BRAF mutation and prognosis (50). BRAF mutation was detected in lung adenocarcinoma (51).

The Mesenchymal to Epithelial Transition (MET) gene is localized at chromosome 7. It was demonstrated *in vitro*, that MET amplification stimulates cell migration, proliferation and developing of metastases in lung cancers samples (52). Patients with increased MET gene expression, has been associated with worst survival (53). MET mutation showed correlation with an older age and mutation persistence, but no correlation in relation with the smoking history (54).

Human epidermal growth factor receptor (HER)-2 is a member of ERBB family, and it is the wild-type of EGFR (55). In some studies, the associations between clinicopathological

characteristics and HER-2 mutation have been shown, however, in other studies there were no correlations. Female gender and nonsmoking history was correlated with HER-2 mutation in lung adenocarcinoma patients (56), but in other studies these correlations were not observed (55). The most common histological type, which correlates with HER2 mutation is adenocarcinoma and its subtypes, like acinar predominant adenocarcinoma (APA), papillary predominant adenocarcinoma (PPA), minimally invasive adenocarcinoma (MIA), and invasive mucinous adenocarcinoma (IMA) (55). Influence of HER2 mutation on prognosis and overall survival is unclear, therefore further studies are needed.

KRAS mutations have been found in different types of cancer, including lung, gastric and gynecologic cancer (57, 58, 59). KRAS mutation correlates with positive smoking status (60). The prognostic influence of KRAS mutations in lung cancer is unknown.

In very rare cases the coexisting of two mutations at the same time are documented. EGFR and ALK mutations are about 1.3% of cases of lung cancer patients (61). Double mutations were associated with decreased response to the therapy with TKIs (62). The presence of two mutations was associated with advanced stage of cancer and worse overall survival (63).

1.1.9. Lung Cancer therapy

After lung cancer detection, the therapy strategy depends on many factors. Patients are usually asymptomatic at the early stage, but in the late stage of the disease the typical complains are chronical cough, hemoptysis, unwanted weight loss and other symptoms**.** The treatment strategy for lung cancer is individual and should be discussed in the tumor board with specialists of pulmonology, oncology, surgery, pathology, radiology and radiotherapy. During the tumor board conference, the best therapy option should be chosen, taking into account the age of the patient, cancer histology, molecular and genetic characteristics, cancer stage, patients' clinical condition after Eastern Cooperative Oncology Group (ECOG) performance status, and comorbidities. It is known that the surgical resection is the most effective treatment for NSCLC, with the longest survival. In stages I (T1N0, T2N0) and II (T1N1, T2N1) patients can be curatively operated and, therefore, considered as "early stage**".** Unfortunately, only 30% of the patients can be curatively operated because most cases of lung cancer are diagnosed at the late stage and in this case the surgical therapy is often impossible. In the most cases the surgery is possible in the stages I, II and sometimes in IIIA**.** The indication for surgery based on lung cancer stage, functional operability of the patient and cancer histology is determined by a

pulmonologist and a surgeon. In early stage of NSCLC, lobectomy is recommended (64). Both surgical techniques, VATS lobectomy and thoracotomy were compared to analyze overall survival. Five year survivals showed similar results, however, thoracotomy was associated with more complications and longer hospitalization (65).

For inoperable patients in stages I or II radiotherapy is recommended, but after curative resection radiotherapy is not recommended, because of the unclear benefit (66). In case when the operation is impossible in stage I, the stereotactic ablative body radiotherapy (SABR) is the alternative therapy option without expanded complications in comparison to standard radiotherapy (67).

The aim of neoadjuvant therapy is to decrease volume of tumor improving radiological response, as well as to raise the curative rate after surgical treatment. There was shown, that neoadjuvant chemotherapy and neoadjuvant-targeted therapy increased the response (68).

Adjuvant platinum- based chemotherapy is recommended for stages IIB-III (20). For adjuvant chemotherapy, a combination of two substances with cisplatin in three to four cycles is recommended (69). Adjuvant treatment with targeted therapies is also an option. For example, there was shown that adjuvant therapy with osimertinib can improve overall survival for NSCLC patients with EGFR-mutation (70).

In advanced lung cancer stage and good performance status, chemotherapy can be offered. The combination of platinum-based chemotherapeutics with other substances (for example pemetrexed, gemcitabine and other) leads to longer survival (71), (72).

Due to new therapy options, the lung cancer classification became more detailed, using molecular and immunohistochemical description. The consequences of finding the EGFR gene mutation, ALK gene and other gene mutations are the new therapy options of targeted therapy using TKIs. Patients with detected EGFR mutation were responsive to the TKI therapy and had improved overall survival compared to patients without EGFR mutation (73, 74, 75). Kwak et al. showed that patients with lung adenocarcinoma and activated ALK kinase have long survival under ALK kinase inhibition (76).

Another way of lung cancer treatment is immunotherapy. The aim of this therapy is the activation of the immune system against tumor cells. The mechanism of this therapy is based on activation of checkpoints inhibitors on T-cells. Programmed cell death 1 (PD-1) or programmed death ligand 1 (PDL-1) proteins work as immunoregulators and regulate T-cell activation and immune system response (77). The receptor PD-1 and its ligand PD-L1 and

cytotoxic T-lymphocyte antigen 4 (CTLA-4) work as checkpoints and some produced antibodies activate the immune response by targeting these molecules, e.g. nivolumab and pembrolizumab target PD-1 (78, 79). Immunotherapy for advanced NSCLC is used in the clinical practice as the first-line or second-line therapy, depending on the PDL-1 status. Patients with PD-L1 expression of >50% are more sensitive and have longer survival than patients who received only platinum-based chemotherapy (80). The combination of immunotherapy and chemotherapy showed the significant increased progression free survival (81). The investigation of nivolumab combined with platinum-based chemotherapy as neoadjuvant strategy in patients with stage IB-IIIA showed significantly improved response compared with chemotherapy alone (82). For control of surveillance, a visit including physical examination and chest and abdominal CT at least every 6 months for 2 years is recommended (83). The discovery of new genetic and immunohistochemical features is the basis for personalized medicine in oncology.

1.2. MicroRNAs

For the first time microRNA let-7 was described in 1993 during the genetic analysis of the worm *Caenorhabditis elegans* (84). Later it was found that let-7 takes part in developmental gene regulation in *C. elegans* (85). Small let-7 and lin-4 molecules were found in *C. elegans* as gene regulators and were named as microRNA (86). MiRNAs are small non-coding RNA molecules that consist of 21-25 nucleotides and play an important role in different essential biological processes. MiRNAs are transcribed from the DNA molecules without translating into proteins. Noncoding miRNAs regulate development in many organisms including high eukaryotes (87). MiRNAs take part in post-transcriptional gene regulation and are included in many biological processes, like differentiation, cell proliferation, apoptosis, normal and abnormal cell growth (88), and each miRNA can target different mRNAs (89). It was shown that almost 30% of all human genes are regulated by miRNAs, which means that miRNAs have an essential role in all biological processes (90). Due to their binding to the 3`-UTR (untranslated region) of target genes, miRNAs are able to have multiple targets (91). The expression profiles of miRNAs are different in healthy and in cancer tissues (92). One of the first discoveries of miRNAs association with cancer development was in year 2002, when Calin et al. described downregulation and/or missing of miRNA-15 and miRNA-16 in patients with B cell chronic lymphocytic leukemia (93).

1.2.1. MicroRNA in biological fluids

Many studies showed that microRNA detection is possible in different body tissues and fluids. Cabral et al. investigated association between circulated miRNAs in serum and developing of hepatocellular carcinoma. Differential miRNAs expression in patients with mild fibrosis and cirrhosis was identified (94). Another scientific group also identified a significant difference of microRNAs expression in serum of patients with hepatocellular carcinoma compared with patients with liver cirrhosis (95). Circulating miRNAs were found in cerebrospinal fluid (96), saliva (97), in breast milk (98), pleura fluid, tears and urine (99). Weber et al. analyzed miRNAS expression in 12 different biofluids and described that plasma contained the highest number of miRNAs (99). MiRNAs expression level of patients with prostate cancer and healthy control group was also compared. Patients with cancer demonstrated significant increased expression of miRNAs in serum (100). Chen et al. studied

circulating miRNAs applying Solexa. Solexa is a high- throughput sequencing technology for analysis of small RNAs, including miRNAs. Due to this technology, miRNA expression in serum was compared between patients with lung cancer and colorectal cancer, and it was found that most miRNAs were involved in tumorigenesis in general, identifying a special miRNAs expression for each type of cancer (101). It has been demonstrated that miRNAs are stable in plasma and serum, this providing the minimal invasive approach to investigate miRNAs and their target genes.

1.2.2. MicroRNA as tumor suppressor or oncogene

Cancer is one of the leading deaths causes worldwide and its development is multifactorial, including gene regulation disturbances. Uncontrolled cell proliferation and apoptosis failure lead to carcinogenesis. It was shown that miRNAs take part in tumorigenesis acting as tumor suppressors or oncogenes (102) and this varies in different types of cancer (103). For example, miRNA-29a functions as an oncogene in breast cancer, but as a suppressor in chronic lymphocytic leukemia (104). Some microRNAs are involved in development of different cancer types. For example, the upregulation of miRNA-217 takes part in glioblastoma, breast cancer, skin cancer, but on the other hand, it can inhibit the cancer cell proliferation and metastasis in osteosarcoma and cervical cancer (105), thus functioning both as a tumor suppressor and as an oncogene. It has been described in the lung cancer cells that the KRAS oncogene can be reduced by overexpression of miR-217, this miRNA acting as a tumor suppressor (106). MiRNA-218 was observed to act as a tumor suppressor in lung cancer and it was downregulated in malignant cells. MiRNA-218 overexpression led to the reduction of tumor growth, directly targeting the IL-6/STAT3 pathway (107). By targeting the TFIIB-related factor 2, miRNA-425-5p reduced lung cancer cell proliferation acting as a tumor suppressor (108). The overexpression of miRNA-503 was demonstrated in retinoblastoma acting as oncogene, but knockdown of this miRNA led to decreased cell proliferation and invasion, directly targeting the PTPN12 (109). Hepatocellular carcinoma (HCC) is the malignant liver tumor and there is an association between HCC and miRNA-485-5p expression level. Upregulated miRNA-485-5p acts as a tumor suppressor inhibiting HCC cell proliferation and reducing metastasis development. The blocked Wnt/β-catenin signaling pathway is included in this cellular mechanism in HCC (110). The Wnt/β-catenin signaling pathway is also involved in the development of prostate cancer. It was found that miRNA-155-5p acts as a tumor suppressor due to the inhibition of the cell invasion and migration via downregulating its direct

target SPOCK1, which is involved in the Wnt/β-catenin signaling pathway (111). Dysregulation of miRNAs expression was also investigated in a colorectal cancer. MiRNA-216a was indicated as a tumor suppressor in colorectal cancer activating autophagy via the TGF-β/MAP1S pathway (112). MiRNA-335 was also observed as a tumor suppressor in colon cancer, inhibiting the distant metastasis development via deregulation of the MEK/ERK signaling pathway (113).

1.2.3. Biogenesis of microRNA

For better understanding of miRNA biology, it is important to study their regulations and functions. Multiple studies showed that miRNAs play a role in cell regulation, differentiation, apoptosis, and growth. MiRNAs act at the post-transcriptional level. One of the interesting aspects of miRNA biogenesis, is that each miRNA can target many mRNAs, controlling different proteins (114). MiRNAs can regulate intracellular traffic of different compartments, from nucleus to cytoplasm and conversely. Primary miRNAs (pri-miRNAs) are transcribed by complex of enzymes, including RNA polymerase, double-stranded RNA-specific endoribonuclease (Drosha) and Dicer. Drosha is mainly localized in the nucleus. Maturation and biosynthesis of miRNAs can be influenced by RNA-binding proteins (RBPs), which interact with different enzymes, including Drosha (Figure 1) (114, 115). Most miRNAs interact with the 3′ UTR of target mRNAs (114). Pri-miRNAs are cleaved into ~70-bp precursor miRNA (pre-miRNAs) by Drosha. After the cleavage, pre-miRNAs are exported from the nucleus to the cytoplasm for the next processes by protein activator Exportin 5 (Exp5) (115). Inside of the cytoplasm, precursors are cleaved by Dicer into a small dsRNAs.

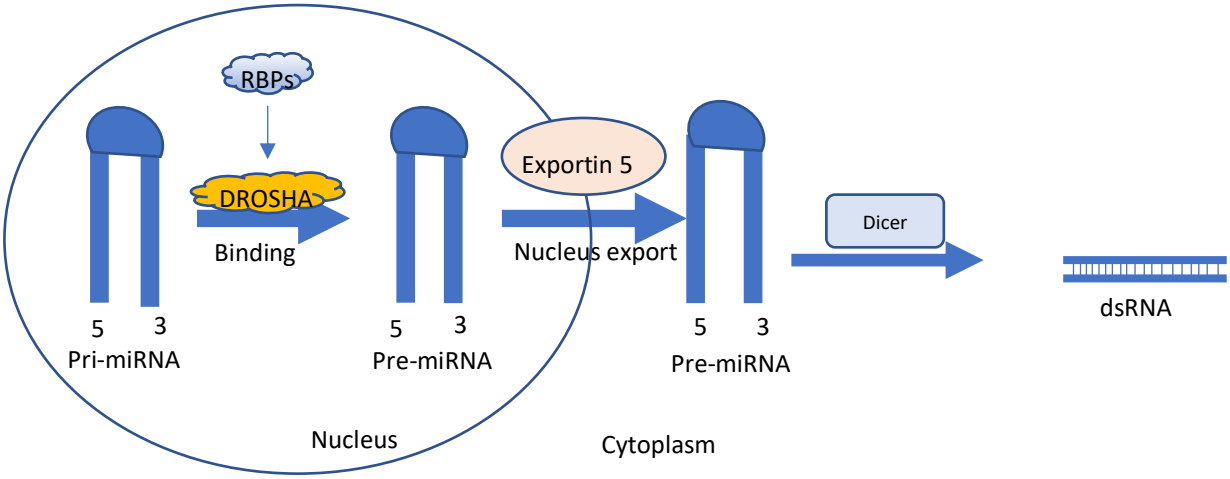


Figure 1. Biogenesis and post-transcriptional suppression of microRNAs.
RBPs can influence miRNA processing and maturation by affecting Drosha activity, nuclear export of pre-miRNAs. The pri-miRNA transcripts are processed into ~70-nucleotide pre-miRNAs by Drosha in the nucleus. Pre-miRNAs are transported to the cytoplasm by Exportin 5 and are processed into dsRNA by Dicer (114, 115). Figure 1 was created by I. Robinson in Power Point.

1.2.4. MicroRNA as a biomarker for early detection of cancer

The ideal biomarker for the early cancer detection should be minimally invasive with less possible complications or body damages, sensitive and analytically stable. There is increasing number of publications about the role of miRNA in detection of different cancer types in early stage, before any symptoms appear. MiRNAs have been described as potential biomarkers in plasma or serum using the RT-qPCR method (116). It was found that serum and plasma are both suitable for miRNAs detection as a circulating biomarker for cancer investigation and miRNAs are stable in both biofluids (117). Some data showed a strong correlation between cancer development and miRNA expression**.** It was described that the expression of two miRNAs, miR-423-5p and miRNA-222-3p, was increased in oral cancer and can be used for the early detection (118). Upregulated miRNA-1246 circulating in serum was found in gastric cancer patients, indicating a role of this miRNA in gastric cancer diagnostics (119). MiRNA-1246 has also been demonstrated as a potential biomarker for pancreatic cancer, because its expression in the serum of healthy donors was lower than in patients with pancreatic

cancer (120). Expression of miRNA-221-3p was elevated in pancreatic cancer in comparison to normal pancreatic tissues, indicating that miRNA-221-3p may be used for early detection of pancreatic cancer (121). The meta-analysis of six different studies with more than 500 patients showed the association of miRNA-92a expression and colorectal cancer, supposing that this miRNA might be a potential biomarker for the diagnostics of colorectal cancer (122). Another potential biomarker for colorectal cancer detection is miRNA-1290. The overexpression of miRNA-1290 in the serum of patients with colorectal cancer was associated with fast tumor growth and decreased overall survival (123). Depending on the cancer type, miRNAs can be down- or upregulated. For instance, miRNA-16 was downregulated in esophageal squamous cell carcinoma, gastric cardia adenocarcinoma and gastric non-cardia adenocarcinoma in comparison to healthy donors, but it was upregulated in patients with pancreatic ductal adenocarcinoma, lung adenocarcinoma, lung squamous cell carcinoma and endometrioid endometrial cancer. There are different circulating biomarkers that can be used in the future as potential screening methods for early cancer detection. MiRNA-376c was detected to be overexpressed in plasma of gastric cancer (GC) patients (124). Expression of circulating miRNA-196a and miRNA-196b showed differences before and after surgical therapy in patients with GC (124). Usuba et al. found 7 different miRNAs (miRNA-6087, miRNA-6724-5p, miRNA-3960, miRNA-1343-5p, miRNA-1185-1-3p, miRNA-6831-5p and miRNA-4695-5p) with increased expression in patients with bladder cancer in comparison to healthy group, which could be a potential biomarkers for the early detection of bladder cancer (125).

1.2.5. MicroRNA as a marker for therapy efficiency

Treatment resistance development is a significant problem in cancer therapy that leads to the therapy failure and poor prognosis. MiRNAs are involved in many cellular regulatory processes, including the tumor cell response to the therapy. It is known that miRNAs are involved in the drug resistance mechanisms. Overexpression of miRNA-155 is associated with the development of chemotherapy resistance in many types of cancer, such as B cell lymphoma, colon cancer, breast cancer and cervical cancer (126). There is a different expression level of miRNA-155 in radio-resistant and radio-sensitive tissues in patients with nasopharyngeal cancer (126). Gefitinib is a well-known substance for lung cancer treatment that works as EGFR tyrosine kinase inhibitor. Downregulating the FOXO3A by miRNA-155 leads to the gefitinib resistance development (126). A member of the Forkhead box O transcription factors (FOXO)

family is the FOXO3A, that regulates many essential processes including cell proliferation, metabolism, and apoptosis. Qiu at al. showed that FOXO3A was targeted by miRNA-155 and it was included in the regulation of the CD 8+T cells function (127). Another study reported that the downregulating of miRNA-221 in breast cancer cells increased chemo-sensitivity, directly targeting Phosphatase and tensin homolog (PTEN) pathway and augmenting apoptosis (128). There was an association between the enhanced expression of miRNA-663b and therapy resistance in patients with breast cancer and the silencing of miRNAs-663b expression led to increased sensitivity to therapy. It was shown that different miRNAs, such as miRNA-2013 and miRNA-150, may play a role in the development of radiotherapy resistance in nasopharyngeal carcinoma (129). In addition, miRNA-191 may lead to radiation resistance in prostate cancer (130). Salim et al. demonstrated that upregulated miRNA-214 in lung cancer cells correlated with apoptosis inhibition and it caused radiotherapy resistance (131). Another study showed the role of miRNA-608 in cisplatin sensitivity by direct regulation of TEAD2 in patients with lung cancer. Overexpression of miRNA-608 leads to increased apoptosis of lung cancer cells and conversely the downregulation of miRNA-608 reduces sensitivity of lung cancer cells to cisplatin (132).

1.2.6. Normalization of microRNA

MicroRNAs can be used as circulating biomarkers in plasma or serum for different diseases, including cancer. To investigate miRNAs, first of all they should be isolated from cell culture, fresh or frozen tissues. Isolation from human fluids is the most technically challenging step because of the global miRNA instability. To prevent the molecular changes, the correct preservation methods, like anticoagulation and freezing are required. It is possible to analyze miRNA expression profiles using the real-time quantitative polymerase chain reaction (RT-qPCR), microarray technology and sequencing (133). RT-qPCR is a very sensitive and specific method, which is commonly used even in case of a low level of gene expression. There are common available and specially prepared ready-to-use kits, which we also used in our study. Data normalization is an important part of the analysis, but there is no final agreement about the best normalization method. The aim of every normalization is to determine the gene specific characteristics and exclude the sample variety. Normalization is an especially important part of data analysis and must be performed to exclude possible bias and technical failures, which might influence final results. Technical failures during the pipetting can appear, and to avoid

them at least three repetitions are needed. To minimalize the methodological failures, the detailed protocols and comparison of different methods are of advantage.

The positive control or the housekeeping gene is commonly used for normalization. Because of the varying expression of the housekeeping gene in different tissues, especially if the analysis is made not only in one cell line, but in the biofluids of different individuals, this method has limitations and cannot be always applied (134). Thanks to the software packages, like geNorm and Normfinder, it is possible to determine stably expressed miRNAs and use them as normalizers. This method is based on conversion of row expression data into relative expression data (135).

Under *in vivo* conditions, normalization is especially challenging, because of the biological variability of patients/donors, the collection of biomaterials, miRNA isolation and storage. There are different methods to normalize miRNAs. Some authors advice to use particular miRNAs as normalizer. For example, miRNA-191, miRNA-25 and miRNA-17–5p were determined to be very stably expressed in lung tumor and in normal tissues (136). MiRNA-1228-3p and miRNA-16-5p were shown as possible endogenous controls for liquid biopsy from patients with different types of cancer (137).

One of the methods is absolute data normalization, which can be used only for high quality RNA samples and this method is not suitable for precise miRNA quantification (138). This method is based on quantified miRNA expression using standard calculation curves (138).

Another commonly used method is global mean normalization method, which is based on the mean expression calculation of the total miRNA (138). MCR (mean-centering restricted) normalization (139) is a method, that is based on the mean of all miRNAs expressed / detected in all samples. This is in contrast to the global mean normalization method, where absolutely all miRNAs, including undetermined and missing miRNAs, are included in the data normalization (139). Cycle of threshold (Ct) is the cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold, a fluorescent signal significantly above the background fluorescence. Ct is inversely proportional to the nucleic acid amount and to the original relative expression level of the gene of interest, e.g. of particular miRNA. The higher the Ct value is, the lower the expression of particular miRNA. The miRNAs with the Ct value ≥ 37 are interpreted as negative and are not included in the MCR calculation. The $\Delta\Delta$Ct method can be used for detection of the expression level of miRNAs (140).

2. Aim of the project

The aim of this study was to analyze the global expression of 752 miRNAs in malignant A549 and in non-malignant bronchial epithelial cells *in vitro* and to identify the most deregulated miRNAs. Based on those *in vitro* data, we wanted to examine the miRNAs expression in plasma samples of lung cancer patients and healthy donors to find out a potential circulating miRNA, that could be promising as a diagnostic biomarker for early lung cancer detection. Finally, by different *in silico* tools, we wanted to identify the target genes influenced by deregulated circulating miRNAs and determine their assignments to specific pathways relevant for biology of lung adenocarcinoma.

3. Materials and Methods of research

3.2. Overview of the study

This study consists of two parts. The figure 2 and 3 graphically show the laboratory workflow of both parts of this study. The aim of the first screening part was to examine the global expression profile of miRNAs in malignant A549 cells and in non-malignant BE cells under standard normoxic (21% O2) conditions, and to compare the miRNAs dysregulation in BE cells and in NSCLC cell lines. In the second part of our study, the most deregulated miRNAs selected in vitro were analysed in patient material. The aim of the second part of our study was to discover differentially expressed miRNAs in the peripheral blood from patients with histologically diagnosed lung adenocarcinoma and healthy donors, to identify the stable microRNAs in plasma, to compare the most dysregulated miRNAs expression from the first part of the research in the plasma samples of patients with histologically diagnosed lung adenocarcinoma with health donors.

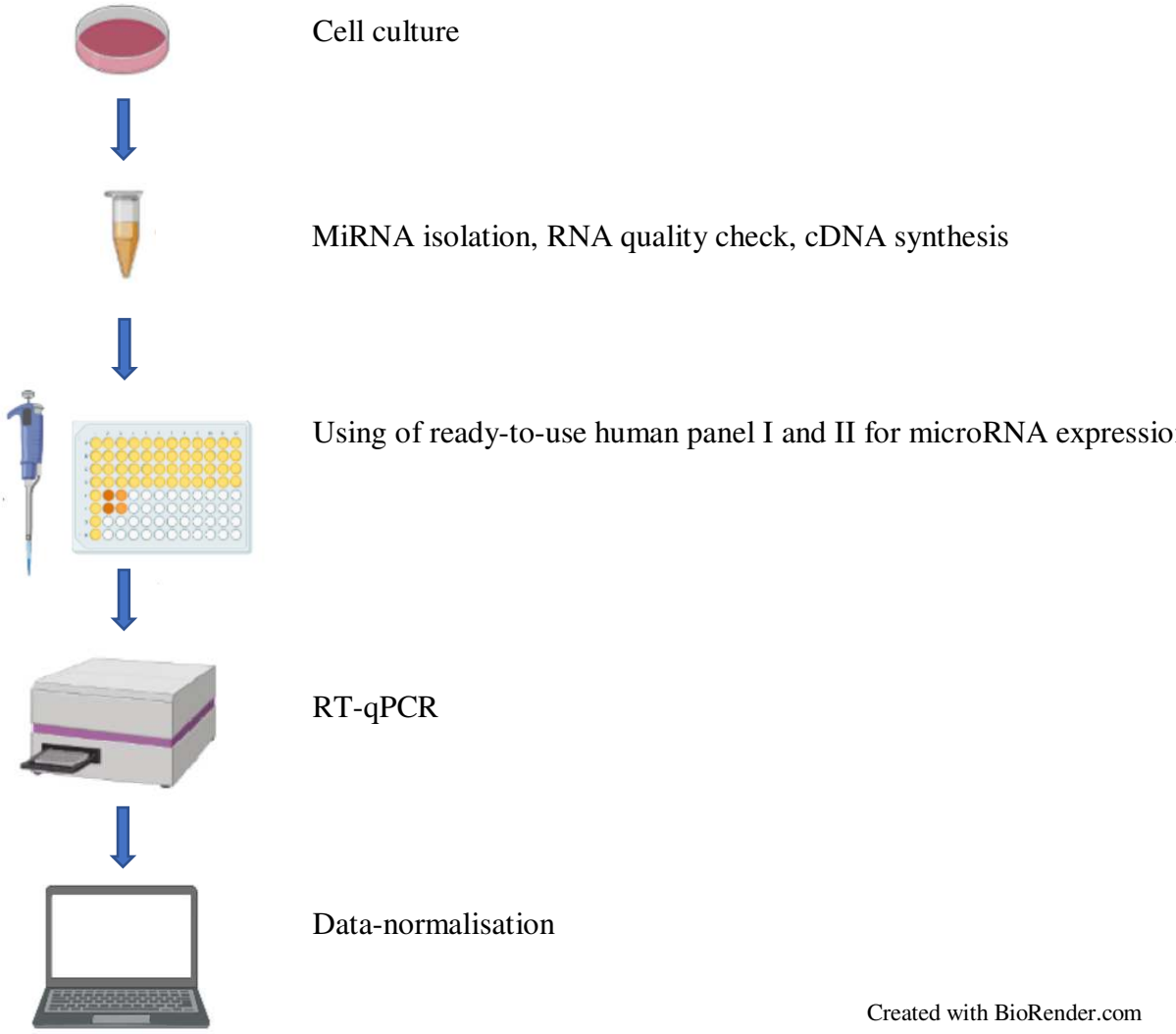
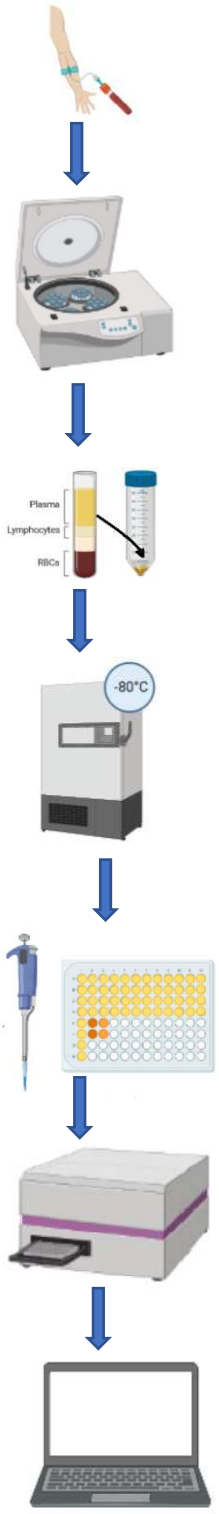


Figure 2. The laboratory workflow of the first phase of our study.

Cells were incubated in normoxic conditions. Then the total RNA including microRNA were isolated and the cDNA synthesis was performed. Using the ready-to-use white 384-well PCR human panel I and II plate for microRNAs, the real-time PCR amplifications were performed. Finally, the data was normalized using MCR method and analysed. Figure 2. was created by I. Robinson with BioRender.com.



Collection of peripheral venous blood (3 ml)

Sample centrifugation (15 min at 1000 x g and 4°C), two times

Sample separation into plasma and cells

Plasma samples storage at -80°C

MicroRNA isolation and cDNA

RT-qPCR

Data normalization using stably expressed miRNAs

Created with BioRender.com

Figure 3. The laboratory workflow of the second part of our study.

The peripheral venous blood was collected from every individual, who was enrolled in the study after checking the including criteria. After blood collection, it was centrifugated twice

immediately. Finally, plasma samples were frozen at -80°C. For the further analysis, the samples were thawing on ice in the room temperature. MiRNA were isolated and the cDNA synthesis was performed. Based on our analysis data from the first part of research, we chose 18 the most deregulated (upregulated or downregulated) miRNAs and used specific primers to determine the statistical difference of miRNAs expression in patient and control group. Based on our previous results and on literature, two stably expressed miRNAs were used as control. Figure 3. was created by I. Robinson with BioRender.com.

3.2. Laboratory analysis

3.2.1. Cell lines

In this study, the human NSCLC cell line A549 was used. A549 human NSCLC cells were obtained from ATCC and grown in DMEM/F12 medium supplemented with 10% fetal calf serum (FCS; Gibco, Paisley, UK).

At the beginning of our study, we examined expression of 752 miRNAs in A549cells compared to BE cells. Because A549 is widely applied human NSCLC cell line, and we had good experiences with this cell line before, we used this cell line for our further experiments. Before starting the experiments, the cells were passaged several times and washed with PBS. To harvest adherent cells, the Trypsin-EDTA solution was used und its action was stopped with media containing 10% FBS after five minutes incubation at 37°C. Cells were collected into sterile tubes and centrifuged at 400 g for five minutes. After that cell were resuspended in PBS and stored at -80°C. Regular mycoplasma tests were performed by the Core Facility Alternative Biomodels & Preclinical Imaging, Medical University of Graz using the Venor GeM Mycoplasma Detection Kit (Bioproducts) and the Hot start MB Taq Polymerase (Bioproducts).

3.2.2. Isolation of non-malignant bronchial epithelial cells

Primary BE cells were acquired from human healthy donor lungs, collected at the Department of Surgery, Medical University of Graz, Austria. This study has been approved by the Institutional Review Board of the Medical University of Graz and it has been conducted according to the Declaration of Helsinki principles. Protocol adapted after Fulscher et al. was applied for human airway epithelial cell isolation. (141). The isolated bronchial tissues were slashed in small segments, washed in phosphate buffered saline buffer (PBS) and cultured in

DMEM/F12 medium with 20% fetal calf serum (FCS), antibiotics (Pen/Strep), and antimycotic (Fungizone, 1:100; Gibco, Waltham, MA). Epithelial cells were resuspended and removed in a new cell culture plate coated with collagen type I surface (rat-tail collagen, 4.5 mg/ml, Sigma Aldrich, diluted 1:100 in PBS). BE cells were grown in special BEGM medium (CC-3170, Lonza, Basel, Switzerland) under standard conditions (37°C, 5% CO_2 and 95% humidity) and were used in low passages (< 4).

3.2.3. Cell growth

Malignant A549 cells and non-malignant bronchial epithelial cells were plated into 25 cm^2 cell culture flasks and grown for three days. Cells were grown in complete media at 37°C under 5% CO2 and 98% humidity under normoxic conditions (21%) of the Lung cell laboratory, Division of Pulmonology, LKH Univ. Clinic Graz, Austria. For RNA isolation 500.000 cells were needed. After three days of growth cells were washed with PBS and trypsin was applied. For stopping trypsin activity, media with 10% FCS was added, and it was centrifuged at 400 g for five minutes and resuspended in 5 mL of fresh media. For cell counting, we used an electric pulse area analysis, called CASY (Innovartis, Reutlingen, Germany). After cell calculation, each sample was centrifuged at 400 g for five minutes, washed again with PBS and finally it was put on ice for the RNA isolation.

3.2.4. Cytokeratin immunofluorescence

For BE cells cultivation, LabRekTM permanox chamber slides (Nalge, Nunc, Rochester, NY) were used. Firstly, cells were washed with PBS and fixed with 4% paraformaldehyde for 10 minutes at room temperature (RT). After that, the permeabilization step for removal of cellular membrane lipids was done with 0.5% Triton-X100 in PBS (Sigma-Aldrich, St. Louis, MO). Undiluted monoclonal mouse cytokeratin antibody (clone MNF116, Dako, Glostrup, Denemark) was used for one hour staining at RT. Detection was performed using Alexa 594 linked anti-IgG antibody (Invitrogen, Carlsbad, CA), diluted 1:600 for one hour at RT. As a negative control, an isotype control antibody was used as primary antibody.

3.2.5. MiRNA isolation

Total RNA including miRNA fraction was extracted from cells using the mirVana™ miRNA Isolation Kit (Invitrogen, by Thermo Fisher Scientific, Vienna, Austria), following the protocol recommended by producer. This special Kit was produced for RNA separation from other biomolecules, which makes it suitable for investigation of small RNA molecules like miRNAs. In contrast to the traditional RNA isolation methods, which are based on chemical extraction with high salt concentration and phenol or phenol-chloroform extraction, this method, using alcohol, ensures pure isolation of RNA in high quality (142). Two different methodologies of RNA isolation are combined in the mirVana™ miRNA isolation: organic and solid-phase extraction. The first step is based on sample disruption and cell lysis in the Acid-Phenol:Chloroform for the future purification. After this step, the cellular extraction is completed, including most DNA molecules. Next is the RNA purification by adding the ethanol and samples are passed through a glass-fiber-containing filter, which binds the RNA. After several washing steps of the filters, the RNA is eluted (Figure 4).

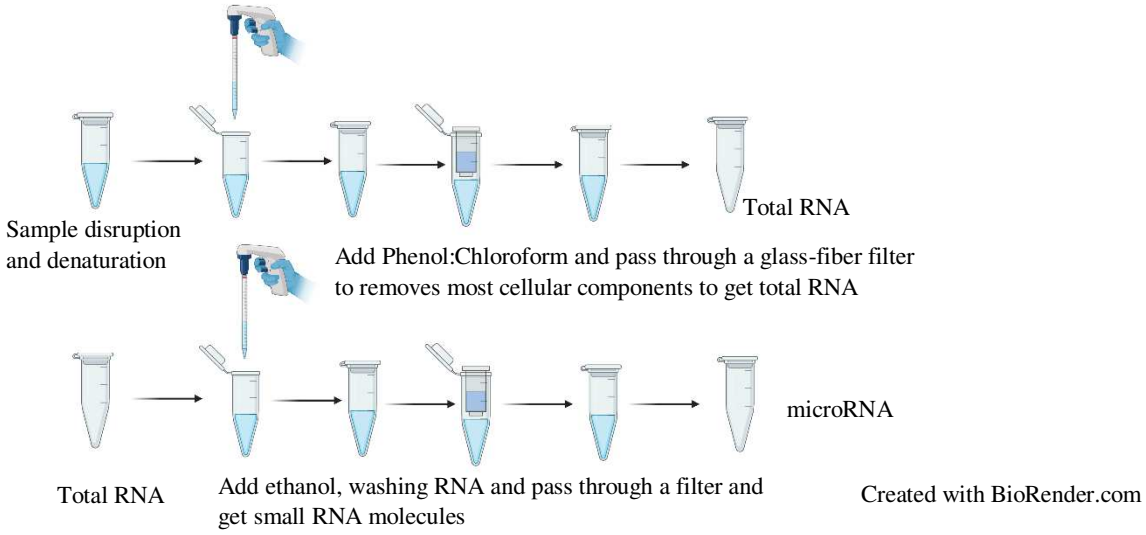


Figure 4. Overview of the miRNA Isolation procedure.

For getting of total RNA, samples were disrupted and denaturated using phenol or phenol-chloroform. After adding the ethanol, samples were passed through a glass-fiber-containing filter getting small RNA molecules. After several washing steps of the filters, the RNA was eluted. The miRNA isolation was done with the mirVana™ miRNA Isolation Kit (Part Numbers AM1560, AM1561, 2011, Life Technologies Corporation). Figure 4 was created by I. Robinson with BioRender.com.

3.2.6. RNA- quality check

Two methods were used for the quality check of isolated RNA including miRNA fraction. The first method was based on the measurement of concentration by NanoDrop 200c UV-Vis Spectrophotometer and the purity was assessed from the ratio A260/A280, this being in range of 1.8 to 2.0. Second method was performed by using the BioAnalyzer BA2100 (Agilent Technologies, Santa Clara, CA). RNA Integrity Number (RIN) was > 9.9 (maximum RIN = 10), indicating high quality of isolated RNA (Figure 5).

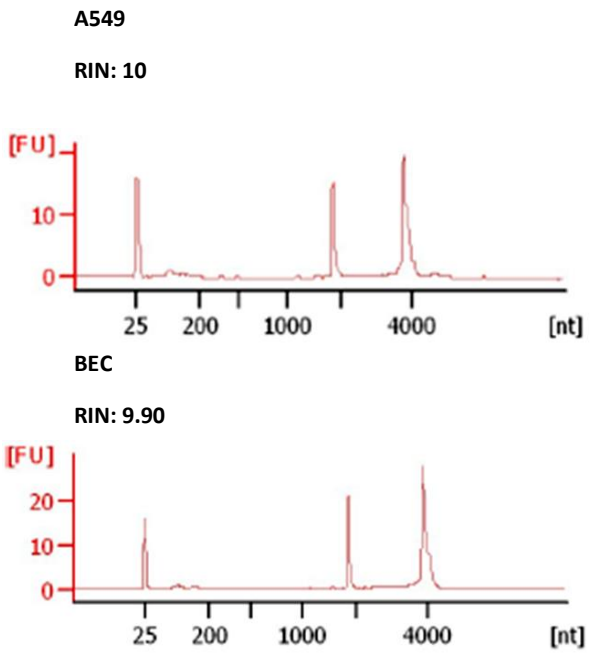


Figure 5. BioAnalyzer electropherograms of the isolated RNAs from A549 and BEC cells. The electropherograms by the BioAnalyzer verified that the total RNA samples included in this study were of highest quality (RIN-values 9.9 to 10). FU = fluorescence unit; nt = nucleotides; RIN = RNA integrity number.

This Figure was reprinted from (143) with permission from IOS Press.

3.2.7. Reverse transcription and SYBR-Green based quantitative RT-qPCR

For RNA transcription and cDNA synthesis, the miRCURY LNA™ Universal cDNA synthesis Kit II was used (Qiagen, Venio, Netherlands). All substances and reactions were kept on ice or at 4°C, as recommended in producer's protocol. To reach a concentration of 5 ng/µl, all RNA samples were pre-diluted with nuclease-free water. All reagents (5x reaction buffer, nuclease-free water, enzyme mix, and pre-diluted RNA) were combined in a master-mix and the cDNA synthesis was performed on the peqSTAR Thermocycler (Peqlab, Erlangen, Germany) at 42°C for 60 minutes, followed by a heat inactivation of the enzyme at 95°C for 5 minutes. After cDNA synthesis, all samples were instantly cooled to 4°C and stored at - 20°C for up to four weeks in special low nucleic acid binding tubes (Eppendorf, Vienna, Austria).

To quantitatively determine and analyse the miRNAs expression in the malignant LUAD and non-malignant BEC cells, the Kit miRCURY LNA™ Universal RT microRNA PCR human panel I and II (Qiagen) were used, according to the instruction and protocol of the producer (miRCURY LNATM Universal RT, microRNA PCR, instruction manual v6.1 #203301, Exiqon).

The combination of PCR master mix, water and cDNA were mixed and spun down. Ten µL of the PCR reaction mix was added to each well of the plate and centrifuged at 15.000 g for one minute. The real-time amplification was performed on the Light Cycler 480 (LC480, Roche).

The figure 6 shows a two-step process: cDNA synthesis and LNA™ PCR amplification. The table 2 displays the RT-qPCR cycle conditions, as recommended by the manufacturer. The RT-qPCR was completed on the LC480 (Roche, Rotkreuz, Germany) using the following protocol: polymerase activation/denaturation, 95°C for 10 min; amplification, 95°C for 10 sec, 60°C for 1 min, ramp-rate 2°C/sec for 45 cycles. For further calculations and evaluations melting temperature-values and cycle threshold (Ct)-values were obtained from the software version LC480 1.5.0.39.

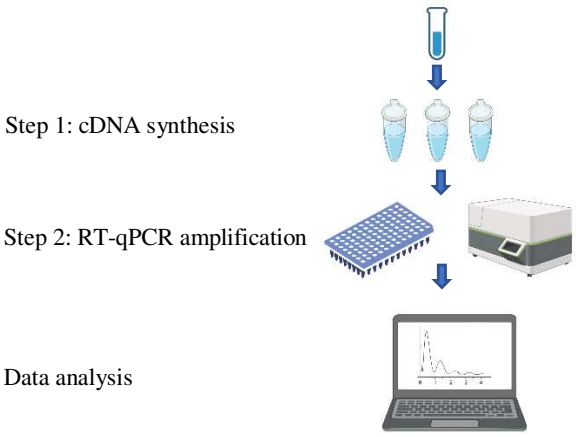


Figure 6. cDNA synthesis and LNA™ PCR amplification.

All reagents (5x reaction buffer, nuclease-free water, enzyme mix, and pre-diluted RNA) were combined in a master-mix and the cDNA synthesis was performed. For RT-qPCR amplification microRNA PCR human panel I and II (Qiagen) were used. Figure 6 was created by I. Robinson with BioRender.com.

Table 2. LC480 cycle conditions for the SYBR-Green based quantitative real-time PCR amplifications.

Steps of the process	**Setting**
Polymerase activation / denaturation	95°C, 10 min
Amplification	45 amplification cycles at 95°C 10 s at 60°C 1 min ramp-rate at 2°C/s Optical read
Melting curve	yes

3.3. Patient analysis

3.3.1. Patient cohort

Thirty-six individuals were enrolled into our analysis: 18 patients with histologically proved lung adenocarcinoma and 18 healthy subjects as control group. All subjects agreed to participate in the study and written informed consent was signed by every participant. Study was approved by the Ethics Committee of the Medical University of Graz. All patients were selected randomly from those who were diagnosed at the Division of Pulmonology of the Medical University of Graz. The including criteria for patients with lung adenocarcinoma was completed pathological finding of histologically diagnosed lung adenocarcinoma by bronchoscopy, by computer tomography or by ultrasound targeted puncture. Exclusion criteria were: i) any other active malignant disease, ii) any antitumor therapy for lung cancer before the histological material was achieved, iii) acute or chronic pulmonary infection. In all patients, lung adenocarcinoma was diagnosed by experienced interdisciplinary team of lung pathologists, radiologists and pulmonologist from the University Hospital in Graz, Austria. Classification and staging have been done following the newest (8^{th}) edition of TNM stage for lung cancer (31). The detailed clinicopathologic characteristics of patients are displayed in Table 3 and Table 4. In the control group individuals without any active malignant diseases and any kind of cancer in the past, without any acute and chronic infections were enrolled. All participants were Caucasian.

Table 3. Clinicopathologic characteristics of NSCLC patients.

Case	Age/Sex	Stage	Localization	Smoking	Mutations	Metastases
1	62/F	Ic	LLL	active	no	no
2	58/F	IV	LLL	active	PD-L1 5% pos.	multiple
3	88/F	IV	RUL	ex	EGFR positive (L858R)	Pleura effusion
4	72/F	Ic	LLL	ex	no	no
5	57/M	IV	LLL	active	no	multiple
6	58/M	IIIa	RUL	ex	PD-L1 30% pos.	lymph nodes
7	82/F	Ia	LLL	ex	ALK positive	no
8	69/M	Ic	RUL	active	PD-L1 30% pos.	n. d.
9	76/F	IIa	LLL	ex	EGFR positive	n. d.
10	56/M	IV	R central	ex	PD-L1 5% pos.	n. d.
11	61/M	IIIa	RUL	ex	PD-L1 100% pos.	n. d.
12	63/M	IV	RUL	ex	EGFR positive, PD-L1 5%	multiple
13	54/F	IV	L central	active	PD-L1 90% pos.	n. d.
14	61/M	IV	LLL	ex	no	multiple
15	74/M	Ic	LUL	ex	PD-L1 100% pos.	no
16	73/M	Ia	RUL	ex	no	no
17	80/M	IV	LUL	ex	PD-L1 100% pos.	bones
18	61/F	IV	L central	ex	no	multiple

F, female; M, male; LLL, left lower lobe; LUL, left upper lobe, RUL, right upper lobe; R, right, L, left, PCT, poly chemotherapy; RTX, radiotherapy, TKI, tyrosine kinase inhibitors; IMT, immunotherapy; n. d., no data; neg., negative; pos., positive; multiple, at least in two organs.

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Table 4. Sex, gender, and anatomical lung cancer localization.

All patients		18
Age (years)		
	Median	66,8
	Range	52-88
Gender		
	Male	10
	Female	8
Lung cancer localization	N	%
left lower lobe	7	39%
left upper lobe	2	11,1%
right upper lobe	6	33,3%
left central	2	11,1%
right central	1	5,5%

3.3.2. Blood collection

First, the peripheral venous blood was collected from every individual to the special EDTA-anticoagulant tubes (Thermo Fisher Scientific, Vienna Austria). In total three millilitres of the whole blood were collected. To avoid RNA damage and reduce the bench time, each sample after blood collection was immediately centrifuged (15 min at 1000 x g and 4°C). After centrifugation the whole blood was separated into plasma and cells. Plasma was placed into sterile DNA LoBind 1.5 ml tubes (Eppendorf, Vienna, Austria). For the elimination of the remained blood cells, the second centrifugation step was performed, and plasma samples were frozen at -80°C for the storage and future analysis.

3.3.3. MiRNA isolation from plasma samples and qPCR expression analysis

For miRNA isolation from plasma samples, miRNeasy Serum/Plasma Advanced Kit (Qiagen) was used. All components of the Kit were stored at room temperature. Isolation of miRNA was performed as recommended by producer, by using 200 µl of plasma samples frozen at -80°C and carefully defrosted on ice just before isolation. Final elution was performed in 20 µl RNase-free water and miRNA aliquots were stored at -20°C immediately after isolation. Each aliquot was defrosted just once for the cDNA synthesis in order to avoid possible miRNA degradation.

Based on *in vitro* analysis data from the first part of our study, we chose the 18 most deregulated (upregulated or downregulated) miRNAs to determine their expression in patients and in control group. Based on our previous results and on literature data, two miRNAs were used as controls (miRNA-16-5p and miRNA-191-5p). These control miRNAs are stable and circulate in the plasma independently of the health status. The most deregulated miRNAs and two control miRNAs are listed in the Table 5.

Table 5. Deregulated and control miRNAs used for patient material.

Nr.	miRNA
1	miRNA-205-5p
2	miRNA-141-3p
3	miRNA-148a-3p
4	miRNA-135b-5p
5	miRNA-200b-3p
6	miRNA-34a-5p
7	miRNA-708-5p
8	miRNA-155-5p
9	miRNA-429
10	miRNA-200c-5p
11	miRNA-15b-3p
12	miRNA-10b-5p
13	miRNA-196b-5p
14	miRNA-193b-3p
15	miRNA-27b-5p
16	miRNA-195-5p
17	miRNA-450a-5p
18	miRNA-196a-5p
Control 1	miRNA-191-5p
Control 2	miRNA-16-5p

3.3.4. Mutational analysis

As recommended by European society for Medical Oncology (ESMO) (144), various genes and their mutations were determined in every sample routinely, using the Ion Torrent S5 system for NGS and two different panels. NGS is commonly used in clinical practice for testing and classification of cancer (145, 146). First panel was the Ion AmpliSeq Colon and Lung Cancer Research Panel v2, containing 92 pairs of primers with an average amplicon length of 162 base pairs. With this DNA-based panel, different genes were analyzed: KRAS, EGFR, BRAF, PIK3CA, AKT1, ERBB2, PTEN, NRAS, STK11, MAP2K1, ALK, DDR2, CTNNB1, MET, TP53, SMAD4, FBX7, FGFR3, NOTCH1, ERBB4, FGFR1, and FGFR2. The second, RNA-based panel was Archer Fusion Plex MUG Pan-TRK Lung panel v1 (custom modified for the Institute of Pathology, Medical University of Graz). This panel was applied for analysis of following genes: ALK, BRAF, EGFR, FGFR1, FGFR2, FGFR3, MET, NRG1, NTRK1, NTRK2, NTRK3, RET, ROS1. Immunohistochemistry was used for determination of PD-L1 expression and calculated as percentage of positive tumor cells compared to all tumor cells.

3.4. Survival analysis

Survival was defined as the period between the first histologically proven lung cancer and the date of death, or last follow-up. Through the social insurance agency of Austria or patient's medical record, date of patient's death was provided. In the medical history of every patient, the date of the diagnosis and the first line of the therapy was documented. Six patients underwent surgical procedures, and their OS was up to 24 months or more. Patients, who have not been treated with specific oncological therapy had the lowest OS. Summarized survival data are displayed in Table 6.

Table 6. Survival data of the patients.

Patient number	First diagnosis	Survival 24 months after first diagnosis (1 = alive, 0 = dead)	Time of dead	First line Therapy	Overall survival in months
1	Oct-19	1		S	25
2	Oct-19	0	Jan-20	N	4, †
3	Oct-19	0	Feb-20	N	5, †
4	Oct-19	1		S	25
5	Oct-19	0	Dec-19	N	3, †
6	Oct-19	1		C, R	25
7	Oct-19	1		S	25
8	Nov-19	1		S	24
9	Nov-19	1		S	24
10	Dec-19	1		C, I	23
11	Dec-19	1		S	23
12	Oct-19	1		T	25
13	Nov-19	1		C, I	24
14	Dec-19	0	Jan-20	C	2, †
15	Dec-19	0	Jan-21	R	14, †
16	Dec-19	1		R	no data
17	Dec-19	0	Jun-20	I	7, †
18	Feb-20	0	Sep-20	C	8, †

S = surgery, C = chemotherapy, R = radiation therapy, I = immunotherapy, T = target therapy, N = no therapy, † = dead

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3.5. *In silico* analysis

Gene expression data of PCK1, PCK2, GLUT1 and LDHB and mutation data was retrieved from the cancer genome atlas program (TCGA) database of AC samples using the UCSC Xena platform (https://xenabrowser.net/). TCGA gene expression data was generated by the TCGA Research Network (https://www.cancer.gov/tcga) or via portal (https://www.cbioportal.org/) (162). Additionally, gene expression data from AC and paired non-involved lung tissue (GSE10072) (163) was analyzed using the GEO (Gene Expression Omnibus) platform (http://ncbi.nlm.nih.gov/geo).

3.6. Statistics

3.6.1. Statistical calculations

For all statistical analyses, we used the software package of GraphPad Prism (v. 5.0). To calculate the significant statistical difference between groups, data were tested for Gaussian distribution and two-sided student´s t-test was performed. P-values smaller than 0.05 were considered statistically significant.

3.6.2. Normalization of RT-qPCR data using mean-centering restricted method

In our working group different normalization methods for miRNAs were compared: NormFinder normalization, Global mean normalization (GenEx by Exiqon), and MCR normalization (147, 148, 149). Based on the results from our previous experiments, the MCR method was used for this study. The MCR is a novel variant of the common mean-centering (MC) normalization (139) (Wylie et al. 2011) strategy. RT-qPCR data examination was done by the Light Cycler 480 (Roche) applying the software version LC480 1.5.0.39. There were three replicates, and the results of measurements were displayed as Ct values.

The Ct- and Tm-values, were transfered from txt-files of the LC480 into Microsoft Excel files. The MCR normalization method considers only miRNAs detectable in all samples and in all three replicates. MiRNA expression, which was not detectable or with Ct values lower than 37, was excluded from the calculation. Blank wales without any amplification reaction were included as a negative control. After MCR normalization, the expression of miRNAs was calculated applying the ΔΔCt comparative threshold method. For this method the following formula was used: ΔCt (sample) = mean reference Ct – mean target Ct. Afterwards, the ΔΔCt representing the difference between the A549 and BE cells samples, was calculated according to the formula: ΔΔCt = ΔCt (A549) - ΔCt (BE cells). Additionally, the mean ΔΔCt of every single sample and the standard deviations were determined. The T-Tests was calculated using Microsoft Excel to determine the p-values and significances. Lastly, miRNA expression level in NSCLC cells (A549) were compared with the expression level in non-malignant BE cells.

4. Results

4.1. MiRNA expression profiles in cell samples

The workflow of this study is shown in Figure 1 and Figure 2. To provide high quality RNA samples, the BioAnalyzer BA2100 electrophoresis from Agilent Technologies was provided. All RNA samples had a RNA integrity number (RIN) value between 9.90 and 10, where 10 indicates the highest RNA quality. Isolated primary BE cells showed almost 100% positivity for cytokeratin (Figure 7).

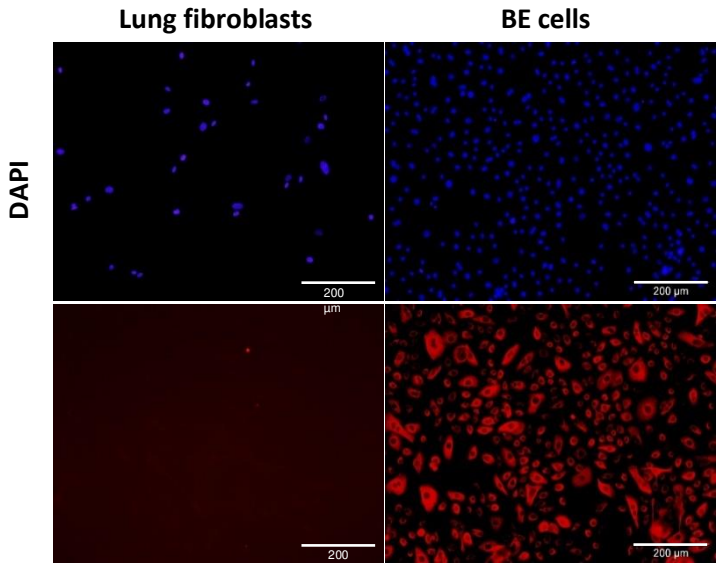

Figure 7. Isolated primary BE cells showed almost 100% positivity for cytokeratin.

Immunofluorescent of cytokeratin in lung fibroblasts and in isolated non-malignant BE cells. DAPI = nuclear staining. Scale bar = 200 µm.

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In total, 752 different miRNAs were analyzed in our screening using the microRNA PCR human panel I and II. One-hundred twenty-three (123) out of the 752 miRNAs were detected in both, A549 and BE cells, using RT-qPCR method. The rest of miRNAs was not detected, because of the lack of their expression, their inappropriate melting temperatures, or technical limitations of this method. After real-time amplifications, melting curves of miRNAs expressions were analyzed using the Light Cycler 480 and the software version LC480 1.5.0.39. For some miRNAs, two melting temperature (Tm)-values were detected. Table 7 shows examples of results of meting temperatures and Ct values on LC480 qRT-PCR. To ensure specificity, miRNAs with two melting temperature were excluded from further analyses. As recommended in the producer´s guidelines (Qiagen) and based on our previous experience, the Ct value of 37 or higher was interpreted as negative.

Table 7. Examples of the results with melting temperatures and cycle threshold values, using LC480 RT-qPCR.

Sample	Tm1	Tm2	Ct
miR-604	65.09	72.21	37.09
miR-615-5p	70.56	85.88	40

Tm = melting temperature; Ct = cycle threshold; miR = microRNA

4.2. Patient characteristics

Eighteen patients and the same number of healthy donors were included in this study. Every patient had the standardised lung cancer diagnostics based on computer tomography, PET-CT scan, and invasive biopsy with bronchoscopy or with CT-targeted puncture. Before being included into this study, the patients did not receive any anticancer therapy. From each participant, 3 ml of the peripheral venous blood were collected. The median age of patients at time of diagnosis was 63 years (range from 52 to 88 years) and of donors 67 years (range from 31 to 84 years). Patient cohort contained 10 males and 8 females, whereas donor cohort contained 13 males and 5 females. Lung adenocarcinoma was confirmed in each patient. Most patients had lung adenocarcinoma in stage IV (44.5%), followed by stage I (33.3%), stage II

(11.1%), and stage III (11.1%). The stage of lung cancer was determined with computer tomography, PET-scan, and lymph nodes biopsy with EBUS using the newest (8^{th}) edition of TNM stage classification. All LUAD patients were either ex- or present smokers. In six patients there were no mutations detected or PD-L1 status was negative. In three samples EGFR mutations were detected and in one sample ALK mutation was found. In four cases PD-L1 with over 50 % positivity was discovered. Five patients were without any metastasis and five patients with multiple metastasis. The most common localization of lung cancer was in left lower lobe (39%) and the rarest localization was right central (5.5%). All clinicopathological characteristics of LUAD patients are summarized in the Table 3 and Table 4 in Methods.

4.3. Normalization of miRNAs using MCR normalization method

RT-qPCR data were analyzed by the MCR normalization method. This method is restricted to all miRNAs that are detectable in all analyzed samples. In total, 58 miRNAs were deregulated in A549 cells compared to BE cells. After setting the cut off to $\Delta\Delta Ct \geq 1.5$, 37 miRNAs were differentially regulated, with 15 up- and 22 down-regulated miRNAs (Table 8). In addition, the table shows the mean $\Delta\Delta Ct$-values of three biological replicates. For further expression analyses in plasma samples of LUAD patient and healthy donors, we selected eighteen most prominently deregulated miRNAs with $\Delta\Delta Ct \geq 2.5$ (Figure 8). Table 8 and Figure 8 show a summary of expressed miRNAs with significant differences (p-value < 0.05) obtained by using the paired t-test.

Table 8. Differentially deregulated miRNAs (ΔΔCt ≥ 1.5) in A549 LUAD cell line compared to the pool of primary, non-malignant bronchial epithelial cells isolated from three healthy donors.

Up		**Down**	
miRNA	ΔΔCt	miRNA	ΔΔCt
miR-196a-5p	5.08	miR-205-5p	-14.58
miR-450a-5p	3.92	miR-141-3p	-8.22
miR-195-5p	3.29	miR-148a-3p	-5.96
miR-27b-5p	3.07	miR-135b-5p	-4.99
miR-193b-3p	2.79	miR-200b-3p	-4.34
miR-196b-5p	2.73	miR-34a-5p	-4.11
miR-10b-5p	2.56	miR-708-5p	-3.88
miR-15b-3p	2.54	miR-155-5p	-3.85
miR-126-3p	2.42	miR-429	-2.87
miR-582-3p	2.16	miR-200c-5p	-2.50
let-7i-5p	2.08	miR-19a-5p	-2.36
miR-589-3p	1.81	miR-210-3p	-2.34
miR-769-5p	1.77	miR-425-3p	-2.18
miR-93-3p	1.68	miR-200a-5p	-2.07
miR-15b-5p	1.59	miR-664a-3p	-2.05
		miR-1248	-1.93
		miR-31-5p	-1.88
		miR-181b-5p	-1.85
		Mir-503-5p	-1.80
		miR-127-3p	-1.73
		miR-23a-3p	-1.65
		miR-944	-1.53

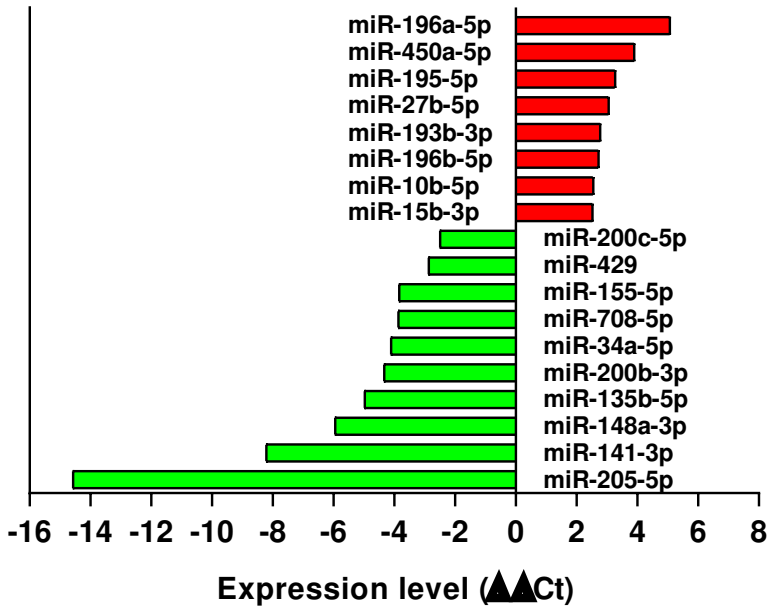

Figure 8. Differentially deregulated miRNAs in A549 cells compared to BE cells.

Eight miRNAs were upregulated, whereas ten were downregulated (cut off was set to $\Delta\Delta Ct \geq 2.5$).

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4.4. Normalization of circulating miRNAs

Normalization of circulating miRNAs MiRNAs are detectable intracellular and extracellular in different human biofluids like serum, plasma, urine, tears, and others. Normalization of circulating miRNAs expression in biofluids is more difficult in comparison to data normalization received in vitro, because of human diversification. Therefore, we investigated the stability of miRNA expression in our cohort very carefully. In all plasma samples from every participation, two miRNAs (miRNA-16-3p and miRNA-191-5p) were stably expressed (Figure 9). In patients, miRNA-16- 3p was expressed at an average Ct = 22.7 and in donors at Ct = 22.6. MiRNA-191-5p was expressed at an average Ct = 29.4 in patients and at an average Ct = 30.1 in donors. Because of the stability of these two miRNAs over all samples, the mean expression value of miRNA-16-3p and miRNA-191-5p was used for data normalization.

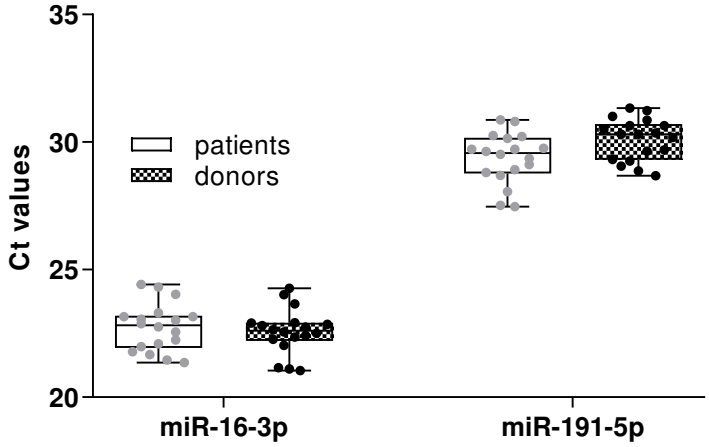

Figure 9. Two stably expressed miRNAs (miRNA-16-3p and miRNA-191-5p).

Two stably expressed miRNAs (miRNA-16-3p and miRNA-191-5p) used for data normalization. Two miRNAs (miRNA-16-3p and miRNA191-5p) were stably expressed in all plasma samples from patients and healthy donors. Thus, the mean expression value of these two miRNAS was used for data normalization.

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4.5. Expression of circulating miRNA in plasma samples

We analyzed expression profile of miRNAs, which were selected based on *in vitro* part of our study and chose the most deregulated miRNAs ($\Delta\Delta Ct \geq 2.5$, Figure 7) for the next step of our research. In total, 18 significantly deregulated miRNAs were chosen (8 were up- and 10 down-regulated in A549 cells compared to BE cells).) As the next, we analyzed their expression in plasma samples of LUAD patients and healthy donors (n = 18 for each group) and compered the expression between two groups (LUAD patients and healthy donors). Eight miRNAs (miRNA-27a-5p, miRNA-135b-5p, miRNA-196a-5p, miRNA-196b-5p, miRNA-200c-5p, miRNA-429, miRNA-450a-5p, and miRNA-708-5p) were not detectable in plasma samples of LUAD patients and in healthy donors. Significant expression of ten miRNAs was found, but six of them (miRNA-10b-5p, miRNA-34a-5p, miRNA-141-3p, miRNA-155-5p, miRNA-200b-3p, and miRNA-205-5p) had a comparable expression level in both groups (Figure 10). Significant deregulation in plasma samples was found for four miRNAs (miRNA-15b-3p, miRNA-148a-3p, miRNA-193b-3p, and miRNA-195-5p) in LUAD patients compared to donors (Figure 11). One miRNA (miRNA-195-5p) was up-regulated and three miRNAs (miRNA-15b-3p, miRNA-148a-3p, and miRNA-193b-3p) were down-regulated in plasma sample of LUAD patients compared to healthy donors. Two miRNAs were used for normalization (miRNA-16-5p and miRNA-191-5p), because of their stability in all samples (Figure 9).

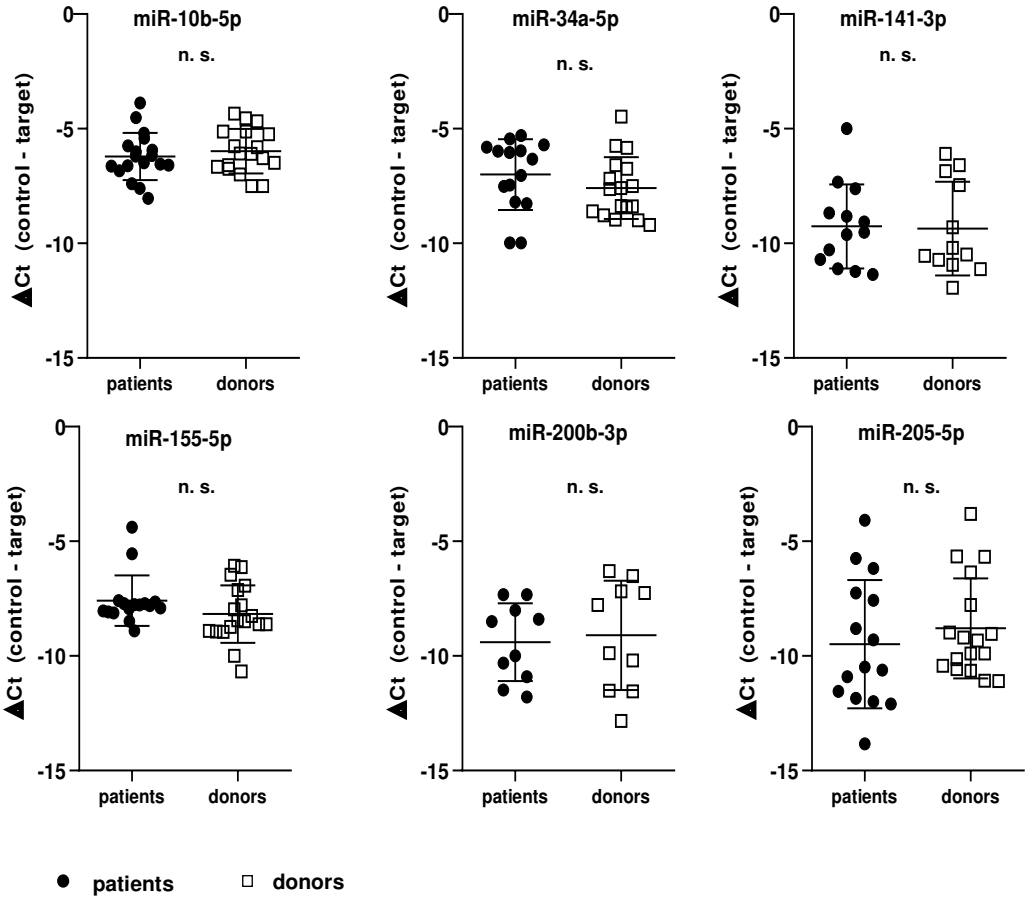

Figure 10. Six miRNAs detectable in plasma samples of patients and healthy participants.

Similar expression levels of six miRNAs were detected in plasma samples of patients and healthy participants. In total, significant expression of ten miRNAs was detected, however, six of them (miRNA-10b-5p, miRNA 34a-5p, miRNA-141-3p, miRNA-155-5p, miRNA-200b-3p, and miRNA-205-5p) had a comparable expression level in both groups (patients and healthy donors). Expression data were normalized to the mean value of control miRNAs (miRNA-16-5p and miRNA-191-5p).This Figure was reprinted from (143) with permission from IOS Press.

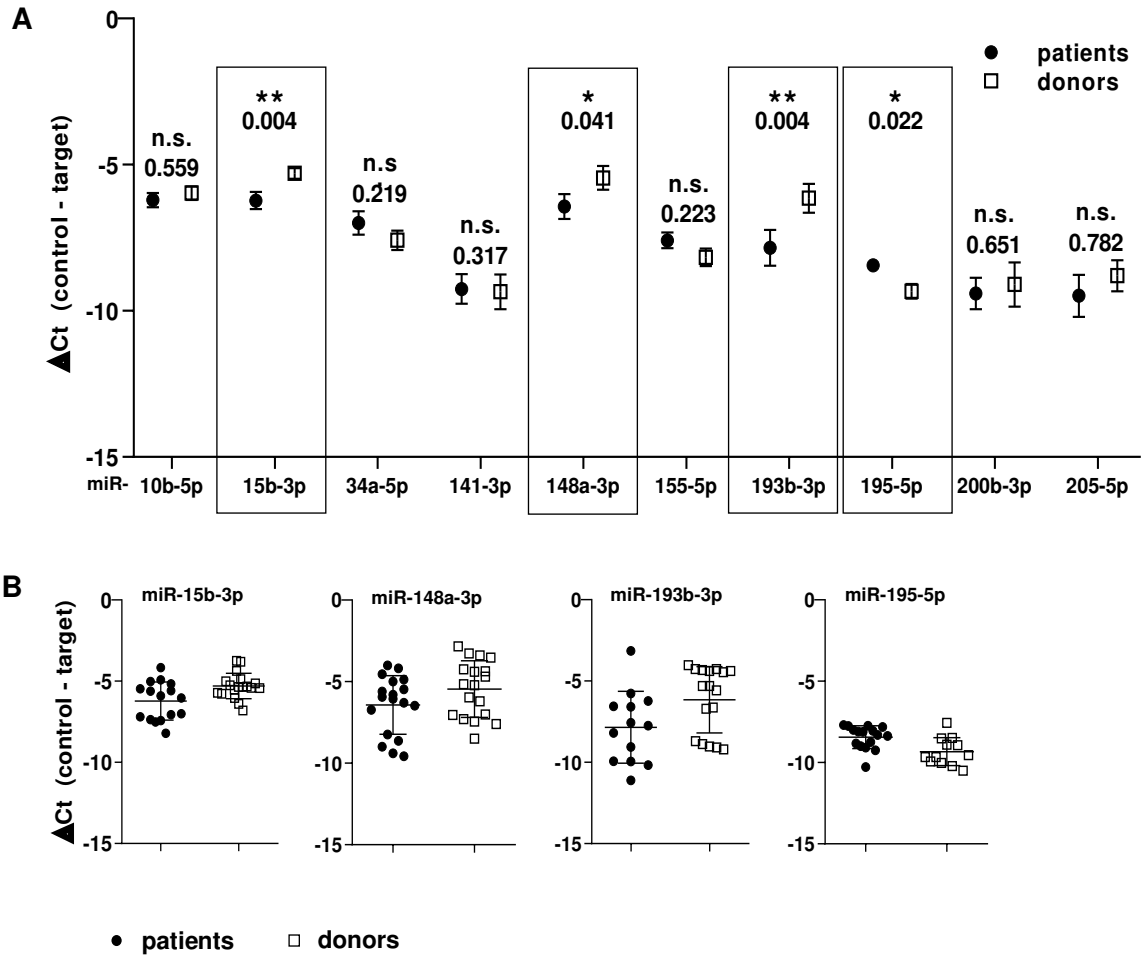

Figure 11. Four significant deregulated miRNAs in plasma samples in LUAD patients compared to donors. Four significant deregulated miRNAs in plasma samples of LUAD patients compared to donors. (A) Four miRNAs (miRNA-15b-3p, miRNA-148a-3p, miRNA-193b-3p, and miRNA-195-5p; framed with rectangles) were significantly deregulated in LUAD patients compared to healthy donors. Expression data were normalized to the mean value of two control miRNAs (miRNA-16-5p and miRNA-191-5p). (B) The distribution of expression data of these four miRNAs in single patients/donors. *, $p < 0.05$; **, $p < 0.01$; n.s. = not significant (two-sided student´s t-test).

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4.6. Deregulated miRNAs and their target genes

A very important part of miRNAs investigation is to understand their function and their involvement in tumorigenesis, because this might lead to discovery of new diagnostics and therapeutic option for NSCLC patients. We screened the literature for target genes and the miRNA of interest (e.g. hsa-miR-X-Xp + target gene; September 2022), with special focus on experimentally validated (e.g. by dual luciferase assay) target genes (*https://pubmed.ncbi.nlm.nih.gov).* Based on the actual literature, 107 validated target genes were found, which are regulated by these four miRNAs (Figure 12). Five target genes, which are well known and used in clinical oncology, were discovered (PTEN, CXCR4, IGF1R, FGF2, and PD-L1). Sixty-one target genes are regulated by miRNA-195-5p, forty-two are regulated by miR-148a-3p, three by miRNA-193-5p and only one validated target gene is regulated by miRNA-15b-3p. Molecular functions of these 107 target genes were summarized in five groups: binding, catalytic activity, molecular function regulator, molecular transducer activity, and transporter activity (Figure 13).

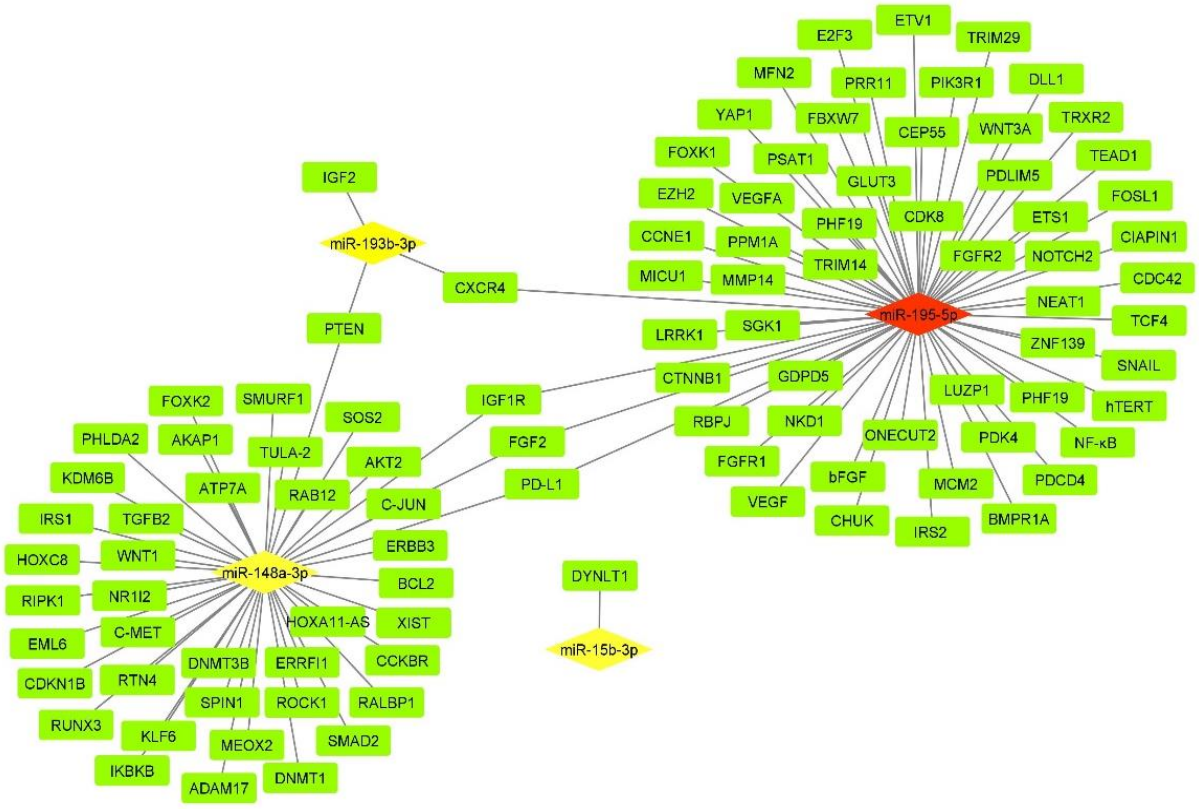

Figure 12. Interactions of four differentially expressed miRNAs and their validated target genes. One-hundred seven (107) validated genes (in green), which are regulated by four selected miRNAs. MiRNA-195-5p (in red) was up-regulated and three miRNAs: miRNA-15b-3p, miRNA-148a-3p, and miRNA-193b-3p (in yellow) were down-regulated. For data visualization, Cytoscape (version 3.8.2) was used.

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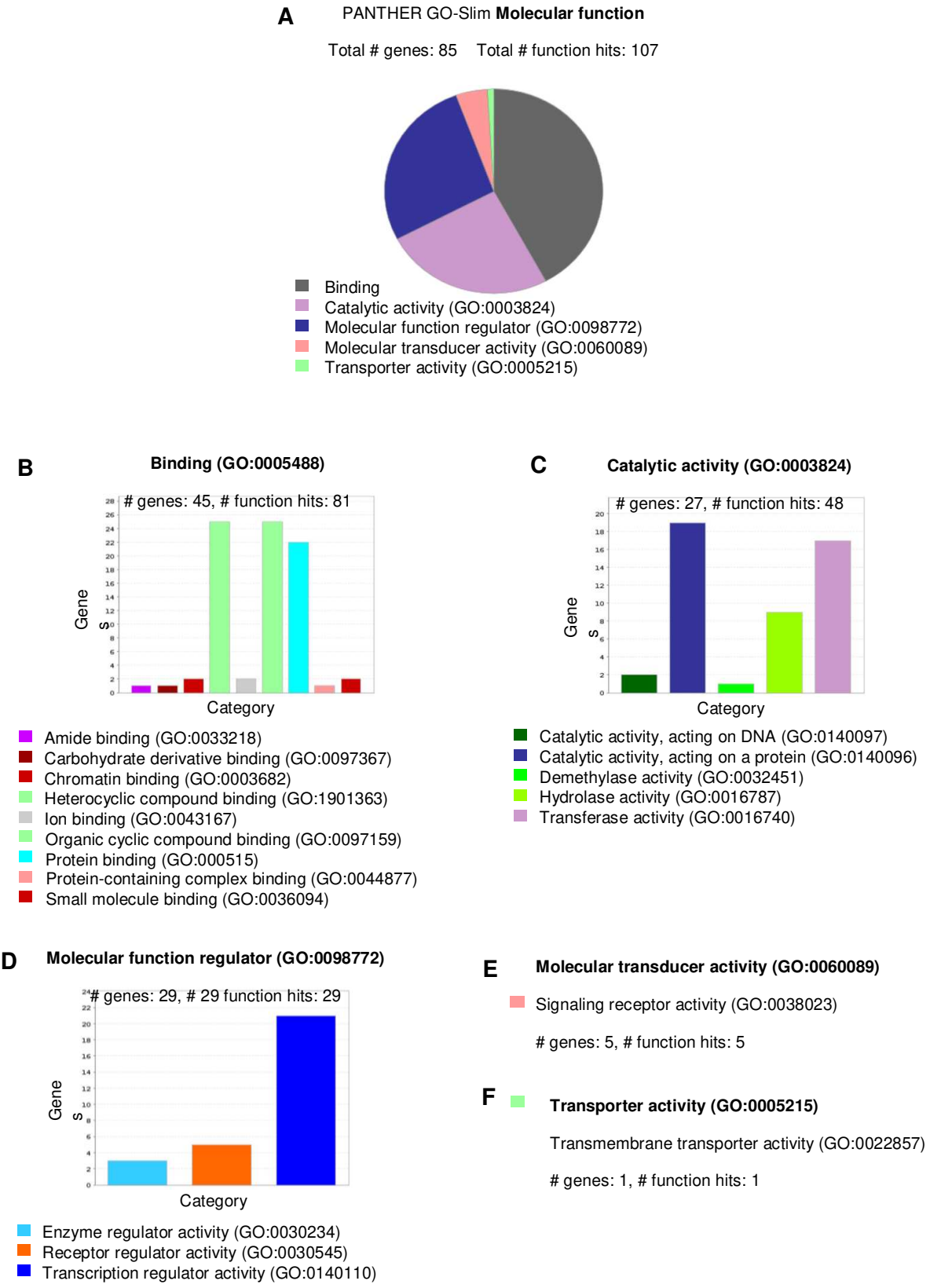

Figure 13. Interactions and molecular functions of validated target genes of four deregulated miRNAs. Interactions and distribution of molecular functions of validated target genes of four deregulated miRNAs (A). The molecular functions of all 107 target genes were annotated into five groups: binding (B), catalytic activity (C), molecular function regulator

(D), molecular transducer activity (E), and transporter activity (F), with the indicated number of genes and function hits.

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4.7. Pathways regulated by deregulated miRNAs

Using the Panther Classification System (*www.pantherdb.org*) we analyzed target genes and their associated pathways. In total, 24 different pathways were found (Figure 14). MiRNA-195-5p was involved in 23 pathways. Some of them are well known as important intracellular pathways and regulators in carcinogenesis. For example, PI3 kinase pathway takes part in cell regulation, growth and activation of it leads to tumor development and resistance to the antitumor treatment (150). MiRNA-148a-3p was involved in 21 pathways, including the p53 pathways, which is associated with the prognosis and response to the therapy (151). MiRNA-193-5p has been associated with 8 different pathways. After this particular analysis, miRNA-15b-3p and its single target gene (DYNLT1, dynein light chain Tctex-type 1) was not associated to any characteristic pathway, but it is known to have an important role in cell regulation, including proliferation, migration, and apoptosis (152).

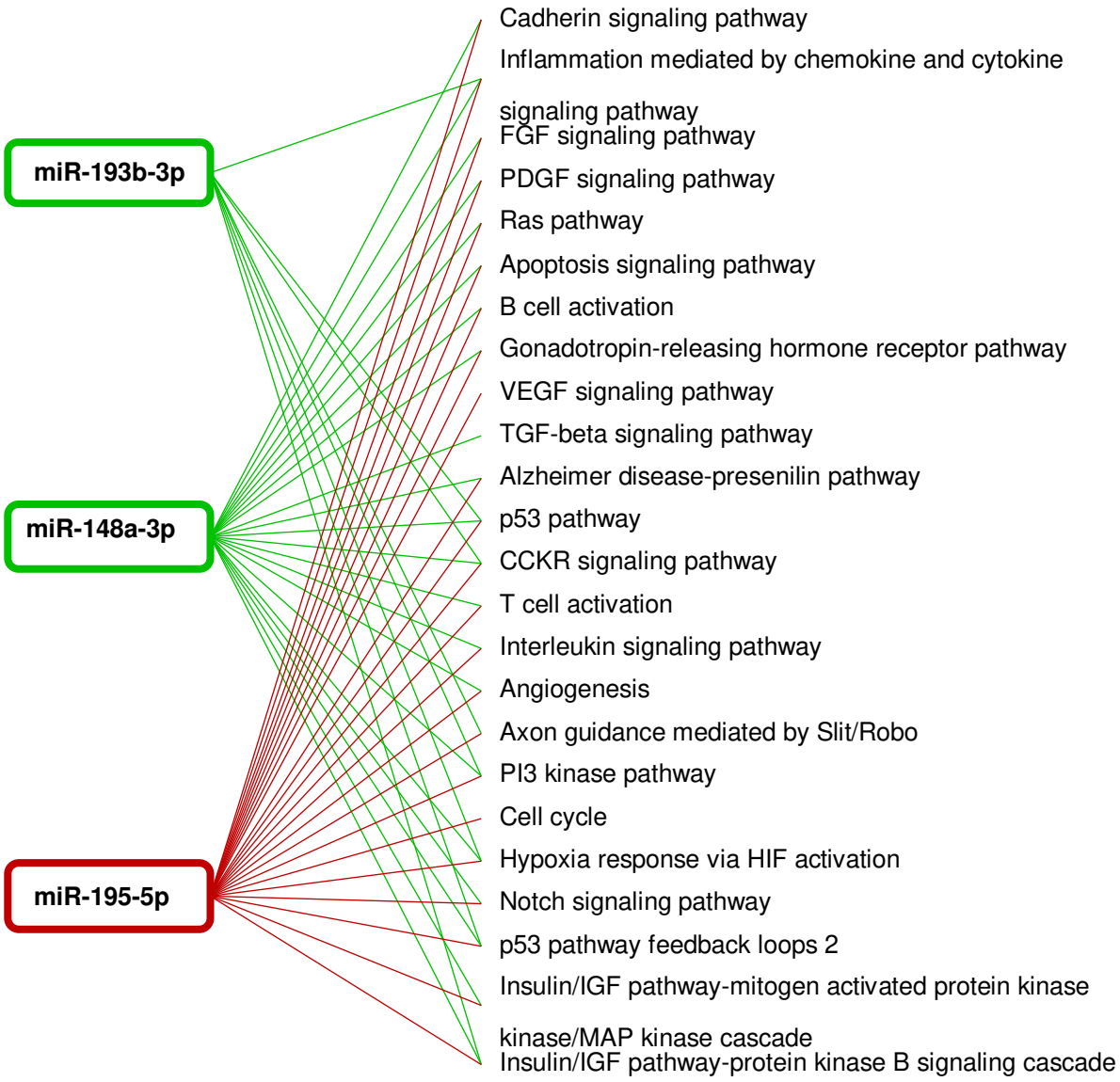

Figure 14. Target genes and pathways of deregulated miRNAs. Twenty-four (24) different pathways were associated with deregulated miRNAs. One miRNA (miRNA-195-5p) was up-regulated, and it is shown in red-lined rectangle. Two miRNAs (miRNA-miR148a-3p, miRNA-193b-3p) were down-regulated and are shown in green-lined rectangles. MiRNA-15b-3p was not annotated to any special pathway. *In silico* analysis has been done using the Panther Classification System (*www.pantherdb.org*).

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5. Discussion

Lung cancer is still the most lethal malignancy worldwide in both sexes. Early detection of NSCLC could reduce mortality and, therefore, one of the research focuses in lung cancer is investigation of tumor markers for early diagnostic. However, to find out the specific biomarker is very challenging. The National Lung Screen Trial (NLST) reported that low dose computed tomography (LDCT) for lung cancer screening can reduce mortality for 20%. However, the use of LDCT has many disadvantages like high false-positive rate that leads to the overdiagnosis, unnecessary radiation exposure and risk of invasive diagnostics (153). Thus, novel minimal invasive methods for lung cancer detection are presently needed.

5.1. Liquid biopsy in lung cancer

Some of the potential biomarkers for lung cancer early detection, which are presently under investigation, are miRNAs. MiRNAs are well-known as endogenous noncoding RNAs, which act as gene regulators at posttranscriptional level. MiRNAs are potential molecular markers for early diagnosis, prognosis and treatment monitoring. Investigation of them is important for clinical practice, to improve the outcome of NSCLC patients.

In the first step of our study, we compared miRNAs expression in malignant A549 NSCLC and non-malignant bronchial epithelial cells. The most deregulated miRNAs *in vitro* were selected for the second part of the study - analysis of these miRNAs in plasma samples from NSCLC patients. Based on the literature, we decided to analyze miRNAs in plasma, because it has been shown that miRNAs expression was generally higher in plasma than in serum (154). Additionally, it was shown that the concentration of circulating miRNAs in serum is lower because of the coagulation processes (155). Plasma samples of eighteen NSCLC patients and eighteen non-cancerous donors were collected and immediately centrifugated in two steps for removing any residual blood cells. All steps including sample collection, storage and miRNA isolation are crucial for getting reliable results and were done very carefully and according to the protocols. Before sample collection, all including and excluding study criteria were proofed.

5.3. Challenges in using circulating miRNAs as biomarkers

Using circulating miRNA in clinical practice is getting more interesting. Due their stability in plasma or serum, they can be applied as minimal invasive biomarkers in different types of cancer. Each biomarker should be easy applicable and conformed to the international standards. However, different possible biases can be a limiting factor. For avoiding the technical biases, standardized methods should be used in multiple steps of experiments, beginning from the blood collection from the patients, samples centrifugation and preparation, storage, miRNA extraction and quantification.

The source of sample is a topic for discussion, because the results from the same patient using plasma or serum could be different, but the use of whole blood without centrifugation is not recommended (156). Already at the first step of the miRNAs investigation, plasma or serum can be differently collected and this can lead to the differences in results. Type of anticoagulant in collection tubes, which is used for plasma or serum, is important. EDTA and citrate tubes are suitable, but the use of heparin as the anticoagulant could negatively influence the miRNAs stability (157). MiRNAs storage at −80°C was shown to be reliable and did not negatively influence the quality of samples, but the maximal duration of storage is unclear (157). RNA extraction step plays an important part in miRNAs investigation. There are two commercial kits, which are available for this purpose: Ambion MiRvana PARIS and the Qiagen miRNeasy Kit. Both protocols have been shown as effective, but Kroh et al. demonstrated that quantity of RNA isolated with Qiagen miRNeasy Kit is higher (157).

A further factor, that can influence miRNAs results both in serum and in plasma, are conditions of centrifugation, like speed, time and temperature (158). Felekkis et al. compared different conditions that led to different outcome of miRNA expression (156). For example, in one protocol centrifugation consisted of two steps. The blood was first centrifuged at 1000× g for 15 min and after that it was centrifuged again at 2500× g for 15 min for removing the platelet contaminants, that can impact the concentrations of miRNAs (156). That was the main reason why we also performed two centrifugation steps, to exclude possible miRNA contaminations from residual blood cells.

Individual related factors of patients could also influence miRNAs expression and, therefore, the final results. There was shown, that anti-platelet treatment may affect the miRNA expression (159), thus, before including participants into the study, administration of different therapy should be evaluated. Other factors like vitamin A and D or inflammatory process may

have an effect on miRNA expression profile. Even individual variabilities, like sex, lifestyle, diet or smoking status could influence the miRNA expression (160).

5.2. Data normalization

One of the most challenging part in research of miRNA is data normalization, especially if data were generated *in vivo*. These difficulties are connected with human variability. In the most studies published so far, expression results were obtained by RT-qPCR. This is very specific and sensitive method, which allows detecting small quantities of miRNAs. Data normalization influences results interpretation, therefore, the normalization method should be carefully chosen. In our previous scientific projects, different normalization strategies were compered and it came out that each method has advantages and disadvantages. For MCR normalization no special software is needed and calculations can be done in Microsoft Excel. The NormFinder normalization approach is also based on Microsoft Excel calculations with additional special application and it can calculate values automatically. The GenEx (Exiqon) normalization requires special software. Based on our previous experiences and present literature, the MCR method was chosen for normalization and following calculation.

In our study, we found that expression levels of two miRNAs (miRNA-191-5p and miRNA-16-3p) were very stable in all samples, both in NSCLC patients and in healthy donors. Based on these results and on present literature, we applied these two miRNAs for internal quality control and for data normalization of circulating miRNAs in plasma. There is no final consensus which reference genes (RGs) should be applied for RT-qPCR data normalization. In studies about data normalization using validated RGs was found, that miR-191-5p was the most stable miRNA in comparison to other studied miRNAs (161). Zheng et al. demonstrated that miR-191 was suitable as RG in study of colorectal adenocarcinoma (162) and Hu et al. showed its stabile expression in breast cancer (163). Another study reported that miR-16, in combination with other microRNAs, can be appropriate as reference miRNA in study of gastric cancer patients and healthy donors. Thus, the use of those two miRNAs suggests their possible role as RG for different diseases.

5.4. MiRNA nomenclature

MiRNA nomenclature includes two species described as -5p and -3p strand. These species are obtained from the 5' and 3' arms of the precursor miRNAs. They are expressed at different levels and can have different target genes and functions (164). All this can lead to confusion. To avoid this, the miRBase (http://www.mirbase.org/) was developed. In this database, it is possible to enter the name of miRNA of interest and check a description of miRNA structure with the 5p and 3p strands. There also other web-based databases, which can be used for miRNA examination. The source Microrna.org (http://www.microrna.org/microrna/) allows to check experimentally identified miRNA and its targets. Starbase (http://starbase.sysu.edu.cn/) is a database that shows interactions of miRNA-mRNA and protein-mRNA. MiRDB (http://mirdb.org/miRDB/) is an online database, where is possible to find miRNA target and their functional annotations. The human microRNA disease database (HMDD) (https://www.cuilab.cn/hmdd) shows the associations of miRNA and disease, based on literature data.

5.5. Differently expressed miRNAs in plasma samples of LUAD compared to donors

The results of our study indicated that four miRNAs were differently expressed in plasma samples of LUAD patients compared to healthy individuals. These are: miRNA-15b-3p, miRNA-148a-3p, miRNA-193b-3p and miRNA-195-5p. It is known that miRNA-15b-3p is located at chromosome 3q25.33. To our knowledge, there are no studies about miRNA-15b-3p expression in lung cancer (August 2022, Pubmed). All data published so far are without specified strand of miRNA-15b (-5p or -3p) or describing only the 5p strand. The role of miRNA-15b-5p in non-cancerous lung diseases was described. Circulating miRNA-15b-5p was found as a potential marker for identifying patients with asthma-COPD overlap (ACO) from patients only with asthma or COPD (165). In different cancer types, miRNA-15b-5p was demonstrated as minimal invasive biomarker for early diagnosis. For example, Toelle et al. showed significant differences of miRNA-15b-5p expression between bladder cancer patients and control group (166). Yi Chen et al determined that miRNA-15b-5p was over-expressed in tumor tissues and preoperative plasmas samples, but its expression level in plasma decreased after surgical therapy (167). Several miRNAs, including miRNA-15b-5p, were identified as potential biomarker for early NSCLC detection using next generation sequencing (168). In another study was shown, that miRNA-15b may play a role in identification of chemotherapy

response in advanced NSCLC patients and it has potential in overall survival prediction (169). It was verified that upregulation of miRNA-15b in LUAD correlated with poor prognosis and decreased overall survival in LUAD patients (170). Results of another research also indicated that overexpression of miRNA-15b is associated with poor prognosis for NSCLC patients and may serve as a biomarker for outcome prediction (171). To summarize, there is no data about miRNA-15b-3p expression in lung cancer and data of miRNA-15b in general showed expression in different diseases including cancers.

Human miRNA-148a-3p is localized at the 7p15.2 chromosomal part and is a member of miRNA-148/152 family. The results of our study showed decreased miRNA-148a-3p expression in plasma of NSCLC patients in comparison to healthy individuals. Using miRNA-148a-3p as additional parameter together with LDCT scan for early lung cancer diagnostics was described in work of He Y. et al. (172). They analyzed the importance of different miRNA, including miRNA-148a-3p, in patients with pulmonary nodules. To morphologically differentiate benign nodules from malignant nodules in CT scan is not always easy and it was shown that combination of miRNAs panel together with LDCT might decrease the false positive results compared to LDCT scan alone. Specificity of miRNAs test was 90.2% for nonmalignant lung diseases, but sensitivity of miRNAs test was 44.7% for adenocarcinoma and 22.2% for other pulmonary diseases (172). In another study was also shown that miRNA-148a-3p was downregulated in NSCLC samples (173), this being in correlation with our data. There was no correlation with overall survival or response to the treatment, but miRNA-148a-3p had significant correlation with tumor stage (173). Different biological functions of miRNA-148a-3p were described in some studies. For example, overexpression of miRNA-148a-3p induced cell proliferation and apoptosis in NSCLC regulating DNMTA (a central DNA methyl transferase in mammalian cells) and promoted tumorigenesis *in vivo* and *in vitro* (174). In another study was demonstrated that knockdown of miRNA-148a-3p increased cell proliferation, migration, tumor growth, and reduced apoptosis, but these mechanisms were destroyed by Y-box binding protein 1 (YBX1) silencing, YBX1 being a frequent oncogene in NSCLC (175). Together, these data demonstrate an important role of miRNA-148a-3p in tumorigenesis of lung cancer.

In our study, miRNA-193b-3p was detected as the most downregulated miRNA. Human miRNA-193b-3p is located at the chromosome 16p13.12. In the literature was described that miRNA-193b-3p expression is decreased in NSCLC samples compared to healthy lung cells (176). This is in line with our results. It was shown that inhibition of miRNA-193b in A549

cells increased cell proliferation, migration and invasion (176). In another study was described that overexpression of miRNA-193b was correlated with poor prognosis in patients with glioma, this being connected to increasing cell proliferation, migration and invasion, whereas its downregulation led to the opposite mechanisms (177). MiRNA-193b was found as a predictive factor for therapy response in patients with esophageal squamous cell carcinoma (ESCC). Serum samples from ESCC and healthy donors were collected and compared before and after the therapy. Overexpression of miRNA-193b was associated with better outcome in ESCC patients (178). To conclude, we can suppose that miRNA-193 may have different functions in tumor development. Downregulated expression level of miRNA-193b was detected in NSCLC cells in comparison to non-malignant cells, and this is in line with our results. However, there are also data, which showed upregulation of miRNA-193b in NSCLC cells compared to the healthy controls (179), therefore, the aberrant expression of miRNA-193b-3p may act as a biomarker for NSCLC.

MiRNA-195-5p is located on human chromosome 17p13.1. Some data showed aberrantly expressed miRNA-195-5p in different cancer types, like colorectal cancer (180) and lung cancer (181, 182). In our study, we found overexpression of miRNA-195-5p in plasma samples of NSCLC patients, but this fact is in contradiction with results of other published studies, where downregulation of miRNA-195-5p in lung cancer cells was shown. The aberrant expression of miRNA-195-5p in our and in other studies probably relates to analyzed material. In the most studies, miRNA-195-5p expression was detected in lung cancer tissue or cell lines, whereas the expression of miRNA-195-5p in plasma of lung cancer patients has not been analyzed. Long et al. detected that increased expression miRNA-195-5p led to inhibition of cell proliferation, migration, and invasion in lung cancer (183). Overexpression of miR-195-5p decreased angiogenesis, migration, and invasion in malignant cells by targeting VeGF and inhibiting the growth of endothelial vascular cells (184). Zhang et al. also demonstrated that inhibition of miR-195-5p induced cell proliferation, migration, and invasion of lung cancer cells (185). Based on the bioinformatic analysis, the involvement of miRNA-195-5p in lung cancer development was reported, regulating the exostosin protein, which manages different biological processes such as cell adhesion and migration (186). Altogether, these data show the aberrant expression of miRNA-195-5p and complexity of its regulation, thus further investigations are needed.

5.6. MicroRNAs *in vivo* and *in vitro*

The direct comparison of the miRNAs expression *in vivo* and *in vitro* is quite difficult. It is known that the cellular microenvironment including different growth factors and cytokines can influence the expression of miRNAs. *In vitro*, it is possible to regulate the presence of these factors, but *in vivo* the situation is different. Riemann et al. showed in their experiments that expression of miRNA-7 in the cell culture and in solid tumors were different. They suppose that it relates to different miRNA-7 binding circular RNA *in vivo* and *in vitro*. Additionally, the difference of miRNAs expression *in vivo* and *in vitro* can be affected by sex hormones (187). The results of another study showed that expression of miRNA-373 in renal cell carcinoma tissues and in the cell lines was identic (188). In our results some deregulated miRNAs have dissimilar expression. MiRNA-195-5p was up-regulated in vitro and in vivo, miRNA- 148a-3p was down-regulated *in vitro* and *in vivo*. However, miRNA-15b-3p and miRNA-193b-3p were up-regulated *in vitro*, but down-regulated in plasma samples. Taken together, these data indicate that miRNAs expression can differ *in vitro* and *in vivo* and it depends on different factors, like cytokines, hormones, and microenvironmental parameters.

5.7. Target genes

For better understanding of the molecular functions of four deregulated miRNAs in tumorigenesis, we *in silico* analyzed target genes, which are regulated by these miRNAs. We discovered five the most prevalent target genes (PTEN, CXCR4, IGF1R, FGF2, and PD-L1). All these target genes have different biological mechanisms in both malignant and nonmalignant cells.

Phosphatase and tensin homolog on chromosome 10 (PTEN) acts as tumor suppressor and was found to be mutated in different kind of cancers and can affect resistance/sensitivity to cancer therapy (189). PTEN gene is located on chromosome 10q23.3 (190). It is known that PTEN protein acts as a lipid phosphatase, converting phosphatidylinositol 3, 4, 5 trisphosphate into phosphatidylinositol 4, 5-bisphosphate, inhibiting the action of the phosphoinositide 3-kinase (PI3K) and leading to the reduction of cell proliferation and migration (189). It was shown that PTEN regulates cell proliferation by activating the PI3K–AKT pathway. Via down-regulating this pathway, PTEN stimulates angiogenesis and cell growth in different tumors (190). It was also demonstrated, that PTEN controls the cell cycle by inhibiting the cyclin D1 activity (191). In one systematic review, the association between expression of PTEN and the survival of patients with different type of cancers was described (192). Decreased expression of PTEN was correlated with poor overall survival (OS) in patients with gastric cancer and with poor OS in NSCLC patients, but some results demonstrated no association between PTEN expression and OS. These may suggest that the correlation between PTEN expression and OS deepens on type of cancer.

Another target gene, which is regulated by miRNAs we described here, is C-X-C chemokine receptor 4 (CXCR4). The relevant role of CXCR4 was initially described in patients with HIV infection and its connection with T-cells. The CXCR4 relation to cancer development was found with increasing research of B-cells and its role in chronic leukemia patients (193). CXCR4 is often overexpressed in different tumors like prostate, breast, and colon. It was shown that increased expression of CXCR4 is correlated with lymph node and distant metastasis. Thus, CXCR4 expression is associated with overall survival and tumor stage. Between NSCLC patients, the most deregulated CXCR4 expression was described in patients with adenocarcinoma (194). CXCL12/CXCR4 pathway may influence cancer development in epithelial cells through CXCR4 expression and its downregulation could be a potential treatment in some hematologic disorders (195). A combination of CXCR4 antagonist with

established chemotherapy was described in breast cancer treatment and positive results in animal models were shown (196). Immunotherapy is very common in anticancer treatment. In some cases, resistance to immunotherapy is developing during the therapy or some patients do not profit from immunotherapeutic approaches in the beginning of treatment. Zhou et al. supposed that the mechanism of resistance to immunotherapy may be connected with the CXCL12/ CXCR4 pathway (197).

Insulin-like growth factor (IGF) is a system of different ligands and receptors, which are involved in regulation of biological mechanisms. This system consists of three ligands (IGF1, IGF2 and insulin) and four membrane receptors (insulin receptor, IGF1R, mannose 6-phosphate receptor and the insulin receptor-related receptor) (198). The central member of the IGF structure is a type 2 tyrosine kinase receptor - IGF1R. It may influence tumor development in numerous types of cancer, like hepatocellular carcinoma, colorectal cancer, and prostate cancer (199). There was shown, that some miRNAs may regulate the expression of IGF1R, inhibiting the cell proliferation, migration and invasion in gastric cancer cells (200), (201). Alfaro-Arnedo et al. demonstrated in mice model that absence of IGF1R led to the inhibited tumor growth and angiogenesis, and its upregulation was correlated with reduced overall survival in lung cancer patients (202). There was shown that overexpression of IGF1R was associated with therapy resistance development, targeting different kinases including the EGFR, which is important in NSCLC treatment. Preclinical data demonstrated correlation between resistance to anti-EGFR therapy and overexpression of IGF1R in pancreas and prostate cancer cell lines, activating of PI3K/AKT signaling pathway. Thus, increased expression of IGF1R led to reduced effect of EGFR targeting (203). Co-mechanism of the IGF1R and EGFR have a potential therapeutic profit because inhibition of IGF1R may depress the resistance development.

Fibroblast growth factor 2 (FGF2) is frequently overexpressed in different types of cancer. FGF family includes 23 FGF signaling polypeptides. It is known that FGF2 may regulate physiological and pathological processes, such as cell growth and differentiation, angiogenesis, and inflammatory responses (204). Angiogenesis is the mechanism of new blood vessels expansion, which is relevant in numerous physiological processes, but also in cancer. The development of circulatory system leads to the growth of tumor and development of metastasis. FGF2 is also known as factor for tissue fibrosis progression. Sakai et al. demonstrated in preclinical mice models, that subcutaneous administration of FGF2 led to increased interstitial fibrosis in pancreatic cancer cells and it was associated with progress of pancreatic cancer (205).

In one work was shown, that upregulation of miR-203 inhibited the FGF2 expression, thereby suppressing the pancreatic cancer cells growth (206). Li et al. demonstrated that FGF2 upregulation was associated with angiogenesis and advanced stage of gastric cancer (207). Based on knowledge about increased expression of FGF2 and worse survival of cancer patients, there is an opportunity to develop the personalized anticancer therapy. Some studies showed positive effects *in vitro*, where inhibition of the FGF2/FGFR pathway act as antiangiogenic factor. Several ongoing clinical studies investigate this effect in patients with advanced tumor stage to find out the influence on downregulation of the FGF2/FGFR (204).

Programmed cell death 1 (PD-1) and programmed cell death ligand 1 (PD-L1) axis is a biomarker for different types of cancer, including lung cancer. PD-L1 is expressed on the surface of tumor and antigen-presenting cells like on T-cells, B-cells, dendritic cells, natural killer T-cells, and very seldom on healthy tissues (208). PD-L1 expression is regulated by different pathways, as well as by miRNAs. One of the known regulating pathways is the mitogen-activated protein kinase (MAPK) signaling pathway. MAPK transmits signaling cascades from extracellular to intracellular part of cells, therefore, MAPK regulates many cell processes including proliferation and differentiation (209). MAPK signaling pathway is activated through tyrosine kinases receptor in healthy tissues, but in case of oncogenic mutations, it leads to cancer development. One such mutation is BRAF (209). Many studies described mutated BRAF in numerous cancers, including NSCLC (210). Based on this knowledge, the specific BRAF inhibitor (vemurafenib) was developed and tested from phase I till phase III clinical trials for melanoma therapy (210). Another regulating pathway for PD-L1 is the PI3K/Akt pathway. Activation of this pathway is induced by many cytokines leading to cancer development. Decreasing of MAPK and PI3K/Akt pathways leads to reduced PD-L1 expression (209).

Numerous clinical studies showed that monoclonal antibodies (mAbs) that target PD-L1/PD-1 might avoid the T cell activation, this leading to the better overall survival in patients with cancer like melanoma, renal cell carcinoma (RCC) and NSCLC (211). PD-1 and PD-L1 are used as therapeutic targets in different tumor treatments. The expression of PD-L1 is increased in many types of cancer, and it can correlate with prognosis. Shen et al. summarized results of many studies in the meta-analysis. NSCLC patients were enrolled in the international, multicenter, randomized controlled studies in advanced stage. In four studies patients received nivolumab, in two studies they received pembrolizumab and in two studies atezolizumab. In all cases PD-1 or PD-L1 inhibitor immunotherapy was applied as second line or later treatment,

and some patients, who were enrolled into the KEYNOTE-006 study, received these inhibitors as a first line treatment. There was shown, that PD-1 or PD-L1 inhibitors as second and later lines of treatment can prolong the overall survival in NSCLC patients in comparison to the use of conventional agents like chemotherapy alone. (212).

It has been described that different miRNAs can be involved in the regulation of PD-1 and PD-L1. For example, the expression of PD-L1 was negatively correlated with the expression of miRNA-155-5p in lung cancer patients and upregulation of miRNA-155-5p decreased expression of PD-L1. MiRNA-34 and miRNA-138-5p may also take part in regulation of PD-L1 expression in different cancers (213).

Altogether, pathway analysis shows that target genes of four deregulated miRNAs in our study are involved in cancer development mechanism. More detailed studies of pathways will lead to better understanding of cancer development and growth and will improve the quality of tumor diagnostics and treatment. Our findings within this study can help to improve lung cancer screening strategy and, subsequently, the survival of lung adenocarcinoma patients.

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