

# Diagnostic Utility of PD-L1 and TP-53 mRNA As Well As microRNAs Expression in Liquid Biopsy of NSCLC Patients – Pilot Study

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## Research Article

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## Abstract

**Introduction** The expression of PD-L1 (programed cell death ligand 1) protein on neoplastic cells is a factor qualifying cancer patients for immunotherapy. However, the status of PD-L1 mRNA expression in plasma or serum has not been well studied in cancer patients. TP-53 (Tumor Protein p53) as tumor suppressor affects the expression of many genes, including microRNAs that regulate PD-L1 expression. Purpose of our study was to evaluate usefulness of analysis of PD-L1 mRNA, microRNAs and TP-53 mRNA expression in liquid biopsy in non-invasive diagnosis of non-small cell lung cancer patients (NSCLC).

**Material and methods** We examined the expression the expression of TP-53 and PD-L1 mRNA as well as seven microRNAs (miR-17, miR-93, miR-142, miR-519, miR-526, miR-34a-5p and miR-34a-3p) in plasma obtained from 80 NSCLC patients and 39 healthy volunteers. We used quantitative PCR preceded by reverse transcription method.

**Results** We observed PD-L1 mRNA expression only in 6 (7.5%) NSCLC patients. We showed that expression of TP-53 mRNA and each tested microRNAs were significantly higher in healthy person than in NSCLC patients. Based on the Receiver Operating Characteristic (ROC) analysis, we found that TP-53 mRNA and each of examined microRNAs expression were good diagnostic markers for non-invasive diagnosis of NSCLC. We found a significant, negative correlation between TP-53 mRNA and miR-17 ( $R=-0.45$ ,  $p=0.0005$ ) as well as miR-34a-5p ( $R=-0.32$ ,  $p=0.04$ ) expression in patients with advanced NSCLC.

**Conclusion** The decreased expression of TP-53 mRNA may influence the disruption of microRNAs which could be involved in the regulation of PD-L1 expression in NSCLC patients

## Introduction

There are many studies on the diagnostic value of microRNAs expression in lung cancer [1, 2, 3, 4, 5]. However, there are still no microRNAs expression assessment panels dedicated for the diagnosis and monitoring of non-small cell lung cancer (NSCLC). One of the problems in determining such miR-panels is which microRNAs molecules should be included in it.

The only validated predictive factor in qualification to immunotherapy in the first line of treatment in NSCLC patients is the expression of the PD-L1 (programed cell death ligand 1) molecule on tumor cells [6, 7, 8, 9]. It is not perfect marker. Clinical trials have shown that patients without this expression also benefit from immunotherapy and some with high PD-L1 expression do not respond to immunotherapy. *PD-L1* (CD 274) gene expression, like almost every gene, is regulated by microRNAs [10, 11, 12, 13, 14]. It is important to see if theses regulatory molecules can be useful as a diagnostic tool for disease diagnosis and monitoring of treatment efficacy in NSCLC patients.

Regulating microRNAs-machinery is quite complicated by a network of molecular dependencies. Additional factor as a tumor suppressor TP-53 (Tumor Protein p53) expression could be implemented to the diagnostic panels. Transcription factor TP-53 can affect the expression directly or indirectly of various genes, including genes for *CD274* and different kinds of microRNAs (Fig. 1) [15, 16, 17, 18]. Moreover, microRNAs expression together with TP-53 and PD-L1 mRNA expression could be assessed in liquid biopsy. It would be beneficial for the next diagnostic and treatment points: for diagnosis, decision about the type of treatment and monitoring of therapy efficacy, without the need to obtain tissue by invasive methods.

Taking the above into account, in our pilot studies we assessed the microRNAs expression which regulated *PD-L1* gene function as well as PD-L1 and TP-53 mRNA level to develop a tool for differentiating NSCLC patients from healthy people.

## Material And Methods

### Patients

We enrolled 80 patients with non-small cell lung cancer. Forty-four (55%) patients had adenocarcinoma, 27 (34%) patients had squamous cell carcinoma, 5 (6%) patients had NSCLC NOS (not otherwise specified) and 4 (5%) patients had large cell carcinoma (5%). There were 37 (46%) women and 43 (54%) men. Clinical, demographical, and pathological features of studied group are shown in Table 1. The control group consisted of 39 healthy people (women – 15, man – 24, median age: 59).

Table 1

Clinical, demographical, and pathological characteristic of NSCLC patients and division into groups above and below the median expression of

Feature (n, %)	TP-53 mRNA expression		miR-17 expression		miR-93 expression		miR-142 expression		miR-519 expression		miR-526 expression		miR-3-	
	n (%)													
	Below median	Above median	Below median	Above median	Below median	Above median	Below median	Above median	Below median	Above median	Below median	Above median	Below median	Above median
	50 (63)	30 (37)	42 (53)	38 (47)	40 (50)	40 (50)	46 (58)	34 (42)	49 (61)	31 (39)	40 (50)	40 (50)	43 (54)	
<b>Age</b>	29 (76)	9 (24)	19 (50)	19 (50)	18 (47)	20 (53)	20 (53)	18 (47)	21 (55)	17 (45)	22 (58)	16 (42)	19 (50)	
< 65 (38, 48)	21 (50)	21 (50)	23 (55)	19 (45)	22 (52)	20 (48)	26 (62)	16 (38)	28 (67)	14 (33)	18 (43)	24 (57)	24 (57)	
≥ 65 (42, 52)														
$\chi^2$	<b>5.895</b>		0.181		0.201		0.702		1.093		1.805		0.409	
p-value	<b>0.015</b>		0.670		0.202		0.402		0.296		0.179		0.522	
<b>Gender</b>	30 (70)	13 (30)	20 (47)	23 (53)	25 (58)	18 (42)	20 (47)	23 (53)	26 (60)	17 (40)	22 (51)	21 (49)	22 (51)	
Male (43, 54)	20 (54)	17 (46)	22 (59)	15 (41)	21 (57)	16 (43)	20 (54)	17 (46)	23 (62)	14 (38)	18 (49)	19 (51)	21 (57)	
Female (37, 46)														
$\chi^2$	2.095		1.337		0.016		0.453		0.024		0.05		0.5	
p-value	0.148		0.247		0.9		0.5		0.877		0.823		0.45	
<b>Histopathology</b>	25 (57)	19 (43)	24 (55)	20 (45)	23 (52)	21 (48)	27 (61)	17 (39)	28 (64)	16 (36)	23 (52)	21 (48)	24 (55)	
ADC (44, 55)	19 (70)	8 (30)	14 (52)	13 (48)	13 (48)	14 (52)	16 (59)	11 (41)	16 (59)	11 (41)	13 (48)	14 (52)	15 (56)	
SqSC (27, 34)	3 (60)	2 (40)	2 (40)	3 (60)	3 (60)	2 (40)	2 (40)	3 (60)	3 (60)	2 (40)	2 (40)	3 (60)	2 (40)	
NOS (5, 6)	3 (75)	1 (25)	2 (50)	2 (50)	1 (25)	3 (75)	1 (25)	3 (75)	2 (50)	2 (50)	2 (50)	2 (50)	2 (50)	
LC (4, 5)														
$\chi^2$	1.6		0.402		1.328		2.658		0.367		0.328		0.449	
p-value	0.659		0.940		0.722		0.447		0.947		0.955		0.93	
<b>Stage</b>	14 (56)	11 (44)	14 (56)	11 (44)	11 (44)	14 (56)	14 (56)	11 (44)	14 (56)	11 (44)	17 (68)	8 (32)	15 (60)	
IA-III A (25, 31)	36 (65)	19 (35)	28 (51)	27 (49)	29 (53)	26 (47)	32 (58)	23 (42)	35 (64)	20 (36)	23 (42)	32 (58)	28 (51)	
IIIB-IV (55, 69)														
$\chi^2$	0.656		0.179		0.524		0.033		0.422		<b>4.713</b>		0.571	
p-value	0.418		0.672		0.469		0.856		0.516		<b>0.03</b>		0.45	
<b>Smoking status</b>	5 (71)	2 (29)	4 (57)	3 (43)	3 (43)	4 (57)	4 (57)	3 (43)	3 (43)	4 (57)	3 (43)	4 (57)	2 (29)	
NO (7, 9)	45 (63)	28 (37)	38 (52)	35 (48)	37 (51)	36 (49)	42 (58)	31 (42)	46 (63)	27 (37)	37 (51)	36 (49)	41 (56)	
YES (73, 91)														
$\chi^2$	0.261		0.066		0.157		0		1.093		0.157		1.956	
p-value	0.609		0.797		0.692		1		0.295		0.692		0.162	
<b>Treatment</b>	8 (53)	7 (47)	9 (60)	6 (40)	8 (53)	7 (47)	10 (67)	5 (33)	7 (47)	8 (53)	10 (67)	5 (33)	10 (67)	
TS, TS + CTH (15, 19)	37 (66)	19 (34)	28 (50)	28 (50)	28 (50)	28 (50)	30 (54)	26 (46)	34 (61)	22 (39)	27 (48)	29 (52)	30 (54)	
CTH (56, 70)	5 (55)	4 (45)	5 (55)	4 (45)	4 (45)	5 (55)	6 (67)	3 (33)	8 (89)	1 (11)	3 (33)	6 (67)	3 (33)	
ITH + CTH (9, 11)														
$\chi^2$	1.028		0.512		0.178		1.179		4.248		2.738		2.517	
p-value	0.598		0.774		0.915		0.555		0.119		0.254		0.284	

TS-thoracic surgery; TS + CTH – thoracic surgery and chemotherapy, CTH – chemotherapy, ITH – immunotherapy, ITH + CTH – immunotherapy and chemoth

Feature (n, %)	TP-53 mRNA expression		miR-17 expression		miR-93 expression		miR-142 expression		miR-519 expression		miR-526 expression		miR-34a-3p expression	
	n (%)													
	Below median	Above median	Below median	Above median	Below median	Above median	Below median	Above median	Below median	Above median	Below median	Above median	Below median	Above median
	50 (63)	30 (37)	42 (53)	38 (47)	40 (50)	40 (50)	46 (58)	34 (42)	49 (61)	31 (39)	40 (50)	40 (50)	43 (54)	
Tumor size	29 (58)	21 (42)	22 (44)	28 (56)	22 (44)	28 (56)	25 (50)	25 (50)	29 (58)	21 (42)	24 (48)	26 (52)	22 (44)	
< 5.7 cm (50, 63)	21 (70)	9 (30)	20 (67)	10 (33)	18 (60)	12 (40)	21 (70)	9 (30)	20 (67)	10 (33)	16 (53)	14 (47)	21 (70)	
≥ 5.7 cm (30, 37)														
χ <sup>2</sup>	1.152		<b>3.863</b>		1.92		3.069		0.593		0.213		<b>5.099</b>	
p-value	0.283		<b>0.049</b>		0.166		0.08		0.441		0.644		<b>0.024</b>	

TS-thoracic surgery; TS + CTH – thoracic surgery and chemotherapy, CTH – chemotherapy, ITH – immunotherapy, ITH + CTH – immunotherapy and chemotherapy

## Plasma samples

We collected 80 plasma samples of newly diagnosed patients for whom clinical information about histopathological diagnosis and type of treatment were collected. We took blood in EDTA tubes. Next we centrifuged the blood in the conditions: 1000 rpm for 10 minutes. Then, we obtained the plasma and stored the plasma at -80°C until RNA isolation. Plasma from healthy people was prepared identically.

## Isolation of RNA

RNA isolation was performed using the miRNeasy Serum/Plasma Kit (Qiagen, Germany) which provides isolation of the total RNA along with the microRNAs fraction. Isolation was conducted according to the manufacturer's instructions. The quantity and quality of RNA was checked using BioPhotometer Plus (Eppendorf, Germany).

## Reverse transcription

Reverse transcription reactions were performed in T Personal Biometra thermocycles (Analytik-Jena Company, Germany). mRNA reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), according to the manufacturer instruction. microRNAs were transcribed to cDNA using TaqMan™ Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, USA), according manufacturer instruction. Both cDNA from microRNAs and mRNA was stored in -20°C until the qPCR (quantitative Polymerase Chain Reaction) was performed.

## Quantitative PCR

Real-time PCR was performed on an Illumina Eco device (Illumina Inc, USA). We assessed expression of PD-L1 (Hs00204257\_m1) and TP-53 (Hs010354249\_m1) mRNA as well as seven microRNAs (miR-17, miR-93, miR-142, miR-519, miR-526, miR-34a-5p and miR-34a-3p). GAPDH (Hs03929097\_g1) mRNA was used as an internal control. The manufacturer of the probes used in this study was Applied Biosystems (USA)

Real-time PCR was performed using TaqMan Fast Advanced Master Mix (Applied Biosystems, USA). The 20 µl of PCR mixture included: 10 µl of TaqMan Fast Advanced Master Mix, 1 µl of TaqMan Gene Expression Assay (PD-L1 or TP-53 or GAPDH in separate wells), 5 µl of RNase free water, and 4 µl of cDNA (transcribed from mRNA). Reaction was conducted in conditions: 95 °C for 20 s and 40 cycles: 95 °C for 3 s, 62 °C for 30 s.

Twenty-microliter PCR mixture for microRNAs assesement contained: 10 µl of TaqMan Fast Advanced Master Mix, 1 µl of TaqMan Advanced miRNA Assay (a separate reaction for each microRNAs), 4 µl of RNase free water, and 5 µl of cDNA (transcribed from microRNAs) diluted in 1:10 proportion according to the manufacturer instruction. QPCR was conducted in conditions: 95 °C for 20 s and 40 cycles: 95 °C for 3 s, 60 °C for 30 s.

Analysis was performed using 2<sup>-ΔCt</sup> method.

## Statistical analysis

The analysis was performed in IBM SPSS Statistics, Statistica and MedCalc softwares. The U-Mann-Whitney test was used to assess the differences in the expression between individual groups. Analyzes of ROC (Receive Operating Characteristic) curves with AUC (Area Under the Curve) was used to assess the usefulness of a diagnostic tests of microRNAs and TP-53 mRNA expression analysis in distinguishing NSCLC patients from healthy people. P-value below 0.05 was considered as significant.

Research have been performed in accordance with the Declaration of Helsinki.

## Results

Originally, goal of our research was to discover the correlation between PD-L1 and TP-53 mRNA expression as well as the microRNAs expression in liquid biopsy from NSCLC patients. Unfortunately, we were unable to perform such an analysis because we found PD-L1 mRNA expression in plasma only in 6 (7.5%) NSCLC patients and any in healthy person. All patients with measurable PD-L1 mRNA expression had advanced NSCLC (1 patient in stage IIIB, 5

patients in stage IV). PD-L1 mRNA expression was detected in 3 patients with squamous cell carcinoma and in 3 patients with adenocarcinoma. In subsequent analyses, we focused on the assessment of TP-53 mRNA expression and expression of epigenetic regulators of *PD-L1* gene function.

We observed that TP-53 mRNA expression and every tested microRNAs are significantly higher in healthy persons than in NSCLC patients (Fig. 2). We found no differences between the expression of these factors in patients with the early and advanced NSCLC (Fig. 2).

In performing the ROC analysis, we found that all tested miRNAs and TP-53 mRNA expression were good predictors for disease diagnosis (Fig. 3). In Table 2 is showed the AUC of Youden index, sensitivity, specificity, p-value and 95% confidence interval for each tested factor.

Table 2  
The values of AUC, Youden index, sensitivity, specificity, *p-value* and 95% confidence interval (95% CI) for each tested factor in ROC analysis performed to distinguish NSCLC patients from healthy controls

Expression of different factors	AUC	Cut-off point (Youden index)	Sensitivity	Specificity	<i>p-value</i>	95% CI
TP53 mRNA	0.742	0.116	0.647	1.000	0.009	0.601–0.931
miR-17	0.770	52.72	0.708	0.760	< 0.001	0.678–0.901
miR-93	0.833	73.14	0.667	0.920	< 0.001	0.753–0.934
miR-142	0.738	5.266	0.697	0.783	0.001	0.652–0.874
miR-519	0.775	0.384	0.788	0.810	< 0.001	0.723–0.908
miR-526	0.958	0.105	0.914	0.913	< 0.001	0.903–0.996
miR-34a-5p	0.810	2.220	0.655	0.946	< 0.001	0.673–0.903
miR-34a-3p	0.820	0.006	0.781	0.786	< 0.001	0.652–0.880

We found no relationship between miRNAs and TP-53 mRNA expression in patients with early stages of NSCLC. However, we found a significant, negative correlation between TP-53 mRNA and miR-17 ( $R = -0.45$ ,  $p = 0.0005$ ) and miR-34a-5p ( $R = -0.32$ ,  $p = 0.04$ ) expression in patients with advanced stages of disease (Fig. 4). We also examined the correlation of microRNAs and TP-53 mRNA expression among healthy people. There was an insignificant positive correlation between TP-53 mRNA and miR-17 as well as miR-34a-5p expression ( $R = +0.57$ ,  $p = 0.06$  and  $R = +0.46$ ,  $p = 0.08$  respectively) in control group.

## Discussion

In our pilot study, we showed that the expression of microRNAs which can act as an epigenetic regulators of PD-L1 expression could be a valuable factor for non-invasive diagnosis of NSCLC. Each of the microRNAs showed lower expression in NSCLC patients compared to healthy subjects. However, there are no statistical differences in expression of these molecules in patients with early and advanced NSCLC. We evaluated microRNAs expression in plasma because it is easily obtainable material compared to tumor tissue. It is also advantageous due to the potential use of microRNAs expression assessment in treatment monitoring.

Initially, our assumption was to assess the expression of PD-L1 and TP-53 mRNA as well as microRNAs. Our interest aroused article Tojyo et al. who found the positive correlation between PD-L1 and TP-53 protein expression examined with immunohistochemistry method in tissue of oral squamous cell carcinoma [16]. However, it turned out that we cannot fully achieve our goals due to the fact that PD-L1 expressions were detected in only 6 of NSCLC patients and in any healthy persons. This may be important because predictive factors for immunotherapy are constantly searched and the value of the liquid biopsy used to evaluate PD-L1 mRNA expression is not fully understood. We analysed PD-L1 mRNA expression with the use of probes which flank the end of the third and fourth exons of NM\_001314029.1 (variant 4), NM\_014143.3 (variant 1) and NR\_052005.1 (variant 3) transcripts (product length 77 nt). Exons 3 and 4 encode the intracellular domain of the PD-L1 protein [13]. The fact that we did not detect PD-L1 mRNA expression in plasma from all patients may indicate the need to use other probes, e.g. complementary to the exons encoding the extracellular domain of PD-L1. However, it seems that the expression of PD-L1 mRNA in serum or plasma is very low. This is definitely an issue that should be developed in further studies on a larger group of patients.

Despite the lack of success in examination of PD-L1 mRNA expression, we have made some remarkably interesting observations regarding TP-53 mRNA and microRNAs expression which seem to be good diagnostic factors in the non-invasive diagnosis of NSCLC. Most of the studies on *TP-53* gene concern the identification of mutations in this gene in tumor tissue or in free-circulating DNA (fc-DNA) [19, 20, 21]. To our best knowledge, there are no reports on TP-53 mRNA expression in liquid biopsy and its diagnostic and clinical significance. Our research showed that TP-53 mRNA expression could differentiate NSCLC patients from healthy subjects. The ROC analysis confirmed that a diagnostic test based on examination of TP-53 mRNA expression is significantly sensitive and specific in distinguishing between these two groups. However, our study has some limitations related to low numbers of study groups and further studies should be conducted using the liquid biopsy methods on an enlarged group of NSCLC patients. The expanding the study group to include patients with other types of cancers also seems to be an important aspect, because the reduction of TP-53 protein expression resulting from mutations or chromosomal aberrations may be observed in many types of cancers, including hematological malignancies.

An interesting observation in our study is occurrence of negative correlation between TP-53 mRNA and microRNA-34a-5p expression only in advanced NSCLC patients. Therefore, this dependence can be related to neoplastic transformation and to increase in tumor aggressiveness. Li et al. examined 60 samples of NSCLC tumors and corresponding para-carcinoma tissues. Authors showed that the miR-34a expression in tumor samples was lower than in corresponding

non-tumor tissues [22]. On the other hand, there are reports about elevated expression of miR-34a in liquid biopsy (serum) in breast cancer patients. The expression of miR-34a was similar in patients and healthy controls [23].

We investigated the expression of two mikroRNAs from miR-34a family: miR-34a-5a and miR-34a-3p. During the formation of microRNAs, mature molecules temporarily form into a double-stranded form, in which one of the strands is called the leading strand and the other is the passenger strand. For some time, only the leader strand was considered to be capable of post-transcriptional silencing, however studies have shown that the passenger strand (usually at the 3' end) is also active. Then, miR-34a-3p is sometimes described as microRNAs from the passenger strand. Expression of miR-34a-3p was a significantly higher in tumors of non-muscle-invasive bladder cancer patients without recurrence after surgery and decreased expression of miR-34a-3p was associated with significantly shorter recurrence-free survival [24]. Fawzy et al. during functional enrichment pathway analysis of miR-34a target genes indicated that both miR-34a were involved in fatty acid biosynthesis and fatty acid metabolism. However, miR-34a-5p was also involved in tumor suppressor or oncogenes function, and may play a role in development of colorectal cancer, thyroid cancer, NSCLC, chronic myeloid leukemia, bladder cancer, pancreatic cancer, glioma, and melanoma. Fawzy et al. conducted their study in tissue of colorectal cancer [25].

Furthermore, we demonstrated reduced expression of miR-17 in plasma from NSCLC patients compared to healthy donors. Jurkovicova et al. showed that the expression of miR-17 in liquid biopsy is similar in non-invasive breast cancer patients and in healthy subjects. Whereas, expression of this molecule is significantly reduced in invasive breast cancer patients compared to healthy volunteers. Moreover, expression of miR-17 is gradually decreased with the decrease in the grade of tumor differentiation [26]. Zeng and colleagues showed that the expression of this microRNA is significantly reduced in patients with benign gastric diseases or with gastric cancer (GC) compared to healthy people. They found that miR-17 can distinguished healthy person and GC patients with AUC of 0.879 and with 80.6% of sensitivity as well as 87.5% of specificity [27]. Hetta et al. showed results different from ours in a group of 40 NSCLC patients and 20 healthy controls. They indicated that miR-17 were significantly upregulated in NSCLC patients [28]. It should be noted that our study group was twice as large as the group analyzed by Hetta et al. It also indicates the need to continue studies with liquid biopsy in a much larger population of patients.

Results of our study showed that level of every analysed microRNA was decreased in plasma of cancer patients compared to healthy donors. It could be assumed that all these molecules play the role of tumor suppressors. However, at least Jurkovicowa indicated that traditional oncomiR with high expression tumor tissue could have low expression in cancer patients' plasma (this may concern e.g. miR-17 expression) [26]. The concentration of microRNAs in serum or plasma may depend on the stage of the disease, the grade of tumor differentiation, the increase in cancer cell apoptosis or the efficiency of the mechanisms of microRNAs secretion into the bloodstream. Thus, the expression of the microRNAs in the tumor tissue will not always be faithfully reflected in the serum or plasma microRNAs expression profile. Nevertheless, the profile of microRNAs expression in liquid biopsy may accurately detect early neoplastic lesions undetectable in imaging diagnostics or serve as a marker of response to treatment or prognosis of the disease course.

miR-93 control the EMT (epithelial to mesenchymal transition) and is overexpressed in several cancers. In the other hand, it has been found that miR-93 inhibited cell proliferation and cell cycle, which suppresses tumor growth and enhanced the therapeutic effect of paclitaxel in cell cultures [29]. There are evidence that miR-93 can acts as a tumor suppressor or oncomiR by enhancing tumor growth, angiogenesis and metastasis [30, 31, 32, 33]. Wang et al. found significantly reduced expression of miRNA-93 in plasma from 55 colorectal cancer patients compared to healthy subjects (57 cases) with AUC = 0.739 for microarray test and AUC = 0.828 for qRT-PCR method of microRNAs assessment [34]. In our study, AUC was 0.833 for qRT-PCR test evaluated this microRNA and it was similar to observation of Wang et al. Therefore, the microRNAs expression and hence the ROC analysis may vary depending on the research method. Ono et al. noted that the results of microRNAs expression analysis and it possible usefulness in diagnostic tests were influenced by many factors, including the testing method [35]. They analysed different types of cancer, including lung, head and neck, liver, stomach, kidney, breast, melanoma and myeloma. They considered the following factors affecting the expression of microRNAs: type of anticoagulant used in blood collection, quantity and quality of RNA, type of microRNA isolation method, and finally research methods, including qRT-PCR, Affymetrix GeneChip miRNA Arrays 4.0, Agilent Oligonucleotides Microarrays, Exiqon miRCURY LNA microRNA arrays,  $\mu$ Paridiclo® Technology, 3D-Gene® and Next-Generation Sequencing (NGS). They found that relatively easy and simple qRT-PCR method is the best method for microRNAs testing with high sensitivity, specificity and accuracy as well as highly reproducible results. One disadvantage of this method was the inability to detect new and unknown microRNAs, which was possible with NGS. However, NGS is still more expensive, complicated and less available compared to the qRT-PCR technique [35].

Kanaan et al. indicated that plasma microRNAs are reliable, non-invasive, and inexpensive markers for colorectal adenomas. Panel of eight microRNAs in liquid biopsy, including miR-142, distinguished polyps from controls with high accuracy with AUC of 0.868. They studied 380 plasma-miRNAs expression using microfluidic array technology in 12 healthy people, 9 patients with colorectal adenomas and 20 patients with colorectal cancer (CRC) [36]. The studied groups were not very numerous, however, microarray analysis is expensive. Moreover, the analysis of 380 microRNAs was a great achievement, especially that there were molecules with diagnostic potential, including miR-142. Gao et al. also drew attention to this microRNA. They testing serum from 363 CRC patients and 156 healthy controls [37]. They found that expression of miR-142 was significantly lower in CRC patients than in healthy volunteers witch AUC of 0.74. Moreover, they proved that low miR-142 expression was associated with a worse prognosis for colorectal cancer patients qualified for surgery [37]. Liu observed that in lung cancer tissue miR-142 expression is reduced. Authors suggested that this molecule acts as a tumor suppressor and promotes NSCLC progression via MALAT1/ $\beta$ -catenin (Metastasis Associated Lung Adenocarcinoma Transcript 1) signalling pathway [38].

There is no evidence in literature, that circulated miR-519 and miR-526 are the markers of non-invasive cancer detection. Nevertheless, expression of miR-519 was low in early breast cancer tissue compared to normal adjacent tissue [39]. Nong et al. indicated that miR-519 directly regulates PD-L1 expression. Application of miR-519 inhibited invasiveness and tumor growth as well as induced apoptosis of pancreatic cancer in a mouse model [40].

In cancer diagnostic panels, not one microRNA but many microRNAs should be used. Disturbances in the expression of various microRNAs are observed in various types of cancer. Therefore, the selection of microRNAs for diagnostic panels is difficult, requires in-depth and extensive studies and must be very

precise. We focused on those that could be related to *PD-L1* gene expression and our results showed that it was the right choice. Our study indicated that seven microRNAs could be used for non-invasive lung cancer diagnosis. Kanaan et al. showed that 8 microRNAs out of 380 molecules may have a diagnostic value in neoplastic diseases [36]. However, their choice became possible after analysis of broad microRNAs panel. Our selection of microRNAs, that potentially regulate PD-L1 expression, is related to the role of PD-L1 in tumor progression. The tumor cells with PD-L1 expression have the ability to escape immune surveillance. This property of PD-L1-positive tumor cells is observed not only in lung cancer but also in other immunogenic cancers such as colon, kidney, stomach, pancreatic cancer. Unfortunately, our study failed to determine an expression of PD-L1 mRNA in liquid biopsy and the existence of a correlation between PD-L1 mRNA and microRNAs expression has not been confirmed. The detection method of mRNA expression analysis should be enhanced, e.g. by PCR pre-amplification method. Further research in this area seems very important.

## Conclusion

To summarise, we would like to indicate that we do not observed PD-L1 mRNA expression in plasma from all NSCLC patients. However, we have made remarkably interesting observations regarding epigenetic regulators of this molecule. In addition, we turned out attention to the TP-53, which was somewhat overlooked in clinical trials due to the lack of therapies targeting this molecule. Nevertheless, more and more reports indicated that, in addition to mutations in *TP-53* gene detected in tumor cells, the TP-53 mRNA and protein expression should also be assessed in cancer patients. The decreased expression of TP-53 may influence the appearance of driver mutations in cancer cells as well as microRNAs disruption [41, 42].

## List Of Abbreviations

**PD-L1** - programmed cell death ligand 1

**TP-53** - Tumor Protein p53

**NSCLC** - non-small cell lung cancer patients

**qPCR** - quantitative Polymerase Chain Reaction

**ROC** - Receive Operating Characteristic

**AUC** - Area Under the Curve

**fc-DNA** - free-circulating DNA

**GC** - gastric cancer

**EMT** - epithelial to mesenchymal transition

**NGS** - Next-Generation Sequencing

**qRT-PCR** – quantitative reverse transcription PCR

**CRC** - colorectal cancer

**MALAT1** - Metastasis Associated Lung Adenocarcinoma Transcript 1

## Declarations

### Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Medical University of Lublin, Poland (No. KE-0254/95/2018) and informed consent was obtained.

### Consent for publication

Not applicable.

### Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.

### Funding

There are no special funding for this publication.

### Authors' contributions

AG wrote the manuscript

BKK collected samples and clinical data

NK prepared data base for analysis

MJ performed a statistical analysis

PK made a critical review

HBG, JM made a critical review

All authors read and approved the final manuscript.

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## Figures

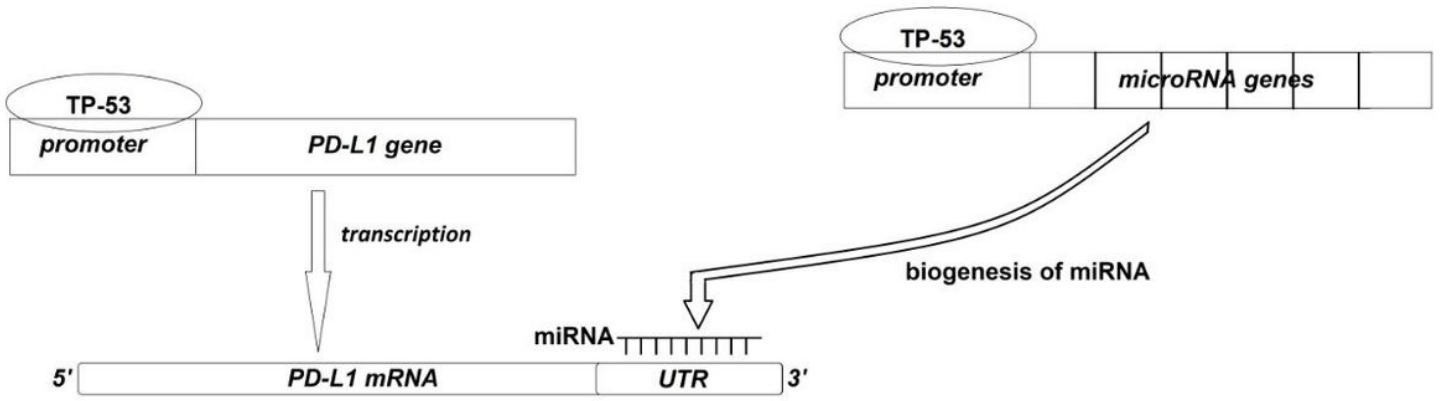


Figure 1

Schematic diagram of the axis of relationship between microRNA, TP-53 and PD-L1

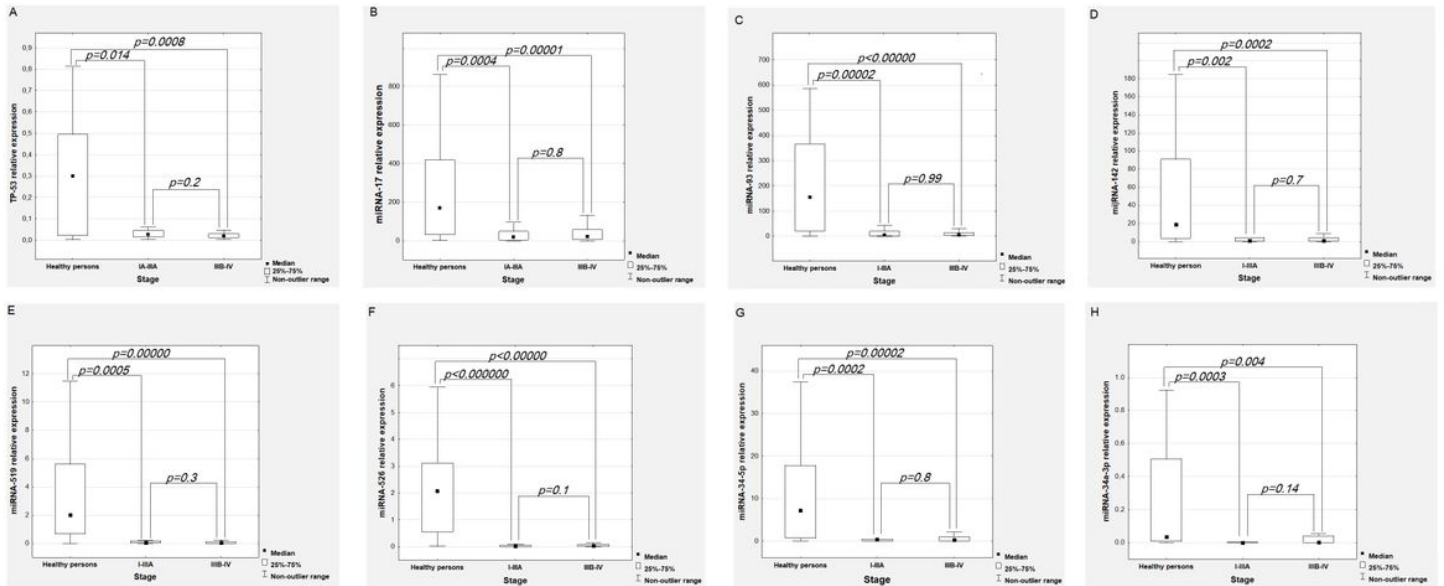


Figure 2

Differences of TP-53 mRNA (A) and microRNAs (B-H) expression in NSCLC patients with early and advanced stages of NSCLC compared to healthy persons

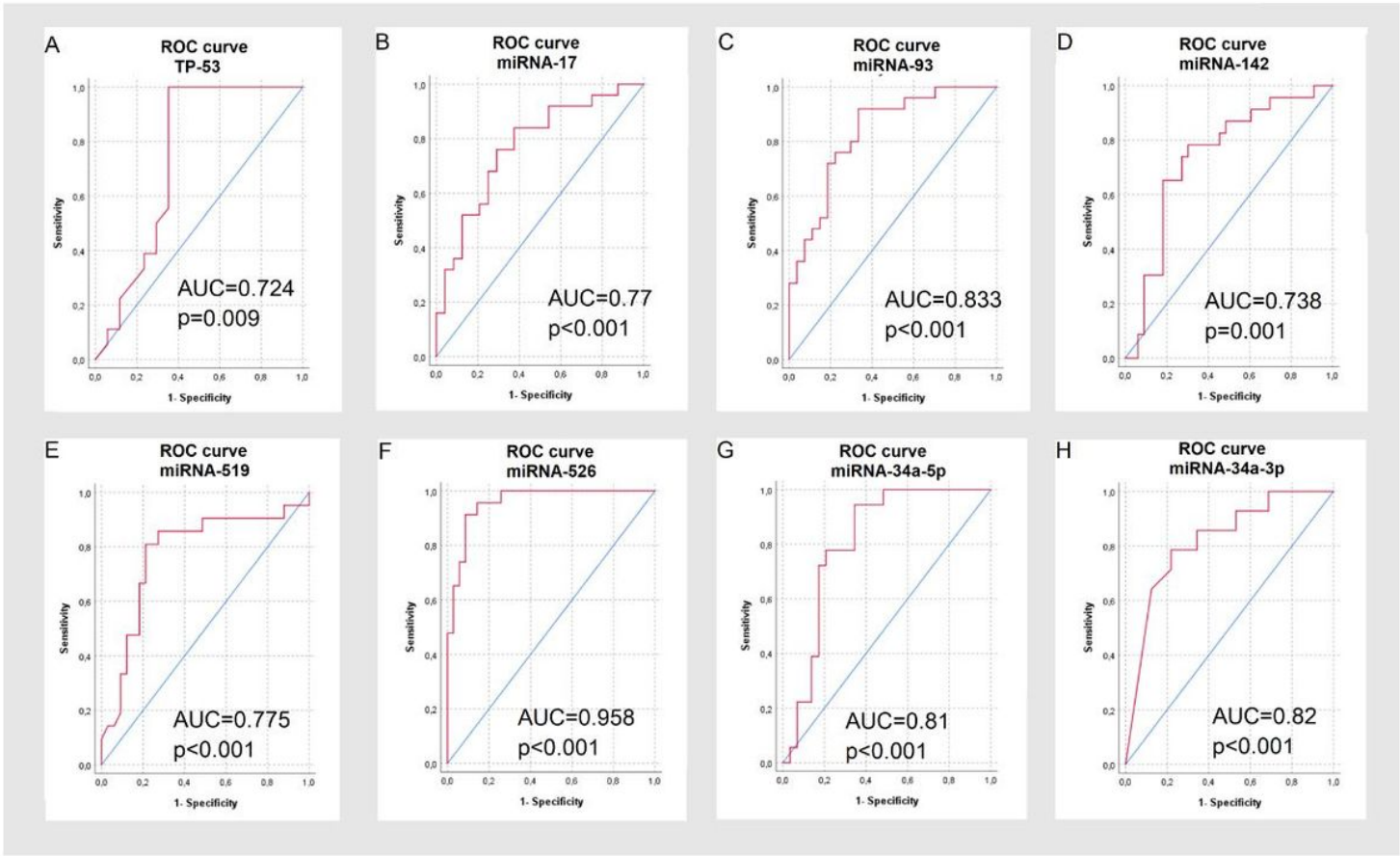


Figure 3

ROC analysis performed to distinguish NSCLC patients from healthy controls

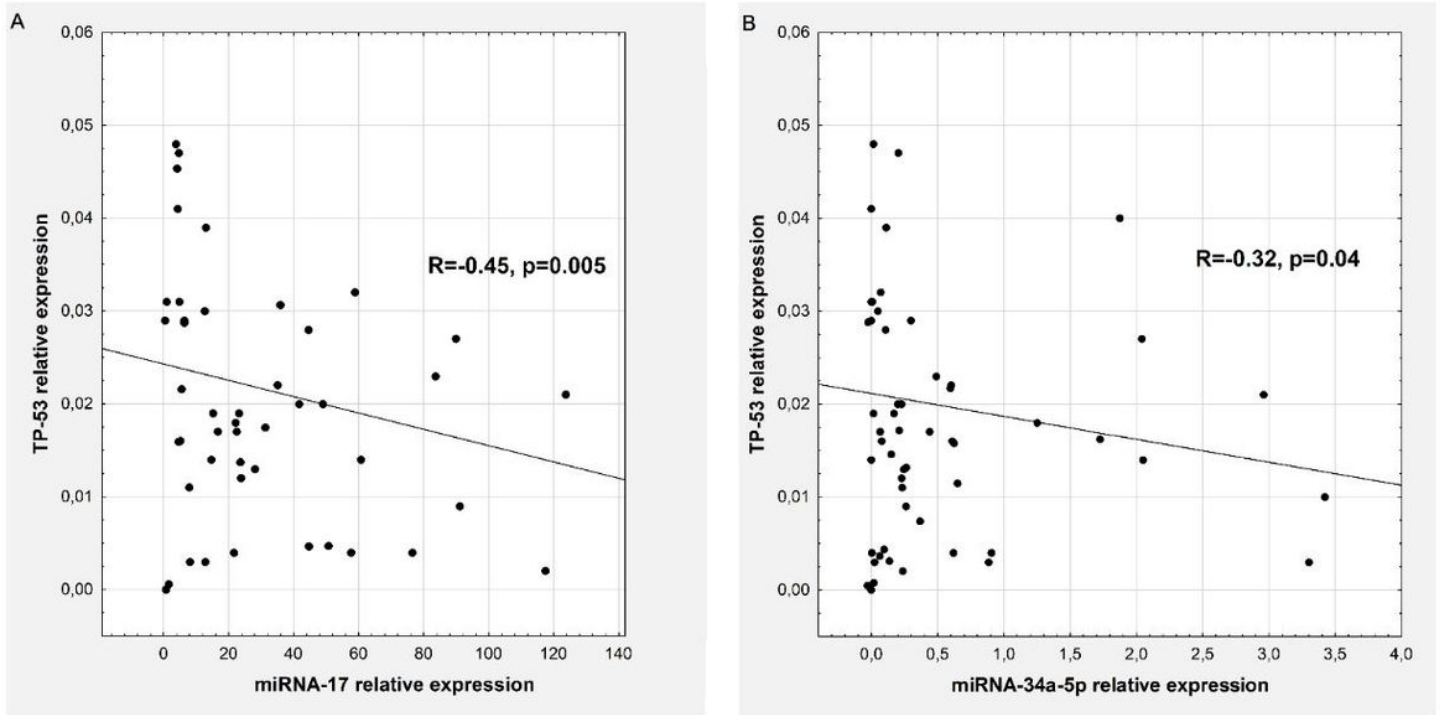


Figure 4

