



RNA DIAGON

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D2.1 Exosomal small ncRNA profile from blood plasma/serum samples

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STUDY 1 PLASMA EXOSOME MIRNA PROFILING FROM NSCLC PATIENTS SAMPLES

1. INTRODUCTION

Lung cancer remains one of the leading causes of cancer-related deaths worldwide, according to GLOBOCAN 2020 [1]. Lung cancer is classified into two main histological groups, non-small cell lung cancer (NSCLC) and small cell lung cancer. NSCLC, which includes adenocarcinoma, squamous cell carcinoma and large cell carcinoma, accounts for over 80% of all lung cancer cases. Recent therapies have improved the prognosis of lung cancer patients, but the 5-year survival remains below 20%, which can be explained by the lack of adequate screening and early diagnosis methods [2]. Improving the outcome of cancer patients would be possible through enhancing the early diagnosis, preferentially with minimally invasive methods.

Exosomes are small vesicles (30–100 nm in size) secreted by most cells into biological fluids, such as plasma, saliva, urine and others, and their contents reflect, to a high degree, the tumor profile, giving vital information regarding cancer development and progression [3]. Via this cell-to-cell communication, exosomes transfer their contents, including miRNAs, RNA, DNA, proteins, and lipids and are involved in many pathological processes, including cancer [4]. Many explored the role of circulating exosomes and exosomal miRNAs in initiating and developing various diseases, including cancer [5].

MicroRNAs (miRNAs) are a class of non-coding RNAs with a length of 21–25 nucleotides involved in regulating gene transcription and translation. Dysregulation of miRNAs is frequently observed in cancer, including NSCLC and is involved in tumor cell proliferation, local invasion, and metastasis. MiRNAs can be evaluated in normal adjacent or tumor tissue or biofluids, making these molecules a promising diagnostic tool. The most frequently upregulated miRNAs in lung cancer (oncomiRNAs) are miR-21, miR-155, miR-30d, and miR-17. In contrast, the most common downregulated miRNA is miR-181a, providing a rationale for evaluating these molecules as diagnostic or prognostic markers in lung cancer patients [6-8].

MiR-21 was identified as a potential circulating biomarker for different cancers. Recent studies described miR-21 overexpression in breast cancer patients as associated with a poor progression-free survival [9]. In NSCLC, miR-21 promotes tumorigenesis by inhibiting negative regulators of the Ras/MEK/ERK signaling pathway [10] and stimulates cell growth and invasion by inhibiting tumor suppressor PTEN [11, 12]. The expression of miR-21 is increased and is associated with poor prognosis in NSCLC. Moreover, a meta-analysis study demonstrated that circulating miR-21 modified levels can be valuable biomarkers of progression and diagnosis in gastric cancer patients [13]. In prostate cancer patients, it was demonstrated that miR-181a, alongside other miRNAs, can be used to separate patients based on the aggressive/nonaggressive types of prostate cancer [14].

Further studies demonstrated that low plasma levels of exosomal miR-181a-5p were associated with organ-invasive primary tumor in colorectal cancer [15]. A previous meta-analysis published by our group indicated that miR-181 downregulation is associated with poor outcomes in lung cancer patients [16]. High circulating miR-155 expression levels were observed in breast cancer patients. This overexpression was significantly diminished after a completed surgery and chemotherapy, and

the study demonstrated that circulating miR-155 can represent diagnostic and therapeutic monitoring markers in the breast cancer [17]. This transcript controls metastasis and cancer progression via TGF- β /SMAD4 signaling [18]. Several reports also indicate that exosomal expression of miR-155 is involved in promoting metastasis in NSCLC [19] and colorectal cancer [20, 21].

In our study, we evaluated the expression levels of a 3miRNA panel including miR-21-5p, miR-155-5p, and miR-181a-5p in normal and tumor tissue combined with their analysis in plasma and plasma exosomes for identifying a panel of miRNAs that could serve as novel biomarkers for the diagnosis of lung cancer. We decided to focus our study on the male population because the cohort of LUSC patients established from Hospitals in Romania mainly consisted of male patients. Therefore, we chose to further investigate the molecular aspects of lung cancer development in male patients.

2. MATERIAL AND METHODS

2.1 Patient selection

Our study included 61 male patients ≥ 18 years of age with a diagnosis of LUSC who presented in the Bronchoscopy Department of the Leon Daniello Pneumology Hospital in Cluj-Napoca, Romania, with suspicion of endobronchial lung cancer at imaging testing (computer tomography or positron emission computed tomography). In addition, 28 healthy male donors were included in the study to evaluate the transcripts in plasma and plasma-derived exosomes.

The study was conducted following the Declaration of Helsinki, and all patients and healthy subjects enrolled in the study were informed about the inclusion and exclusion criteria. They signed the informed consent approved by the hospital and the institutional ethics committee of Leon Daniello Pulmonology Hospital, Cluj-Napoca, Romania no. 264/26.06.2018 and the Ethical Committee of Iuliu Hatieganu University of Medicine and Pharmacy no. 438/24.11.2016. The study cohort comprised 61 patients, from which matched pairs of TT/TN, plasma, and plasma exosome samples was analyzed. Ten out of the 61 patients were common in all three types of samples. This study also assessed the TCGA LUSC dataset (n=279) that included data from male patients (<https://www.cancer.gov/tcga>). The patient's characteristics are detailed in Table 1.

Table 1. Lung squamous cell carcinoma (LUSC) patient cohorts were included in the study.

Cohorts		TCGA LUSC cohort (n=279)	Study cohort (n=61)
		No. of male patients (%)	No. of male patients (%)
Age	<50	10 (3.6)	1 (1.6)
	50-59	34 (12.2)	
	60-69	102 (36.6)	14 (23)
	70-79	107 (38.3)	
	>80	22 (7.9)	23 (37.7)
	unknown	4 (1.4)	17 (27.9)

			6 (9.8)
			0 (0)
Sex	M	279 (100)	61 (100)
T	T1 T2 T3 T4	54 (19.3) 169 (60.6) 47 (16.9) 9 (3.2)	0 (0) 7 (11.5) 16 (26.2) 38 (62.3)
N	N0 N1 N2 Nx/N3	186 (66.7) 67 (24) 20 (7.2) 6 (2.1)	8 (13.1) 4 (6.6) 41 (67.2) 8 (13.1)
M	M0 M1 Mx	209 (74.9) 4 (1.4) 66 (23.7)	36 (59) 25 (41) 0 (0)
Stage	I II III IV	130 (46.6) 105 (37.7) 40 (14.3) 4 (1.4)	0 (0) 3 (4.9) 34 (55.7) 24 (39.4)
Smoking status	Never smoker Current smoker Former smoker unknown	5 (1.8) 94 (33.7) 172 (61.6) 8 (2.9)	4 (6.6) 35 (57.4) 22 (36) 0 (0)

2.2 Sample collection and preparation

The tumor tissue samples were collected using bronchoscopy with endobronchial biopsy, performed under local anaesthesia. From each patient, normal tissue was collected from the contralateral, healthy lungs.

From the selected patients and healthy donors, four ml of peripheral whole-blood samples were collected, and the plasma samples were obtained by centrifugation of the peripheral blood at 4200 rpm for 10 min at room temperature. Exosomes from plasma samples were obtained using the ultra-centrifugation protocol. One ml plasma was centrifuged at $2,000 \times g$ for 20 minutes at $4^\circ C$ followed by another centrifugation at $10,000 \times g$ for 30 minutes at $4^\circ C$ to remove plasma debris; the remaining plasma was ultracentrifuged at $100,000 \times g$ for 70 minutes to pellet exosomes. After ultracentrifugation, the exosomes pellet was eluted in 500 μl PBS 1X.

2.3 RNA extraction

Total RNA was extracted and isolated from 40 NSCLC plasma samples and 28 plasma samples from healthy donors using the Plasma/Serum Circulating and Exosomal RNA Purification Kit - Norgen according to the manufacturer's instructions. The same extraction kit was used for 30 plasma exosome samples from NSCLC patients and 20 plasma samples from healthy controls. Total RNA was isolated from 40 matched paired tissue samples (tumor and adjacent normal tissue) using Tri reagent (Thermo Fisher Scientific) and following the steps described in the manufacturer's protocol. RNA concentration and quality were evaluated using a NanoDrop-2000C spectrophotometer. The concentration ranged from 25 ng/ μl (in plasma and exosomes samples) to 50 ng/ μl (in tissue samples).

2.4 Quantitative reverse transcription PCR (qRT-PCR) analysis

Three miRNAs (miR-21-5p, miR-181a-5p, and miR-155-5p) were selected for assessing their expression level profile in plasma, tissue, and exosome by qRT-PCR. These miRNAs were investigated in a.) 40 LUSC fresh frozen matched paired tissue samples (TT/TN), b.) their plasma samples and plasma from 28 healthy controls, and c.) 30 LUSC patients and 20 healthy control plasma for exosomes.

The cDNA synthesis was performed using a 7.5 μl of reverse transcription mixture containing 0.72 μl of RT primer, 50 ng of total RNA and 0.5 μl of MultiScribe Reverse Transcriptase, 0.75 μl Reverse Transcription Buffer (10 \times), 0.075 μl dNTPs (100 mM), 0.1 μl of RNase Inhibitor according to TaqMan MicroRNA reverse Transcription Kit (Applied Biosystems) protocol. The cDNA mixture was incubated in PCR tubes at $16^\circ C$ 30 min, $42^\circ C$ 30 min, and $85^\circ C$ 5 min.

qRT-PCR was performed in a total volume of 10 μl using 5 μl of cDNA (diluted 1:3 with nuclease-free water), 4.53 TaqMan Fast Advanced Master Mix (Applied Biosystems) and 0.47 μl primer for each miRNA in ViiA7 (Applied Biosystems) PCR machine. The reactions were set up as follows: the initial step included the UNG incubation at $50^\circ C$ for two minutes and polymerase activation at $95^\circ C$ for the 20s, followed by 40 cycles of $95^\circ C$ for 1s (denature), $60^\circ C$ (Anneal/extend) for 20s. The sequences for the miRNA primers are listed in the table (Table 2).

Table 2. MiRNA assays were used in the study.

Assay Name	Assay ID	miRNA Sequence
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hsa-miR-21-5p	000397	UAGCUUAUCAGACUGAUGUUGA
hsa-miR-155-5p	002623	UUA AUGCUAAUCGUGAUAGGGGU
hsa-miR-181a-5p	000480	AACAUUCAACGCUGUCGGUGAGU
has-miR-16-5p	000391	UAGCAGCACGUAAAUAUUGGCG
U6 snRNA	001973	GTGCTCGCTTCGGCAGCACATATACTAAAATTGG AACGATACAGAGAAGATTAGCATGGCCCCTGCGC AAGGATGACACGCAAATTCGTGAAGCGTTCCATA TTT
RNU48	001006	GATGACCCCAGGTA ACTCTGAGTGTGTCGCTGAT GCCATCACCGCAGCGCTCTGACC

2.5 Statistical analysis

The expression level for each miRNA was calculated using the $2^{-\Delta\Delta C_t}$ method; U6, RNU48 and miR-16 were used for normalization; $p < 0.05$ was considered statistically significant. Additionally, a ROC (receiver operating characteristic) graphical representation was performed to assess the sensitivity and specificity of each evaluated transcript at plasma, tissue and exosome levels using GraphPad Prism (<https://www.graphpad.com/>, Version 9), and the combined ROC curves were generated using the CombiROC online tool [22]. Expression level plots for miRNAs in TT vs TN samples and heatmaps were performed in R (version 4.2.1) using *ggplot* and *pheatmap* packages.

3. RESULTS

3.1 Extracellular vesicles isolation and characterization

EVs isolated from the plasma of 30 lung cancer patients and 20 healthy donors were characterized. NanoSight and Transmission Electron Microscopy analysis of exosome samples showed a proper size, concentration distribution and specific disc-like structures. The exosome size ranged from 60–80 nm (Fig. 1A), and the edges of the structures were clear and light, with concentrated staining in the central area (Fig. 1B).

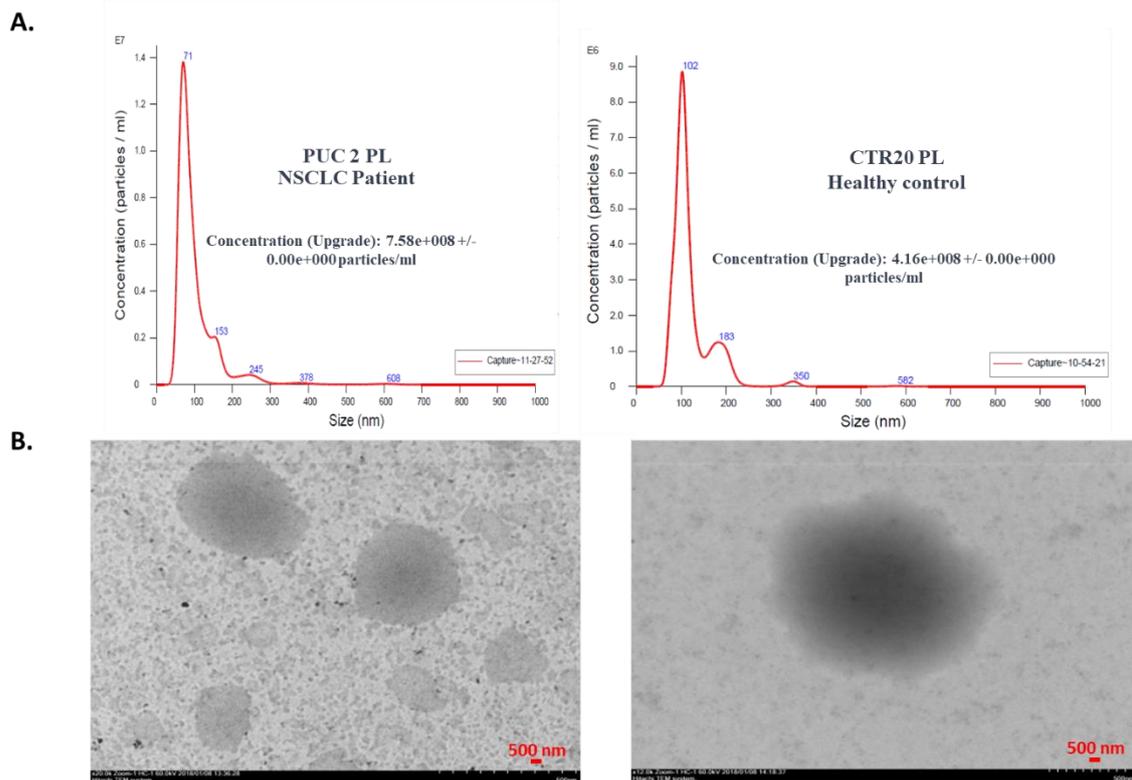


Figure 1. Lung cancer patients' plasma extracellular vesicle size characterization by Nanosight (A) and Transmission electron microscopic (TEM) visualization. (B)

3.2 Expression levels of miR-21-5p, miR-181a-5p, and miR-155-5p in LUSC study cohort

We tested the differential expressions of miR-21-5p, miR-181a-5p, and miR-155-5p in tissue, plasma and EVs of LUSC patients and controls.

Matched pair tissue samples from male LUSC (n=40) patients were used for miRNA expression analysis. All three miRNAs, miR-21-5p, miR-181a-5p, and miR-155-5p, showed statistically significant differential expressions between the tumor tissue (TT) compared with the adjacent normal lung tissue (TN) (log fold change > 1 and $P < 0.05$). MiR-21-5p and miR-155-5p are significantly overexpressed, while miR-181a-5p is underexpressed in LUSC TT versus TN (Fig. 2A).

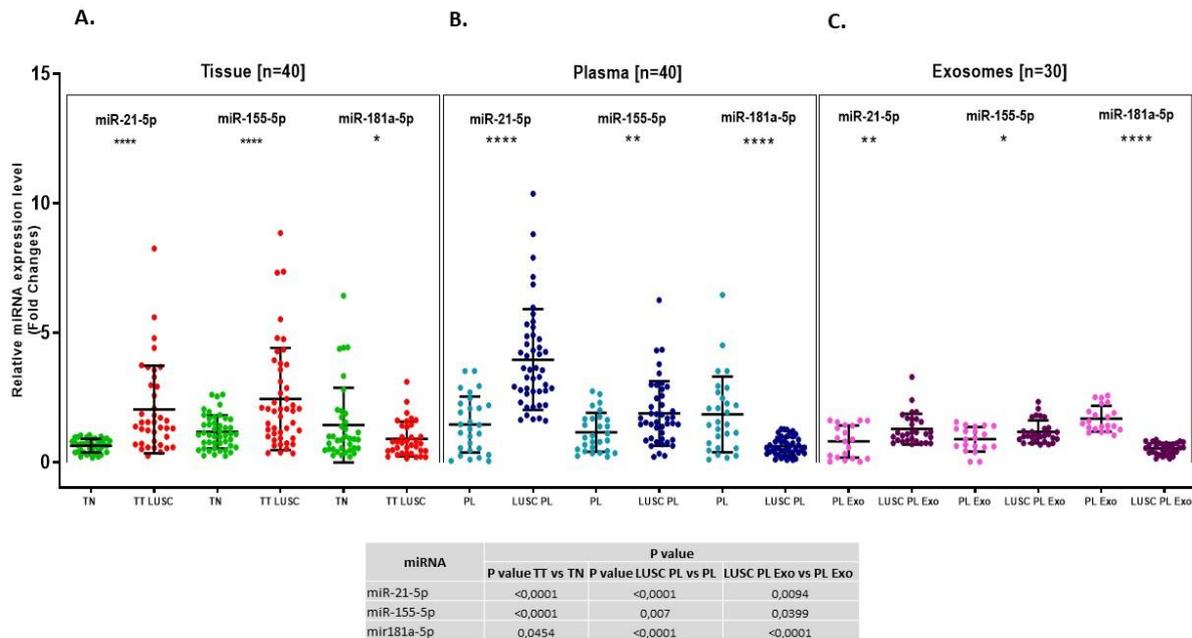


Figure 2. MiR-21-5p, miR-155-5p and MiR181a expression levels in LUSC tissue (A), LUSC plasma (B) and LUSC plasma EVs (C).

To confirm the abundance of the three miRNAs, we quantified the miRNA in plasma samples from 40 male LUSC patients comparing the expression level with the plasma profile from 28 healthy subjects. All three miRNAs, miR-21-5p, miR-181a-5p, and miR-155-5p, confirmed the levels observed in the tissue samples (log fold change > 1 and $P < 0.05$). MiR-21-5p and miR-155-5p are increased, while miR-181a-5p decreases in LUSC plasma versus plasma from healthy controls. (Fig. 2B)

Additional validation was performed by investigating the levels of the three miRNAs in plasma EVs. We assessed the levels in EV samples from 30 male LUSC patients versus exosome miRNA expression from 20 healthy subjects' plasma. We observed the same expression levels of the three miRNAs, miR-21-5p and miR-155-5p are most abundant. In comparison, miR-181a-5p is less abundant in LUSC EVs versus normal plasma EVs (log fold change > 1 and $P < 0.05$) (Fig. 2C). When we compared the levels of the three miRNAs in plasma and exosomes samples from the same patients, we found that the trends of overexpression/underexpression of these miRNAs in plasma are also maintained in exosomes samples across LUSC patients (Fig. 3).

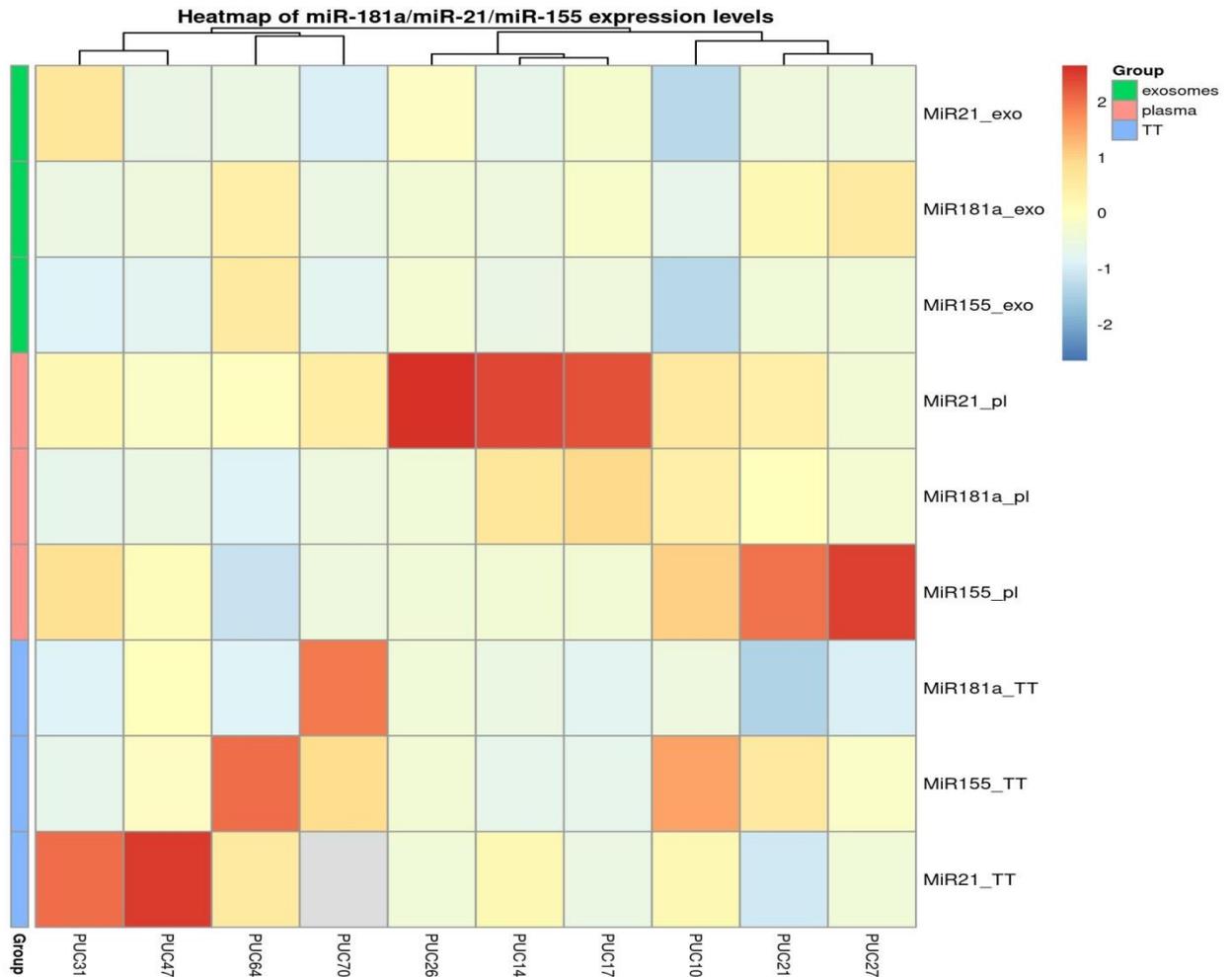


Figure 3. Heatmap of expression levels (FC) of the three miRNAs in TT, plasma, and plasma exosomes from common LUSC patients.

3.1 Expression levels of miR-21-5p, miR-181a-5p, and miR-155-5p in TCGA LUSC datasets

We validated the TCGA LUSC datasets, including only samples from male patients in the analysis. As observed in Table 1, most of the samples were from patients diagnosed in stages I and II, 235 from a total of 279, while stages III and IV were only 44 (Table 1). The analysis of the expression levels of the three miRNAs in the LUSC TCGA datasets identified miR-21-5p, miR-155-5p, and miR-181a-5p dysregulated in lung cancer. The expression levels of miR-21-5p and miR-155-5p are significantly elevated, while miR-181a-5p is reduced in TT compared to TN, considering all stages (Figure 4). These differences are already detectable in stages I+II TT vs TN (Figure 4A) and stages III+IV TT vs TN (Figure 4B), indicating that the expression levels of the three miRNAs are constantly altered in both early- and late-stage LUSC (Figure 4C).

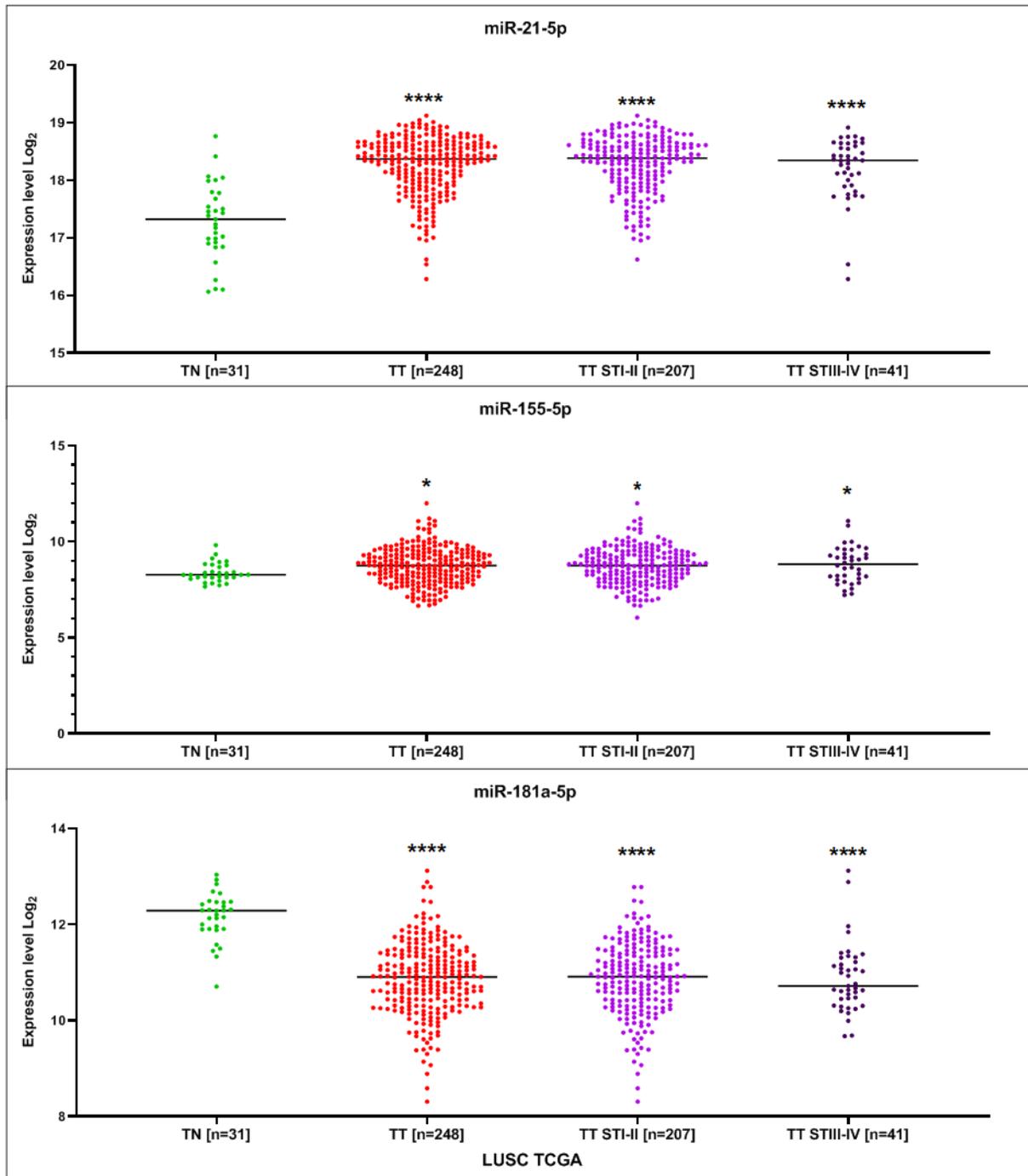


Figure 4. Expression levels of miR-21-5p, miR-155-5p, and miR-181a-5p in TCGA LUSC datasets (male only); A) Expression levels of miR-21-5p, miR-155-5p, and miR-181a-5p in stages I+II lung tumor tissue (TT) vs normal lung tissue (TN); B) Expression levels of miR-21-5p, miR-155-5p, and miR-181a-5p in stages III+IV lung tumor tissue (TT) vs normal lung tissue (TN); C) Expression levels of miR-21-5p, miR-155-5p, and miR-181a-5p in lung tumor tissue (TT) vs normal lung tissue (TN).

3.4 Sensitivity and specificity of miR-21-5p, miR-181a-5p, and miR-155-5p as biomarkers

A ROC curve analysis was performed to evaluate the utility of these miRNAs in lung cancer diagnostics. Figure 5 shows the AUCs, cutoff values, specificity, and sensitivity of the three

miRNAs independently. While miR-21-5p alone recorded a satisfactory AUC (0.8-1) in each sample type, miR-181a-5p and miR-155-5p showed fair and poor AUCs between 0.5 and 0.7, respectively, except miR-181a-5p expression in plasma exosomes, where the AUC=1, indicating miR-181a expression in plasma exosomes has high predictive accuracy for LUSC patients. On the other hand, the combi-roc analysis revealed combinations of these miRNAs with high predictive accuracy for NSCLC. In plasma samples, miR-155-5p+miR-21-5p and miR-155-5p+miR-181a-5p had higher AUC (0.88) than the one obtained on individual transcripts (Fig. 5ABC).

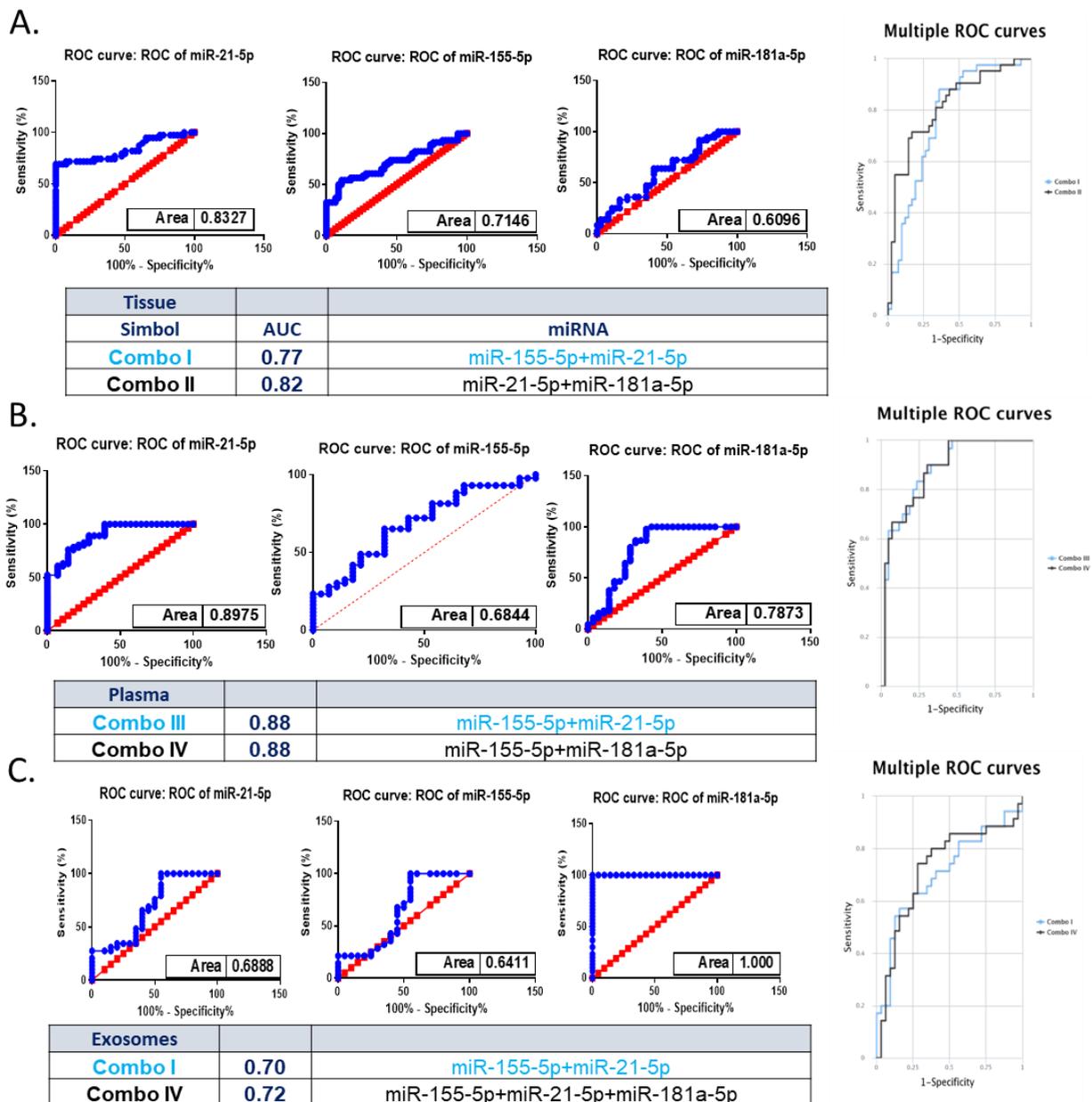


Figure 5. ROC curves analysis using GraphPad Prism and combined ROC curves using the CombiROC online tool for miR-21-5p, miR-155-5p and miR-181a-5p for tissue (A), plasma (B) and exosomes (C).

4. DISCUSSION

Aberrant miRNA expression is considered an essential biomarker for diagnosing, prognosis and predicting therapy resistance in NSCLC. MiRNAs could be exploited as biomarkers in cancer patients due to their stability in various specimen types, high reproducibility, and the availability of assays that can assess their levels, such as qRT-PCR or microarray. Several recent studies on lung cancer patients have suggested finding a valuable miRNA signature for early diagnosis in this pathology [23, 24]. Many studies have been conducted on lung cancer patient's tumor tissue, serum or plasma. Because obtaining tumor tissue represents a challenge, it is essential to find minimally invasive methods with high sensibility and sensitivity to be used either for early diagnosis or to improve the survival of lung cancer patients.

Exosomes are membrane-bound, nano-sized extracellular vesicles released by cells in eukaryotic organisms. Their cargo includes DNA, RNA, proteins, and lipids. Their stability in different biological fluids, proficiency to pass over cell barriers, biocompatibility, and affinity for target cells, make these vesicles useful in various cancer applications, such as drug delivery and immunotherapy [25]. Many miRNAs that are altered in lung cancer can be detected in extracellular vesicles, thus supporting their role as prognostic and diagnostic tools for this malignancy [26-28].

This study aimed to provide new insights into NSCLC biomarkers by analysing the expression levels of the same three miRNAs in different biological samples: tissue, plasma, and exosomes. For ten patients, matching tissue, plasma and EVs were assessed with concordant results regarding miRNA modulations in tumor vs normal/healthy samples. We observed an overall upregulation of miR-21-5p and miR-155-5p in tumors vs normal tissues and a downregulation of miR-181a-5p in our and TCGA LUSC cohorts. We also verified that these differences are detectable in early and late stages, indicating that the expression levels of the three miRNAs are an early alteration in lung cancer. This trend was also confirmed in plasma and exosomes compared to healthy controls.

Indeed, when we investigated the sensitivity and specificity for the three individual miRNAs, we observed that miR-21-5p recorded a satisfactory AUC (0.8-1) in all settings (tissue, plasma, exosomes). At the same time, for miR-181a-5p expression in exosomes vs exosomes from healthy controls, the AUC=1 indicated miR-181a has high predictive accuracy for LUSC patients. The combined ROC analysis of these miRNAs revealed that their combinations could reach high predictive accuracy for NSCLC. In plasma samples, the combinations of miR-155-5p+miR-21-5p, as well as miR-155-5p + miR-181a-5p, had AUC higher (0.88) than the one obtained with individual miRNAs (Fig. 4).

The mirroring of the expression changes of miR-21, miR-181a, and miR-155 in lung tissues, plasma and exosomes emphasizes the utility of these molecules in cancer diagnosis. All these results indicate that the three miRNAs combined – miR-21-5p, miR-155-5p and miR-181a-5p – can be considered for further studies as biomarkers for early detection of LUSC in male patients.

5. CONCLUSION

In this study, we evaluated the combination of three miRNAs – miR-21-5p, miR-155-5p and miR-181a-5p – as potential biomarkers for LUSC diagnosis in male patients. The investigation revealed

upregulation of miR-21-5p and miR-155-5p and downregulation of miR-181a, consistent in both early- and late-stages in LUSC TCGA datasets. This alteration was confirmed in a cohort of male LUSC patients in TT vs TN.

The abundance of circulating miRNAs in plasma and exosomes, with their known regulatory role, makes these RNA biotypes potential clinical biomarkers. This study confirmed that this trend in expression alteration is maintained in plasma and exosomes from LUSC male patients compared to plasma and exosomes from healthy controls.

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