



# Persistent and novel changes in plasma microRNA profiles in patients with non-small cell lung cancer following tumour resection

Maria Sromek<sup>1^</sup>, Maciej Głogowski<sup>2^</sup>, Magdalena Chechlińska<sup>1^</sup>, Mariusz Kulińczak<sup>1^</sup>, Michalina Zajdel<sup>1^</sup>, Natalia Żeber-Lubecka<sup>3,4^</sup>, Aneta Bałabas<sup>3^</sup>, Łukasz M. Szafron<sup>3^</sup>, Maria Kulecka<sup>3^</sup>, Jan K. Siwicki<sup>1^</sup>

<sup>1</sup>Department of Cancer Biology, Maria Skłodowska-Curie National Research Institute of Oncology, Warsaw, Poland; <sup>2</sup>Department of Lung Cancer and Chest Tumors, Maria Skłodowska-Curie National Research Institute of Oncology, Warsaw, Poland; <sup>3</sup>Department of Genetics, Maria Skłodowska-Curie National Research Institute of Oncology, Warsaw, Poland; <sup>4</sup>Department of Gastroenterology, Hepatology and Clinical Oncology, Centre of Postgraduate Medical Education, Warsaw, Poland

**Contributions:** (I) Conception and design: M Sromek, M Głogowski, JK Siwicki; (II) Administrative support: M Chechlińska; (III) Provision of study materials or patients: M Głogowski; (IV) Collection and assembly of data: M Sromek, M Kulińczak, M Zajdel, N Żeber-Lubecka, A Bałabas; (V) Data analysis and interpretation: M Sromek, ŁM Szafron, M Kulecka, JK Siwicki, M Chechlińska; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

**Correspondence to:** Maria Sromek, PhD. Department of Cancer Biology, Maria Skłodowska-Curie National Research Institute of Oncology, W.K. Roentgen 5, 02-781 Warsaw, Poland. Email: Maria.Sromek@nio.gov.pl; Jan K. Siwicki, PhD. Assoc. Prof., Department of Cancer Biology, Maria Skłodowska-Curie National Research Institute of Oncology, W.K. Roentgen 5, 02-781 Warsaw, Poland. Email: Jan.Siwicki@nio.gov.pl.

**Background:** Non-small cell lung cancer (NSCLC) accounts for 80% of lung cancers, the leading cause of cancer mortality. microRNAs (miRNA, miR) have emerged as important components of carcinogenesis and promising biomarkers. We aimed to analyse global plasma miRs in NSCLC patients before and at least one year after tumour resection.

**Methods:** Plasma was collected from the peripheral blood of 24 donors without cancer and of NSCLC patients before surgery (n=36) and at least 1 year after surgery (n=12). Next-generation sequencing (NGS)-based miR profiling was performed. Patients were followed-up for 4 to 12 years after surgery to assess disease recurrence.

**Results:** Untreated NSCLC patients exhibited significant changes in plasma miR levels compared to cancer-free donors (48 up- and 17 down-regulated miRs). miR profiles in patients with adenocarcinoma (ADC) (n=18) and squamous cell carcinoma (SCC) significantly differed (16 and 86 miRs up-, and 15 and 16 miRs down-regulated, respectively). A subset of pre-surgery deregulated miRs was found to be associated with recurrence (49 miRs). Six miRs were shown to have independent prognostic value. After tumour resection, some pre-surgery miR alterations returned to control levels (18 miRs), some others persisted (27 miRs), while also novel plasma miR changes emerged (75 miRs) in patients with no clinical evidence of recurrence.

**Conclusions:** Untreated NSCLC patients present deregulated plasma miRs, some of which may have a potential of prognostic markers. After tumour excision plasma miR profiles change, some miR levels normalise, some changes persist and novel miR changes are observed despite no clinical symptoms of recurrence. Plasma miR profiles in NSCLC patients may suggest systemic abnormalities predisposing to

<sup>^</sup> ORCID: Maria Sromek, 0000-0001-8636-5542; Maciej Głogowski, 0000-0002-0850-0566; Magdalena Chechlińska, 0000-0001-6139-1234; Mariusz Kulińczak, 0000-0002-2517-0116; Michalina Zajdel, 0000-0003-1868-6402; Natalia Żeber-Lubecka, 0000-0003-4036-3191; Aneta Bałabas, 0000-0003-0228-7650; Łukasz M. Szafron, 0000-0003-1670-0274; Maria Kulecka, 0000-0002-9830-8892; Jan K. Siwicki, 0000-0002-5083-3022.

lung cancer and/or reflect a systemic response to pre-cancer/dormant cancer cells.

**Keywords:** Non-small cell lung cancer (NSCLC); circulating microRNA; systemic; tumour resection

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## Introduction

Lung cancer is the leading cause of cancer morbidity and mortality, accounting for 12.4% of all cancer diagnoses and responsible for 18.7% of cancer-related deaths. Lung cancer ranks first in incidence and mortality among men and second (after breast cancer) in women. The overall 5-year survival rate of lung cancer patients remains low (<20%), primarily because most cases are diagnosed at an advanced stage. The incidence of lung cancer varies by gender (male: 15.3%, female: 9.4%) (1), and by age, with 47% of lung cancer patients being  $\geq 70$  years old and 15% being

$\geq 80$  years old (2). Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer cases. Factors such as tobacco and environmental exposures (especially asbestos and air pollution), also significantly increase lung cancer risk (1,3).

Tumour resection is the primary treatment for early-stage NSCLC. However, approximately 40% of patients experience recurrence after surgery, mainly due to distant metastases (4–6). Therefore, a better understanding of the molecular mechanisms underlying NSCLC progression is urgently needed to develop new diagnostic approaches for early NSCLC diagnosis and monitoring. Various blood-based approaches have been developed to improve lung cancer diagnosis, including evaluation of circulating cell-free DNA and RNA [including microRNAs (miRs), long non-coding RNAs (lncRNA), messenger RNAs (mRNAs)], circulating extracellular vesicles, tumour-educated platelets, proteins, and metabolites (7,8). Circulating miRs have become a significant focus in lung cancer research in recent years. They are linked to cancer development, progression, and response to treatment. They have the potential to serve as diagnostic and prognostic biomarkers. Additionally, they are involved in the regulation of inflammatory processes and key epigenetic mechanisms (9).

Primary malignant tumours influence not only their nearby stroma, the microenvironment, but also the macroenvironment, by inducing various alterations in benign parts of the organ of origin, in the regional lymph nodes, and in the distant tissues or organs. Some of the systemic cancer-driven changes are associated with tumour aggressiveness and outcome (10–13). Increasing data also show that the host macroenvironment, including intestinal microbiota, metabolism, tensional homeostasis, morphostasis, tissue bioelectric properties, and nervous system, play a crucial role in cancer progression (14).

Changes in circulating miRs following NSCLC resection have been previously analysed, but in all studies, except one (15), post-surgical levels of circulating miRs were analysed shortly after tumour resection (1–2 weeks) (15–17), and may be significantly related to surgical

### Highlight box

#### Key findings

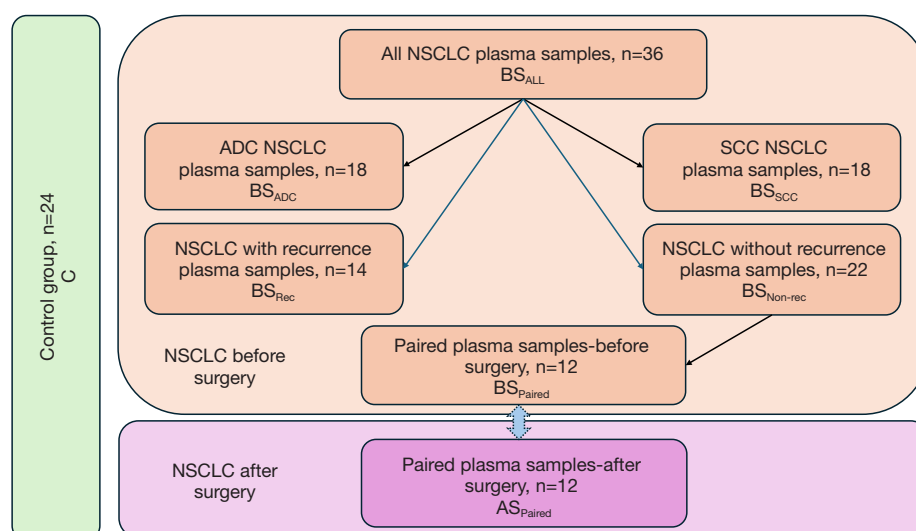
- The pre-surgery plasma microRNA profile in untreated non-small cell lung cancer (NSCLC) patients significantly differs from that of normal blood donors, with distinct pattern in adenocarcinoma versus squamous cell carcinoma patients. Some of the pre-surgery alterations may have a potential of a marker of recurrence. After tumour excision plasma microRNA profiles change, some microRNA levels normalise, but the majority of pre-surgery changes persist, suggesting systemic abnormalities predisposing to lung cancer and/or systemic response to pre-cancer/dormant cancer cells. Interestingly, novel microRNA changes are observed following long-term follow-up in patients with no clinical symptoms of recurrence.

#### What is known and what is new?

- The alterations in plasma microRNA levels in NSCLC patients have been reported previously, however only limited data are available on circulating microRNAs long-term after tumor excision, especially in patients with no clinical symptoms of recurrence.
- To our knowledge this is the first analysis of post-surgery plasma microRNA profiles in NSCLC patients who showed no clinical recurrence following long-term follow-up.

#### What is the implication, and what should change now?

- Our observations support the rationale for the development of new therapeutic strategies targeting both cancer cell-autonomous and non-autonomous effectors in the tumour ecosystem.



**Figure 1** A diagram showing plasma samples of NSCLC divided into analysed groups. ADC, adenocarcinoma; AS, after surgery; BS, before surgery; C, control group; Rec, recurrence; Non-rec, without recurrence; NSCLC, non-small cell lung cancer; SCC, squamous cell carcinoma.

treatment and wound healing. Long-term systemic miR changes seem biologically and clinically important. Our previously published data suggested that deregulated plasma miR-9-5p, miR-16-5p, miR-205-5p, and miR-486-5p levels may reflect not only the presence of primary NSCLC, but also a systemic response to the disease (18). Our current study extends these findings by analysing global plasma miRs in NSCLC patients before and at least one year after tumour resection with a focus on patients showing no clinical evidence of recurrence during long-term follow-up. We present the following article in accordance with the MDAR reporting checklist (available at <https://tclr.amegroups.com/article/view/10.21037/tclr-24-626/rc>).

## Methods

### Patients and samples

Peripheral blood samples were collected at the Maria Skłodowska-Curie National Research Institute of Oncology in Warsaw from previously untreated patients with histologically confirmed NSCLC, undergoing surgery at our institution between 2011 and 2015. Patients with concomitant lung diseases and those with other tumours were excluded. Finally, out of initial over 100 patients only 36 met our inclusion criteria [group BS<sub>All</sub>, median age 62.5 (range, 43–77) years, 23 men and 13 women] (Figure 1,

Table S1). Blood samples from donors without cancer [group C, n=24, median age 58.5 (range, 36–70) years, 15 men and 9 women] were also taken (Table S2). The histopathological diagnosis was confirmed as adenocarcinoma (ADC) (n=18) and squamous cell carcinoma (SCC) (n=18), according to the 2021 World Health Organization (WHO) classification (19). Blood was collected from all NSCLC patients one day before surgery (BS<sub>All</sub>, n=36, including BS<sub>Paired</sub>, n=12) and in 12 patients also at least 1 year (2 years on average) after surgery (AS<sub>Paired</sub>, n=12, ADC n=7 and SCC n=5, median age 61 (range, 43–76) years, 8 men and 4 women) (Figure 1). The patients from the AS<sub>Paired</sub> group had not received any additional treatment before the second blood sample collection. In 24 patients, blood samples were either not analysed or not collected after surgery (AS). These included samples from patients with serious concomitant diseases or recurrences, and individuals who did not consent to the second collection or continued their follow-up at a different facility. Some samples were also excluded due to pre-analytical reasons. To assess disease recurrence, patients were followed-up for 4 to 12 years after surgery (Table S1). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Bioethics Committee of Maria Skłodowska-Curie National Research Institute of Oncology in Warsaw (No. 4/2011/1/2012), and written informed consent was obtained from sample donors.

### RNA extraction

Total RNA was isolated from plasma samples (300 µL) using mirVana PARIS RNA and Native Protein Purification Kit (Thermo Fisher Scientific, Waltham, MA USA), according to the protocols described earlier by Sromek *et al.* (18), and stored at  $-70^{\circ}\text{C}$ .

### Next-generation sequencing (NGS)

Small RNA fractions were checked for concentration and quality on Bioanalyzer 2100 using a Small RNA Kit Chip (Agilent, Santa Clara, CA, USA), and assessed with Qubit RNA HS Assay Kits (Thermo Fisher Scientific). miR libraries were prepared from 1 µg of total RNA with an Ion Total RNA-Seq Kit v2 for Small RNA Libraries and Ion Xpress RNA-Seq Barcode Kit (Thermo Fisher Scientific), according to the manufacturer's protocol. Briefly, reverse transcription of RNA, subsequent complementary DNA (cDNA) purification, and library size selection were performed with Nucleic Acid Binding Beads. Next, cDNA was amplified by polymerase chain reaction (PCR), purified and size selected. For sample quality control, the yield and size distribution DNA constructs were then assessed on the Agilent 2100 Bioanalyzer system using a High Sensitivity DNA Kit (Agilent, Santa Clara, CA, USA). The length of ligation products in barcoded libraries of miR ranged between 94 and 114 bp.

Template preparation procedure for clonal amplification of up to 16 miR libraries at a concentration of 18 pM and loading of the Ion PI Chip v3 were achieved using the Ion Chef instrument, with Ion PI<sup>TM</sup> Hi-Q<sup>TM</sup> Chef Kit (Thermo Fisher Scientific). Sequencing was performed using the Ion Torrent Proton sequencer (Thermo Fisher Scientific).

### Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The reverse transcription reaction was performed for selected 8 up-regulated and 7 down-regulated miRs with the TaqMan Advanced miR cDNA Synthesis Kit (Applied Biosystems CN A28007) (Table S3). qPCR was performed using the TaqMan Fast Advanced Master Mix (Applied Biosystems Carlsbad, CA USA) on QuantStudio<sup>TM</sup> 7 Flex Real Time PCR System (Thermo Fisher Scientific). Specific TaqMan Advanced miR Assays (Applied Biosystems Carlsbad, CA USA) were used to measure miRs specified in Table S3. miR-101 and miR-361 were selected as internal

controls based on the NormFinder algorithm. All qPCR reactions were carried out in triplicates, and the relative miR quantities were calculated with the  $2^{-\Delta\text{Ct}}$  method. The data were analysed with the QuantStudio<sup>TM</sup> Real-Time PCR Software (Thermo Fisher Scientific). The differences in miR levels between samples revealed by the RT-qPCR were assessed by the Mann-Whitney *U* test.

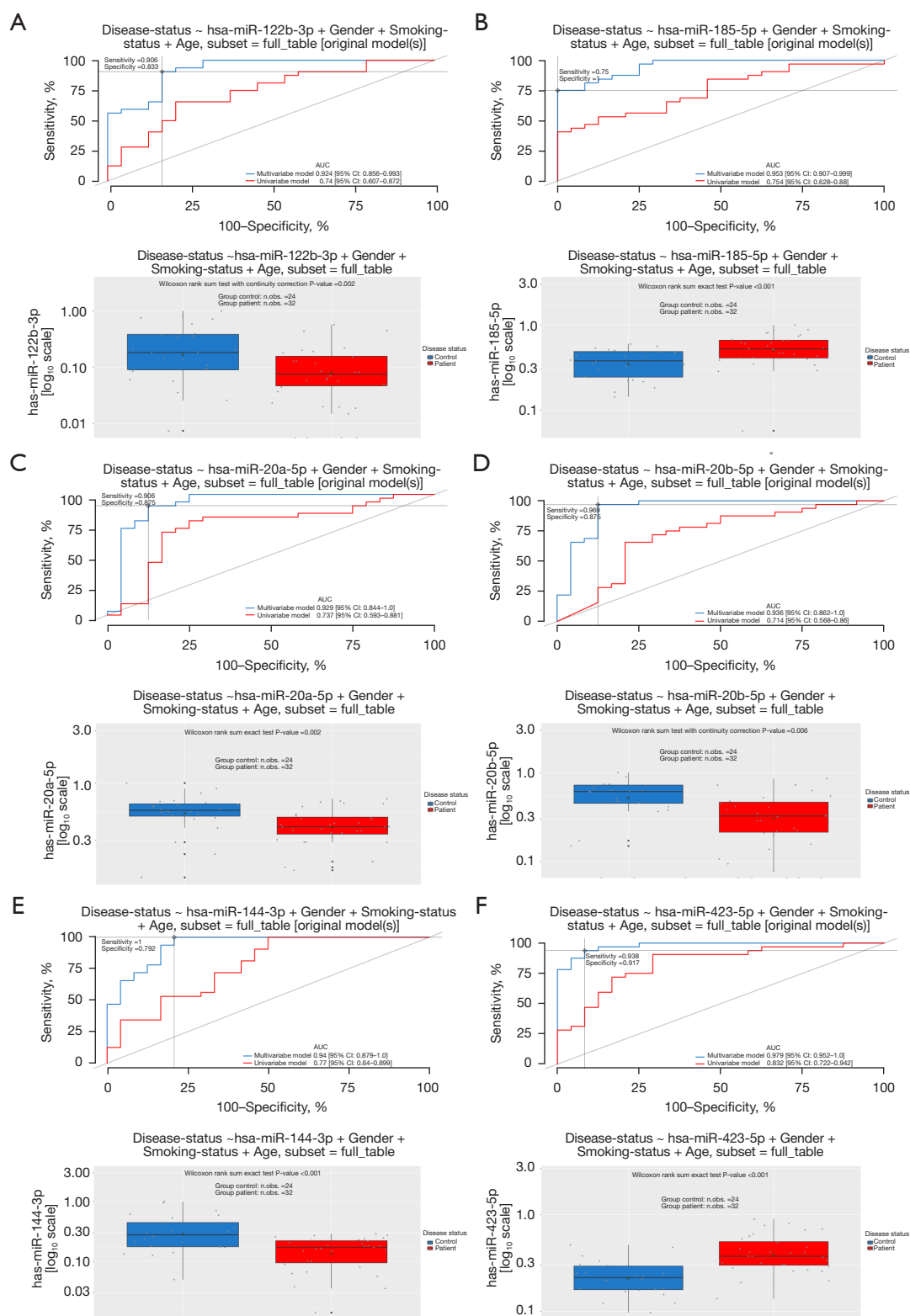
### Statistical analysis

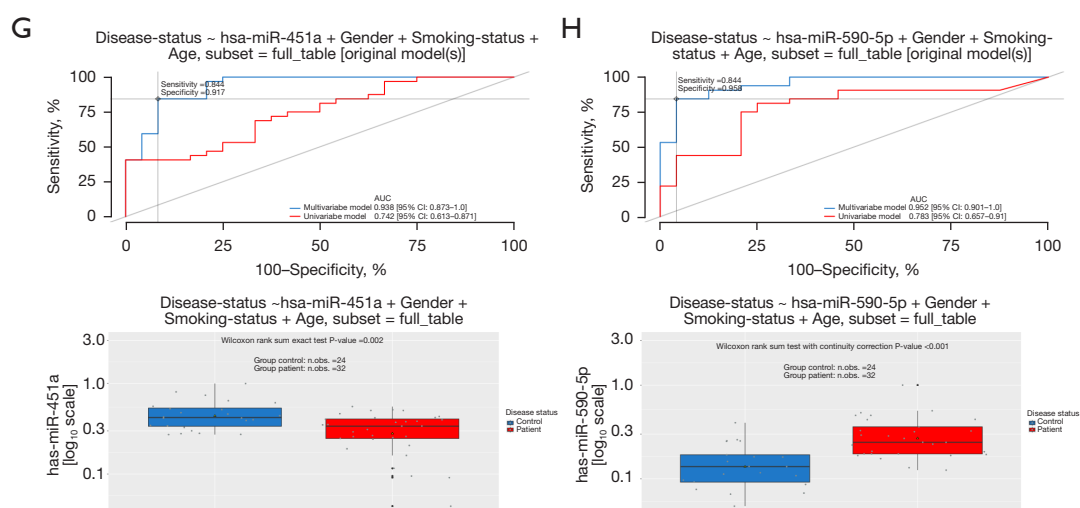
The miRDeep2 application was used to map NGS reads to the human genome hg19, quantify the known miRs (miRBase V21/22), and predict novel miRs. Differential miR expression was analysed using the DESeq2 package, with a significance threshold set to 0.05 without the Benjamini-Hochberg correction. The obtained gene expression data were normalised (according to the internal DESeq2 normalisation mechanisms) and, after  $\log_2$ -transformation, used for principal component analysis (PCA) and hierarchical clustering in the R environment, to identify similarities in miR expression patterns between the samples.

The lists of genes with significantly altered expression levels between the studied groups were subsequently used in ontological analyses performed with the miR enrichment analysis and annotation tool (miEAA, <https://ccb-compute2.cs.uni-saarland.de/mieaa/>, version 2.1). This tool was employed to perform the over-representation analysis of mature miRs in 28 different categories (Table S4). The enrichment analysis was performed with the nonparametric Kolmogorov-Smirnov (KS) test. The minimum hits per sub-category threshold was set to 2. The term was classified as enriched if the KS test P value after the Benjamini-Hochberg adjustment was lower than 0.05. The enriched ontological terms were visualised in word clouds, and the statistical significance of each term along with the over-represented miRs were shown on heatmaps.

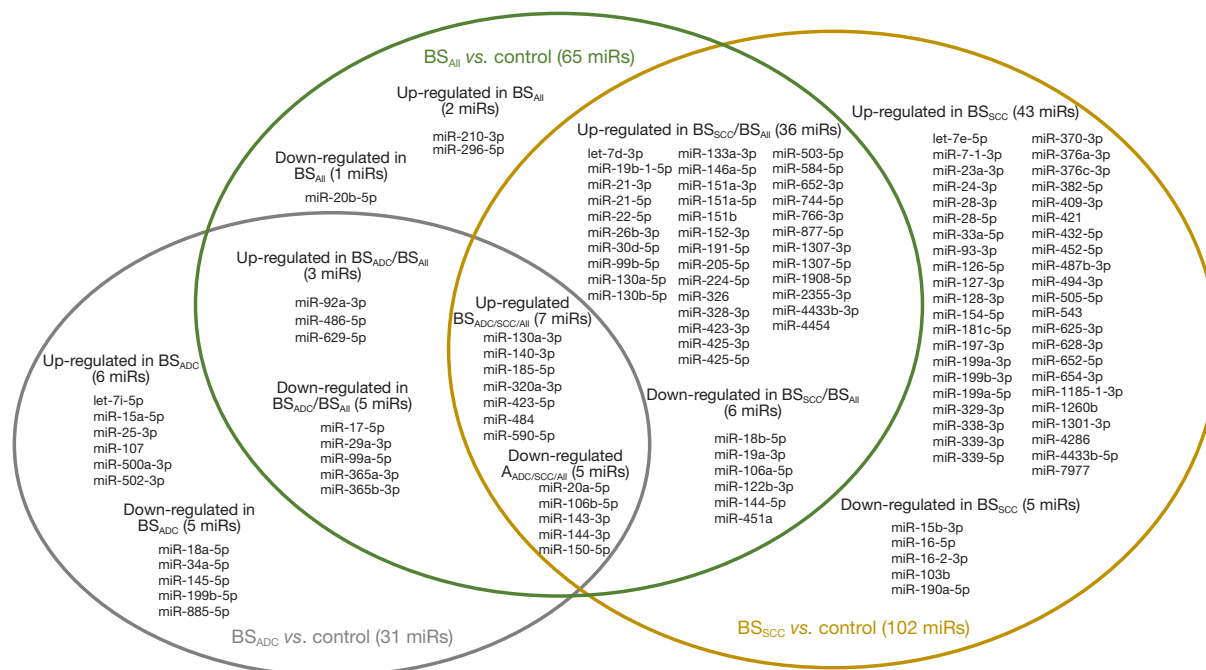
A list of 125 miRs identified in the present study as differentially expressed was subjected to detailed statistical inference in the R environment (v. 4.0.2). We used univariable and multivariable Cox proportional hazard models (package: survival, v. 3.5.7) to assess the value of these miRs as potential novel prognostic biomarkers. In each model, overall survival/death and disease-free survival/relapse were used as dependent variables. All Cox models were also checked with respect to the proportionality of hazards for each variable used. The same list of miRs was utilised to assess the risk of altered expression on the development of NSCLC for the tumours compared to the



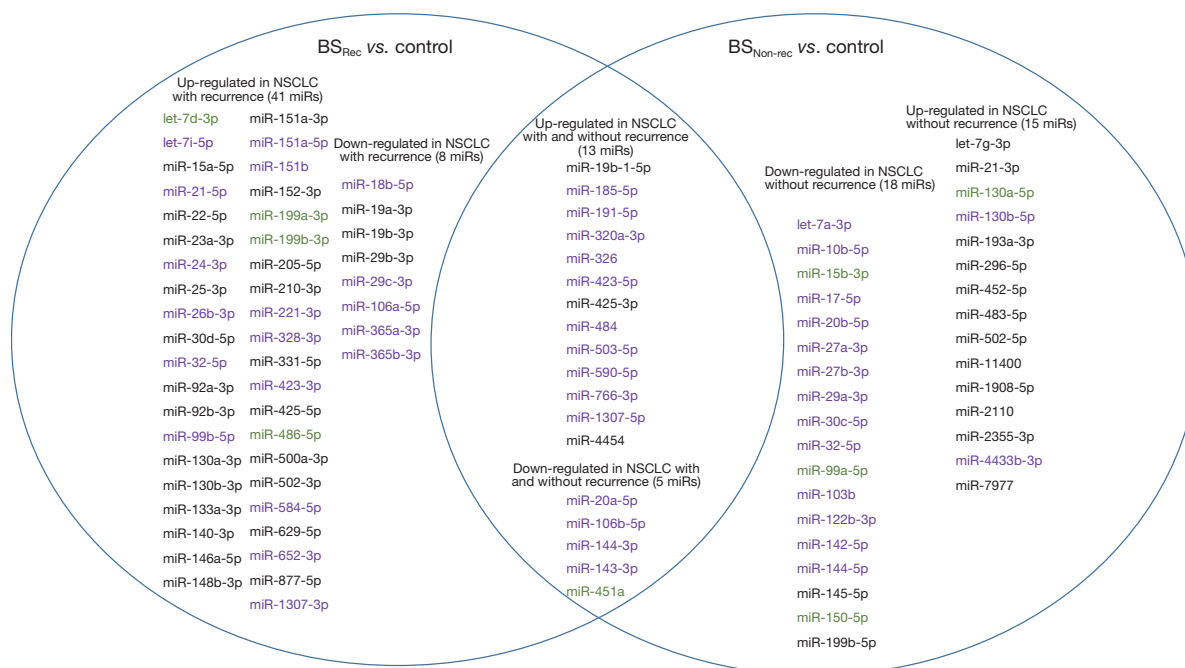




**Figure 3** The ROC curves representing the sensitivity and specificity of plasma miRs levels in differentiating NSCLC patients from the control group, along with plots of individual miR levels: 122b-3p (A), 185-5p (B), 20a-5p (C), 20b-5p (D), 144-3p (E), 423-5p (F), 451a (G), and 590-5p (H), generated using a linear regression model adjusted for sex, smoking status, and age. Values are accompanied by 95% CI shown in square brackets. AUC, area under the curve; CI, confidence interval; miRs, microRNAs; NSCLC, non-small cell lung cancer; ROC, receiver operating characteristic.



**Figure 4** Plasma miRs in patients BS, differentiating those with non-small cell lung adenocarcinoma (BS<sub>ADC</sub>; n=18) and squamous cell carcinoma (BS<sub>SCC</sub>; n=18) from controls (C; n=24). The green circle delineates miRs with significantly altered levels in both histopathological subtypes (BS<sub>All</sub>; n=36). The gray and yellow circles delineate miRs with significantly altered plasma levels in BS<sub>ADC</sub> and BS<sub>SCC</sub>, respectively. ADC, adenocarcinoma; BS, before surgery; miRs, microRNAs; SCC, squamous cell carcinoma.



**Figure 5** Plasma miRs differentiating non-small cell lung cancer patients before surgery with (BS<sub>Rec</sub>; n=14) and without (BS<sub>Non-rec</sub>; n=22) later recurrence, and the control group (C; n=24). In a subgroup of twelve non-small cell lung cancer patients (without recurrence) with paired plasma samples before surgery (BS<sub>Paired</sub>) and after surgery (AS<sub>Paired</sub>), the purple color indicates plasma miRs, the levels of which are also altered after tumour removal (AS<sub>Paired</sub>). The green color indicates miRs with the altered levels before surgery (BS<sub>Paired</sub>) that normalise after tumour removal (AS<sub>Paired</sub>). The black colour indicates plasma miRs with normal levels either before (BS<sub>Paired</sub>) or after surgery (AS<sub>Paired</sub>). AS, after surgery; BS, before surgery; Rec, recurrence; miRs, microRNAs.

of NSCLC, BS<sub>SCC</sub> and BS<sub>ADC</sub>, with 4 out of 7 up-regulated and 3 out of 6 down-regulated SCC-specific miRs, and 4 out of 5 up-regulated ADC-specific miRs. None of the 5 miRs selected from those demonstrated by NGS as down-regulated in ADC were confirmed by RT-qPCR (Table S11). miR-144-3p, miR-423-5p, miR-451a and miR-590-5p were also identified in a logistic regression model in the context of lung cancer occurrence (Figure 3, Table S6).

The ontological analysis of selected significantly up-regulated miRs in NSCLC patients revealed their involvement in proliferation, cell cycle, and the regulation of metalloendopeptidase activity (Table S12). In the latter process, there were also strongly associated some miRs down-regulated in NSCLC patients. Additionally, these miRs influence apoptosis, cell organisation and differentiation, as well as pulmonary blood vessel remodelling. This group is closely related to important genes (*TP53*, *PTEN*, *BCL2*) and signalling pathways (PI3K AKT, TGFbeta, mTOR) (Table S13).

### Pre-surgery plasma miR profiles associated with NSCLC recurrence and overall survival

In NSCLC patients before surgery with (BS<sub>Rec</sub>; n=14) and without (BS<sub>Non-rec</sub>; n=22) later recurrence, we identified 49 and 33 plasma miRs, respectively, that significantly differentiated patients from controls. These included 41 plasma miRs with higher and 8 miRs with lower levels in BS<sub>rec</sub> series than in controls (Figure 5, Table S14), and 15 with higher and 18 with lower levels in BS<sub>Non-rec</sub> series vs. controls (Figure 5, Table S15). Interestingly, we found up-regulation of miR-32-5p in BS<sub>Rec</sub> patients, but in opposite down-regulation of the molecule in BS<sub>Non-rec</sub> patients.

To identify prognostic value of miR levels, multivariable Cox regression analysis was performed, considering DFS and OS. The following variables were included: miR levels, gender, age, histological tumour type, tumour grade, clinical stage. The elevated miR-21-5p and miR-32-5p levels were associated with a higher risk of recurrence, while decreased miR-122b-3p and miR-150-5p levels were associated with

OS (Figure S3). Elevated levels of miR-502-3p and miR-92a-3p were linked to both a higher risk of recurrence and reduced OS in NSCLC patients (Figures S4,S5, Table S7). Therefore, the above miRs may independently influence prognosis.

#### ***Analysis of the changes in plasma miR levels in NSCLC patients' paired samples before and after tumour resection***

Paired plasma samples collected from NSCLC patients one day before surgery (BS<sub>Paired</sub>; n=12), and from the same patients at least 1 year (2 years on average) after surgery (AS<sub>Paired</sub>; n=12) were analysed. The patients showed no clinical recurrence during follow-up (Table S1).

#### **Plasma miR levels before NSCLC resection in a subgroup of patients with plasma miR levels assessed also after tumour resection (BS<sub>Paired</sub> vs. C)**

A 47 plasma miR profile, comprising 6 and 41 miRs increased and decreased, respectively, in BS<sub>Paired</sub> series vs. controls, differentiated BS<sub>Paired</sub> subgroup of NSCLC patients before surgery from controls (Table 1, Figure 6). Thirty-six percent of those miRs belonged to the altered plasma miR profiles identified in the whole group of 36 patients (Table S5). A substantial set of 24 molecules associated with recurrences does not show any deregulations in plasma levels in BS<sub>Paired</sub> series as compared to the control group (up-regulated in BS<sub>Rec</sub> patients: 15a-5p, 22-5p, 23a-3p, 25-3p, 30d-5p, 92a-3p, 92b-3p, 130b-3p, 140-3p, 146a-5p, 148b-3p, 151a-3p, 152-3p, 205-5p, 210-3p, 331-5p, 425-5p, 500a-3p, 502-3p, 629-5p, 877-5p; down-regulated in BS<sub>Rec</sub> patients: 19a-3p, 19b-3p, 29b-3p) (Figure 5).

#### **Changes in plasma miR levels in NSCLC patients after tumour resection (BS<sub>Paired</sub> vs. AS<sub>Paired</sub>)**

Following surgery, plasma levels of 24 miRs increased, while levels of 15 decreased according to the analysis of paired samples from individual patients (Table 2, Figures S6-S8).

#### **Plasma miR levels in NSCLC patients after tumour resection compared to normal levels (AS<sub>Paired</sub> vs. C)**

In the AS<sub>Paired</sub> samples from NSCLC patients, the levels of 51 plasma miRs were higher and of 51 were lower, compared to that in controls (Table 3, Figure 7). Some of these miRs were also identified in the plasma of BS<sub>Rec</sub> patients with recurrences (up-regulated miRs: 7i-5p, 21-5p, 24-3p, 26b-3p, 99b-5p, 151a-5p, 151b, 221-3p, 328-3p, 423-3p, 584-5p, 652-3p, 1307-3p and down-regulated miRs: 18b-

5p, 29c-3p, 106a-5p, 365a-3p, 365b-3p) (Figure 5).

#### ***Pre-surgery plasma miR alterations that normalized after NSCLC resection***

After tumour resection, one of six miRs with increased pre-surgery plasma levels (miR-130a-5p), and 17 out of the 41 miRs with decreased pre-surgery plasma levels in BS<sub>Paired</sub> subgroup returned to the levels comparable to those in controls (normalised) (Figure 8). However, based on the analyses comprising paired samples, out of the 17 mentioned above, 9 miRs increased in individual patients (BS<sub>Paired</sub> vs. AS<sub>Paired</sub>) (Table 2, Figure 7, Figure S6A).

The functional analyses showed that the miRs whose levels normalised after surgery (n=18) are specific to blood, predominantly to leukocytes. They are associated with many important carcinogenesis-related biological, immunological, inflammatory processes. They are involved in cellular responses to stress, injury, hypoxia (FoxO, HIF-1 pathways), and cigarette smoking (AhR receptors). The genes most strongly associated with these miRs play critical roles in proliferation, inhibition of apoptosis, angiogenesis, and cell migration. Notable genes include *BCL2*, *AKT1*, *MTOR*, *PTEN*, *MEG3*, and *SSX2IP*. Important terms acquired in functional analysis of this set of miRs are summarised in Table 4, full list is available in supplementary materials (table available at <https://cdn.amegroups.cn/static/public/tlcr-24-626-1.xlsx>).

#### ***Some of NSCLC-associated plasma miR alterations persisted after surgery***

In the AS<sub>Paired</sub> samples from patients without recurrence, some of the NSCLC-associated plasma miR changes observed before surgery persisted despite tumour resection. These included 5 out of all the 6 miRs (83%) that were overexpressed before surgery, and 22 out of all the 41 pre-surgery down-regulated miRs (53.7%) (Figure 8).

The functional analysis showed that NSCLC-associated plasma miRs (n=27) whose alterations persisted after surgery are predominantly linked to tissues such as blood vessels, lung, and pleura, with additional sources including myocardium, bladder, prostate, and spinal cord. These miRs are associated with various processes, in lung tissue as well as lung diseases [chronic obstructive pulmonary disease (COPD), lung cancer, NSCLC, fibrosis], processes occurring in bones and cartilage and the functioning of the heart and its blood vessels. They take part in the immune system processes and development, activity of platelets (platelet dense granule organization/activation), hypoxia, inflammation, wound healing. In this group, there are miRs associated with mitochondrial activity involved in the

**Table 1** Changes in plasma levels of miRs in a subgroup of NSCLC patients before surgery (n=12) and in the control group (n=24)

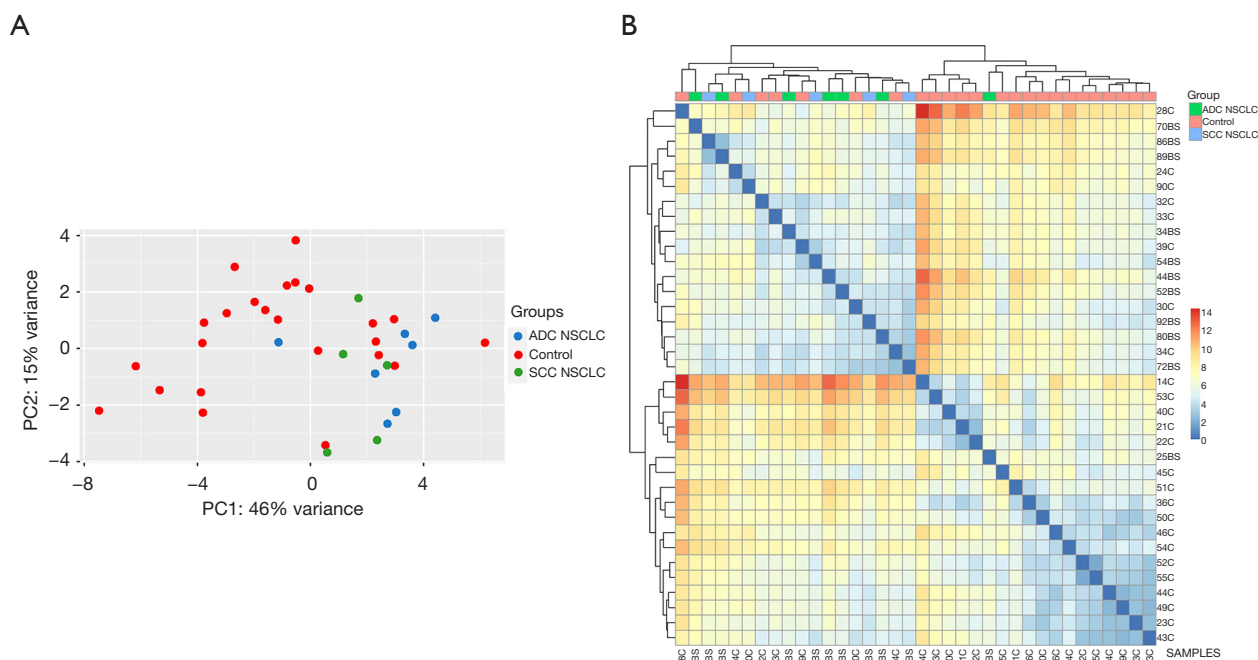
miRs	BaseMean	Log <sub>2</sub> FC	P value	P adj
Plasma miRs up-regulated in NSCLC patients (6 miRs)				
hsa-miR-1307-5p	7.25	1.27	0.009	0.08
hsa-miR-130a-3p	45.01	0.69	0.01	0.10
hsa-miR-423-5p	96.77	0.49	0.03	0.19
hsa-miR-503-5p	3.57	1.23	0.04	0.24
hsa-miR-133a-3p	3.74	1.44	0.049	0.24
hsa-miR-130a-5p	0.30	2.85	0.049	NA
Plasma miRs down-regulated in NSCLC patients (41 miRs)				
hsa-miR-6887-5p	7.88	-3.78	<0.001	<0.001
hsa-miR-20b-5p	20.74	-1.42	<0.001	0.004
hsa-miR-122b-3p	46.51	-2.00	<0.001	0.004
hsa-miR-93-5p	141.86	-0.87	<0.001	0.004
hsa-miR-17-5p	200.82	-1.15	<0.001	0.004
hsa-miR-106a-5p	35.40	-1.00	<0.001	0.007
hsa-miR-150-5p	84.49	-1.42	<0.001	0.01
hsa-miR-486-5p	834.54	-1.10	<0.001	0.01
hsa-miR-222-3p	11.63	-1.46	<0.001	0.01
hsa-miR-27b-3p	23.19	-1.33	<0.001	0.01
hsa-miR-125b-5p	22.48	-1.44	<0.001	0.01
hsa-miR-142-5p	56.61	-1.00	<0.001	0.01
hsa-miR-181b-5p	4.37	-2.07	0.001	0.02
hsa-miR-106b-5p	319.60	-0.72	0.001	0.02
hsa-miR-125a-5p	11.06	-1.64	0.002	0.03
hsa-miR-15b-3p	18.00	-0.96	0.002	0.04
hsa-miR-26b-5p	84.65	-0.80	0.004	0.055
hsa-miR-20a-5p	361.38	-0.57	0.004	0.055
hsa-miR-126-3p	142.28	-0.86	0.004	0.058
hsa-miR-144-3p	1,091.07	-0.88	0.005	0.058
hsa-miR-27a-3p	56.33	-0.94	0.005	0.058
hsa-miR-30a-5p	12.30	-1.05	0.005	0.06
hsa-miR-10b-5p	12.73	-1.29	0.006	0.06
hsa-miR-197-3p	9.04	-1.36	0.007	0.06
hsa-miR-194-5p	6.08	-1.49	0.007	0.06
hsa-miR-99a-5p	8.29	-1.42	0.007	0.06
hsa-miR-181a-5p	37.77	-1.16	0.01	0.08

**Table 1** (continued)

Table 1 (continued)

miRs	BaseMean	Log <sub>2</sub> FC	P value	P adj
hsa-miR-634	2.70	-2.16	0.01	0.09
hsa-miR-192-5p	13.55	-0.77	0.01	0.09
hsa-miR-122-5p	161.52	-0.99	0.01	0.10
hsa-miR-221-3p	76.75	-0.79	0.01	0.12
hsa-miR-26a-5p	384.20	-0.88	0.01	0.13
hsa-miR-374b-5p	7.61	-1.15	0.02	0.16
hsa-miR-143-3p	21.32	-0.99	0.02	0.18
hsa-miR-139-5p	11.71	-0.96	0.03	0.21
hsa-miR-18a-5p	72.97	-0.51	0.03	0.21
hsa-let-7d-3p	4.73	-0.93	0.03	0.21
hsa-miR-32-5p	8.67	-0.83	0.03	0.21
hsa-miR-451a	53,735.61	-0.37	0.04	0.24
hsa-miR-199a-3p	121.88	-0.94	0.049	0.24
hsa-miR-199b-3p	121.88	-0.94	0.049	0.24

miRs, microRNAs; FC, fold change; NA, not available; NSCLC, non-small cell lung cancer.



**Figure 6** Differences in plasma miR levels in a subgroup of 12 NSCLC patients BS and C. (A) The PCA exhibiting differences between NSCLC patients with SCC (n=5) and ADC (n=7) and the C (n=24). (B) Heatmap showing sample grouping according to the statistically significant differences in miR levels identified by NGS (patient and control samples are marked as BS and C, respectively). ADC, adenocarcinoma; BS, before surgery; C, controls; miR, microRNA; NGS, next-generation sequencing; NSCLC, non-small cell lung cancer; PCA, principal component analysis; SCC, squamous cell carcinoma.

**Table 2** Changes in plasma levels of miRs in a subgroup of NSCLC patients (n=12) before and after surgery

miRs	Base mean	Log <sub>2</sub> FC	P value	P adj
Plasma miRs up-regulated in NSCLC patients after surgery (24 miRs)				
hsa-miR-191-5p	196.39	1.13	<0.001	0.007
hsa-miR-199a-3p	132.94	1.22	<0.001	0.007
hsa-miR-199b-3p	132.94	1.22	<0.001	0.007
hsa-miR-21-5p	421.05	1.09	<0.001	0.01
hsa-miR-126-5p	145.33	1.29	0.001	0.02
hsa-miR-486-5p	577.45	0.89	0.002	0.03
hsa-miR-181a-5p	33.96	1.31	0.002	0.03
hsa-miR-223-3p	1,228.53	0.98	0.002	0.03
hsa-miR-126-3p	138.00	0.97	0.002	0.03
hsa-miR-221-3p	87.03	1.07	0.004	0.052
hsa-let-7i-5p	143.96	0.59	0.005	0.052
hsa-miR-26a-5p	310.34	0.78	0.006	0.052
hsa-let-7f-5p	177.08	0.63	0.007	0.052
hsa-miR-484	210.50	0.65	0.01	0.08
hsa-miR-423-3p	36.31	1.01	0.01	0.08
hsa-miR-151a-5p	81.76	0.63	0.01	0.09
hsa-miR-197-3p	9.93	1.42	0.01	0.10
hsa-miR-24-3p	232.86	0.75	0.01	0.10
hsa-miR-150-5p	59.08	0.82	0.02	0.14
hsa-miR-744-5p	10.08	1.37	0.02	0.14
hsa-miR-151b	94.94	0.54	0.03	0.16
hsa-miR-4286	33.08	0.97	0.04	0.18
hsa-miR-181b-5p	3.53	1.60	0.04	NA
hsa-let-7g-5p	205.82	0.46	0.047	0.20
Plasma miRs down-regulated in NSCLC patients after surgery (15 miRs)				
hsa-miR-15b-5p	264.76	-1.41	<0.001	0.007
hsa-miR-122b-3p	8.98	-2.08	<0.001	0.01
hsa-miR-29c-3p	15.38	-1.27	0.005	0.052
hsa-miR-144-3p	552.85	-0.74	0.006	0.052
hsa-miR-7-5p	66.76	-1.01	0.006	0.052
hsa-miR-450a-5p	2.18	-2.36	0.009	NA
hsa-miR-205-5p	5.99	-1.48	0.01	0.08
hsa-miR-369-3p	1.76	-1.77	0.02	NA
hsa-miR-106b-5p	176.76	-0.52	0.03	0.14

**Table 2** (continued)

Table 2 (continued)

miRs	Base mean	Log <sub>2</sub> FC	P value	P adj
hsa-miR-885-5p	2.18	-1.92	0.03	NA
hsa-miR-365a-3p	2.74	-1.57	0.03	NA
hsa-miR-365b-3p	2.74	-1.57	0.03	NA
hsa-miR-101-5p	1.53	-1.69	0.03	NA
hsa-miR-219a-5p	0.61	-3.73	0.04	NA
hsa-miR-22-3p	127.77	-0.46	0.04	0.20

miRs, microRNAs; FC, fold change; NA, not available; NSCLC, non-small cell lung cancer.

Table 3 Changes in plasma levels of miRs in a subgroup of NSCLC patients after surgery (n=12) and in the control group (n=24)

miRs	Base mean	Log <sub>2</sub> FC	P value	P adj
Plasma miRs up-regulated in NSCLC patients after surgery (51 miRs)				
hsa-miR-191-5p	249.49	1.50	<0.001	<0.001
hsa-miR-130a-3p	77.32	0.98	<0.001	<0.001
hsa-miR-423-5p	146.98	0.80	<0.001	<0.001
hsa-miR-423-3p	50.24	1.37	<0.001	<0.001
hsa-miR-151a-5p	121.29	0.81	<0.001	<0.001
hsa-miR-151b	142.68	0.71	<0.001	<0.001
hsa-miR-484	305.00	0.79	<0.001	<0.001
hsa-miR-744-5p	13.28	1.59	<0.001	<0.001
hsa-let-7f-5p	263.21	0.71	<0.001	<0.001
hsa-miR-33a-5p	15.00	1.16	<0.001	0.001
hsa-let-7i-5p	233.98	0.61	<0.001	0.001
hsa-let-7a-5p	467.04	0.58	<0.001	0.001
hsa-miR-326	18.15	1.16	<0.001	0.002
hsa-miR-24-3p	344.51	0.83	<0.001	0.006
hsa-miR-652-3p	31.44	0.77	<0.001	0.007
hsa-miR-766-3p	7.12	1.18	0.001	0.01
hsa-miR-21-5p	669.96	0.76	0.001	0.01
hsa-miR-130b-5p	2.23	1.31	0.001	0.01
hsa-miR-126-5p	241.67	0.75	0.001	0.01
hsa-miR-185-5p	427.49	0.50	0.001	0.01
hsa-miR-1307-5p	10.67	0.97	0.001	0.01
hsa-miR-1307-3p	9.01	0.94	0.001	0.01
hsa-miR-133a-3p	6.23	1.48	0.002	0.02
hsa-miR-1260b	30.28	0.96	0.004	0.02
hsa-miR-320a-3p	158.73	0.64	0.004	0.03
hsa-miR-223-3p	1,863.12	0.78	0.005	0.03
hsa-miR-221-3p	152.49	0.71	0.005	0.03

Table 3 (continued)

Table 3 (continued)

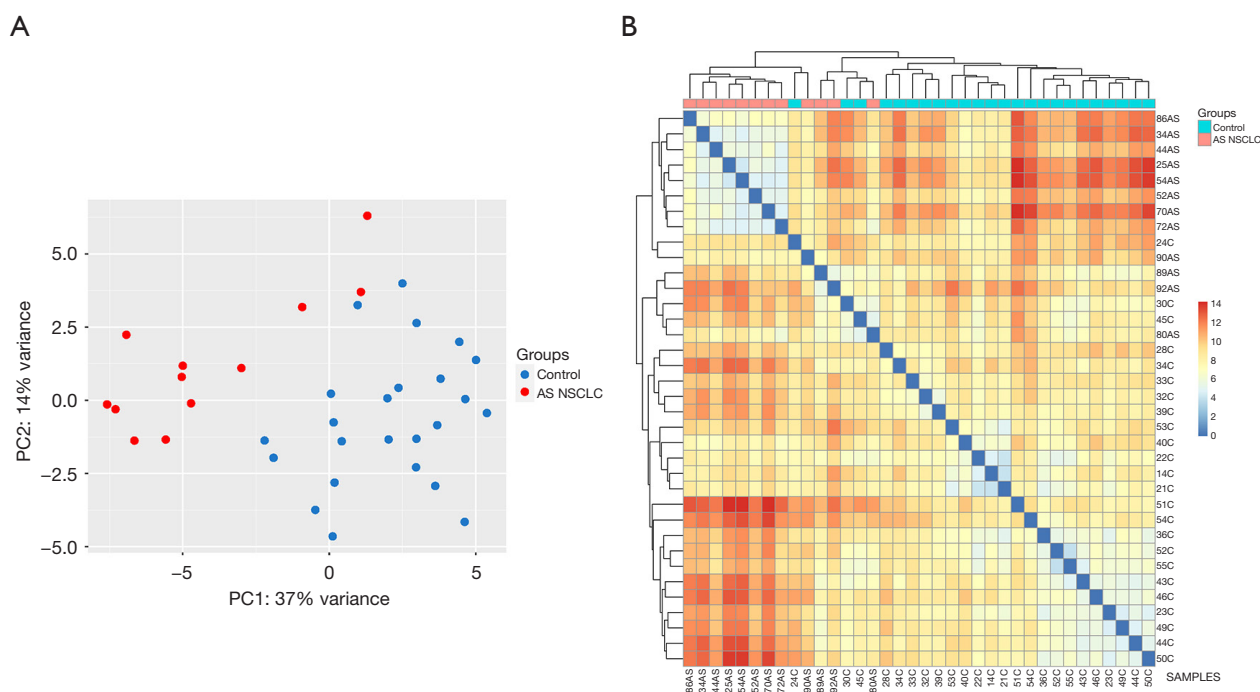
miRs	Base mean	Log <sub>2</sub> FC	P value	P adj
hsa-miR-584-5p	11.28	1.06	0.005	0.03
hsa-miR-4286	51.03	0.91	0.005	0.03
hsa-miR-4433b-3p	0.99	1.80	0.006	0.04
hsa-let-7c-5p	5.87	0.83	0.009	0.054
hsa-miR-99b-5p	3.28	1.15	0.01	0.06
hsa-miR-128-3p	57.55	0.44	0.01	0.07
hsa-let-7d-5p	52.82	0.48	0.01	0.07
hsa-miR-7-1-3p	2.34	1.35	0.01	0.07
hsa-miR-197-3p	19.59	0.85	0.01	0.07
hsa-let-7b-5p	307.71	0.44	0.01	0.08
hsa-miR-186-5p	41.29	0.41	0.01	0.09
hsa-miR-4433b-5p	30.12	0.95	0.02	0.09
hsa-miR-590-5p	11.63	0.59	0.02	0.09
hsa-miR-486-3p	9.23	0.68	0.02	0.09
hsa-miR-376c-3p	33.60	0.80	0.02	0.09
hsa-miR-625-3p	4.52	1.05	0.02	0.11
hsa-miR-26b-3p	1.75	0.92	0.02	0.11
hsa-miR-328-3p	2.31	1.10	0.02	0.12
hsa-let-7g-5p	340.57	0.29	0.03	0.15
hsa-miR-339-5p	38.46	0.50	0.03	0.15
hsa-miR-199a-5p	20.16	0.76	0.03	0.15
hsa-miR-503-5p	3.42	0.95	0.04	0.17
hsa-miR-376b-3p	5.79	0.84	0.049	0.19
hsa-miR-98-5p	12.04	0.42	0.049	0.19
Plasma miRs down-regulated in NSCLC patients after surgery (51 miRs)				
hsa-miR-122-5p	210.56	-1.98	<0.001	<0.001
hsa-miR-30a-5p	16.54	-1.51	<0.001	<0.001
hsa-miR-122b-3p	68.32	-3.42	<0.001	<0.001
hsa-miR-194-5p	8.71	-2.21	<0.001	<0.001
hsa-miR-106b-5p	457.90	-1.22	<0.001	<0.001
hsa-miR-27b-3p	32.57	-1.28	<0.001	<0.001
hsa-miR-103b	2.21	-4.58	<0.001	<0.001
hsa-miR-365a-3p	6.45	-1.70	<0.001	<0.001
hsa-miR-365b-3p	6.45	-1.70	<0.001	<0.001
hsa-miR-143-3p	29.41	-1.60	<0.001	<0.001
hsa-miR-32-5p	13.79	-1.15	<0.001	<0.001
hsa-miR-125b-5p	32.72	-1.22	<0.001	<0.001
hsa-miR-27a-3p	85.02	-0.87	<0.001	<0.001
hsa-miR-375-3p	2.80	-2.60	<0.001	0.001

Table 3 (continued)

Table 3 (continued)

miRs	Base mean	Log <sub>2</sub> FC	P value	P adj
hsa-miR-106a-5p	54.24	-0.83	<0.001	0.003
hsa-miR-144-3p	1,686.92	-1.38	<0.001	0.004
hsa-miR-20b-5p	34.82	-0.96	<0.001	0.004
hsa-miR-363-3p	19.53	-0.75	<0.001	0.004
hsa-miR-192-5p	21.40	-0.65	<0.001	0.006
hsa-miR-214-3p	2.30	-1.74	<0.001	0.01
hsa-miR-30e-5p	116.58	-0.51	0.001	0.01
hsa-miR-144-5p	84.71	-0.77	0.002	0.01
hsa-miR-20a-5p	560.94	-0.50	0.002	0.02
hsa-miR-6887-5p	12.45	-1.68	0.003	0.02
hsa-miR-7-5p	116.11	-0.76	0.003	0.02
hsa-miR-93-5p	230.53	-0.53	0.003	0.02
hsa-miR-16-5p	2,978.59	-0.49	0.003	0.02
hsa-miR-634	4.47	-1.71	0.003	0.02
hsa-miR-17-5p	323.07	-0.74	0.004	0.02
hsa-miR-205-5p	10.86	-0.99	0.004	0.02
hsa-miR-30b-5p	64.23	-0.54	0.004	0.03
hsa-miR-15b-5p	519.64	-1.04	0.005	0.03
hsa-miR-30c-5p	160.81	-0.54	0.006	0.04
hsa-miR-374a-5p	1.76	-1.34	0.006	0.04
hsa-miR-340-5p	18.68	-0.74	0.007	0.04
hsa-miR-145-3p	0.76	-1.82	0.007	0.04
hsa-let-7a-3p	14.32	-0.88	0.01	0.05
hsa-miR-18a-3p	0.41	-2.32	0.01	0.06
hsa-miR-10b-5p	19.78	-0.85	0.01	0.07
hsa-miR-29a-3p	78.11	-0.67	0.01	0.07
hsa-miR-219b-3p	0.45	-2.91	0.01	0.07
hsa-miR-18b-5p	17.74	-0.69	0.01	0.07
hsa-miR-143-5p	1.79	-1.91	0.01	0.08
hsa-miR-885-5p	3.46	-1.20	0.01	0.09
hsa-miR-26b-5p	133.84	-0.46	0.01	0.09
hsa-miR-29c-3p	29.68	-0.85	0.02	0.09
hsa-miR-9985	0.94	-1.64	0.02	0.09
hsa-miR-16-1-3p	0.29	-1.88	0.02	NA
hsa-miR-142-5p	84.18	-0.40	0.03	0.13
hsa-miR-125b-2-3p	0.45	-2.09	0.03	0.15
hsa-miR-5010-3p	0.48	-1.80	0.04	0.17

miRs, microRNAs; FC, fold change; NA, not available; NSCLC, non-small cell lung cancer.



**Figure 7** Differences in plasma miR levels in a subgroup of 12 NSCLC patients AS. (A) The PCA exhibiting differences between NSCLC patients AS (n=12) and the C (n=24). (B) Heatmap showing sample grouping according to the statistically significant differences in miR levels identified by NGS (patient and control samples are marked as AS and C, respectively). AS, after surgery; C, controls; miR, microRNA; NGS, next-generation sequencing; NSCLC, non-small cell lung cancer; PCA, principal component analysis.

apoptotic processes [activation of Phorbol-12-myristate-13-acetate-induced protein 1 (*Noxa*) and translocation to mitochondria]. The genes these miRs are most closely associated with are responsible for the following processes: cell proliferation and differentiation (*HOXD11*, *MAPK9*, *UBR5*), histone modification (*KMT5B*) and circulation and blood pressure in the lungs (*BMPR2*). Important terms identified in functional analysis are summarised in *Table 5*, the full list is available in supplementary materials (table available at <https://cdn.amegroups.cn/static/public/tlcr-24-626-2.xlsx>)

#### **Novel plasma miR alterations in NSCLC patients after tumour resection**

Forty six out of 51 (90%) miRs with increased, and 29 out of 51 (57%) with decreased plasma levels after surgery, exhibited similar levels to those in blood donors without cancer before surgery. Additionally, plasma levels of miR-221-3p and miR-197-3p, found to be significantly lower in untreated NSCLC patients than in normal donors, increased after surgery and significantly exceeded normal levels (*Figure 8*, *Figure S6B*).

Functional analysis showed that the miRs presenting novel alterations in plasma levels after tumour resection (n=75) were sperm-, neuroepithelial stem cell-, mesenchymal stromal cell (MSC)- and myotube-specific. They were associated with many critical signalling pathways responsible for cell proliferation and differentiation. Furthermore, they play roles in epigenetic processes, such as remodelling and organisation of chromatin (SWI/SNF family chromatin remodeling complexes), nucleosome organisation, histone modification, and in the functioning of stem cells, including somatic stem cell population maintenance, pluripotency of stem cell. In addition, these miRs influence different cell death mechanisms. They are related to mitochondrial activity and activation of p53 up-regulated modulator of apoptosis (PUMA) pathway. This set of miRs is also involved in the immune system processes and is associated with the formation and development of cardiac vascular smooth muscle cell, coronary vein morphogenesis and cardiovascular disease. miRs associated with platelet formation were also included. The genes most strongly regulated by these miRs are responsible for mRNA

**Table 4** The key ontological terms derived from the functional analysis of plasma microRNAs, with levels normalised following non-small cell lung cancer tumour excision (full list of terms in table available at <https://cdn.amegroups.cn/static/public/tlcr-24-626-1.xlsx>)

Category	Important ontological terms (q-value)
Cell/tissue specific	Blood
	Leukocyte
Carcinogenesis	Proteoglycans in cancer
	Cancer pathways
Cell death	Autophagia/macrophautophagia
	Apoptosis
	Anoikis
Cell proliferation	Proliferation
	Cell cycle
	Epithelial cell proliferation
Cell plasticity	Regulation of epithelial-mesenchymal transition (EMT)
Inter-cells contact/cell migration/motility	Cell migration/motility
	Focal adhesion
	Negative regulation of metalloproteinase activity
Response to external factors	Cellular response to stress
	Cigarette smoking [e.g., aryl hydrocarbon receptor (AhR)]
	DNA damage response
	Hypoxia (FoxO, HIF-1 pathways)
	Response to wounding
	Senescence/oxidative stress
	Starvation
Blood cells	B-cell receptor sig. path/B-cell proliferation/differentiation
	T cell receptor sig. path/T cell death/differentiation/proliferation
	Hemopoietic stem cell gene regulation by GABP complex
	Leukocyte transendothelial migration
Mitochondria	Copper homeostasis
	Mitochondrial depolarization
	Mitochondrial membrane potential
	Protein localization to mitochondria
	Release of cytochrome c from mitochondria during apoptosis

**Table 4** (continued)

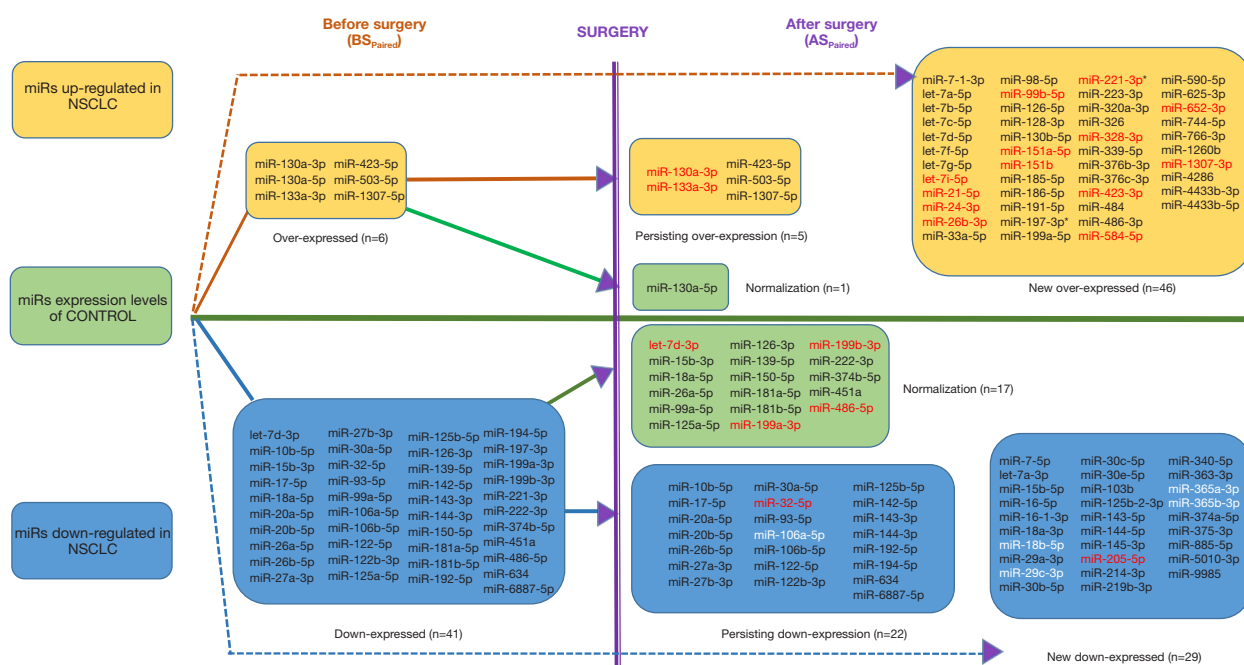
Table 4 (continued)

Category	Important ontological terms (q-value)
Immune system response	Chemokine
	Inflammation/infection/prostaglandin
	Interleukin
	Regulation of immune system response, process/development
	Response to cytokine/cytokine signaling
	Tumor necrosis factor (TNF)
Metabolism	Adipocyte signaling path/adipogenesis
	Carbohydrate digestion and absorption
	Glucose metabolic process
	Lipid metabolism/lipolysis in adipocytes
	Metabolism of RNA
	Nitrogen compound metabolic process
	Nitric oxide synthases (NOS) regulator activity
	Protein metabolic process/modification/amino acids
Blood vessels	Angiogenesis
	Endothelial cell proliferation/angiogenesis
	Neovascularization
Bones and cartilage tissue	Bone morphogenesis
	Endochondral ossification
	Osteoclast differentiation
Nervous system	Negative regulation of neuron projection development
	Nervous system
	Neurogenesis
Heart	Positive regulation of cardiac muscle cell proliferation
	Cardiac hypertrophic response
	Adrenergic signaling in cardiomyocytes
Growth factor/growth factor receptors	Insulin-like growth factor/1 receptor (IGF/1R)
	Nerve growth factor (NGF)
	Transforming growth factor $\beta$ (TGF $\beta$ )
	Vascular endothelial growth factor (VEGF)
Hormones	Estrogene
	Insulin
	Thyroid hormone signaling pathway/thyroid stimulating hormone (TSH)

Table 4 (continued)

Table 4 (continued)

Category	Important ontological terms (q-value)
Notable genes	<i>BCL2</i>
	<i>AKT1</i>
	<i>MTOR</i>
	<i>PTEN</i>
	<i>MEG3</i>
	<i>SSX2IP</i>
Signalling pathways	MAPK
	PI3K/AKT1
	mTOR
	TP 53 network



**Figure 8** Changes in plasma miR levels in NSCLC patients with paired plasma samples before surgery (BS<sub>paired</sub>; n=12) and after surgery (AS<sub>paired</sub>; n=12), in relation to the controls (C; n=24). The red font marks plasma miRs up-regulated in NSCLC patients with recurrence. The white font marks plasma miRs down-regulated in NSCLC patients with recurrence. The asterisk marks post-surgery up-regulated miRs with down-regulated pre-surgery level. The yellow, blue and green boxes mark up-regulated, down-regulated and normalised miRs, respectively. miR, microRNA; NSCLC, non-small cell lung cancer.

**Table 5** The key ontological terms derived from the functional analysis of plasma microRNAs, permanently altered following the excision of non-small cell lung cancer tumour (full list of terms in table available at <https://cdn.amegroups.cn/static/public/tlcr-24-626-2.xlsx>)

Category	Ontological terms (q-value)
Cell/tissue specific	Artery
	Bladder
	Brain/arachnoid mater
	Lung
	Lymph node
	Myocardium
	Muscle
	Nerve
	Pleura
	Prostate
	Spinal cord
	Vein
Carcinogenesis	Proteoglicans in cancer
	Cancer pathways
Cell death	Autophagia/macrophagia
	Apoptosis
	Anoikis
Cell proliferation	Proliferation
	Neuroblast proliferation
	Cell differentiation/maturation
	Cell cycle
Cell plasticity	Positive regulation of epithelial-mesenchymal transition (EMT)
Inter-cells contact/cell migration/motility	Cell matrix adhesion/cell substrate adhesion
	Cell migration/motility
	Extracellular matrix receptor (ECM-receptor)
	Focal adhesion
Chromatin organisation	Histone modification/acetylation
Response to external factors	Cellular response to stress
	DNA damage response
	Hipoxia
	Negative regulation of connective tissue replacement involved in inflammatory response wound healing
	Senescence/oxidative stress
	Starvation

**Table 5** (continued)

Table 5 (continued)

Category	Ontological terms (q-value)
Blood cells	B-cell/T-cell proliferation/differentiation
	Hematopoietic cell lineage
	Hemopoetic stem cell gene regulation by GABP complex
	Leukocyte transendothelial migration
	Monocyte differentiation
	Platelet degranulation
	Platelet dense granule organization/activation
Mitochondria	Activation of NOXA and translocation to mitochondria
	Copper homeostasis
	Mitochondrial membrane potential/protein localization to mitochondria
	Mitochondrial premeability transition pore complex (MPTP complex)
	Positive regulation of parkin-mediated stimulation of mitophagy in response to mitochondrial depolarization/mitophagy
Immune response	Chemokine
	Inflammation/infection/prostaglandin
	Interleukins
	Modulation by host of viral process regulation of immune system response
	Regulation of immune system response process/development
	Response to cytokine/cytokine signaling
Metabolic processes	Lipid metabolism/biosynthetic/lipolysis in adipocytes
	Negative regulation of lipid storage/lipid binding
	Positive regulation of low-density lipoprotein receptor activity
	Positive regulation of high-density lipoprotein particle assembly
	Adipocyte signaling path/adipogenesis
	Biosynthetic process
	Catabolic process
	Cholesterol transport/apolipoprotein A-I receptor activity
	Protein metabolic process/modification/amino acids
	Nitrogen compound metabolic process
Blood vessels	Endothelial cell proliferation/angiogenesis
	Vascular associated smooth muscle cell proliferation (VSMC)
Bones and cartilage tissue	Bone morphogenesis
	Chondrocyte proliferation/development
	Endochondral ossification
	Negative regulation of osteoblast differentiation
	Osteoclast differentiation

Table 5 (continued)

Table 5 (continued)

Category	Ontological terms (q-value)
Heart	Cardiac myofibril assembly
	Cardiac muscle hypertrophy
	Cardiovascular disease
	Coronary artery morphogenesis
	Dilated cardiomyopathy
	Endocardial cell differentiation
	Heart development
	Heart morphogenesis
	Left ventricular cardiac muscle tissue morphogenesis
	microRNAs in cardiomyocyte hypertrophy
	Pathogenesis of cardiovascular diseases
	Regulation of cell proliferation involved in heart valve morphogenesis
	Semi-lunar valve development
Lung	Chronic obstructive pulmonary disease (COPD)
	Lung blood pressure
	Lung alveolus development
	Positive regulation of pulmonary blood vessel remodeling
Nervous system	Nervous system development
Growth factor	Epidermal growth factor receptor (EGFR)
	Insulin-like growth factor/1 receptor (IGF/1R)
	Platelet-derived growth factor (PDGF)
	Transforming growth factor $\beta$ (TGF $\beta$ )
	Vascular endothelial growth factor (VEGF)
Hormones	Androgen/receptor signaling pathway
	Estrogen
	Insulin
	Prolactin signaling pathway
Notable genes	<i>HOXD11</i>
	<i>MAPK9</i>
	<i>UBR5</i>
	<i>BMPR2</i>
	<i>KMT5B</i>

Table 5 (continued)

Table 5 (continued)

Category	Ontological terms (q-value)
Selected signalling pathways	ATM dependent DNA damage/damage response
	BMP
	DDX58IFIH1
	ERB 2
	Embryonic stem cells (ESC) pluripotency and self-renewal signaling pathway
	NIKNF-kappa B
	PI3K/AKT1
	PTEN
	RUNX3
	SMAD23
	SMAD/SMAD2SMAD3SMAD4
	mTOR
	TP 53 network

degradation processes (*EDC3*), Nonsense Mediated Decay NMD independent of the Exon Junction Complex (EJC)-post-transcriptional gene regulation (*EEF1A1*), apoptosis (*CASP3*) and cell proliferation (*MYC*). Important terms identified in functional analysis are summarised in *Table 6*, the full list is available in supplementary materials (table available at <https://cdn.amegroups.cn/static/public/tlcr-24-626-3.xlsx>).

## Discussion

Untreated patients with NSCLC exhibited notable changes in plasma miR levels compared to cancer-free donors. A subset of those miRs was found to be associated with recurrence. Interestingly, even after at least one year post NSCLC resection, patients who showed no clinical evidence of recurrence retained some of the pre-surgery changes while also manifesting new changes.

Alterations in plasma miR levels may reflect various tumour biology mechanisms. Plasma miRs in NSCLC patients can originate not only directly from primary tumours, circulating cancer cells, dormant micrometastases, and pre-cancer cells in distant organs but also from stimulated neighbouring tissues. This has been substantiated by multiple studies.

First, a substantial body of evidence indicates that the dissemination of cancer cells, including NSCLC cells, can occur early (21,22). In 60% of nodule-negative

NSCLC patients without clinical evidence of distant metastases, haematogenous dissemination of cancer cells was demonstrated (23). The early disseminated cancer cells can colonise various organs and persist for many years as dormant cells in patients without any clinical features of metastasis (24). Furthermore, epithelial non-transformed cells have been suggested to disseminate and colonise various organs before the onset of malignant transformation, thereby contributing to metastasis (25). Circulating tumour cells with clear malignant features have been found in patients with chronic obstructive pulmonary disease, a risk factor for lung cancer (26). In patients undergoing surgery for benign pulmonary diseases Nordgård *et al.* detected microscopic NSCLC metastases in control lymph nodes (27).

Second, pre-surgery plasma miR alterations may represent a systemic response to cancer, involving the phenomenon of tumour education of cells from other tissues, including platelets and other blood cells (28). For example, whole blood cells from NSCLC patients have been shown to present deregulated miR expression (29,30).

In NSCLC patients, the levels of some of the pre-surgery deregulated plasma miRs returned to levels comparable to those in cancer-free donors after tumour resection. The post-surgery normalisation suggests that the abnormal levels of those plasma miRs were influenced by the presence of the primary tumour and may have potential as a marker.

**Table 6** The key ontological terms derived from the functional analysis of plasma microRNAs, showing novel level changes in non-small cell lung cancer patients after surgery (full list of terms in table available at <https://cdn.amegroups.cn/static/public/tlcr-24-626-3.xlsx>)

Category	Ontological terms (q-value)
Cell/tissue specific	Leukocyte
	Neuroepithelial stem cell
	Mesenchymal stromal cell (MSC)
	Myotube specific
	Sperm specific
Carcinogenesis	Proteoglycans in cancer
	Cancer pathways
Cell death	Autophagia/macrophagy
	Apoptosis
	Anoikis
	Pyroptosis
	Necroptotic process
	Ferroptosis
Cell proliferation	Fibroblast apoptotic process
	Cell cycle
Cell plasticity	Positive regulation of epithelial-mesenchymal transition (EMT)
	Somatic stem cell population maintenance
	Pluripotency of stem cell
Inter-cell contact/cell migration/motility	Regulation of stress fiber assembly
	Focal adhesion
	Cell matrix adhesion/cell substrate adhesion
Chromatin organisation	Histone modification
	Nucleosome organization
	Pericentric/subtelomeric heterochromatin formation
	Remodeling and organization chromatin (SWI/SNF complex)
Response to external factors	Cellular response to stress
	Starvation
	DNA damage response
	Senescence/oxidative stress
Blood cells	B-cell proliferation/differentiation
	T cell death/differentiation/proliferation
	Leukocyte transendothelial migration
	Macrophage differentiation
	Platelet formation

**Table 6** (continued)

Table 6 (continued)

Category	Ontological terms (q-value)
Mitochondria	Activation of p53 upregulated modulator of apoptosis (PUMA) and translocation to mitochondria Mitochondrial activity (rRNA/tRNA in mitochondrion) Positive regulation of parkin-mediated stimulation of mitophagy in response to mitochondrial depolarization/mitophagy
Immune response	Inflammation/infection Interleukins (IL2, IL3, IL4, IL5, IL10, IL13) Modulation by host of viral process regulation of immune system response Response to cytokine/cytokine signaling Tumor necrosis factor (TNF) Translational silencing of ceruloplasmin expression Virus infection/viral process
Metabolism	Adipocyte signaling path/adipogenesis Biosynthetic process Catabolic process Fatty acid synthase activity/biosynthesis/degradation Protein metabolic process/modification/amino acids Nitrogen compound metabolic process
Blood vessels	Angiogenesis Blood vessel endothelial cell migration
Heart	Arrhythmogenic right ventricular cardiomyopathy (ARVC) Cardiac vascular smooth muscle cell development Cardiovascular disease Coronary artery morphogenesis Coronary vein morphogenesis Heart morphogenesis
Nervous system	Negative regulation of neuron projection development Nervous system Neurogenesis
Growth factor/growth factor receptors	Insulin-like growth factor/1 receptor (IGF/1R) Transforming growth factor $\beta$ (TGF $\beta$ ) Vascular endothelial growth factor (VEGF)
Hormones	Cortisol synthesis and secretion Insulin Prolactin signaling pathway Thyroid hormone signaling pathway/thyroid stimulating hormone (TSH)

Table 6 (continued)

Table 6 (continued)

Category	Ontological terms (q-value)
Notable genes	<i>EDC3</i>
	<i>CASP3</i>
	<i>EEF1A1</i>
	<i>MYC</i>
Selected signalling pathways	FoxO signaling pathway
	TP 53 network
	Hippo-Merlin
	Yap/TAZ
	ROBO/SLITs
	Longevity signaling pathway
	Wnt (pluripotency and self-renewal signaling pathway)/Wnt/B-catenin
	SWI/SNF complex

This is in line with the understanding of the tumour as an abnormal tissue interacting with the entire organism (31), and with previous observations in patients with gastric, hepatocellular, and breast carcinoma, where increased pre-operative levels of some plasma miRs returned to a normal levels after tumour resection (32-34).

This study revealed a set of pre-surgery plasma miRs associated with recurrence, which showed altered levels compared to those in cancer-free donors. In addition, some miRs were shown to have independent prognostic value. These included miR-21-5p, miR-32-5p, miR-122b-3p, miR-150-5p, miR-502-3p, and miR-92a-3p. Interestingly, pre-surgery plasma miR-32-5p was up-regulated in patients with recurrence, while it was down-regulated in those without recurrence. It is worth noting that a recent study has suggested a tumour suppressor role for miR-32-5p in NSCLC (35).

We found some recurrence-associated alterations both in plasma of BS<sub>Paired</sub> as well in AS<sub>Paired</sub> patients without recurrence during long-term follow-up (from 4 to 12 years after surgery). Approximately 90% of NSCLC recurrences develop within the first 2–3 years after curative resection (36). Additionally, NSCLC patients are at a higher risk for developing a second cancer (37). Noteworthy, in long-term monitoring of patients without clinical evidence of recurrence at the second collection of plasma samples, four patients developed a second primary malignancy, including: two patients with NSCLC (ADC) and two patients with prostate cancer. It is possible that these recurrence-

associated alterations in the plasma of BS<sub>Paired</sub> patients reflect some systemic changes or pre-cancer effects.

The presented study also showed that the majority of the pre-surgery plasma miR changes persisted despite tumour removal and the absence of clinical evidence of recurrence. The persisted plasma miRs alterations can be associated with the occult/dormant lymph node metastases (23,27) and/or the disseminated non-cancer/pre-cancer cells that can secrete miRs and/or stimulate secretion by neighbouring tissues (25).

Additionally, since all post-surgery-examined patients were smokers, smoking-related plasma miRs may have contributed to some of the plasma miR level changes persisting after surgery. Noteworthy, half of the miRs exhibiting persisted plasma level changes have been previously linked to smoking (38,39), and four of those miRs (miR-10b-5p, miR-106b-5p, miR-106a-5p and miR-125b-5p) have been found to be differentially expressed in bronchial epithelium in smokers and non-smokers (40).

The alterations persisting after tumour removal, may represent “*field cancerisation*” or “*field of injury*” phenomena (41), perhaps associated with smoking. A context of “*tissue organization field theory*” (TOFT), perceiving cancer as a tissue-based disease that arises following the disruption of reciprocal interactions between parenchyma and stroma resulting in altered tissue organisation, may also be considered (42). Finally, since histologically normal lung tissue from lung cancer patients exhibits a progression-

associated inflammatory gene signature (43), activation of inflammatory pathways in the NSCLC macroenvironment may have also contributed to the permanently altered plasma miR profile described here.

Post-surgery profiling revealed novel plasma miR alterations not observed before surgery, differentiating patients from cancer-free donors. This phenomenon may be linked to several factors. Cancer surgery, including that in NSCLC patients, on the one hand has been shown to generate an increased number of circulating cancer cells (44) that can convert into dormant micrometastases in distant organs. On the other hand, it has been shown to modulate tumour dormancy (45). Dormant micrometastases, as already mentioned above, can contribute to the observed new plasma miR profiles. Another mechanism linked to lung cancer resection that contributes to new plasma miR changes, may be related to systemic postoperative hypoxemia resulting from the partial removal of a significant portion (20–50%) of lung tissue (46). The removal can lead to systemic oxygen deficiency—hypoxemia—resulting in reduced oxygenation of various tissues (hypoxia), with further changes relating to biogenesis of miRs being influenced by hypoxia. It has been demonstrated that hypoxia can induce or downregulate the expression of various miRs (47). We observed that the group of miRs demonstrating new post-surgery alterations included 6 overexpressed miRs (miR-21-5p, let7-b-5p, let-7a-5p, miR-98-5p, let-7c-5p and let-7i-5p), whose expression is upregulated by hypoxia. In addition, the new post-surgery alterations included three overexpressed miRs, whose expression is downregulated by hypoxia (miR-17b-5p, miR-16-5p and miR144-5p).

The observed novel post-surgery plasma miR profiles were also associated with stem cells, megakaryocytes and platelets, and may reflect some systemic changes after full or partial lobectomy during lung cancer surgery. This is in line with the suggested physiological role of lung megakaryocytes in thrombopoiesis, as well as with the discovery of abundant megakaryocytes found in pulmonary artery blood in lung cancer patients (48,49).

We found that terms related to mitochondria and their participation in apoptosis also appeared in the functional analysis of the novel post-surgery plasma miR alterations. Thus, new post-surgery alterations in mitochondrial function-associated plasma miRs (mitochondrial activity, rRNA/tRNA in mitochondrion) may reflect a post-surgery systemic change linked to mitochondria transfer (50).

The strength of our study is that for the first time the

analysis of changes in plasma miRs profiles was performed in carefully defined group of NSCLC patients showing no clinical evidence of recurrence during long-term follow-up (from 4 to 12 years) after surgery. In addition, for the first time it was found that in NSCLC patients after tumour excision, some circulating miR profile changes persist and novel miR changes emerge despite the absence of clinical symptoms of recurrence. One of the limitations of our study is the small size of the patients' cohort. However, the statistical power of our tests was sufficient to identify potential biomarkers of significant relevance, and our preliminary analyses showed some promising diagnostic/prognostic associations, but the confirmation of their application potential will require further studies including a larger cohort of patients. Another limitation is the lack of analysis of the observed plasma miR profiles in the context of environmental exposures.

## Conclusions

In summary, the study provides new insights into the dynamics of plasma miR alterations following NSCLC resection. The pre-surgery plasma miR profile in NSCLC patients significantly differs from that of normal blood donors. It encompasses recurrence-associated set of miRs, including miR-21-5p, miR-32-5p, miR-122b-3p, miR-150-5p, miR-502-3p, and miR-92a-3p which show independent prognostic value. Furthermore, miR-32-5p exhibited notable opposite changes in patients with and without recurrence, what additionally suggesting its biomarker potential. Noteworthy, we demonstrated for the first time that in NSCLC patients with no clinical evidence of recurrence during long-term follow-up after tumour resection (from 4 to 12 years after surgery) some pre-surgery alterations normalised, novel changes emerged, but the majority of the pre-surgery alterations persisted. The long-term persistence of the NSCLC-related plasma miRs abnormalities despite tumour resection may indicate the existence of permanent systemic alterations that predispose or are induced by lung cancer. The observations provide further support of the postulated (51) development of new therapeutic strategies targeting both cancer cells and the tumour macroenvironment.

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## Footnote

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**Ethical Statement:** The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Bioethics Committee of Maria Skłodowska-Curie National Research Institute of Oncology in Warsaw (No. 4/2011/1/2012), and written informed consent was obtained from sample donors.

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