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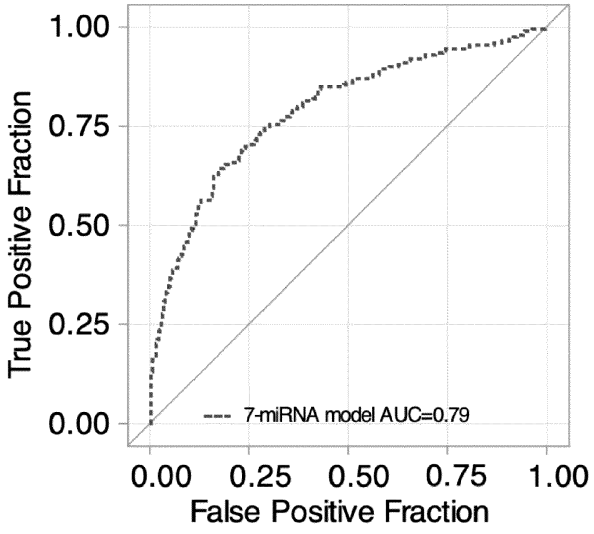
(54) PROGNOSTIC METHOD FOR AGGRESSIVE LUNG ADENOCARCINOMAS

(57) The present invention relates to methods based on miRNA biomarkers for the prognosis of aggressive lung adenocarcinoma (ADC) including early-stage (stage I) disease, preferably in fresh-frozen or in formalin-fixed, paraffin-embedded (FFPE) specimens. More in particular, the invention refers to the use of 7-miRNA, 14-miRNA or 19-miRNA prognostic signatures in a method for prognostic risk stratification of ADC, preferably for identify patients with aggressive early-stage lung adenocarcinoma (namely C1-ADC).

Figures 3.a), b), c), d), e) and f). Validation of the 7-miRNAs model.

(Figure 3a)



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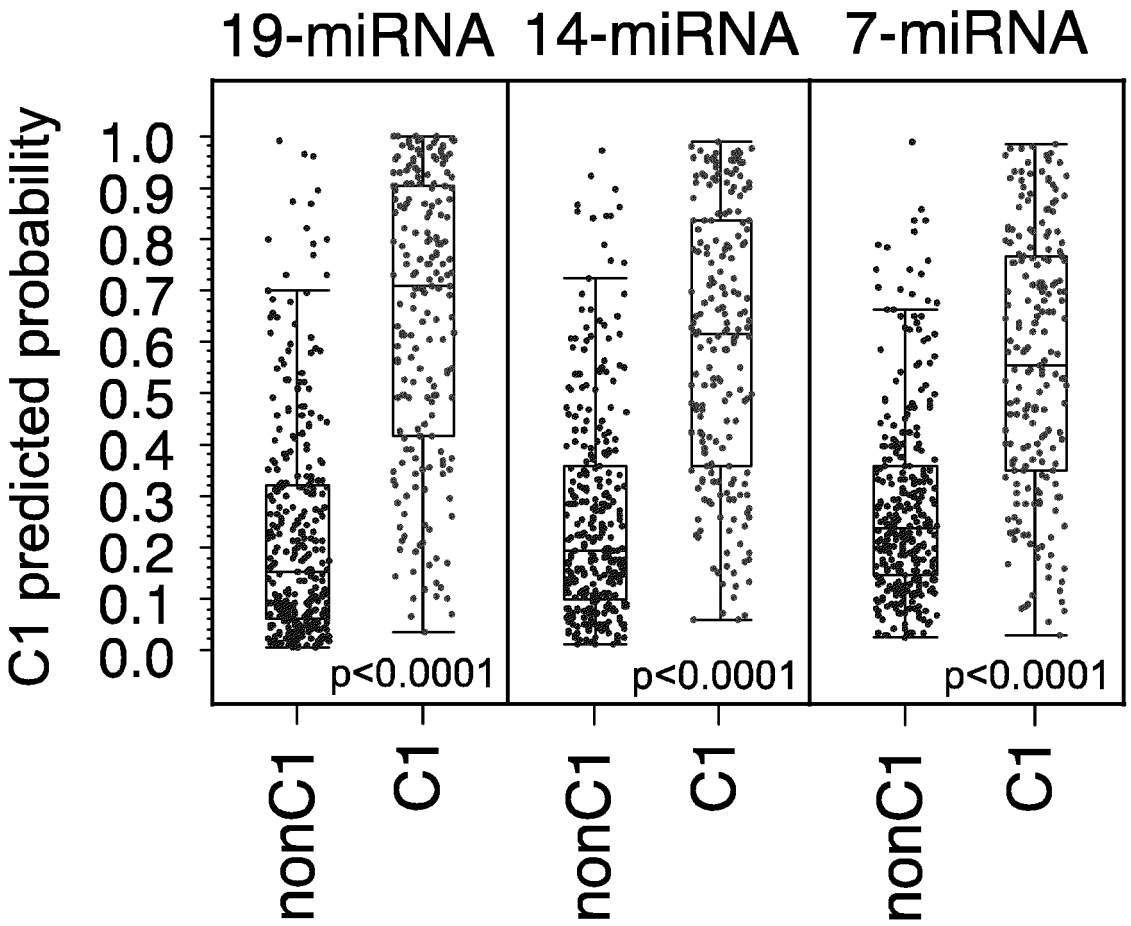
(Figure 3b)


19-miRNA
14-miRNA
7-miRNA
C1 predicted probability
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
p<0.0001
p<0.0001
p<0.0001
nonC1
C1
nonC1
C1
nonC1
C1

Description

[0001] The present invention relates to methods based on miRNA biomarkers for the prognosis of aggressive lung adenocarcinoma (ADC) including early-stage (stage I) disease, preferably in fresh-frozen or in formalin-fixed, paraffin-embedded (FFPE) specimens. More in particular, the invention refers to the use of 7-miRNA, 14-miRNA or 19-miRNA prognostic signatures in a method for prognostic risk stratification of ADC, preferably for identify patients with aggressive early-stage lung adenocarcinoma (namely C1-ADC).

Background of the invention

[0002] Latest global lung cancer data indicate a burden of 2.09 million new cases and 1.76 million deaths in 2018 [1]. The main type of lung cancer is represented by Non-Small-Cell Lung Cancer (NSCLC) (80-85%) including several heterogeneous tumor subtypes, among which lung adenocarcinoma (ADC) accounts for ~40% of all lung cancer cases. Primary and secondary prevention strategies such as anti-smoking campaigns and the implementation of large CT screening programs resulted in a reduction of lung cancer mortality of a ~20% (as observed in NELSON and NLST trials) and progressive lung cancer stage-shift [2,3]. However, the high level of molecular heterogeneity of lung cancer enhances the metastatic dissemination of a large fraction of aggressive early stage tumors (-30-50%) [4].

[0003] In-depth molecular and functional characterization of ADC could help to contextualize tumor heterogeneity in specific molecular subtypes which may suggest alternative therapeutic options. We recently described a 10-gene prognostic signature for stage I ADC which identified a subset of tumors, namely C1-ADC [5,6], with peculiar gene/protein expression and genetic alterations resembling more advanced cancer.

[0004] This prognostic gene signature can be measured by quantitative real-time PCR (qRT-PCR) or digital PCR (dPCR), Affymetrix or RNA-sequencing, or direct digital detection (e.g. Nanostring technology), in fresh-frozen or in formalin-fixed, paraffin-embedded (FFPE) specimens [6].

[0005] To foster clinical translation of this 10-gene signature, here we present a miRNA signature as a surrogate of the 10 genes, for prognostic risk stratification of ADC, in particular to identify patients with aggressive early-stage lung adenocarcinomas. A miRNA-based prognostic signature would overcome the problem of using low-quality mRNA when extracted from FFPE samples, which are routinely used for diagnostic purposes. Indeed, shorter non-coding RNA molecules such as miRNA are more resistant to harsh conditions [7,8] and compatible with most of the expression profiling methods including qRT-PCR.

[0006] Some prior art discloses the use of detecting miRNAs to diagnose cancer, such as lung cancer. However, no prior art document discloses a prognostic method based on a specific miRNA signature that effectively works for detecting patients with the aggressive ADC subtype i.e. the C1-ADC, and that can be applied also by using fresh-frozen or in formalin-fixed, paraffin-embedded (FFPE) specimens.

[0007] For example, WO2012/089630A1 discloses a method to identify asymptomatic high-risk individual with early-stage lung cancer in biologic fluids, by means of detecting at least 5 miRNAs within a list of 34 miRNA.

[0008] WO2016/038119 and Bianchi F. et al. [14] discloses a method for diagnosing lung cancer in a subject by detecting a decrease and an increased abundance of different miRNAs in a blood sample obtained from that patient, the presence of which provides an earlier indication of cancer than alternative art-recognized methods, including, but not limited to, low-dose computed tomography (LDCT).

[0009] There is therefore an urgent need of prognostic biomarkers and of a method to identify patients with early-stage aggressive lung cancer, who could eventually benefit from systemic adjuvant chemotherapy (i.e. platinum-based) rather than molecular targeted/immune-therapies and that can be applied to different kind of body fluids or tissue samples, including FFPE specimens.

Brief description of the figures.

[0010]

Figure 1. Flow chart of study design with data sets and analysis.

Figure 2. mRNA and miRNA expression profile analysis of the TCGA-LUAD cohort.
(a) Hierarchical clustering analysis of the 10-gene expression signature. C1-C4 clusters are colored as per the legend. Age, gender, smoking status and stage are colored as per the legend. (b) Kaplan-Meier curves for 3-years overall survival stratified by C1-C4 clusters. Log-rank p-values are shown for C1 vs nonC1 patients (C2-C4) comparison. (c) Receiver operating characteristic (ROC) curves showing the False Positive Fraction and True Positive Fraction of the 19- (solid line) and 14-miRNA (dashed line) models. The areas under curve (AUC) are reported. (d) Networks of miRNA derived from 19-, 14- and 7-miRNAs model and corresponding target genes. Rectangles represent genes (mRNA); ellipses represent miRNA from 19-miRNA model; circles represent miRNA from 14-miRNA

model; hexagons represent miRNA from both 14- and 19-miRNA model. (e) Hierarchical clustering of 7-miRNAs in the TCGA-LUAD cohort. C1 and nonC1 tumors (defined according to the 10-gene signature) are colored as per the legend. Predicted C1 and nonC1 tumors (defined according the 7-miRNA logistic model) are colored as per the legend.

Figure 3. Validation of the 7-miRNA model.

(a) ROC curve showing the False Positive Fraction and True Positive Fraction of the 7-miRNA model. The AUC is reported. (b) Box-plot for C1 predicted probability in C1 (light grey dots) and nonC1 (black dots) patients. Predicted probabilities are calculated through the 19-, 14- and 7-miRNA models. Wilcoxon-Mann-Whitney test p-values are reported. (c) Bubble plot of top 10 GeneSets found significantly overlapping with gene networks targeted by the 7-miRNA signature. Bubbles size is proportional to statistical significance (-Log of FDR q-value) and color codes refer to number of genes found in the overlap. In X-axis, ratios (k/K) of overlap of the query set of genes (k) with overlapping GeneSet size (K). (d) Heatmap of the 10-gene expression of CSS cohort. C1 and nonC1 tumors are colored as per the legend. Risk scores are calculated based on the 10-gene risk model. (e) ROC curves showing the False Positive Fraction and True Positive Fraction of the 7-miRNA model in the CSS cohort, for all stages (solid black line) or only stage I tumors (dashed light grey line). The AUC are reported. (f) Box-plot for C1 predicted probability in C1 (light grey dots) and nonC1 (black dots) tumors in CSS cohort, for all stages tumors and stage I tumors. Predicted probabilities are calculated through the 7-miRNA model. Wilcoxon-Mann-Whitney test p-values are reported.

Figure 4. AUC obtained from signatures of 14 and 19 miRNAs.

Flow-chart of the design applied to compare the AUC of the 14- and 19-miRNA models to random signatures of equal lengths. For random list of 14 and 19 miRNAs: median, first quartile (Q1), third quartile (Q3), minimum (min) and maximum (max) AUC are reported. Bell distributions of AUC for 100 random signatures; dashed vertical line at AUC = 0.5; solid vertical line at AUC of 14- and 19-miRNA models; filled black area corresponds to min-max AUC range for 100 random signatures. DE stands for differentially expressed.

Figure 5. Table S1A.

TCGA-LUAD cohort. 200 miRNAs significantly regulated by DESeq2 in C1 vs. nonC1 patients comparison.

Figure 6. Table S1B.

TCGA-LUAD cohort. 90 miRNAs significantly regulated by BRB-ArrayTools in C1 vs. nonC1 patients comparison.

Definitions

[0011] Unless otherwise defined, all terms of art, notations and other scientific terminology used herein are intended to have the meanings commonly understood by those persons skilled in the art to which this disclosure pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference; thus, the inclusion of such definitions herein should not be construed to represent a substantial difference over what is generally understood in the art.

[0012] The term *"microRNA"* or *"miRNA"* used herein refers to a small non-coding RNA molecule of about 22 nucleotides found in plants, animals and some viruses, that has role in RNA silencing and post-transcriptional regulation of gene expression. miRNA exerts its functions via base-pairing with complementary sequences within mRNA molecules.

[0013] The term *"signature"* herein refers to an expression pattern derived from combination of several miRNA (i.e. transcripts) used as biomarkers.

[0014] The term *"FFPE specimen"* herein refers to a tissue sample fixed in formalin and embedded in paraffin.

[0015] The term *"AUC"* herein refers to the area under the ROC curve (Receiver Operating Characteristic curve), that is a graph showing the performance of a binary classification model at various classification thresholds.

[0016] The term patients in group *"C1"* or *"C1-ADC"* used herein include patients affected by aggressive lung adenocarcinoma with experience poor-prognosis patients (i.e. with shorter overall survival, and/or with shorter disease-free survival, and/or responsive to a treatment, and/or with metastatic disease) which can include, but not limited to, patients with early-stage disease (i.e. stage I); while patients included in the group *"nonC1"* or *"nonC1-ADC"* are affected by a non-aggressive lung adenocarcinoma or good-prognosis patients (i.e. with longer overall survival, and/or with longer disease-free survival, and/or responsive to treatment, and/or without metastatic disease) which can include, but not limited to, patients with early-stage disease (i.e. stage I).

[0017] The term "aggressive" herein refers to a cancer diagnosed in patients with an adverse prognosis (i.e. with shorter overall survival, and/or with shorter disease-free survival, and/or responsive to a treatment, and/or with metastatic disease).

[0018] The term "prognostic" herein refers to the ability to discriminate patients with good/poor prognosis.

[0019] The term "biomarkers" (short for biological markers) herein refers to biological indicators (for example a transcript, i.e. miRNA) and/or measures of some biological state or condition.

[0020] The terms *"comprising", "having", "including"* and *"containing"* should be understood as 'open' terms (i.e. meaning "including, but not limited to") and should also be deemed a support for terms such as *"consist essentially of"*,

"consisting essentially of", *"consist of"*, or *"consisting of"*.

[0021] The term "TCGA" herein refers to The Cancer Genome Atlas database, where molecular data (e.g. gene and protein expression, gene mutations, methylation profile, copy number variation) for a total of 33 different type of tumors were made available to public (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga).

[0022] The term "TCGA-LUAD" herein refers to the specific cohort of lung adenocarcinoma (LUAD) patients which data are available in The Cancer Genome Atlas database.

[0023] The term "CSS" herein refers to the cohort of lung adenocarcinoma patients enrolled in IRCSS Casa Sollievo della Sofferenza Hospital.

Description of the invention

[0024] It has been surprisingly found that a 19-miRNA or 14-miRNA signature, or preferably a 7-miRNA signature, is able to identify patients affected by aggressive lung adenocarcinoma including early-stage disease, otherwise stated as "C1-ADC" or "C1". The expression profile and therefore the quantity of such miRNAs can be measured by RNA sequencing (RNA-seq), by quantitative real-time PCR (qRT-PCR) or digital PCR (dPCR), by Affymetrix or direct digital detection (e.g. Nanostring technology), and can be applied to different body fluids or tissue samples, also to FFPE samples, overcoming the drawbacks related to instability/degradation of mRNA mainly in FFPE samples.

[0025] Here, the inventors applied a multi-tiered approach relying on RNA-seq (mRNA and miRNA profile) data analysis of a large cohort of lung cancer patients (TCGA-LUAD; N=510), which enabled them to identify prognostic miRNA signatures in lung adenocarcinoma patients. Such signatures showed high accuracy (AUC ranging between 0.79 and 0.85) in scoring aggressive disease and can be used in a molecular multi-biomarkers classifier method. Importantly, using a network-based approach the inventors rewired miRNA-mRNA regulatory networks, identifying a minimal signature of 7 miRNAs, which works also in FFPE samples, and controls a variety of genes overlapping with cancer relevant pathways.

[0026] The obtained results further demonstrate the reliability of miRNA-based biomarkers for lung cancer prognostication and their use in a classification method based on the application of miRNA biomarkers in the clinical routine.

[0027] More in details, the inventors obtained surrogate miRNA signatures of 7-, 14- and 19-miRNA which recapitulate a previously described 10-gene prognostic signature in ADC including stage I disease [6]. The 7-, 14- and 19-miRNA signatures were all effective to identify aggressive "C1-ADC" disease (AUC=0.79-0.85). Notably, all miRNAs in the 7-miRNA signature were detected in most of the FFPE samples (Ct<40; Table S2) which confirmed the proven higher stability when used in low-quality mRNA [9].

[0028] Importantly, in their approach the inventors adopted a network-rewiring strategy by specifically select miRNA-mRNA pairs characterizing aggressive stage I tumors ("C1"). Such approach allowed to select a core of 7 miRNAs capable to stratify C1 from nonC1 tumors with an accuracy comparable to the 14- and 19-miRNA models (Figure 2c and Figure 3a), and, importantly, interacting with the "C1" transcriptome. This is relevant for capturing molecular mechanisms controlled by miRNAs which are associated to lung cancer progression.

[0029] As a matter of fact, the inventors observed a large overlap between the '7-miRNA networks' with several gene sets representing cancer relevant pathways (Figure 3c).

[0030] The advantages of the new method identified is that of being a reliable prognostic method for the screening of aggressive lung adenocarcinoma including early-stage disease. In particular, said method displays several characteristics that are desirable in a routine clinical setting:

- it can overcome the problem of using low-quality mRNA when extracted for example from FFPE samples, which are routinely used for diagnostic purposes;
- it could distinguish between a fraction of patients with ADC after treatment (i.e. surgery, or surgery and adjuvant chemotherapy, or surgery and radiotherapy, or a combination thereof) that could experience relapse and an adverse prognosis, with subsequent reduction of mortality;
- it can be used with multiple gene expression profiling platforms (i.e. RNAseq, quantitative real-time PCR, digital PCR, Affymetrix, digital detection through molecular barcoding).
- it analyses specific transcript sequences which augment the specificity of the said method.
- it can be implemented in a point-of-care testing (POCT) for miRNA-based diagnostics.
- It can measure quantities of transcripts using small amount of biological material (up to 1-10 nanograms of purified nucleic acids).

[0031] It is therefore an embodiment of the present invention a method *in-vitro* or *ex-vivo* for identifying patients affected by aggressive lung adenocarcinoma (C1-ADC), comprising the steps of:

a. detecting the amount of each of the 19 miRNAs having sequence hsa-miR-193b-5p (SEQ ID NO. 3), hsa-miR-

31-3p (SEQ ID NO. 11), hsa-miR-31-5p (SEQ ID NO. 12), hsa-miR-550a-5p (SEQ ID NO. 15), hsa-miR-196b-5p (SEQ ID NO. 5), hsa-miR-584-5p (SEQ ID NO. 17), hsa-miR-30d-5p (SEQ ID NO. 10), hsa-miR-582-3p (SEQ ID NO. 16), hsa-miR-9-5p (SEQ ID NO. 19), hsa-let-7c-3p (SEQ ID NO. 1), hsa-miR-138-5p (SEQ ID NO. 2), hsa-miR-196a-5p (SEQ ID NO. 4), hsa-miR-203a-3p (SEQ ID NO. 6), hsa-miR-215-5p (SEQ ID NO. 7), hsa-miR-2355-3p (8SEQ ID NO. 3); hsa-miR-30d-3p (SEQ ID NO. 9), hsa-miR-4709-3p (SEQ ID NO. 13), hsa-miR-548b-3p (SEQ ID NO. 14) and hsa-miR-675-3p (SEQ ID NO. 18); or of each of the 14 miRNA having sequence hsa-miR-196b-5p (SEQ ID NO. 5), hsa-miR-584-5p (SEQ ID NO. 17), hsa-miR-30d-5p (SEQ ID NO. 10), hsa-miR-582-3p (SEQ ID NO. 16), hsa-miR-9-5p (SEQ ID NO. 19), hsa-miR-193b-3p (SEQ ID NO. 23), hsa-miR-135b-5p (SEQ ID NO. 20), hsa-miR-187-3p (SEQ ID NO. 21), hsa-miR-192-5p (SEQ ID NO. 22), hsa-miR-210-3p (SEQ ID NO. 24), hsa-miR-29b-2-5p (SEQ ID NO. 25), hsa-miR-3065-3p (SEQ ID NO. 26), hsa-miR-375-3p (SEQ ID NO. 27) and hsa-miR-708-5p (SEQ ID NO. 28); or of each of the 7 mi-RNA having sequence hsa-miR-31-5p (SEQ ID NO. 12), hsa-miR-31-3p (SEQ ID NO. 11), hsa-miR-193b-3p (SEQ ID NO. 23), hsa-miR-193b-5p (SEQ ID NO. 3), hsa-miR-196b-5p (SEQ ID NO. 5), hsa-miR-550a-5p (SEQ ID NO. 15) and hsa-miR-584-5p (SEQ ID NO. 17)in a biological sample from a subject.

[0032] According to a further preferred embodiment, the method of the present invention further comprises step b) wherein the data obtained in step a) is normalized.

[0033] According to a further preferred embodiment, the method of the present invention further comprises step c) wherein the patients are classified either in the class of subjects affected by aggressive lung adenocarcinoma, or in the class of subjects affected by non-aggressive lung adenocarcinoma (nonC1-ADC).

[0034] Preferably, the class of subjects affected by aggressive lung adenocarcinoma comprises patients at an early-stage disease (stage I).

[0035] More preferably, the lung adenocarcinoma is a non-small cell lung adenocarcinoma (NSCLC).

[0036] According to the present invention, the detection of said miRNA is performed by means on hybridization with primers and/or probes, each one selective for the sequence of one miRNA.

[0037] According to a preferred embodiment of the present invention, the quantity of said miRNAs in step a) is calculated by quantitative RT-PCR (qRT-PCR), digital PCR, RNA sequencing, Affymetrix microarray, custom microarray or digital detection through molecular barcoding, selected from NanoString technology.

[0038] Preferably, the quantitative RT-PCR (qRT-PCR) of step a) is performed by using specific primers and/or probers for each of miRNA to be detected.

[0039] Preferably, said specific primers and probes are designed in order to retro-transcribe (i.e. RT reaction) and then amplify (i.e. qPCR) each miRNA present in the 19-miRNA, 14-miRNA o 7-miRNA signature. The RT reaction can be based on 3' poly-A tailing and 5' ligation of an adaptor sequence to extend the mature miRNAs present in the sample [16], or by using a miRNA specific stem-loop primers [17].

[0040] Preferably, miRNA quantities are measured as total RNA, comprising mRNA and miRNA, extracted using conventional RNA extraction methods, selected from AllPrep DNA/RNA FFPE kit (QIAGEN) or other RNA extraction methods from FFPE blocks and RNA extractions methods from other body fluid samples or tissue samples.

[0041] Preferably, the RNA sequencing is performed using 10ng of total RNA, from which miRNAs are selected according to size. MiRNA sequencing libraries are constructed ligating miRNAs with specific sequencing adapters and converting them into cDNA. Sequencing by synthesis, using preferably Illumina sequencing platform, is then applied to the miRNA library preparation.

[0042] Preferably, qRT-PCR analysis is performed using 10 ng of total RNA which is reverse-transcribed using 3' poly-A tailing and 5' ligation of an adaptor sequence, or using specific stem-loop primers, followed by qRT-PCR analysis using miRNA specific primers and probes selected for any miRNA signature analyzed according to the present invention.

[0043] More preferable, the qRT-PCR analysis is performed by using the TaqMan Advanced miRNA cDNA Synthesis Kit (ThermoFisher) and TaqMan Advanced miRNA Assays or with an analogue method of quantitative RT-PCR, by using the specific primers and probes selected for any miRNA signature analyzed according to the present invention

[0044] Preferably, Poly(A) tailing, adapter ligation, RT reaction and miR-Amp are performed following instructions of TaqMan Advanced miRNA Assay (ThermoFisher) or of an analogue method.

[0045] Preferably, the hsa-miR16-5p (MIMAT0000069; SEQ ID N: 29 UAGCAGCACGUAAAUAUUGGCG) is used as standard reference in the qRT-PCR reaction.

[0046] According to the present invention, the normalization of the data reported in step b) is made according to the scheme reported in below, when the miRNAs are quantified by RNA sequencing:

	RNA sequencing		
	For 14-miRNAs signature	**For 19-miRNAs signature**	**For 7-miRNA signature**
STEP b-raw data normalization	For miRNA *i* in sample *j*: Normalized $count_{ij}$=raw $count_{ij}$/$SizeFactor_j$ Where: $SizeFactor_j$=$median_j$	For miRNA *i* in sample *j*: Normalized $count_{ij}$=raw $count_{ij}$/$SizeFactor_j$ Where: $SizeFactor_j$=$median_j$	For miRNA *i* in sample *j*: Normalized $count_{ij}$=raw $count_{ij}$/$SizeFactor_j$ Where: $SizeFactor_j$=$median_j$
	(across all $RatioFactor_{ij}$), $RatioFactor_{ij}$=raw $count_{ij}$/ $geomean_i$ (across all $samples_{ij}$)	(across all $RatioFactor_{ij}$), $RatioFactor_{ij}$=raw $count_{ij}$/ $geomean_i$ (across all $samples_{ij}$)	(across all $RatioFactor_{ij}$), $RatioFactor_{ij}$=raw $count_{ij}$/ $geomean_i$ (across all $samples_{ij}$)

wherein

- the normalized $count_{ij}$ of each miRNA (*i*) of each sample (*j*) is calculated as ratio of the raw $count_{ij}$ and a $size\ factor_j$ specific for each $sample_j$; the $size\ factor_j$ specific for each sample is calculated as the median of all $ratio\ factors_{ij}$ of that sample; $ratio\ factors_{ij}$ represents the ratio between raw $count_{ij}$ and the geometric $mean_j$ of all raw $count_{ij}$ relative to a specific miRNA $_j$,

[0047] According to the present invention, the normalization of the data reported in step b) is made according to the scheme reported in below, when the miRNAs are quantified by RT-PCR:

	qRT-PCR		
	For 14-miRNAs signature	**For 19-miRNAs signature**	**For 7-miRNAs signature**
STEP b-raw data normalization	For miRNA *i* in sample *j*: Normalized Ct_{ij}=raw Ct_{ij}-SF_j where SF_j=hsa-miR-16-5p$_j$-21.87	For miRNA *i* in sample *j*: Normalized Ct_{ij}=raw Ct_{ij}-SF_j where SF_j=hsa-miR-16-5p$_j$-21.87	For miRNA *i* in sample *j*: Normalized Ct_{ij}=raw Ct_{ij}-SF_j where SF_j=hsa-miR-16-5p$_j$-21.87

wherein:

- the normalized Ct_{ij} (cycle threshold) of each miRNA (*i*) of each sample (*j*) is calculated as difference between the raw Ct_{ij} and a $scaling\ factor_j$ (SF) specific for each $sample_j$; the scaling factor represents the difference between the raw Ct of the miRNA "hsa-miR-16-5p" used as a reference in the sample and a constant equal to 21.87.

[0048] According to the present invention, the normalization of the raw data reported in step b) when the miRNAs are quantified by Affymetrix microarray is made according to Gene Chip miRNA Arrays, where Affymetrix oligonucleotide microarrays are used to interrogate the expression of all mature miRNA sequences in last miRBase Release. According to the present invention, the normalization of the raw data reported in step b) when the miRNAs are quantified by direct digital quantification (e.g. Nanostring, nCounter analysis) is made according to digital color-coded barcode technology that is based on direct multiplexed measurement of miRNA expression.

[0049] According to the method of the present invention, the classification of the predicted class of subjects affected by aggressive lung adenocarcinoma or of subjects affected by non-aggressive lung adenocarcinoma of step c) is calculated by the following formula:

$$\text{predicted class} = \begin{Bmatrix} \text{C1}, & \frac{1}{1+e^{-z}} \geq 0.5 \\ \text{nonC1}, & \frac{1}{1+e^{-z}} < 0.5 \end{Bmatrix}$$

wherein

- in a model of 19-miRNAs analyzed by RNA sequencing:

$$z = -8.2029+(-0.2651*\text{hsa-let-7c-3p})+(0.1709*\text{hsa-miR-138-5p})+(0.1443*\text{hsa-miR-193b-5p})+(0.0200*\text{hsa-miR-196a-5p})+(0.0464*\text{hsa-miR-196b-5p})+(0.2297*\text{hsa-miR-203a-3p})+(0.1285*\text{hsa-miR-215-5p})+(0.3933*\text{hsa-miR-2355-3p})+(-0.2220*\text{hsa-miR-30d-3p})+(-0.1874*\text{hsa-miR-30d-5p})+(0.1535*\text{hsa-miR-31-3p})+(0.0326*\text{hsa-miR-31-5p})+(-0.3032*\text{hsa-miR-4709-3p})+(-0.1672*\text{hsa-miR-548b-3p})+(0.1529*\text{hsa-miR-550a-5p})+(0.1132*\text{hsa-miR-582-3p})+(0.5229*\text{hsa-miR-584-5p})+(0.1314*\text{hsa-miR-675-3p})+(0.0497*\text{hsa-miR-9-5p});$$

- in a model of 14-miRNAs analyzed by RNA sequencing:

$$z = -5.4414+(-0.1028*\text{hsa-miR-135b-5p})+(-0.0486*\text{hsa-miR-187-3p})+(0.2828*\text{hsa-miR-192-5p})+(0.1977*\text{hsa-miR-193b-3p})+(0.0201*\text{hsa-miR-196b-5p})+(0.1908*\text{hsa-miR-210-3p})+(-0.5074*\text{hsa-miR-29b-2-5p})+(0.0384*\text{hsa-miR-3065-3p})+(-0.3312*\text{hsa-miR-30d-5p})+(-0.0475*\text{hsa-miR-375-3p})+(0.1895*\text{hsa-miR-582-3p})+(0.5606*\text{hsa-miR-584-5p})+(0.2663*\text{hsa-miR-708-5p})+(0.0435*\text{hsa-miR-9-5p});$$

and

- in a model 7-miRNAs analyzed by RNA sequencing:

$$z = -8.5210+(0.1171*\text{hsa-miR-193b-3p})+(0.2233*\text{hsa-miR-193b-5p})+(0.1341*\text{hsa-miR-196b-5p})+(0.1554*\text{hsa-miR-31-3p})+(0.0584*\text{hsa-miR-31-5p})+(0.3622*\text{hsa-miR-550a-5p})+(0.4683*\text{hsa-miR-584-5p});$$

or in alternative,

- in a model of 7-miRNAs analyzed by qRT-PCR :

$$z= 2.2920 +(\text{hsa-miR-193b-3p}*0.1295)+(\text{hsa-miR-193b-5p}*0.0920)+(\text{hsa-miR-196b-5p}*-0.1310)+(\text{hsa-miR-31-3p}*-0.2116)+(\text{hsa-miR-31-5p}*-0.2724)+(\text{hsa-miR-550a-5p}*0.2717)+(\text{hsa-miR-584-5p}*0.0413).$$

[0050] According to a preferred embodiment, step c) classify the patients in the predicted class of subjects affected by aggressive lung adenocarcinoma which can include, but not limited to, early-stage disease (stage I).

[0051] According to a preferred embodiment, the method of the present invention identifies patients included in the group of aggressive lung adenocarcinoma with a poor-prognosis, selected from patients with shorter overall survival and and/or patients with shorter disease-free survival, and/or patients responsive to a treatment, and/or with patients

with metastatic disease which can include, but not limited to, patients with early-stage disease (stage I).

[0052] According to a further preferred embodiment, the method of the present invention identifies patients included in the group of non aggressive lung adenocarcinoma with a good-prognosis, selected from patients with longer overall survival, and/or patients with longer disease-free survival, and/or patients responsive to treatment, patients and/or without metastatic disease which can include, but not limited to, patients with early-stage disease (stage I).

[0053] According to a further preferred embodiment, the method of the present invention is used for the prognostic risk stratification of patients with lung adenocarcinoma and/or to identify alternative therapeutic options after surgery, selected from systemic adjuvant chemotherapy, selected from platinum-based combinations, preferably cisplatin, carboplatin plus a third generation agents such as gemcitabine, vinorelbine, a taxane or camptothecin, molecular targeted therapeutics, immunoterapeutics, radiotherapy, or a combination thereof.

[0054] Preferably, in the method of the present invention the biological sample is a tissue sample or a body fluid.

[0055] More preferably said tissue sample is a fresh tissue sample, a frozen tissue sample or a FFPE tissue sample.

[0056] More preferably said body fluid is serum or plasma.

[0057] A further embodiment is a microarray, a quantitative polymerase chain reaction, a sequencing-based technology or a digital molecular barcoding-based technology, to perform the method according to the present invention.

[0058] A further embodiment is a kit to perform the method according to the present invention, comprising a multi-well plate and specific primers and/or probers for each of miRNAs to be detected.

[0059] Preferably the primers and probes used in the method of the present invention to amplify each of miRNAs to be detected correspond to the primers and probes used in the assays listed in Table 12.

Results

1. miRNA-signature identification

[0060] We developed a multi-tiered approach summarized in Figure 1, which allowed us to identify a surrogate miRNA-based signature for prognostication of ADC patients.

Table 1. Patients and tumors characteristics.

	TCGA-LUAD cohort N=515	CSS cohort N=44
Age [years]		
Median (first quartile;third quartile)	66 (59;73)[1]	73 (67;77)
Gender		
Male	238 (46.2%)	27 (61.4%)
Female	277 (53.8%)	17 (38.6%)
Smoking status		
Current/former smoker	367 (71.3%)	20 (45.5%)
Never smoker	63 (12.2%)	11 (25.0%)
Missing smoking status	85 (16.5%)	13 (29.5%)
Stage		
Stage I	279 (54.2%)	31 (70.5%)[2]
Stage II-IV	235 (45.6%)	13 (29.5%)
Missing stage	1 (0.2)	-
Follow-up[3]		
Survivors length of follow-up		
<1 yr	52 (10.3%)	13(31.7%)
1-2 yrs	128 (25.3%)	11 (26.8%)
2-3 yrs	56 (11.1%)	10 (24.4%)
>3 yrs	133 (26.3%)	5 (12.2%)

(continued)

Follow-up[3]		
Deaths within 3 years	137 (27.1%)	2 (4.9%)[4]
Percentages could not add up to 100 due to rounding; [1] 19 patients with missing information on age; [2] 1 patient with adenocarcinoma *in situ;* [3] 9 patients with missing follow-up in the TCGA-LUAD cohort; [4] 3 deaths were excluded: 1 without date of dead, and 2 within 30 days from surgery.		

[0061] Hierarchical clustering analysis using the 10-gene signature of the TCGA-LUAD cohort (N=515) patients revealed 4 main branches, namely C1 (N=201), C2 (N=98), C3 (N=39), and C4 (N=177) clusters (Figure 2a) that are consistent with previous findings [6]. Analysis of the 3-years overall survival showed non-significant differences between C2, C3 and C4 clusters (log-rank test p-value=0.90 and p=0.48 in stage I and advanced stages, respectively), that were therefore collapsed into nonC1 clusters. C1 patients displayed the worse prognosis both in stage I (p-value=0.0010) and in more advanced stages (p=0.0061) (Figure 2b).

[0062] We then performed miRNA expression profile of 510 out of the 515 ADC of the TCGA-LUAD cohort, with miRNAs expression data available. We used both DESeq2 R package and BRB-ArrayTools (see methods) as alternative statistical approaches in order to identify differentially expressed miRNAs in C1 and nonC1 patients. We analyzed a total of 382 miRNAs of which 200 were found differentially expressed by DESeq2 and 90 by BRB-ArrayTools (Table S1A and Table S1B, respectively, see Figures 5 and 6).

[0063] A total of 87 miRNAs were overlapping in the two sets. Lasso regularization was then applied to identify optimized miRNA-based signatures capable of stratifying C1 from nonC1 tumors. Two signatures of 14-miRNA (from the 90 miRNA set) and 19-miRNA (from the 200 miRNA set) were derived (5 miRNA overlapping; Table 2), which displayed an high accuracy in C1/nonC1 cancer patients stratification (cross-validated AUC=0.81 and AUC=0.85, respectively; Figure 2c).

[0064] To further reduce complexity of these miRNA-based biomarkers, we looked for a minimal set of miRNAs capable of the same accuracy of the 14- and 19-miRNA signatures to identify C1 aggressive disease.

[0065] The following assumptionts were made: i) the molecular function of a miRNA is dependent to the network of targeted mRNAs which, in this case, are those differentially expressed in C1/nonC1 tumors; ii) a prognostic biomarkers should be functionally linked to mechanisms involved in tumor progression. Accordingly, we explored the miRNA-mRNA interactome characterizing C1 tumors by performing ARACNe (Algorithm for the Reconstruction of Accurate Cellular Networks)(see methods) using the set of 200 miRNA, and a set of 2900 mRNA genes found significantly regulated in C1-ADC ($p<0.05$) by DESeq2 (see methods). Our analysis was restricted to genes identified by DESeq2 in order to reduce technical variability.

[0066] The following rules were applied to rewire C1 miRNA-mRNA interactome: 1) we selected miRNA-mRNA pairs generated in only C1 tumors and specific, but not exclusive, for stage I (N=2858); 2) we selected miRNA predicted to target C1-genes (N=1787), and 3) with an opposite trend of expression than C1-genes (N=598); 4) we selected miRNA interacting with a least three C1-genes (N=528).

[0067] Among the miRNA-mRNA networks identified, we found a set of interacting networks with 7 miRNA as "HUBs" which derived from both the 19-miRNA and 14-miRNA signatures (Table 2 and Figure 2d). Hierarchical clustering analysis of this 7-miRNA signature (Table S2) showed an overall increased expression in the more aggresive C1 tumors (Figure 2e). Importantly, the 7-miRNA signature had a cross-validated AUC of 0.79 in C1/nonC1 patients stratification, which is comparable to the other two signatures (Figure 3a) as well as when we considered differences in C1 predicted probability (Figure 3b). The predicted C1 class from all the 3 signatures (7-, 14- and 19-miRNA) presented significantly increased hazard of death at 3 years in patients of all stages, with an increased risk comparable to the C1 patients identified by the 10 genes (Table 3). However, when we focused the analysis to stage I ADC patients, we scored that the best risk-stratification was held by the 7-miRNA signature with approximately 2-fold increased risk of death for C1 patients (HR=2.11; 95% Confidence Interval: 1.11-4.00; p=0.0223) (Table 3). Interestingly enough, the networks of genes targeted by these 7 miRNAs were found significantly (FDR q-value<0.0001) enriched in gene sets representing molecular mechanisms related to cancer progression, which fulfilled our initial hypotheses (Figure 3c).

[0068] Despite most of 90 miRNAs identified by BRB-ArrayTools (87/90, 97%) were comprised in the 200-miRNA set found by DESeq2, including 12 out of 14 miRNAs of the BRB-derived model, we performed ARACNe as well by using this 90-miRNAs set. Among the three not overlapping miRNAs, only hsa-miR-210-3p passed all the selection filters we described previously. However, when we added this additional miRNA to the 7-miRNA signature and perfomed cross-validation in C1/nonC1 patients stratification, the prediction performance remained the same (i.e. AUC=0.79).

Table 2. TCGA-LUAD cohort. Differentially expressed miRNAs composing the 3 signatures of 19, 14 and 7 miRNAs.

miRNA	Signature	*TCGA-LUAD cohort - C1 vs nonC1 patients*		
		Fold Change	Wald test adjusted p-value[1]	C1 regulation
hsa-mi R-193b-5p	19- and 7-miRNA	1.5	3.3E-07	↑
hsa-miR-31-3p	19- and 7-miRNA	3.2	1.9E-20	↑
hsa-miR-31-5p	19- and 7-miRNA	3.1	1.7E-18	↑
hsa-mi R-550a-5p	19- and 7-miRNA	1.5	6.0E-09	↑
hsa-mi R-196b-5p	19-, 14-miRNA and 7-miRNA	3.2	9.8E-21	↑
hsa-miR-584-5p	19-, 14-miRNA and 7-miRNA	2.8	1.2E-40	↑
hsa-miR-30d-5p	19- and 14-miRNA	0.6	4.8E-16	↓
hsa-miR-582-3p	19- and 14-miRNA	2.2	2.5E-18	↑
hsa-miR-9-5p	19 and 14-miRNA	1.8	1.7E-06	↑
hsa-let-7c-3p	19-miRNA	0.8	1.9E-02	↓
hsa-mi R-138-5p	19-miRNA	1.9	1.2E-10	↑
hsa-miR-196a-5p	19-miRNA	1.4	2.7E-02	↑
hsa-miR-203a-3p	19-miRNA	1.4	3.1 E-04	↑
hsa-miR-215-5p	19-miRNA	5.0	1.2E-37	↑
hsa-miR-2355-3p	19-miRNA	1.3	5.4E-05	↑
hsa-miR-30d-3p	19-miRNA	0.6	2.5E-15	↓
hsa-miR-4709-3p	19-miRNA	0.5	1.3E-19	↓
hsa-miR-548b-3p	19-miRNA	0.6	7.2E-10	↓
hsa-miR-675-3p	19-miRNA	2.1	1.5E-08	↑
hsa-miR-193b-3p	14- and 7-miRNA	1.4	8.6E-06	↑
hsa-miR-135b-5p	14-miRNA	0.7	3.7E-06	↓
hsa-miR-187-3p	14-miRNA	0.6	2.3E-04	↓
hsa-mi R-192-5p	14-miRNA	3.1	9.8E-21	↑
hsa-miR-210-3p	14-miRNA	1.2	6.4E-02	↑
hsa-miR-29b-2-5p	14-miRNA	0.7	1.2E-07	↓
hsa-miR-3065-3p	14-miRNA	0.7	4.2E-05	↓
hsa-miR-375-3p	14-miRNA	1.2	1.7E-01	↑
hsa-miR-708-5p	14-miRNA	1.3	2.7E-03	↑
[1] Benjamini-Hochberg method from DESeq2 tool				

Table S2. Distributions of expression for the 7 miRNAs in TCGA-LUAD and CSS cohorts.

	TCGA-LUAD N=510			*CSS cohort N=44*		
	normalized counts (log2)			*normalized CT*		
miRNA	**Median**	**Q1**	**Q3**	**Median**	**Q1**	**Q3**
hsa-miR-193b-3p	7.70	6.90	8.62	23.58	21.94	24.38
hsa-miR-193b-5p	4.27	3.55	5.00	30.44	24.59	36.55
hsa-miR-196b-5p	6.42	5.14	8.67	29.10	27.96	30.19
hsa-miR-31-3p	2.94	1.53	4.95	26.40	25.14	27.53
hsa-miR-31-5p	3.62	1.87	5.75	26.88	24.21	28.15
hsa-miR-550a-5p	4.01	3.33	4.67	24.86	23.89	25.83
hsa-miR-584-5p	6.46	5.87	7.29	23.02	21.64	24.72
Q1, first quartile of distribution; Q3, third quartile of distribution						

Table 3. TCGA-LUAD cohort. Univariate and multivariable Cox regression analyses for 3-years overall survival in patients of all stages and stratified by stage. Hazard Ratio (HR) and 95% Confidence Interval (95% CI) are reported.

		Univariate analysis		**Multivariable analysis[1]**	
	N (Number of deaths)	**HR (95% CI)**	**Wald test p-value**	**HR (95% CI)**	**Wald test p-value**
ALL STAGES	**501 (135)[2]**				
10-gene	194 (75)	2.21 (1.57-3.10)	<0.0001	2.03 (1.43-2.87)	<0.0001
19-miRNA	169 (66)	2.13 (1.52-2.99)	<0.0001	1.85 (1.31-2.61)	0.0005
14-miRNA	165 (67)	2.17 (1.55-3.04)	<0.0001	2.06 (1.46-2.91)	<0.0001
7-miRNA	146 (67)	2.90 (2.07-4.06)	<0.0001	2.69 (1.91-3.78)	<0.0001
STAGE I	**274 (40)**				
10-gene	92 (23)	2.86 (1.53-5.36)	0.0010	2.96 (1.55-5.65)	0.0010
19-miRNA	73 (11)	1.07 (0.54-2.15)	0.8462	1.12 (0.55-2.26)	0.7529
14-miRNA	79 (17)	1.90 (1.01-3.56)	0.0451	1.99 (1.05-3.79)	0.0359
7-miRNA	65 (15)	2.11 (1.11-4.00)	0.0223	2.14 (1.11-4.12)	0.0235
STAGE II-IV	**226 (95)**				
10-gene	101 (52)	1.69 (1.13-2.54)	0.0108	1.64 (1.08-2.49)	0.0207
19-miRNA	95 (55)	2.27 (1.51-3.41)	<0.0001	2.18 (1.43-3.31)	0.0003
14-miRNA	86 (50)	2.00 (1.34-3.00)	0.0007	2.04 (1.35-3.08)	0.0007
7-miRNA	80 (52)	2.89 (1.93-4.33)	<0.0001	2.91 (1.93-4.39)	<0.0001
[1] all stages analyses were adjusted for age, sex, smoking status and stage; analyses stratified by stage were adjusted for age, sex and smoking status; [2]1 patient with missing stage and 9 patients with missing follow-up.					

2. Seven-miRNA-signature validation

[0069] Finally, we performed a validation of the 7 miRNA-signature in an external cohort of 44 lung adenocarcinoma patients, which was collected at the IRCCS Casa Sollievo della Sofferenza Hospital (CSS). Table 1 shows patients and tumors characteristics of CSS cohort, highlighting an overrepresentation of stage I tumors in CSS (70%) with respect

to the TCGA-LUAD cohort (54%). We performed qRT-PCR analysis of FFPE samples using the 10-gene signature and calculated relative risk-score to stratify the cohort into a C1 (N=16) and nonC1 (N=28) groups (Figure 3d) (see methods). Next, we performed qRT-PCR analysis using TaqMan Advanced miRNA Assay to profile also the 7-miRNA signature in the same cohort of 44 ADC and, using logistic regression, we rederived a model based on the expression profile of the 7-miRNA signature (Table S2). The 7-miRNA model stratified C1 from nonC1 tumors with an AUC of 0.76 (Figure 3e) and with significant difference (p=0.0028) in C1 predicted probability (Figure 3f). Remarkably, when we limited the analysis to stage I tumors, we scored an AUC of 0.81 (Figure 3e) and a significant difference (p=0.0108) in C1 predicted probability (Figure 3f). Tables 4-7 reported below presents the details of the miRNAs signatures identified, their accession number (mirbase.org), their sequences, the regulation trends (C1 versus nonC1) and the model weight used.

Table 4. The 19-miRNA signature (detected by RNA sequencing).

Assay refers to the code used to identify each miRNA in the TCGA-LUAD RNA sequencing experiment; Acc. mature-miRNA refers to the miRBase accession number of the mature miRNA; Sequence refers to the nucleotide sequence of the mature miRNA; miRbase ID refers to the name of the mature miRNA in the miRbase database; Ratio refers to the fold change calculated dividing the median expression of each miRNA in C1-ADC samples by the median expression in nonC1-ADC samples; p-values were calculated from Wald test of DESeq2 comparing C1-ADC vs. nonC1-ADC samples. All miRNAs have q-value < 0.05 based on Benjamini-Hochberg adjustment; Model weight refers to the coefficient in the equation used to calculate the predicted class.

The 19-miRNA signature (RNA-seq)						
Assay	**Acc. mature-miRNA**	**Sequence (SEQ ID N.)**	**miRbase ID**	**Ratio**	**P-value**	**Model weight**
hsa-let-7c-3p	MIMAT0026472	CUGUACAACCUUCUAGCUUUCC (SEQ ID NO. 1)	hsa-let-7c-3p	0.82	8.45E-03	-0.2651
hsa-miR-138-5p	MIMAT0000430	AGCUGGUGUUGUGAAUCAGGCCG (SEQ ID NO. 2)	hsa-miR-138-5p	1.76	5.81E-12	0.1709
hsa-miR-193b-5p	MIMAT0004767	CGGGGUUUUGAGGGCGAGAUGA (SEQ ID NO. 3)	hsa-miR-193b-5p	1.44	3.01E-08	0.1443
hsa-miR-196a-5p	MIMAT0000226	UAGGUAGUUUCAUGUUGUUGGG (SEQ ID NO. 4)	hsa-miR-196a-5p	2.82	1.28E-02	0.0200
hsa-miR-196b-5p	MIMAT0001080	UAGGUAGUUUCCUGUUGUUGGG (SEQ ID NO. 5)	hsa-miR-196b-5p	3.15	1.28E-22	0.0464
hsa-miR-203a-3p	MIMAT0000264	GUGAAAUGUUUAGGACCACUAG (SEQ ID NO. 6)	hsa-miR-203a-3p	1.48	7.33E-05	0.2297
hsa-miR-215-5p	MIMAT0000272	AUGACCUAUGAAUUGACAGAC (SEQ ID NO. 7)	hsa-miR-215-5p	1.57	6.34E-40	0.1285
hsa-miR-2355-3p	MIMAT0017950	AUUGUCCUUGCUGUUUGGAGAU (SEQ ID NO. 8)	hsa-miR-2355-3p	1.34	9.39E-06	0.3933
hsa-miR-30d-3p	MIMAT0004551	CUUUCAGUCAGAUGUUUGCUGC (SEQ ID NO. 9)	hsa-miR-30d-3p	0.63	7.06E-17	-0.2220
hsa-miR-30d-5p	MIMAT0000245	UGUAAACAUCCCCGACUGGAAG (SEQ ID NO. 10)	hsa-miR-30d-5p	0.57	1.25E-17	-0.1874
hsa-miR-31-3p	MIMAT0004504	UGCUAUGCCAACAUAUUGCCAU (SEQ ID NO. 11)	hsa-miR-31-3p	5.96	2.92E-22	0.1535
hsa-miR-31-5p	MIMAT0000089	AGGCAAGAUGCUGGCAUAGCU (SEQ ID NO. 12)	hsa-miR-31-5p	5.39	3.57E-20	0.0326
hsa-miR-4709-3p	MIMAT0019812	UUGAAGAGGAGGUGCUCUGUAGC (SEQ ID NO. 13)	hsa-miR-4709-3p	0.44	2.32E-21	-0.3032

(continued)

Assay refers to the code used to identify each miRNA in the TCGA-LUAD RNA sequencing experiment; Acc. mature-miRNA refers to the miRBase accession number of the mature miRNA; Sequence refers to the nucleotide sequence of the mature miRNA; miRbase ID refers to the name of the mature miRNA in the miRbase database; Ratio refers to the fold change calculated dividing the median expression of each miRNA in C1-ADC samples by the median expression in nonC1-ADC samples; p-values were calculated from Wald test of DESeq2 comparing C1-ADC vs. nonC1-ADC samples. All miRNAs have q-value < 0.05 based on Benjamini-Hochberg adjustment; Model weight refers to the coefficient in the equation used to calculate the predicted class.

The 19-miRNA signature (RNA-seq)

Assay	Acc. mature-miRNA	Sequence (SEQ ID N.)	miRbase ID	Ratio	P-value	Model weight
hsa-miR-548b-3p	MIMAT0003254	CAAGAACCUCAGUUGCUUUU GU (SEQ ID NO. 14)	hsa-miR-548b-3p	0.54	3.59E-11	-0.1672
hsa-miR-550a-5p	MIMAT0004800	AGUGCCUGAGGGAGUAAGAG CCC (SEQ ID NO. 15)	hsa-miR-550a-5p	1.48	3.60E-10	0.1529
hsa-miR-582-3p	MIMAT0004797	UAACUGGUUGAACAACUGAA CC (SEQ ID NO. 16)	hsa-miR-582-3p	1.45	5.92E-20	0.1132
hsa-miR-584-5p	MIMAT0003249	UUAUGGUUUGCCUGGGACUG AG (SEQ ID NO. 17)	hsa-miR-584-5p	1.50	3.24E-43	0.5229
hsa-miR-675-3p	MIMAT0006790	CUGUAUGCCCUCACCGCUCA (SEQ ID NO. 18)	hsa-miR-675-3p	1.75	1.07E-09	0.1314
hsa-miR-9-5p	MIMAT0000441	UCUUUGGUUAUCUAGCUGUA UGA (SEQ ID NO. 19)	hsa-miR-9-5p	2.42	1.78E-07	0.0497

Table 5. The 14-miRNA signature (detected by RNA sequencing).

Assay refers to the code used to identify each miRNA in the TCGA-LUAD RNA sequencing experiment; Acc. mature-miRNA refers to the miRBase accession number of the mature miRNA; Sequence refers to the nucleotide sequence of the mature miRNA; miRbase ID refers to the name of the mature miRNA in the miRbase database; Ratio refers to the fold change calculated dividing the median expression of each miRNA in C1-ADC samples by the median expression in nonC1-ADC samples; p-values were calculated from parametric test of BRB-ArrayTools comparing C1-ADC vs. nonC1-ADC samples. All miRNAs have q-value < 0.05 based on 100 permutation; Model weight refers to the coefficient in the equation used to calculate the predicted class.

The 14-miRNA signature (RNA-seq)

Assay	Acc. mature-miRNA	Sequence	miRbase ID	Ratio	P-value	Model weight
hsa-miR-135b-5p	MIMAT0000758	UAUGGCUUUUCAUUCCUAU GUGA (SEQ ID NO. 20)	hsa-miR-135b-5p	0.62	4.40E-06	-0.1028
hsa-miR-187-3p	MIMAT0000262	UCGUGUCUUGUGUUGCAG CCGG (SEQ ID NO. 21)	hsa-miR-187-3p	0.56	1.42E-03	-0.0486
hsa-miR-192-5p	MIMAT0000222	CUGACCUAUGAAUUGACAG CC (SEQ ID NO. 22)	hsa-miR-192-5p	1.45	1.00E-07	0.2828
hsa-miR-193b-3p	MIMAT0002819	AACUGGCCCUCAAAGUCCC GCU (SEQ ID NO. 23)	hsa-miR-193b-3p	1.57	5.00E-07	0.1977
hsa-miR-196b-5p	MIMAT0001080	UAGGUAGUUUCCUGUUGU UGGG (SEQ ID NO. 5)	hsa-miR-196b-5p	3.15	1.00E-07	0.0201

(continued)

Assay refers to the code used to identify each miRNA in the TCGA-LUAD RNA sequencing experiment; Acc. mature-miRNA refers to the miRBase accession number of the mature miRNA; Sequence refers to the nucleotide sequence of the mature miRNA; miRbase ID refers to the name of the mature miRNA in the miRbase database; Ratio refers to the fold change calculated dividing the median expression of each miRNA in C1-ADC samples by the median expression in nonC1-ADC samples; p-values were calculated from parametric test of BRB-ArrayTools comparing C1-ADC vs. nonC1-ADC samples. All miRNAs have q-value < 0.05 based on 100 permutation; Model weight refers to the coefficient in the equation used to calculate the predicted class.

The 14-miRNA signature (RNA-seq)

Assay	Acc. mature-miRNA	Sequence	miRbase ID	Ratio	P-value	Model weight
hsa-miR-210-3p	MIMAT0000267	CUGUGCGUGUGACAGCGG CUGA (SEQ ID NO. 24)	hsa-miR-210-3p	1.43	4.06E-05	0.1908
hsa-miR-29b-2-5p	MIMAT0004515	CUGGUUUCACAUGGUGGC UUAG (SEQ ID NO. 25)	hsa-miR-29b-2-5p	0.72	1.00E-07	-0.5074
hsa-miR-3065-3p	MIMAT0015378	UCAGCACCAGGAUAUUGUU GGAG (SEQ ID NO. 26)	hsa-miR-3065-3p	0.56	1.40E-06	0.0384
hsa-miR-30d-5p	MIMAT0000245	UGUAAACAUCCCCGACUGG AAG (SEQ ID NO. 10)	hsa-miR-30d-5p	0.57	1.00E-07	-0.3312
hsa-miR-375-3p	MIMAT0000728	UUUGUUCGUUCGGCUCGC GUGA (SEQ ID NO. 27)	hsa-miR-375-3p	0.66	4.53E-05	-0.0475
hsa-miR-582-3p	MIMAT0004797	UAACUGGUUGAACAACUGA ACC (SEQ ID NO. 16)	hsa-miR-582-3p	1.45	1.00E-07	0.1895
hsa-miR-584-5p	MIMAT0003249	UUAUGGUUUGCCUGGGAC UGAG (SEQ ID NO. 17)	hsa-miR-584-5p	1.50	1.00E-07	0.5606
hsa-miR-708-5p	MIMAT0004926	AAGGAGCUUACAAUCUAGC UGGG (SEQ ID NO. 28)	hsa-miR-708-5p	1.28	2.18E-03	0.2663
hsa-miR-9-5p	MIMAT0000441	UCUUUGGUUAUCUAGCUG UAUGA (SEQ ID NO. 19)	hsa-miR-9-5p	2.42	7.40E-06	0.0435

Table 6. The 7-miRNA signature (detected by qRT-PCR).

Assay refers to the code used to identify each miRNA in the CSS qRT-PCR sequencing experiment; Acc. mature-miRNA refers to the miRBase accession number of the mature miRNA; Sequence refers to the nucleotide sequence of the mature miRNA; miRbase ID refers to the name of the mature miRNA in the miRbase database; Ratio refers to the fold change calculated dividing the median expression of each miRNA in C1-ADC samples by the median expression in nonC1-ADC samples; p-values were calculated from Wilcoxon test comparing C1-ADC vs. nonC1-ADC samples; Model weight refers to the coefficient in the equation used to calculate the predicted class.

The 7-miRNA signature (qRT-PCR)						
Assay	**Acc. mature-miRNA**	**Sequence**	**miRbase ID**	**Ratio**	**P-value**	**Model weight**
478314 mir	MIMAT0002819	AACUGGCCCUCAAAGUCCCGC U (SEQ ID NO. 23)	hsa-miR-193b-3p	0.79	0.8933	0.1295
478742_mir	MIMAT0004767	CGGGGUUUUGAGGGCGAGAU GA (SEQ ID NO. 3)	hsa-miR-193b-5p	0.005	0.0482	0.0920
478585_mir	MIMAT0001080	UAGGUAGUUUCCUGUUGUUG GG (SEQ ID NO. 5)	hsa-miR-196b-5p	1.60	0.2670	-0.1310
478012_mir	MIMAT0004504	UGCUAUGCCAACAUAUUGCCA U (SEQ ID NO. 11)	hsa-miR-31-3p	1.84	0.2089	-0.2116
478015_mir	MIMAT0000089	AGGCAAGAUGCUGGCAUAGCU (SEQ ID NO. 12)	hsa-miR-31-5p	6.89	0.0769	-0.2724
477852_mir	MIMAT0004800	AGUGCCUGAGGGAGUAAGAGC CC (SEQ ID NO. 15)	hsa-miR-550a-5p	1.19	0.5830	0.2717
478167_mir	MIMAT0003249	UUAUGGUUUGCCUGGGACUG AG (SEQ ID NO. 17)	hsa-miR-584-5p	0.60	0.1643	0.0413

Table 7. The miRNA signature (detected by RNA sequencing).

Assay refers to the code used to identify each miRNA in the TCGA-LUAD RNA sequencing experiment; Acc. mature-miRNA refers to the miRBase accession number of the mature miRNA; Sequence refers to the nucleotide sequence of the mature miRNA; miRbase ID refers to the name of the mature miRNA in the miRbase database; Ratio refers to the fold change calculated dividing the median expression of each miRNA in C1-ADC samples by the median expression in nonC1-ADC samples; p-values were calculated from Wald test of DESeq2 comparing C1-ADC vs. nonC1-ADC samples. All miRNAs have q-value < 0.05 based on Benjamini-Hochberg adjustment; Model weight refers to the coefficient in the equation used to calculate the predicted class.

The 7-miRNA signature (RNAseq)						
Assay	**Acc. mature-miRNA**	**Sequence**	**miRbase ID**	**Ratio**	**P-value**	**Model weight**
hsa-miR-193b-3p	MIMAT0002819	AACUGGCCCUCAAAGUCCCGCU (SEQ ID NO. 23)	hsa-miR-193b-3p	1.57	1.12E-06	0.1171
hsa-miR-193b-5p	MIMAT0004767	CGGGGUUUUGAGGGCGAGAUGA (SEQ ID NO. 3)	hsa-miR-193b-5p	1.44	3.01E-08	0.2233
hsa-miR-196b-5p	MIMAT0001080	UAGGUAGUUUCCUGUUGUUGGG (SEQ ID NO. 5)	hsa-miR-196b-5p	3.15	1.28E-22	0.1341
hsa-miR-31-3p	MIMAT0004504	UGCUAUGCCAACAUAUUGCCAU (SEQ ID NO. 11)	hsa-miR-31-3p	5.96	2.92E-22	0.1554
hsa-miR-31-5p	MIMAT0000089	AGGCAAGAUGCUGGCAUAGCU (SEQ ID NO. 12)	hsa-miR-31-5p	5.39	3.57E-20	0.0584
hsa-miR-550a-5p	MIMAT0004800	AGUGCCUGAGGGAGUAAGAGCCC (SEQ ID NO. 15)	hsa-miR-550a-5p	1.48	3.60E-10	0.3622
hsa-miR-584-5p	MIMAT0003249	UUAUGGUUUGCCUGGGACUGAG (SEQ ID NO. 17)	hsa-miR-584-5p	1.50	3.24E-43	0.4683

[0070] The parameters of logistic regression of the three signatures and the formula used to include the groups of patients in C1 or nonC1 group are represented here below:

Model 19-miRNA (RNA sequencing):

[0071]

z=-8.2029+(-0.2651*hsa-let-7c-3p)+(0.1709*hsa-miR-138-5p)+(0.1443*hsa-miR-193b-5p)+(0.0200*hsa-miR-196a-5p)+(0.0464*hsa-miR-196b-5p)+(0.2297*hsa-miR-203a-3p)+(0.1285*hsa-miR-215-5p)+(0.3933*hsa-miR-2355-3p)+(-0.2220*hsa-miR-30d-3p)+(-0.1874*hsa-miR-30d-5p)+(0.1535*hsa-miR-31-3p)+(0.0326*hsa-miR-31-5p)+(-0.3032*hsa-miR-4709-3p)+(-0.1672*hsa-miR-548b-3p)+(0.1529*hsa-miR-550a-5p)+(0.1132*hsa-miR-582-3p)+(0.5229*hsa-miR-584-5p)+(0.1314*hsa-miR-675-3p)+(0.0497*hsa-miR-9-5p)

Model 14-miRNA (RNA sequencing):

[0072]

z=-5.4414+(-0.1028*hsa-miR-135b-5p)+(-0.0486*hsa-miR-187-3p)+(0.2828*hsa-miR-192-5p)+(0.1977*hsa-miR-193b-3p)+(0.0201*hsa-miR-196b-5p)+(0.1908*hsa-miR-210-3p)+(-0.5074*hsa-miR-29b-2-5p)+(0.0384*hsa-miR-3065-3p)+(-0.3312*hsa-miR-30d-5p)+(-0.0475*hsa-miR-375-3p)+(0.1895*hsa-miR-582-3p)+(0.5606*hsa-miR-584-5p)+(0.2663*hsa-miR-708-5p)+(0.0435*hsa-miR-9-5p)

Model 7-miRNA (RNA sequencing):

[0073]

z=-8.5210+(0.1171*hsa-miR-193b-3p)+(0.2233*hsa-miR-193b-5p)+(0.1341*hsa-miR-196b-5p)+(0.1554*hsa-miR-31-3p)+(0.0584*hsa-miR-31-5p)+(0.3622*hsa-miR-550a-5p)+(0.4683*hsa-miR-584-5p)

Model 7-miRNA (qRT-PCR):

[0074]

z=2.2920 +(hsa-miR-193b-3p*0.1295)+(hsa-miR-193b-5p*0.0920)+(hsa-miR-196b-5p*-0.1310)+(hsa-miR-31-3p*-0.2116)+(hsa-miR-31-5p*-0.2724)+(hsa-miR-550a-5p*0.2717)+(hsa-miR-584-5p*0.0413)

[0075] The formula shown herein is used to identify the predicted class of patient selected by using the models reported above:

$$Predicted\ class = \begin{cases} C1, & \frac{1}{1+e^{-z}} \geq 0.5 \\ nonC1, & \frac{1}{1+e^{-z}} < 0.5 \end{cases}$$

4. Evaluation of the AUC obtained from other 14- and 19-miRNAs signatures.

[0076] To demonstrate that the 14- and 19-miRNAs signatures, from which the 7 miRNAs signature derived, are the most effective to identify the more aggressive subtype of lung cancer (C1), we compared the AUC of the 14- and 19-miRNA models to the expected AUC distribution derived from random signatures of equal lengths (14 and 19 miRNAs length) (Figure 4). In particular, we generated:

- 100 random lists of 14 miRNAs from 292 miRNAs not differentially expressed according to BRB-Array Tools (Table 8);
- 100 random lists of 14 miRNAs from 76 miRNAs differentially expressed according to BRB-Array Tools excluding the 14 miRNAs of the model (Table 9);

- 100 random lists of 19 miRNAs from 182 miRNAs not differentially expressed according to DESeq2 tool (Table 10);

- 100 random lists of 19 miRNAs from 181 miRNAs differentially expressed according to DESeq2 tool excluding the 19 miRNAs of the model (Table 11).

Table 8. List of 292 miRNAs not differentially expressed according to BRB-Array Tools.

miRNAID				
hsa-miR-215-5p	hsa-miR-22-3p	hsa-miR-501-3p	hsa-miR-324-3p	hsa-let-7a-2-3p
hsa-miR-4709-3p	hsa-miR-222-3p	hsa-miR-27a-5p	hsa-miR-378a-5p	hsa-miR-133a-3p
hsa-miR-30d-3p	hsa-miR-99b-3p	hsa-miR-454-3p	hsa-miR-503-5p	hsa-miR-93-3p
hsa-miR-138-5p	hsa-mi R-500b-5p	hsa-miR-758-3p	hsa-miR-320a	hsa-miR-3934-3p
hsa-miR-548b-3p	hsa-miR-212-3p	hsa-miR-3615	hsa-miR-16-2-3p	hsa-miR-224-3p
hsa-miR-675-3p	hsa-mi R-136-5p	hsa-miR-653-5p	hsa-miR-30a-5p	hsa-miR-25-3p
hsa-miR-2355-3p	hsa-miR-758-5p	hsa-let-7f-5p	hsa-let-7b-3p	hsa-miR-15a-5p
hsa-miR-203a-3p	hsa-mi R-500a-5p	hsa-let-7d-5p	hsa-miR-3913-5p	hsa-miR-642a-5p
hsa-let-7c-3p	hsa-miR-532-3p	hsa-miR-382-5p	hsa-let-7a-5p	hsa-miR-1-3p
hsa-miR-196a-5p	hsa-miR-509-3p	hsa-miR-151a-3p	hsa-let-7b-5p	hsa-miR-1180-3p
hsa-miR-31-3p	hsa-miR-3065-5p	hsa-miR-221-5p	hsa-miR-214-5p	hsa-miR-877-5p
hsa-miR-31-5p	hsa-miR-577	hsa-miR-424-3p	hsa-miR-200c-3p	hsa-miR-221-3p
hsa-miR-550a-5p	hsa-miR-1247-3p	hsa-miR-24-3p	hsa-miR-374b-5p	hsa-miR-363-3p
hsa-miR-193b-5p	hsa-mi R-132-5p	hsa-miR-744-3p	hsa-miR-3607-3p	hsa-miR-493-3p
hsa-miR-664b-3p	hsa-miR-1287-3p	hsa-miR-369-3p	hsa-miR-142-3p	hsa-miR-337-3p
hsa-miR-153-5p	hsa-miR-217	hsa-miR-200a-3p	hsa-miR-451a	hsa-miR-32-5p
hsa-miR-664a-5p	hsa-miR-106a-5p	hsa-miR-3130-5p	hsa-miR-143-3p	hsa-miR-5698
hsa-miR-421	hsa-miR-17-5p	hsa-miR-342-5p	hsa-miR-150-3p	hsa-miR-26b-5p
hsa-miR-582-5p	hsa-miR-508-3p	hsa-miR-214-3p	hsa-miR-20a-5p	hsa-miR-99b-5p
hsa-miR-18a-5p	hsa-miR-514a-3p	hsa-miR-574-3p	hsa-miR-361-3p	hsa-miR-889-3p
hsa-miR-432-5p	hsa-miR-455-5p	hsa-let-7e-3p	hsa-miR-425-3p	hsa-miR-185-5p
hsa-miR-942-5p	hsa-miR-590-3p	hsa-miR-5586-5p	hsa-miR-30c-2-3p	hsa-miR-3127-5p
hsa-miR-3677-3p	hsa-mi R-3074-5p	hsa-miR-16-5p	hsa-miR-33a-5p	hsa-miR-28-3p
hsa-miR-362-5p	hsa-miR-181a-2-3p	hsa-miR-378a-3p	hsa-miR-144-5p	hsa-miR-370-3p
hsa-mi R-766-3p	hsa-mi R-141-5p	hsa-miR-103a-3p	hsa-miR-199a-5p	hsa-miR-139-3p
hsa-miR-1306-5p	hsa-mi R-29a-5p	hsa-miR-505-5p	hsa-miR-598-3p	hsa-miR-98-5p
hsa-miR-30b-3p	hsa-miR-410-3p	hsa-miR-3200-3p	hsa-let-7f-1-3p	hsa-miR-27b-5p
hsa-miR-320b	hsa-miR-625-5p	hsa-miR-181d-5p	hsa-miR-301a-5p	hsa-miR-10b-5p
hsa-miR-101-5p	hsa-miR-4326	hsa-miR-378c	hsa-miR-452-3p	hsa-miR-96-5p
hsa-miR-629-3p	hsa-mi R-149-5p	hsa-miR-15b-5p	hsa-miR-19b-1-5p	hsa-miR-429
hsa-miR-362-3p	hsa-miR-1307-5p	hsa-miR-3614-5p	hsa-miR-224-5p	hsa-miR-29b-1-5p
hsa-miR-151b	hsa-miR-4677-3p	hsa-miR-3613-5p	hsa-miR-301a-3p	hsa-miR-34b-5p
hsa-mi R-205-5p	hsa-mi R-125a-3p	hsa-miR-203b-3p	hsa-miR-744-5p	hsa-miR-145-3p
hsa-miR-338-3p	hsa-miR-181c-5p	hsa-miR-1269a	hsa-let-7e-5p	hsa-miR-34c-5p

(continued)

miRNAID				
hsa-miR-576-5p	hsa-miR-199b-5p	hsa-miR-7-1-3p	hsa-miR-107	hsa-miR-10a-5p
hsa-miR-4772-3p	hsa-miR-501-5p	hsa-miR-142-5p	hsa-miR-145-5p	hsa-miR-16-1-3p
hsa-miR-4661-5p	hsa-miR-19a-3p	hsa-miR-376c-3p	hsa-miR-330-3p	hsa-miR-423-3p
hsa-miR-20b-5p	hsa-mi R-152-3p	hsa-miR-190a-5p	hsa-miR-335-5p	hsa-miR-379-5p
hsa-miR-195-3p	hsa-miR-345-5p	hsa-miR-409-3p	hsa-miR-21-5p	hsa-miR-1247-5p
hsa-mi R-95-3p	hsa-mi R-100-5p	hsa-let-7i-5p	hsa-miR-424-5p	hsa-let-7a-3p
hsa-mi R-493-5p	hsa-mi R-671-5p	hsa-miR-92a-3p	hsa-miR-140-5p	hsa-miR-126-5p
hsa-mi R-222-5p	hsa-mi R-671-3p	hsa-miR-450b-5p	hsa-miR-181c-3p	hsa-miR-505-3p
hsa-miR-431-3p	hsa-miR-148b-3p	hsa-miR-1468-5p	hsa-miR-3653-3p	hsa-miR-183-5p
hsa-miR-331-5p	hsa-miR-338-5p	hsa-miR-452-5p	hsa-miR-181b-3p	hsa-miR-374a-5p
hsa-miR-185-3p	hsa-mi R-136-3p	hsa-miR-34c-3p	hsa-miR-26b-3p	hsa-miR-144-3p
hsa-miR-193a-3p	hsa-mi R-200b-5p	hsa-miR-155-5p	hsa-miR-340-3p	hsa-miR-33b-5p
hsa-miR-1296-5p	hsa-mi R-140-3p	hsa-miR-129-5p	hsa-miR-6842-3p	hsa-miR-126-3p
hsa-miR-495-3p	hsa-miR-204-5p	hsa-miR-409-5p	hsa-miR-374a-3p	hsa-miR-342-3p
hsa-mi R-125b-2-3p	hsa-miR-937-3p	hsa-miR-127-3p	hsa-miR-423-5p	hsa-miR-17-3p
hsa-mi R-146a-5p	hsa-miR-484	hsa-let-7i-3p	hsa-miR-92b-3p	hsa-miR-181b-2-3p
hsa-miR-130b-5p	hsa-mi R-450a-5p	hsa-miR-28-5p	hsa-miR-651-5p	hsa-miR-652-3p
hsa-miR-550a-3p	hsa-miR-455-3p	hsa-miR-141-3p	hsa-miR-130a-3p	hsa-miR-34b-3p
hsa-miR-502-3p	hsa-miR-20a-3p	hsa-miR-628-5p	hsa-miR-381-3p	hsa-miR-296-5p
hsa-miR-4662a-5p	hsa-miR-10a-3p	hsa-let-7d-3p	hsa-miR-19b-3p	hsa-miR-511-5p
hsa-miR-200a-5p	hsa-miR-335-3p	hsa-miR-199a-3p	hsa-let-7g-3p	hsa-miR-191-5p
hsa-miR-26a-2-3p	hsa-miR-589-5p	hsa-miR-199b-3p	hsa-miR-361-5p	hsa-miR-30a-3p
hsa-mi R-128-1-5p	hsa-mi R-340-5p	hsa-miR-143-5p	hsa-miR-186-5p	
hsa-miR-487b-3p	hsa-miR-127-5p	hsa-miR-654-3p	hsa-miR-200b-3p	
hsa-miR-486-5p	hsa-miR-324-5p	hsa-miR-125b-5p	hsa-miR-411-5p	

Table 9. List of 76 miRNAs differentially expressed according to BRB-Array Tools excluding the 14 miRNAs of the model.

miRNAID				
hsa-miR-326	hsa-miR-330-5p	hsa-mi R-30c-5p	hsa-miR-30e-3p	hsa-miR-769-5p
hsa-miR-1266-5p	hsa-miR-195-5p	hsa-miR-34a-5p	hsa-miR-365a-3p	hsa-mi R-139-5p
hsa-miR-1976	hsa-miR-331-3p	hsa-miR-181a-5p	hsa-miR-146b-5p	hsa-miR-328-3p
hsa-miR-1301-3p	hsa-miR-22-5p	hsa-miR-181 b-5p	hsa-miR-500a-3p	hsa-miR-148a-5p
hsa-miR-590-5p	hsa-miR-2355-5p	hsa-mi R-182-5p	hsa-miR-532-5p	hsa-miR-101-3p
hsa-miR-24-1-5p	hsa-miR-660-5p	hsa-miR-181a-3p	hsa-miR-542-3p	hsa-miR-29b-3p
hsa-miR-874-3p	hsa-miR-15b-3p	hsa-miR-223-3p	hsa-miR-425-5p	hsa-miR-197-3p
hsa-miR-130b-3p	hsa-miR-29c-5p	hsa-let-7g-5p	hsa-miR-21-3p	hsa-miR-148a-3p
hsa-miR-24-2-5p	hsa-let-7c-5p	hsa-miR-23b-3p	hsa-miR-193a-5p	hsa-mi R-194-5p

(continued)

miRNAID				
hsa-miR-497-5p	hsa-miR-23a-3p	hsa-miR-27b-3p	hsa-miR-106b-3p	hsa-miR-106b-5p
hsa-miR-664a-3p	hsa-miR-26a-5p	hsa-miR-30b-5p	hsa-miR-151a-5p	hsa-mi R-29c-3p
hsa-miR-200c-5p	hsa-miR-27a-3p	hsa-mi R-128-3p	hsa-miR-146b-3p	hsa-miR-30e-5p
hsa-miR-339-3p	hsa-miR-29a-3p	hsa-mi R-132-3p	hsa-miR-625-3p	
hsa-miR-339-5p	hsa-miR-93-5p	hsa-miR-125a-5p	hsa-miR-629-5p	
hsa-miR-218-5p	hsa-miR-99a-5p	hsa-mi R-150-5p	hsa-miR-1287-5p	
hsa-miR-134-5p	hsa-miR-365b-3p	hsa-miR-1307-3p	hsa-miR-708-3p	

Table 10. List of 182 miRNAs not differentially expressed according to DESeq2 tool.

miRNAID				
hsa-miR-210-3p	hsa-miR-24-3p	hsa-miR-29b-1-5p	hsa-miR-744-5p	hsa-miR-758-3p
hsa-miR-375	hsa-miR-744-3p	hsa-miR-34b-5p	hsa-let-7e-5p	hsa-miR-3615
hsa-miR-134-5p	hsa-miR-369-3p	hsa-mi R-145-3p	hsa-miR-107	hsa-miR-653-5p
hsa-miR-671-3p	hsa-miR-200a-3p	hsa-miR-34c-5p	hsa-miR-145-5p	hsa-let-7f-5p
hsa-miR-148b-3p	hsa-miR-3130-5p	hsa-miR-10a-5p	hsa-miR-330-3p	hsa-let-7d-5p
hsa-miR-338-5p	hsa-miR-342-5p	hsa-miR-16-1-3p	hsa-miR-335-5p	hsa-miR-382-5p
hsa-miR-136-3p	hsa-miR-214-3p	hsa-miR-423-3p	hsa-miR-21-5p	hsa-miR-151a-3p
hsa-miR-200b-5p	hsa-miR-574-3p	hsa-miR-379-5p	hsa-miR-424-5p	hsa-miR-221-5p
hsa-miR-140-3p	hsa-let-7e-3p	hsa-miR-1247-5p	hsa-miR-140-5p	hsa-miR-424-3p
hsa-miR-204-5p	hsa-miR-5586-5p	hsa-let-7a-3p	hsa-miR-181c-3p	hsa-let-7i-3p
hsa-miR-937-3p	hsa-miR-16-5p	hsa-mi R-126-5p	hsa-miR-3653-3p	hsa-miR-28-5p
hsa-miR-484	hsa-miR-378a-3p	hsa-miR-505-3p	hsa-miR-181b-3p	hsa-miR-141-3p
hsa-miR-450a-5p	hsa-miR-103a-3p	hsa-mi R-183-5p	hsa-miR-26b-3p	hsa-miR-628-5p
hsa-miR-455-3p	hsa-miR-505-5p	hsa-miR-374a-5p	hsa-miR-340-3p	hsa-let-7d-3p
hsa-miR-20a-3p	hsa-miR-3200-3p	hsa-miR-324-3p	hsa-miR-6842-3p	hsa-miR-199a-3p
hsa-miR-10a-3p	hsa-miR-181d-5p	hsa-miR-378a-5p	hsa-miR-374a-3p	hsa-miR-199b-3p
hsa-miR-335-3p	hsa-miR-378c	hsa-miR-503-5p	hsa-miR-423-5p	hsa-miR-143-5p
hsa-miR-589-5p	hsa-miR-15b-5p	hsa-miR-320°	hsa-miR-92b-3p	hsa-miR-654-3p
hsa-miR-340-5p	hsa-miR-3614-5p	hsa-mi R-16-2-3p	hsa-miR-651-5p	hsa-miR-144-5p
hsa-miR-127-5p	hsa-miR-3613-5p	hsa-miR-30a-5p	hsa-miR-130a-3p	hsa-miR-199a-5p
hsa-miR-32-5p	hsa-miR-203b-3p	hsa-let-7b-3p	hsa-miR-381-3p	hsa-miR-598-3p
hsa-miR-5698	hsa-miR-1269a	hsa-miR-3913-5p	hsa-miR-19b-3p	hsa-let-7f-1-3p
hsa-miR-26b-5p	hsa-miR-7-1-3p	hsa-let-7a-5p	hsa-let-7g-3p	hsa-miR-301a-5p
hsa-miR-99b-5p	hsa-miR-142-5p	hsa-let-7b-5p	hsa-miR-361-5p	hsa-miR-452-3p
hsa-miR-889-3p	hsa-miR-376c-3p	hsa-miR-214-5p	hsa-miR-186-5p	hsa-miR-19b-1-5p
hsa-miR-185-5p	hsa-miR-190a-5p	hsa-mi R-200c-3p	hsa-miR-200b-3p	hsa-miR-224-5p
hsa-miR-3127-5p	hsa-miR-409-3p	hsa-miR-374b-5p	hsa-miR-33b-5p	hsa-miR-301a-3p

(continued)

miRNAID				
hsa-miR-28-3p	hsa-let-7i-5p	hsa-miR-3607-3p	hsa-miR-126-3p	hsa-miR-486-5p
hsa-miR-370-3p	hsa-miR-92a-3p	hsa-mi R-142-3p	hsa-miR-342-3p	hsa-miR-324-5p
hsa-miR-139-3p	hsa-miR-450b-5p	hsa-miR-451°	hsa-miR-17-3p	hsa-mi R-125b-5p
hsa-miR-98-5p	hsa-miR-1468-5p	hsa-mi R-143-3p	hsa-miR-181b-2-3p	hsa-miR-337-3p
hsa-miR-27b-5p	hsa-miR-452-5p	hsa-mi R-150-3p	hsa-miR-652-3p	hsa-miR-429
hsa-miR-10b-5p	hsa-miR-34c-3p	hsa-miR-20a-5p	hsa-miR-34b-3p	hsa-miR-144-3p
hsa-miR-96-5p	hsa-miR-155-5p	hsa-miR-361-3p	hsa-miR-296-5p	hsa-miR-411-5p
hsa-miR-501-3p	hsa-miR-129-5p	hsa-miR-425-3p	hsa-miR-511-5p	
hsa-miR-27a-5p	hsa-miR-409-5p	hsa-miR-30c-2-3p	hsa-miR-191-5p	
hsa-miR-454-3p	hsa-miR-127-3p	hsa-miR-33a-5p	hsa-miR-30a-3p	

Table 11. List of 181 miRNAs differentially expressed according to DESeq2 tool excluding the 19 miRNAs of the model.

miRNAID				
hsa-miR-192-5p	hsa-miR-362-3p	hsa-miR-4662a-5p	hsa-miR-590-5p	hsa-miR-101-5p
hsa-miR-29b-2-5p	hsa-miR-151b	hsa-miR-200a-5p	hsa-miR-200c-5p	hsa-miR-26a-5p
hsa-miR-135b-5p	hsa-miR-29c-5p	hsa-miR-625-3p	hsa-miR-3065-5p	hsa-miR-181a-3p
hsa-miR-3065-3p	hsa-miR-125a-5p	hsa-miR-26a-2-3p	hsa-miR-577	hsa-miR-497-5p
hsa-miR-187-3p	hsa-miR-660-5p	hsa-miR-218-5p	hsa-miR-1247-3p	hsa-miR-30e-5p
hsa-miR-708-5p	hsa-miR-2355-5p	hsa-miR-128-1-5p	hsa-miR-151a-5p	hsa-miR-139-5p
hsa-miR-193b-3p	hsa-miR-205-5p	hsa-miR-487b-3p	hsa-miR-1287-5p	hsa-miR-30e-3p
hsa-miR-194-5p	hsa-miR-23a-3p	hsa-miR-148a-3p	hsa-miR-132-5p	hsa-miR-146b-3p
hsa-miR-664a-3p	hsa-miR-338-3p	hsa-let-7a-2-3p	hsa-miR-1287-3p	hsa-miR-629-3p
hsa-miR-664b-3p	hsa-miR-106b-5p	hsa-miR-133a-3p	hsa-miR-217	hsa-miR-146a-5p
hsa-miR-181a-5p	hsa-miR-1301-3p	hsa-miR-197-3p	hsa-miR-106a-5p	hsa-miR-130b-5p
hsa-miR-30b-5p	hsa-miR-223-3p	hsa-miR-93-3p	hsa-miR-29b-3p	hsa-miR-29c-3p
hsa-miR-153-5p	hsa-miR-365b-3p	hsa-miR-874-3p	hsa-miR-17-5p	hsa-miR-27a-3p
hsa-miR-664a-5p	hsa-miR-365a-3p	hsa-mi R-132-3p	hsa-miR-508-3p	hsa-miR-550a-3p
hsa-miR-99a-5p	hsa-miR-576-5p	hsa-mi R-182-5p	hsa-miR-514a-3p	hsa-miR-15b-3p
hsa-miR-421	hsa-miR-193a-5p	hsa-miR-3934-3p	hsa-miR-455-5p	hsa-miR-502-3p
hsa-miR-582-5p	hsa-miR-4772-3p	hsa-miR-224-3p	hsa-miR-22-5p	hsa-miR-328-3p
hsa-miR-30c-5p	hsa-miR-4661-5p	hsa-miR-25-3p	hsa-miR-590-3p	hsa-miR-330-5p
hsa-miR-128-3p	hsa-miR-769-5p	hsa-miR-21-3p	hsa-miR-3074-5p	hsa-miR-136-5p
hsa-miR-101-3p	hsa-miR-34a-5p	hsa-miR-15a-5p	hsa-miR-181a-2-3p	hsa-miR-758-5p
hsa-miR-18a-5p	hsa-miR-148a-5p	hsa-miR-642a-5p	hsa-miR-141-5p	hsa-miR-500a-5p
hsa-miR-432-5p	hsa-miR-20b-5p	hsa-miR-339-3p	hsa-miR-708-3p	hsa-miR-93-5p
hsa-miR-942-5p	hsa-miR-1266-5p	hsa-miR-1-3p	hsa-miR-29a-5p	hsa-miR-339-5p
hsa-miR-130b-3p	hsa-miR-195-3p	hsa-miR-1180-3p	hsa-miR-410-3p	hsa-miR-500a-3p

(continued)

miRNAID				
hsa-let-7c-5p	hsa-miR-95-3p	hsa-miR-425-5p	hsa-miR-625-5p	hsa-miR-532-3p
hsa-miR-3677-3p	hsa-miR-493-5p	hsa-miR-877-5p	hsa-miR-24-1-5p	hsa-miR-509-3p
hsa-miR-362-5p	hsa-miR-222-5p	hsa-miR-221-3p	hsa-miR-331-3p	hsa-miR-1976
hsa-miR-195-5p	hsa-miR-431-3p	hsa-miR-23b-3p	hsa-miR-4326	hsa-miR-152-3p
hsa-miR-629-5p	hsa-miR-29a-3p	hsa-miR-363-3p	hsa-miR-149-5p	hsa-miR-326
hsa-miR-181 b-5p	hsa-miR-27b-3p	hsa-mi R-24-2-5p	hsa-miR-1307-5p	hsa-miR-345-5p
hsa-miR-106b-3p	hsa-miR-331-5p	hsa-miR-493-3p	hsa-let-7g-5p	hsa-miR-100-5p
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hsa-miR-30b-3p	hsa-miR-1296-5p	hsa-miR-99b-3p	hsa-miR-181c-5p	
hsa-miR-146b-5p	hsa-miR-495-3p	hsa-miR-500b-5p	hsa-miR-199b-5p	
hsa-miR-1307-3p	hsa-miR-532-5p	hsa-miR-542-3p	hsa-miR-19a-3p	
hsa-miR-320b	hsa-miR-125b-2-3p	hsa-miR-212-3p	hsa-miR-501-5p	

[0077] The same logistic regression used for the selected signature was applied to each random list, and corresponding model performances were evaluated through AUC.

[0078] As shown in Figure 4, none of the AUC obtained using these random signatures were as high as the AUC obtained using the 14- and 19-miRNAs models, which demonstrate that the method applied for model selection was the most effective to identify the best set of miRNAs diagnostic for C1 subtype of lung cancer (Figure 4).

MATERIALS AND METHODS

[0079] We selected the cohort of 515 patients with lung adenocarcinomas from the TCGA data portal (https://portal.gdc.cancer.gov/) at 2018. A total of 510 tumors were profiled for both gene and miRNA expression. Log2 read counts were used for expression analysis. Patients follow-up information was used for survival analysis: overall survival was defined as the time from the date of tumor resection until death from any cause.

[0080] Follow-up was truncated at 3 years to reduce the potential overestimation of overall mortality with respect to lung cancer-specific mortality.

[0081] Hierarchical clustering analysis was performed on the 10-gene signature for the entire cohort of 510 patients. Clustering was done by using Cluster 3.0 for Mac OS X (C Clustering Library 1.56) with uncentered correlation and centroid linkage, and Java TreeView software environment (version 1.1.6r4; http://jtreeview.sourceforge.net). Four main branches were selected to build clusters. Kaplan-Meier survival curves were stratified by clusters and log-rank test p-values were calculated. C1 cluster was associated to the worse prognosis, and all other clusters were pooled together (nonC1 clusters).

[0082] To reduce the complexity of the TCGA-LUAD dataset (2237 miRNAs) and extract the most informative data to use, we selected the most transcriptionally regulated miRNAs. We selected those miRNAs with raw counts > 0 in at least the 50% of patients either in C1 or nonC1, identifying a total of 382 miRNAs. We applied the complexity reduction also to genes and we selected the most varying across all samples (standard deviation in the top 25%), identifying a total of 4899 genes. Using DESeq2 R package, we identified a total of 2900 differentially expressed genes between C1 and nonC1 tumors.

[0083] BRB-ArrayTools [10] and DESeq2 (R package) [11] tools were used for class prediction (C1 cluster vs nonC1 clusters) according to miRNA expression. BRB-ArrayTools uses statistics based on two-sample T-test with multivariate permutations test (1000 random permutations); confidence level of false discovery rate assessment, 80%; maximum allowed proportion of false-positive genes, 0.05. DESeq2 is based on Wald test statistics to identify differentially expressed transcripts. Lists of miRNAs differentially expressed obtained from BRB-ArrayTools and DESeq2 tools were subsequently reduced via Lasso regularization. In details, a penalized unconditional logistic regression was applied considering cluster as discrete outcome (C1 cluster vs. nonC1 clusters) and miRNA expressions as explanatory variables. Cross-validated (10-fold) log-likelihood with optimization (50 simulations) of the tuning penalty parameter was used to control for potential overfitting.

[0084] Starting from differentially expressed genes (identified with DESeq2) and miRNAs (identified with both DESeq2 and BRB-ArrayTools), we used ARACNe [12] with 1000 bootstraps to infer direct regulatory relationships between transcriptional regulators (i.e. miRNAs) and target genes. ARACNe was performed using all patients, stage I patients and stage II-IV patients. miRNA target genes were retrieved using miRWalk 3.0 [13].

[0085] Probability of being in the C1 cluster was estimated using the unconditional logistic regression for the 3 signatures of 19, 14 and 7 miRNAs. Model performance was assessed using the cross-validated area under the receiver operating curve, and assessing the difference in C1 predicted probability between C1 and nonC1 patients (Wilcoxon-Mann-Whitney test). Cox regression model was used to evaluate the prognostic role of these miRNA signatures and their ability to recapitulate the risk-stratification of the original 10-genes signature.

[0086] To get insights in the biology of the 7-miRNA model, we verify the enrichment of cancer-relevant pathways associated to their target genes. We investigated the Molecular Signature Database (MSigDB; v7.2) (https://www.gsea-msigdb.org/gsea/msigdb/annotate.jsp) using the list of 87 targeted genes by interrogating the CGP (chemical and genetic perturbations, 3358 gene sets). Bubble plot analysis was performed using JMP 15.2.1 (SAS) software.

[0087] Hierarchical clustering analysis was performed on the 7-miRNA signature for 510 patients, those with available miRNA expression data. Clustering was done by using Cluster 3.0 for Mac OS X (C Clustering Library 1.56) with uncentered correlation and centroid linkage, and Java TreeView software environment (version 1.1.6r4; http://jtreeview.sourceforge.net).

[0088] The 7 miRNAs signature was identified as the best for risk-stratification and therefore validated in an external cohort of patients from IRCCS Casa Sollievo della Sofferenza Hospital (CSS, San Giovanni Rotondo, Italy). Between February 2017 and February 2020, 44 patients with lung adenocarcinoma underwent surgery at the CSS. Written informed consent was obtained from all study patients. None of these patients received preoperative chemotherapy. Clinical information was obtained through review of medical records. Vital status was assessed through the Vital Records Offices of the patients' towns of residence or by contacting directly the patients or their families.

[0089] One tissue core (1.5 mm in diameter) from FFPE blocks, in representative tumor areas with adequate tumor cellularity (>60%) selected by a pathologist, was processed for total RNA extraction. The AllPrep DNA/RNA FFPE kit (QIAGEN) was used for Total RNA extraction. Quantitative real-time PCR (qRT-PCR) was performed to analyze the 10-genes signature as described in Dama et al [6]. Briefly, RNA was quantified using Nanodrop ND-10000 Spectrophotometer and a total of 200 ng was retro-transcribed using SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific) and pre-amplified for 10 cycles with PreAmp Master Mix Kit (ThermoFisher Scientific), following manufacturer's instructions. qRT-PCR analysis was performed starting from 1:10 diluted pre-amplified cDNA, using the TaqMan Fast Advance Master Mix and hydrolysis probes (ThermoFisher Scientific; for primers see Dama et al [6]), in a QuantStudio 12k Flex (ThermoFisher Scientific). Thermal cycling amplification was performed with an initial incubation at 95°C for 30 seconds, followed by 45 cycles of 95°C for 5 seconds and 60°C for 30 seconds. For miRNA expression analysis, a total of 10 ng RNA was reverse-transcribed using the TaqMan Advanced miRNA cDNA Synthesis Kit (ThermoFisher Scientific). Poly(A) tailing, adapter ligation, RT reaction and miR-Amp (using TaqMan Advanced miRNA assays) were performed following manufacturer's instructions [15], i.e.: 95°C for 30 seconds, 45 cycles of 95°C for 5 seconds, and 60°C for 30 seconds, using a Card Custom Advance (ThermoFisher Scientific) in a QuantStudio 12k Flex (ThermoFisher Scientific).

[0090] The hsa-miR-16-5p was used as standard reference for CT normalization using a previously described methodology [6]. Briefly, the normalized CT of each miRNA (i) of each sample (j) was calculated as difference between the raw CTij and a scaling factor (SF) specific for each sample (j); the SFj represented the difference between the raw CT of the miRNA "hsa-miR-16-5p" used as a reference in the sample (j) and a constant equal to 21.87.

[0091] Risk-scores were assigned to each patient based to the 10-gene risk model described in Dama et al. [6]. Before applying the risk-model, data were rescaled (q1-q3 normalization). Patients with risk-scores higher than the 66^{th} percentiles (6) were classified as C1 tumors. Next, unconditional logistic regression (C1 vs nonC1 tumors) with 7 miRNAs as explanatory variables was applied, and the area under the receiver operating curve was calculated. Difference in C1 predicted probability between C1 and nonC1 patients was evaluated through Wilcoxon-Mann-Whitney test.

[0092] All statistical analyses were performed using SAS software, version 9.4 (SAS Institute, Inc., Cary, NC) and R 3.3.1 (R Core Team, 2016) and JMP 15 (SAS). P-values less than 0.05 were considered statistically significant.

Table 12. Commercial assays used to amplify each miRNA of the 19-, 14- and 7-miRNA signatures.

Assay refers to the code used to identify each miRNA in the qRT-PCR experiment; "Acc. mature-miRNA" refers to the miRBase accession number of the mature miRNA; "miRbase ID" refers to the name of the mature miRNA in the miRbase database; "Signature" refers to the specific signature each miRNA belongs to; "house-keeping miRNA" is the miRNA used to normalized the qRT-PCR experiments; "spike-in" miRNA is the miRNA used to control the performance of the qRT-PCR experiments.

Assay	Acc. mature-miRNA	miRbase ID	Signature
479365_mir	MIMAT0026472	hsa-let-7c-3p	19-miRNA
478582_mir	MIMAT0000758	hsa-miR-135b-5p	14-miRNA
477905_mir	MIMAT0000430	hsa-miR-138-5p	19-miRNA
477941_mir	MIMAT0000262	hsa-miR-187-3p	14-miRNA
478262_mir	MIMAT0000222	hsa-miR-192-5p	14-miRNA
478314_mir	MIMAT0002819	hsa-miR-193b-3p	14- and 7-miRNA
478742_mir	MIMAT0004767	hsa-miR-193b-5p	19- and 7-miRNA
478230_mir	MIMAT0000226	hsa-miR-196a-5p	19-miRNA
478585_mir	MIMAT0001080	hsa-miR-196b-5p	19-, 14-miRNA and 7-miRNA
478316_mir	MIMAT0000264	hsa-miR-203a-3p	19-miRNA
477970_mir	MIMAT0000267	hsa-miR-210-3p	14-miRNA
478516_mir	MIMAT0000272	hsa-miR-215-5p	19-miRNA
477989_mir	MIMAT0017950	hsa-miR-2355-3p	19-miRNA
478003_mir	MIMAT0004515	hsa-miR-29b-2-5p	14-miRNA
479605_mir	MIMAT0015378	hsa-miR-3065-3p	14-miRNA
479362_mir	MIMAT0004551	hsa-miR-30d-3p	19-miRNA
478606_mir	MIMAT0000245	hsa-miR-30d-5p	19- and 14-miRNA
478012_mir	MIMAT0004504	hsa-miR-31-3p	19- and 7-miRNA
478015_mir	MIMAT0000089	hsa-miR-31-5p	19- and 7-miRNA
478074_mir	MIMAT0000728	hsa-miR-375-3p	14-miRNA
478928_mir	MIMAT0019812	hsa-miR-4709-3p	19-miRNA
479018_mir	MIMAT0003254	hsa-miR-548b-3p	19-miRNA
477852_mir	MIMAT0004800	hsa-miR-550a-5p	19- and 7-miRNA
479064_mir	MIMAT0004797	hsa-miR-582-3p	19- and 14-miRNA
478167_mir	MIMAT0003249	hsa-miR-584-5p	19-, 14-miRNA and 7-miRNA
478195_mir	MIMAT0006790	hsa-miR-675-3p	19-miRNA
478197_mir	MIMAT0004926	hsa-miR-708-5p	14-miRNA
478214_mir	MIMAT0000441	hsa-miR-9-5p	19- and 14-miRNA
477860_mir	MIMAT0000069	hsa-miR-16-5p	house-keeping miRNA
478411_mir	MIMAT0000177	ath-miR159a	spike-in miRNA

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[0093]

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<220>
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<400> 24
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<400> 26
ucagcaccag gauauuguug gag 23

<210> 27
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<212> RNA
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<400> 27
uuuguucguu cggcucgcgu ga 22

<210> 28
<211> 23
<212> RNA
<213> Unknown

<220>
<223> hsa-miR-708-5p

<400> 28
aaggagcuua caaucuagcu ggg 23

<210> 29
<211> 22

<212> RNA
<213> Unknown

<220>
<223> hsa-miR16-5p

<400> 29
uagcagcacg uaaauauugg cg 22

Claims

1. A method *in-vitro* or *ex-vivo* for identifying patients affected by aggressive lung adenocarcinoma (C1-ADC), comprising the steps of:

   a) detecting the amount of each of the **19 miRNAs** having sequence hsa-miR-193b-5p (SEQ ID NO. 3), hsa-miR-31-3p (SEQ ID NO. 11), hsa-miR-31-5p (SEQ ID NO. 12), hsa-miR-550a-5p (SEQ ID NO. 15), hsa-miR-196b-5p (SEQ ID NO. 5), hsa-miR-584-5p (SEQ ID NO. 17), hsa-miR-30d-5p (SEQ ID NO. 10), hsa-miR-582-3p (SEQ ID NO. 16), hsa-miR-9-5p (SEQ ID NO. 19), hsa-let-7c-3p (SEQ ID NO. 1), hsa-miR-138-5p (SEQ ID NO. 2), hsa-miR-196a-5p (SEQ ID NO. 4), hsa-miR-203a-3p (SEQ ID NO. 6), hsa-miR-215-5p (SEQ ID NO. 7), hsa-miR-2355-3p (SEQ ID NO. 3); hsa-miR-30d-3p (SEQ ID NO. 9), hsa-miR-4709-3p (SEQ ID NO. 13), hsa-miR-548b-3p (SEQ ID NO. 14) and hsa-miR-675-3p (SEQ ID NO. 18); or of each of the **14 miRNAs** having sequence hsa-miR-196b-5p (SEQ ID NO. 5), hsa-miR-584-5p (SEQ ID NO. 17), hsa-miR-30d-5p (SEQ ID NO. 10), hsa-miR-582-3p (SEQ ID NO. 16), hsa-miR-9-5p (SEQ ID NO. 19), hsa-miR-193b-3p (SEQ ID NO. 23), hsa-miR-135b-5p (SEQ ID NO. 20), hsa-miR-187-3p (SEQ ID NO. 21), hsa-miR-192-5p (SEQ ID NO. 22), hsa-miR-210-3p (SEQ ID NO. 24), hsa-miR-29b-2-5p (SEQ ID NO. 25), hsa-miR-3065-3p (SEQ ID NO. 26), hsa-miR-375-3p (SEQ ID NO. 27) and hsa-miR-708-5p (SEQ ID NO. 28), or of each of the 7 **mi-RNAs** having sequence hsa-miR-31-5p (SEQ ID NO. 12), hsa-miR-31-3p (SEQ ID NO. 11), hsa-miR-193b-3p (SEQ ID NO. 23), hsa-miR-193b-5p (SEQ ID NO. 3), hsa-miR-196b-5p (SEQ ID NO. 5), hsa-miR-550a-5p (SEQ ID NO. 15) and hsa-miR-584-5p (SEQ ID NO. 17) in a biological sample from a subject.

2. Method according to claim 1 further comprising step b), wherein the data obtained in step a) is normalized.

3. Method according to claim 2 further comprising step c), wherein the patients are classified either in the predicted class of subjects affected by aggressive lung adenocarcinoma or in the class of subjects affected by non-aggressive lung adenocarcinoma (nonC1-ADC).

4. Method according to claim 3, wherein the patients affected by aggressive lung adenocarcinoma comprises patients at an early-stage of the disease (stage I).

5. Method according to any of the previous claims, wherein the biological sample is a tissue sample or a body fluid, preferably said tissue sample is a fresh tissue sample, a frozen tissue sample or a FFPE tissue sample, and said body fluid is serum or plasma.

6. Method according to any of previous claims wherein the lung adenocarcinoma is a non-small cell lung carcinoma (NSCLC).

7. Method according to claim 1, wherein the miRNAs in step a) is detected by quantitative RT-PCR (qRT-PCR), or digital PCR, or RNA sequencing, or Affymetrix microarray, or custom microarray, or digital detection through molecular barcoding selected from NanoString technology.

8. Method according to claim 1, wherein the normalization of the data reported in step b) is made according to the scheme reported in below, when the miRNAs are detected by RNA sequencing:

	RNA sequencing		
	For 14-miRNAs signature	**For 19-miRNAs signature**	**For 7-miRNAs signature**
STEP b-raw data normalization	For miRNA *i* in sample *j*: Normalized $count_{ij}$=raw $count_{ij}$/$SizeFactor_j$ Where: $SizeFactor_j$=$median_j$ (across all $RatioFactor_{ij}$), $RatioFactor_{ij}$=raw $count_{ij}$/ $geomean_i$ (across all $samples_{ij}$)	For miRNA *i* in sample *j*: Normalized $count_{ij}$=raw $count_{ij}$/$SizeFactor_j$ Where: $SizeFactor_j$=$median_j$ (across all $RatioFactor_{ij}$), $RatioFactor_{ij}$=raw $count_{ij}$/ $geomean_i$ (across all $samples_{ij}$)	For miRNA *i* in sample *j*: Normalized $count_{ij}$=raw $count_{ij}$/$SizeFactor_j$ Where: $SizeFactor_j$=$median_j$ (across all $RatioFactor_{ij}$), $RatioFactor_{ij}$=raw $count_{ij}$/ $geomean_i$ (across all $samples_{ij}$)

wherein

- the normalized $count_{ij}$ of each miRNA (*i*) of each sample (*j*) is calculated as ratio of the raw $count_{ij}$ and a size $factor_j$ specific for each $sample_j$; the size $factor_j$ specific for each sample is calculated as the median of all ratio $factors_{ij}$ of that sample; ratio $factors_{ij}$ represents the ratio between raw $count_{ij}$ and the geometric $mean_j$ of all raw $count_{ij}$ relative to a specific $miRNA_i$,

9. Method according to claim 1, wherein the normalization of the data reported in step b) is made according to the scheme reported in below, when the miRNAs are detected by qRT-PCR:

	qRT-PCR		
	For 14-miRNA signature	**For 19-miRNA signature**	**For 7-miRNA signature**
STEP b-raw data normalization	For miRNA *i* in sample *j*: Normalized Ct_{ij}=raw Ct_{ij}-SF_j where SF_j=hsa-miR-16-5p$_j$-21.87	For miRNA *i* in sample *j*: Normalized Ct_{ij}=raw Ct_{ij}-SF_j where SF_j=hsa-miR-16-5p$_j$-21.87	For miRNA *i* in sample *j*: Normalized Ct_{ij}=raw Ct_{ij}-SF_j where SF_j=hsa-miR-16-5p$_j$-21.87

wherein:

- the normalized Ct_{ij} (cycle threshold) of each miRNA (*i*) of each sample (*j*) is calculated as difference between the raw Ct_{ij} and a scaling $factor_j$ (SF) specific for each $sample_j$; the scaling factor represents the difference between the raw Ct of the miRNA "hsa-miR-16-5p" used as a reference in the sample and a constant equal to 21.87.

10. Method according to any of the previous claims, wherein the classification of the class of subjects affected by aggressive lung adenocarcinoma or of subjects affected by non-aggressive lung adenocarcinoma of step c) is calculated by the following formula:

$$\text{predicted class} = \begin{cases} \text{C1}, & \dfrac{1}{1+e^{-z}} \geq 0.5 \\ \text{nonC1}, & \dfrac{1}{1+e^{-z}} < 0.5 \end{cases}$$

wherein:

- in a model of 19-miRNAs analyzed by RNA sequencing :

z=-8.2029+(-0.2651*hsa-let-7c-3p)+(0.1709*hsa-miR-138-5p)+(0.1443*hsa-miR-193b-5p)+(0.0200*hsa-miR-196a-5p)+(0.0464*hsa-miR-196b-5p)+(0.2297*hsa-miR-203a-3p)+(0.1285*hsa-miR-215-5p)+(0.3933*hsa-miR-2355-3p)+(-0.2220*hsa-miR-30d-3p)+(-0.1874*hsa-miR-30d-5p)+(0.1535*hsa-miR-31-3p)+(0.0326*hsa-miR-31-5p)+(-0.3032*hsa-miR-4709-3p)+(-0.1672*hsa-miR-548b-3p)+(0.1529*hsa-miR-550a-5p)+(0.1132*hsa-miR-582-3p)+(0.5229*hsa-miR-584-5p)+(0.1314*hsa-miR-675-3p)+(0.0497*hsa-miR-9-5p);

- in a model of 14-miRNAs analyzed by RNA sequencing:

z=-5.4414+(-0.1028*hsa-miR-135b-5p)+(-0.0486*hsa-miR-187-3p)+(0.2828*hsa-miR-192-5p)+(0.1977*hsa-miR-193b-3p)+(0.0201*hsa-miR-196b-5p)+(0.1908*hsa-miR-210-3p)+(-0.5074*hsa-miR-29b-2-5p)+(0.0384*hsa-miR-3065-3p)+(-0.3312*hsa-miR-30d-5p)+(-0.0475*hsa-miR-375-3p)+(0.1895*hsa-miR-582-3p)+(0.5606*hsa-miR-584-5p)+(0.2663*hsa-miR-708-5p)+(0.0435*hsa-miR-9-5p);

and
- in model 7-miRNAs analyzed by RNA sequencing:

z = -8.5210+(0.1171*hsa-miR-193b-3p)+(0.2233*hsa-miR-193b-5p)+(0.1341*hsa-miR-196b-5p)+(0.1554*hsa-miR-31-3p)+(0.0584*hsa-miR-31-5p)+(0.3622*hsa-miR-550a-5p)+(0.4683*hsa-miR-584-5p);

or in alternative,
- in a model of 7-miRNAs analyzed by qRT-PCR :

z= 2.2920 +(hsa-miR-193b-3p*0.1295)+(hsa-miR-193b-5p*0.0920)+(hsa-miR-196b-5p*-0.1310)+(hsa-miR-31-3p*-0.2116)+(hsa-miR-31-5p*-0.2724)+(hsa-miR-550a-5p*0.2717)+(hsa-miR-584-5p*0.0413).

11. Method according to any of the previous claims, for the identification of patients included in the group of aggressive lung adenocarcinoma with a poor-prognosis, selected from patients with shorter overall survival, and/or patients with shorter disease-free survival, and/or patients responsive to a treatment, and/or with patients with metastatic disease which can include, but not limited to, patients with early-stage disease (stage I).

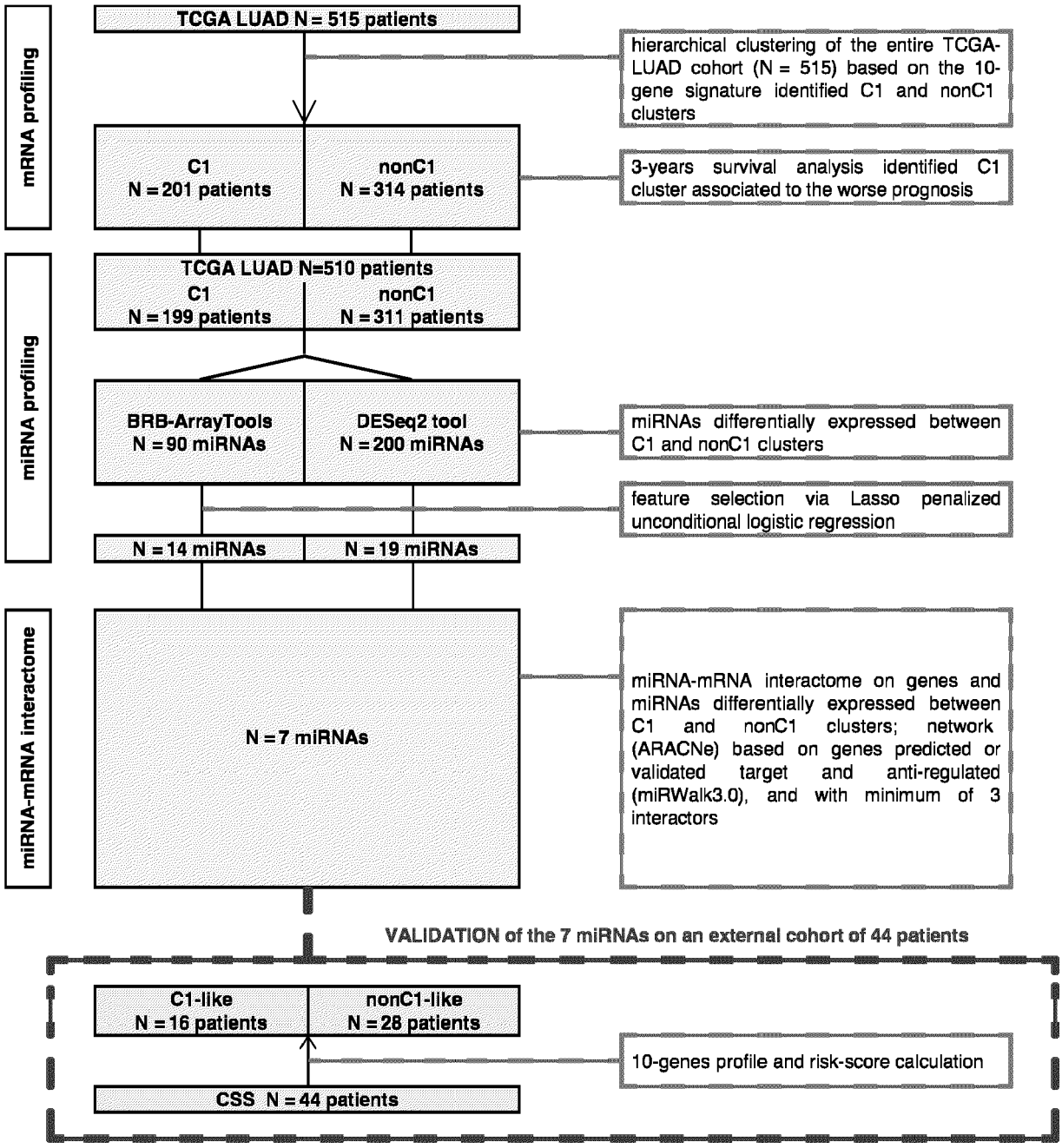
12. Method according to any of the previous claims, for the identification of patients included in the group of non aggressive

lung adenocarcinoma with a good-prognosis, selected from patients with longer overall survival, and/or patients with longer disease-free survival, and/or patients responsive to treatment, patients and/or without metastatic disease which can include, but not limited to, patients with early-stage disease (stage I).

13. Method according to any of the previous claims for the prognostic risk stratification of lung adenocarcinomas (C1-ADC) and/or for the identification of alternative therapeutic options after surgery, selected from systemic adjuvant chemotherapy, selected from platinum-based combinations, preferably cisplatin, carboplatin plus a third generation agents such as gemcitabine, vinorelbine, a taxane, or camptothecin, molecular targeted therapeutics, immunotherapy, radiotherapy, or a combination thereof.

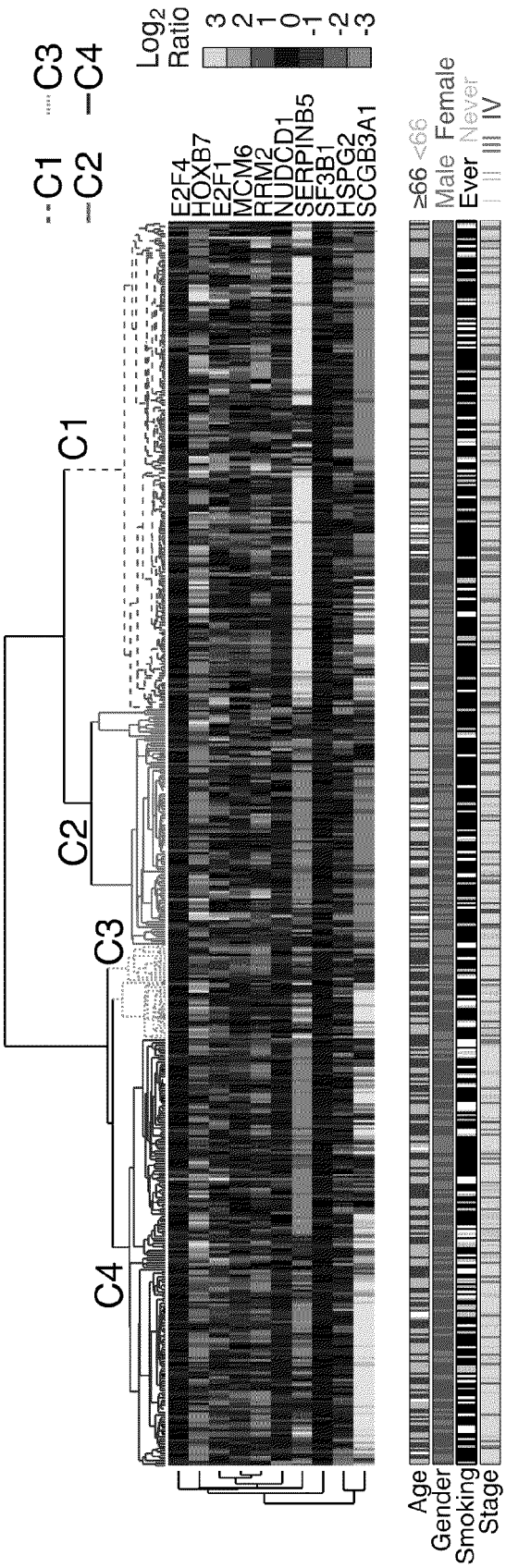
14. A kit to perform the method according to any of the previous claims, comprising a multi-well plate and specific primers and/or probers for each of miRNAs to be detected.

Figure 1. Flow chart of study design with data sets and analysis.

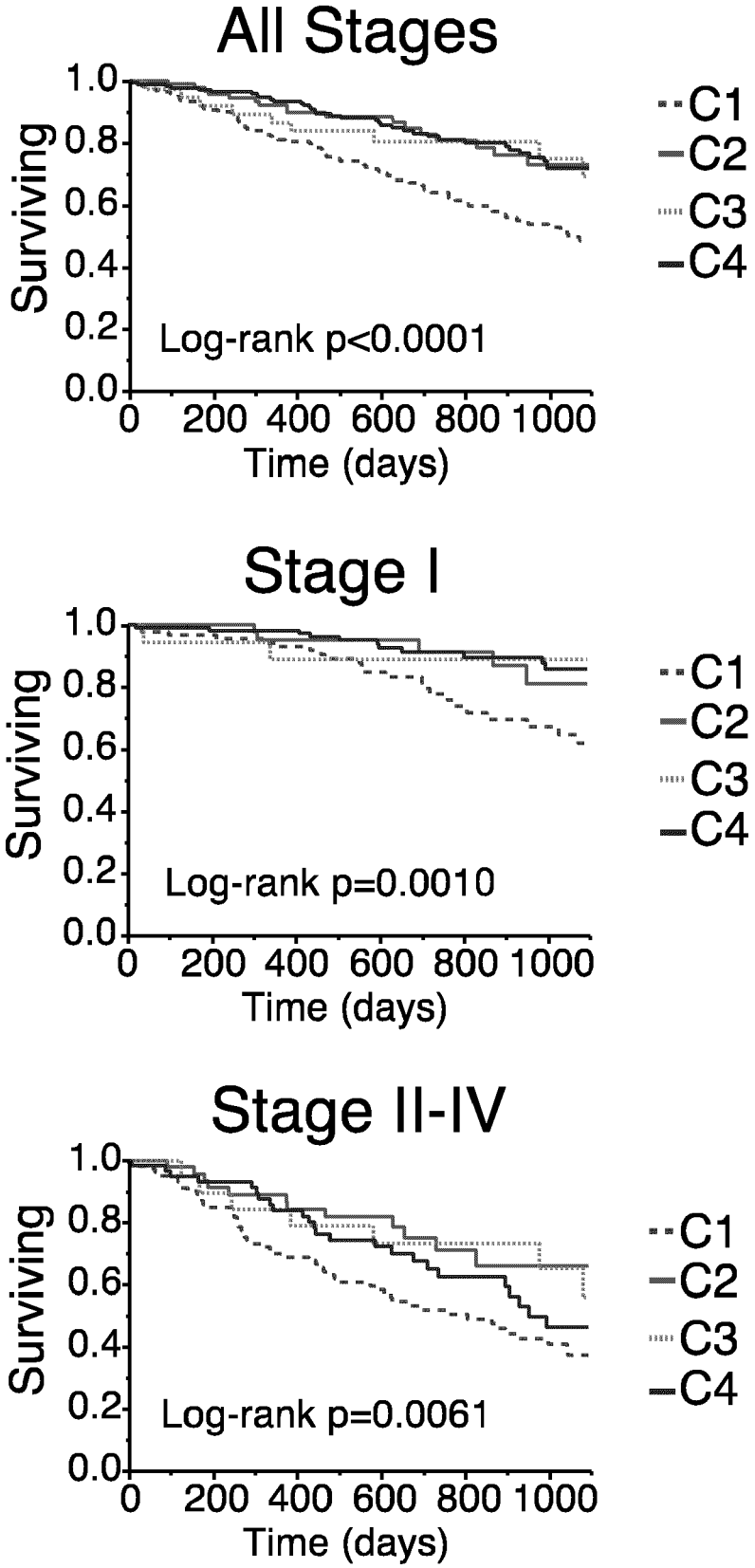


Figures 2.a), b), c), d) and e) mRNA and miRNA expression profile analysis of the TCGA-LUAD cohort.

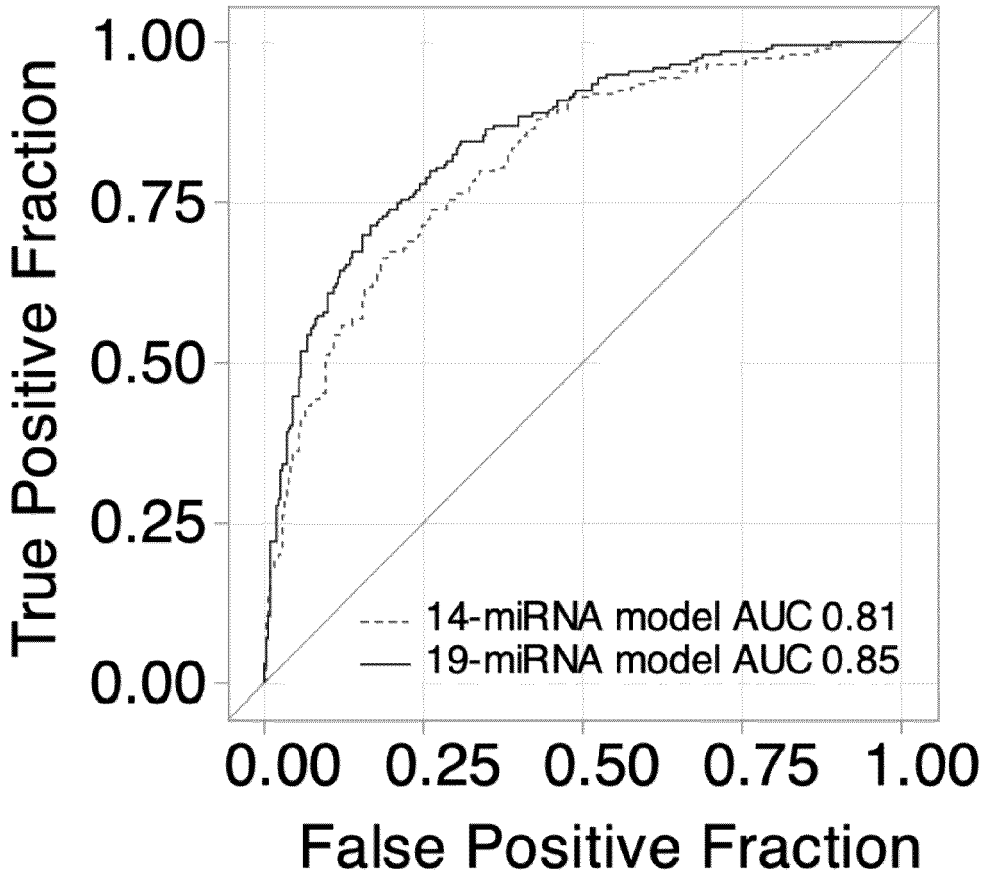
(Figure 2a)



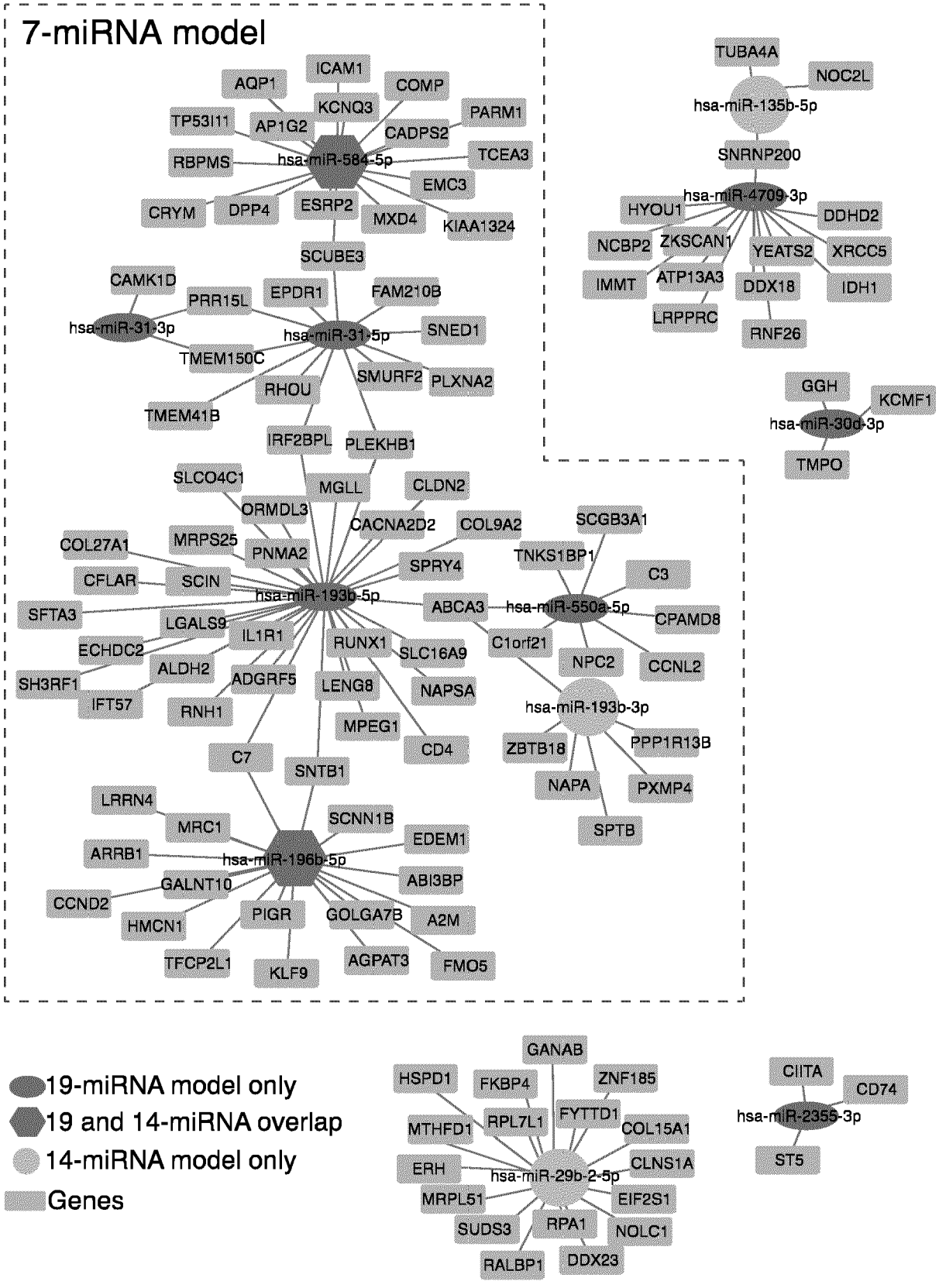
(Figure 2b)


All Stages
Surviving
1.0
0.8
0.6
0.4
0.2
0.0
Log-rank p<0.0001
0
200
400
600
800
1000
Time (days)
C1
C2
C3
C4
Stage I
Surviving
Log-rank p=0.0010
Time (days)
C1
C2
C3
C4
Stage II-IV
Surviving
Log-rank p=0.0061
Time (days)
C1
C2
C3
C4

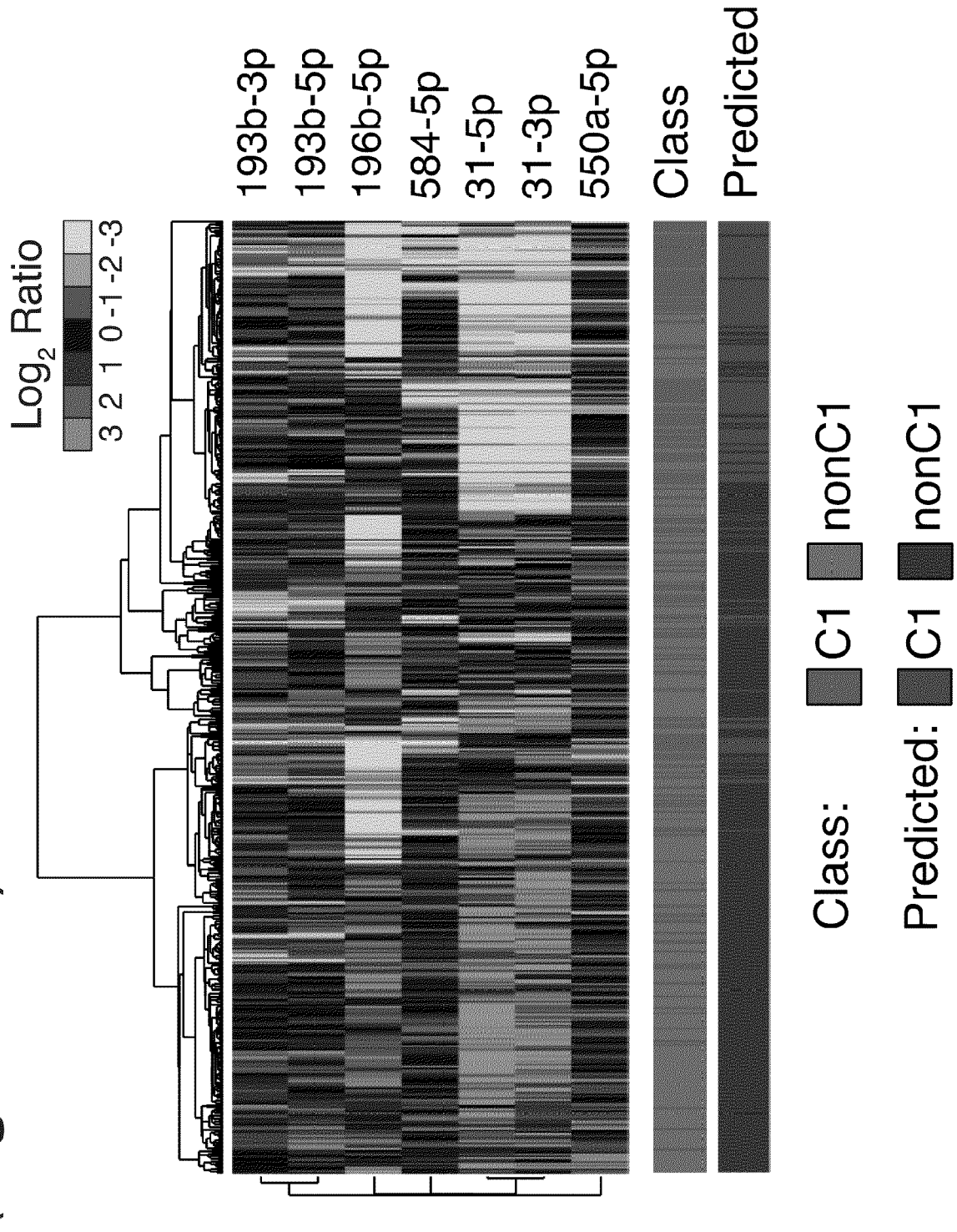
(Figure 2c)


True Positive Fraction
1.00
0.75
0.50
0.25
0.00
14-miRNA model AUC 0.81
19-miRNA model AUC 0.85
0.00
0.25
0.50
0.75
1.00
False Positive Fraction

(Figure 2d)

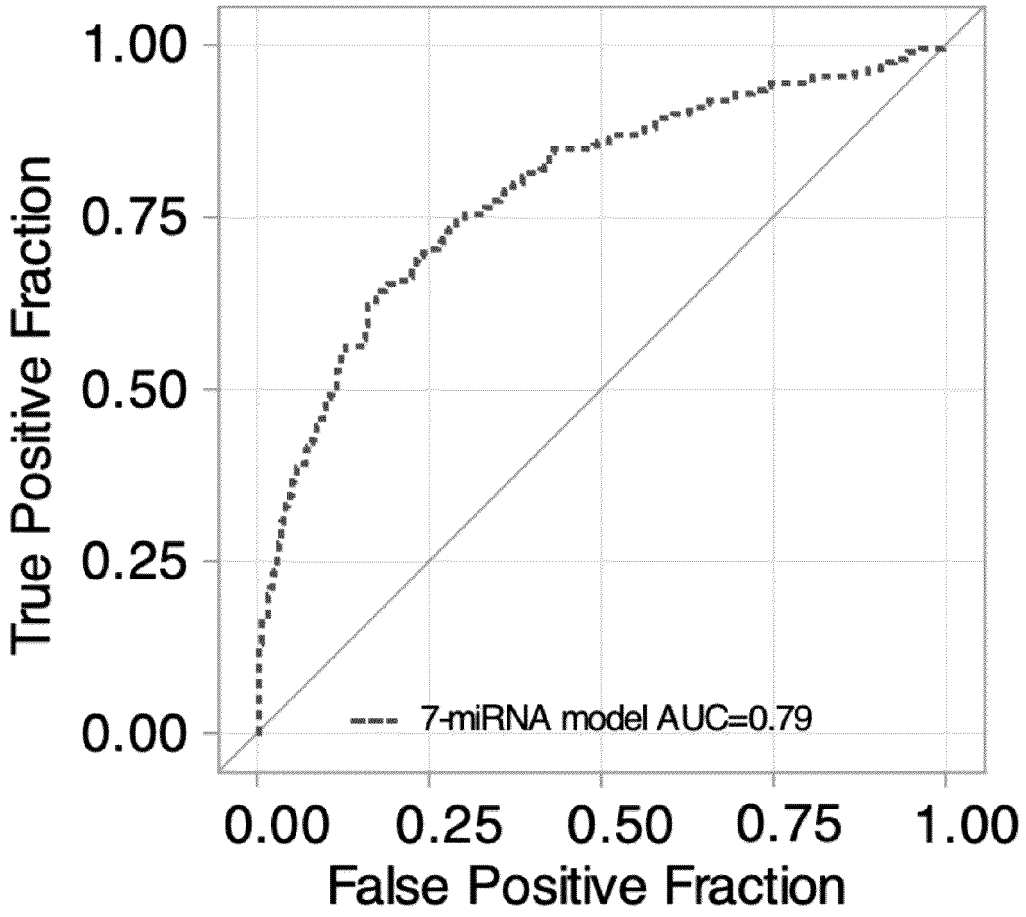

7-miRNA model
ICAM1
AQP1
COMP
KCNQ3
PARM1
TP53I11
AP1G2
CADPS2
RBPMS
hsa-miR-584-5p
TCEA3
EMC3
CRYM
DPP4
ESRP2
MXD4
KIAA1324
SCUBE3
CAMK1D
PRR15L
EPDR1
FAM210B
hsa-miR-31-3p
hsa-miR-31-5p
SNED1
TMEM150C
SMURF2
PLXNA2
RHOU
TMEM41B
IRF2BPL
PLEKHB1
SLCO4C1
MGLL
CLDN2
ORMDL3
CACNA2D2
COL9A2
SCGB3A1
COL27A1
MRPS25
PNMA2
TNKS1BP1
SPRY4
CFLAR
SCIN
hsa-miR-193b-5p
C3
SFTA3
ABCA3
hsa-miR-550a-5p
CPAMD8
LGALS9
IL1R1
RUNX1
SLC16A9
C1orf21
ECHDC2
NPC2
CCNL2
ALDH2
ADGRF5
LENG8
NAPSA
SH3RF1
IFT57
RNH1
hsa-miR-193b-3p
MPEG1
CD4
ZBTB18
PPP1R13B
C7
SNTB1
NAPA
PXMP4
LRRN4
SPTB
SCNN1B
MRC1
EDEM1
ARRB1
hsa-miR-196b-5p
ABI3BP
GALNT10
CCND2
PIGR
GOLGA7B
A2M
HMCN1
AGPAT3
FMO5
TFCP2L1
KLF9
TUBA4A
NOC2L
hsa-miR-135b-5p
SNRNP200
hsa-miR-4709-3p
HYOU1
DDHD2
NCBP2
ZKSCAN1
YEATS2
XRCC5
IMMT
ATP13A3
DDX18
IDH1
LRPPRC
RNF26
GGH
KCMF1
hsa-miR-30d-3p
TMPO
19-miRNA model only
19 and 14-miRNA overlap
14-miRNA model only
Genes
GANAB
HSPD1
FKBP4
ZNF185
FYTTD1
MTHFD1
RPL7L1
COL15A1
ERH
hsa-miR-29b-2-5p
CLNS1A
MRPL51
EIF2S1
SUDS3
RPA1
NOLC1
RALBP1
DDX23
CIITA
CD74
hsa-miR-2355-3p
ST5

(Figure 2e)

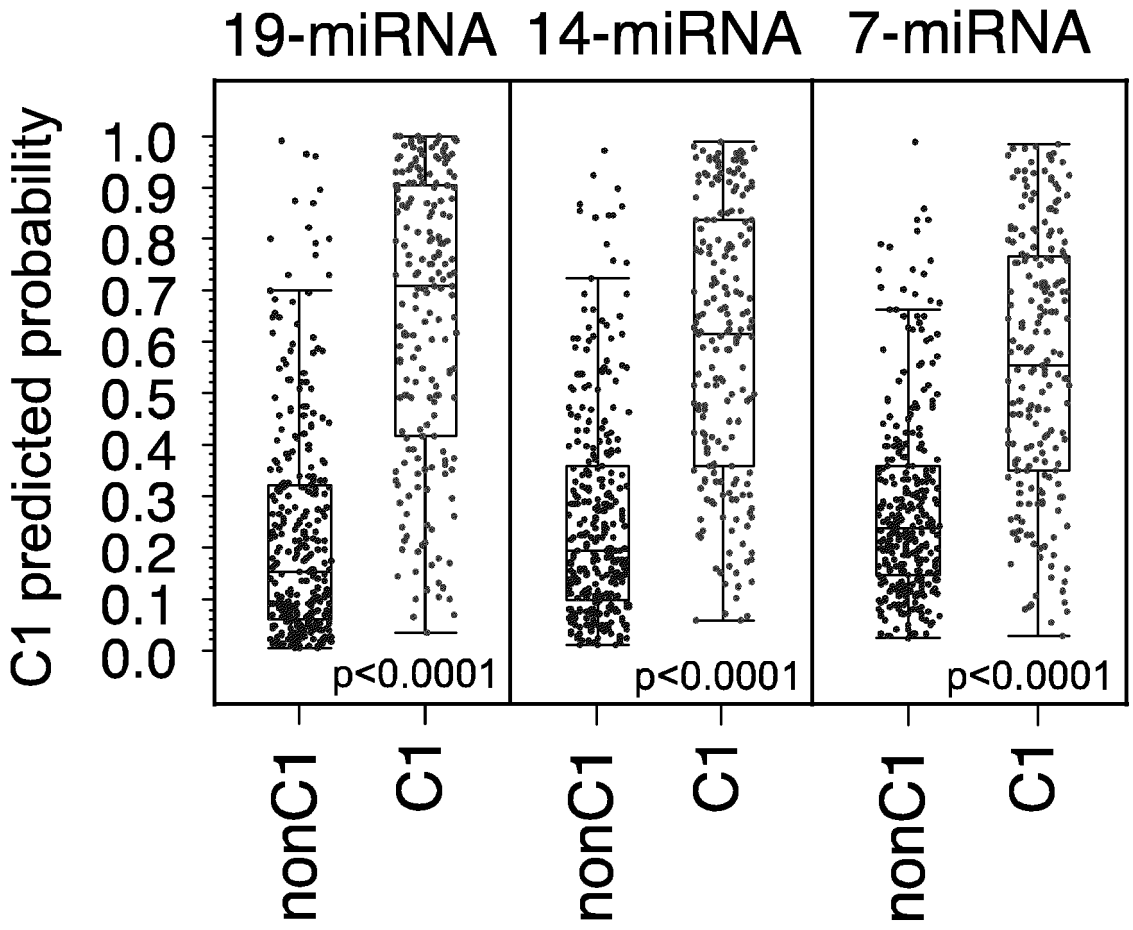

Log2 Ratio
3 2 1 0 -1 -2 -3
193b-3p
193b-5p
196b-5p
584-5p
31-5p
31-3p
550a-5p
Class
Predicted
Class: C1 nonC1
Predicted: C1 nonC1

Figures 3.a), b), c), d), e) and f). Validation of the 7-miRNAs model.

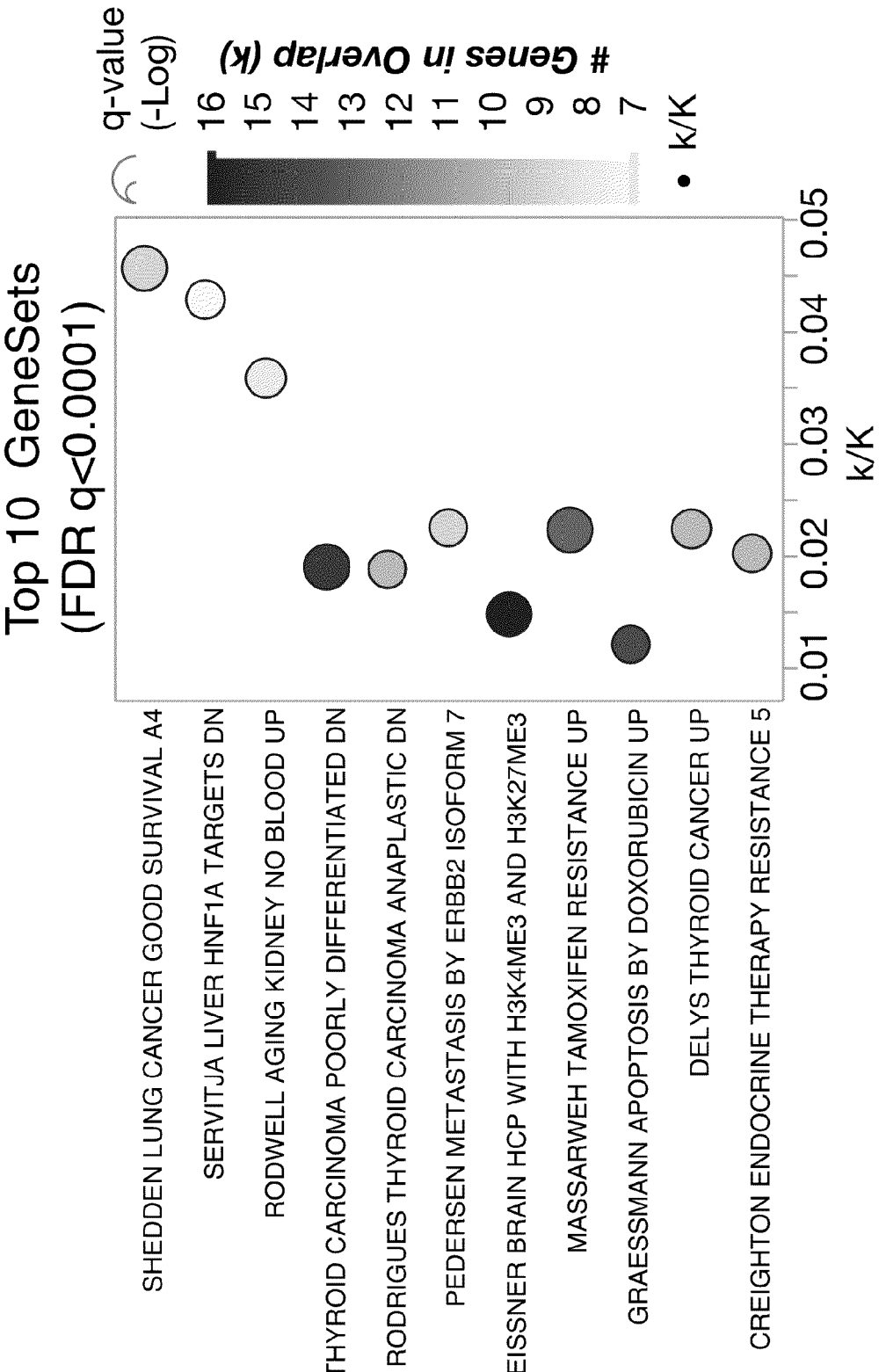
(Figure 3a)



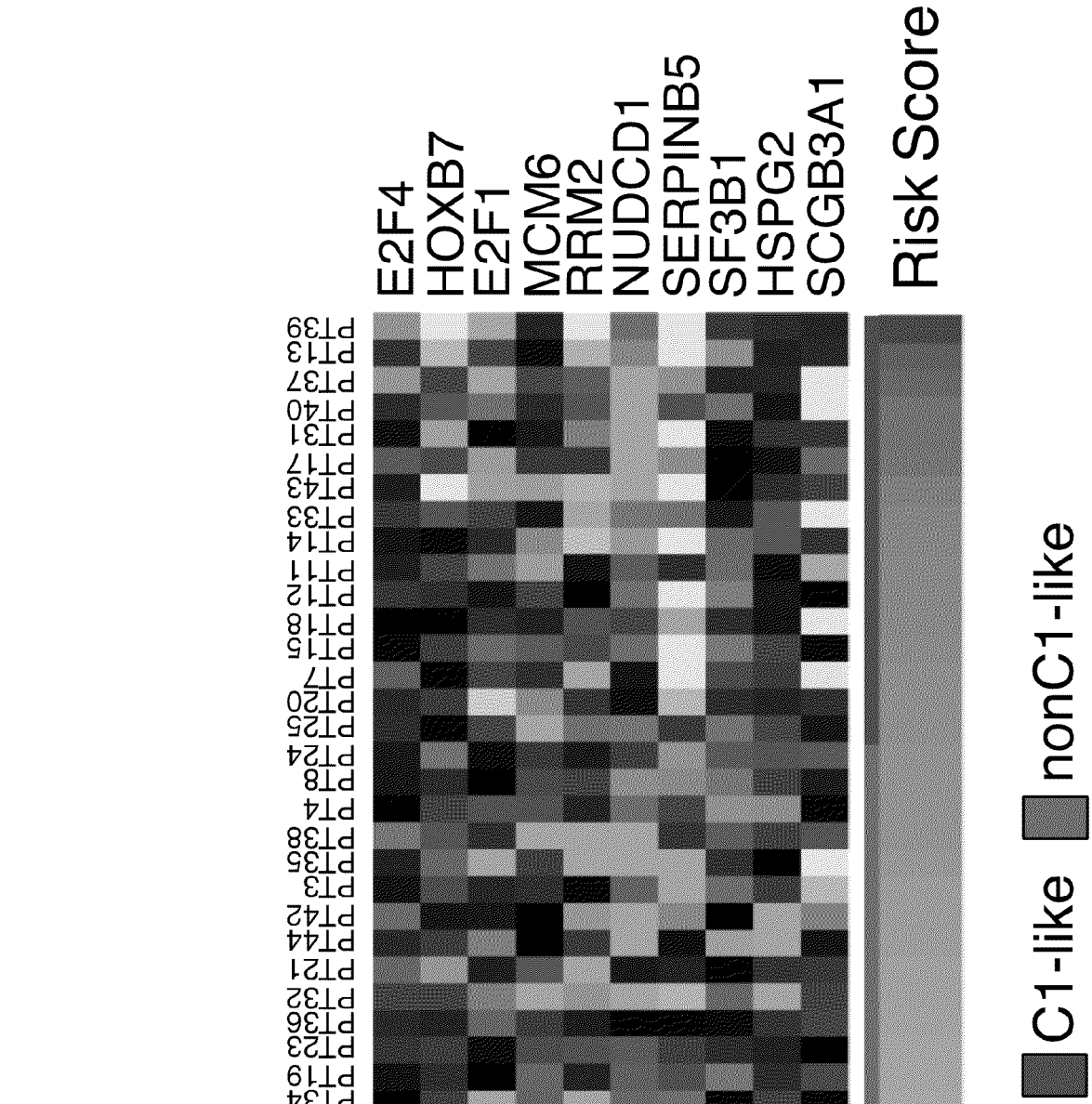
(Figure 3b)


19-miRNA
14-miRNA
7-miRNA
C1 predicted probability
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
p<0.0001
p<0.0001
p<0.0001
nonC1
C1
nonC1
C1
nonC1
C1

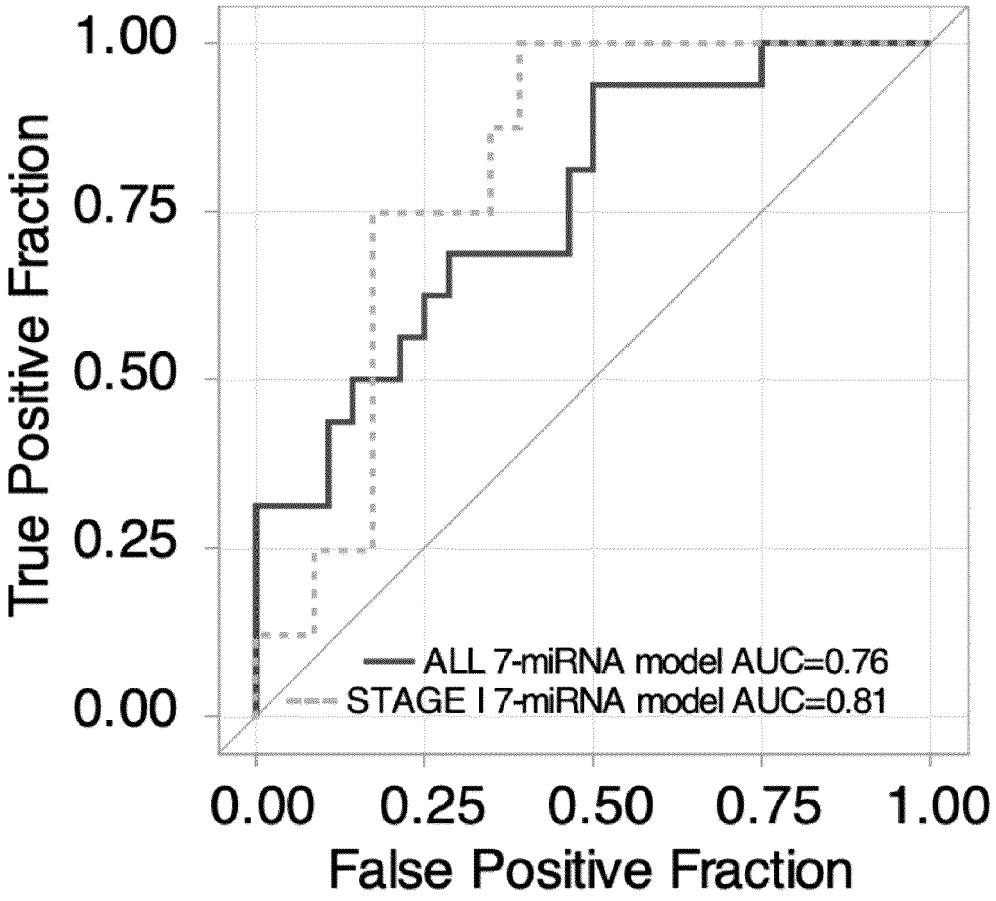
(Figure 3c)


Top 10 GeneSets
(FDR q<0.0001)
q-value
(-Log)
Genes in Overlap (k)
k/K
16
15
14
13
12
11
10
9
8
7
0.01
0.02
0.03
0.04
0.05
k/K
SHEDDEN LUNG CANCER GOOD SURVIVAL A4
SERVITJA LIVER HNF1A TARGETS DN
RODWELL AGING KIDNEY NO BLOOD UP
RODRIGUES THYROID CARCINOMA POORLY DIFFERENTIATED DN
RODRIGUES THYROID CARCINOMA ANAPLASTIC DN
PEDERSEN METASTASIS BY ERBB2 ISOFORM 7
MEISSNER BRAIN HCP WITH H3K4ME3 AND H3K27ME3
MASSARWEH TAMOXIFEN RESISTANCE UP
GRAESSMANN APOPTOSIS BY DOXORUBICIN UP
DELYS THYROID CANCER UP
CREIGHTON ENDOCRINE THERAPY RESISTANCE 5

(Figure 3d)


Log2 Ratio
3 2 1 0 -1 -2 -3
E2F4
HOXB7
E2F1
MCM6
RRM2
NUDCD1
SERPINB5
SF3B1
HSPG2
SCGB3A1
Risk Score
C1-like
nonC1-like

(Figure 3e)


1.00
0.75
0.50
0.25
0.00
True Positive Fraction
ALL 7-miRNA model AUC=0.76
STAGE I 7-miRNA model AUC=0.81
0.00
0.25
0.50
0.75
1.00
False Positive Fraction

(Figure 3f)

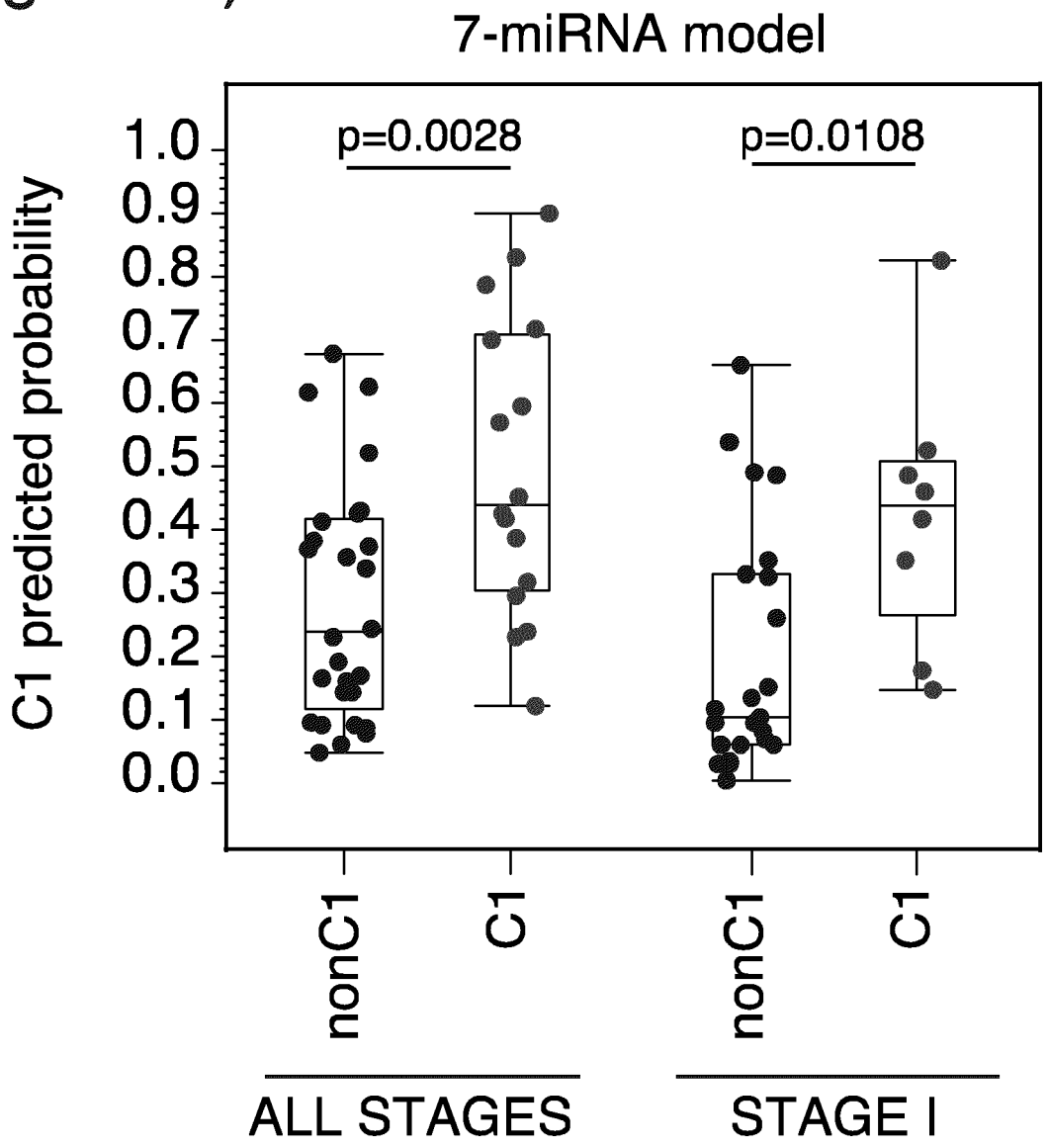

7-miRNA model
C1 predicted probability
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
p=0.0028
p=0.0108
nonC1
C1
nonC1
C1
ALL STAGES
STAGE I

Figure 4. AUC obtained from signatures of 14 and 19 miRNAs.

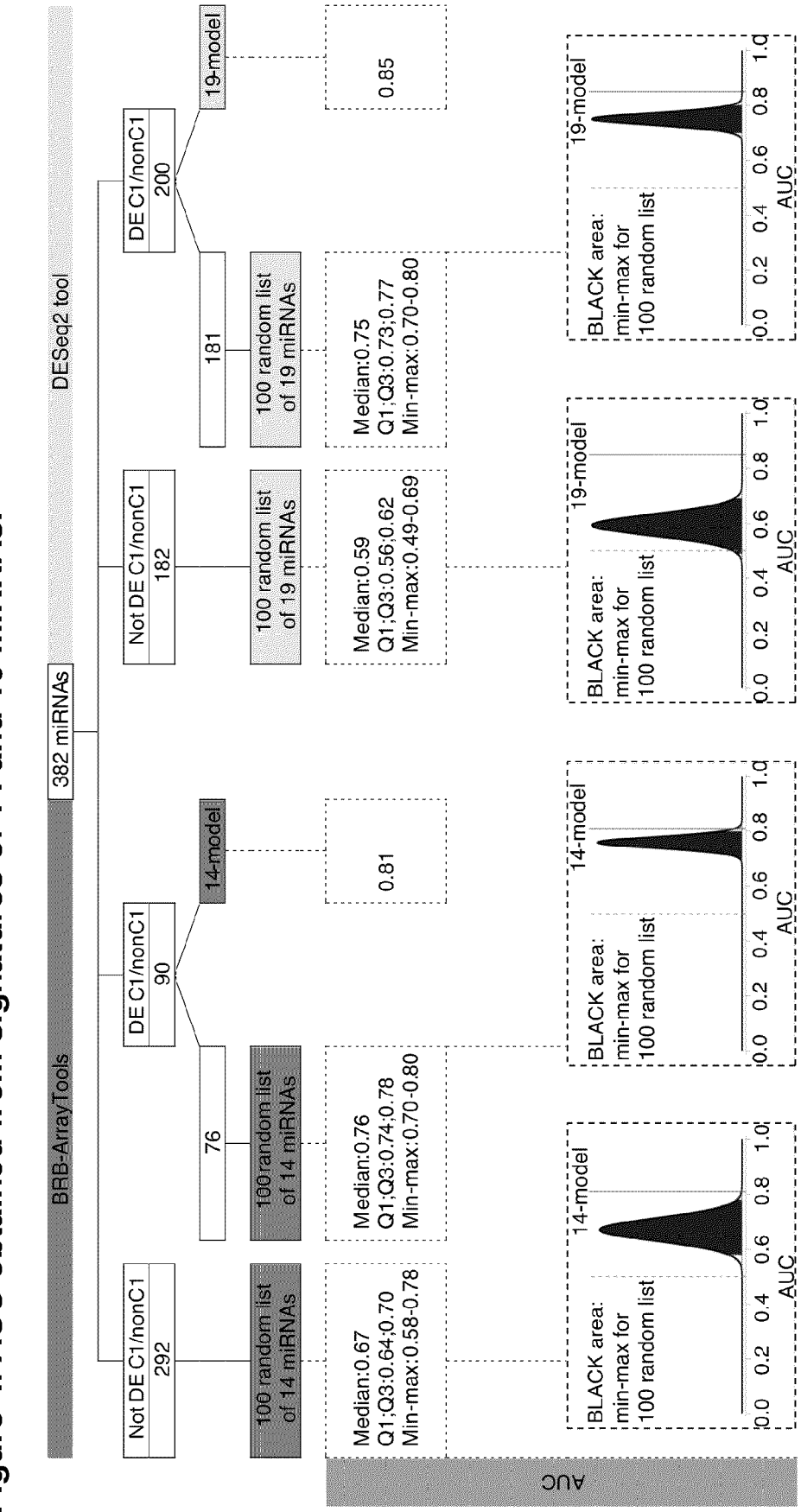


Figure 5. Table S1A. TCGA-LUAD cohort. 200 miRNAs significantly regulated by DESeq2 in C1 vs. nonC1 patients comparison.

miRNA Name	Fold Change C1 vs. nonC1 patients	Wald test p-value	Wald test adjusted p-value (Benjamini-Hochberg method)
hsa-miR-584-5p	2.78	3.24E-43	1.24E-40
hsa-miR-215-5p	4.99	6.34E-40	1.21E-37
hsa-miR-194-5p	3.31	1.35E-26	1.72E-24
hsa-miR-192-5p	3.10	1.04E-22	9.81E-21
hsa-miR-196b-5p	3.15	1.28E-22	9.81E-21
hsa-miR-31-3p	3.21	2.92E-22	1.86E-20
hsa-miR-4709-3p	0.48	2.32E-21	1.26E-19
hsa-miR-31-5p	3.15	3.57E-20	1.71E-18
hsa-miR-582-3p	2.22	5.92E-20	2.51E-18
hsa-miR-30d-5p	0.61	1.25E-17	4.79E-16
hsa-miR-30d-3p	0.65	7.06E-17	2.45E-15
hsa-miR-664a-3p	0.68	1.15E-15	3.65E-14
hsa-miR-664b-3p	0.70	5.78E-14	1.70E-12
hsa-miR-181a-5p	0.70	1.05E-13	2.87E-12
hsa-miR-30b-5p	0.66	4.61E-13	1.17E-11
hsa-miR-153-5p	1.93	3.34E-12	7.61E-11
hsa-miR-664a-5p	0.69	3.39E-12	7.61E-11
hsa-miR-138-5p	1.90	5.81E-12	1.23E-10
hsa-miR-548b-3p	0.59	3.59E-11	7.21E-10
hsa-miR-99a-5p	0.72	1.07E-10	2.04E-09
hsa-miR-421	1.51	1.84E-10	3.35E-09
hsa-miR-550a-5p	1.51	3.60E-10	5.98E-09
hsa-miR-582-5p	1.67	3.50E-10	5.98E-09
hsa-miR-30c-5p	0.74	5.25E-10	8.35E-09
hsa-miR-128-3p	1.31	7.67E-10	1.17E-08
hsa-miR-675-3p	2.13	1.07E-09	1.51E-08
hsa-miR-101-3p	0.76	1.05E-09	1.51E-08

hsa-miR-18a-5p	1.47	1.33E-09	1.82E-08
hsa-miR-432-5p	0.56	1.51E-09	1.98E-08
hsa-miR-942-5p	1.37	3.77E-09	4.80E-08
hsa-miR-29b-2-5p	0.72	1.02E-08	1.23E-07
hsa-miR-130b-3p	1.43	1.06E-08	1.23E-07
hsa-let-7c-5p	0.72	1.06E-08	1.23E-07
hsa-miR-3677-3p	1.50	2.64E-08	2.96E-07
hsa-miR-193b-5p	1.46	3.01E-08	3.29E-07
hsa-miR-362-5p	0.69	4.33E-08	4.59E-07
hsa-miR-195-5p	0.75	5.90E-08	6.09E-07
hsa-miR-629-5p	1.29	8.99E-08	9.03E-07
hsa-miR-181b-5p	0.78	1.17E-07	1.15E-06
hsa-miR-106b-3p	1.34	1.37E-07	1.31E-06
hsa-miR-9-5p	1.83	1.78E-07	1.66E-06
hsa-miR-766-3p	1.30	2.03E-07	1.85E-06
hsa-miR-1306-5p	1.33	3.56E-07	3.16E-06
hsa-miR-135b-5p	0.67	4.26E-07	3.70E-06
hsa-miR-30b-3p	0.73	5.23E-07	4.44E-06
hsa-miR-146b-5p	0.74	5.47E-07	4.54E-06
hsa-miR-1307-3p	1.28	6.02E-07	4.90E-06
hsa-miR-320b	1.33	7.61E-07	6.06E-06
hsa-miR-101-5p	0.79	7.87E-07	6.14E-06
hsa-miR-193b-3p	1.43	1.12E-06	8.55E-06
hsa-miR-26a-5p	0.82	1.15E-06	8.62E-06
hsa-miR-181a-3p	0.81	1.22E-06	8.98E-06
hsa-miR-497-5p	0.81	1.75E-06	1.26E-05
hsa-miR-30e-5p	0.83	2.05E-06	1.45E-05
hsa-miR-139-5p	0.74	2.35E-06	1.63E-05
hsa-miR-30e-3p	0.82	2.47E-06	1.68E-05
hsa-miR-146b-3p	0.77	3.02E-06	2.02E-05

hsa-miR-629-3p	1.37	4.10E-06	2.70E-05
hsa-miR-362-3p	0.76	5.03E-06	3.25E-05
hsa-miR-151b	0.77	5.12E-06	3.26E-05
hsa-miR-29c-5p	0.79	5.32E-06	3.33E-05
hsa-miR-125a-5p	0.83	5.55E-06	3.42E-05
hsa-miR-3065-3p	0.67	6.90E-06	4.18E-05
hsa-miR-660-5p	0.79	7.88E-06	4.70E-05
hsa-miR-2355-5p	1.27	8.33E-06	4.89E-05
hsa-miR-205-5p	1.75	9.15E-06	5.30E-05
hsa-miR-2355-3p	1.30	9.39E-06	5.36E-05
hsa-miR-23a-3p	1.15	9.87E-06	5.50E-05
hsa-miR-338-3p	0.68	9.94E-06	5.50E-05
hsa-miR-106b-5p	1.21	1.01E-05	5.51E-05
hsa-miR-1301-3p	1.28	1.11E-05	5.99E-05
hsa-miR-223-3p	1.34	1.17E-05	6.23E-05
hsa-miR-365b-3p	1.28	1.40E-05	7.30E-05
hsa-miR-365a-3p	1.28	1.43E-05	7.35E-05
hsa-miR-576-5p	1.25	1.44E-05	7.35E-05
hsa-miR-193a-5p	1.25	1.81E-05	9.08E-05
hsa-miR-4772-3p	1.31	1.88E-05	9.34E-05
hsa-miR-4661-5p	1.37	2.01E-05	9.73E-05
hsa-miR-769-5p	1.20	1.99E-05	9.73E-05
hsa-miR-34a-5p	0.82	2.12E-05	1.01E-04
hsa-miR-148a-5p	0.77	2.50E-05	1.18E-04
hsa-miR-20b-5p	1.51	2.62E-05	1.22E-04
hsa-miR-1266-5p	1.33	2.98E-05	1.37E-04
hsa-miR-195-3p	0.81	3.04E-05	1.38E-04
hsa-miR-95-3p	1.39	4.35E-05	1.96E-04
hsa-miR-493-5p	0.71	4.48E-05	1.99E-04
hsa-miR-187-3p	0.65	5.35E-05	2.35E-04

hsa-miR-222-5p	1.30	6.66E-05	2.89E-04
hsa-miR-203a-3p	1.39	7.33E-05	3.15E-04
hsa-miR-431-3p	0.68	7.49E-05	3.18E-04
hsa-miR-29a-3p	0.82	7.61E-05	3.19E-04
hsa-miR-27b-3p	0.85	8.41E-05	3.49E-04
hsa-miR-331-5p	1.18	8.52E-05	3.50E-04
hsa-miR-185-3p	1.24	9.59E-05	3.90E-04
hsa-miR-193a-3p	1.23	9.88E-05	3.97E-04
hsa-miR-1296-5p	1.24	1.19E-04	4.72E-04
hsa-miR-495-3p	0.69	1.26E-04	4.98E-04
hsa-miR-532-5p	0.83	1.66E-04	6.47E-04
hsa-miR-125b-2-3p	0.79	2.03E-04	7.83E-04
hsa-miR-146a-5p	0.79	2.38E-04	9.09E-04
hsa-miR-130b-5p	1.25	2.61E-04	9.88E-04
hsa-miR-29c-3p	0.81	2.84E-04	1.06E-03
hsa-miR-27a-3p	1.16	3.18E-04	1.18E-03
hsa-miR-550a-3p	1.26	3.33E-04	1.22E-03
hsa-miR-15b-3p	1.19	3.59E-04	1.31E-03
hsa-miR-502-3p	0.85	3.81E-04	1.37E-03
hsa-miR-328-3p	0.83	3.92E-04	1.40E-03
hsa-miR-330-5p	1.20	4.03E-04	1.43E-03
hsa-miR-4662a-5p	1.31	4.80E-04	1.68E-03
hsa-miR-200a-5p	1.24	4.91E-04	1.69E-03
hsa-miR-625-3p	1.20	4.92E-04	1.69E-03
hsa-miR-26a-2-3p	0.83	5.06E-04	1.73E-03
hsa-miR-218-5p	0.84	5.12E-04	1.73E-03
hsa-miR-128-1-5p	1.19	5.23E-04	1.75E-03
hsa-miR-487b-3p	0.72	5.34E-04	1.77E-03
hsa-miR-148a-3p	0.83	5.67E-04	1.87E-03
hsa-let-7a-2-3p	1.34	5.81E-04	1.90E-03

hsa-miR-133a-3p	0.74	6.01E-04	1.94E-03
hsa-miR-197-3p	1.15	6.28E-04	2.02E-03
hsa-miR-93-3p	1.19	6.56E-04	2.07E-03
hsa-miR-874-3p	1.18	6.56E-04	2.07E-03
hsa-miR-132-3p	1.16	7.00E-04	2.19E-03
hsa-miR-182-5p	0.83	8.16E-04	2.53E-03
hsa-miR-3934-3p	0.81	8.80E-04	2.70E-03
hsa-miR-224-3p	0.76	8.83E-04	2.70E-03
hsa-miR-708-5p	1.25	8.98E-04	2.72E-03
hsa-miR-25-3p	1.15	9.72E-04	2.92E-03
hsa-miR-21-3p	1.18	1.08E-03	3.21E-03
hsa-miR-15a-5p	0.88	1.08E-03	3.21E-03
hsa-miR-642a-5p	1.25	1.13E-03	3.33E-03
hsa-miR-339-3p	0.86	1.18E-03	3.44E-03
hsa-miR-1-3p	0.75	1.26E-03	3.64E-03
hsa-miR-1180-3p	1.23	1.29E-03	3.71E-03
hsa-miR-425-5p	1.17	1.56E-03	4.46E-03
hsa-miR-877-5p	1.25	1.62E-03	4.54E-03
hsa-miR-221-3p	1.20	1.62E-03	4.54E-03
hsa-miR-23b-3p	0.88	1.74E-03	4.85E-03
hsa-miR-363-3p	1.32	1.85E-03	5.12E-03
hsa-miR-24-2-5p	1.16	2.02E-03	5.56E-03
hsa-miR-493-3p	0.76	2.08E-03	5.67E-03
hsa-miR-22-3p	1.10	2.28E-03	6.19E-03
hsa-miR-222-3p	1.18	2.61E-03	6.98E-03
hsa-miR-99b-3p	1.18	2.62E-03	6.98E-03
hsa-miR-500b-5p	0.84	2.65E-03	6.98E-03
hsa-miR-542-3p	0.82	2.63E-03	6.98E-03
hsa-miR-212-3p	1.22	2.72E-03	7.01E-03
hsa-miR-136-5p	0.77	2.71E-03	7.01E-03

hsa-miR-758-5p	0.75	2.70E-03	7.01E-03
hsa-miR-500a-5p	0.84	2.76E-03	7.08E-03
hsa-miR-93-5p	1.17	2.86E-03	7.27E-03
hsa-miR-339-5p	0.86	2.94E-03	7.43E-03
hsa-miR-500a-3p	0.86	3.07E-03	7.72E-03
hsa-miR-532-3p	0.86	3.26E-03	8.14E-03
hsa-miR-509-3p	0.70	3.36E-03	8.34E-03
hsa-miR-1976	0.88	3.48E-03	8.57E-03
hsa-miR-590-5p	1.15	3.74E-03	9.16E-03
hsa-miR-200c-5p	1.18	3.83E-03	9.31E-03
hsa-miR-3065-5p	0.78	4.00E-03	9.68E-03
hsa-miR-577	1.38	4.03E-03	9.68E-03
hsa-miR-1247-3p	1.33	4.26E-03	1.02E-02
hsa-miR-151a-5p	0.89	4.46E-03	1.06E-02
hsa-miR-1287-5p	0.86	4.75E-03	1.12E-02
hsa-miR-132-5p	1.15	6.45E-03	1.51E-02
hsa-miR-1287-3p	0.83	6.66E-03	1.55E-02
hsa-miR-217	0.80	6.86E-03	1.59E-02
hsa-miR-106a-5p	1.24	6.92E-03	1.59E-02
hsa-miR-29b-3p	0.87	7.78E-03	1.78E-02
hsa-miR-17-5p	1.15	7.89E-03	1.79E-02
hsa-miR-508-3p	0.74	8.32E-03	1.88E-02
hsa-let-7c-3p	0.82	8.45E-03	1.90E-02
hsa-miR-514a-3p	0.73	9.70E-03	2.17E-02
hsa-miR-455-5p	0.88	9.79E-03	2.17E-02
hsa-miR-22-5p	1.14	1.03E-02	2.27E-02
hsa-miR-590-3p	1.15	1.08E-02	2.38E-02
hsa-miR-3074-5p	1.18	1.09E-02	2.39E-02
hsa-miR-181a-2-3p	0.89	1.11E-02	2.40E-02
hsa-miR-141-5p	1.16	1.14E-02	2.45E-02

hsa-miR-708-3p	1.17	1.15E-02	2.46E-02
hsa-miR-29a-5p	0.87	1.16E-02	2.47E-02
hsa-miR-410-3p	0.78	1.18E-02	2.50E-02
hsa-miR-196a-5p	1.40	1.28E-02	2.71E-02
hsa-miR-625-5p	1.15	1.39E-02	2.92E-02
hsa-miR-24-1-5p	0.88	1.43E-02	2.98E-02
hsa-miR-331-3p	1.13	1.49E-02	3.09E-02
hsa-miR-4326	1.26	1.52E-02	3.14E-02
hsa-miR-149-5p	1.23	1.57E-02	3.23E-02
hsa-miR-1307-5p	1.18	1.63E-02	3.33E-02
hsa-let-7g-5p	0.90	1.64E-02	3.34E-02
hsa-miR-4677-3p	1.11	1.73E-02	3.50E-02
hsa-miR-125a-3p	1.13	1.74E-02	3.51E-02
hsa-miR-181c-5p	0.88	1.76E-02	3.52E-02
hsa-miR-199b-5p	0.88	1.84E-02	3.65E-02
hsa-miR-501-5p	0.87	1.87E-02	3.70E-02
hsa-miR-19a-3p	1.17	2.10E-02	4.13E-02
hsa-miR-152-3p	1.12	2.17E-02	4.25E-02
hsa-miR-326	0.84	2.24E-02	4.37E-02
hsa-miR-345-5p	1.16	2.32E-02	4.49E-02
hsa-miR-100-5p	1.18	2.35E-02	4.50E-02
hsa-miR-150-5p	0.86	2.34E-02	4.50E-02
hsa-miR-671-5p	1.13	2.40E-02	4.58E-02

Figure 6. Table S1B. TCGA-LUAD cohort. 90 miRNAs significantly regulated by BRB-ArrayTools in C1 vs. nonC1 patients comparison.

miRNA Name	Fold Change C1 vs nonC1 patients	Parametric p-value
hsa-miR-30d-5p	0.59	< 1.00E-07
hsa-miR-30b-5p	0.62	< 1.00E-07
hsa-miR-584-5p	1.79	< 1.00E-07
hsa-miR-664a-3p	0.70	< 1.00E-07
hsa-miR-192-5p	2.17	< 1.00E-07
hsa-miR-181a-5p	0.73	< 1.00E-07
hsa-miR-196b-5p	2.53	< 1.00E-07
hsa-miR-29b-2-5p	0.70	< 1.00E-07
hsa-miR-99a-5p	0.73	< 1.00E-07
hsa-miR-128-3p	1.28	< 1.00E-07
hsa-miR-101-3p	0.78	< 1.00E-07
hsa-miR-30c-5p	0.77	< 1.00E-07
hsa-miR-582-3p	1.63	1.00E-07
hsa-miR-194-5p	1.84	5.00E-07
hsa-miR-193b-3p	1.49	5.00E-07
hsa-miR-30e-5p	0.82	7.00E-07
hsa-let-7c-5p	0.73	8.00E-07
hsa-miR-181b-5p	0.78	1.20E-06
hsa-miR-193a-5p	1.31	1.30E-06
hsa-miR-3065-3p	0.60	1.40E-06
hsa-miR-629-5p	1.25	3.70E-06
hsa-miR-135b-5p	0.63	4.40E-06
hsa-miR-9-5p	1.93	7.40E-06
hsa-miR-106b-3p	1.30	8.60E-06
hsa-miR-26a-5p	0.83	9.80E-06
hsa-miR-497-5p	0.81	1.03E-05
hsa-miR-29c-5p	0.79	1.20E-05
hsa-miR-2355-5p	1.27	1.26E-05
hsa-miR-34a-5p	0.81	3.06E-05
hsa-miR-125a-5p	0.84	3.82E-05
hsa-miR-210-3p	1.52	4.06E-05
hsa-miR-1307-3p	1.26	4.47E-05
hsa-miR-375	0.62	4.53E-05
hsa-miR-29a-3p	0.81	5.19E-05
hsa-miR-146b-3p	0.79	5.72E-05
hsa-miR-195-5p	0.80	6.41E-05
hsa-miR-181a-3p	0.83	6.53E-05
hsa-miR-365b-3p	1.25	1.05E-04
hsa-miR-27b-3p	0.84	1.07E-04
hsa-miR-365a-3p	1.25	1.07E-04
hsa-miR-1301-3p	1.24	1.83E-04
hsa-miR-23a-3p	1.13	1.93E-04
hsa-miR-660-5p	0.81	1.94E-04
hsa-miR-769-5p	1.16	2.52E-04

hsa-miR-1976	0.84	2.68E-04
hsa-miR-590-5p	1.21	2.72E-04
hsa-miR-21-3p	1.21	2.73E-04
hsa-miR-146b-5p	0.79	2.91E-04
hsa-miR-30e-3p	0.86	2.93E-04
hsa-miR-328-3p	0.83	3.63E-04
hsa-miR-223-3p	1.28	3.70E-04
hsa-miR-197-3p	1.16	4.18E-04
hsa-miR-130b-3p	1.24	4.30E-04
hsa-miR-29c-3p	0.81	6.43E-04
hsa-miR-106b-5p	1.15	9.18E-04
hsa-miR-24-2-5p	1.18	1.21E-03
hsa-miR-15b-3p	1.17	1.25E-03
hsa-miR-23b-3p	0.88	1.37E-03
hsa-miR-27a-3p	1.14	1.41E-03
hsa-miR-187-3p	0.64	1.42E-03
hsa-miR-532-5p	0.86	1.57E-03
hsa-miR-148a-3p	0.83	1.74E-03
hsa-miR-218-5p	0.84	1.84E-03
hsa-miR-132-3p	1.15	2.05E-03
hsa-miR-182-5p	0.83	2.15E-03
hsa-miR-708-5p	1.26	2.18E-03
hsa-miR-542-3p	0.81	2.19E-03
hsa-miR-29b-3p	0.84	2.30E-03
hsa-miR-148a-5p	0.81	2.48E-03
hsa-miR-134-5p	1.33	2.54E-03
hsa-miR-425-5p	1.17	2.73E-03
hsa-miR-139-5p	0.82	3.01E-03
hsa-miR-339-3p	0.87	3.17E-03
hsa-miR-22-5p	1.17	3.53E-03
hsa-miR-339-5p	0.85	3.87E-03
hsa-miR-24-1-5p	0.85	4.15E-03
hsa-miR-874-3p	1.17	4.33E-03
hsa-miR-625-3p	1.17	4.59E-03
hsa-miR-326	0.79	4.71E-03
hsa-miR-1287-5p	0.85	5.46E-03
hsa-miR-708-3p	1.21	5.54E-03
hsa-miR-150-5p	0.81	6.66E-03
hsa-miR-330-5p	1.16	7.09E-03
hsa-miR-151a-5p	0.90	8.81E-03
hsa-let-7g-5p	0.89	1.09E-02
hsa-miR-200c-5p	1.17	1.13E-02
hsa-miR-1266-5p	1.20	1.15E-02
hsa-miR-500a-3p	0.88	1.15E-02
hsa-miR-93-5p	1.14	1.20E-02
hsa-miR-331-3p	1.14	1.23E-02

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EUROPEAN SEARCH REPORT

Application Number
EP 20 21 3323

DOCUMENTS CONSIDERED TO BE RELEVANT

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A	SIRIWARDHANA CHATHURA ET AL: "Development of a miRNA-seq based prognostic signature in lung adenocarcinoma", BMC CANCER, vol. 19, no. 1, 1 December 2019 (2019-12-01), XP055803659, DOI: 10.1186/s12885-018-5206-8 Retrieved from the Internet: URL:https://bmccancer.biomedcentral.com/track/pdf/10.1186/s12885-018-5206-8.pdf> * figure 1; table 3 *	1-13	TECHNICAL FIELDS SEARCHED (IPC) C12Q

-/--

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
The Hague	12 May 2021	Santagati, Fabio

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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EUROPEAN SEARCH REPORT

Application Number
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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
A	PENG ZHUO ET AL: "Identification of microRNAs as potential biomarkers for lung adenocarcinoma using integrating genomics analysis", ONCOTARGET, vol. 8, no. 38, 8 September 2017 (2017-09-08), pages 64143-64156, XP055803811, United States ISSN: 1949-2553, DOI: 10.18632/oncotarget.19358 * figure 7 *	1-13	
A	YERUKALA SATHIPATI SRINIVASULU ET AL: "Identifying the miRNA signature associated with survival time in patients with lung adenocarcinoma using miRNA expression profiles", SCIENTIFIC REPORTS, vol. 7, no. 1, 1 December 2017 (2017-12-01), XP055804115, DOI: 10.1038/s41598-017-07739-y Retrieved from the Internet: URL:https://www.nature.com/articles/s41598-017-07739-y.pdf> * table 2 *	1-13	TECHNICAL FIELDS SEARCHED (IPC)
X	Anonymous: "miProfile TM Human miRNome miRNA qPCR Array(96-well)", , 1 January 2016 (2016-01-01), XP055803970, Retrieved from the Internet: URL:https://www.biocat.com/genomics/microrna/microrna-qpcr-arrays [retrieved on 2021-05-12] * the whole document *	14	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
The Hague	12 May 2021	Santagati, Fabio

CATEGORY OF CITED DOCUMENTS

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12-05-2021

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AU 2012238336 B2	10-04-2014	NONE	
CN 104818334 B	25-05-2018	NONE	

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