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(54) Title: SERUM MIRNA-142-3P AS PROGNOSTIC CANCER MARKER

(57) Abstract: The present invention relates to a method for predicting the risk of cancer recurrence in a subject afflicted with cancer comprising the steps of determining in a sample from said subject afflicted with cancer the amount of at least miR-142-p3 and comparing said amount with a reference, whereby the risk of cancer recurrence is predicted. The present invention also relates to methods of recommending a cancer therapy, of treating a subject afflicted with cancer, of predicting the efficacy of standard cancer treatment, and of monitoring cancer therapy as well as to the use of miR-142-p3 for diagnosing a severe form of cancer. The present invention also relates to a device for predicting the risk of cancer recurrence and to a kit for carrying out a method according to the present invention.



Serum miRNA-142-3p as prognostic cancer marker

The present invention relates to a method for predicting the risk of cancer recurrence in a subject afflicted with cancer comprising the steps of determining in a sample from said subject afflicted with cancer the amount of at least miR-142-p3 and comparing said amount with a reference, whereby the risk of cancer recurrence is predicted. The present invention also relates to methods of recommending a cancer therapy, of treating a subject afflicted with cancer, of predicting the efficacy of standard cancer treatment, and of monitoring cancer therapy as well as to the use of miR-142-p3 for diagnosing a severe form of cancer. The present invention also relates to a device for predicting the risk of cancer recurrence and to a kit for carrying out a method according to the present invention.

The outcome of resectable non-small cell lung cancer (NSCLC) is critically determined by metastatic spread: About 30-50% of early-stage NSCLC patients encounter tumour recurrence within 5 years after surgery. The identification of suitable biomarkers may help to evaluate the risk of disease recurrence in early stage lung cancer patients. Gene and miRNA expression profiling in NSCLC tissues indicated molecular signatures associated with prognosis (Chen et al. "A five-gene signature and clinical outcome in non-small-cell lung cancer." *N Engl J Med* 2007; 356: 11-20; Lee et al., "Prediction of recurrence-free survival in postoperative non-small cell lung cancer patients by using an integrated model of clinical information and gene expression." *Clin Cancer Res* 2008; 14: 7397-7404; Patnaik et al., "Evaluation of microRNA expression profiles that may predict recurrence of localized stage I non-small cell lung cancer after surgical resection." *Cancer Res* 2010; 70: 36-45; Yu et al., "MicroRNA signature predicts survival and relapse in lung cancer." *Cancer Cell* 2008; 13: 48-57).

Several studies reported variable abundance of circulating miRNAs in lung cancer patients and healthy individuals, which may be useful for diagnosis and prognosis (Fosset et al., "miR-1254 and miR-574-5p: serum-based microRNA biomarkers for early-stage non-small cell lung cancer." *J Thorac Oncol* 2011; 6: 482-488; Bianchi et al., "A serum circulating miRNA diagnostic test to identify asymptomatic high-risk individuals with early stage lung cancer." *EMBO Mol Med* 2011; 3: 495-503; Boeri et al., "MicroRNA signatures in tissues and plasma predict development and prognosis of computed tomography detected lung cancer." *Proc Natl Acad Sci U S A* 2011; 108: 3713-3718; Hu et al., "Serum microRNA signatures identified in a genome-wide serum microRNA expression profiling predict survival of non-small-cell lung cancer." *J Clin Oncol* 2010; 28: 1721-1726). For example, Bianchi and colleagues tested a population of asymptomatic high-risk individuals and identified a 34 miRNA signature in serum for the diagnosis of early-stage NSCLC. Furthermore, a signature of four 'high-risk' serum miRNAs (miR-486, miR-1, miR-499, miR-30d) was reported to predict overall survival of early-stage NSCLC patients after surgery and adjuvant chemotherapy (Hu et al, loc. cit.).

miRNAs are small, non-coding RNAs (~18-25 nucleotides in length) that regulate gene expression on a post-transcriptional level by degrading mRNA molecules or blocking their translation (Bartel DP.: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281-97). Hence, they play an essential role in the regulation of a large number of biological processes, including cancer (Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, Rassenti L, Kipps T, et al. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A 2002;99:15524-9). Under the standard nomenclature system, names are assigned to experimentally confirmed miRNAs. The prefix "mir" is followed by a dash and a number. The uncapitalized "mir-" refers to the pre-miRNA, while a capitalized "miR-" refers to the mature form. miRNAs with nearly identical sequences bar one or two nucleotides are annotated with an additional lower case letter. Species of origin is designated with a three-letter prefix, e.g. hsa for Homo sapiens (human). Two mature miRNAs originating from opposite arms of the same pre-miRNA are denoted with a -3p or -5p suffix.

Circulating miRNAs are defined as miRNAs present in the cell-free component of body fluids like plasma, serum, and the like. Lawrie et al. (Lawrie CH, Gal S, Dunlop HM, Pushkaran B, Liggins AP, Pulford K, Banham AH, Pezzella F, Boulwood J, Wainscoat JS, Hatton CS, Harris AL. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. Br J Haematol 2008;141:672-5) were among the first to demonstrate the presence of miRNAs in bodily fluids. Since then, circulating miRNAs have been reported as aberrantly expressed in blood plasma or serum in different types of cancer, e.g. prostate, colorectal or esophageal carcinoma (Brase JC, Johannes M, Schlomm T, Falth M, Haese A, Steuber T, Beissbarth T, Kuner R, Sultmann H. Circulating miRNAs are correlated with tumor progression in prostate cancer. Int J Cancer 2011;128:608-16.; Huang Z, Huang D, Ni S, Peng Z, Sheng W, Du X. Plasma microRNAs are promising novel markers for early detection of colorectal cancer. Int J Cancer 2010;127:118-26.; Zhang C, Wang C, Chen X, Yang C, Li K, Wang J, Dai J, Hu Z, Zhou X, Chen L, Zhang Y, Li Y, et al. Expression profile of microRNAs in serum: a fingerprint for esophageal squamous cell carcinoma. Clin Chem 2010;56:1871-9.). Their most important advantages include the possibility to be measured repeatedly in a minimally invasive manner as well as their remarkable stability in plasma/serum, where they circulate mostly outside of exosomes and are stable due to their binding to Argonaute proteins (Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Brian KC, Allen A, Lin DW, Urban N, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A 2008;105:10513-8; Turchinovich A, Weiz L, Langhein A, Burwinkel B. Characterization of extracellular circulating microRNA. Nucleic Acids Res 2011;39:7223-33; Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, Mitchell PS, Bennett CF, Pogosova-Agadjanyan EL, Stirewalt DL, Tait JF, Tewari M. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A 2011;108:5003-8).

A tissue biomarker-driven stratification of cancer with a high risk of recurrence may improve therapy management and patient care. Insufficient amounts and quality of tumor sample material, as well as a lack of patient compliance to highly invasive sampling methods, however,

may limit the future application of tissue biomarkers in routine diagnostics. Furthermore, it is unlikely that tissue biomarkers are also useful to monitor the disease course after surgery. There is, thus, a need in the art for suitable, less-invasive biomarkers from surrogates which may help to evaluate the risk of disease recurrence in subjects afflicted with cancer.

The technical problem underlying the present invention, thus, could be seen as the provision of means and methods for complying with the aforementioned needs. The said technical problem is solved by the embodiments characterized in the claims and herein below.

Accordingly, the present invention relates to a method for predicting the risk of cancer recurrence in a subject afflicted with cancer comprising the steps of:

- a) determining in a sample from said subject afflicted with cancer the amount of at least miR-142-p3; and
- b) comparing said amount with a reference, whereby the risk of cancer recurrence is predicted.

The methods of the present invention, preferably, are in vitro methods. Moreover, they may comprise steps in addition to those explicitly mentioned above. Moreover, one or more of the steps of the methods of the present invention may be performed or assisted by automated equipment. More preferably, the methods of the present invention comprise the further step of staging of the cancer.

The term "cancer staging" is known to the skilled person and relates to assessing spread of the cancer from the primary tumor, i.e. the original source. Preferably, for solid tumors cancer staging is performed according to the TNM Classification of Malignant Tumours (TNM), based on the size of the primary tumor, lymph node involvement, and distant metastasis and, more preferably, assigning stages 0, IA, IB, IIA, IIB, IIIA, IIIB, and IV.

In the method for predicting the risk of cancer recurrence, preferably, further steps may relate, e.g., to sample pretreatment for step a), or to obtaining a reference as described herein below in step b).

The term "cancer" in the context of this invention refers to a disease of an animal, including man, characterized by uncontrolled growth by a group of body cells ("cancer cells"). This uncontrolled growth may be accompanied by intrusion into and destruction of surrounding tissue (invasion) and possibly spread of cancer cells to other locations in the body (metastasis). Preferably, the cancer is selected from the list consisting of acute lymphoblastic leukemia, acute myeloid leukemia, adrenocortical carcinoma, aids-related lymphoma, anal cancer, appendix cancer, astrocytoma, atypical teratoid, basal cell carcinoma, bile duct cancer, bladder cancer, brain stem glioma, breast cancer, burkitt lymphoma, carcinoid tumor, cerebellar astrocytoma, cervical cancer, chordoma, chronic lymphocytic leukemia, chronic myelogenous leukemia, colon cancer, colorectal cancer, craniopharyngioma, endometrial cancer, ependymblastoma, ependymoma, esophageal cancer, extracranial germ cell tumor, extragonadal germ cell tumor, extrahepatic bile duct cancer, gallbladder cancer, gastric cancer, gastrointestinal stromal tumor, gestational trophoblastic tumor, hairy cell leukemia, head and neck cancer, hepatocellular cancer, hodgkin lymphoma, hypopharyngeal cancer, hypothalamic and visual pathway glioma,

intraocular melanoma, kaposi sarcoma, laryngeal cancer, medulloblastoma, medulloepithelioma, melanoma, merkel cell carcinoma, mesothelioma, mouth cancer, multiple endocrine neoplasia syndrome, multiple myeloma, mycosis fungoides, nasal cavity and paranasal sinus cancer, nasopharyngeal cancer, neuroblastoma, non-hodgkin lymphoma, non-small cell lung cancer, oral cancer, oropharyngeal cancer, osteosarcoma, ovarian cancer, ovarian epithelial cancer, ovarian germ cell tumor, ovarian low malignant potential tumor, pancreatic cancer, papillomatosis, paranasal sinus and nasal cavity cancer, parathyroid cancer, penile cancer, pharyngeal cancer, pheochromocytoma, pituitary tumor, pleuropulmonary blastoma, primary central nervous system lymphoma, prostate cancer, rectal cancer, renal cell cancer, retinoblastoma, rhabdomyosarcoma, salivary gland cancer, sézary syndrome, small cell lung cancer, small intestine cancer, soft tissue sarcoma, squamous cell carcinoma, squamous neck cancer, testicular cancer, throat cancer, thymic carcinoma, thymoma, thyroid cancer, urethral cancer, uterine sarcoma, vaginal cancer, vulvar cancer, waldenström macroglobulinemia, and wilms tumor. More preferably, the cancer is lung cancer; even more preferably, the cancer is non-small cell lung cancer (NSCLC), most preferably NSCLC grade I, grade II, or grade IIIa.

The term "cancer recurrence" as used herein relates to a re-growth of cancer cells in a subject after a primary tumor has been removed (relapse). It is understood by the skilled artisan that cancer recurrence, preferably, relates to a local re-growth of cancer cells (local relapse) and/or to metastasis, including both local and distant metastasis.

As used herein, the term "predicting the risk" refers to assessing the probability according to which a subject will be suffering from a disease or condition referred to herein within a defined time window (predictive window) in the future. The predictive window is an interval in which the subject shall develop the disease or condition according to the predicted probability. The predictive window may be the entire remaining lifespan of the subject upon analysis by the method of the present invention. Preferably, however, the predictive window is an interval of one month, six months or one, two, three, four, five or ten years after the sample to be analyzed by the method of the present invention has been obtained. As will be understood by those skilled in the art, such an assessment is usually not intended to be correct for 100% of the subjects to be analyzed. The term, however, requires that the assessment will be valid for a statistically significant portion of the subjects to be analyzed. Whether a portion is statistically significant can be determined without further ado by the person skilled in the art using various well known statistic evaluation tools, e.g., determination of confidence intervals, p-value determination, Student's t-test, Mann-Whitney test, etc.. Details are found in Dowdy and Wearden, Statistics for Research, John Wiley & Sons, New York 1983. Preferred confidence intervals are at least 90%, at least 95%, at least 97%, at least 98% or at least 99 %. The p-values are, preferably, 0.1, 0.05, 0.01, 0.005, or 0.0001. Preferably, the probability envisaged by the present invention allows that the prediction will be correct for at least 60%, at least 70%, at least 80%, or at least 90% of the subjects of a given cohort or population.

Accordingly, the term "predicting the risk of cancer recurrence" relates to assessing the probability according to which a subject will be afflicted with a recurrence of cancer as defined herein above.

The term "subject", as referred to herein, encompasses animals, preferably mammals, and, more preferably, humans. Preferably, the subject comprises a gene encoding a miR-142-p3 or a homolog thereof as described herein below. Preferably, said subject is afflicted with cancer, more preferably with lung cancer, even more preferably with NSCLC, and most preferably with a NSCLC grade I, grade II, or grade IIIa. Preferably, the subject is a subject wherein the primary tumor has not or not yet been removed. Subjects which suffer from the said cancer can be identified by the accompanying symptoms known for the disease. These symptoms are known in the art and described, e.g., in Patel and Peters (1993), Mayo Clin Proc., 68, 273-7.505.

The term "miRNA" or "microRNA" is understood by the skilled artisan and relates to a short ribonucleic acid (RNA) molecule found in eukaryotic cells and in body fluids of metazoan organisms. It is to be understood that the present invention preferably also encompasses pri-miRNAs, and the pre-miRNAs of the miRNAs of the present invention. Thus preferably, a miRNA-precursor consists of 25 to several thousand nucleotides, more preferably 40 to 130 nucleotides, even more preferably 50 to 120 nucleotides, or, most preferably 60 to 110 nucleotides. Preferably, a miRNA consists of 5 to 100 nucleotides, more preferably 10 to 50 nucleotides, even more preferably 12 to 40 nucleotides, or, most preferably 18 to 26 nucleotides. Preferably, the miRNAs of the present invention are miRNAs of human origin, i.e. they are encoded in the human genome. Also preferably, the term miRNA relates to the "guide" strand which eventually enters the RNA-induced silencing complex (RISC) as well as to the "passenger" strand complementary thereto. Moreover, any relation to a specific miRNA in this specification is, preferably, to be understood to include variants of the specific miRNA. Said variants may represent orthologs, paralogs or other homologs. Further, variants include polynucleotides comprising nucleic acid sequences which are at least 95%, at least 98% or at least 99% identical to the nucleic acid sequences of the specific miRNA sequences. The percent identity values are, preferably, calculated over the entire nucleic acid sequence region. A series of programs based on a variety of algorithms is available to the skilled worker for comparing different sequences. In this context, the algorithms of Needleman and Wunsch or Smith and Waterman give particularly reliable results. To carry out the sequence alignments, the program PileUp (J. Mol. Evolution., 25, 351-360, 1987, Higgins et al., CABIOS, 5 1989: 151-153) or the programs Gap and BestFit (Needleman and Wunsch (J. Mol. Biol. 48; 443-453 (1970)) and Smith and Waterman (Adv. Appl. Math. 2; 482-489 (1981))), which are part of the GCG software packet (Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711 (1991)), are to be used. The sequence identity values recited above in percent (%) are to be determined, preferably, using the program GAP over the entire sequence region with the following settings: Gap Weight: 50, Length Weight: 3, Average Match: 10.000 and Average Mismatch: 0.000, which, unless otherwise specified, shall always be used as standard settings for sequence alignments.

The term "miR-142-p3" relates to a polynucleotide being the miRNA excised from the 3' arm of a mir-142 stem loop. Preferably, the miR-142-p3 is the human miR-142-p3, having a nucleotide sequence 5'-uguaguguuuccuacuuuauugga-3', (SEQ ID NO:1; miRBase (Griffiths-Jones S., NAR 2004 32(Database Issue):D109-D111; Kozomara A, Griffiths-Jones S., NAR 2011 39(Database Issue):D152-D157; Griffiths-Jones et al. (2006), NAR 34, Database Issue: D140-D144) Acc.

number: MIMAT0000434), excised from the 3' arm of hsa-miR-142 (SEQ ID NO:2; miRBase Acc. number: MI0000458, Genbank Acc. number: NR_029683.1 GI:262205315).

The term "sample", as used herein, refers to a sample from a tissue or an organ; more preferably, the term relates to a sample of a body fluid, to a sample of wash/rinse fluid obtained from an outer or inner body surface, or to a breath condensate. More preferably, samples of body fluids are samples of, e.g., preferably, blood, plasma, serum, urine, saliva, lacrimal fluid, fluids obtainable from the breast glands, e.g. milk, endobronchial epithelial liquid, or sputum. More preferably, the samples of body fluids are free of cells of the subject or have been treated to be free of cells of the subject, e.g. by centrifugation or by filtration. Samples can be obtained by well-known techniques and include, preferably, scrapes, swabs or biopsies from the digestive tract, liver, pancreas, anal canal, the oral cavity, the upper aerodigestive tract and the epidermis. Such samples can be obtained by use of brushes, (cotton) swabs, spatula, rinse/wash fluids, punch biopsy devices, puncture of cavities with needles or surgical instrumentation. Tissue or organ samples may be obtained from any tissue or organ by, e.g., biopsy or other surgical procedures. Separated cells may be obtained from the body fluids or the tissues or organs by separating techniques such as filtration, centrifugation or cell sorting. Preferably, samples are obtained from those body fluids, cells, tissues or organs which are known or suspected to contain at least one miRNA of the present invention. More preferably, samples are obtained from those body fluids, cells, tissues or organs described herein below to contain the miRNAs of the present invention. Preferably, the sample is a blood sample, more preferably a plasma sample, most preferably a plasma sample processed as described herein below.

The term "amount" as used herein encompasses the absolute amount of a miRNA referred to herein, the relative amount or concentration of a miRNA referred to herein, as well as any value or parameter which correlates thereto. Such values or parameters comprise intensity signal values from all specific physical or chemical properties obtained from the a miRNA referred to herein by measurements, e.g., intensity signals obtained from specifically bound ligands. It is to be understood that values correlating to the aforementioned amounts or parameters can also be obtained by all standard mathematical operations.

The term "determining the amount" relates to ascertaining an amount of a compound present in a sample. The amount of a miRNA can be determined in a sample of a subject by techniques well known in the art and/or described herein in the examples. Measuring the amount of a miRNA is, preferably, accomplished by, e.g. mass spectrometry, Northern Blots, or, more preferably, PCR-based determination techniques. Preferably, the amount of a miRNA of the present invention is determined using a detection agent. As used herein, the term "detection agent" relates to an agent specifically interacting with, and thus recognizing, a miRNA of the present invention. Preferably, said detection agent is a polynucleotide or an oligonucleotide. Preferably, the detection agent is labeled in a way allowing detection of said detection agent by appropriate measures. Labeling can be done by various techniques well known in the art and depending of the label to be used. Preferred labels to be used are fluorescent labels comprising, inter alia, fluorochromes such as fluorescein, rhodamin, or Texas Red. However, the label may also be an enzyme or an antibody. It is envisaged that an enzyme to be used as a label will generate a detectable signal by reacting with a substrate. Suitable enzymes, substrates and techniques are

well known in the art. An oligonucleotide to be used as label may specifically recognize a target molecule which can be detected directly (e.g., a target molecule which is itself fluorescent) or indirectly (e.g., a target molecule which generates a detectable signal, such as an enzyme). The labeled detection agents of the sample will be contacted to the sample to allow specific interaction of the labeled detection agent with the miRNAs in the sample. Washing may be required to remove nonspecifically bound detection agent which otherwise would yield false values. After this interaction step is complete, a researcher will place the detection device into a reader device or scanner. A device for detecting fluorescent labels, preferably, consists of some lasers, preferably a special microscope, and a camera. The fluorescent labels will be excited by the laser, and the microscope and camera work together to create a digital image of the sample. These data may be then stored in a computer, and a special program will be used, e.g., to subtract out background data. The resulting data are, preferably, normalized, and may be converted into a numeric and common unit format. The data will be analyzed to compare samples to references and to identify significant changes. It is to be understood that the labeled detection agent need not necessarily detect the specific miRNA molecule isolated from the sample; the detection agent may also detect the amplification product obtained from said miRNA molecule, e.g., preferably, by PCR. It is, however, also envisaged that the detection agent is used without a label. Preferably, the detection agent is bound to a solid surface and the sample, comprising miRNAs from a sample which have been labeled are contacted to with said surface-bound detection agent. Preferably, in addition to miR-142-p3 at least one marker selected from the list consisting of miR-29b-3p (SEQ ID NO:5; miRBase Acc. number: MIMAT0000100), miR-331-3p (SEQ ID NO:6; miRBase Acc. number: MIMAT0000760), miR-486-5p (SEQ ID NO:9; miRBase Acc. number: MIMAT0002177), miR-20b-5p (SEQ ID NO:4; miRBase Acc. number: MIMAT0001413), miR-338-3p (SEQ ID NO:7; miRBase Acc. number: MIMAT0000763), miR-183-3p (SEQ ID NO:3; miRBase Acc. number: MIMAT0004560), miR-517a-3p (SEQ ID NO:10; miRBase Acc. number: MIMAT0002852) and miR-380-5p (SEQ ID NO:8; miRBase Acc. number: MIMAT0000734) is determined.

“Comparing” as used herein encompasses comparing the amount of the miRNA referred to herein which is comprised by the sample to be analyzed with an amount of the said miRNA in a suitable reference sample as specified elsewhere herein in this description. It is to be understood that comparing as used herein refers to a comparison of corresponding parameters or values, e.g., an absolute amount of the miRNA as referred to herein is compared to an absolute reference amount of said miRNA; a concentration of the miRNA as referred to herein is compared to a reference concentration of said miRNA; an intensity signal obtained from the miRNA as referred to herein in a test sample is compared to the same type of intensity signal of said miRNA in a reference sample. The comparison referred to in the methods of the present invention may be carried out manually or computer assisted. For a computer assisted comparison, the value of the determined amount may be compared to values corresponding to suitable references which are stored in a database by a computer program. The computer program may further evaluate the result of the comparison by means of an expert system. Accordingly, the result of the identification referred to herein may be automatically provided in a suitable output format. It is understood by the skilled person that an amount of a specific marker, preferably miRNA, determined in a sample will preferably be compared to a matching reference, i.e. to a reference

obtained by determining the amount of said same marker, preferably miRNA, in a sample from at least one reference subject. Thus, e.g., a value of miR-142-p3 determined in a sample of a subject is, preferably, compared to a reference value of miR-142-p3 determined in at least one sample from at least one reference subject.

5 The term "reference", "reference value", or "reference amount" as used herein refers to an amount of miRNA, which allows assessing if a high risk of cancer recurrence or a low risk of cancer recurrence is to be assumed for the subject from which the sample is derived. A suitable reference value may be determined from a reference sample to be analyzed together, i.e. simultaneously or subsequently, with the sample.

Reference amounts can, in principle, be calculated for a group or cohort of subjects as specified herein based on the average or median values for a given miRNA by applying standard methods of statistics. In particular, accuracy of a test such as a method aiming to diagnose an event, or not, is best described by its receiver-operating characteristics (ROC) (see especially Zweig 1993, Clin. Chem. 39:561-577). The ROC graph is a plot of all of the sensitivity versus specificity pairs resulting from continuously varying the decision threshold over the entire range of data observed. The clinical performance of a diagnostic method depends on its accuracy, i.e. its ability to correctly allocate subjects to a certain prognosis or diagnosis. The ROC plot indicates the overlap between the two distributions by plotting the sensitivity versus 1-specificity for the complete range of thresholds suitable for making a distinction. On the y-axis is sensitivity, or the true-positive fraction, which is defined as the ratio of number of true-positive test results to the sum of number of true-positive and number of false-negative test results. This has also been referred to as positivity in the presence of a disease or condition. It is calculated solely from the affected subgroup. On the x-axis is the false-positive fraction, or 1-specificity, which is defined as the ratio of number of false-positive results to the sum of number of true-negative and number of false-positive results. It is an index of specificity and is calculated entirely from the unaffected subgroup. Because the true- and false-positive fractions are calculated entirely separately, by using the test results from two different subgroups, the ROC plot is independent of the prevalence of the event in the cohort. Each point on the ROC plot represents a sensitivity/-specificity pair corresponding to a particular decision threshold. A test with perfect discrimination (no overlap in the two distributions of results) has an ROC plot that passes through the upper left corner, where the true-positive fraction is 1.0, or 100% (perfect sensitivity), and the false-positive fraction is 0 (perfect specificity). The theoretical plot for a test with no discrimination (identical distributions of results for the two groups) is a 45° diagonal line from the lower left corner to the upper right corner. Most plots fall in between these two extremes. If the ROC plot falls completely below the 45° diagonal, this is easily remedied by reversing the criterion for "positivity" from "greater than" to "less than" or vice versa. Qualitatively, the closer the plot is to the upper left corner, the higher the overall accuracy of the test. Dependent on a desired confidence interval, a threshold can be derived from the ROC curve allowing for the diagnosis or prediction for a given event with a proper balance of sensitivity and specificity, respectively. Accordingly, the reference to be used for the methods of the present invention can be generated, preferably, by establishing a ROC for said cohort as described above and deriving a threshold amount there from. Dependent on a desired sensitivity and specificity for a diagnostic method, the ROC plot allows deriving suitable thresholds. Preferably, the

reference amounts lie within the range of values that represent a sensitivity of at least 75% and a specificity of at least 45%, or a sensitivity of at least 80% and a specificity of at least 40%, or a sensitivity of at least 85% and a specificity of at least 33%, a sensitivity of at least 90% and a specificity of at least 25%, a sensitivity of at least 80% and a specificity of at least 70%, a sensitivity of at least 80% and a specificity of at least 80%, a sensitivity of at least 80% and a specificity of at least 90%, a sensitivity of at least 70% and a specificity of at least 70%, a sensitivity of at least 70% and a specificity of at least 80%, or a sensitivity of at least 70% and a specificity of at least 90%.

Preferably, the reference amount as used herein is derived from samples of subjects for which it is known if their donors were being afflicted with cancer recurrence within the predictive window, or not (reference subjects). It is understood by the skilled person that it is preferable to derive a reference value from more than one subject, preferably, more than five, more than ten, more than 25, more than 50, or more than 100 reference subjects. More preferably, the reference value is derived from a population of reference subjects sufficiently large to allow for a statistically significant prediction. Thus, a deviation, i.e. a decrease or an increase of the miRNA amounts referred to herein is, preferably, a statistically significant deviation, i.e. a statistically significant decrease or a statistically significant increase.

The reference amount level may be a discrete figure or may be a range of figures. Evidently, the reference level or amount may vary between individual species of miRNA. The measuring system therefore, preferably, is calibrated with a sample or with a series of samples comprising known amounts of each specific miRNA. It is understood by the skilled person that in such case the amount of miRNA can preferably be expressed as arbitrary units (AU). Thus, preferably, the amounts of miRNA are determined by comparing the signal obtained from the sample to signals comprised in a calibration curve. The reference amount applicable for an individual subject may vary depending on various physiological parameters such as age or subpopulation. Thus, a suitable reference amount may be determined by the methods of the present invention from a reference sample to be analyzed together, i.e. simultaneously or subsequently, with the test sample. Moreover, a threshold amount can be preferably used as a reference amount. More preferably, the reference amounts are reference ranges which represent the 75th, the 80th, the 85th, the 90th, the 91st, the 92nd, the 93rd, the 94th, the 95th, the 96th, the 97th, the 98th, or the 99th percentile of amounts found in reference subjects. Also preferably, the reference amounts are reference ranges which represent the average or mean values ± 1 , 1.5, 2, 2.5, 3, 3.5, 4, 4.5 standard deviations of amounts found in reference subjects for a given population or cohort of subjects.

In a preferred embodiment of the present invention, the reference amount is derived from at least one reference subject not afflicted with the cancer for which the risk of recurrence is to be predicted or from at least one reference subject not afflicted with cancer at all. More preferably, the reference amount is derived from at least one reference subject afflicted with the cancer for which the risk of recurrence is to be predicted, but known not to have a high risk of cancer recurrence. Most preferably, the reference amount is derived from at least one reference subject afflicted with the cancer for which the risk of recurrence is to be predicted, but having survived without cancer recurrence for at least 5, 7, 10, 20, 30, or 40 months after removal of said cancer.

In such case, it has been found that an amount of miRNA increased relative to the reference

amount or reference range is, preferably, indicative of a subject being at high risk of cancer recurrence, while an amount of miRNA equal to or decreased relative to said reference is indicative for a low risk of tumor recurrence.

- 5 In another preferred embodiment, the reference amount is obtained from at least one reference subject afflicted with the cancer for which the risk of recurrence is to be predicted and known to have a high risk of cancer recurrence. More preferably, the reference amount is obtained from at least one reference subject afflicted with the cancer for which the risk of recurrence is to be predicted and afflicted with cancer recurrence at least 5, 7, 10, 20, 30, or 40 months after
10 removal of said cancer. In such case, it has been found that an amount of miRNA equal to or increased relative to the reference amount or reference range is, preferably, indicative of a subject being at high risk of cancer recurrence, while a decreased amount of miRNA is indicative for a low risk of tumor recurrence.
- 15 Advantageously, it was found in the studies underlying the present invention, that a determination of the amount of miR-142-p3 in a serum sample of a patient afflicted with NSCLC allows for predicting the risk of said patient to suffer from relapse, i.e. recurrence of the tumor and/or metastasis. Specifically, it was found that an increase in miR-142-p3 was highly predictive for recurrence within 24 months. Moreover, increased levels of miR-142-p3 were associated
20 with decreased overall and disease-free survival. Thus, the present invention provides for a prediction marker of disease progress, which can be measured in samples accessible with low invasive methods.

The definitions made above apply mutatis mutandis to the following:

- 25 The present invention further relates to a method of recommending a cancer therapy to a subject comprising the steps of:
- i) the method for predicting the risk of cancer recurrence of the present invention; and
 - ii) the further step of recommending a cancer therapy to the subject depending on the result of
30 the comparison of step b).

As used herein, the term "therapy" refers to all measures applied to a subject to ameliorate the diseases or disorders referred to herein or the symptoms accompanied therewith to a significant extent. Said therapy as used herein also includes measures leading to an entire restoration of the
35 health with respect to the diseases or disorders referred to herein. It is to be understood that therapy as used in accordance with the present invention may not be effective in all subjects to be treated. However, the term shall require that a statistically significant portion of subjects being afflicted with a disease or disorder referred to herein can be successfully treated. Whether a portion is statistically significant can be determined without further ado by the person skilled in
40 the art using various well known statistic evaluation tools discussed herein above.

The term "cancer therapy", as used herein, relates to applying to a subject afflicted with cancer measures to remove cancer cells from the subject, to inhibit growth of cancer cells, to kill cancer cells, or to cause the body of a patient to inhibit the growth of or to kill cancer cells. Preferably,
45 cancer therapy in the context of the method of recommending a cancer therapy according to the

present invention relates to measures to prevent cancer recurrence and/or to measures for early detection of cancer recurrence. Thus, it is understood that recommending cancer therapy includes follow-up examinations, preferably after removal of the primary tumor, radiation therapy or surgery, alone or combination with other therapy regimens. It is understood by the skilled person that the selection of the cancer therapy may depend on several factors, like age of the subject, tumor staging, and receptor status of tumor cells. It is, however, also understood by the person skilled in the art, that the selection of the cancer therapy can be assisted by the methods of the present invention: if, e.g. a low risk of recurrence is diagnosed by the method for predicting the risk of cancer recurrence, surgical removal of tumor may be sufficient. If, on the other hand, a high risk of cancer recurrence is determined, therapy measures in addition to surgery, e.g. chemotherapy and/or targeted therapy and/or immunotherapy may be required. Treatment protocols appropriate for a subject with a high risk of cancer recurrence and treatment protocols appropriate for a subject with a low risk of cancer recurrence are known in the art, e.g., in the case of NSCLC, surgery alone in the low-risk group or surgery with adjuvant chemo- and radiotherapy in the high-risk group.

As used herein, the term "chemotherapy" relates to treatment of a subject with an antineoplastic drug. Preferably, chemotherapy is a treatment including alkylating agents (e.g. cyclophosphamide), platinum (e.g. carboplatin), anthracyclines (e.g. doxorubicin, epirubicin, idarubicin, or daunorubicin) and topoisomerase II inhibitors (e.g. etoposide, irinotecan, topotecan, camptothecin, or VP16), anaplastic lymphoma kinase (ALK)-inhibitors (e.g. Crizotinib or AP26130), aurora kinase inhibitors (e.g. N-[4-[4-(4-Methylpiperazin-1-yl)-6-[(5-methyl-1H-pyrazol-3-yl)amino]pyrimidin-2-yl]sulfanylphenyl]cyclopropanecarboxamide (VX-680)), antiangiogenic agents (e.g. Bevacizumab), or Iodine131-1-(3-iodobenzyl)guanidine (therapeutic metaiodobenzylguanidine), histone deacetylase (HDAC) inhibitors, alone or any suitable combination thereof. It is to be understood that chemotherapy, preferably, relates to a complete cycle of treatment, i.e. a series of several (e.g. four, six, or eight) doses of antineoplastic drug or drugs applied to a subject separated by several days or weeks without such application.

The term "targeted therapy", as used herein, relates to application to a patient a chemical substance known to block growth of cancer cells by interfering with specific molecules known to be necessary for tumorigenesis or cancer or cancer cell growth. Examples known to the skilled artisan are small molecules like, e.g. PARP-inhibitors (e.g. Iniparib), or monoclonal antibodies like, e.g., Trastuzumab.

The term "immunotherapy" as used herein relates to the treatment of cancer by modulation of the immune response of a subject. Said modulation may be inducing, enhancing, or suppressing said immune response. The term "cell based immunotherapy" relates to a cancer therapy comprising application of immune cells, e.g. T-cells, preferably tumor-specific NK cells, to a subject.

The terms "radiation therapy" or "radiotherapy" is known to the skilled artisan. The term relates to the use of ionizing radiation to treat or control cancer. The skilled person also knows the term "surgery", relating to operative measures for treating breast cancer, e.g. excision of tumor tissue.

As used herein, the term "recommending a cancer therapy" relates to proposing a cancer therapy to a subject or to a medical practitioner treating said subject. It is to be understood that the decision of how said subject is to be actually treated is not part of recommending a cancer therapy. It is also understood that recommending a cancer therapy may, preferably, relate to proposing one or more alterations to an established treatment schedule.

The present invention also relates to a method of treating a subject afflicted with cancer comprising the steps of:

- a) determining in a sample from said subject afflicted with cancer the amount of at least miR-142-p3;
- b) comparing said amount with a reference; and
- c) treating said subject with a treatment protocol appropriate for a patient with a high risk of cancer recurrence or with a treatment protocol appropriate for a patient with a low risk of tumor recurrence and/or metastasis, depending on the result of the comparison of step b).

The term "treating" relates to selecting and applying a cancer therapy as described herein above to a subject. Thus, in the method of treating a subject afflicted with cancer according to the present invention, the aforementioned steps, preferably, are steps contributing to the decision which cancer therapy shall be applied to a subject. It is understood that treating a subject is typically the duty of the medical practitioner, who will be in charge of prescribing and/or applying and/or surveying the relevant methods of cancer therapy.

The present invention further relates to a method of predicting the efficacy of standard cancer treatment in preventing cancer recurrence in a subject being treated with said standard cancer treatment comprising the steps of:

- a) determining in a sample from said subject afflicted with cancer the amount of at least miR-142-p3; and
- b) comparing said amount with a reference, whereby the efficacy of standard cancer treatment is predicted.

The term "standard cancer treatment" relates to the treatment of a cancer by an established treatment protocol. Preferably, the standard treatment is a treatment recommended by at least one relevant agency, e.g. in clinical practice guidelines, e.g. in the case of operable lung cancer (stage I-IIIa): Tumor resection, four cycles cisplatin-based combination of chemotherapy; or combined radio-/chemotherapy (simultaneously or sequentially) dependent on further clinical parameters, as recommended in Goeckenjan et al, German Respiratory Society; German Cancer Society. "Prevention, diagnosis, therapy, and follow-up of lung cancer: interdisciplinary guideline of the German Respiratory Society and the German Cancer Society." *Pneumologie*. 2011 Jan;65(1):39-59.

As used herein, the term "predicting the efficacy" of a treatment relates to assessing the probability according to which a therapy will be effective, i.e. lead to the absence of disease, preferably cancer recurrence, at least within the predictive window, or not. Thus, the term relates to assessing the probability according to which a subject will be afflicted with cancer recurrence despite standard treatment, or not. Preferably, a reference is obtained from at least one patient

- selected from the list consisting of: a patient not afflicted with said cancer, a patient not afflicted with cancer, a patient known not to have a high risk of cancer recurrence and/or metastasis, and a patient having survived without cancer recurrence for at least 10, 20, 30, or 40 months after removal of said cancer, and it is predicted that a standard cancer treatment will not be effective in case the amount of the miRNA according to the present invention is higher than the reference amount, and it is predicted that a standard cancer treatment will be effective in case said amount is essentially equal to or lower than the reference amount. Also preferably, a reference is obtained from at least one patient known to have a high risk of cancer recurrence or from a patient afflicted with cancer recurrence at least 5, 7, 10, 20, 30, or 40 months after removal of said cancer, and it is predicted that a standard cancer treatment will not be effective in case the amount of the miRNA according to the present invention is essentially equal to or higher than the reference amount, and predicting that a standard cancer treatment will be effective in case said amount is lower than the reference amount.
- 15 The present invention further relates to a method of monitoring cancer therapy in a subject being treated against cancer comprising the steps of:
- a) determining in a sample from said subject afflicted with cancer the amount of at least miR-142-p3;
 - b) comparing said amount with a reference; and
 - 20 c) recommending and/or instituting follow-up examination at short intervals and/or modified treatment or not, depending on the result of the comparison according to step b).

The term "monitoring" is known to the skilled person and relates to surveilling, observing, recording, and/or detecting the progress of disease or of recovery while treatment is applied to a subject. Preferably, the term relates to surveilling if therapy is effective in a subject. Accordingly, the term "monitoring cancer therapy" relates to testing a subject, preferably a subject from which the primary tumor has been removed, under cancer treatment for signs of cancer recurrence. Preferably, a reference is obtained from at least one patient selected from the list consisting of: a patient not afflicted with said cancer, a patient not afflicted with cancer, a patient known not to have a high risk of cancer recurrence and/or metastasis, and a patient having survived without cancer recurrence for at least 10, 20, 30, or 40 months after removal of said cancer, and follow-up examination at short intervals and/or modified treatment is recommended and/or instituted in case the amount of the miRNA according to the present invention is higher than the reference amount, and follow-up examination at short intervals and/or modified treatment is not recommended and/or instituted in case said amount is essentially equal to or lower than the reference amount. Also preferably, a reference is obtained from at least one patient known to have a high risk of cancer recurrence or from a patient afflicted with cancer recurrence at least 5, 7, 10, 20, 30, or 40 months after removal of said cancer, and follow-up examination at short intervals and/or modified treatment is recommended and/or instituted in case the amount of the miRNA according to the present invention is essentially equal to or higher than the reference amount, and follow-up examination at short intervals and/or modified treatment is not recommended and/or instituted in case said amount is lower than the reference amount.

The present invention also relates to miR-142-p3 for use in the diagnosis of a severe form of cancer.

The term "severe form of cancer", as used herein, relates to a form of cancer wherein a subject is afflicted with a high risk of cancer recurrence, has decreased overall survival probability and/or has a decreased disease-free survival probability, preferably despite treatment, more preferably despite standard treatment. Preferably, the term relates to a form of cancer wherein a subject is afflicted with cancer recurrence, within 6, 12, 18, 24, 30, or 36 months after removal of a primary tumor and/or start of treatment.

The present invention further relates to the use of miR-142-p3 for the manufacture of a diagnostic composition for the diagnosis of a severe form of cancer.

Moreover, the present invention relates to the use of miR-142-p3 in a sample of a subject for the diagnosis of a severe form of cancer.

Further, the present invention relates to a device for predicting the risk of cancer recurrence in a patient afflicted with cancer or for diagnosing a severe form of cancer comprising:

a) an analyzing unit comprising a detection agent for determining the amount of at least miR-142-3p in a sample of a subject; and

b) an evaluation unit comprising a data processor having tangibly embedded an algorithm for carrying out a comparison of the amount determined by the analyzing unit with a reference and which is capable of generating an output file containing a prediction and/or diagnosis established based on the said comparison.

The term "device", as used herein, relates to a system of means comprising at least the aforementioned means operatively linked to each other as to allow the diagnosis. Preferred means for determining the amount of the miRNAs of the present invention, and means for carrying out the comparison are disclosed above in connection with the methods of the invention. How to link the means in an operating manner will depend on the type of means included into the device. For example, where means for automatically determining the amount of the miRNAs of the present invention are applied, the data obtained by said automatically operating means can be processed by, e.g., a computer program in order to establish a diagnosis. Preferably, the means are comprised by a single device in such a case. Said device may accordingly include an analyzing unit for the measurement of the amount of the miRNAs of the present invention in a sample and an evaluation unit for processing the resulting data for the diagnosis. Preferred means for detection are disclosed in connection with embodiments relating to the methods of the invention above. In such a case, the means are operatively linked in that the user of the system brings together the result of the determination of the amount and the diagnostic value thereof due to the instructions and interpretations given in a manual. The means may appear as separate devices in such an embodiment and are, preferably, packaged together as a kit. The person skilled in the art will realize how to link the means without further inventive skills. Preferred devices are those which can be applied without the particular knowledge of a specialized clinician, e.g., test stripes or electronic devices which merely require loading with a sample. The results may be given as output of parametric diagnostic raw data, preferably, as absolute or relative amounts. It is to be understood that these data will need interpretation by the clinician.

However, also envisaged are expert system devices wherein the output comprises processed diagnostic raw data the interpretation of which does not require a specialized clinician. Further preferred devices comprise the analyzing units/devices (e.g., biosensors, arrays, solid supports coupled to ligands specifically recognizing the miRNAs of the present invention, Plasmon surface resonance devices, NMR spectro-meters, mass- spectrometers etc.) or evaluation units/devices referred to above in accordance with the methods of the invention.

Also, the present invention relates to a kit for carrying out a method according to any one of claims 1 to 15, wherein said kit comprises instructions for carrying out said method, a detection agent for determining the amount of at least miR-142-3p in a sample of a subject, and standards for a reference.

The term "kit" as used herein refers to a collection of the aforementioned compounds, means or reagents of the present invention which may or may not be packaged together. The components of the kit may be comprised by separate vials (i.e. as a kit of separate parts) or provided in a single vial. Moreover, it is to be understood that the kit of the present invention is to be used for practicing the methods referred to herein above. It is, preferably, envisaged that all components are provided in a ready-to-use manner for practicing the methods referred to above. Further, the kit preferably contains instructions for carrying out the said methods. The instructions can be provided by a user's manual in paper- or electronic form. For example, the manual may comprise instructions for interpreting the results obtained when carrying out the aforementioned methods using the kit of the present invention.

All references cited in this specification are herewith incorporated by reference with respect to their entire disclosure content and the disclosure content specifically mentioned in this specification.

Figure Legends

Fig. 1: Study design of microRNA quantification in the serum of pulmonary adenocarcinoma patients.

664 miRNAs were screened in sera of 40 patients for association with recurrence, and validated in an independent cohort of 114 patients.

Fig. 2: Validation of miR-142-3p and miR-29b serum levels in independent patient cohorts.

A) miRNA levels in benign individuals (n= 21), early-stage (stage I-II, n= 71) adenocarcinoma with recurrent (Rec) or non-recurrent (Non-Rec) disease course, and advanced adenocarcinoma (stage III-IV, n= 29). Circulating miR-142-3p and miR-29b levels were distinctly elevated in adenocarcinoma patients with recurrence compared to all other subgroups. Statistically significant p-values were determined using the Wilcoxon test (*: p< 0.05, **: p< 0.01, ***: p< 0.001). B) Kaplan-Meier plots indicated poor outcome in the recurrence-free survival of patients (stage I-IIIa, n=114) with higher miR-142-3p (p= 0.002) or miR-29b (p= 0.05) serum levels, respectively. The upper (dark grey) and lower (light grey) patient subgroups were defined according to the median Cp miRNA expression level.

P-values were determined using the Log-rank test.

Fig. 3: Kaplan-Meier plots of overall survival

Kaplan-Meier plots indicated poor outcome in the overall survival of patients with higher miR-142-3p (A) or miR-29b (B) serum levels, respectively. The upper (dark grey) and lower (light grey) patient subgroups were defined according to the median Cp miRNA expression level. P-values were determined using the Log-rank test.

Fig. 4: Kaplan-Meier plots of recurrence-free survival

Kaplan-Meier plot indicated poor outcome in the recurrence-free survival of patients receiving adjuvant therapy with higher miR-142-3p. The upper (dark grey) and lower (light grey) patient subgroups were defined according to the median Cp miRNA expression level. P-value was determined using the Log-rank test.

Fig. 5: ROC curve predicting a recurrence event in patients by using either miR-142-3p levels, staging or both variables.

The Receiver Operating Curve (ROC) plot is the result of a linear logistic regression model using miR-142-3p, staging, or both. The endpoint was if a patient suffered from a relapse within 24 months. A 10-fold cross-validation was repeated 5 times. The median of the area under the curve (AUC) iterations was used as a stable error measurement.

The following Examples shall merely illustrate the invention. They shall not be construed, whatsoever, to limit the scope of the invention.

Example 1: Serum samples and patient characteristics

Patient cohorts were collected at the Thoraxklinik at Heidelberg University following written patient consent. The use of sera for this study was approved by the local ethnics committee. Serum samples were obtained from early-stage lung adenocarcinoma patients (stage I-IIIa, UICC 6th edition) before surgery. In this retrospective study only patients were included, who had a complete tumour resection (local R0 status). Patients in the screening (n = 40) and the validation (n = 114) cohorts were divided into a recurrence and non-recurrence group according to the relapse event in the follow-up period of 24 months after surgery. The majority of disease recurrences were classified as distant metastasis (51/ 66) and less frequent as local tumour recurrence. Detailed patient cohort description and follow-up data are listed in Table 1. Additionally, sera from age-matched individuals with benign diagnosis (benign pulmonary nodules, n= 21) and patients with advanced tumours (stage III- IV, n= 29) were included in this study.

Table 1: Epidemiology and clinical characteristics of operable pulmonary adenocarcinoma patients.

Patient characteristics	Recurrence ≤ 24 months (n=56)	Recurrence-free / Recurrence > 24 months * (n=98)
Primary tumour		
Stage I	20	68
Stage II	11	12
Stage IIIa	25	18
Adjuvant Therapy		
Chemotherapy	30	39
Radiotherapy	17	13
Time to relapse, m (MAD)	8.2 (2.9)	34.4 (7.1)*
Follow up-time, m (MAD)	29.8 (12.3)	39.2 (9.4)
Metastatic site		
Local recurrence	7	1
Distant metastases	41	10
Second tumour \ Unknown	8	3
Epidemiology		
Age, y (MAD)	61.5 (6.5)	62.0 (5.5)
Male	36	51
Female	20	47
Smoking, packyear (MAD)	35 (15.0)	30.0 (20.0)
Deaths	40	6
Time to death (MAD)	19.5 (10.6)	36.5 (8.8)

Values are calculated as median; abbreviations: MAD, Median absolute deviation; m, months; y, years; *14 recurrence events were diagnosed after 24 months follow-up time

Example 2: Total RNA extraction from serum

miRNA extraction from serum samples was conducted as previously described in Brase et al. ("Circulating miRNAs are correlated with tumor progression in prostate cancer." Int J Cancer 2011; 128: 608-616), including minor modifications. Briefly, 400 (100) µl serum was used in the screening (validation) study, and 12 (3) µg glycogen was added to the denatured serum in order to enhance the recovery of total RNA. Two *C. elegans* miRNAs (cel-miR-39 and cel-miR-54) were spiked into each serum sample to check for RNA recovery. The extraction was done by using TRI Reagent BD (Sigma, Munich, Germany), following miRNA purification by using the miRNeasy 96 Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Example 3: qRT-PCR of circulating miRNAs

In the screening study, miRNA fractions of 40 serum samples were analysed by quantitative real-time PCR (ABI Prism 7900HT Sequence Detection System; Applied Biosystems, Foster City,

CA, USA). Briefly, miRNAs were reversely transcribed, preamplified and measured on TaqMan Array Human miRNA A and B Cards v.2.0 comprising 664 human miRNAs. miRNA cycle threshold (Ct) values were calculated from each array using the SDS-Software (Applied Biosystems). Quality filtering of miRNA data was done as previously described (Brase et al.,
 5 "Circulating miRNAs are correlated with tumor progression in prostate cancer." Int J Cancer 2011; 128: 608-616). Briefly, miRNAs were discarded from further analyses, if their abundance was very low (Ct > 35) in more than 70% (28/ 40) of the samples. qRT-PCR miRNA array raw data from both plates were separately processed using median normalization.

10 In the validation study, selected miRNAs were reversely transcribed and amplified. The Second Derivative Maximum Method was used to determine the crossing point (Cp) using the LightCycler 480 system (Roche, Mannheim, Germany). Briefly, miRNAs were reversely transcribed using miRNA-specific RT primer and amplified by specific Taqman miRNA assays (Applied Biosystems; Table 2). The median of three technical replicates was calculated. Relative
 15 quantification was applied using two spiked *C. elegans* miRNAs (cel-miR-39, cel-miR-54) as internal controls.

Table 2: miRNA assay name, miRBase 18.0 ID and sequence information

miRNA Assay (Applied Biosystems)	miRBase 18.0 ID	Sequence	Assay ID (AB)	miRBase Accession Number	SEQ ID NO:
hsa-miR-142-3p	hsa-miR-142-3p	UGUAGUGUUUCCUACUUUAUGGA	484	MIMAT0000434	1
hsa-miR-183*	hsa-miR-183-3p	GUGAAUUACCGAAGGGCCAUAA	2270	MIMAT0004580	3
hsa-miR-20b	hsa-miR-20b-5p	CAAAGUGCUCUAGUGCAGGUAG	1014	MIMAT0001413	4
hsa-miR-29b	hsa-miR-29b-3p	UAGCACCAUUUGAAUCAGUGUU	413	MIMAT0000100	5
hsa-miR-331-3p	hsa-miR-331-3p	GCCCCUGGGCCU AUCCUAGAA	545	MIMAT0000760	6
hsa-miR-338-3p	hsa-miR-338-3p	UCCAGCAUCAGUGAUUUUGUUG	2252	MIMAT0000783	7
hsa-miR-380*	hsa-miR-380-5p	UGGUUGACCAUAGAACAUGCGC	570	MIMAT0000734	8
hsa-miR-488-5p	hsa-miR-488-5p	UCCUGUACUGAGCUGCCCCGAG	1278	MIMAT0002177	9
hsa-miR-517a	hsa-miR-517a-3p	AUCGUGCAUCCCUUAGAGUGU	2402	MIMAT0002852	10
hsa-miR-818	hsa-miR-818	AAACUCUACUUGUCCUUCUGAGU	1593	MIMAT0003287	11

E.

All statistical analyses were performed using R (Ihaka and Gentleman, "R: A Language for Data Analysis and Graphics." Journal of Computational and Graphical Statistics 1996; 5: 299-314).

25 Differential expression of miRNAs between patient subgroups with and without recurrence was conducted using the LIMMA test (Smyth, "Linear models and empirical bayes methods for assessing differential expression in microarray experiments." Stat Appl Genet Mol Biol 2004; 3: Article3). miRNAs were ranked according to the adjusted and nonadjusted p-values. In the validation study, group-wise analysis of single miRNAs was done using Wilcoxon rank sum test.
 30 Kaplan-Meier curves were calculated for recurrence-free survival as endpoint. Tumour staging is the standard prognostic variable. Cox proportional hazard model (endpoint relapse) was applied using miRNA expression, tumour staging or both factors. Similarly, a linear discriminant

analysis and linear logistic regression model was carried out to determine the predictive power for correct relapse group assignment using miRNA expression, staging, or both variables. Here, a 10-fold cross-validation was repeated 5 times. The median of the resulting 5 AUCs was used as a stable error measurement.

Example 5: Experimental design and miRNA screening in serum of adenocarcinoma patients

In total, 40 early-stage adenocarcinoma patients (stage I and II) were included in the miRNA screening study to identify putative prognostic miRNAs (Fig. 1). Circulating miRNA levels were compared between patients with (n= 20, median time to recurrence: 7.7 months) and without recurrence (n= 20, median observation time: 46.7 months). From a total of 664 measured miRNAs, 316 miRNAs remained evaluable after quality filtering. The majority of detectable circulating miRNAs ranged within 6 log levels with a median Ct value of 29.8. The efficiency and heterogeneity of miRNA recovery across the serum samples was also tested by qRT-PCR of two spike-in controls. The median normalized miRNA levels showed a high correlation compared with the spike-in normalized data (r= 0.997). In total, 37 differentially expressed miRNAs were identified between the recurrence and non-recurrence group (p< 0.05, nonadjusted). Ten miRNA candidates were selected for validation based on expression intensity and fold change (Table 3).

Table 3: miRNA expression analysis in serum between pulmonary adenocarcinoma patients with and without recurrence after 24 months.

miRNA	Screening		Validation			
	pT I-II (n=40)		pT I-II (n=71)		pT I-IIIa (n=114)	
	p-value*	FC	p-value†	FC	p-value†	FC
miR-142-3p	0.0270	1.30	0.0052	1.75	0.0046	1.46
miR-29b	0.0110	1.71	0.0161	1.72	0.0707	1.31
miR-331-3p	0.0211	0.86	0.0179	1.46	0.0693	1.36
miR-517a	0.0022	0.01	0.0818	0.84	0.1573	0.86
miR-338-3p	0.0372	3.17	0.1085	1.57	0.5938	0.96
miR-20b	0.0044	0.70	0.1373	1.32	0.3834	1.16
miR-486-5p	0.0223	0.70	0.1549	1.37	0.0544	1.29
miR-183*	0.0496	0.40	0.2592	1.22	0.1201	1.18
miR-618	0.0120	0.50	0.2943	1.33	0.6852	0.98
miR-380*	0.0167	1.47	0.8550	1.15	0.7721	1.15

* LIMMA analysis (nonadjusted p-value), † Wilcoxon test; abbreviation: pT, pathological stage; FC, fold change (>1 increased, <1 decreased in patients with vs. without recurrence)

Example 6: Validation of circulating miR-142-3p and miR-29b associated with adenocarcinoma disease recurrence

- 5 The validation cohort comprised 114 early-stage adenocarcinoma patients, 36 (median time to recurrence: 9.3 months) with recurrence and 78 (median observation time: 36.8 months) without recurrence (Fig. 1). The number of absent values was high ($> 30\%$) for miR-380*, miR-517a and miR-618, low (5- 10%) for miR-338-3p and miR-183*, and zero for miR-142-3p, miR-29b, miR-486-5p, miR-331-3p and miR-20b. First, we analysed patients with stage I-II tumours (n= 71), similar to our screening cohort. miR-142-3p ($p = 0.005$) and miR-29b ($p = 0.016$) levels were shown to be increased in the recurrence compared with the non-recurrence group (Table 2). All other miRNA candidates could not be confirmed in the validation study. For example, miR-331-3p was significant, but discordantly expressed in the screening and validation cohort.
- 15 We also accounted for individuals with benign diagnosis and patients with advanced tumours: Serum levels of miR-142-3p and miR-29b were exclusively increased in patients with early-stage adenocarcinoma developing recurrence (Fig. 2A). The Kaplan-Meier curves further indicated that patients with elevated miR-142-3p (stage I, II: $p = 0.004$; stage I-IIIa: $p = 0.002$) and elevated miR-29b (stage I, II: $p = 0.02$; stage I-IIIa: $p = 0.05$) serum levels displayed a higher risk of recurrence (Figure 2B). A significant association was also observed for both miRNAs, if overall survival or disease-free survival was used as clinical endpoint (Fig. 3).

Concerning further clinical parameters, miR-142-3p levels, but not miR-29b levels, remained significantly increased in the recurrence group if only patients with distant metastasis (Wilcoxon-test, $p = 0.021$), or patients without adjuvant therapy ($p = 0.027$) were considered, respectively. Kaplan-Meier curves indicated increased miR-142-3p levels in patients with poor outcome, who received adjuvant therapy ($p = 0.01$; Fig. 4). Taken together, increased levels of miR-142-3p in serum of early-stage adenocarcinomas with disease recurrence was a robust finding after miRNA biomarker screening and validation.

Example 7: Multivariate analyses using miR-142-3p serum level and tumour staging

- A higher tumour stage, the standard prognostic variable in pulmonary adenocarcinoma, was associated with a higher risk of tumour recurrence. In a likelihood-ratio test, tumour stage (I, II, and IIIa) alone significantly stratified high-risk and low-risk patients ($p = 1.54 \times 10^{-6}$). We further tested if circulating miR-142-3p can act as an independent predictor of metastatic spread. Here, we observed an improvement in evaluating the probability of the relapse event by using both tumour stage and serum level of miR-142-3p ($p = 0.007$). This result was corroborated in a linear regression model using staging information, miR-142-3p expression, or both variables (Fig. 5). We observed similar receiver operating curves (ROCs) if stage (AUC = 0.66) and miR-142-3p expression (AUC = 0.64) were separately analysed. Of note, the area under the curve (AUC) increased when stage and miR-142-3p expression were combined into one model (AUC = 0.78). None of the additionally analyzed miRNAs, like miR-29b, was able to improve this model. Thus, miR-142-3p together with tumour stage improved risk stratification of early-stage adenocarcinoma patients in our cohort.

Example 8: Comparison of miRNA levels in the sera and tumor tissues of adenocarcinoma patients

- 5 In order to compare miRNA abundance in tumour cells and surrogates, we analysed miR-142-3p expression in serum, tumour and corresponding normal lung tissues from 46 early-stage adenocarcinoma patients. miR-142-3p was equally expressed in tumour and corresponding benign tissues, and not deregulated in tumours of patient with and without disease recurrence ($p = 0.252$). Poor correlation was found between serum and tumour tissue samples for miR-142-3p
10 ($r = 0.07$). Taken together, the expression of miR-142-3p in NSCLC tissues did not reveal an association with metastatic spread as was observed for the circulating form in the sera of early-stage NSCLC patients.

Claims

1. A method for predicting the risk of cancer recurrence in a subject afflicted with cancer comprising the steps of:
 - 5 a) determining in a sample from said subject afflicted with cancer the amount of at least miR-142-p3; and
 - b) comparing said amount with a reference, whereby the risk of cancer recurrence is predicted.
- 10 2. The method according to claim 1, wherein
 - a) the reference is obtained from at least one subject selected from the list consisting of: a subject not afflicted with said cancer, a subject not afflicted with cancer, a subject known not to have a high risk of cancer recurrence and/or metastasis, and a subject having survived without cancer recurrence for at least 5, 7, 10, 20, 30, or 40 months after removal of said cancer, and
 - 15 b) a high risk of cancer recurrence is predicted in case said amount is higher than the reference amount, and a low risk of tumor recurrence is predicted in case said amount is essentially equal to or lower than the reference amount.
- 20 3. The method according to claim 1, wherein
 - a) the reference is obtained from at least one subject known to have a high risk of cancer recurrence or from a subject afflicted with cancer recurrence at least 5, 7, 10, 20, 30, or 40 months after removal of said cancer, and
 - b) a high risk of cancer recurrence is predicted in case said amount is essentially equal to
 - 25 or higher than the reference amount, and a low risk of cancer recurrence is predicted in case said amount is lower than the reference amount.
4. A method of recommending a cancer therapy to a subject comprising the steps of:
 - i) the method for predicting the risk of cancer recurrence of claim 1; and
 - 30 ii) the further step of recommending a cancer therapy to the subject depending on the result of the comparison of step b).
5. The method according to claim 4, wherein

a) the reference is obtained from at least one subject selected from the list consisting of:
a subject not afflicted with said cancer, a subject not afflicted with cancer, a subject
known not to have a high risk of of cancer recurrence and/or metastasis, and a subject
having survived without cancer recurrence for at least 10, 20, 30, or 40 months after
removal of said cancer, and

b) a treatment protocol appropriate for a subject with a high risk of cancer recurrence is
recommended in case said amount is higher than the reference amount, and a
treatment protocol appropriate for a subject with a low risk of tumor recurrence is
recommended in case said amount is essentially equal to or lower than the reference
amount.

6. The method according to claim 4, wherein

a) the reference is obtained from at least one subject known to have a high risk of cancer
recurrence or from a subject afflicted with cancer recurrence at least 5, 7, 10, 20, 30,
or 40 months after removal of said cancer, and b)

b) a treatment protocol appropriate for a subject with a high risk of cancer recurrence is
recommended in case said amount is essentially equal to or higher than the reference
amount, and a treatment protocol appropriate for a subject with a low risk of cancer
recurrence is recommended in case said amount is lower than the reference amount.

7. A method of treating a subject afflicted with cancer comprising the steps of:

a) determining in a sample from said subject afflicted with cancer the amount of at least
miR-142-p3;

b) comparing said amount with a reference; and

c) treating said subject with a treatment protocol appropriate for a subject with a high
risk of cancer recurrence or with a treatment protocol appropriate for a subject with a
low risk of tumor recurrence and/or metastasis, depending on the result of the
comparison of step b).

8. The method according to claim 7, wherein

a) the reference is obtained from at least one subject selected from the list consisting of:
a subject not afflicted with said cancer, a subject not afflicted with cancer, a subject
known not to have a high risk of of cancer recurrence and/or metastasis, and a subject
having survived without cancer recurrence for at least 10, 20, 30, or 40 months after
removal of said cancer, and

- b) treating is treatment according to a treatment protocol appropriate for a subject with a high risk of cancer recurrence in case said amount is higher than the reference amount, and treating is treatment according to a treatment protocol appropriate for a subject with a low risk of tumor recurrence in case said amount is essentially equal to or lower than the reference amount.

9. The method according to claim 7, wherein

- a) the reference is obtained from at least one subject known to have a high risk of cancer recurrence or from a subject afflicted with cancer recurrence at least 5, 7, 10, 20, 30, or 40 months after removal of said cancer, and b)

- b) treating is treatment according to a treatment protocol appropriate for a subject with a high risk of cancer recurrence in case said amount is essentially equal to or higher than the reference amount, and treating is treatment according to a treatment protocol appropriate for a subject with a low risk of tumor recurrence in case said amount is lower than the reference amount.

10. A method of predicting the efficacy of standard cancer treatment in preventing cancer recurrence in a subject being treated with said standard cancer treatment comprising the steps of:

- a) determining in a sample from said subject afflicted with cancer the amount of at least miR-142-p3; and
b) comparing said amount with a reference, whereby the efficacy of standard cancer treatment is predicted.

11. The method according to claim 10, wherein

- a) the reference is obtained from at least one subject selected from the list consisting of: a subject not afflicted with said cancer, a subject not afflicted with cancer, a subject known not to have a high risk of cancer recurrence and/or metastasis, and a subject having survived without cancer recurrence for at least 10, 20, 30, or 40 months after removal of said cancer, and
b) predicting that said standard cancer treatment will not be effective in case said amount is higher than the reference amount, and predicting that said standard cancer treatment will be effective in case said amount is essentially equal to or lower than the reference amount.

12. The method according to claim 10, wherein

a) the reference is obtained from at least one subject known to have a high risk of cancer recurrence or from a subject afflicted with cancer recurrence at least 5, 7, 10, 20, 30, or 40 months after removal of said cancer, and b)

5 b) predicting that said standard cancer treatment will not be effective in case said amount is essentially equal to or higher than the reference amount, and predicting that said standard cancer treatment will be effective in case said amount is lower than the reference amount.

10 13. A method of monitoring cancer therapy in a subject being treated against cancer comprising the steps of:

a) determining in a sample from said subject afflicted with cancer the amount of at least miR-142-p3;

b) comparing said amount with a reference; and

15 c) recommending and/or instituting follow-up examination at short intervals and/or modified treatment or not, depending on the result of the comparison according to step b).

14. The method according to claim 13, wherein

20 a) the reference is obtained from at least one subject selected from the list consisting of: a subject not afflicted with said cancer, a subject not afflicted with cancer, a subject known not to have a high risk of cancer recurrence and/or metastasis, and a subject having survived without cancer recurrence for at least 10, 20, 30, or 40 months after removal of said cancer, and

25 b) follow-up examination at short intervals and/or modified treatment is recommended and/or instituted in case said amount is higher than the reference amount, and follow-up examination at short intervals and/or modified treatment is not recommended and/or instituted in case said amount is essentially equal to or lower than the reference amount.

30

15. The method according to claim 13, wherein

a) the reference is obtained from at least one subject known to have a high risk of cancer recurrence or from a subject afflicted with cancer recurrence at least 5, 7, 10, 20, 30, or 40 months after removal of said cancer, and

35 b) follow-up examination at short intervals and/or modified treatment is recommended and/or instituted in case said amount is essentially equal to or higher than the reference

amount, and follow-up examination at short intervals and/or modified treatment is not recommended and/or instituted in case said amount is lower than the reference amount.

- 5 16. MiR-142-p3 for use in the diagnosis of a severe form of cancer.
17. MiR142-3p for use according to claim 16, wherein a severe form of cancer is a cancer having a high risk of recurrence and/or metastasis.
- 10 18. Use of miR-142-p3 for the manufacture of a diagnostic composition for the diagnosis of a severe form of cancer.
19. The use according to claim 18, wherein a severe form of cancer is a cancer having a high risk of recurrence and/or metastasis.
- 15
20. Use of miR-142-p3 in an sample of a subject for the diagnosis of a severe form of cancer.
21. The use according to claim 20, wherein a severe form of cancer is a cancer having a high risk of recurrence and/or metastasis.
- 20
22. A device for predicting the risk of cancer recurrence in a subject afflicted with cancer or for diagnosing a severe form of cancer comprising:
- 25 a) an analyzing unit comprising a detection agent for determining the amount of at least miR-142-3p in a sample of a subject; and
- b) an evaluation unit comprising a data processor having tangibly embedded an algorithm for carrying out a comparison of the amount determined by the analyzing unit with a reference and which is capable of generating an output file containing a prediction and/or diagnosis established based on the said comparison.
- 30 23. A kit for carrying out a method according to any one of claims 1 to 15, wherein said kit comprises instructions for carrying out said method, a detection agent for determining the amount of at least miR-142-3p in a sample of a subject, and standards for a reference.

24. The method according to any one of claims 1 to 15, wherein additionally at least one marker selected from the list consisting of miR-29b-3p (SEQ ID NO:5), miR-331-3p (SEQ ID NO:6), miR-486-5p (SEQ ID NO:9), miR-20b-5p (SEQ ID NO:4), miR-338-3p (SEQ ID NO:7), miR-183-3p (SEQ ID NO:3), miR-517a-3p (SEQ ID NO:10) and miR-380-5p (SEQ ID NO:8) is determined and wherein said marker(s) is or are compared to a matching reference.
25. The method according to any one of claims 1 to 15, wherein additionally staging of the cancer is obtained.
26. The method according to any one of claims 1 to 15, miR-142-p3 for use according to claim 16 or 17, the use of any one of claims 18 to 21, the device of claim 22, or the kit of claim 23, wherein said cancer is a solid cancer.
27. The method according to any one of claims 1 to 15, miR-142-p3 for use according to claim 16 or 17, the use of any one of claims 18 to 21, the device of claim 22, or the kit of claim 23, wherein the cancer is non-small cell lung cancer (NSCLC).
28. The method according to any one of claims 1 to 15, miR-142-p3 for use according to claim 16 or 17, the use of any one of claims 18 to 21, the device of claim 22, or the kit of claim 23, wherein the cancer is NSCLC stage I or II.
29. The method according to any one of claims 1 to 15, the use of claim 20 or 21, or the device of claim 22, wherein the sample is a body fluid sample.
30. The method according to any one of claims 1 to 15, the use of claim 20 or 21, or the device of claim 22, wherein the sample is an endobronchial epithelial liquid, bronchial lavage liquid, sputum, breath condensate, blood, serum, or plasma sample.

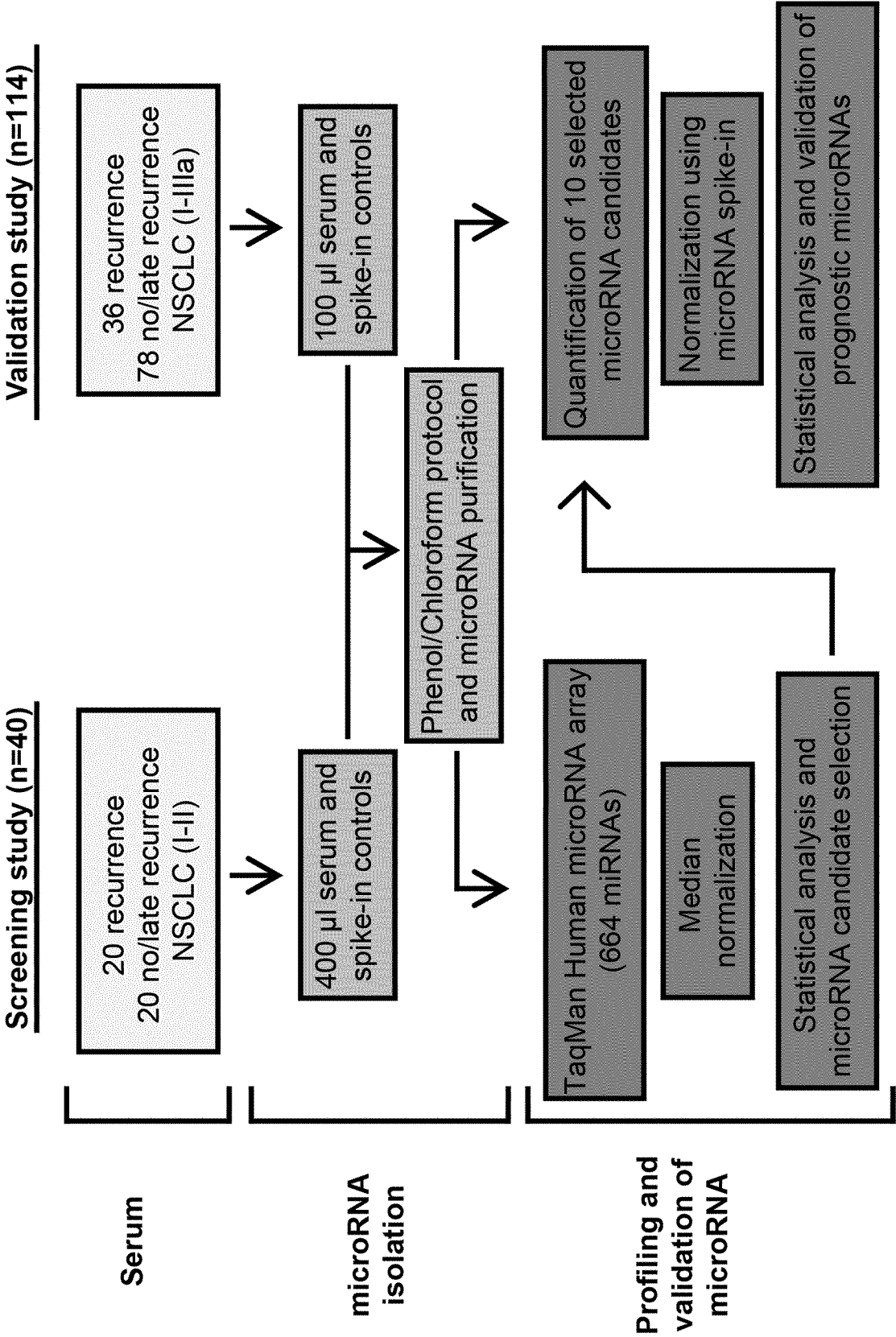


Fig. 1

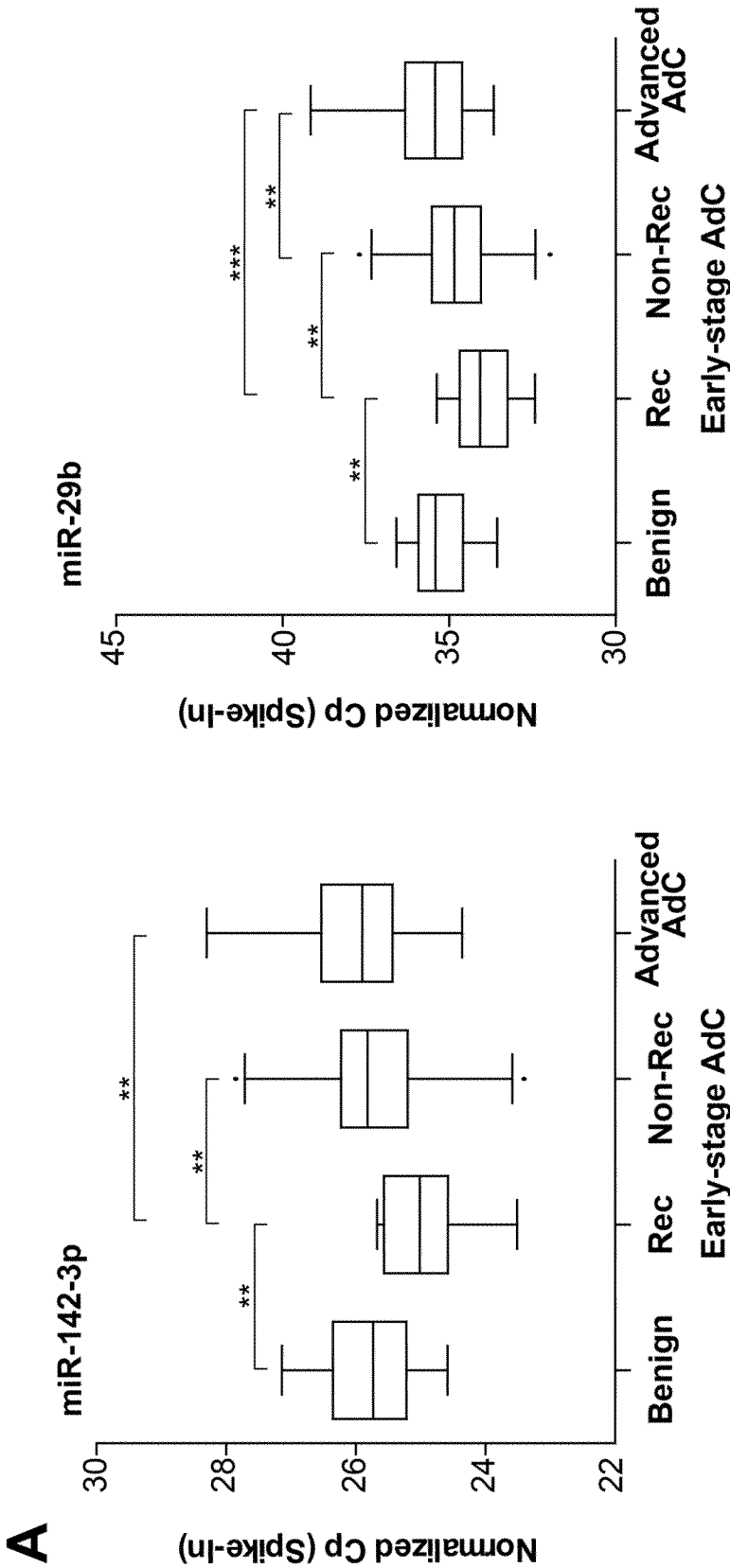


Fig. 2A

B

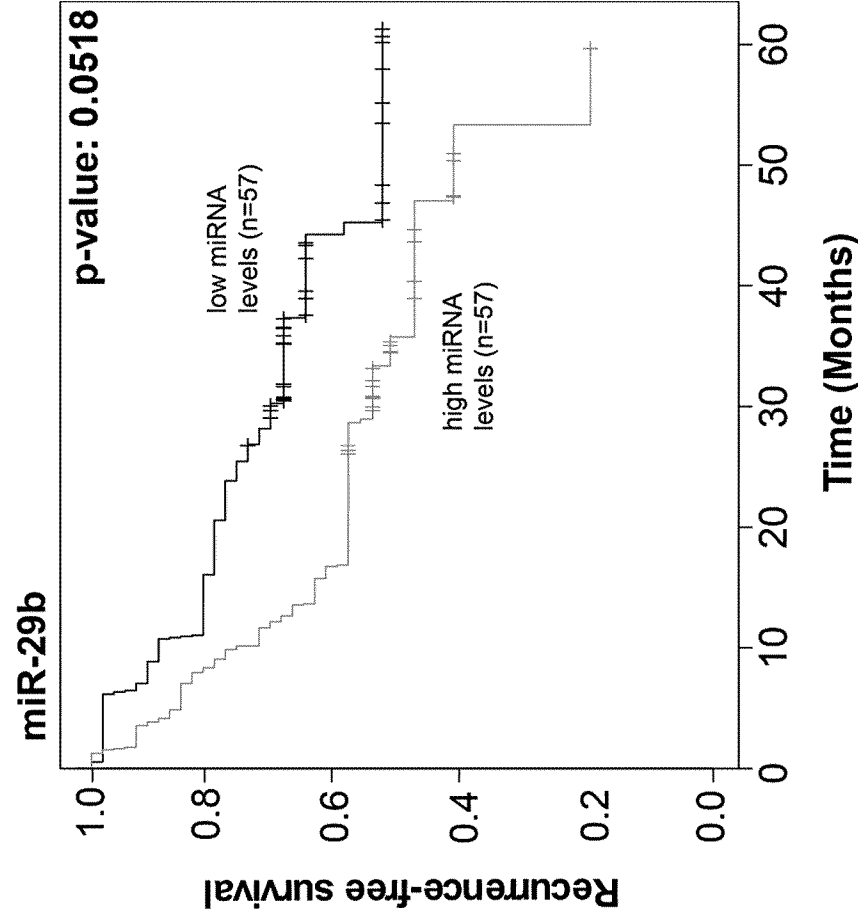
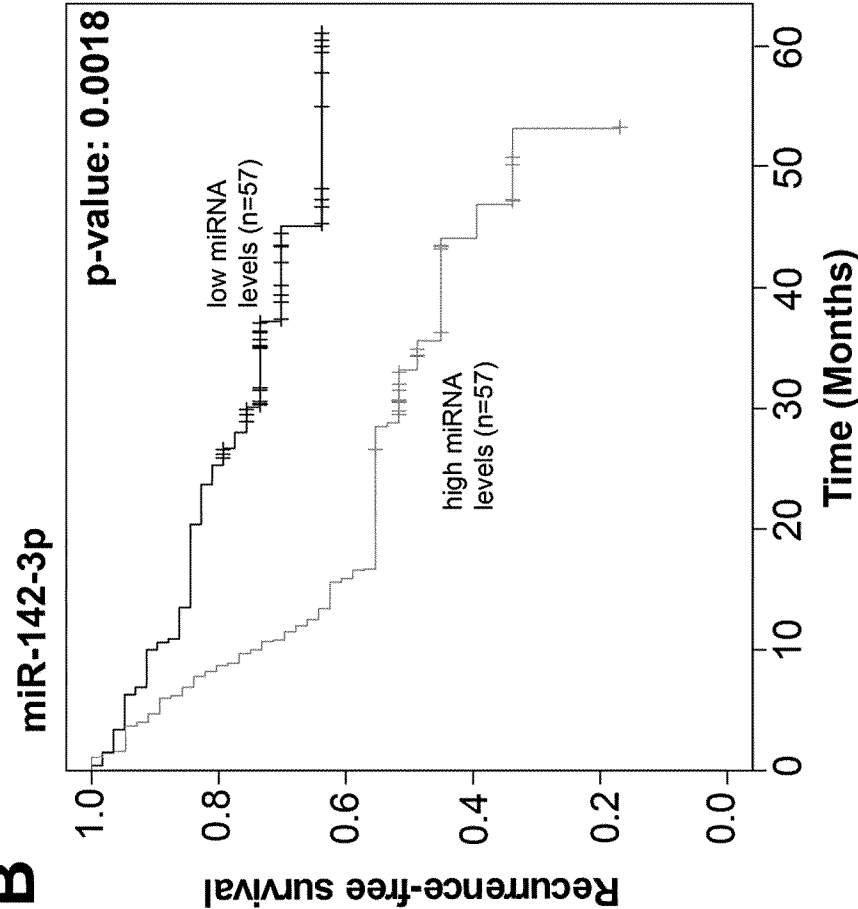


Fig. 2B

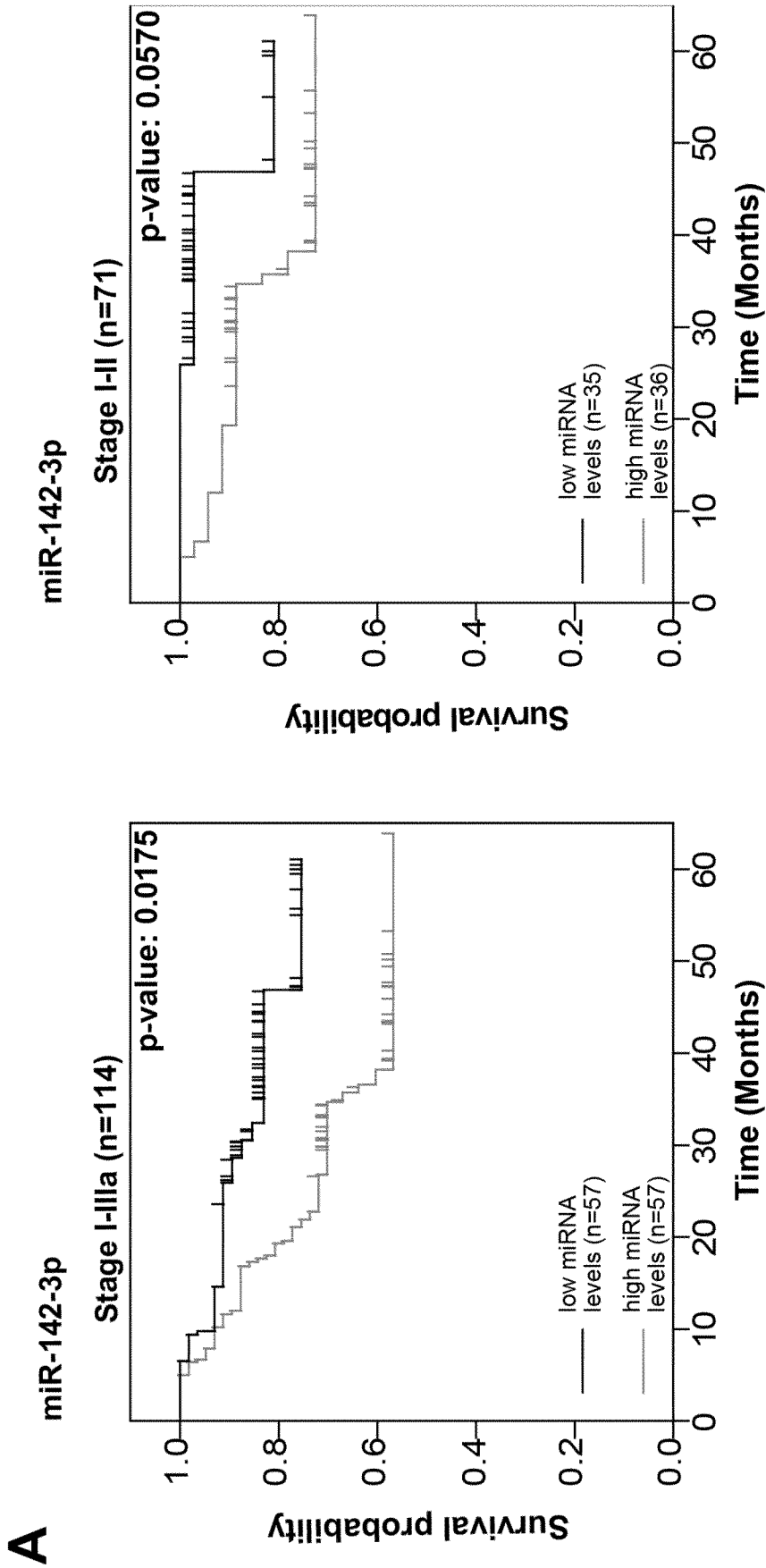


Fig. 3A

B

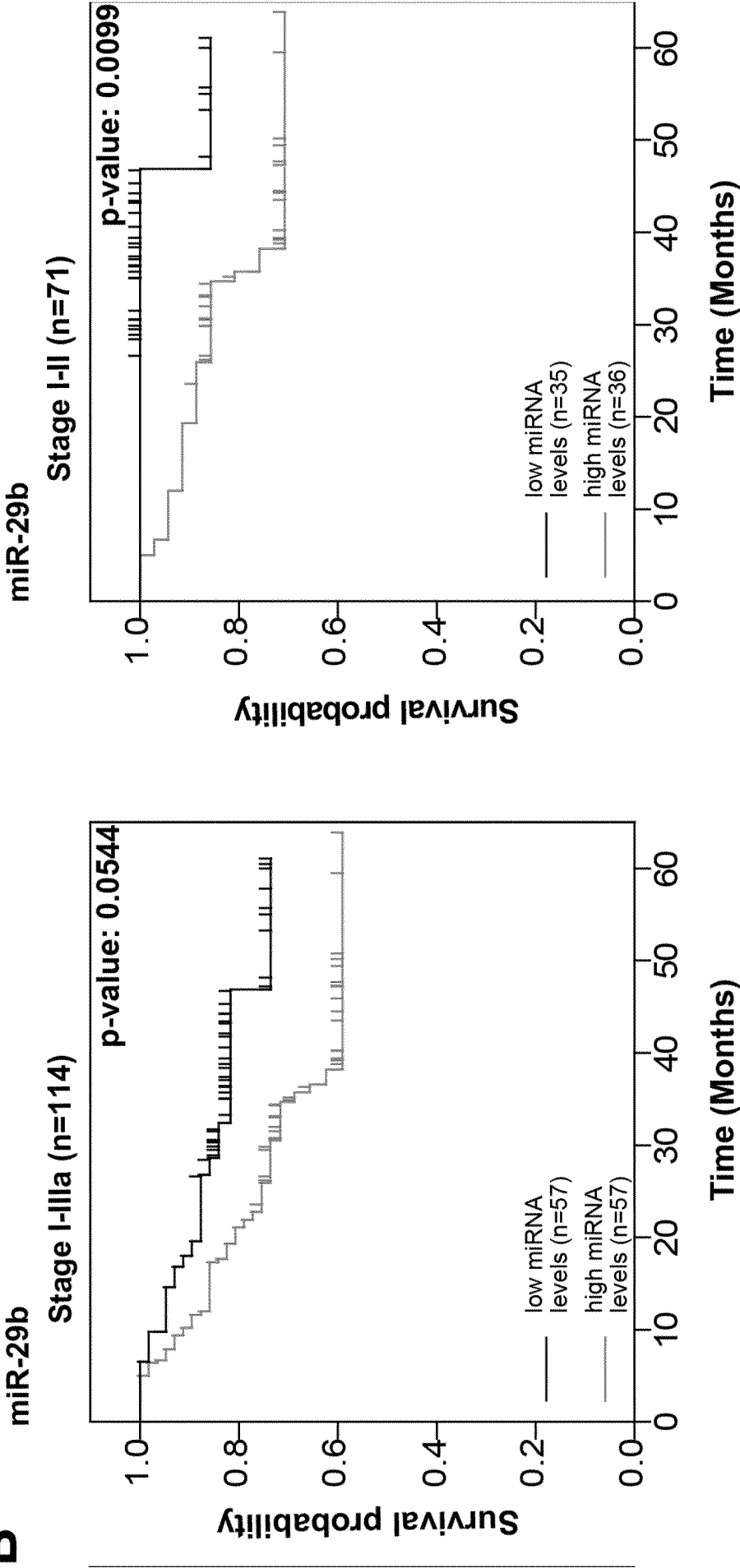


Fig. 3B

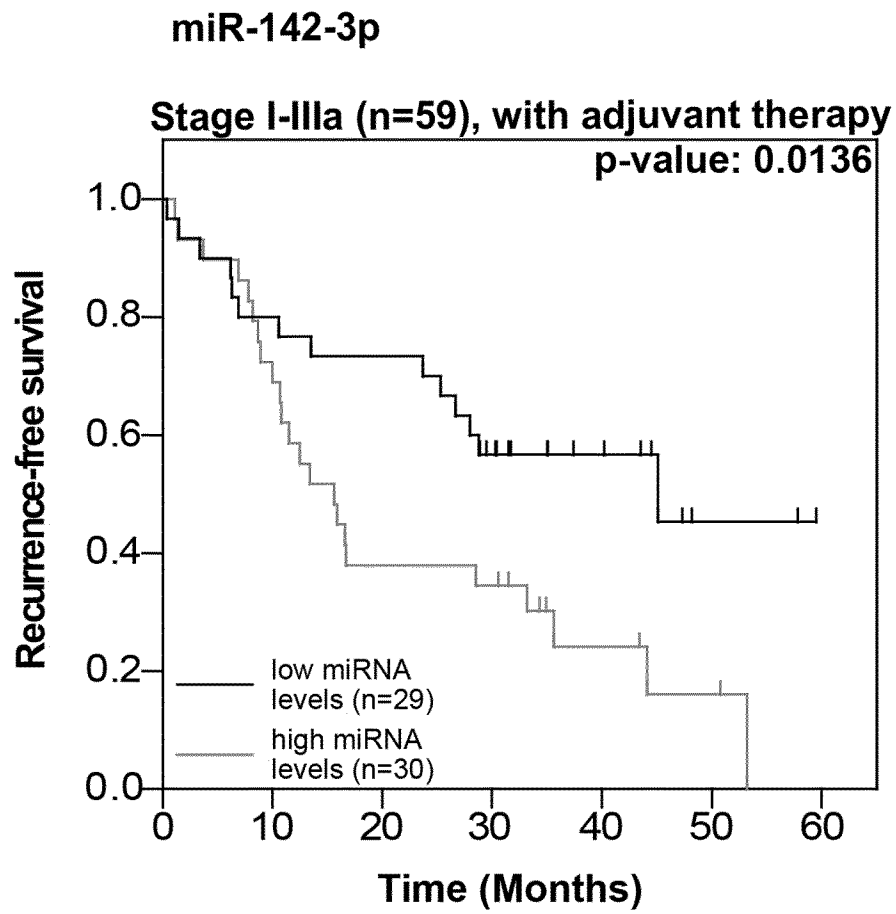


Fig. 4

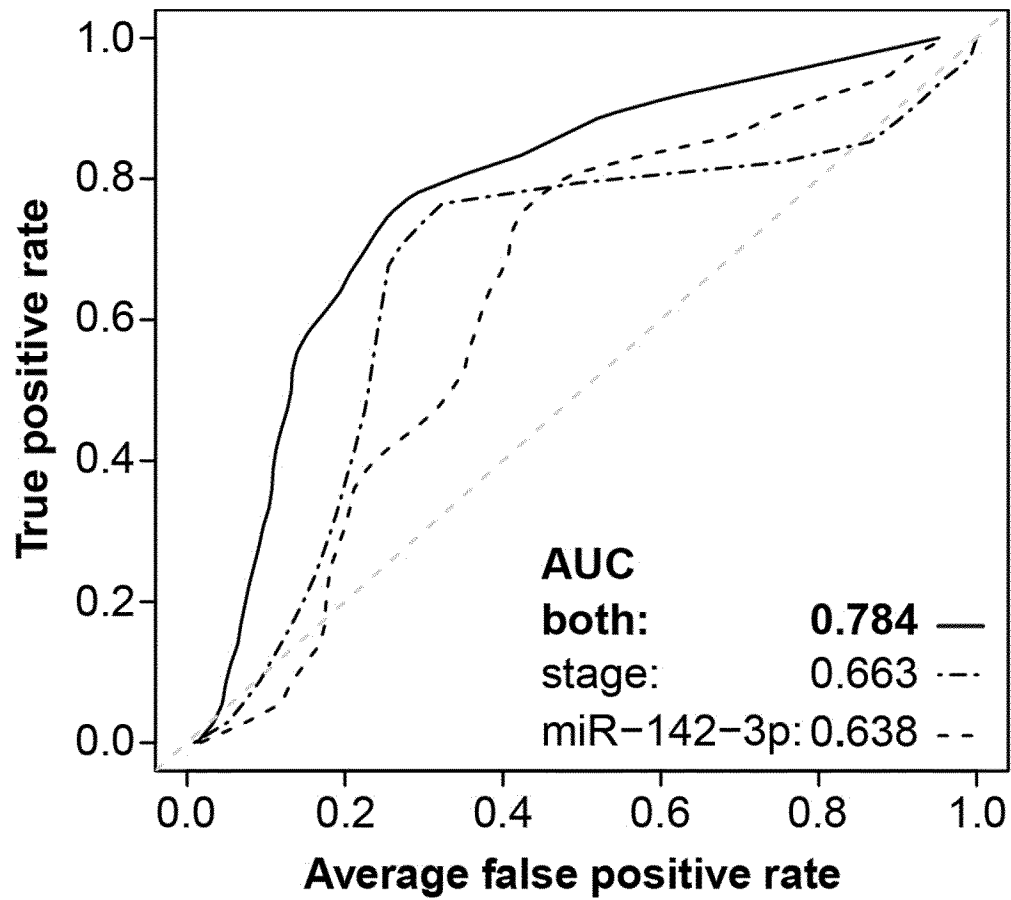


Fig. 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/051004

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, MEDLINE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	WO 2010/073721 A1 (UNIV TOKUSHIMA [JP]; TAIHO PHARMACEUTICAL CO LTD [JP]; SHIMADA MITSUO) 1 July 2010 (2010-07-01) the whole document	10-24
X	WO 2012/083969 A2 (HERLEV HOSPITAL [DK]; RUPRECHT KARLS UNIVERSITY OF HEIDELBERG [DE]; JO) 28 June 2012 (2012-06-28) the whole document	1-6,13, 16-23, 26,29,30
X	WO 2011/057304 A2 (UNIV YALE [US]; WEIDHAAS JOANNE B [US]) 12 May 2011 (2011-05-12) the whole document	16-23, 26,29,30
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Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

20 February 2014

Date of mailing of the international search report

05/03/2014

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Botz, Jürgen

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2014/051004

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	HOTCHI M ET AL: "microRNA expression is able to predict response to chemoradiotherapy in rectal cancer", MOLECULAR AND CLINICAL ONCOLOGY 2013 SPANDIDOS PUBLICATIONS GBR, vol. 1, no. 1, January 2013 (2013-01), pages 137-142, XP002720629, ISSN: 2049-9450 the whole document -----	16-23, 26,29,30
X,P	WO 2013/190091 A1 (UNIV RUPRECHT KARLS HEIDELBERG [DE]) 27 December 2013 (2013-12-27) the whole document -----	1-30

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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