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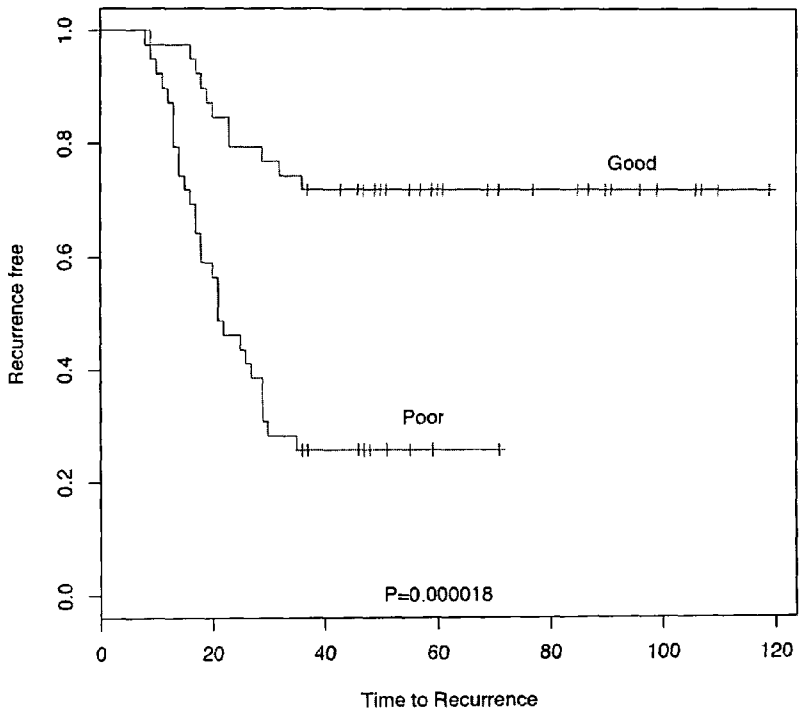
(54) Title: METHODS AND DEVICES FOR PROGNOSIS OF CANCER RELAPSE

Figure 1



(57) Abstract: The present invention features microRNAs as biomarkers for prognosing cancer relapse in cancer patient. The present invention also features methods, devices, and kits for this purpose.

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METHODS AND DEVICES FOR PROGNOSIS OF CANCER RELAPSE

FIELD OF THE INVENTION

The invention features methods, devices, and kits for prognosing cancer relapse in a cancer patient.

BACKGROUND OF THE INVENTION

Gene expression analysis in tumor samples from patients has been used to facilitate cancer prognosis and diagnosis. Gene expression patterns can reveal the presence of cancer in a patient, its type, stage, and origin, and whether genetic mutations are involved. Gene expression may even have a role in predicting the efficacy of chemotherapy.

In recent years a new class of regulatory molecules, microRNAs, has been discovered. Determining their concentration, or expression, in cancer cells has revealed a role in cancer. It has been demonstrated that the detection of microRNAs can be used to determine the site of origin of cancers and can be used to differentiate between aggressive and non-aggressive cancers. Information contained in the expression level of genes and microRNAs is complementary, and combining this information in methods of prognosis or diagnosis may produce results that are more clinically accurate and useful.

Lung cancer is a disease with high mortality. Even after surgery, the majority of lung cancer patients suffer a relapse and die. If the removed tumor is more than 3 cm in diameter, the standard of care is to offer the patient chemotherapy to prevent relapse. If the tumor is less than 3 cm in diameter, and no spreading of the tumor is observed (also referred to as Stage Ia), the patient is offered no further treatment. Yet more than half of lung cancer patients suffer a relapse and die within 5 years.

There is a need for methods for prognosing cancer relapse in a patient with a cancer after one or more medical treatments for cancer, including surgery.

SUMMARY OF THE INVENTION

The invention includes a method for prognosing cancer relapse in a cancer patient before or after one or more cancer treatments (e.g., surgery, radiation therapy, and/or chemotherapy) by determining the level of expression of at least one biomarker (e.g., more than one biomarker, such as 2, 3, or 4 or more biomarkers), in which the biomarker has at least 85% (e.g., 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of any one of hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 (SEQ ID NOs: 1-4, respectively). In one embodiment, the method involves determining the expression level of a biomarker having the sequence of any one of hsa-miR-650, hsa-miR-324-3p, hsa-

miR-513b, and hsa-miR-1307, either singly or in any combination of 2, 3, or all 4 biomarkers (either simultaneously or in sequence).

In another embodiment, the methods of the invention may include determining the levels of expression of pair-wise combinations of the hsa-miR-650, hsa-miR-324-3p, hsa-miR-513b, and hsa-miR-1307 biomarkers (or a biomarker having at least 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence of any one of the hsa-miR-650, hsa-miR-324-3p, hsa-miR-513b, and hsa-miR-1307 biomarkers). In another embodiment, the methods of the invention may include determining the levels of expression of triplet or quadruplet combinations of the hsa-miR-650, hsa-miR-324-3p, hsa-miR-513b, and hsa-miR-1307 biomarkers (or a biomarker having at least 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence of any one of the hsa-miR-650, hsa-miR-324-3p, hsa-miR-513b, and hsa-miR-1307 biomarkers). The methods of the invention may include determining the level of expression of two or more biomarkers (e.g., 2, 3, or 4 biomarkers) simultaneously or in sequence.

The methods of the invention include determining the level of expression of the biomarker(s) in a sample from a cancer patient. The sample may be a blood sample or a tissue sample, e.g., a tumor sample. The methods of the invention can be used for prognosing relapse of any type of cancer, e.g., lung cancer, such as a non-small cell lung carcinoma, before or after a first cancer treatment in a cancer patient. In one embodiment of the invention, the methods of prognosing cancer relapse in a cancer patient may occur after a first cancer treatment. Alternatively, the prognosis may occur prior to a first cancer treatment. In another embodiment, the prognosis may occur after a first treatment but before a second treatment. Alternatively, the prognosis may occur after the second cancer treatment. The cancer treatment described in the invention may include one or more of surgery, radiation therapy, and chemotherapy and/or any other therapy known in the art for treating cancer. In one aspect of the invention, the chemotherapeutic agent may include one or more of a drug, an antibody, and an oligonucleotide.

In one aspect of the method, an increase or a decrease in the level of expression of at least one biomarker (e.g., a biomarker having at least 85% (e.g., 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of any one of hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 (SEQ ID NOs: 1-4, respectively)) indicates a good prognosis of no cancer relapse. Alternatively, an increase or a decrease in the level of expression of one or more biomarkers (e.g., a biomarker having at least 85% (e.g., 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of any one of hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 (SEQ ID NOs: 1-4, respectively)) indicates a poor prognosis of cancer relapse.

The methods of the invention may include prognosing cancer relapse based on level of expression of at least one biomarker (e.g., a biomarker having at least 85% (e.g., 85%, 90%, 95%, 97%, 99%, or

100%) sequence identity to the sequence of any one of hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 (SEQ ID NOs: 1-4, respectively)) in a cancer patient sample relative to level of expression of the biomarker(s) in a sample from a normal patient, or from a sample from a patient after a first (or subsequent) cancer treatment. Alternatively, the detection of expression of one or more biomarkers would alone provide sufficient information for a cancer relapse prognosis.

The methods of the invention may include collecting nucleic acid molecules from a patient (e.g., cancer patient) sample (e.g., a tissue sample, such as a tumour sample) and, optionally, using a quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to amplify the nucleic acid molecules, followed by detection of one or more biomarkers (e.g., 1, 2, 3, or 4 biomarkers) in the sample or determining the expression level of at least one biomarker (e.g.,1, 2, 3, or 4 biomarkers) in the sample.

The invention features devices that can be used to detect the expression of, or determine the expression level of, at least one biomarker (e.g., more than one biomarker, such as 2, 3, or 4 or more biomarkers) and may include at least one (e.g., more than one, such as 2, 3, or 4 or more) single-stranded nucleic acid molecule (also referred to as an oligonucleotide probe) having at least 85% (e.g., 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of a biomarker or its complement sequence. The sequence of the biomarker includes at least 5 (e.g., 5, 6, 7, 8, 10, 12, 15, 20, or 22) consecutive nucleotides of the sequence of any one of hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 (SEQ ID NOs: 1 to 4 respectively). For example, the devices may include oligonucleotide probes that can be used to detect the expression of any one of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers (SEQ ID NOs: 1-4, respectively), or sequences complementary to these biomarkers, in a tissue sample from a patient (e.g., a cancer patient).

In one embodiment, the device includes oligonucleotide probes having at least 100% sequence identity to the sequence of any one or more of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers, or their complement sequences. The device can include pair-wise, triple, or quadruple combinations of oligonucleotide probes having at least 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence of any one of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers (SEQ ID NOs: 1-4, respectively), or their complement sequences.

The device allows specific hybridization between single stranded nucleic acid molecules of the device (e.g., oligonucleotide probes) and the biomarker(s) or its complement sequence(s). The device includes at least one single-stranded nucleic acid molecule having a length in the range of 10 to 100 nucleotides (e.g., a length of 10, 20, 25, 30, 40, 60, 80, or 100 nucleotides or a length in the range of 5-50, 20-50, or 20-100 nucleotides). When the device is contacted with a diverse population of nucleic acid molecules prepared from a sample under conditions that allow hybridisation, the oligonucleotide probes in the device hybridize with their target biomarker and can detect the presence of at least one biomarker

(e.g., 1, 2, 3, or 4 biomarkers) in a sample (e.g., a patient tissue sample). Alternatively, the device can be used to determine the expression level of one or more of (e.g., 1, 2, 3, or 4) the above-mentioned biomarkers. In one embodiment of the invention, the device is a microarray device.

The invention includes methods for prognosing cancer relapse in a cancer patient by using the devices described above for detecting, or for determining the level of expression of, at least one biomarker (e.g., a biomarker having at least 85% (e.g., 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of any one of hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 (SEQ ID NOs: 1-4, respectively)) in a patient sample (e.g., a tumor sample), such that the detection of, or the level of expression of one or more (e.g., 1, 2, 3, or 4) biomarkers is prognostic of cancer relapse in the patient. The sample can be from a patient diagnosed with any one of the cancers described herein (e.g., a lung cancer, more specifically, non-small cell lung cancer). The device can be used for prognosis of cancer relapse in a cancer patient before or after a first cancer treatment. Alternatively, the device can be used for prognosis of cancer relapse after a first cancer treatment, but before a second treatment. In yet another aspect of the invention, the device can be used for prognosis of cancer relapse after a second cancer treatment.

The device of the method can be used to detect an increase or a decrease in the level of expression of at least one of the above-mentioned biomarkers (e.g., 1, 2, 3, or 4 biomarkers) indicating a good prognosis of no cancer relapse. Alternatively, the device can be used to detect an increase or a decrease in the level of expression of one or more of the above-mentioned biomarkers (e.g., 1, 2, 3, or 4 biomarkers) indicating a poor prognosis of cancer relapse.

The device can be used for prognosing cancer relapse based on level of expression of one or more biomarkers in a cancer patient sample relative to level of expression in a sample from a normal patient, or from a sample from a patient after a first cancer treatment. Alternatively, detection of expression of one or more biomarkers by the device can be used to provide a prognosis for cancer relapse.

The invention also features a kit that may include reagents for collecting nucleic acid molecules from a sample from a cancer patient, reagents for amplifying nucleic acid molecules collected from the sample to produce an amplified sample, and at least one device for detecting the level of expression of at least one biomarker (e.g., 1, 2, 3, or 4 biomarkers) having the sequence of any one of SEQ ID NOs: 1 to 4 in the amplified sample. In one embodiment, a quantitative reverse transcription-polymerase chain reaction (qRT-PCR) may be used to produce the amplified sample. The kit may further include instructions for prognosing cancer relapse in a cancer patient based on the level of expression of the at least one biomarker (e.g., one or more, or all, of the biomarkers having the sequence of any one of SEQ ID NOs: 1 to 4).

In one embodiment, the kit may include the device described above (e.g., a microarray device) to detect at least one (e.g., 1, 2, 3, or 4) biomarker (e.g., a biomarker having the sequence of any one of SEQ ID NOs: 1 to 4) in the sample or to determine the expression level of at least one (e.g., 1, 2, 3, or 4) biomarkers in the sample. The kit may further include instructions for applying nucleic acid molecules collected from the sample to the device, and/or instructions for detecting hybridization of at least one oligonucleotide probe with at least one biomarker or its complement sequence in order to detect the expression of, or to determine the expression level of the at least one biomarker in the sample. The kit may further include instructions for prognosing cancer relapse in a cancer patient based on the level of expression of the at least one biomarker as detected using the device.

Biomarkers relevant for prognosing cancer relapse are identified as those that are differentially expressed between the relevant groups for which prognosis is warranted. For example, samples obtained from cancer patients may be assayed for the biomarkers of the invention to group patients according to whether or not the patient experiences a relapse after a cancer treatment e.g., surgery, radiation therapy, and/or chemotherapy. Total RNA, including mRNA and microRNA is extracted from the samples and labeled according to standard procedures. The amount of mRNA from each known gene, or microRNA from each microRNA species known, is measured with one or more DNA microarrays containing probes complementary to the mRNAs and/or microRNAs.

This approach can be used for mRNA biomarkers, as well as for microRNA biomarkers. Prognosis is based on mRNA biomarkers, microRNA biomarkers, or combinations thereof, all measured using one or more DNA microarrays (or RT-PCR) on labeled RNA extracted from a sample from the patient's tumor.

The method of the invention can be applied for prognosis of cancer (e.g., lung cancer) relapse prior to or after treatment. There is currently no good and accurate method for determining whether a cancer will relapse or not. The methods described herein can be used by, e.g., an oncologist, to choose the most appropriate treatment for the patient based on the genetic makeup of the individual tumor. Knowing the likelihood of relapse will allow the oncologist to select one or more appropriate chemotherapy regimens, or a combination of surgery, chemotherapy, and radiation therapy.

"Cancer patient" as used herein refers to a subject, e.g., a human subject, who has, or has had a cancer and may or may not have been treated for the cancer.

"Complement" of a nucleic acid sequence or a "complementary" nucleic acid sequence as used herein refers to an oligonucleotide which is in "antiparallel association" when it is aligned with the nucleic acid sequence such that the 5' end of one sequence is paired with the 3' end of the other. Nucleotides and other bases may have complements and may be present in complementary nucleic acids. Bases not commonly found in natural nucleic acids that may be included in the nucleic acids of the

present invention include, for example, inosine and 7-deazaguanine. "Complementarity" may not be perfect; stable duplexes of complementary nucleic acids may contain mismatched base pairs or unmatched bases. Those skilled in the art of nucleic acid technology can determine duplex stability empirically considering a number of variables including, for example, the length of the oligonucleotide, percent concentration of cytosine and guanine bases in the oligonucleotide, ionic strength, and incidence of mismatched base pairs.

When complementary nucleic acid sequences form a stable duplex, they are said to be "hybridized" or to "hybridize" to each other or it is said that "hybridization" has occurred. Nucleic acids are referred to as being "complementary" if they contain nucleotides or nucleotide homologues that can form hydrogen bonds according to Watson-Crick base-pairing rules (e.g., G with C, A with T or A with U) or other hydrogen bonding motifs such as for example diaminopurine with T, 5-methyl C with G, 2-thiothymidine with A, inosine with C, pseudoisocytosine with G, etc. Anti-sense RNA may be complementary to other oligonucleotides, e.g., mRNA.

"Biomarker" as used herein indicates a gene or other portion of a subject's genetic material that is expressed in a form that can be measured (e.g., as an mRNA, microRNA, or protein) and whose expression indicates good or poor prognosis of cancer relapse in a patient.

"Marker gene" or "biomarker gene" as used herein means a gene in a cell the expression of which correlates to sensitivity or resistance of the cell (and thus the patient from which the cell was obtained) to a treatment (e.g., exposure to a compound).

"Microarray" as used herein means a device employed by any method that quantifies one or more subject oligonucleotides, e.g., DNA or RNA, or analogues thereof, at a time. One exemplary class of microarrays consists of DNA probes attached to a glass or quartz surface. For example, many microarrays, including those made by Affymetrix, use several probes for determining the expression of a single gene. The DNA microarray may contain oligonucleotide probes that may be, e.g., full-length cDNAs complementary to an RNA or cDNA fragments that hybridize to part of an RNA. The DNA microarray may also contain modified versions of DNA or RNA, such as locked nucleic acids or LNA. Exemplary RNAs include mRNA, miRNA, and miRNA precursors. Exemplary microarrays also include a "nucleic acid microarray" having a substrate-bound plurality of nucleic acids, hybridization to each of the plurality of bound nucleic acids being separately detectable. The substrate may be solid or porous, planar or non-planar, unitary or distributed. Exemplary nucleic acid microarrays include all of the devices so called in Schena (ed.), DNA Microarrays: A Practical Approach (Practical Approach Series), Oxford University Press (1999); Nature Genet. 21(1)(suppl.):1-60 (1999); Schena (ed.), Microarray Biochip: Tools and Technology, Eaton Publishing Company/BioTechniques Books Division (2000). Additionally, exemplary nucleic acid microarrays include substrate-bound plurality of nucleic acids in which the

plurality of nucleic acids are disposed on a plurality of beads, rather than on a unitary planar substrate, as is described, inter alia, in Brenner et al., Proc. Natl. Acad. Sci. USA 97(4):1665-1670 (2000). Examples of nucleic acid microarrays may be found in U.S. Pat. Nos. 6,391,623, 6,383,754, 6,383,749, 6,380,377, 6,379,897, 6,376,191, 6,372,431, 6,351,712 6,344,316, 6,316,193, 6,312,906, 6,309,828, 6,309,824, 6,306,643, 6,300,063, 6,287,850, 6,284,497, 6,284,465, 6,280,954, 6,262,216, 6,251,601, 6,245,518, 6,263,287, 6,251,601, 6,238,866, 6,228,575, 6,214,587, 6,203,989, 6,171,797, 6,103,474, 6,083,726, 6,054,274, 6,040,138, 6,083,726, 6,004,755, 6,001,309, 5,958,342, 5,952,180, 5,936,731, 5,843,655, 5,814,454, 5,837,196, 5,436,327, 5,412,087, 5,405,783, the disclosures of which are incorporated herein by reference in their entireties.

Exemplary microarrays may also include "peptide microarrays" or "protein microarrays" having a substrate-bound plurality of polypeptides, the binding of a oligonucleotide, a peptide, or a protein to each of the plurality of bound polypeptides being separately detectable. Alternatively, the peptide microarray, may have a plurality of binders, including but not limited to monoclonal antibodies, polyclonal antibodies, phage display binders, yeast 2 hybrid binders, aptamers, which can specifically detect the binding of specific oligonucleotides, peptides, or proteins. Examples of peptide arrays may be found in WO 02/31463, WO 02/25288, WO 01/94946, WO 01/88162, WO 01/68671, WO 01/57259, WO 00/61806, WO 00/54046, WO 00/47774, WO 99/40434, WO 99/39210, WO 97/42507 and U.S. Pat. Nos. 6,268,210, 5,766,960, 5,143,854, the disclosures of which are incorporated herein by reference in their entireties.

"Gene expression" as used herein means the amount of a gene product in a cell, tissue, organism, or subject, e.g., amounts of DNA, RNA, or proteins, amounts of modifications of DNA, RNA, or protein, such as splicing, phosphorylation, acetylation, or methylation, or amounts of activity of DNA, RNA, or proteins associated with a given gene.

"Treatment" or "medical treatment" means administering to a subject or living organism or exposing to a cell or tumor a compound (e.g., a drug, a protein, an antibody, an oligonucleotide, a chemotherapeutic agent, and a radioactive agent) or some other form of medical intervention used to treat or prevent cancer (e.g., lung cancer) or the symptoms of cancer (e.g., cryotherapy and radiation therapy). Radiation therapy includes the administration to a patient of radiation generated from sources such as particle accelerators and related medical devices that emit X-radiation, gamma radiation, or electron (beta radiation) beams. A treatment may further include surgery, e.g., to remove a tumor from a subject or living organism.

Other features and advantages of the invention will be apparent from the following Detailed Description, the drawings, and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing a Kaplan-Meier plot of recurrence in 78 non-small cell lung carcinoma (NSCLC) patients predicted in a leave-one-out cross-validation using a 60-microRNA model.

Figure 2 is a graph showing a Kaplan-Meier plot of overall survival in 30 NSCLC patients predicted in an independent validation using a 4-microRNA model.

DETAILED DESCRIPTION

The invention features methods for determining the expression level of one or more biomarkers from a patient sample for prognosing cancer relapse in a cancer patient before or after a cancer treatment (e.g., surgery and/or treatment with one or more, and preferably two or more, chemotherapeutic agents and/or radiation). The invention also features devices (e.g., a microarray) that include nucleic acid probes that can detect the expression of one or more biomarkers from a patient sample. The devices can be used for prognosing whether a cancer in a patient will relapse before or after a treatment. The invention also features kits to determine the level of expression of one or more biomarkers from a patient sample for prognosing cancer relapse in a cancer patient. The methods according to the present invention can be implemented using software that is run on an apparatus for measuring gene expression in connection with a detection device, such as a microarray. The detection device (e.g., a microarray, such as a DNA microarray), which is included in a kit for processing a tumor sample from a subject, and the apparatus for reading the device and turning the result into a prognosis for the subject, may be used to implement the methods of the invention.

Cancers

The methods, devices, and kits of the invention can be used for prognosing cancer relapse in a patient suffering from, or diagnosed with, cancer, for example, a cancer of the breast, prostate, lung (e.g., non small cell lung carcinoma), bronchus, colon, rectum, urinary bladder, skin, kidney, pancreas, oral cavity, pharynx, ovary, thyroid, parathyroid, stomach, brain, esophagus, liver, intrahepatic bile duct, cervix larynx, heart, testis, small intestine, large intestine, anus, anal canal, anorectum, vulva, gallbladder, pleura, bone, joint, hypopharynx, eye and/or orbit, nose, nasal cavity, middle ear, nasopharynx, ureter, peritoneum, omentum, mesentery, and/or gastrointestines, as well as any form of cancer including, e.g., chronic myeloid leukemia, acute lymphocytic leukemia, non-Hodgkin lymphoma, melanoma, carcinoma, basal cell carcinoma, malignant mesothelioma, neuroblastoma, multiple myeloma, leukemia, retinoblastoma, acute myeloid leukemia, chronic lymphocytic leukemia, Hodgkin lymphoma, carcinoid tumors, acute tumor, and/or soft tissue sarcoma (e.g., preferably the cancer is a cancer of the bladder,

breast, colon, rectum, uterus, kidney, lung, skin (e.g., melanoma), pancreas, prostate, blood and/or bone marrow (e.g., leukemia), lymphocytes (e.g., non-Hodgkin lymphoma), and/or thyroid).

Cancer Treatments

The methods, devices, and kits of the invention can be used to determine the potential for relapse of a cancer in a cancer patient, e.g., a lung cancer patient, before or after a first treatment. The first treatment may include, e.g., one or more of surgery, radiation therapy, and/or chemotherapy. The chemotherapy may include administration of one or more of (e.g., two or more of) the following agents: vincristine, cisplatin, etoposide, azaguanine, carboplatin, adriamycin, aclarubicin, mitoxantrone, mitoxantrone, mitomycin, paclitaxel, gemcitabine, taxotere, dexamethasone, ara-c, methylprednisolone, methotrexate, bleomycin, methyl-gag, belinostat, carboplatin, 5-fu (5-fluorouracil), idarubicin, melphalan, IL4-PR38, valproic acid, all-trans retinoic acid (ATRA), cytoxan, topotecan, suberoylanilide hydroxamic acid (SAHA, vorinostat), depsipeptide (FR901228), bortezomib, leukeran, fludarabine, vinblastine, busulfan, dacarbazine, oxaliplatin, hydroxyurea, tegafur, daunorubicin, estramustine, mechlorethamine, streptozocin, carmustine, lomustine, mercaptopurine, teniposide, dactinomycin, tretinoin, ifosfamide, tamoxifen, irinotecan, floxuridine, thioguanine, PSC 833, erlotinib, herceptin, bevacizumab, celecoxib, fulvestrant, iressa, anastrozole, letrozole, cetuximab, rituximab, radiation, histone deacetylase (HDAC) inhibitors, and 5-Aza-2'-deoxycytidine (decitabine).

A beneficial aspect of the invention is that the methods, devices, and kits can be used for prognosing cancer relapse in a cancer patient before or after one or more treatments for cancer (e.g., two, three, four, five, ten, twenty, thirty, or more treatments for cancer) by assaying the level of expression of one or more (e.g., two, three, or four) biomarkers selected from the group consisting of hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307, either simultaneously or in sequence. The expression of each of these biomarkers has been determined to be prognostic for cancer relapse in a patient. Other biomarkers that can be used for prognosing cancer relapse in a patient include one or more of the biomarkers listed in Tables 1 and 2 below.

The methods, devices, and kits of the invention can also be used to identify patient subpopulations that are responsive to one or more treatments thought to be ineffective for treating disease (e.g., cancer) in the general population. More generally, prognosis of cancer relapse in a cancer patient treated with one or more treatments can be done using biomarker expression regardless of knowledge about patient's prior cancer treatments. The methods of the present invention can be implemented using software that is run on an apparatus for measuring gene expression in connection with a microarray. Devices of the invention (e.g., a microarray, such as a DNA and/or RNA microarray) can be included in a kit for processing a tumor sample from a subject (e.g., a cell, tissue, or organ sample containing a tumor

or a cell thereof), and the apparatus for reading the device and turning the result into a prognosis profile for the subject may be used to implement the methods of the invention.

Biomarkers of the Invention

The invention features biomarkers having at least 85% (e.g., 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of any one of hsa-miR-513b (5' UUCACAAGGAGGUGUCAUUUAU3'; SEQ ID NO:1); hsa-miR-650 (5' AGGAGGCAGCGCUCUCAGGAC3'; SEQ ID NO: 2); hsa-miR-324-3p (5' ACUGCCCCAGGUGCUGCUGG3'; SEQ ID NO: 3); and hsa-miR-1307 (ACUCGGCGUGGCGUCGGUCGUG; SEQ ID NO 4). Additional biomarkers that can be used in the methods, devices, and kits of the invention are listed in Tables 1 and 2 below. These additional biomarkers can be used either independently or in combination with the biomarkers having 85% or more sequence identity (e.g., 100% sequence identity) to the sequence of any one of hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307. Preferably, the biomarkers of the invention have the sequence of hsa-miR-650, hsa-miR-324-3p, hsa-miR-513b, and hsa-miR-1307 and may be used in any combination (simultaneously or in sequence) as described below. Furthermore any combination of these four biomarkers may be used with one or more of biomarkers listed in Tables 1 and 2 (simultaneously or in sequence).

The biomarkers of the methods may be used in methods, devices and kits, as described below, to determine the potential for relapse of a cancer (e.g., lung cancer) in a patient before or after one or more treatments for cancer, such as the cancer treatments listed above.

Methods for prognosing cancer relapse in a cancer patient using the biomarkers of the invention

The invention features methods for prognosing cancer relapse in a patient with a cancer before or after one or more treatments for cancer, e.g., surgery, radiation therapy, and/or chemotherapy, by measuring the level of expression of one or more (e.g., 1, 2, 3, or 4) biomarkers having at least 85% (e.g., 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of any one of hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307. Preferably, the method involves determining the expression level of a biomarker having the sequence of any one of hsa-miR-650, hsa-miR-324-3p, hsa-miR-513b, and hsa-miR-1307 (SEQ ID NOs: 1-4, respectively), either singly or in any combination of 2, 3, or all 4 (either simultaneously or in sequence). Preferably, the method is performed in a patient after at least one treatment for cancer.

For example, the methods of the invention may include determining the levels of expression of pair-wise combinations of the hsa-miR-650, hsa-miR-324-3p, hsa-miR-513b, and hsa-miR-1307

biomarkers (or a biomarker having at least 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence of any one of the hsa-miR-650, hsa-miR-324-3p, hsa-miR-513b, and hsa-miR-1307 biomarkers). In particular, the levels of expression of the following pair-wise combinations of biomarkers can be measured, either simultaneously or in sequence:

1) hsa-miR-513b and hsa-miR-650;
2) hsa-miR-513b and hsa-miR-324-3p;
3) hsa-miR-513b and hsa-miR-1307;
4) hsa-miR-650 and hsa-miR-513b;
5) hsa-miR-650 and hsa-miR-324-3p;
6) hsa-miR-650and hsa-miR-1307;
7) hsa-miR-324-3p and hsa-miR-513b;
8) hsa-miR-324-3p and hsa-miR-650;
9) hsa-miR-324-3p and hsa-miR-1307;
10) hsa-miR-1307 and hsa-miR-513b;
11) hsa-miR-1307 and hsa-miR-650; and
12) hsa-miR-1307 and hsa-miR-324-3p.

The methods of the invention may also include determining the levels of expression of triple combinations of the hsa-miR-650, hsa-miR-324-3p, hsa-miR-513b, and hsa-miR-1307 biomarkers (or a biomarker having at least 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence of any one of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers). In particular, the levels of expression of the following triple combinations of biomarkers can be measured, either simultaneously or in sequence:

1) hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p;
2) hsa-miR-513b, hsa-miR-650, hsa-miR-1307;
3) hsa-miR-513b, hsa-miR-324-3p, hsa-miR-650;
4) hsa-miR-513b, hsa-miR-324-3p, hsa-miR-1307;
5) hsa-miR-513b, hsa-miR-1307, hsa-miR-650;
6) hsa-miR-513b, hsa-miR-1307, hsa-miR-324-3p;
7) hsa-miR-650, hsa-miR-513b, hsa-miR-324-3p;
8) hsa-miR-650, hsa-miR-513b, hsa-miR-1307;
9) hsa-miR-650, hsa-miR-324-3p, hsa-miR-513;
10) hsa-miR-650, hsa-miR-324-3p, hsa-miR-1307;
11) hsa-miR-650, hsa-miR-1307, hsa-miR-513b;
12) hsa-miR-650, hsa-miR-1307, hsa-miR-324-3p;

13) hsa-miR- 324-3p, hsa-miR-513b, hsa-miR-650;

14) hsa-miR-324-3p, hsa-miR-513b, hsa-miR-1307;

15) hsa-miR-324-3p, hsa-miR-650, hsa-miR-513;

16) hsa-miR-324-3p, hsa-miR-650, hsa-miR-1307;

17) hsa-miR-324-3p, hsa-miR-1307, hsa-miR-513b;

18) hsa-miR-324-3p, hsa-miR-1307, hsa-miR-650;

19) hsa-miR-1307, hsa-miR-513b, hsa-miR-650;

20) hsa-miR-1307, hsa-miR-513b, hsa-miR-324-3p;

21) hsa-miR-1307, hsa-miR-650, hsa-miR-513b;

22) hsa-miR-1307, hsa-miR-650, hsa-miR-324-3p;

23) hsa-miR-1307, hsa-miR-324-3p, hsa-miR-513b; and

24) hsa-miR-1307, hsa-miR-324-3p, hsa-miR-650.

The methods of the invention may also include determining the levels of expression of quadruple combinations of the hsa-miR-650, hsa-miR-324-3p, hsa-miR-513b, and hsa-miR-1307 biomarkers (or a biomarker having at least 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence of any one of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers). In particular, the levels of expression of the following quadruple combinations of biomarkers can be determined, either simultaneously or in sequence:

1) hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, hsa-miR-1307;

2) hsa-miR-513b, hsa-miR-650, hsa-miR-1307, hsa-miR-324-3p;

3) hsa-miR-513b, hsa-miR-324-3p, hsa-miR-650, hsa-miR-1307;

4) hsa-miR-513b, hsa-miR-324-3p, hsa-miR-1307, hsa-miR-650;

5) hsa-miR-513b, hsa-miR-1307, hsa-miR-650, hsa-miR-324-3p;

6) hsa-miR-513b, hsa-miR-1307, hsa-miR-324-3p, hsa-miR-650;

7) hsa-miR-650, hsa-miR-513b, hsa-miR-324-3p, hsa-miR-1307;

8) hsa-miR-650, hsa-miR-513b, hsa-miR-1307, hsa-miR-324-3p;

9) hsa-miR-650, hsa-miR-324-3p, hsa-miR-513b, hsa-miR-1307;

10) hsa-miR-650, hsa-miR-324-3p, hsa-miR-1307, hsa-miR-513b;

11) hsa-miR-650, hsa-miR-1307, hsa-miR-513b, hsa-miR-324-3p;

12) hsa-miR-650, hsa-miR-1307, hsa-miR-324-3p, hsa-miR-513b;

13) hsa-miR-324-3p, hsa-miR-513b, hsa-miR-650, hsa-miR-1307;

14) hsa-miR-324-3p, hsa-miR-513b, hsa-miR-1307, hsa-miR-1307;

15) hsa-miR-324-3p, hsa-miR-650, hsa-miR-513b, hsa-miR-1307;

16) hsa-miR-324-3p, hsa-miR-650, hsa-miR-1307, hsa-miR-513b;

17) hsa-miR-324-3p, hsa-miR-1307, hsa-miR-513b, hsa-miR-650;

18) hsa-miR-324-3p, hsa-miR-1307, hsa-miR-650, hsa-miR-513b;

19) hsa-miR-1307, hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p;

20) hsa-miR-1307, hsa-miR-513b, hsa-miR-324-3p, hsa-miR-650;

21) hsa-miR-1307, hsa-miR-650, hsa-miR-513b, hsa-miR-324-3p;

22) hsa-miR-1307, hsa-miR-650, hsa-miR-324-3p, hsa-miR-513b;

23) hsa-miR-1307, hsa-miR-324-3p, hsa-miR-513b, hsa-miR-650; and

24) hsa-miR-1307, hsa-miR-324-3p, hsa-miR-650m, hsa-miR-513b.

The methods of the invention may include collecting nucleic acid samples from a sample, e.g., a tissue sample. The sample is preferably a tumor sample from a cancer patient. For example, the sample may be from a lung cancer patient, such as a patient suffering from a non-small cell lung carcinoma. The methods of the invention may optionally include amplifying the nucleic acid molecules using, e.g., polymerase chain reaction (PCR), to produce an amplified solution. The methods of the invention may further include performing qRT-PCR in a thermal cycler using the nucleic acid molecules collected from a sample or using the amplified solution described above to measure the level of expression of one or more of the biomarkers in the sample. Procedures for performing qRT-PCR are described in, e.g., U.S. Patent No. 7,101,663 and U.S. Patent Application Nos. 2006/0177837 and 2006/0088856, each of which is incorporated herein by reference. The level of expression of two or more of the hsa-miR-650, hsa-miR-324-3p, hsa-miR-513b, and hsa-miR-1307 biomarkers (and, optionally, one or more additional biomarkers listed in Tables 1 and 2) in the sample can be determined simultaneously in the same reaction. Alternatively, the level of expression of two or more of the hsa-miR-650, hsa-miR-324-3p, hsa-miR-513b, and hsa-miR-1307 biomarkers (and, optionally, one or more additional biomarkers listed in Tables 1 and 2, if desired) in the sample can be determined simultaneously in different reactions. Furthermore, the level of expression of two or more of the hsa-miR-650, hsa-miR-324-3p, hsa-miR-513b, and hsa-miR-1307 biomarkers (as well as one or more additional biomarkers listed in Tables 1 and 2, if desired) can be determined one after the other in the same or separate reactions.

The methods of the invention may also include prognosing cancer relapse in a cancer patient after one or more cancer treatments, e.g., surgery, radiation therapy, and/or chemotherapy, based on the level of expression of one or more of the hsa-miR-650, hsa-miR-324-3p, hsa-miR-513b, and hsa-miR-1307 biomarkers (and, optionally, one or more additional biomarkers listed in Tables 1 and 2, if desired) in the sample.

For example, an increase in the level of expression of one or more of the biomarkers may indicate a good prognosis of no relapse after one or more cancer treatments, such as those treatments described above. Alternatively, a decrease in the level of expression of one or more of the biomarkers may indicate

a good prognosis of no relapse after one or more cancer treatments, such as those described above. Furthermore, an increase in the level of expression of one or more of the biomarkers may indicate a poor prognosis of cancer relapse after one or more cancer treatments. Alternatively, a decrease in the level of expression of one or more of the biomarkers may indicate a poor prognosis of cancer relapse after one or more cancer treatments. Alternatively the detection of expression alone of any of the biomarkers may be an indication of the prognosis of the relapse of a cancer in a cancer patient after a cancer treatment.

A good prognosis refers to a case where the patient will be alive at least 5 years (e.g., 4, 5, 6, 7, 8, 10, or 12 or more years) after a first cancer treatment, and a poor prognosis refers to a case where the patient will not likely survive for at least 5 years after a first cancer treatment. Kaplan-Meier curves can be used to compare survival over time, as shown in figures 1 and 2.

In the methods for prognosing cancer relapse, the expression level of one or more of the biomarkers can be determined relative to that in a normal cell or relative to a cancer cell from a patient who has undergone a first course of treatment.

Devices and methods for cancer prognosis using biomarkers of the invention

The invention features devices that include one or more oligonucleotide probes having a sequence that is identical, or complementary, to at least 5 (e.g., 5, 6, 7, 8, 10, 12, 15, 20, or 22; preferably 22) consecutive nucleotides (or nucleotide analogues) of the sequence of one or more of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers. The oligonucleotide probes of the devices may also include sequences having at least 85% (e.g., 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of any one of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers (SEQ ID NOs: 1-4, respectively), or their complements, over at least about 5 (e.g., 5, 6, 7, 8, 10, 12, 15, 20, or 22; preferably 22) consecutive nucleotides (or nucleotide analogues). For example, the devices may include oligonucleotide probes that can be used to detect the presence of, or the level of expression of, any one or more (e.g., any combination of) the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers (SEQ ID NOs: 1-4, respectively), or sequences complementary to these biomarkers, in a tissue sample from a patient.

Preferably, a device of the invention includes oligonucleotide probes having a sequence with at least 85% sequence identity to the sequence of one or more of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers (SEQ ID NOs: 1-4, respectively), or their complements, over at least about 22 consecutive nucleotides (or nucleotide analogues). More preferably, the device includes oligonucleotide probes having at least 100% sequence identity to the sequence of any one or more of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers, or their complements. The

devices may include probes that can be used to detect the presence, or level of expression, of only one of the biomarkers, or they may include probes that can be used to detect the presence, or level of expression, of combinations of 2, 3, or 4 of the biomarkers.

For example, the device can include the following pair-wise combinations of oligonucleotide probes having at least 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence of any one of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers (SEQ ID NOs: 1-4, respectively), or their complements, over at least about 5 (e.g., 5, 6, 7, 8, 10, 12, 15, 20, or 22; preferably 22) consecutive nucleotides (or nucleotide analogues):

1) hsa-miR-513b and hsa-miR-650;

2) hsa-miR-513b and hsa-miR-324-3p;

3) hsa-miR-513b and hsa-miR-1307;

4) hsa-miR-650 and hsa-miR-324-3p;

5) hsa-miR-650and hsa-miR-1307;

6) hsa-miR-324-3p and hsa-miR-1307.

Preferably, the device includes pair-wise combinations of oligonucleotide probes that have at least 85% sequence identity to the sequence of any one of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers (SEQ ID NOs: 1-4, respectively), or their complements, over at least about 22 consecutive nucleotides (or nucleotide analogues). More preferably, the device includes pair-wise combinations of oligonucleotide probes that have at least 100% sequence identity to the sequence of any one of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers (SEQ ID NOs: 1-4, respectively), or their complements, over at least about 22 consecutive nucleotides (or nucleotide analogues).

The device can also include the following triplet combinations of oligonucleotide probes having at least 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence of any one of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers (SEQ ID NOs: 1-4, respectively), or their complements, over at least about 5 (e.g., 5, 6, 7, 8, 10, 12, 15, 20, or 22) consecutive nucleotides (or nucleotide analogues):

1) hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p;

2) hsa-miR-513b, hsa-miR-650, hsa-miR-1307;

3) hsa-miR-513b, hsa-miR-324-3p, hsa-miR-1307; and

4) hsa-miR-650, hsa-miR-324-3p, hsa-miR-1307.

Preferably, the device includes triplet combinations of oligonucleotide probes that have at least 85% sequence identity to the sequence of any one of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers (SEQ ID NOs: 1-4, respectively), or their complements, over at least about

22 consecutive nucleotides (or nucleotide analogues). More preferably, the device includes triplet combinations of oligonucleotide probes that have at least 100% sequence identity to the sequence of any one of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers (SEQ ID NOs: 1-4, respectively), or their complements, over at least about 22 consecutive nucleotides (or nucleotide analogues).

The device can also include oligonucleotide probes having at least 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to the sequence of each of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers (SEQ ID NOs: 1-4, respectively), or their complements, over at least about 5 (e.g., 5, 6, 7, 8, 10, 12, 15, 20, or 22) consecutive nucleotides (or nucleotide analogues). Preferably, the device includes oligonucleotide probes that have at least 85% sequence identity to the sequence of each of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers (SEQ ID NOs: 1-4, respectively), or their complements, over at least about 22 consecutive nucleotides (or nucleotide analogues). More preferably, the device includes oligonucleotide probes that have at least 100% sequence identity to the sequence of each of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers (SEQ ID NOs: 1-4, respectively), or their complements, over at least about 22 consecutive nucleotides (or nucleotide analogues).

The oligonucleotide probes of the devices described above may have a length of, e.g., 5-20, 25, 5-50, 5-100, 25-100, 50-100, or over 100 nucleotides. The oligonucleotide probes may be deoxyribonucleic acids (DNA) or ribonucleic acids (RNA).

The invention also features methods of using the devices described above to detect the expression of or determine the level of expression of one of the hsa-miR-650, hsa-miR-324-3p, hsa-miR-513b, and hsa-miR-1307 biomarkers, or any combination of two or more of the hsa-miR-650, hsa-miR-324-3p, hsa-miR-513b, and hsa-miR-1307 biomarkers, in a patient sample for prognosing cancer relapse after a cancer treatment.

The device of the invention containing one or more oligonucleotide probes can be a microarray device. The microarray device may contain oligonucleotide probes that may be, e.g., cDNAs corresponding to or complementary to an RNA (e.g., an mRNA) or a microRNA, or the oligonucleotide probes may be cDNA fragments that hybridize to part of an RNA (e.g., an mRNA) or a microRNA. Exemplary RNAs include miRNA, and miRNA precursors. Exemplary microarrays also include a "nucleic acid microarray" having a substrate-bound plurality of nucleic acids, hybridization to each of the plurality of bound nucleic acids being separately detectable.

The microarrays of the invention can include one or more oligonucleotide probes that have nucleotide sequences that are identical to or complementary to, e.g., at least 5, 8, 12, 20, 22, 30, 40, 60, 80, 100, 150, or 200 consecutive nucleotides (or nucleotide analogues) of the hsa-miR-650, hsa-miR-324-

3p, hsa-miR-513b, and hsa-miR-1307 biomarkers and/or to one or more of the biomarkers listed in Tables 1 and 2 below. The oligonucleotide probes may have a length in the range of, e.g., 5-20, 5-50, 25-50, 5-100, 25-100, 50-100, or over 100 nucleotides long. The oligonucleotide probes may be deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) or analogues thereof, such as LNA. Consecutive nucleotides within the oligonucleotide probes (e.g., 5-20, 25, 5-50, 50-100, or over 100 consecutive nucleotides), which are used as biomarkers of responsiveness to a cancer treatment, may also appear as consecutive nucleotides in one or more of the genes described herein beginning at or near, e.g., the first, tenth, twentieth, thirtieth, fortieth, fiftieth, sixtieth, seventieth, eightieth, ninetieth, hundredth, hundred-fiftieth, two-hundredth, five-hundredth, or one-thousandth nucleotide of the genes listed in Tables 1 and 2 below.

When a diverse population of nucleic acid molecules prepared from a sample, e.g., a patient sample is applied to the devices described above, the target nucleic acid molecule(s) in the sample hybridizes with the probe(s) on the device. This hybridization allows the detection of, and/or a determination of the quantity of, a target nucleic acid molecule(s) in the sample (e.g., one or more of the biomarkers described herein), and provides a readout of the level of expression of that target nucleic acid molecule(s). The target nucleic acid molecule(s) may be any one of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers described above, any may also include any one or more of the biomarkers described in Tables 1 and 2 below. The devices described above may be used to simultaneously (or sequentially) detect, or determine the level of expression of, one or more of these biomarkers. Optionally, the nucleic acid molecules isolated from the sample may be amplified prior to detection using the device of the invention using, e.g., PCR, to produce an amplified sample. The amplified sample can then be applied to a device of the invention.

The devices of the invention can be used in methods to determine the expression level of one or more of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers in a sample for prognosing cancer relapse in a cancer patient before and/or after one or more cancer treatments. The devices can be used to simultaneously (or sequentially) determine the expression level of multiple biomarkers, for example, 2, 3, or 4 biomarkers, and to use this information to determine a for cancer relapse prognosis for a patient.

In one example, cell/tissue samples are snap frozen in liquid nitrogen until processing. RNA may be extracted using, e.g., Trizol Reagent from Invitrogen following the manufacturer's instructions. RNA can be amplified using, e.g., MessageAmp kit from Ambion Inc. following the manufacturer's instructions. MicroRNA can be extracted from formalin-fixed paraffin embedded samples using, e.g., RecoverAll (Ambion Inc.) and labeled using, e.g., Genisphere HSR (GenisPhere Inc.). Amplified RNA can be quantified using, e.g., the HG-U133A GeneChip from Affymetrix Inc and a compatible apparatus,

e.g., the GCS3000Dx from Affymetrix, using the manufacturer's instructions. MicroRNA can be quantified using Affymetrix miRNA version 1.0 or 2.0.

The resulting gene expression measurements can be further processed for example, as described in examples 1-3. The procedures described can be implemented using R software available from R-Project and supplemented with packages available from Bioconductor.

For lung cancer prognosis any one of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers may be sufficient to give an accurate prediction. Preferably two or more of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers are used. In addition, 3 to 50 mRNA or microRNA biomarkers, such as those listed in Tables 1 and 2, can be used to provide an even more accurate prediction. Given the relatively small number of biomarkers required, procedures such as quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) may be performed to determine, with greater precision, the amount of biomarkers expressed in a sample. This will provide an alternative to or a complement to the use of devices described above. For example, qRT-PCR may be performed alone or in combination with a microarray described herein.

Kits for prognosing cancer relapse after a cancer treatment in cancer patients

The invention also features kits for prognosing cancer relapse in a cancer patient (e.g., a lung cancer patient) after one or more cancer treatments. The kits may include reagents for collecting nucleic acid molecules from a sample from a patient. For example, the kits may include reagents for lysis of patient samples and/or for isolating and purifying RNA from patient samples. The kits may further include reagents for amplifying the nucleic acid molecules isolated from the patient sample, for example, by PCR. The kits may include reagents for determining the level of expression of one or more biomarkers having at least 85% (e.g., 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of any one of the hsa-miR-650, hsa-miR-324-3p, hsa-miR-513b, and hsa-miR-1307 biomarkers, or their complements, using assays known in the art, e.g., qRT-PCR. The kits may include primers and probes for performing qRT-PCR to determine the expression level of the biomarkers described above. The kits may include instructions prognosing cancer relapse based on the level of expression of one or more biomarkers determined using the kits.

The kits may further include any one of the devices described above, to which a nucleic acid sample from a patient or an amplified solution may be applied, so that the probes on the device can hybridize with target biomarkers in the sample and provide a readout of the level of expression of one or more biomarkers (e.g., one or more of the hsa-miR-513b, hsa-miR-650, hsa-miR-324-3p, and hsa-miR-1307 biomarkers) in the sample. The device allows the simultaneous (or sequential) measurement of the level of expression of one or more of the biomarkers in a sample. The device in the kits may be a

microarray device. The kits may further include instructions for prognosing cancer relapse in a cancer patient, e.g., a good prognosis or a poor prognosis, based on the level of expression of one or more the biomarkers determined using the devices described above. Additionally, the device of the kits can be used in combination with qRT-PCR based assays to determine the level of expression of one or more the biomarkers. Furthermore, the kits may include software programs for prognosing cancer relapse based on the expression level of the biomarkers.

In one example, the kits may include reagents for RNA extraction from tumors (e.g., Trizol from Invitrogen Inc), reagents for RNA amplification (e.g., MessageAmp from Ambion Inc), a microarray for determining gene expression (e.g., the HG-U133A GeneChip from Affymetrix Inc), a microarray hybridization station and scanner (e.g., the GeneChip System 3000Dx from Affymetrix Inc), and software for analyzing the expression levels of biomarkers, as described herein (e.g., implemented in R from R-Project or S-Plus from Insightful Corp.).

EXAMPLES

Example 1: MicroRNAs useful for lung cancer prognosis

Formalin fixed paraffin embedded (FFPE) tissue specimens from 79 patients with pathologic stage 1 NSCLC were used for analysis. Clinical data was collected from Roswell Park Cancer Institute's tumor registry and was validated by chart review. Tissue was deparaffinized and miRNA extracted. After quality control assessments of the extracted RNA, hybridization was performed to a locked nucleic acid (LNA) based array platform (Exiqon Inc.) containing probes for all miRs in miRBase version 11. Data from the arrays was background corrected and Loess normalized. In a leave-one-out cross validation, miRNAs differentially expressed between patients with recurrence and patients without, were selected with a t-test, using a multiple testing correction leaving a false discovery rate of 0.1%. The resulting miRNAs were subjected to Principal Component Analysis and the five most important components used to train a multivariate classifier using classification algorithms K nearest neighbor, nearest centroid, neural network and support vector machine. The left out sample was predicted by majority vote among the classification algorithms into Good or Poor prognosis. A Kaplan-Meier plot was prepared of the time to recurrence for the Good and Poor prognosis groups. A log-rank test for statistical significance of the difference between the two groups was performed.

RESULTS – Of 79 samples, 78 samples passed the quality control conditions for hybridization. Data analysis performed as detailed above led to 60 microRNAs being selected for all 78 classifiers. The 60 microRNAs are shown in Table 1 together with a total of 157 microRNAs that are statistically significant (FDR=1%) in an analysis of all 78 samples and for which P-value and log2 fold change is calculated. The 60-gene model predicted outcome in a statistically significant fashion (Figure 1).

Example 2: MicroRNAs useful for lung cancer prognosis

It is possible to use as few as 2 or 3 microRNAs to obtain classification of lung cancer samples as described in Example 1. If the 2 or 3 microRNAs are selected from the list of hsa-miR-141 hsa-miR-22 hsa-miR-200b* hsa-miR-630 hsa-miR-27a hsa-miR-510 hsa-miR-30c-1*, classification performance similar to that shown in Figure 1 can be achieved. The First Principal Component with a cutoff of 0 can be used alone to predict recurrence or non-recurrence. Other classification methods based on the expression of 2 or 3 microRNAs selected from Table 1 can be used as well.

Example 3: Using Affymetrix arrays with DNA probes complementary to microRNAs.

Examples 1 and 2 involved the use of a locked nucleic acids platform from Exiqon to identify microRNAs that can be used to distinguish between patients with a good and a poor prognosis. A DNA-based platform such as Affymetrix has different physical and chemical properties, and will result in a different list of optimal microRNAs for the same purpose. The same FFPE samples used for Example 1 were analyzed on the Affymetrix *GeneChip® miRNA 1.0* array. Normalization was performed using constant totalRNA for each sample. A support vector machine svm from the library e1071 from www.bioconductor.org with default parameters was used to train a predictor. In cross-validation experiments, the following 3 microRNA probes on the Affy platform were best in separating poor prognosis from good prognosis patients: hsa- hsa-miR-650, hsa-miR-324-3p, hsa-miR-513b. Of these, hsa-miR-513b contributes most to the prognosis, followed by hsa-miR-650, followed by hsa-miR-324-3p, which is least important. If a fourth miR is desired, hsa-miR-1307 can be used and may improve performance on some datasets.

Example 4: Independent validation of 4-microRNA profile from example 3.

The 3-microRNA profile from Example 3 was independently verified on a cohort of 31 NSCLC patients in Stage Ia (Figure 2). This cohort was normalized in the same manner as the cohort in example 3. Using the support vector machine trained on the cohort from Example 3, the patients were predicted with either good prognosis or poor prognosis. The Kaplan-Meier curve in Figure 2 shows the overall survival in the two prediction groups. There is a statistically significant difference in survival between the good and poor prognosis groups (P=0.0001 in a logrank test).

TABLE 1. List of probe IDs referring to the miRNAs on miRCURY LNA arrays (v. 10.0, Exiqon). 60-gene refers to whether or not the miRNA was selected for all classifiers in leave-one-out cross-validation. A value of TRUE means that the miRNA is more reliable and important.

ID	name	log2(FC)	P-value	60-gene
17327	hsa-miR-630	-0.421	2.74e-09	TRUE
17859	hsa-miR-200b*	-0.438	8.78e-09	TRUE
42834	hsa-miR-219-2-3p	-0.606	1.11e-07	FALSE
42682	hsa-miR-25	0.979	1.47e-07	FALSE
42957	hsa-miR-323-3p	-0.536	2.99e-07	FALSE
42702	hsa-miR-30c-1*	-0.426	3.22e-07	TRUE
42593	hsa-miR-623	-0.551	3.23e-07	TRUE
5250	hsa-miR-105	1.14	3.75e-07	FALSE
42524	hsa-miR-21*	0.77	1.46e-06	FALSE
17752	hsa-let-7f	1.13	1.5e-06	FALSE
10986	hsa-miR-193a-3p	1.1	2.03e-06	FALSE
42811	hsa-miR-542-5p	-0.539	2.45e-06	TRUE
33596	hsa-miR-126*	1.17	2.48e-06	FALSE
19593	hsa-miR-27a	1.28	2.51e-06	TRUE
27720	hsa-miR-15a	1.23	2.82e-06	FALSE
30687	hsa-miR-93	1.14	2.85e-06	FALSE
11065	hsa-miR-335	1.17	3.29e-06	FALSE
11142	hsa-miR-510	-0.325	3.36e-06	TRUE
42458	hcmv-miR-US25-1*	-0.51	3.59e-06	TRUE
14302	hsa-miR-374b	1.01	4.21e-06	FALSE
27537	ebv-miR-BART13	-0.432	4.5e-06	TRUE
13132	hsa-miR-519e*	-0.325	5.12e-06	TRUE
27378	hsa-miR-374a	1.23	5.17e-06	FALSE
10985	hsa-miR-191	1.16	5.35e-06	TRUE
10995	hsa-miR-199a-3p/ hsa-miR-199b-3p	1.26	5.37e-06	FALSE
10138	hsa-miR-130a	1.13	5.92e-06	FALSE
11078	hsa-miR-365	0.661	6.44e-06	FALSE
27551	hsa-miR-612	-0.325	6.58e-06	TRUE
13143	hsa-miR-301a	1.11	7.03e-06	FALSE
17552	hsa-miR-617	-0.433	7.07e-06	TRUE
11022	hsa-miR-221	0.931	8.4e-06	FALSE

17836	hsa-miR-30b*	-0.392	8.52e-06	TRUE
10972	hsa-miR-181b	0.596	9.57e-06	FALSE
42513	hsa-miR-300	-0.339	1.16e-05	TRUE
42533	hiv1-miR-H1	-0.408	1.16e-05	FALSE
29562	hsa-miR-199a-5p	1.22	1.18e-05	TRUE
27541	hcmv-miR-UL70-3p	-0.517	1.18e-05	FALSE
13175	hsa-miR-27b	1.26	1.2e-05	TRUE
42838	miRPlus_42838	-0.387	1.28e-05	TRUE
10998	hsa-miR-19b	1.34	1.42e-05	FALSE
10967	hsa-miR-16	1.35	1.62e-05	TRUE
11020	hsa-miR-22	1.03	1.74e-05	TRUE
10306	hsa-miR-146b-5p	1.02	1.75e-05	FALSE
42467	hsa-miR-129-5p	-0.485	1.88e-05	TRUE
42843	hsa-miR-654-5p	-0.411	2.11e-05	TRUE
42865	hsa-miR-181a	0.795	2.11e-05	TRUE
4610	hsa-miR-126	1.08	2.11e-05	TRUE
4700	hsa-miR-140-5p	0.923	2.17e-05	FALSE
11023	hsa-miR-222	0.881	2.21e-05	TRUE
19011	hsa_SNORD10	0.903	2.22e-05	TRUE
17541	ebv-miR-BART1-5p	-0.268	2.84e-05	FALSE
5740	hsa-miR-21	1.5	2.91e-05	TRUE
19015	hsa-miR-142-5p	1.14	3.03e-05	FALSE
11182	hsa-miR-98	0.837	3.12e-05	FALSE
11151	hsa-miR-516b	-0.265	3.17e-05	TRUE
17608	hsa-miR-425	0.753	3.35e-05	FALSE
17460	hsa-miR-657	-0.359	3.48e-05	TRUE
19580	hsa-let-7i	1.03	3.63e-05	TRUE
10997	hsa-miR-19a	1.35	3.67e-05	FALSE
28191	hsa-miR-30e	1.13	3.71e-05	FALSE
11104	hsa-miR-422a	0.423	3.78e-05	FALSE
42717	hsa-miR-92b*	-0.447	3.94e-05	TRUE
27565	hsa-miR-423-5p	-0.238	4.03e-05	TRUE
42929	hsa-miR-25*	-0.279	4.33e-05	TRUE
17445	hsa-miR-610	-0.352	4.94e-05	TRUE
11279	U6-snRNA-2	0.587	5.54e-05	TRUE
42532	hsa-miR-22*	0.455	5.73e-05	FALSE
19005	hsa_SNORD118	0.665	5.91e-05	TRUE
42738	hsa-miR-340*	-0.397	6.04e-05	TRUE

19602	hsa-let-7g	0.859	6.81e-05	FALSE
42831	hsa-miR-28-5p	0.953	7.32e-05	FALSE
31026	hsa-miR-101	1.01	7.48e-05	FALSE
19591	hsa-miR-199b-5p	1.04	7.51e-05	FALSE
42758	hsa-miR-640	-0.389	7.78e-05	TRUE
29460	hsa-miR-553	-0.249	7.94e-05	FALSE
17328	ebv-miR-BART8*	-0.465	7.99e-05	TRUE
42744	hsa-miR-23a	1.25	8.62e-05	TRUE
11040	hsa-miR-29b	1.17	8.91e-05	FALSE
42832	hsa-miR-638	-0.381	9.56e-05	TRUE
42570	hsa-miR-194*	-0.448	9.65e-05	TRUE
19604	hsa_SNORD4A	0.728	0.000105	TRUE
42795	kshv-miR-K12-3	-0.326	0.000108	TRUE
10962	hsa-miR-154	0.719	0.000127	FALSE
42902	hsa-miR-185	0.375	0.000129	TRUE
42754	hsa-miR-586	-0.31	0.000135	TRUE
42887	hsa-miR-331-3p	0.473	0.000139	TRUE
17561	ebv-miR-BART6-3p	-0.452	0.00014	TRUE
19585	hsa-miR-148b	0.757	0.000146	FALSE
17567	kshv-miR-K12-1	-0.206	0.00015	FALSE
42650	hsa-miR-17	1.13	0.000153	TRUE
32884	hsa-miR-342-3p	0.974	0.000162	FALSE
17358	ebv-miR-BART16	-0.345	0.000164	TRUE
19582	hsa-miR-106b	0.96	0.000167	TRUE
42652	hsa-miR-584	-0.438	0.000178	TRUE
42802	hsa-miR-150	-0.236	0.000187	TRUE
10928	hsa-miR-125a-5p	0.531	0.000189	TRUE
33430	hsa-miR-548b-3p	-0.307	0.000191	TRUE
42739	hsa-miR-339-5p	0.533	0.000192	TRUE
13485	hsa-miR-10a	0.882	0.000195	FALSE
13148	hsa-miR-195	0.892	2e-04	FALSE
11030	hsa-miR-26a	1.19	0.000203	FALSE
42693	hsa-miR-326	-0.414	0.000209	TRUE
10946	hsa-miR-141	1.08	0.000209	TRUE
17646	ebv-miR-BHRF1-3	-0.222	0.000211	TRUE
42648	hsa-miR-106a	1.16	0.000213	TRUE
42564	hsa-miR-26b	1.18	0.000237	FALSE
10925	hsa-miR-10b	0.781	0.00025	FALSE

42700	hsa-miR-631	-0.532	0.000254	FALSE
11024	hsa-miR-223	0.727	0.000273	FALSE
19581	hsa-miR-100	0.796	0.000273	FALSE
17280	hsa-miR-15b	1.06	0.000291	FALSE
42442	hsa-miR-498	-0.252	0.000325	FALSE
19008	hsa_SNORD2	0.48	0.000357	FALSE
27533	hsa-miR-320a	0.543	0.00036	FALSE
10919	hsa-miR-103	0.427	0.000361	FALSE
42528	hsa-miR-296-3p	-0.335	0.000372	FALSE
42609	hsa-miR-135a*	-0.295	0.000373	FALSE
42951	ebv-miR-BHRF1-2	-0.345	0.000398	FALSE
17506	hsa-miR-24	1.26	0.000411	FALSE
17718	hsa-miR-92b	0.547	0.000425	FALSE
29872	hsa-miR-340	0.338	0.000431	FALSE
28431	miRPlus_28431	-0.167	0.000436	FALSE
11053	hsa-miR-32	0.881	0.000448	FALSE
42603	hsa-miR-424*	-0.291	0.00045	FALSE
42965	hsa-miR-424	0.629	0.000518	FALSE
42529	hsa-miR-939	-0.229	0.00053	FALSE
19606	hsa_SNORD12	0.264	0.000534	FALSE
17952	miRPlus_17952	-0.231	0.000534	FALSE
42630	hsa-miR-140-3p	0.744	0.00056	FALSE
11027	hsa-miR-23b	1.14	0.000573	FALSE
42640	hsa-miR-20b	1.01	0.000582	FALSE
42649	hsa-miR-20a	0.995	0.000596	FALSE
11048	hsa-miR-30a	0.83	0.000605	FALSE
42679	hsa-miR-642	-0.29	0.000615	FALSE
42527	hsa-miR-935	-0.447	0.000622	FALSE
11134	hsa-miR-502-5p	-0.152	0.000651	FALSE
17613	hsa-miR-645	-0.346	0.000655	FALSE
42751	hsa-miR-720	0.501	0.000717	FALSE
11224	hsa-miR-30e*	0.687	0.000725	FALSE
17822	hsa-miR-490-5p	-0.363	0.000729	FALSE
42695	hsa-miR-596	-0.36	0.000743	FALSE
42486	hsa-miR-149*	-0.238	0.000744	FALSE
10978	hsa-miR-184	-0.21	0.000749	FALSE
11041	hsa-miR-29c	0.858	0.000763	FALSE
42782	hcmv-miR-UL148D	-0.303	0.00078	FALSE

10947	hsa-miR-142-3p	0.962	0.000794	FALSE
28302	miRPlus_28302	0.387	0.000857	FALSE
42573	hsa-miR-1	0.323	0.000871	FALSE
42899	hsa-miR-377*	-0.233	0.000896	FALSE
42845	hsa-miR-125b-2*	-0.206	0.00091	FALSE
17463	hsa-miR-151-3p	0.597	0.000956	FALSE
30787	hsa-miR-125b	0.753	0.000967	FALSE
17470	kshv-miR-K12-2	-0.399	0.001	FALSE
42812	hsa-miR-508-5p	-0.272	0.00106	FALSE
17493	hsa-miR-622	-0.358	0.0011	FALSE
42853	hsa-miR-433	-0.358	0.00116	FALSE
11175	hsa-miR-525-5p	-0.188	0.00116	FALSE

Table 2, The sequences of the mature microRNAs listed in Table 1

hsa-let-7f UGAGGUAGUAGAUUGUAUAGUU
hsa-miR-15a UAGCAGCACAUAAUGGUUUGUG
hsa-miR-16 UAGCAGCACGUAAAUAUUGGCG
hsa-miR-17 CAAAGUGCUUACAGUGCAGGUAG
hsa-miR-19a UGUGCAAAUCUAUGCAAAACUGA
hsa-miR-19b UGUGCAAAUCCAUGCAAAACUGA
hsa-miR-20a UAAAGUGCUUAUAGUGCAGGUAG
hsa-miR-21 UAGCUUAUCAGACUGAUGUUGA
hsa-miR-22 AAGCUGCCAGUUGAAGAACUGU
hsa-miR-23a AUCACAUUGCCAGGGAUUUCC
hsa-miR-24 UGGCUCAGUUCAGCAGGAACAG
hsa-miR-25 CAUUGCACUUGUCUCGGUCUGA
hsa-miR-26a UUCAAGUAAUCCAGGAUAGGCU
hsa-miR-26b UUCAAGUAAUUCAGGAUAGGU
hsa-miR-27a UUCACAGUGGCUAAGUUCCGC
hsa-miR-28-5p AAGGAGCUCACAGUCUAUUGAG
hsa-miR-30a UGUAAACAUCCUCGACUGGAAG
hsa-miR-32 UAUUGCACAUUACUAAGUUGCA
hsa-miR-93 CAAAGUGCUGUUCGUGCAGGUAG
hsa-miR-98 UGAGGUAGUAAGUUGUAUUGUU
hsa-miR-100 AACCCGUAGAUCCGAACUUGUG

hsa-miR-101 UACAGUACUGUGAUAACUGAA
hsa-miR-29b UAGCACCAUUUGAAAUCAGUGUU
hsa-miR-103 AGCAGCAUUGUACAGGGCUAUGA
hsa-miR-105 UCAAAUGCUCAGACUCCUGUGGU
hsa-miR-106a AAAAGUGCUUACAGUGCAGGUAG
hsa-miR-199a-5p CCCAGUGUUCAGACUACCUGUUC
hsa-miR-129-5p CUUUUUGCGGUCUGGGCUUGC
hsa-miR-10a UACCCUGUAGAUCCGAAUUUGUG
hsa-miR-10b UACCCUGUAGAACCGAAUUUGUG
hsa-miR-181a AACAUUCAACGCUGUCGGUGAGU
hsa-miR-181b AACAUUCAUUGCUGUCGGUGGGU
hsa-miR-199b-5p CCCAGUGUUUAGACUAUCUGUUC
hsa-miR-221 AGCUACAUUGUCUGCUGGGUUUC
hsa-miR-222 AGCUACAUCUGGCUACUGGGU
hsa-miR-223 UGUCAGUUUGUCAAAUACCCCA
hsa-let-7g UGAGGUAGUAGUUUGUACAGUU
hsa-let-7i UGAGGUAGUAGUUUGUGCUGUU
hsa-miR-1 UGGAAUGUAAAGAAGUAUGUAU
hsa-miR-15b UAGCAGCACAUCAUGGUUUACA
hsa-miR-23b AUCACAUUGCCAGGGAUUACC
hsa-miR-27b UUCACAGUGGCUAAGUUCUGC
hsa-miR-125b UCCCUGAGACCCUAACUUGUGA
hsa-miR-130a CAGUGCAAUGUUAAAAGGGCAU
hsa-miR-140-5p CAGUGGUUUUACCCUAUGGUAG
hsa-miR-140-3p UACCACAGGGUAGAACCACGG
hsa-miR-141 UAACACUGUCUGGUAAAGAUGG
hsa-miR-142-5p CAUAAAGUAGAAAGCACUACU
hsa-miR-142-3p UGUAGUGUUUCCUACUUUAUGGA
hsa-miR-191 CAACGGAAUCCCAAAAGCAGCUG
hsa-miR-125a-5p UCCCUGAGACCCUUUAACCUGUGA
hsa-miR-126 UCGUACCGUGAGUAAUAAUGCG
hsa-miR-150 UCUCCCAACCCUUGUACCAGUG
hsa-miR-154 UAGGUUAUCCGUGUUGCCUUCG
hsa-miR-184 UGGACGGAGAACUGAUAAGGGU

hsa-miR-185 UGGAGAGAAAGGCAGUUCCUGA
hsa-miR-193a-3p AACUGGCCUACAAAGUCCCAGU
hsa-miR-195 UAGCAGCACAGAAAUAUUGGC
hsa-miR-320a AAAAGCUGGGUUGAGAGGGCGA
hsa-miR-106b UAAAGUGCUGACAGUGCAGAU
hsa-miR-29c UAGCACCAUUUGAAAUCGGUUA
hsa-miR-219-2-3p AGAAUUGUGGCUGGACAUCUGU
hsa-miR-301a CAGUGCAAUAGUAUUGUCAAAGC
hsa-miR-296-3p GAGGGUUGGGUGGAGGCUCUCC
hsa-miR-30e UGUAAACAUCCUUGACUGGAAG
hsa-miR-365 UAAUGCCCCUAAAAAUCCUUAU
hsa-miR-374a UUAUAAUACAACCUGAUAAGUG
hsa-miR-340 UUAUAAAGCAAUGAGACUGAUU
hsa-miR-342-3p UCUCACACAGAAAUCGCACCCGU
hsa-miR-323-3p CACAUUACACGGUCGACCUCU
hsa-miR-326 CCUCUGGGCCCUUCCUCCAG
hsa-miR-151-3p CUAGACUGAAGCUCCUUGAGG
hsa-miR-148b UCAGUGCAUCACAGAACUUUGU
hsa-miR-331-3p GCCCCUGGGCCUAUCCUAGAA
hsa-miR-339-5p UCCCUGUCCUCCAGGAGCUCACG
hsa-miR-335 UCAAGAGCAAUAACGAAAAAUGU
ebv-miR-BHRF1-2 UAUCUUUUGCGGCAGAAAUUGA
ebv-miR-BHRF1-3 UAACGGGAAGUGUGUAAGCACA
ebv-miR-BART1-5p UCUUAGUGGAAGUGACGUGCUGUG
hsa-miR-422a ACUGGACUUAGGGUCAGAAGGC
hsa-miR-423-5p UGAGGGGCAGAGAGCGAGACUUU
hsa-miR-424 CAGCAGCAAUUCAUGUUUUGAA
hsa-miR-425 AAUGACACGAUCACUCCCGUUGA
hsa-miR-20b CAAAGUGCUCAUAGUGCAGGUAG
hcmv-miR-UL148D UCGUCCUCCCCUUCUUCACCG
hsa-miR-433 AUCAUGAUGGGCUCCUCGGUGU
kshv-miR-K12-1 AUUACAGGAAACUGGGUGUAAGC
kshv-miR-K12-2 AACUGUAGUCCGGGUCGAUCUG
kshv-miR-K12-3 UCACAUUCUGAGGACGGCAGCGA

hsa-miR-490-5p CCAUGGAUCUCCAGGUGGGU
hsa-miR-146b-5p UGAGAACUGAAUUCCAUAGGCU
hsa-miR-498 UUUCAAGCCAGGGGGCGUUUUUC
hsa-miR-525-5p CUCCAGAGGGAUGCACUUUCU
hsa-miR-516b AUCUGGAGGUAAGAAGCACUUU
hsa-miR-502-5p AUCCUUGCUAUCUGGGUGCUA
hsa-miR-508-5p UACUCCAGAGGGCGUCACUCAUG
hsa-miR-510 UACUCAGGAGAGUGGCAAUCAC
hsa-miR-553 AAAACGGUGAGAUUUUGUUUU
hsa-miR-92b UAUUGCACUCGUCCCGGCCUCC
hsa-miR-584 UUAUGGUUUGCCUGGGACUGAG
hsa-miR-586 UAUGCAUUGUAUUUUUAGGUCC
hsa-miR-548b-3p CAAGAACCUCAGUUGCUUUUGU
hsa-miR-596 AAGCCUGCCCGGCUCCUCGGG
hsa-miR-610 UGAGCUAAAUGUGUGCUGGGA
hsa-miR-612 GCUGGGCAGGGCUUCUGAGCUCCUU
hsa-miR-617 AGACUUCCCAUUUGAAGGUGGC
hsa-miR-622 ACAGUCUGCUGAGGUUGGAGC
hsa-miR-623 AUCCCUUGCAGGGGCUGUUGGGU
hsa-miR-630 AGUAUUCUGUACCAGGGAAGGU
hsa-miR-631 AGACCUGGCCCAGACCUCAGC
hsa-miR-638 AGGGAUCGCGGGCGGGUGGCGGCCU
hsa-miR-640 AUGAUCCAGGAACCUGCCUCU
hsa-miR-642 GUCCCUCUCCAAAUGUGUCUUG
hsa-miR-645 UCUAGGCUGGUACUGCUGA
hsa-miR-654-5p UGGUGGGCCGCAGAACAUGUGC
hsa-miR-657 GGCAGGUUCUCACCCUCUCUAGG
hsa-miR-542-5p UCGGGGAUCAUCAUGUCACGAGA
hcmv-miR-UL70-3p GGGGAUGGGCUGGCGCGCGG
ebv-miR-BART6-3p CGGGGAUCGGACUAGCCUUAGA
ebv-miR-BART13 UGUAACUUGCCAGGGACGGCUGA
ebv-miR-BART16 UUAGAUAGAGUGGGUGUGUGCUCU
hsa-miR-300 UAUACAAGGGCAGACUCUCUCU
hsa-miR-374b AUAUAAUACAACCUGCUAAGUG

hsa-miR-935 CCAGUUACCGCUUCCGCUACCGC
hsa-miR-939 UGGGGAGCUGAGGCUCUGGGGGUG
hiv1-miR-H1 CCAGGGAGGCGUGCCUGGGC
hsa-miR-720 UCUCGCUGGGGCCUCCA
hsa-miR-21* CAACACCAGUCGAUGGGCUGU
hsa-miR-22* AGUUCUUCAGUGGCAAGCUUUA
hsa-miR-25* AGGCGGAGACUUGGGCAAUUG
hsa-miR-200b* CAUCUUACUGGGCAGCAUUGGA
hsa-miR-30b* CUGGGAGGUGGAUGUUUACUUC
hsa-miR-135a* UAUAGGGAUUGGAGCCGUGGCG
hsa-miR-125b-2* UCACAAGUCAGGCUCUUGGGAC
hsa-miR-126* CAUUAUUACUUUUGGUACGCG
hsa-miR-149* AGGGAGGGACGGGGGCUGUGC
hsa-miR-194* CCAGUGGGGCUGCUGUUAUCUG
hsa-miR-30c-1* CUGGGAGAGGGUUGUUUACUCC
hsa-miR-30e* CUUUCAGUCGGAUGUUUACAGC
hsa-miR-377* AGAGGUUGCCCUUGGUGAAUUC
hsa-miR-340* UCCGUCUCAGUUACUUUAUAGC
hsa-miR-424* CAAAACGUGAGGCGCUGCUAU
hcmv-miR-US25-1* UCCGAACGCUAGGUCGGUUCUC
hsa-miR-519e* UUCUCCAAAAGGGAGCACUUUC
hsa-miR-92b* AGGGACGGGACGCGGUGCAGUG
ebv-miR-BART8* GUCACAAUCUAUGGGGUCGUAGA

OTHER EMBODIMENTS

While certain novel features of this invention shown and described are pointed out in the annexed claims, the invention is not intended to be limited to the details specified, since a person of ordinary skill in the relevant art will understand that various omissions, modifications, substitutions and changes in the forms and details of the invention illustrated and in its operation may be made without departing in any way from the spirit of the present invention. No feature of the invention is critical or essential unless it is expressly stated as being "critical" or "essential."

Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed in the scope of the present invention.

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

CLAIMS

1. A method for prognosing cancer relapse in a cancer patient comprising determining the level of expression of a biomarker having at least 85% sequence identity to the sequence of SEQ ID NO: 1 in a sample from the patient, wherein the level of expression of said biomarker is prognostic of cancer relapse in said patient.

2. The method of claim 1, wherein said biomarker comprises the sequence of SEQ ID NO: 1.

3. A method for prognosing cancer relapse in a cancer patient comprising determining the level of expression of a biomarker having at least 85% sequence identity to the sequence of SEQ ID NO: 2 in a sample from the patient, wherein the level of expression of said biomarker is prognostic of cancer relapse in said patient.

4. The method of claim 3, wherein said biomarker comprises the sequence of SEQ ID NO: 2.

5. A method for prognosing cancer relapse in a cancer patient comprising determining the level of expression of a biomarker having at least 85% sequence identity to the sequence of SEQ ID NO: 3 in a sample from the patient, wherein the level of expression of said biomarker is prognostic of cancer relapse in said patient.

6. The method of claim 5, wherein said biomarker comprises the sequence of SEQ ID NO: 3.

7. A method for prognosing cancer relapse in a cancer patient comprising determining the level of expression of at least one biomarker having at least 85% sequence identity to the sequence of any one of SEQ ID NOs: 1 to 3 in a sample from the patient, wherein the level of expression of said biomarker is prognostic of cancer relapse in said patient.

8. The method of claim 7, wherein said biomarker comprises the sequence of any one of SEQ ID NOs: 1 to 3.

9. The method of any one of claims 1 to 8, further comprising determining the level of expression of a biomarker having at least 85% sequence identity to the sequence of SEQ ID NO: 4.

10. The method of any one of claims 1 to 9, further comprising determining the level of expression of a biomarker having the sequence of SEQ ID NO: 4.

11. The method of any one of claims 1 to 10, wherein the sample is a tissue sample.

12. The method of claim 11, wherein the sample is a tumor sample.

13. The method of any one of claims 1 to 12, wherein the cancer is a lung cancer.

14. The method of claim 13, wherein the lung cancer is a non-small cell lung cancer.

15. The method of any one of claims 1 to 14, wherein the prognosis occurs in said patient after a first cancer treatment.

16. The method of any one of claims 1 to 14, wherein the prognosis occurs in said patient prior to a first cancer treatment.

17. The method of any one of claims 1 to 14, wherein the prognosis occurs in said patient after a first cancer treatment, but before a second cancer treatment.

18. The method of any one of claims 1 to 14, wherein the prognosis occurs in said patient after a second cancer treatment.

19. The method of any one of claims 15 to 18, wherein said treatment comprises one or more of surgery, radiation therapy, and chemotherapy.

20. The method of any one of claims 1 to 19, wherein an increase in the level of expression of said biomarker indicates a good prognosis of no cancer relapse, or wherein a decrease in the level of expression of said one or more biomarkers indicates a good prognosis of no cancer relapse.

21. The method of any one of claims 1 to 19, wherein an increase in the level of expression of said biomarker indicates a poor prognosis of cancer relapse, or wherein a decrease in the level of expression of said one or more biomarkers indicates a poor prognosis of cancer relapse.

22. The method of any one of claims 1 to 21, wherein the level of expression of said biomarker in said sample is determined by collecting nucleic acid molecules from said sample and, optionally, using a quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to amplify said nucleic acid molecules.

23. A device for detecting the level of expression of at least one biomarker comprising at least one single-stranded nucleic acid molecule having at least 85% sequence identity to the sequence of said biomarker or its complement sequence, wherein the sequence of said biomarker comprises at least 5 consecutive nucleotides of the sequence of SEQ ID NO: 1, and wherein the device allows specific hybridization between said single stranded nucleic acid molecule and said biomarker or its complement sequence, respectively.

24. The device of claim 23, wherein the at least one single-stranded nucleic acid molecule comprises at least 5 consecutive nucleotides of the sequence of SEQ ID NO: 1, or its complement sequence.

25. A device for detecting the level of expression of at least one biomarker comprising at least one single-stranded nucleic acid molecule having at least 85% sequence identity to the sequence of said biomarker or its complement sequence, wherein the sequence of said biomarker comprises at least 5 consecutive nucleotides of the sequence of SEQ ID NO: 2, and wherein the device allows specific hybridization between said single stranded nucleic acid molecule and said biomarker or its complement sequence, respectively.

26. The device of claim 25, wherein the at least one single-stranded nucleic acid molecule comprises at least 5 consecutive nucleotides of the sequence of SEQ ID NO: 2, or its complement sequence.

27. A device for detecting the level of expression of at least one biomarker comprising at least one single-stranded nucleic acid molecule having at least 85% sequence identity to the sequence of said biomarker or its complement sequence, wherein the sequence of said biomarker comprises at least 5 consecutive nucleotides of the sequence of SEQ ID NO: 3, and wherein the device allows specific hybridization between said single stranded nucleic acid molecule and said biomarker or its complement sequence, respectively.

28. The device of claim 27, wherein the at least one single-stranded nucleic acid molecule comprises at least 5 consecutive nucleotides of the sequence of SEQ ID NO: 3, or its complement sequence.

29. A device for detecting the level of expression of at least one biomarker comprising at least one single-stranded nucleic acid molecule having at least 85% sequence identity to the sequence of said biomarker or its complement sequence, wherein the sequence of said biomarker comprises at least 5 consecutive nucleotides of the sequence of any one of SEQ ID NOs:1 to 3, and wherein the device allows specific hybridization between said single stranded nucleic acid molecule and said biomarker or its complement sequence, respectively.

30. The device of claim 27, wherein the at least one single-stranded nucleic acid molecule comprises at least 5 consecutive nucleotides of the sequence of any one of SEQ ID NOs: 1 to 3, or its complement sequence.

31. The device of any one of claims 23 to 30, further comprising at least one single-stranded nucleic acid molecule having at least 85% sequence identity to the sequence of said biomarker or its complement sequence, wherein the sequence of said biomarker comprises at least 5 consecutive nucleotides of the sequence of SEQ ID NO: 4, and wherein the device allows specific hybridization between said single stranded nucleic acid molecule and said biomarker or its complement sequence, respectively.

32. The device of claim 31, wherein the at least one single-stranded nucleic acid molecule comprises at least 5 consecutive nucleotides of the sequence of SEQ ID NO: 4, or its complement sequence.

33. The device of any one of claims 23 to 32, wherein said at least one single-stranded nucleic acid molecule has a length in the range of 10 to 100 nucleotides.

34. The device of any one of claims 23 to 33, said device allowing, when contacted with a diverse population of nucleic acid molecules prepared from a sample under conditions allowing hybridisation to occur, the determination of the level of expression of said at least one biomarker.

35. The device of any one of claims 23 to 34, wherein the device is a microarray device.

36. A method for prognosing cancer relapse in a cancer patient comprising determining the level of expression of at least one biomarker in a patient sample using the device of any one of claims 23 to 35, wherein the level of expression of said biomarker is prognostic of cancer relapse in said patient.

37. The method of claim 36, wherein the sample is a tissue sample.

38. The method of claim 37, wherein the sample is a tumor sample.

39. The method of claim 36, wherein the cancer is a lung cancer.

40. The method of claim 39, wherein the cancer is a non-small cell lung cancer.

41. The method of any one of claims 36 to 40, wherein the prognosis occurs in said patient after a first cancer treatment.

42. The method of any one of claims 36 to 40, wherein the prognosis occurs in said patient prior to a first cancer treatment.

43. The method of any one of claims 36 to 40, wherein the prognosis occurs in said patient after a first cancer treatment, but before a second treatment.

44. The method of any one of claims 36 to 40, wherein the prognosis occurs in said patient after a second cancer treatment.

45. The method of any one of claims 41 to 44, wherein said treatment comprises any combination of one or more of surgery, radiation therapy, and chemotherapy.

46. The method of any one of claims 36 to 45, wherein an increase in the level of expression of said at least one biomarker indicates a good prognosis of no cancer relapse, or wherein a decrease in the level of expression of said at least one biomarker indicates a good prognosis of no cancer relapse.

47. The method of any one of claims 36 to 45, wherein an increase in the level of expression of said at least one biomarker indicates a poor prognosis of cancer relapse, or wherein a decrease in the level of expression of said at least one biomarker indicates a poor prognosis of cancer relapse.

48. A kit comprising reagents for collecting nucleic acid molecules from a sample from a patient, reagents for amplifying said nucleic acid molecules collected from said sample to produce an amplified sample, and at least one device for detecting the level of expression of at least one biomarker having the sequence of any one of SEQ ID NOs: 1 to 4 in said amplified sample.

49. The kit of claim 48, wherein a quantitative reverse transcription-polymerase chain reaction (qRT-PCR) is used to produce said amplified sample.

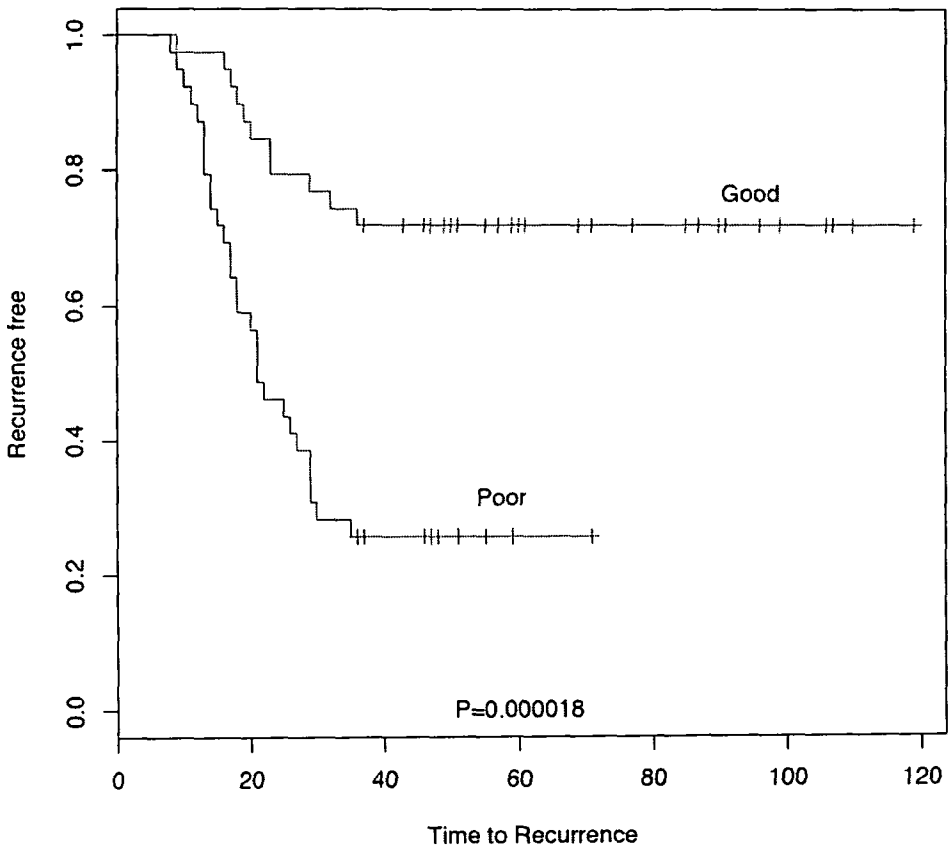
50. The kit of any one of claims 48 to 49, further comprising instructions for prognosing cancer relapse in said cancer patient based on the level of expression of said at least one biomarker.

51. The kit of any one of claims 48 to 50, wherein said device is the device of any one of claims 23 to 47.

52. The kit of claim 51 further comprising instructions for applying nucleic acid molecules collected from the sample to said device, and/or instructions for determining the level of expression of said at least one biomarker by detecting hybridization of said at least one single-stranded nucleic acid molecule to said biomarker or its complement sequence.

53. The kit of claim 52, further comprising instructions for prognosing cancer relapse in a cancer patient based on the level of expression of said at least one biomarker as detected using the device.

Figure 1


Recurrence free
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0.2
0.4
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1.0
Good
Poor
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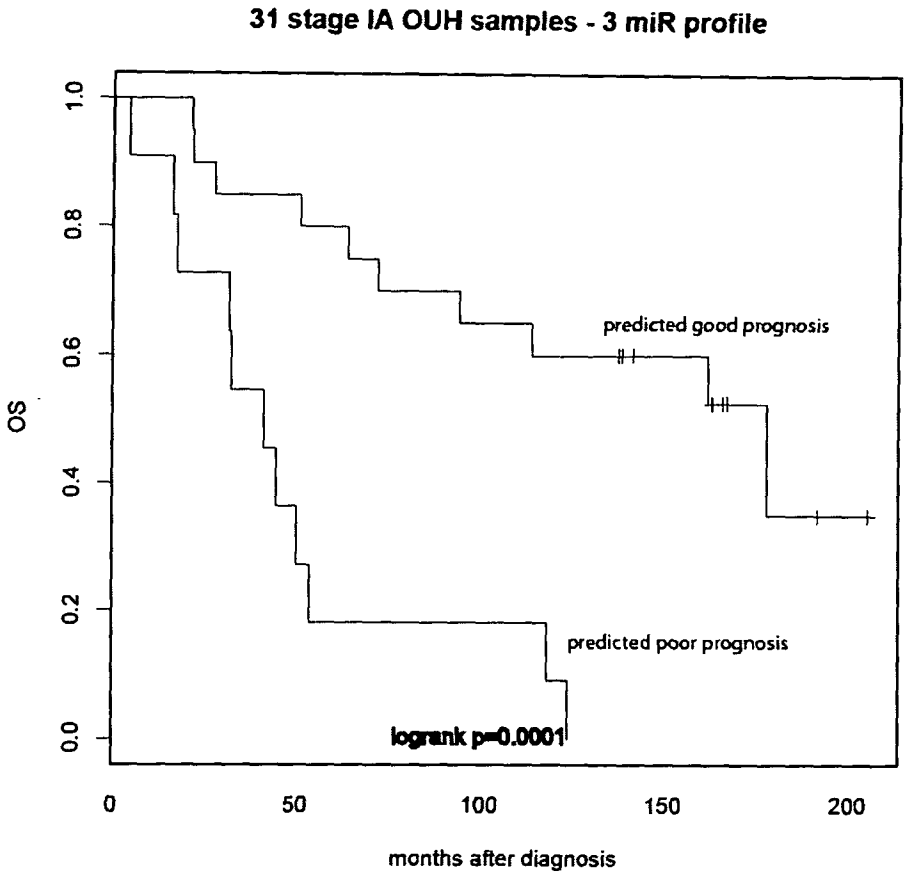
0
20
40
60
80
100
120
Time to Recurrence

Figure 2


31 stage IA OUH samples - 3 miR profile
OS
0.0
0.2
0.4
0.6
0.8
1.0
predicted good prognosis
predicted poor prognosis
logrank p=0.0001
0
50
100
150
200
months after diagnosis

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/002332

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CASTELLI E C ET AL: "In silico analysis of microRNAS targeting the HLA-G 3' untranslated region alleles and haplotypes", HUMAN IMMUNOLOGY, NEW YORK, NY, US, vol. 70, no. 12, 1 December 2009 (2009-12-01), pages 1020-1025, XP026750950, ISSN: 0198-8859, DOI: 10.1016/J.HUMIMM.2009.07.028 [retrieved on 2009-08-05] abstract ----- -/--	1,2, 7-24,29, 31-47

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 August 2012	08/11/2012

Name and mailing address of the ISA/	Authorized officer
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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GALLARDO ELENA ET AL: "miR-34a as a prognostic marker of relapse in surgically resected non-small-cell lung cancer.", CARCINOGENESIS NOV 2009 LNKD-PUBMED:19736307, vol. 30, no. 11, November 2009 (2009-11), pages 1903-1909, XP002680754, ISSN: 1460-2180	1,2, 7-12, 15-22, 36-47
A	abstract; figure 2	13,14, 23,24, 29,31-35

X	CN 102 002 490 A (SHANGHAI CANCER INST) 6 April 2011 (2011-04-06)	1,2, 7-12, 15-22, 36-47
A	abstract page 12	13,14, 23,24, 29,31-35

X,P	WO 2011/135459 A2 (MEDICAL PROGNOSIS INST A S [DK]; KNUDSEN STEEN [DK]) 3 November 2011 (2011-11-03) abstract page 53; claim 1; figure 1	23,24, 29,31-35

A	XU LING ET AL: "[Association of miRNAs expression profiles with prognosis and relapse in childhood acute lymphoblastic leukemia].", ZHONGHUA XUE YE XUE ZA ZHI = ZHONGHUA XUEYEXUE ZAZHI MAR 2011 LNKD-PUBMED:21535956, vol. 32, no. 3, March 2011 (2011-03), pages 178-181, XP009161486, ISSN: 0253-2727 abstract	1,2, 7-22,29, 31-47

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2012/002332

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1, 2, 23, 24(completely); 7-22, 29, 31-47(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1, 2, 23, 24(completely); 7-22, 29, 31-47(partially)

   subject matter of the above claims, as characterised by SEQ ID NO: 1

   ---

2. claims: 3, 4, 25, 26(completely); 7-22, 29, 31-47(partially)

   subject matter of the above claims, as characterised by SEQ ID NO: 2

   ---

3. claims: 5, 6, 27, 28, 30(completely); 7-22, 29, 31-47(partially)

   subject matter of the above claims, as characterised by SEQ ID NO: 3

   ---

4. claims: 48-53

   subject matter of the above claims.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/002332

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CN 102002490 A	06-04-2011	NONE	
WO 2011135459 A2	03-11-2011	NONE	