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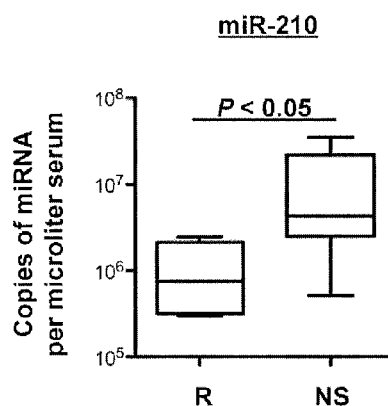
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- as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))

[Continued on next page]

(54) Title: USE OF BLOOD *MIR-210* FOR CANCER PROGNOSIS

Figure 2A



(57) Abstract: A method of prognosing cancer during anti-angiogenic therapy by measuring *miR-210* levels in the blood is provided. The prognosis is based on changes in levels of *miR-210* that result from the therapy. This method also provides a means of predicting the outcome of anti-angiogenic therapy.

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USE OF BLOOD *MIR-210* FOR CANCER PROGNOSIS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 61/353,414, filed June 10, 2010, the disclosure of which is incorporated herein by reference in its entirety.

BACKGROUND

[0002] MicroRNAs (miRNA) are small RNAs, typically 22 nucleotides in length that influence gene expression networks by repressing target messenger RNAs via specific base-pairing interactions. Over-expression and silencing of specific miRNA have been described in a number of diseases, including cancer. The continuing discovery of mechanisms by which microRNA dysregulation contributes to cancer diagnosis and therapy and the identification of specific diagnostic biomarkers and therapeutic targets is needed to provide new and useful tools for improved management of disease.

[0003] Hypoxia was reported to induce overexpression of microRNA-210 (*miR-210*) in human cancer cell lines cultured in vitro (Kulshreshtha et al. 2007, Mol Cell Biol 27:1859-67), in tumors from breast cancer patients (Camps et al. 2008, Clin Cancer Res 14:1340-48); and in blood of pancreatic adenocarcinoma patients (Ho et al. 2009, J Clin Oncol 27:15s (Suppl)). Gee et al., 2010, Cancer 116:2148-58, reported that hsa-miR-210 was significantly correlated with other markers of hypoxia, and high levels of hsa-miR-210 were associated with locoregional disease recurrence. Further, cellular levels of *miR-210* were suggested to be useful in diagnosing cancer (Croce et al., U.S. Patent No. 7,670,840; Croce et al., US2008/0306006, and Croce et al. US2008/0306017).

[0004] Copending application PCT/US2009/044906, hereby incorporated by reference in its entirety, describes detection of extracellular microRNA in blood and its use to measure disease, including cancer. There is a need to apply this discovery in a clinical setting as a means to guide therapy.

SUMMARY

[0005] Applicant discovered using a murine cancer model that an increased level of *miR-210* in blood resulting from treatment by an anti-angiogenic therapeutic correlates with a decrease in the mass of the cancer, thereby providing a favorable prognosis.

[0006] One embodiment of the invention is a method of prognosing cancer comprising the steps of collecting at least one first blood sample from a subject with cancer; administering to the subject an anti-angiogenic treatment; collecting at least one second blood sample following said treatment; measuring levels of *miR-210* in the samples; comparing levels of *miR-210* before and after treatment; and providing a prognosis, wherein the prognosis is based on the difference in the levels of *miR-210* between the samples.

[0007] One aspect of the method, where the levels of *miR-210* change following the treatment, is to provide a favorable or unfavorable prognosis, depending on the direction of the change. Another aspect of the invention, where the levels of *miR-210* do not increase following the treatment, is to provide no change in the prognosis of the subject.

[0008] Another aspect of the method is where measuring levels of *miR-210* comprises the steps of extracting RNA from the first and second samples; and contacting said RNA with at least one nucleic acid probe to measure blood levels of *miR-210*. The RNA is preferably extracted from serum or plasma obtained from each sample, most preferably from serum. The levels of *miR-210* may be measured by quantitative PCR. The RNA may be contacted with a microarray comprising a multiplicity of single stranded oligonucleotides to measure blood levels of *miR-210*. A control RNA can be included in the measurement, either added to the samples prior to measurements, or chosen from among RNA known to be present in the samples.

[0009] Another aspect of the method is where a multiplicity of blood samples are used to determine a prognosis. The multiplicity of blood samples may be obtained from a multiplicity of subjects. The subjects can be ones having the same type of cancer, or may be healthy normal subjects. The method may preferably include calculating an average level of *miR-210* in the multiplicity of samples. In one instance, the method further comprises a step of comparing the level of *miR-210* in the first blood sample of the subject with cancer to the average level of *miR-210* in a normal subject, and a further step of providing a prognosis. Where the level of *miR-210* in the subject with cancer is statistically the same as the average value for a normal subject, the method provides a favorable prognosis for the subject when undergoing an anti-angiogenic treatment. Where the level of *miR-210* in the subject with cancer is statistically substantially higher than the average value for a normal subject, the method provides an unfavorable prognosis for an anti-angiogenic treatment. In another aspect, where the level of *miR-210* in the subject is statistically substantially higher than the average value for a normal subject indicating a maximal hypoxic response near a tipping

point, the method provides a favorable prognosis for response to an anti-angiogenic treatment.

[0010] Another aspect of the method involves collection of a multiplicity of samples that are collected from the subject with cancer at various times following anti-angiogenic treatment. The method further comprises comparing the level of *miR-210* in the first sample with the level of *miR-210* in each of the multiplicity of second samples. Where the levels of *miR-210* increase over time following anti-angiogenic treatment, the method provides a favorable prognosis. Where levels do not increase, the method provides an alternate treatment modality, preferably comprising administering a pro-angiogenic treatment and/or vascular-stabilizing agent, followed by administering a cytotoxic drug.

[0011] Another aspect of the method is where a blood sample is taken from the subject before treatment, and the level of *miR-210* in the pre-treatment sample is used to determine a prognosis for response to anti-angiogenic therapy. In one aspect of this method, if the level of *miR-210* in the pre-treatment sample is elevated, the elevated level denotes that the tumor has already adapted to hypoxia, and will be less sensitive to anti-angiogenic therapy. Such an indication of reduced sensitivity to anti-angiogenic therapy indicates a different initial therapeutic approach, for example, treatment with an agent that will render the cancer more sensitive to subsequent therapy with an anti-angiogenic agent, or with agents that increase tumor perfusion. Optionally, a decrease in *miR-210* levels following such sensitizing treatment may be used to indicate that the subject is ready to receive anti-angiogenic therapy. In another aspect of this method, a high level of *miR-210* in the pre-treatment sample indicates that the tumor has already maximally adapted to hypoxia and has reached a tipping point, and that anti-angiogenic therapy will lead to a favorable response.

[0012] Another aspect of the method uses a nucleic acid probe that is one or more single stranded nucleic acid molecules, preferably one or more nucleic acids that hybridize with the nucleic acid having the sequence of SEQ ID NO:1 or SEQ ID NO:2.

[0013] Another aspect of the method is where the prognosis is in the form of a report, preferably a result of a computer calculation, most preferably, a computer that causes the report to be produced in a tangible medium. Optionally, a computing device may be used as to store data to be configured into a prognosis in the form of a report. The report may be generated as the result of data indicative of, among other things *miR-210* levels in various samples, the data having been subject to a method of prognosing by the computing device, the result being displayed on a display.

[0014] Another aspect of the method is where, instead of blood, another bodily fluid is used, which fluid may be selected from the group consisting of urine, semen, seminal fluid, seminal plasma, prostatic fluid, pre-ejaculatory fluid (Cowper's fluid), pleural effusion, tears, saliva, sputum, sweat, biopsy, ascites, cerebrospinal fluid, amniotic fluid, lymph, marrow, cervical secretions, vaginal secretions, endometrial secretions, gastrointestinal secretions, bronchial secretions, breast secretions, ovarian cyst secretions, or fluid extracted from a tissue sample.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] **Figures 1A-F** shows levels of miRNA in blood from patients with prostate cancer.

[0016] Fig. 1A is a graph showing circulating miRNAs in sera pooled from patients with advanced prostate cancer or healthy donors by TLDA profiling. Inset: *mir-100*, *miR-141*, *miR-148a*, *miR-200a*, *miR-200c*, *miR-210*, *miR-222*, *miR-375*, and *miR-425-5p* met biomarker criterion of >5-fold change (unadjusted $P < 0.05$, student's t-test) in pooled sera from CaP patients relative to normal controls.

[0017] Figs. 1B-1F: The graphs in the upper panels show miRNA levels by TaqMan® miRNA qRT-PCR (P value assigned by Wilcoxon signed-rank test), miRNA copies/ μ l serum. Horizontal bars: mean value $\pm$ SEM of miRNA copies/ μ l serum for each group. The graphs in lower panels show receiver operating characteristic (ROC) curves. AUC, area under the curve; CaP, prostate cancer patient sera; FC, fold-change; N, normal sera (PSA_{neg}/DRE_{neg}).

[0018] **Figures 2A-E** show *miR-210* levels (copies per microliter serum) compared to *miR-141*, *miR-200a*, *miR-200c*, and *miR-375* for prostate cancer responders to therapy (i.e., in a period of declining PSA) or not responding (i.e., in a period of not changing or increasing PSA).

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Definitions

[0019] The terminology described herein is for the purpose of describing particular embodiments of the disclosure and is not intended to be limiting.

[0020] The singular forms "a", "an", and "the" as used herein include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to a composition containing "a compound" includes a mixture of two or more compounds. It should also be noted that the term "or" is generally employed in its sense including "and/or" unless the content clearly dictates otherwise.

[0021] As used herein, "microRNA" or "miRNA" means a small, noncoding RNA sequence of 5 to 40 nucleotides in length that can be detected in a biological specimen. Some microRNAs are derived from hairpin precursors processed, for example, by the enzyme DICER to a mature species, for example, about 18-28 nucleotides, preferably 21-23 nucleotides.

[0022] In the canonical pathway, complementary codes found in the miRNA can bind to corresponding mRNA. This process can lead to inhibition of protein translation or degradation of the mRNA itself. Non-canonical modes of action have yet to be identified.

[0023] "Biological fluid" or "body fluid" can be used interchangeably and refer to a fluid isolated from a mammal. Such fluids include, but are not limited to, blood fluid, a blood fluid fraction, serum, plasma, urine, saliva, lymph, tears, pleural effusion, mucus, ascitic fluid, respiratory secretions such as bronchial secretions, amniotic fluid, cerebrospinal fluid, breast secretions, ovarian cyst fluid, and fluid isolated from a tissue.

[0024] The term "biological sample" refers to all biological fluids and excretions isolated from any given subject. In the context of the invention such samples include, but are not limited to, blood and fractions thereof, blood serum, blood plasma, urine, excreta, semen, seminal fluid, seminal plasma, prostatic fluid, pre-ejaculatory fluid (Cowper's fluid), pleural effusion, tears, saliva, sputum, sweat, biopsy, ascites, cerebrospinal fluid, amniotic fluid, lymph, marrow, cervical secretions, vaginal secretions, endometrial secretions, gastrointestinal secretions, bronchial secretions, breast secretions, ovarian cyst secretions, hair, and tissue fluid samples.

[0025] "MicroRNA Variants" are common, for example, among different animal species. In addition, variation at the 5' and 3' ends of microRNAs are common, and can be the result of imprecise cleavage by enzymes such as DICER during maturation as well as non-templated addition following maturation. These variants demonstrate a scope of acceptable variation in the sequence of the microRNAs that does not impair function or the ability to detect the microRNA(s). Some simple 3' end modification (addition of one or a few nucleotides to the 3' end) may affect the ability to detect the miRNA by qRT-PCR directed

against a canonical species, but not by microarray. For example, we have observed single nucleotide additions to the *miR-210* miRNA of A, U, C or G, as well as additions of more than 1 nucleotide at the 3' end. As another example, we have observed 3' additions of A or U to the *miR-141* miRNA sequence and found that this influences the sensitivity of TaqMan® qRT-PCR in detecting the miRNA species.

[0026] The terms "polynucleotide", "oligonucleotide", or "nucleic acid" can be used interchangeably and refer to nucleotide sequences of any length, including DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a nucleotide sequence, for example by DNA or RNA polymerase, or by chemical reaction. Nucleic acids may be single stranded or double stranded, or may contain portions of both double and single stranded sequence. A single strand can provide a probe that hybridizes to a target sequence. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imparted before or after assembly of the nucleotide sequence. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications include, for example, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl. phosphonates, phosphotriesters, phosphoamidates, cabamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (e.g., nucleases, antibodies, signal peptides, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide(s). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping groups moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-

azido-ribose, carbocyclic sugar analogs, .alpha.-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S("thioate"), P(S)S ("dithioate"), "(O)NR₂ ("amidate"), P(O)R, P(O)OR, CO or CH₂ ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C). optionally containing an ether (--O--) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical.

[0027] An "anti-angiogenic" factor or an "angiogenic inhibitor" are compounds used to stop the growth of tumors and progression of cancers by limiting the pathologic formation of new blood vessels (angiogenesis). These compounds include bevacizumab, cetuximab, erlotinib, serofenib, sunitinib, temsirolimus, everolimus, lenalidomide, thalidomide, alitretinoin, imiquimod, polyphenon, carboxyamidotriazole, TNP-470, CM101, IFN-alpha, IL-12, platelet factor-4, suamin, SU5416, thrombospondin, VEGFR antagonists, soluble VEGFR-1, RP-1, angiopoietin-2, TSP-1, TSP-2, TIMP, CDA1, Meth-1, Meth-2, prothrombin, antithrombin III, VEG-1, SPARC, osteopontin, maspin, canstatin, proliferin-related protein, restin, antiostatic steroids plus heparin, cartilage-derived angiogenesis inhibitory factor, matrix metalloproteinase inhibitors, angiostatin, endostatin, rhendostatin, 2-methoxyestradiol, tecogalan, prolactin, linomide, and matrix metalloproteinase inhibitors such as batimastat, and marimastat. In addition, many standard chemotherapeutic agents used in the treatment of cancer also inhibit angiogenesis, as do ionizing radiation-based treatments and hormonal modulation therapies such as androgen deprivation therapy, and are therefore also "anti-angiogenic".

[0028] An "isolated" polynucleotide is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in its natural source. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from the specific nucleic acid molecule as it exists in natural cells.

[0029] "Complement" or "complementary" as used herein in reference to a nucleic acid sequence means Watson and Crick or Hoogsteen base pairing between nucleotides or nucleotide analogs.

[0030] "Percent (%) nucleic acid sequence identity" as used herein means the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in a nucleic acid sequence of interest, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software. When comparing DNA and RNA, thymine (T) and uracil (U) may be considered equivalent.

[0031] As used herein, "differential expression" means qualitative or quantitative differences in the expression pattern of one or more polynucleotides, including miRNA, in a body fluid, cell, or tissue. Expression of the one or more polynucleotides may be upregulated, resulting in an increased amount of transcripts, or downregulated, resulting in a decreased amount of transcripts. Expression of the one or more polynucleotides may be upregulated or downregulated in a particular state, such as a disease state, relative to a reference state, such as a normal state, thus permitting comparison of two or more states. The one or more polynucleotides may exhibit a pattern of expression in said body fluid, cell, or tissue that is detectable by standard techniques, including but not limited to expression arrays, quantitative reverse transcriptase PCR, northern analysis, and real-time PCR. Some of the polynucleotides may be expressed in one state but not another.

[0032] As used herein, "gene" includes any polynucleotide sequence or portion thereof with a functional role in encoding or transcribing a protein or regulating other gene expression or producing a non-coding RNA such as a microRNA. The gene may consist of all the nucleic acids responsible for encoding a functional protein or only a portion of the nucleic acids responsible for encoding or expressing a protein. The polynucleotide sequence may contain a genetic abnormality within exons, introns, initiation or termination regions, promoter sequences, other regulatory sequences or unique adjacent regions to the gene.

[0033] "Stringency" of hybridization reactions is readily determinable by one of ordinary skill in the art, and generally is an empirical calculation dependent upon probe length, washing temperature, and salt concentration. In general, longer probes can be annealed at higher temperatures, whereas shorter probes anneal well only at lower temperatures. Hybridization generally depends on the ability of denatured DNA or RNA to reanneal when complementary strands are present in an environment below their melting temperature. The higher the degree of desired homology between the probe and hybridizable

sequence, the higher the relative temperature that can be used. As a result, it follows that higher relative temperatures would tend to make the reaction conditions more stringent, while lower temperatures less so.

[0034] "Stringent conditions" or "high stringency conditions" are sequence dependent and can vary dependent upon circumstances. Stringent conditions can be selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m can be the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium. Stringent conditions include those in which the salt concentration is less than about 1.0 M sodium ion, such as about 0.01-1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., about 10-50 nucleotides) and at least about 60°C for long probes (e.g., greater than about 50 nucleotides). Stringent conditions can also be achieved with the addition of destabilizing agents such as formamide. Exemplary stringent condition include those that: (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C; (2) employ during hybridization a denaturing agent, such as formamide, for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C; or (3) overnight hybridization in a solution that employs 50% formamide, 5xSSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5x Denhardt's solution, sonicated salmon sperm DNA (50 pg/ml), 0.1 % SDS, and 10% dextran sulfate at 42°C, with a 10 minute wash at 42°C in 0.2x SSC (sodium chloride/sodium citrate) followed by a 10 minute high-stringency wash consisting of 0.1x SSC containing EDTA at 55°C.

[0035] "Moderately stringent conditions" may be identified as described by Sambrook et al., *Molecular Cloning: A Laboratory Manual*, New York: Cold Spring Harbor Press, 1989, and include the use of washing solution and hybridization conditions (e.g., temperature, ionic strength and % SDS) less stringent than those described above. One example of moderately stringent conditions is overnight incubation at 37°C in a solution comprising: 20% formamide, 5x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 mg/ml denatured sheared salmon sperm DNA, followed by washing the filters in 1 x SSC at about

37-50°C. The skilled artisan will recognize how to adjust the temperature, ionic strength, etc. as necessary to accommodate factors such as probe length and the like.

[0036] The term "cancer" refers to or describes the physiological condition in animals, including humans, that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer (e.g., epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, cancer of the urinary tract, hepatoma, breast cancer, colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, melanoma, multiple myeloma and B-cell lymphoma, brain, as well as head and neck cancer, and associated metastases.

[0037] "Tumor", as used herein, refers to malignant neoplastic cell growth and proliferation, and all pre-cancerous and cancerous cells and tissues.

[0038] "Carriers" as used herein include pharmaceutically acceptable carriers, excipients, or stabilizers that are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN™, polyethylene glycol (PEG), and PLURONICS™.

[0039] "Treatment" is an intervention performed with the intention of preventing the development or altering the pathology of a disease or disorder. Accordingly, "treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disease or disorder as well as those in which the

disease or disorder is to be prevented. In tumor (e.g., cancer) treatment, a therapeutic agent may directly decrease the pathology of tumor cells, *or* render the tumor cells more susceptible to treatment by other therapeutic agents, e.g., radiation and/or chemotherapy.

Modes for Carrying Out the Invention

[0040] MicroRNAs (miRNAs) are small, noncoding RNAs that influence gene regulatory networks by post-transcriptional regulation of specific messenger RNA targets. MicroRNA expression is dysregulated in human malignancies, frequently leading to overexpression or loss of expression of certain microRNAs.

MiRNAs associated with specific cancer include those listed in Table 1:

Table 1

miRNA
<i>miR-100</i>
<i>miR-141</i>
<i>miR-148a</i>
<i>miR-200a</i>
<i>miR-200c</i>
<i>miR-210</i>
<i>miR-222</i>
<i>miR-375</i>
<i>miR-425-5p</i>

[0041] Specifically, human *miR-210* (hsa-*miR-210*) has the symbol HGNC:MIR210 and the precursor sequence:

ACCCGGCAGUGCCUCCAGGCGCAGGGCAGCCCCUGCCCACCGCACACUGCGCUGCCCCAGACC
CACUGUGCGUGUGACAGCGGCUGAUCUGUGCCUGGGCAGCGCGACCC .
(SEQ ID NO: 1)

The mature sequence, hsa-*miR-210*, has the following structure

CUGUGCGUGUGACAGCGGCUGA
(SEQ ID NO: 2)

[0042] The precursor sequence of hsa-*miR-210* has the following stem-loop structure (in which bold nucleotides indicate the segment that is selected during processing to yield the mature molecule, hsa-*miR-210*):

```

5'  accc  ca   -c          gg      c   cc   -      c   c   -
      gg  gugc  uccagggcgag  cagcc  cug  cac  cgcaca  ug  g  cugc
      ||  ||||  ||||| |||||  |||||  |||  |||  |||||  ||  |  |||  c
      cc  cgcg  ggguccguguc  gucgg  gac  gug  gcgugu  ac  c  gacc
3'  ---c  ag   ac          ua      c   -a   u      c   c   a

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Hsa-*miR-210* occurs chromosomally at 11: 568089-568198.

[0043] MicroRNAs are also involved in the development and function of the cardiovascular system. For example, specific miRNAs have been implicated in vascular angiogenesis and cardiomyocyte apoptosis, and also in the development of cardiac hypertrophy, arrhythmia, and heart failure.

[0044] The sequences in Table 2 are sequences of mature forms of the miRNAs listed. DNA or RNA corresponding to the reverse complement of these could be used as probes to measure miRNAs in the blood.

Table 2

SEQ ID NOS:	Name	Type	Species	Sequence
3	<i>miR-100</i>	RNA	Human	aacccguagauccgaacuugug
4	<i>miR-141</i>	RNA	Human	uaacacugucugguaaagaugg
5	<i>miR-148a</i>	RNA	Human	ucagugcaucacagaacuugu
6	<i>miR-200a</i>	RNA	Human	uaacacugucugguaacgaugu
7	<i>miR-200c</i>	RNA	Human	uaauacugccggguaaugauga
8	<i>miR-210</i>	RNA	Human	cugugcgugacagcggcuga
9	<i>miR-222</i>	RNA	Human	agcuacaucuggcuacugggu
10	<i>miR-375</i>	RNA	Human	uuuguucguucggcucgcguga

[0045] As is shown in the Examples, blood levels of *mir-100*, *miR-141*, *miR-148a*, *miR-200a*, *miR-200c*, *miR-210*, *miR-222*, *miR-375*, and *miR-425-5p* correlated incidence of cancer, and levels of *mir-210* correlated with successful anti-cancer therapy that resulted from the use of treatments that have anti-angiogenic effects. As such, one or more of these miRNAs can be useful as a guide for anti-angiogenic therapy (including hormonal therapy, chemotherapy, or radiation therapy, all of, which have been shown to function through anti-angiogenic effects). Further, levels of *mir-100*, *miR-141*, *miR-148a*, *miR-200a*, *miR-200c*, *miR-210*, *miR-222*, *miR-375*, or *miR-425-5p* can be useful in predicting non-responsiveness.

miRNA Variation

[0046] Mature miRNAs are described herein as useful biomarkers having specific nucleotide sequences. The sequence of each biomarker can also be varied, for example, due to genetic variation between individuals, *i.e.*, SNPs, known to exist for some miRNAs and more likely to be discovered. MicroRNA variation can result from changes due to RNA editing.

[0047] MicroRNA variants can have up to 3 substituted or deleted nucleotides at one or both of the 5' and 3' ends of the miRNA. MicroRNA sequences can vary at the 5' and 3' end (though more commonly at the 3' end), due to various factors, including imprecise cleavage by enzymes such as the Dicer enzyme during miRNA maturation, as well as non-templated addition of nucleotides.

[0048] A variant of *mir-100*, *miR-141*, *miR-148a*, *miR-200a*, *miR-200c*, *miR-210*, *miR-222*, *miR-375*, or *miR-425-5p* as referred to herein preferably has a sequence with at least 80%, 85%, 90% 95%, or more identity with mature sequence of *mir-100*, *miR-141*, *miR-148a*, *miR-200a*, *miR-200c*, *miR-210*, *miR-222*, *miR-375*, or *miR-425-5p*.

Methods of Detecting and Identifying microRNAs

[0049] The disclosure provides methods for detecting and identifying extracellular miRNA, for example, in a body fluid. In one example, miRNA is isolated from a body fluid such as serum or plasma, the isolated miRNA is converted to cDNA, and amplified.

[0050] Generally, microRNA can be detected by various methods known to detect mRNA, including northern blotting, ribonuclease protection assay (RPA), reverse transcription polymerase chain reaction (RT-PCR), and *in situ* hybridization (ISH). One new method for detecting mRNA is QuantiGene® (Panomics, Fremont, CA), where RNA can be quantitated directly from whole blood lysates or paraffin-embedded (FFPE) tissue homogenates (Warrior *et al.* 2000, *J. Biomol. Screen.* 5:343-52). With QuantiGene®, no RNA purification, no reverse transcription, and no target amplification is required. New methods and systems for non-PCR-based RNA detection that does not require purification of the RNA is available, for example, from Gen-Probe, Inc. Third Wave Technologies also has described a miRNA detection technology that does not involve PCR, and that can be performed directly in lysates of a biological samples.

[0051] Kits for isolating RNA, and in particular miRNA, from a biological sample are known and commercially available, such as the *mirVana*™ miRNA isolation kits (Ambion, Austin, TX). However, not all RNA isolation methods retain the small RNA fraction. The method for isolating RNA from a body fluid should be adapted to retain the small RNA fraction, for example, less than 40 nucleotides. Examples of suitable kits include, but are not limited to, the *mirVana*™ PARIS™ and *mirVana*™ miRNA isolation kits (Ambion, Austin, TX). Preferably, the miRNA isolation method retains the small RNA fraction in a background of total RNA or as an enriched fraction of RNA species. In specific

embodiments, the retained RNA species are 200 nucleotides or smaller, 150 nucleotides or smaller, 100 nucleotides or smaller, 50 nucleotides or smaller, 40 nucleotides or smaller, 30 nucleotides or smaller, 25 nucleotides or smaller.

[0052] cDNA can be generated by reverse transcription of isolated miRNA using reverse transcription conventional techniques. miRNA reverse transcription kits are known and commercially available. Examples of suitable kits include, but are not limited to, *mirVana*TM TaqMan® miRNA transcription kit (Ambion, Austin, TX) and the TaqMan® miRNA transcription kit (Applied Biosystems, Foster City, CA). Specific primers, including miRNA-specific stem-loop primers, are known and commercially available, for example, from Applied Biosystems (Foster City, Ca), Ambion (Austin, TX), and Qiagen (Valencia, CA).

[0053] The reverse transcript of the miRNA can be amplified using conventional PCR techniques including, but not limited to, real time PCR. Kits for quantitative real time PCR of RNA and miRNA are known and commercially available. Examples of suitable kits include, but are not limited to, the TaqMan® MicroRNA Assay (Applied Biosystems, Foster City, CA) and the *mirVana*TM qRT-PCR miRNA Detection Kit (Ambion, Austin, TX). One example of a suitable primer set is the *mirVana*TM qRT-PCR primer set (Ambion, Austin, TX). The RNA can be ligated to a single stranded oligonucleotide containing universal primer sequences, a polyadenylated sequence, or adaptor sequence prior to reverse transcriptase and amplified using a primer complementary to the universal primer sequence, poly(T) primer, or primer comprising a sequence that is complementary to the adaptor sequence.

[0054] No established endogenous small RNA control is known for normalization of technical variations in sample processing or of potential variation in sample quality. One example of variation in sample quality of a body fluid, such as plasma, may be the presence of PCR inhibitors due to occult red blood cell lysis in plasma samples. Normalizing by matching the amount of input RNA into the reverse transcription reaction is not an appropriate approach because the RNA content of the body fluid can vary considerably and may vary with disease states. Therefore, a fixed volume of RNA eluate from a given volume of starting body fluid, rather than a fixed mass of RNA, can be used as input into the reverse transcription reaction. For example, for a sample in which the starting body fluid volume was 400 p1, an input of 1.67 µl of eluted RNA (taken from a total RNA eluate volume of

approximately 80.4 p1) into the reverse transcription reaction corresponds to the mass of RNA derived from approximately 8.3 µl of starting body fluid.

[0055] The data can be normalized by spiking-in one or more RNA oligonucleotide that is sufficiently distinct from the sequences normally present in the sample, for example, an miRNA obtained from a non-human species where an identical or similar human species miRNA does not exist or a synthetic sequence designed to be distinct in the sample. Any sequence that is sufficiently distinct from the sequences to be measured, but which can be measured by similar assays, is a viable candidate for a spike-in control sequence. RNAs that do not cross hybridize with probes for known human miRNA, such as *C. elegans* miRNAs *cel-miR-39*, *cel-miR-54*, and *cel-miR-238*, are spiked-in after addition of the denaturing solution to the body fluid to avoid degradation by endogenous plasma RNases. For each RNA sample, the spiked-in miRNAs can be measured using, for example, TaqMan® qRT-PCR assays.

[0056] The data can be normalized across samples using a median normalization procedure. For each sample, the Ct values obtained for the three spiked-in miRNAs are averaged to generate SpikeIn_Average_Ct value. The median of the SpikeIn_Average_Ct values obtained from all the samples to be compared is calculated (designated here as the Median_SpikeIn_Ct value). A Normalization_Factor is then calculated for each sample based on the following formula: $\text{Normalization_Factor} = 1/[2^{(\text{Median_SpikeIn_Ct value}) - (\text{SpikeIn_Average_Ct value of the given sample})}]$.

[0057] The number of copies of a given miRNA in each sample, calculated using a standard curve, can be multiplied by the Normalization_Factor corresponding to the sample to obtain a normalized copy number value. In cases where it is desirable to apply normalization directly to Raw_Ct values corresponding to miRNAs of interest the Raw_Ct for a given miRNA in a given sample can be adjusted as follows: $\text{Normalized-Ct value for the miRNA in the sample} = \text{Raw_Ct value} - [(\text{SpikeIn_Average_Ct value of the given sample}) - (\text{Median_SpikeIn_Ct value})]$.

[0058] In some instances, novel miRNAs may require the development of custom qRT-PCR assays for their measurement. Custom qRT-PCR assays to measure novel micro miRNAs in a body fluid can be developed using a robust method that involves an extended reverse transcription primer and locked nucleic acid modified PCR (Raymond *et al.*, 2005, *RNA* 11(11):1737-44). Although several qRT-PCR methods have been reported for measuring miRNAs, extended reverse transcription primer and locked nucleic acid modified

PCR is likely to provide the greatest specificity. Custom miRNA assays can be tested by running it on a dilution series of chemically synthesized miRNA corresponding to the target sequence. This permits determination of the limit of detection and linear range of quantitation of each assay. Furthermore, when used as a standard curve, these data permit an estimate of the absolute abundance of endogenous miRNAs measured in body fluid samples.

[0059] A body fluid sample, such as blood, serum, or plasma, can be obtained from a single individual or pooled, for example, from a group of individuals suffering from a particular disease or disorder. MicroRNA can be isolated from the sample by any number of methods, for example, as described herein, and the abundance of one or more microRNA can be determined by any of a number of methods, for example by calculating average Ct values. Data for each candidate miRNA in a given sample can be normalized by subtracting the Reference Ct for that sample. Normalized data can be represented by a Δ Ct value (Normalized = Average Ct of the miRNA assayed - Reference Ct). Given that Ct values are on a $\log_2$ scale, the value $2^{\Delta Ct}$ represents linear scale expression values that can be compared directly for subsequent statistical analyses.

[0060] Amplification curves are checked to verify that Ct values are assessed in the linear range of each amplification plot. Typically, the linear range spans several orders of magnitude. For each candidate biomarker miRNA assayed, whether known or novel, a chemically synthesized version of the miRNA can be obtained and analyzed in a dilution series to determine the limit of sensitivity of the assay, the linear range of quantitation, and to estimate the absolute abundance of the candidate miRNAs measured.

Prognosis

[0061] The methods described herein provide a rapid and non-invasive assay for prognosing the outcome of a cancer treatment in an individual.

[0062] "Prognosis" as used herein refers to the probable outcome of a disease, preferably when a patient is diagnosed as having a tumor, and, more preferably, when the patient is diagnosed as having a cancer. The prognosis of a cancer includes the probable outcomes of using a treatment modality, preferably when the treatment involves use of angiogenesis therapeutic drug, most preferably when the treatment involves use of an anti-angiogenic therapeutic drug. The prognosis can include an outcome in which the cancer is refractory to a possible treatment modality, preferably where the treatment modality will improve the prospects for recovery, increase the chances of survival, reduce the recovery

period for the cancer, or minimize the probability of recurrence of the cancer. Most preferably the method of prognosis will identify a suitable treatment modality to improve the probability of a favorable outcome for the patient. A favorable outcome is one in which the cancer patient has at least a 70% chance, and preferably an 80% chance that the cancer will not recur or metastasize within 2, 3, 4, 5, 6, 7, 8, 9, 10 or more years from beginning a therapeutic regime.

[0063] The treatment regime is related to the level of *mir-100*, *miR-141*, *miR-148a*, *miR-200a*, *miR-200c*, *miR-210*, *miR-222*, *miR-375*, or *miR-425-5p* in the blood, serum or bodily fluid of the patient.

- In a first instance, one can observe that treatment causes a change in blood *mir-100*, *miR-141*, *miR-148a*, *miR-200a*, *miR-200c*, *miR-210*, *miR-222*, *miR-375*, or *miR-425-5p* levels in cancer patient. In that instance, the prognosis and the outcome of the treatment is favorable or unfavorable, depending on the direction of the change.
- In another instance, anti-angiogenic treatment does not cause a change in levels of *mir-100*, *miR-141*, *miR-148a*, *miR-200a*, *miR-200c*, *miR-210*, *miR-222*, *miR-375*, or *miR-425-5p*. In this instance, the prognosis is not favorable, because the cancer may be refractory to treatment.
- In another instance, a cancer patient presents with high levels of blood *mir-100*, *miR-141*, *miR-148a*, *miR-200a*, *miR-200c*, *miR-210*, *miR-222*, *miR-375*, or *miR-425-5p* and is refractory to an anti-angiogenic therapy. In this instance, the patient should be switched to a pro-angiogenic drug to stabilize the blood supply to the cancer and then to treat with a cytotoxic agent, e.g., a chemotherapy.
- In another instance, a cancer patient presents with a low level of blood *mir-100*, *miR-141*, *miR-148a*, *miR-200a*, *miR-200c*, *miR-210*, *miR-222*, *miR-375*, or *miR-425-5p*. This patient likely will have a favorable prognosis when an anti-angiogenic drug is administered.

[0064] Whether a level of *mir-100*, *miR-141*, *miR-148a*, *miR-200a*, *miR-200c*, *miR-210*, *miR-222*, *miR-375*, or *miR-425-5p* is high for a specific cancer may be determined by the ordinary skilled worker from a clinical trial conducted with that cancer. In one instance, whether a blood level of *mir-100*, *miR-141*, *miR-148a*, *miR-200a*, *miR-200c*, *miR-210*, *miR-222*, *miR-375*, or *miR-425-5p* is low or high depends from a standard value which is

predetermined for the specific cancer. In another instance, blood levels of *miR-210* are low or high relative to an average value obtained from normal, healthy subjects. In another instance, whether blood levels of *mir-100*, *miR-141*, *miR-148a*, *miR-200a*, *miR-200c*, *miR-210*, *miR-222*, *miR-375*, or *miR-425-5p* are low depends on an average value obtained from different patients with the same cancer. In another instance, the reference value is a median obtained by factoring the average value from similar cancer patients. The level of *miR-210* may be normalized by comparing it to the level of a control gene. The skilled person can realize that the reference value may be chosen depending on factors as the normalization method and the control values used.

[0065] In general, the methods include determining the relative amount of *miR-210* in an extracellular sample, for example, body fluid obtained from an individual. The amount of *miR-210* in a body fluid can be compared to a control, for example a matched sample of normal body fluid, a previously analyzed sample, or a suitable standard control developed for the particular assay.

Diagnostic Kits

[0066] Diagnostic kits adapted for the determination of microRNA biomarkers and diagnoses of disease are provided herein. Such kits may include materials and reagents adapted to specifically determine the presence and/or amount of a microRNA biomarker or group of miRNA biomarkers selected to be diagnostic of disease in a sample of body fluid. The kit can include nucleic acid molecules or probes in a form suitable for the detection of said miRNA biomarkers. The nucleic acid molecules can be in any composition suitable for the use of the nucleic acid molecules according to the instructions. The kit can include a detection component, such as a microarray, a labeling system, a cocktail of components (e.g., suspensions required for any type of PCR, especially real-time quantitative RT-PCR), membranes, color-coded beads, columns and the like. Furthermore, the kit can include a container, pack, kit or dispenser together with instructions for use.

[0067] A diagnostic kit may contain, for example, forward and reverse primers designed to amplify and detect the microRNA in body fluid. Many different PCR primers can be designed and adapted as necessary to amplify one or more miRNA that are differentially expressed in a body fluid and correlate to a particular disease or disorder. In one embodiment, the primers are designed to amplify a miRNA or group of miRNA that are differentially expressed in a body fluid of an individual having cancer or at risk of developing

cancer. The diagnostic kit may also contain single stranded oligonucleotide containing universal primer sequences, polyadenylated sequences, or adaptor sequences prior and a primer complementary to said sequences. The miRNA isolated from the body fluid is ligated to the single stranded oligonucleotide containing universal primer sequence, polyadenylated sequence, or adaptor sequence prior to reverse transcription and amplified with said complementary primers. In an embodiment, the kit comprises primers that amplify one of more of the miRNA shown above. In another embodiment, poly-A-tailing is used to generate a sequence that can then be hybridized to a poly-T primer that is used for reverse transcription. See, for example, Shi and Chiang. 2005. *BioTechniques*. 39: 5 19-525.

MicroArrays

[0068] Another aspect of the invention is microarrays comprising the miRNA biomarkers of the invention. Microarrays can be used to measure the expression levels of large numbers of miRNAs simultaneously. Microarrays can be fabricated using a variety of technologies, including printing with fine-pointed pins onto glass slides, photolithography using pre-made masks, photolithography using dynamic micromirror devices, ink-jet printing, or electrochemistry on microelectrode arrays. Also useful are microfluidic TaqMan® Low-Density Arrays based on an array of microfluidic qRT-PCR reactions as well as related microfluidic qRT-PCR based methods.

[0069] Microarrays can be used for the expression profiling of miRNAs in diseases, such as cancer. For this purpose, RNA is extracted from the body fluid and the miRNAs are size-selected from total RNA. Oligonucleotide linkers are attached to the 5' and 3' ends of the miRNAs and the resulting ligation products are used as templates for an RT-PCR reaction. The sense strand PCR primer can have a fluorophore attached to its 5' end, thereby labeling the sense strand of the PCR product. The PCR product is denatured and then hybridized to the microarray. A PCR product, referred to as the target nucleic acid that is complementary to the corresponding miRNA capture probe sequence on the array will hybridize, via base pairing, to the spot at which the, capture probes are affixed. The spot will then fluoresce when excited using a microarray laser scanner. The fluorescence intensity of each spot is then evaluated in terms of the number of copies of a particular miRNA, using a number of positive and negative controls and array data normalization methods, which will result in assessment of the level of expression of a particular miRNA.

[0070] Alternatively, total RNA containing the miRNA extracted from a body fluid sample can be used directly without size-selection of the miRNAs, and the RNA is 3' end labeled using T4 RNA ligase and a fluorophore-labeled short RNA linker. Fluorophore-labeled miRNAs complementary to the corresponding miRNA capture probe sequences on the array hybridize, via base pairing, to the spot at which the capture probes are affixed. The fluorescence intensity of each spot is then evaluated in terms of the number of copies of a particular miRNA, using a number of positive and negative controls and array data normalization methods, which will result in assessment of the level of expression of a particular miRNA.

[0071] Several types of microarrays can be employed including, but not limited to, spotted oligonucleotide microarrays, pre-fabricated oligonucleotide microarrays or spotted long oligonucleotide arrays.

[0072] Additional methods for miRNA detection and measurement include, for example, strand invasion assay (Third Wave Technologies, Inc.), surface plasmon resonance, cDNA, MTDNA (metallic DNA; Adavance Technologies, Saskatoon, SKI, single-molecule methods such as the one developed by US Genomics, etc.

EXAMPLES

[0073] The invention is further described by reference to the following Examples. These are intended as exemplifying embodiments of the invention, and are not to limit the invention.

METHODS

RNA isolation procedures

[0074] RNA was isolated from cultured cells generally using the following steps: Cells were washed twice with PBS. LysisBinding buffer (600 μ l) from the *mirVana*TM miRNA isolation kit (Ambion #1560) was added directly to the culture plate or flask to lyse the cells. Lysates were harvested manually with a sterile cell scraper and transferred to a 2 ml tube. Samples were stored at -80°C or RNA was immediately extracted using the manufacturer's recommended protocol for total RNA isolation.

[0075] RNA was isolated from mouse plasma samples generally using the following steps: Mouse plasma (100 μ l) was thawed on ice and transferred to a tube containing 100 μ l H₂O and 200 μ l 2X Denaturing Solution (Gel loading Buffer II, Denaturing PAGE AM-

8546G, Ambion, Austin, TX). To allow for normalization of sample-to-sample variation in the RNA isolation step, the synthetic *C. elegans* miRNAs *cel-miR-39*, *cel-miR-54* and *cel-miR-238* (Qiagen, Inc., Valencia, CA) were added to each denatured sample, as a mixture of 25 fmol of each oligonucleotide in a 5 µl total volume (i.e., *after* combining the plasma sample with Denaturing Solution). RNA was isolated using the *mirVana*™ PARIS™ kit following the manufacturer's protocol for liquid samples (Ambion, Austin, TX), modified such that samples were extracted twice with an equal volume of Acid-Phenol Chloroform (as supplied by the Ambion kit). FWA was eluted with 105 µl water. The average volume of eluate recovered from each column was 80.4 µl.

Mouse xenografts

[0076] The primary prostate epithelial and primary prostate stromal cells can be cultured by the methods described in Gmyrek 2001, *Am J. Pathol.* 159:579-90 (Normal and malignant prostate epithelial cells differ in their response to hepatocyte growth factor/scatter factor.) . Human prostate cancer-derived cells (22Rv1 cells) were cultured in standard plastic tissue culture plates in RPMI 1640 (GIBCO®) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a 5% CO₂ incubator. Xenografts were established in NOD/SCID mice by subcutaneous injection of 7.5×10^5 22Rv1 cells per mouse. Mouse blood was collected by cardiac puncture at 28 days following injection.

qRT-PCR

[0077] miRNAs that were quantified using TaqMan® miRNA qRT-PCR assays with modifications as described below:

[0078] For miRNA assay of a sample of RNA isolated from serum or plasma, a fixed volume of 1.67 µl of RNA solution from the approximate 80.4 µl eluate from RNA isolation of a given sample was input into the reverse transcription (RT) reaction. RNA is isolated from a 400 µl plasma or serum sample. For example, a 1.67 µl RNA solution represents the RNA corresponding to $(1.67/80.4) \times 400$ or 8.3 µl of plasma or serum. To generate standard curves of the chemically synthesized RNA oligonucleotides corresponding to known miRNAs, varying : dilutions of each oligonucleotide were made in water such that the final input into the RT reaction has a volume of 1.67 µl.

[0079] Input RNA is reverse transcribed using the TaqMan® miRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Applied BioSystems, Inc.) in a small-scale RT reaction containing 1.387 µl H₂O, 0.5 µl 10X Reverse-Transcription Buffer,

0.063 μ l RNase-Inhibitor (20 U/ μ l), 0.05 μ l 100 mM dNTPs with dTTP, 0.33 μ l MultiscribeTM Reverse-Transcriptase and 1.67 μ l input RNA. Components other than the input RNA are prepared as a larger volume master mix using a Tetrad[®] 2 Peltier Thermal Cycler (BioRad) at 16°C for 30 minutes, at 42°C for 30 minutes, and at 85°C for 5 minutes.

[0080] Standard curves were generated for each miRNA assay using a dilution series of known input amounts of synthetic miRNA oligonucleotide corresponding to the target of the assay. The dilution series samples were run with common RT and PCR enzyme master mixes on the same plate as experimental samples. Data points where measured Ct was interpreted to be below the linear range of the assay were not used for derivation of the trend line.

[0081] Synthetic single-stranded RNA oligonucleotides corresponding to the mature miRNA sequence (miRBase Release v. 10.1) can be purchased, *e.g.*, from IDT, Sigma, or Qiagen. Synthetic miRNAs are input into the RT reaction over an empirically-derived range of copies to generate standard curves for each of the miRNA TaqMan[®] assays listed above.

[0082] In general, the lower-limit of accurate quantification for each assay is designated based on the minimal number of copies input into an RT reaction that resulted in a Ct value within the linear range of the standard curve and that was also not equivalent to or higher than a Ct obtained from an RT input of a lower copy number. A line was fit to data from each dilution series using Ct values within the linear range, from which $y = m\ln(x) + b$ equations were derived for quantification of the absolute miRNA copies (x) from each sample Ct (y). The absolute copies of miRNA input into the RT reaction are converted to copies of miRNA per microliter of plasma (or serum) based on the knowledge that the material input into the RT reaction corresponds to RNA from 2.1% of the total starting volume of plasma (*i.e.*, 1.67 μ l of the total RNA eluate volume (80.4 μ l on average) was input into the RT reaction).

[0083] The layout of the samples in the reverse transcription, pre-amplification, and real-time PCR reactions in the strip tubes and multi-well plates are designed to minimize the chance of cross-contamination by the standard curve samples and to minimize systematic bias when comparing groups, such as xenografted mice and control mice. Experimental samples can be separated from standard curves by at least one empty row. In the mouse xenograft experiments, RNA samples from the xenografts and the controls were blinded and randomized in the layout on the PCR plate. In the human xenograft experiments, case and

control samples were alternated in sequence to avoid geographic location bias on the PCR plate.

Normalization of experimental qRT-PCR data

[0084] Given the early stage of plasma/serum miRNA research, no established endogenous small RNA control exists for normalization of technical variations in sample processing or of potential variation in sample quality (presence of PCR inhibitors due to occult red blood cell lysis in plasma samples, for example). Normalizing by matching the amount of input RNA into the RT reaction is not an appropriate approach because the RNA content of plasma can vary considerably and in fact has been suggested to vary with disease states. Therefore, a fixed volume of RNA eluate (1.67 μ l) from a given volume of starting plasma was used, rather than a fixed mass of RNA, as input into the RT reaction. For example, for a sample in which the starting plasma volume was 400 μ l, an input of 1.67 μ l of eluted RNA (taken from a total RNA eluate volume of approximately 80.4 μ l) into the RT reaction corresponds to the mass of RNA derived from approximately 8.3 μ l of starting plasma.

[0085] An approach for data normalization was devised based upon spiking-in three synthetic RNA oligonucleotides corresponding to miRNAs that do not exist in the mouse or human genomes. These RNAs were synthesized to match the sequence of three *C. elegans* miRNAs, *cel-miR-39*, *cel-miR-54*, and *cel-miR-238* (purchased as custom RNA oligonucleotide syntheses from Qiagen) and were confirmed empirically as not cross-hybridizing with probes for known human miRNAs on a locked nucleic acid-probe-based microRNA microarray (data not shown). The spiked-in oligos were introduced (as a mixture of 25 fmo1 of each oligonucleotide in a 5 μ l total volume of water) *after* addition of 2X Denaturing Solution (Ambion) to the plasma or serum sample to avoid degradation by endogenous plasma RNases. For each RNA sample, the *C. elegans* spiked-in miRNAs were measured using TaqMan® qRT-PCR assays (Applied BioSystems, Inc.) as described herein.

[0086] The data were normalized across samples using a median normalization procedure. For each sample, the Ct values obtained for the three spiked-in *C. elegans* miRNAs were averaged to generate SpikeIn_Average_Ct value. The median of the SpikeIn_Average_Ct values obtained from all the samples to be compared was next calculated (designated here as the Median_SpikeIn_Ct value). A Normalization_Factor was

then calculated for each sample based on the following formula: Normalization-Factor = $1/[2^{(\text{Median_SpikeIn_Ct value}) - (\text{SpikeIn_Average_Ct value of the given sample})}]$.

[0087] The number of copies of a given miRNA in each sample (calculated using the standard curves described earlier) was multiplied by the Normalization-Factor corresponding to the sample to obtain a normalized copy number value. In cases where it was desirable to apply normalization directly to Raw_Ct values corresponding to miRNAs of interest, the Raw_Ct for a given miRNA in a given sample was adjusted as follows: Normalized_Ct value for the miRNA in the sample = Raw_Ct value - [(SpikeIn_Average_Ct value of the given sample) - (Median_SpikeIn_Ct value)]

EXAMPLES

EXAMPLE 1

Xenograft experiments

NOD/SCID mice (Strain NOD.CB17-*Prkdc*^{scid}/J, stock/ number 0013031), were purchased from Jackson Labs (Bar Harbor, ME) and bred in-house. The mice were housed at a density of five mice per cage in an SPF environment in laminate airflow units changed once per week.

22Rv1 cells were harvested for injection with trypsin (0.05%)-EDTA solution, washed with culture medium and resuspended in a solution of ice-cold 50% basement membrane matrix (BD Matrigel™) in Hank's Balanced Salt Solution (GIBCO®) at a concentration of 7.5×10^6 cells/ml.

Xenografts were established in NOD/SCID mice by subcutaneous injection at the hip of 7.5×10^5 cells/mouse in 200 μ l total volume cell-Matrigel™-HBSS suspension. All xenograft mice developed grossly visible tumors.

Mice were treated with a control antibody or with an anti-angiopoietin 2 antibody (Anti-AGPT2 Ab) (Amgen Inc.). All mice were sacrificed 28 days post-injection. Prior to sacrifice, the mice were anesthetized by an intra-peritoneal injection of 500 μ l Avertin® (Sigma-Aldrich, St. Louis, MO) and blood was collected by cardiac puncture. This was followed by euthanasia and cervical dislocation. Each tumor was harvested in its entirety, snap frozen in liquid nitrogen, and stored at -80°C.

Ct values were converted to absolute number of copies/ μ l plasma using a dilution series of known input quantities of synthetic target miRNA run simultaneously (on the same

plate) as the experimental samples. Values shown have been normalized using measurements of *C. elegans* synthetic miRNA controls spiked into plasma after denaturation for RNA isolation.

Table 3 shows results of measurement of serum *miR-210* in mice bearing prostate cancer xenografts, either treated with control Ab or with anti-Angiopoietin 2 therapeutic antibody.

Table 3

SHAM-TREATED (Control Ab)			ANTI-AGPT2 Ab TREATED		
Tumor Weight in mg	<i>miR-210</i> copies/uL serum	<i>miR-210</i> copies/uL serum/mg tumor	Tumor Weight in mg	<i>miR-210</i> copies/uL serum	<i>miR-210</i> copies/uL serum/mg tumor
548	780	1.42	416	73545	177
1155	53610	46.4	514	50475	98.2
1017	2724	2.68	978	126635	129.
973	82549	84.8	1048	55310	52.8
1152	47430	41.2	1120	5115	4.57
1001	87105	87.0	1127	25939	23.0
682	1223	1.79	788	137464	174
547	51026	93.3	294	87167	296
Average:		44.8	365	52499	144
			Average:		122

These results show that the number of copies of *miR-210* in serum on average is higher in Anti-AGPT2 Ab treated animals. Similarly, copies of *miR-210* per mg of tumor mass is higher in mice treated with the anti-AGPT2 antibody than in control Ab treated mice.

EXAMPLE 2

Identification of Plasma miRNA Biomarkers of Disease

[0088] An ideal extracellular biomarker of a disease or disorder, for example, to be detected in extracellular fluid such as plasma or serum, is one that is highly disease specific. The extracellular ideal biomarker would show very low or absent expression in normal extracellular fluid, and its detection and/or abundance in extracellular fluid would be indicative of disease.

[0089] Table 4 lists results of TLDA TaqMan® qRT-PCR assay of human normal blood for the listed microRNAs, which have nucleotide sequences corresponding to those listed in Table 2, above.

Table 4**Cancer MicroRNA Marker: Low/absent expression in healthy donor human plasma.**

miRNA	Ct
hsa-miR-222-4373076	27.214977
hsa-miR-425-5p-4380926	29.179981
hsa-miR-148a-4373130	31.928728
hsa-miR-200c-4373096	32.412086
hsa-miR-375-4373027	33.360348
hsa-miR-210-4373089	33.956635
hsa-miR-100-4373160	Not detected
hsa-miR-141-4373137	Not detected
hsa-miR-200a-4373273	Not detected
hsa-miR-200a-4378069	Not detected

[0090] Ct values represents cycle threshold values obtained from qRT-PCR analysis of the indicated miRNA. The miRNA is expressed at extremely low/absent level in normal plasma, as Ct values greater than 30 indicate extremely low or absent expression.

These miRNA expressed at very low or absent levels in healthy donor human plasma can be evaluated for correlation to disease and detection in extracellular fluids of individuals suffering from the disease or disorder.

In particular, miR-210 in normal subjects is at low levels or is absent.

EXAMPLE 3

Global profiling of serum miRNAs identifies elevation of microRNAs in serum of prostate cancer patients.

[0091] Figures 1A-1F show the results of measuring miRNA in blood from patients with prostate cancer. Figure 1A shows results of measurement of circulating miRNAs in sera pooled from patients with advanced prostate cancer or healthy donors by TLDA profiling. Figure 1A (inset) shows that *mir-100*, *miR-141*, *miR-148a*, *miR-200a*, *miR-200c*, *miR-210*, *miR-222*, *miR-375*, and *miR-425-5p* met biomarker criterion of >5-fold change (unadjusted $P < 0.05$, student's t-test) in pooled sera from CaP patients relative to normal controls. The data are presented in Table 5.

Table 5

<u>microRNA</u>	<u>Fold-Change</u>	<u>p-value</u>
hsa-miR-210	55.2	<0.001
hsa-miR-100	42.9	<0.001
hsa-miR-200c	27.2	<0.001
hsa-miR-200a	25.7	<0.001
hsa-miR-141	8.3	<0.001
hsa-miR-222	0.01	<0.001
hsa-miR-148a	5.5	0.04
hsa-miR-375	123.5	0.03
hsa-miR-425-5p	39.5	0.02

[0092] Figures 1B-F upper panels, show results of measuring miRNA biomarker candidates levels in individual samples by TaqMan® miRNA qRT-PCR (*P* value assigned by Wilcoxon signed-rank test). Levels of miRNA is given in terms of miRNA copies/μl serum. The horizontal bars show the mean value +/- SEM of miRNA copies/μl serum for each group. The lower panels show receiver operating characteristic (ROC) curves plot sensitivity vs. (1 - specificity) to assess the ability of each miRNA biomarker to distinguish CaP and control sera. AUC, area under the curve; CaP, prostate cancer patient sera; FC, fold-change; N, normal sera (PSA_{neg}/DRE_{neg}). Data are provided in Figures 1B-F for the 5 microRNAs that showed the most significant difference in abundance in serum between cancer cases vs. controls. Remarkably, serum *miR-210* is elevated in a subset of advanced prostate cancer patients relative to levels in serum of healthy controls, showing that it distinguishes patients with hypoxic tumors from ones whose tumors exhibit lower levels of hypoxia.

EXAMPLE 9

Serum levels of miR-210 distinguish patients with advanced prostate cancer that are either responding or not responding to therapy.

[0093] Therapies being administered to patients varied and typically included androgen deprivation therapy and/or chemotherapy. Figures 2A-2E show copies of *miR-210* per microliter serum plotted for individuals with whose prostate cancer was responding to therapy (defined as being in a period of declining PSA at the time the blood was drawn for microRNA measurement) or not responding (defined as being in a period of not changing or increasing PSA when the blood was drawn for microRNA measurement). Serial serum PSA values define treatment response, using PSA as a measure of tumor burden. Remarkably, serum *miR-210* levels distinguish patients responding to therapy from those who are not

responding, whereas serum levels of *miR-141*, *miR-200a*, *miR-200c*, and *miR-375*, other prostate cancer-associated microRNA biomarkers, do not. The other biomarkers reflect disease burden and efficiency of overall miRNA release by the cancer. Results of measuring these miRNAs could be used singly or as a means to calculate *miR-210* levels in a given patient normalized to disease burden.

PROCESSING, STORAGE AND DATA MANIPULATION RELATED TO *miR-210*

[0094] In an embodiment, certain aspects of the present disclosure may take place on a general computing device. Aspects of storing, manipulating, calculating, configuring, or displaying data, prognosing and/or any other computing operations may be performed by a computing device. Examples of well known computing systems, environments, and/or configurations that may be suitable include, but are not limited to, personal computers, server computers, hand held or laptop devices, smartphones, multiprocessor systems, microprocessor-based systems, set top boxes, programmable consumer electronics, network PCs, minicomputers, mainframe computers, distributed computing environments and the like.

[0095] An example computing device may comprise one or more processors and one or more memories. The memories may be coupled to the one or more processors. As one example, the memories may be coupled to the one or more processors by a system bus, which may be of a type of bus structure known in the art, including a memory bus or memory controller, a peripheral bus, and a local bus using any of a variety of bus architectures. The memories may be read only memory (ROM), random access memory (RAM) and the like. A basic input/output system (BIOS) may be used to transfer data and information between elements within the computing device.

[0096] A computing device may include disks and drives for writing and reading data. These may be hard drives, floppy drives, removable storage, optical disks, magnetic discs, magnetic cassettes, flash memory, or any other disk or drive for storing data. The disks and drives may be computer readable media and may have stored thereon instructions that when executed by a processor cause the processor to perform one or more actions included herein.

[0097] A computing device may be associated with one or more user inputs, such as, for example, a mouse, a keyboard, a voice recorder, touchscreen, joystick, a camera, a medical device with digital output and the like. These and other peripheral input devices may be connected via serial or parallel port to the system bus or any other interface. A monitor,

tv, touchscreen, or other type of display device may also be connected to the system bus via an interface such as a video adapter. The same may be true for a printer, a video output, or speakers.

[0098] A computing device may be in a network environment with one or more logical connections to one or more computers. These may include servers, routers, PCs, network nodes and the like. Data and information may be sent and received by the computing device and may be manipulated.

[0099] In an embodiment, a computing device may be configured to receive data related to one or more measurements of miR-210. The data related to one or more measurements of miR-210 may be stored in a memory associated with the computing device. As one example the one or more measurements may be stored in a database. These data may be configured to provide indications of the efficacy of the prognosis for various levels of miR-210 as indicated above.

[0100] In an embodiment, a series of trials related to miR-210 may provide information indicative of the one or more levels of miR-210 based on one or more trials, as well as information indicative of one or more prognoses. Other information indicative of one or more elements related to each prognosis may also be included. The information indicative of the one or more levels of miR-210, the information indicative of the one or more prognoses and the information indicative of one or more elements related to the prognosis may be stored in a database.

[0101] In one embodiment, information indicative of one or more elements related to clinical markers of the cancer.

[0102] The database may be configured as a tool for prognosis. For example, the database may be used as a comparison for data indicative of the levels of miR210. A patient may have their levels of miR210 measured. This measurement may be compared to one or more outputs of the database, such as, for example, calculations, similar prognosis factors, patient similarities and the like. In another embodiment, the database may be configured to provide a graphical display of the levels of miR-210 and prognosis.

[0103] Further, the data in the database may be displayed on a display device, or it may be used in one or more calculations. As one example, a calculation may comprise a prognosis of a patient associated with the data. In other words, the computing device may be configured to provide an output related to the prognosis of a patient as a response to receiving one or more measurements of miR-210.

[0104] In an embodiment, computer readable medium may have stored thereon instructions that when executed by a processor may cause the processor to receive data indicative of one or more levels of miR-210, manipulate the data, store the data, place the data in a database, subject the data to one or more calculations such as those described herein, and provide a prognosis.

[0105] Further, a computing device or computer readable medium may be configured to send data indicative of one or more levels of miR-210; receive, as a response to the sending, an indication of the prognosis of a subject; and display, on a display, the prognosis of the subject.

[0106] In another embodiment, the database above may be used to instruct a researcher or a person in control of choosing an appropriate therapy on the basis of the prognosis of cancer patient.

* * *

[0107] All publications and patent applications in this specification are indicative of the level of ordinary skill in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated by reference.

[0108] The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

What is Claimed:

1. A method of prognosing cancer comprising the steps of:
 - a. collecting at least one first blood sample from a subject with cancer;
 - b. administering to the subject an anti-angiogenic treatment;
 - c. collecting at least one second blood sample following said treatment;
 - d. measuring levels of *miR-210* in the samples;
 - e. comparing levels of *miR-210* before and after treatment;
 - f. providing a prognosis, wherein the prognosis is based on the difference in the levels of *miR-210* between the samples.
2. The method of claim 1, wherein the levels of *miR-210* change following the treatment.
3. The method of claim 2, wherein the prognosis is favorable.
4. The method of claim 1, wherein the levels of *miR-210* do not change following the treatment.
5. The method of claim 4, wherein the prognosis is unchanged following treatment.
6. The method of claim 1, wherein the measuring of *miR-210* comprises the steps of:
 - a. extracting RNA from the first and second samples;
 - b. contacting said RNA with at least one nucleic acid probe to measure blood levels of *miR-210*.
7. The method of claim 6, wherein the RNA is extracted from serum or plasma obtained from each sample.
8. The method of claim 7, wherein the RNA is extracted from serum.
9. The method of claim 6, further comprising measuring levels of *miR-210* by quantitative PCR.
10. The method of claim 6, wherein the RNA is contacted with a microarray comprising a multiplicity of single stranded oligonucleotides to measure blood levels of *miR-210*.
11. The method of claim 6, further comprising contacting said RNA with at least one nucleic acid probe to measure levels of a control RNA.
12. The method of claim 6, wherein the control RNA is added to the RNA extracted from the first and second samples.
13. The method of claim 6, wherein the RNA extracted from the first and second samples comprise the control RNA

14. The method of claim 1, wherein the first blood sample consists of a multiplicity of blood samples.
15. The method of claim 14, wherein the multiplicity of blood samples is obtained from a multiplicity of subjects.
16. The method of claim 1, wherein the second blood sample consists of a multiplicity of blood samples.
17. The method of claim 16, wherein the multiplicity of blood samples is obtained from a multiplicity of subjects.
18. The method of claim 15, further comprising a step of calculating an average level of *miR-210* in the multiplicity of samples.
19. The method of claim 17, further comprising a step of calculating an average level of *miR-210* in the multiplicity of samples.
20. The method of claim 1, further comprising steps of collecting blood samples from a multiplicity of normal subjects, extracting RNA from the samples, and measuring and calculating the average level of *miR-210* in the samples, and providing an average level of *miR-210* in a normal subject.
21. The method of claim 20, further comprising a step of comparing the level of *miR-210* in the first blood sample of the subject with cancer to the average level of *miR-210* in a normal subject.
22. The method of claim 21, further comprising a step of providing a prognosis.
23. The method of claim 22, wherein the prognosis is favorable for an anti-angiogenic treatment.
24. The method of claim 23, where the level of *miR-210* in the subject with cancer is statistically the same as the average value for a normal subject.
25. The method of claim 22, wherein the prognosis is not favorable for an anti-angiogenic treatment.
26. The method of claim 23, where the level of *miR-210* in the subject with cancer is statistically substantially different than the average value for a normal subject.
27. The method of claim 16, wherein the samples are collected from the subject with cancer at various times before and after anti-angiogenic treatment.
28. The method of claim 17, further comprising comparing the level of *miR-210* in the first sample with the level of *miR-210* in each of the multiplicity of second samples.
29. The method of claim 28, wherein the prognosis is favorable.

30. The method of claim 29, wherein the levels of *miR-210* changes over time following anti-angiogenic treatment.
31. The method of claim 26, further comprising providing an alternate treatment modality.
32. The method of claim 31, wherein the alternate treatment modality comprises administering a pro-angiogenic treatment.
33. The method of claim 32, further comprising administering a cytotoxic drug.
34. The method of claim 1, wherein the nucleic acid probe is a single stranded nucleic acid.
35. The method of claim 34, wherein the single stranded probe hybridizes with the nucleic acid having the sequence of SEQ ID NO:1 or SEQ ID NO:2.
36. The method of claim 1, wherein the prognosis is in a report.
37. The method of claim 1, wherein the prognosis is a result of a computer calculation.
38. The method of claim 1, consisting of the additional step of causing said report to be produced in a tangible medium.
39. The method of claim 1, wherein said body sample is blood or a fractions thereof, blood serum, blood plasma, urine, semen, seminal fluid, seminal plasma, prostatic fluid, pre-ejaculatory fluid (Cowper's fluid), pleural effusion, tears, saliva, sputum, sweat, biopsy, ascites, cerebrospinal fluid, amniotic fluid, lymph, marrow, cervical secretions, vaginal secretions, endometrial secretions, gastrointestinal secretions, bronchial secretions, breast secretions, ovarian cyst secretions, or fluid extracted from a tissue sample.
40. The method of claim 1, wherein, prior to contacting said nucleic acid probe, the RNA is converted to cDNA using a reverse transcriptase.
41. The method of claim 1, wherein, prior to contacting said nucleic acid probe, the cDNA is amplified in a polymerase chain reaction.
42. The method of claim 1, wherein the cancer is of epithelial cell origin.
43. The method of claim 1, wherein the cancer is a of prostate or ovarian origin.
44. A computing device, comprising a means to store data to be configured in a prognosis in the form of a report, wherein the prognosis is calculated by comparing a level of expression of *miR-210* in blood of a patient to a reference value.

45. The computing device of claim 44, wherein the report is generated as the result of data indicative of miR-210 levels in various samples, the data having been subject to a method of prognosis by the computing device.
46. The computing device of claim 44 or 45, wherein the report is displayed.
47. The computing device of claim 44, comprising one or more elements selected from the group consisting of data storage, manipulation, processing, configuration, prognosis, display, and calculation.
48. The computing device of claim 44, consisting of a personal computers, server computers, hand held or laptop devices, smartphones, multiprocessor systems, microprocessor-based systems, set top boxes, programmable consumer electronics, network PCs, minicomputers, mainframe computers, or distributed computing environments.
49. The computing device of claim 44, comprising one or more processors and one or more memories.
50. The computing device of claim 44, wherein the memories may be read only memory (ROM) or random access memory (RAM).
51. The computing device of claim 44, comprising disks and drives for writing and reading data.
52. The computing device of claim 44, comprising one or more user inputs.
53. The computing device of claim 44, comprising a network environment with one or more logical connections to one or more computers, wherein data and information may be sent and received by the computing device and may be manipulated.
54. The computing device of claim 44, wherein the device is configured to receive data related to one or more measurements of miR-210.
55. The computing device of claim 54, wherein the data is related to one or more measurements of miR-210 that are stored in a memory associated with the computing device.
56. The computing device of claim 54, wherein the data relates to a miR-210 based on one or more clinical trials, or is indicative of one or more prognosis.
57. Use of a computing device according to any of claims 44-56 in a prognosis of a disease based on a measurement of a level of expression of miR-210.

Figure 1A

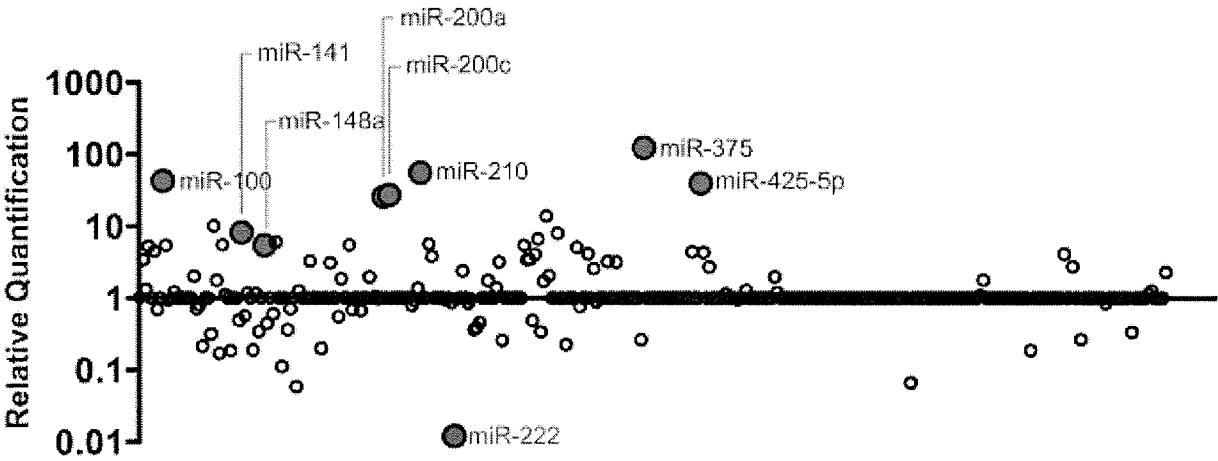


Figure 1B

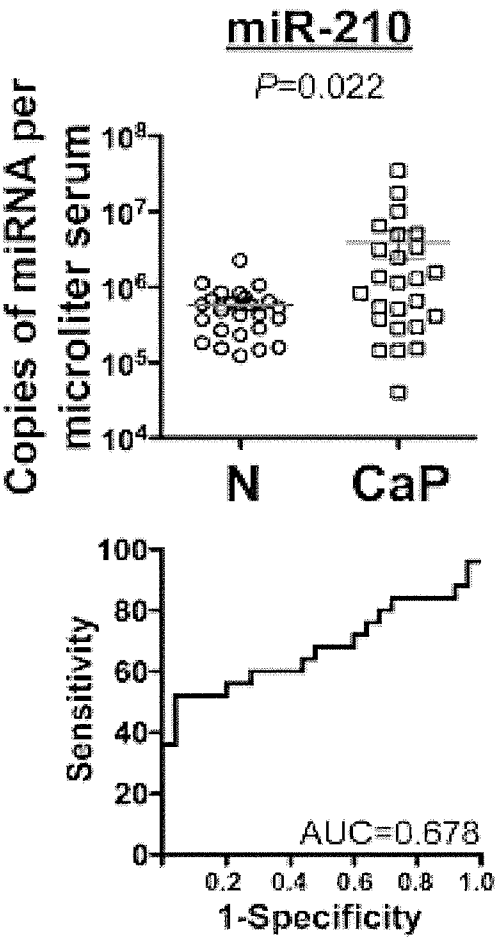


Figure 1C

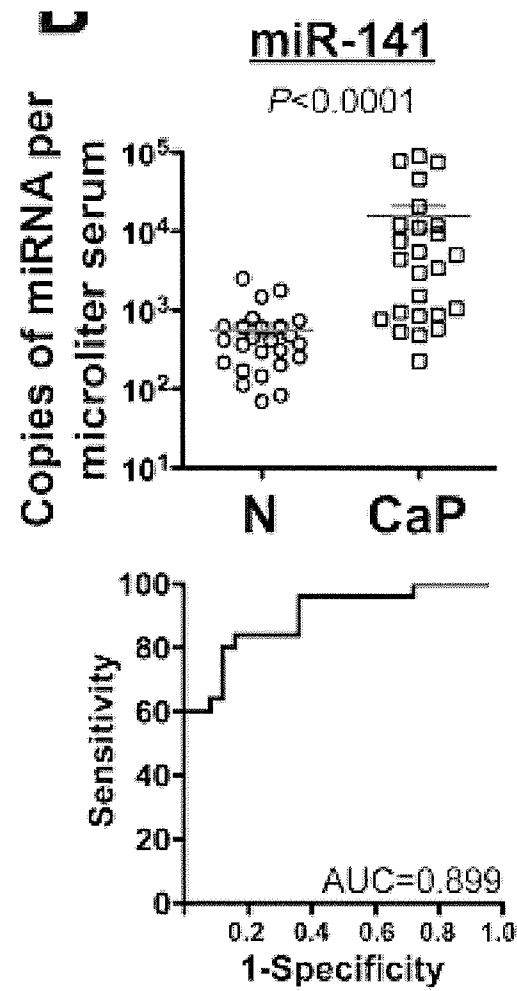


Figure 1D

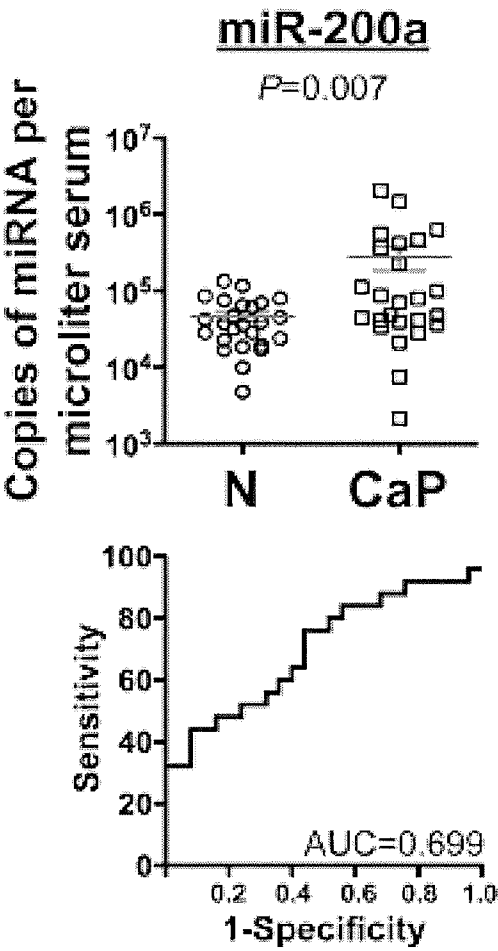


Figure 1E

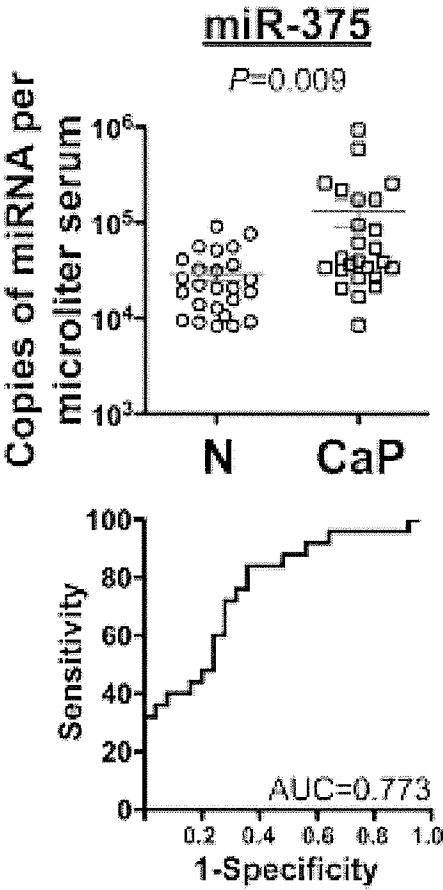
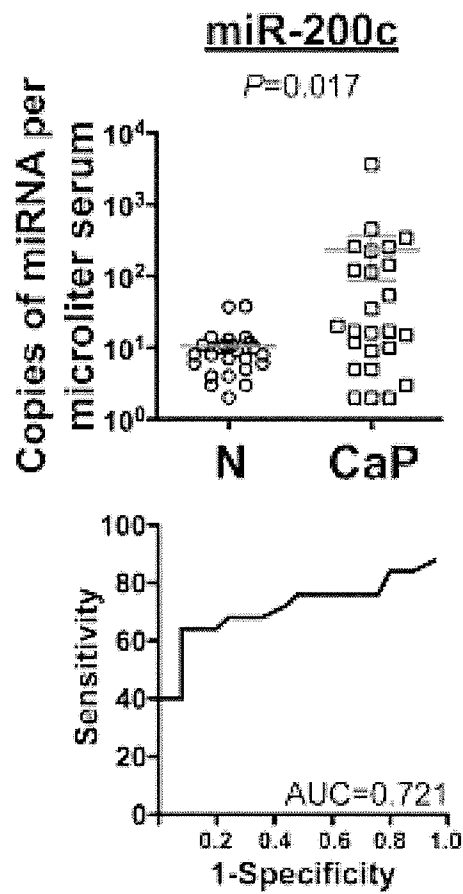


Figure 1F



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Figure 2A

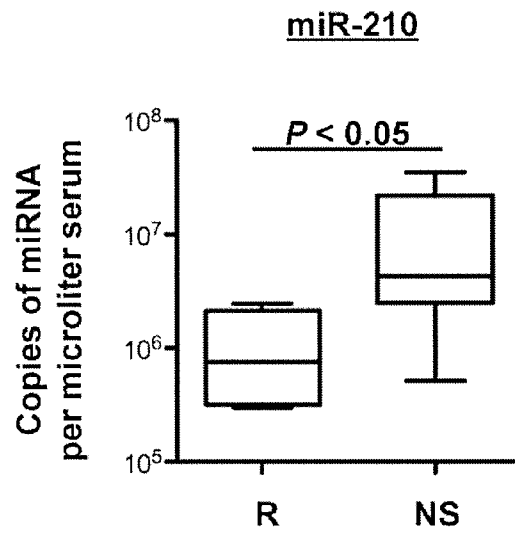
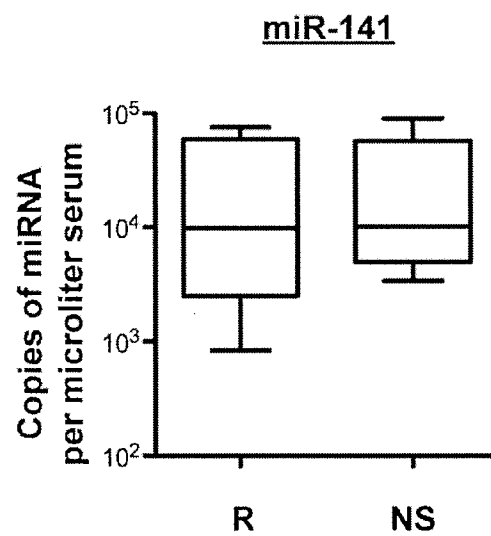


Figure 2B



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Figure 2C

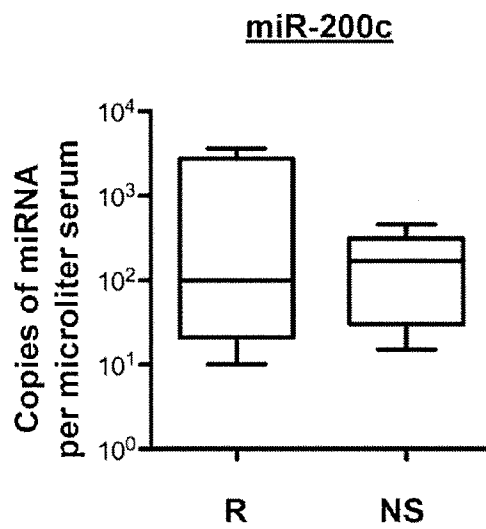


Figure 2D

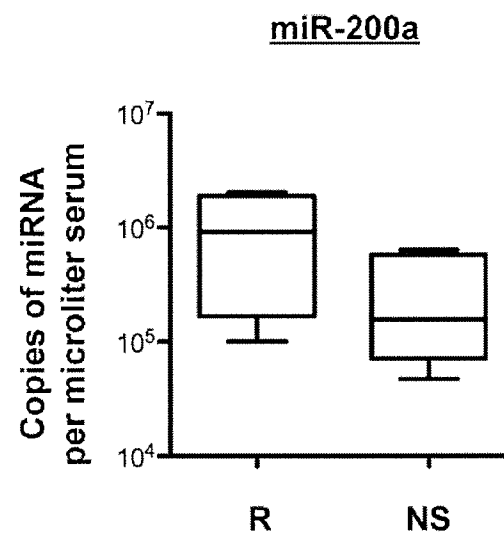
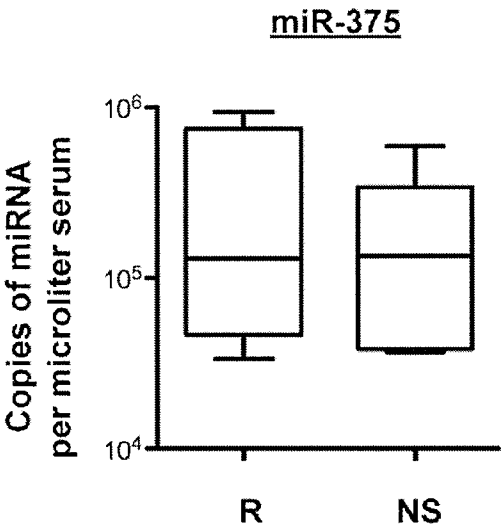


Figure 2E



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/040078

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 practical, search terms used)

EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009/143379 A2 (HUTCHINSON FRED CANCER RES [US]; TEWARI MUNEESH [US]) 26 November 2009 (2009-11-26)	44-57
Y	page 27 - page 28; example 8 -----	1-43
Y	HARRIS A: "Resistance to anti-angiogenic therapy induced by hypoxia and notch signalling", EJC SUPPLEMENTS, vol. 8, no. 3, March 2010 (2010-03), pages 183-184, XP002652946, & EUROPEAN BREAST CANCER CONFERENCE; BARCELONA, SPAIN; MARCH 24 -24, 2010 ISSN: 1359-6349 the whole document ----- -/--	1-43



Further documents are listed in the continuation of Box C.



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

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

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"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

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"&" document member of the same patent family

Date of the actual completion of the international search

28 July 2011

Date of mailing of the international search report

12/08/2011

Name and mailing address of the ISA/

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Authorized officer

Cornelis, Karen

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/040078

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ADRIAN M. JUBB ET AL: "Assessment of tumour hypoxia for prediction of response to therapy and cancer prognosis", JOURNAL OF CELLULAR AND MOLECULAR MEDICINE, vol. 14, no. 1-2, 1 January 2010 (2010-01-01), pages 18-29, XP55003427, ISSN: 1582-1838, DOI: 10.1111/j.1582-4934.2009.00944.x the whole document	1-43
A	HARRIET E. GEE ET AL: "hsa-miR-210 is a marker of tumor hypoxia and a prognostic factor in head and neck cancer", CANCER, 1 January 2010 (2010-01-01), pages NA-NA, XP55003217, ISSN: 0008-543X, DOI: 10.1002/cncr.25009 cited in the application the whole document	1-57
A	CAMPS CARME ET AL: "hsa-miR-210 Is induced by hypoxia and is an independent prognostic factor in breast cancer", CLINICAL CANCER RESEARCH, THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, US, vol. 14, no. 5, 1 March 2008 (2008-03-01), pages 1340-1348, XP002507074, ISSN: 1078-0432, DOI: DOI:10.1158/1078-0432.CCR-07-1755 the whole document	1-57
A	WO 2009/131887 A2 (MERCK & CO INC [US]; ZHANG ZHAN [US]; SUN HONG [US]; DAI HONGYUE [US];) 29 October 2009 (2009-10-29) the whole document	1-57
A	LAWRIE CHARLES H ET AL: "Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma", BRITISH JOURNAL OF HAEMATOLOGY, BLACKWELL PUBLISHING LTD, vol. 141, no. 5, 1 June 2008 (2008-06-01), pages 672-675, XP002545705, ISSN: 1365-2141, DOI: DOI:10.1111/J.1365-2141.2008.07077.X [retrieved on 2008-03-03] the whole document	1-57

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2011/040078

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W0 2009131887 A2	29-10-2009	US 2011166200 A1	07-07-2011