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(54) **Title**: CIRCULATING miR-10b AND/OR miR-21 AS MARKERS FOR RESPONSE TO ANTIANGIOGENIC TREATMENT IN BRAIN CANCER PATIENTS

(57) **Abstract**: Provided are methods and kits for assessment of treatment efficacy of antiangiogenic drugs by determining the circulating levels of mi R-10b and/or mi R-21 in serum of treated subjects having brain cancer.

CIRCULATING miR-10b AND/OR miR-21 AS MARKERS FOR RESPONSE TO ANTIANGIOGENIC TREATMENT IN BRAIN CANCER PATIENTS

FIELD OF THE INVENTION

The invention relates to methods and kits for assessment of treatment efficacy of antiangiogenic drugs by determining the levels of specific miRNA molecules, miR-10b and/or miR-21 in serum of treated subjects having brain cancer.

BACKGROUND OF THE INVENTION

High Grade gliomas (WHO grade III–IV) are the most frequent and most devastating of the primary central nervous system tumors. Few patients with stage IV gliomas- Glioblastoma multiforme (GBM) live beyond 2 years. Therapeutic modalities for treating gliomas, such as, glioblastoma (GBM), include surgical resection, radiation and chemotherapy, as well as antiangiogenic drug therapy that targets, for example, vascular endothelial growth factor (VEGF). Antiangiogenic therapy can produce early marked decrease in contrast enhancement in imaging studies and consequently results in a high rate of radiologic response. For instance, the antiangiogenic agent Bevacizumab can produce a high rate of radiologic response due to early decrease in contrast enhancement on imaging studies. However, such radiologic responses should be interpreted with caution because they are partly a result of normalization of abnormally permeable tumor vessels and not always necessarily indicative of a true anti-tumor effect of the antiangiogenic therapy. Moreover, antiangiogenic therapy may increase the tendency of tumor cells to co-opt existing blood vessels, resulting in an invasive non-enhancing growth phenotype. Progressive non-enhancing tumor may sometimes be detected by continuous increase in T2 or fluid attenuated inversion recovery signals (FLAIR) on MRI, which are suggestive of infiltrative tumor. The presence of progressive non-enhancing tumor is often associated with clinical deterioration that helps in determining the true course of the disease despite the absence of an enhancing mass. The multi facets of clinical and radiological appearance create circumstances which are defined as pseudoprogression or pseudoresponse. Standardized response criteria for clinical trials are therefore needed and the Response Assessment in Neuro-Oncology (RANO)

working group developed such new criteria for brain tumors that assemble radiologic and clinical parameters into four categories of response (36).

Various tumor-associated circulating biomarkers have been investigated (17) including circulating nucleic acids (DNA, RNA and microRNA). It has been previously demonstrated that cell free circulating DNA of patients harboring glial tumors can be used to identify genetic and epigenetic alterations that are present in the tumor tissue (18). In another study, the profile of microRNA (miRNA) expression of glial tumors was determined and identified miRNAs that were up-regulated in gliomas as compared to normal brain (19). Circulating, tumor-specific miRNAs have been detected in the blood of GBM patients in several studies (9, 11, 29, 35, 39). Any tumor-derived marker is expected to have its highest concentrations within the tumor itself, followed by surrounding extracellular fluid, followed by peripheral blood. CSF analysis may serve as a close reflection of the tumor's extracellular space, but this is not practical in high-grade gliomas. One study did attempt to distinguish between GBM and brain metastases (breast and lung primaries) through miRNAs profiling of patients' CSF (32).

Currently, the response of gliomas (such as GBM) to therapy, and consequently the tumor status is evaluated only by clinical parameters standardized by the RANO criteria. Chemo and radio-resistance can be a pivotal factor in the prognosis of patients with gliomas. The ability to predict response to treatment would greatly improve clinical decision-making, improve health outcomes and markedly reduce costs.

There is thus a need in the art for reliable and robust biomarkers for detecting the presence of tumor, tumor activity, and response to treatment that would serve as an essential diagnostic tool. Preferably, such biomarkers should be detected in the bloodstream to provide a considerable advantage due to their relatively easy accessibility, which facilitates repetitive assessment, while reducing costs and increasing efficiency and accuracy. Further, the use of such biomarkers in clinical trials can be used to identify patient's response to new anti-cancer therapies, thereby accelerating the development of novel therapeutics.

SUMMARY OF THE INVENTION

The present invention in embodiments thereof provides methods and kits for prognosis or determination or assessment of treatment efficacy of antiangiogenic drug in patients having brain cancer, by identifying specific miRNA biomarkers in serum samples of the subject, the miRNA are selected from miR-21 and/or miR10b. In some embodiments, the antiangiogenic drug is a VEGF-A inhibitor. In some embodiments, the antiangiogenic drug is Bevacizumab. In some embodiments, the glioma is a high grade glioma. In some embodiments, there is a correlation between the quantification levels of the specific miRNA in the circulating blood (serum) of the subject and the effect of the antiangiogenic drug. In some exemplary embodiments, the specific miRNAs is miR-21. In some embodiments, the specific miRNA is miR10b. In some embodiments, the specific miRNAs are miR-21 and miR-10b.

Advantageously, the invention provides simple, reliable, sensitive and cost effective kits and methods for determining the efficacy of antiangiogenic treatment (such as Bevacizumab) for glioma cancer, such as, for example, glioblastoma (GBM), by determining the level of miR-21 and/or miR10b in circulating blood (serum) of the subject.

In some embodiments, the present invention is based in part on the surprising finding that specific circulating miRNA molecules, namely, miR-21 and/or miR10b, detected in the circulation (i.e., in the subjects serum and not localized to the brain region) are indicative of treatment efficacy of antiangiogenic drugs in subjects afflicted with glioma and in particular, glioblastoma. The present invention is further based in part on the surprising finding that the specific circulating miRNAs identified (miR-21 and Mir-10b) are indicative of treatment efficacy, albeit the fact they were previously identified in the cancer tissue along with other hypoxia related miRNAs (such as, miR-196b, miR-210 and miR-1271), which were found not to be significantly indicative of treatment efficacy based on their presence in the circulation. Further, the present invention is further based in part on the surprising finding that the method for identifying treatment efficacy is specific, as it provides indication with respect to antiangiogenic treatment, but not necessarily for any type of treatment (such as, for example, treatment with alkylating agents).

According to some embodiments, the present invention provides a method for determining efficacy of antiangiogenic treatment for a brain cancer in a subject in need thereof, said method comprising:

a) determining the levels of circulating miR-21 and/or miR-10b in a first serum sample of said subject;

b) treating said subject with an anti-angiogenic treatment;

c) determining the levels of circulating miR-21 and/or miR-10b in a second serum sample of said subject; and

d) comparing the levels of circulating miR-21 and/or miR-10b in said first and second serum samples,

wherein a modulation in the levels of circulating miR-21 and/or miR-10b from the first to the second serum sample is indicative of said treatment efficacy.

According to some embodiments, step a) is conducted at a time point prior to or during step b). According to another embodiment, steps a) and c) are conducted at distinct time points during step b).

According to some embodiments the method may further include repeating step c) for one or more times to determine the levels of circulating miR-21 and/or miR-10b in consecutive serum samples, obtained at designated time intervals; and comparing the levels of circulating miR-21 and/or miR-10b between said consecutive serum samples, wherein a modulation in the levels of circulating miR-21 and/or miR-10b between consecutive serum samples is indicative of said treatment efficacy.

In some embodiments, the time intervals between consecutive serum samples is in the range of 1-10 weeks. In some embodiments, the time intervals between obtaining serum samples may be identical or different between consecutive samples.

In some embodiments, an increase in the levels of circulating miR-21 and/or miR-10b from the first to the second serum sample is indicative of the treatment being efficacious.

In some embodiments, an increase in the levels of circulating miR-21 and/or miR-10b from an earlier second serum sample and a later consecutive second serum sample is indicative of the treatment being efficacious.

In some embodiments, a decrease in the levels of circulating miR-21 and/or miR-10b from an earlier second serum sample and a later consecutive second serum sample is indicative of a reduction in treatment efficacy.

In some embodiments, a decrease in the levels of circulating miR-21 and/or miR-10b from the first serum sample and the second serum sample is indicative of a reduction in treatment efficacy.

According to some embodiments, an increase in the levels of circulating miR-21 and/or miR-10b between consecutive second serum samples is indicative of the treatment being efficacious.

According to some embodiments, as long as the levels of circulating miR-21 and/or miR-10b between consecutive serum samples is increased, the treatment is being efficacious.

According to some embodiments, if the levels of circulating miR-21 and/or miR-10b between first serum sample and second serum sample is not modulated or reduced, it is indicative of reduction in treatment efficacy.

According to some embodiments, if the levels of circulating miR-21 and/or miR-10b between consecutive serum samples is not modulated or reduced, it is indicative of reduction of treatment efficacy.

According to some embodiments, the antiangiogenic treatment may include administration of an antiangiogenic drug selected from: Bevacizumab (Avastin), itraconazole, carboxyamidotriazole, TNP-470, CM101, IFN-α, IL-12, platelet factor-4, suramin, SU5416, thrombospondin, VEGFR antagonists, angiostatic steroids and heparin, Cartilage-Derived Angiogenesis Inhibitory Factor, matrix metalloproteinase inhibitors, angiostatin, endostatin, -2methoxyestradiol, tecogalan, tetrathiomolybdate, thalidomide, thrombospondin and prolactin. According to some embodiments the antiangiogenic drug is Bevacizumab.

According to some embodiments the brain tumor is glioma. In some embodiments, the glioma is high grade glioma.

According to some embodiments, the method may further include a step of modulating the antiangiogenic treatment regime (for example, reducing or increasing dosage, ceasing treatment, replacing or adding a drug, and the like), based on the determined treatment efficacy.

In some embodiments, the method may further include a step of isolating RNA from the first serum sample and/or the second serum sample(s) prior to identification of the miRNA.

In some embodiments, the level of miRNA in the first serum sample and/or the second serum sample is determined and/or quantified by a method selected from amplification reaction, a sequencing reaction, microarray, or combination thereof.

In some embodiments, the amplification reaction comprises PCR (such as, real-time PCR, RT-PCT and the like), using specific nucleic-acid primers for miR-21 and/or miR-10b.

In some embodiments, the sequencing reaction is next-generation sequencing, using specific nucleic-acid probes to identify miR-21 and/or miR-10b.

According to some embodiments, suitable methods to detect and/or quantify miRNA may include such methods as, but not limited to: amplification reaction, such as, PCR, RT-PCR, real time PCR, and the like; Gene expression microarrays, such as Affymetrix, Agilent, and Illumina microarray platforms; NanoString technology, such as nCounter technology which employs unique fluorescent–tagging of individual miRNA species followed by two-dimensional display and optical scanning and counting of miRNA molecules; Sequencing, such as, next Generation Sequencing (NGS) using specific adaptors and/or probes; and the like, or combinations thereof.

According to some embodiments, there is provided a method for assessing treatment efficacy of antiangiogenic drug in a cancer subject having brain tumor, the method comprising determining the level of circulating miRNA molecules in serum samples of said subjects obtained before and after treatment with the antiangiogenic drug, and comparing the levels of the circulating miRNA molecules between the serum samples, wherein an increased level of expression of the miRNA between the serum samples obtained before and after treatment is indicative of increased treatment efficacy, wherein the miRNA molecules are selected from miR-10b, miR-21 or both.

In some embodiments, the level of the circulating miRNA mir-10B and/or miR-21 is determined at one or more time points after initiation of treatment with the antiangiogenic drug on consecutive serum samples.

In some embodiments, the method may further include comparing the circulating levels of miRNA mir-10B and/or miR-21 between the consecutive serum samples, wherein an increased level of expression of the miRNA between consecutive serum samples is indicative of increased treatment efficacy.

In some embodiments, the consecutive serum samples are obtained at time intervals in the range of 1-10 weeks.

In some embodiments, the antiangiogenic drug may be selected from: Bevacizumab (Avastin), itraconazole, carboxyamidotriazole, TNP-470, CM101, IFN-α, IL-12, platelet factor-4, suramin, SU5416, thrombospondin, VEGFR antagonists, angiostatic steroids and heparin, Cartilage-Derived Angiogenesis Inhibitory Factor, matrix metalloproteinase inhibitors, angiostatin, endostatin, -2 methoxyestradiol, tecogalan, tetrathiomolybdate, thalidomide, thrombospondin and prolactin. According to some embodiments the antiangiogenic drug is Bevacizumab.

In some embodiments, the brain tumor is glioma. In some embodiments, the glioma is glioblastoma (GBM). In some embodiments, the glioma is high grade glioma.

In some embodiments, the RNA is isolated from the serum samples prior to identification of the miRNA.

In some embodiments, the level of miRNA in the serum is determined by a method selected from: amplification reaction, sequencing reaction, microarray, or combinations thereof.

In some embodiments, the amplification reaction comprises PCR using specific nucleic acid primers for miR-21 and/or miR-10b.

In some embodiments, the sequencing reaction is selected from next-generation sequencing, using specific nucleic-acid probes.

In some embodiments, the method may further include evaluating the tumor status by clinical parameters.

In some embodiments, the level of the circulating miRNA mir-10b and/or miR-21 is determined at one or more time points after initiation of treatment with the antiangiogenic drug.

According to some embodiments, there is provided a kit for determining efficacy of an antiangiogenic treatment for brain cancer, comprising means for determining the levels of miR-21

and/or miR-10b in serum samples of a subject obtained before and after the antiangiogenic treatment, and instructions for using the kit in the determining efficacy of the antiangiogenic treatment for the brain cancer. In some embodiments, an increase in the levels of miR-21 and/or miR-10b between the serum samples obtained before and after treatment in indicative of the treatment being efficacious. In some embodiments, the antiangiogenic treatment comprises administration of Bevacizumab to the subject. In some embodiments, the cancer is glioma.

According to some embodiments the means for determining the levels of miR-21 and/or miR-10b comprises specific nucleic acid molecules for identification of miR-21 and/or miR-10b in the serum samples.

In some embodiments, the nucleic acid molecules comprise specific primers for identification of miR-21 and/or miR-10b in an amplification reaction performed on RNA isolated from the serum sample. In some embodiments, the amplification reaction is selected from, PCR, RT-PCR, real-time PCR, or combinations thereof.

In some embodiments, the nucleic acid molecules comprise specific probes for identification of miR-21 and/or miR-10b in a sequencing reaction performed on RNA or DNA isolated from the serum sample.

Other objects, features and advantages of the present invention will become clear from the following description and drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 - Expression of Circulating miRNAs in subjects harboring high-grade gliomas and in healthy normal controls. The scatter plots presented show the expression of tested miRNAs in serum samples of healthy controls (n=10) and in brain tumor patients (n=30). Serum samples were obtained from patients harboring high-grade gliomas prior to the diagnostic surgical procedure. Analysis was performed by real time RT PCR.

Figs. 2A-C - Dynamics of circulating miRNAs expression and enhancing tumor diameters during bevacizumab treatment. **Fig. 2A**- Graphs showing fold changes over time in the sum expression

of circulating miR-10b and miR-21. **Fig. 2B**- Graphs showing fold changes over time in the sum of products of cross sectional enhancing diameters of the tumor measured on enhanced T1-weighted MR images. Each patient is represented by a separate line. Only patients who had more than two follow up serum samples during treatment are represented in the graphs.

Fig. 3 - Longitudinal evaluations of circulating miRNAs quantification and enhancing tumor diameters during bevacizumab treatment in 10 individual patients. The line graphs show the Percentage fold changes over time in the sum quantification of circulating miR-10b and miR-21 (light gray line) and in sum products of cross sectional enhancing diameters of the tumor measured on MRI (dark gray line) in 10 individual patients that had more than 2 measurements. Shown is fold change from pre-bevacizumab values for the sum of both miRNA quantification and for the sum of products of perpendicular diameters of the enhancing tumor.

Fig. 4 - RANO assessment of tumor response during bevacizumab treatment. RANO assessment was performed on the day of clinic visit within 14 days of MRI study. Each patient is represented by a separate line (as in Figs. 2A-B). Only patients who had more than two follow up serum samples during treatment for evaluation of miRNAs are represented in the graphs. RANO = response assessment in neuro-oncology; CR=complete response; PR=partial response; SD=stable disease; PR=progressive disease.

DETAILED DESCRIPTION

According to some embodiments, the present invention provides methods and kits for determining treatment efficacy of antiangiogenic drug in a patient afflicted with brain cancer, wherein circulating levels of the miR-21 and/or miR-10b, are used as biomarkers.

The present invention discloses for the first time the unexpected discovery that the detection of circulating miR-21 and/or miR-10b in serum of a brain cancer patient treated with an antiangiogenic drug reflects the treatment efficacy of the treated subject. The methods currently used for assessing treatment efficacy are complicated and cumbersome and in many cases provides false or non-accurate results. Hence, the use of detecting circulating levels of specific miRNAs, namely, miR-21 and/or miR-10b as a biomarker of treatment efficacy of antiangiogenic

drugs, overcomes the drawbacks of currently used methods in providing a more sensitive, reliable, simple and cost effective method for determine efficacy of such treatment.

Thus, in some embodiments, the present invention provides methods, kits and compositions for assessing or determining the effectiveness of an antiangiogenic treatment for brain cancer, in particular, glioma, based on the modulation in the circulating levels of miR-21 and/or miR-10b.

Definitions

To facilitate an understanding of the present invention, a number of terms and phrases are defined below. It is to be understood that these terms and phrases are for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

As referred to herein, the terms "polynucleotide molecules", “oligonucleotide”, "polynucleotide", "nucleic acid" and "nucleotide" sequences may interchangeably be used. The terms are directed to polymers of deoxyribonucleotides (DNA), ribonucleotides (RNA), and modified forms thereof in the form of a separate fragment or as a component of a larger construct, linear or branched, single stranded (ss), double stranded (ds), triple stranded (ts), or hybrids thereof. The term also encompasses RNA/DNA hybrids. The polynucleotides may be, for example, sense and antisense oligonucleotide or polynucleotide sequences of DNA or RNA. The DNA or RNA molecules may be, for example, but are not limited to: complementary DNA (cDNA), genomic DNA, synthesized DNA, recombinant DNA, or a hybrid thereof or an RNA molecule such as, for example, mRNA, shRNA, siRNA, miRNA, and the like. Accordingly, as used herein, the terms "polynucleotide molecules", “oligonucleotide”, "polynucleotide", "nucleic acid" and "nucleotide" sequences are meant to refer to both DNA and RNA molecules. The terms further include oligonucleotides composed of naturally occurring bases, sugars, and covalent inter nucleoside linkages, as well as oligonucleotides having non-naturally occurring portions, which function similarly to respective naturally occurring portions.

The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more

amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

As referred to herein, the term "Treating a disease" or "treating a condition" is directed to administering a composition, which comprises at least one reagent (which may be, for example, one or more polynucleotide molecules, one or more expression vectors, one or more substance/ingredient, and the like), effective to ameliorate symptoms associated with a disease, to lessen the severity or cure the disease, or to prevent the disease from occurring. Administration may include any administration route.

As used herein the terms "diagnosing" or "diagnosis" refer to the process of identifying/detecting cancer in a subject. Furthermore, the term encompasses screening for a cancer, determining the grade of the cancer, determining the stage of the cancer, distinguishing a cancer from other cancers including those cancers that may feature one or more similar or identical symptoms, providing prognosis of a cancer, monitoring cancer progression or relapse, assessing treatment efficacy, selecting a therapy for a cancer, optimization of a given therapy for a cancer, monitoring the treatment of a cancer, and/or predicting the suitability of a therapy for specific patients or subpopulations or determining the appropriate dosing of a therapeutic product in patients or subpopulations.

The term "organism" refers to a mammal. In some embodiments, the organism is human. In some embodiments, the organism is selected from a pet, a rodent, a farm animal, and a lab animal.

As used herein the term "subject" is interchangeable with an individual or patient. According to some embodiments, the subject is a mammal. According to some embodiments, the subject is a human. According to some embodiments, the subject is symptomatic. According to other embodiments, the subject is asymptomatic. According to some embodiments, the subject is suspected of having a brain cancer, such as, glioma. According to some embodiments, the subject is treated with an antiangiogenic drug.

As used herein the term “small interfering RNA” and “siRNA” are used interchangeably and refer to a nucleic acid molecule mediating RNA interference or gene silencing. The siRNA inhibits expression of a target gene and provides effective gene knock-down.

The terms "microRNA" and "miRNA" are directed to a small non-coding RNA molecule that can function in transcriptional and post-transcriptional regulation of target gene expression.

The terms “microRNA 21”, “hsa-mir-21”, “hsa-miR-21-5p” and “miR-21” may interchangeably be used. The terms are directed to a microRNA that is encoded by the MIR21 gene. In some embodiments, the human miR-21 (5p arm) has the nucleotide sequence :5 – UAGCUUAUCAGACUGAUGUUGA (SEQ ID NO:1).

The terms “microRNA 10b”, “hsa-mir-10b”, hsa-miR-10b-5p and “miR-10b” may interchangeably be used. In some embodiments, the human miR-10b (5p arm) has the nucleotide sequence: UACCCUGUAGAACCGAAUUUGUG (SEQ ID NO:2).

The term “circulating” with respect to miRNA molecules is directed to the presence of the miRNA in circulating blood (serum) and not localized to an organ or localized to a specific organ (such as the brain).

The term “brain cancer” and brain tumor” may interchangeably be used. The terms are directed to a primary tumor in the brain (that is, a tumor that originates from the brain tissue). In some embodiments, a brain tumor may include: Anaplastic astrocytoma, Astrocytoma, Central neurocytoma, Choroid plexus carcinoma, Choroid plexus papilloma, Choroid plexus tumor, Dysembryoplastic neuroepithelial tumour, Ependymal tumor, Fibrillary astrocytoma, Giant-cell glioblastoma, Glioblastoma multiforme, Gliomatosis cerebri, Gliosarcoma, Hemangiopericytoma, Medulloblastoma, Medulloepithelioma, Meningeal carcinomatosis, Neuroblastoma, Neurocytoma, Oligoastrocytoma, Oligodendroglioma, Optic nerve sheath meningioma, Pediatric ependymoma, Pilocytic astrocytoma, Pinealoblastoma, Pineocytoma, Pleomorphic anaplastic neuroblastoma, Pleomorphic xanthoastrocytoma, Primary central nervous system lymphoma, Sphenoid wing meningioma, Subependymal giant cell astrocytoma, Subependymoma, Trilateral retinoblastoma. In some embodiments, the cancer is glioma (which originates from astrocytes). In some embodiments, the cancer is high grade glioma. In sums embodiments, the cancer is Glioblastoma multiforme (GBM).

The term “antiangiogenic drug” or antiangiogenic treatment” may interchangeably be used. The terms are directed to a regent that function as an angiogenesis inhibitor, which may interfere with various steps in the process of angiogenesis (which is the formation of new blood vessels). For example, in some embodiments, an antiangiogenic drug is bevacizumab (Avastin), which is a

monoclonal antibody that specifically recognizes and binds to VEGF. When VEGF is attached to bevacizumab, it is unable to activate the VEGF receptor. Other angiogenesis inhibitors, including sorafenib and sunitinib, bind to receptors on the surface of endothelial cells or to other proteins in the downstream signaling pathways, blocking their activities. In some embodiments, an antiangiogenic drug may be selected from: Bevacizumab, itraconazole, carboxyamidotriazole, TNP-470, CM101, IFN-α, IL-12, platelet factor-4, suramin, SU5416, thrombospondin, VEGFR antagonists, angiostatic steroids and heparin, Cartilage-Derived Angiogenesis Inhibitory Factor, matrix metalloproteinase inhibitors, angiostatin, endostatin, -2methoxyestradiol, tecogalan, tetrathiomolybdate, thalidomide, thrombospondin and prolactin. Each possibility is a separate embodiment.

As used herein the term "biological sample" refers to any sample obtained from the subject being tested. According to some embodiments, the biological sample is a biopsy. According to some embodiments, the biological sample is selected from: cells, tissue and bodily fluid. Each possibility represents a separate embodiment of the invention. According to some embodiments, the biological sample is a fluid sample. According to some embodiments, the fluid sample is selected from the group consisting of: whole blood, plasma, serum, and the like. Each possibility represents a separate embodiment of the invention. In some embodiments, the biological sample is obtained or collected from the subject in any method known in the art. The sample may be collected from the subject by noninvasive, invasive or minimal invasive means. Each possibility represents a separate embodiment of the invention. According to some embodiments, the sample may be treated prior to being subjected to the methods of the present invention. According to some embodiments, the sample is a fluid sample and is substantially free of residual cells or debris of cells. For removing cells or debris of cells from within a bodily fluid sample, said cells or debris of cells may be precipitated by centrifugation and the supernatant is taken for determining the specific biomarkers levels. Typically, centrifugation of up to 2500 revolutions per minute (rpm) for up to 20 min is performed. A common centrifugation procedure is associated with centrifugation of 1100 rpm for 7 min, or centrifugation of 2000 rpm for 5 min. Alternatively, cells can be removed by filtration. According to some embodiments, the liquid sample undergoes concentration with a suitable membrane pore cut-off size. In accordance with some embodiments, a fluid sample treated with such filter is concentrated, namely, any molecule, particle below the size of the cut-off of the

filter is removed. According to some embodiments, the liquid sample is concentrated by up to 100 times the initial concentration.

According to some embodiments, the sample is a fluid sample, such as, serum, and the miRNA molecules within said fluid sample are analysed. In accordance with some embodiments, the sample is collected, centrifuged, the supernatant is removed and analysed for specific miRNA molecules. According to some embodiments, the sample is reconstituted (e.g. with fluids, such as, PBS or media). According to alternative embodiments the fluid sample is analyzed together with the cells present therein without prior separation. According to additional embodiments, the sample may conveniently be frozen after being collected from the subject and thawed before use. In some embodiments, the biological sample may also optionally comprise a sample that has not been physically removed from the subject.

According to some embodiments, the methods of the invention encompass determining the circulating levels of miR-10b and/or miR-21 in serum samples of a subject afflicted with glioma, wherein the samples are obtained before and after treatment with an antiangiogenic drug. In some embodiments, the serum samples may be obtained at various time points before commencement of treatment. In some embodiments, the time points before commencement of treatment may be any time point of between 1 hour to 6 months. In some embodiments, the time points before commencement of treatment may be any time point of between 1-2 months. In some embodiments, the serum samples may be obtained at various time points after commencement of treatment. In some embodiments, the time points after commencement of treatment may be any time point of between 1 hour to 24 months. In some embodiments, the time points after commencement of treatment may be any time point of between 1 month to 24 months. In some embodiments, the time points after commencement of treatment may be any time point of between 2 to 12 weeks. In some embodiments, the time points after commencement of treatment may be any time point of between 1 hour to 12 months. In some embodiments, the intervals between time points obtaining of serum samples may be predetermined. In some embodiments, the intervals between time points of obtaining of serum samples may be identical or different. For example, the intervals may include intervals of 1 day, one week, one month (i.e., one month between consecutive samples). For example, the intervals may include intervals of 2-12 weeks. For example, the intervals may include intervals of 1-4 weeks. For example, the intervals may include

intervals of 4-10 weeks. In some embodiments, the number of samples obtained before and/or after treatment as well as the time intervals between obtaining the samples may be predetermined or may be determined based on the patient condition, treatment regime, and the like.

According to some embodiments, the method of assessing treatment efficacy of antiangiogenic drug of patient with brain cancer may encompass, apart for the determining the levels of circulating miR-21 and/or miR-10b, any known methodologies used to assess the brain cancer condition and/or treatment efficacy.

As used herein the term "elevation" or "increase" of circulating levels of miR-21 and/or miR-10b refers, according to some embodiments, to a statistically significant elevation. The term is interchangeable with an increase. According to some embodiments, level is amount. According to some embodiments, levels of miR-21 and/or miR-10b reflect the expression of these nucleotide molecules in a serum sample of a subject. According to some embodiments, levels of miR-21 and/or miR-10b reflect the secretion of these polynucleotides from within cells and presence in the serum.

As used herein the term "modulation" of circulating levels of miR-21 and/or miR-10b refers to the change in the levels. The change may be increase or decrease. In some embodiments, the changes may be between various measurements obtained at different time points. In some embodiments, the changes may be between consecutive measurements.

According to some embodiments, there are provided methods for the detection of brain cancer condition in a subject and treatment efficacy thereof, the methods comprise detecting or determining circulating miR-21 and/or miR-10b levels in a serum sample of the subject, wherein an elevation in the levels of the miR-21 and/or miR-10b compared to a control value is indicative of degree of brain cancer in said subject. In some embodiments, the control value is zero (i.e., non detectable level). In some embodiments, the control value is any predetermined value. In some embodiments, the methods are qualitative. In some embodiments, the methods are quantitative.

According to some embodiments, the method for detection and assessing treatment efficacy of antiangiogenic drug in a subject afflicted with brain cancer comprise the steps of: a) collecting or obtaining a serum sample from the subject; b) detecting or determining the presence (level) of mir-10b and/or miR-21 in the serum sample; c) comparing the level of said mir-10b

and/or miR-21 to a control value; and d) determining the presence, stage, or efficacy of antiangiogenic treatment of brain cancer in the subject.

According to some embodiments, the methods of the invention may comprise one or more steps that may be performed in various orders.

For example, the method may be performed by the steps comprising:

a) collecting or obtaining a biological sample (for example, serum) from the subject;

b) extracting RNA from the sample; optionally, inducing cDNA formation; amlyfying miR-21 and/or miR-10b using specific primers;

c) determining, detecting and/or quantifying the amount of miR-21 and/or miR-10b in the sample; and

d) comparing the level of the miRNAs in the sample to a control value.

According to some embodiments, the method for determining the effectiveness of a treatment of a subject with an antiangiogenic treatment, may include one or more of the steps of:

a) determining the levels of circulating miR-21 and/or miR-10b in a first serum sample of the subject;
b) subjecting the subject with an antiangiogenic treatment of cancer;
c) determining the levels of circulating miR-21 and/or miR-10b in a second serum sample of said subject; and
d) comparing said levels of circulating miR-21 and/or miR-10b in the first and second biological samples,

wherein a significant increase in of circulating miR-21 and/or miR-10b levels from the first to the second biological samples is indicative of said treatment being efficacious.

In some embodiments, the method may further include repeating step c) one or more times at designated time intervals to obtain consecutive serum samples and further comparing the levels of the miRNA levels between consecutive samples, to determine if the levels are modulated to determine the efficacy of treatment. For example, is the levels between an earlier sample and a later (consecutive) sample is increased, it is indicative that the treatment is effective. For example, if the levels between an earlier samples and a later (consecutive) sample has not changed or has decreased, it is indicative that the treatment efficacy is reduced.

According to some embodiments, one or more first samples are taken at a time point prior to initiation of the treatment for cancer and one or more second samples are taken at a time point during or after the treatment. According to some embodiments, one or more first samples are taken at a time point during the treatment (step b) and one or more second samples are taken at a time point during the treatment and subsequent to the time point of the one or more first samples. According to some embodiments, one or more first samples are taken at a time point during the treatment (step b) and one or more second samples are taken at a time point after the treatment has been discontinued. According to some embodiments, one or more second samples may be obtained consecutively, at designated time intervals (such as, in the range of 1 -10 weeks). According to some embodiments, an increase in the level of the miR-21 and/or miR-10b exhibited in the at least one second sample as compared to that determined for the first sample or compared to a previous consecutive second serum sample, is indicative that the treatment is effective. According to some embodiments, a decrease or no change in the level of miR-21 and/or miR-10b in the at least one second samples as compared to the first sample or to a previously consecutively obtained second sample, is indicative that the treatment is ineffective (or unsuccessful).

According to some embodiments, the antiangiogenic treatment may include administration of an antiangiogenic drug selected from: Bevacizumab (Avastin), itraconazole, carboxyamidotriazole, TNP-470, CM101, IFN-α, IL-12, platelet factor-4, suramin, SU5416, thrombospondin, VEGFR antagonists, angiostatic steroids and heparin, Cartilage-Derived Angiogenesis Inhibitory Factor, matrix metalloproteinase inhibitors, angiostatin, endostatin, -2 methoxyestradiol, tecogalan, tetrathiomolybdate, thalidomide, thrombospondin and prolactin. According to some embodiments the antiangiogenic drug is Bevacizumab.

According to some embodiments the brain tumor is glioma. In some embodiments, the glioma is high grade glioma.

In some embodiments, the level of miRNA in the first serum sample and/or the second serum sample is determined by amplification reaction. In some embodiments, the amplification reaction comprises real-time PCR using specific primers for miR-21 and/or miR-10b. In some embodiments, the method may further include a step of isolating total RNA from the first serum sample and/or the second serum prior to identification of the miRNA.

According to some embodiments, there is provided a method for assessing treatment efficacy of antiangiogenic drug in a cancer subject having brain tumor, the method comprising determining the level of circulating miRNA molecules in serum of said subjects before and after treatment with the antiangiogenic drug, wherein an increased level of expression of the miRNA in the serum is indicative of increased treatment efficacy, wherein the miRNA molecules are selected from miR-10b, miR-21 or both.

In some embodiments, the antiangiogenic drug may be selected from: Bevacizumab (Avastin), itraconazole, carboxyamidotriazole, TNP-470, CM101, IFN-α, IL-12, platelet factor-4, suramin, SU5416, thrombospondin, VEGFR antagonists, angiostatic steroids and heparin, Cartilage-Derived Angiogenesis Inhibitory Factor, matrix metalloproteinase inhibitors, angiostatin, endostatin, -2methoxyestradiol, tecogalan, tetrathiomolybdate, thalidomide, thrombospondin and prolactin. According to some embodiments the antiangiogenic drug is Bevacizumab.

In some embodiments, the brain tumor is glioma. In some embodiments, the glioma is glioblastoma (GBM). In some embodiments, the glioma is high grade glioma.

In some embodiments, the level of miRNA in the serum is determined and/or quantified by amplification reaction. In some embodiments, RNA (for example, total RNA) is isolated from the serum prior to identification of the miRNA.

According to some embodiments, amplification reactions/methods include polymerase chain reaction (PCR). PCR may be quantitative real-time PCR (qRT-PCR), reverse transcription PCR (RT-PCR), or combinations thereof. Each possibility represents a separate embodiment of the invention.

In some embodiments, identification and/or quantification of the specific miRNAs in the serum may be performed by use of specific probes or tags capable of specifically interacting with the miRNA molecules. In some embodiments, such tags or probes may include nucleic acid molecules. In some embodiments, such tags or probes may include nucleic acid probe, nucleic acid primers, peptidic-tags, and the like.

According to some embodiments, suitable methods to detect and/or quantify miRNA may include such methods as, but not limited to: amplification reaction, such as, PCR, RT-PCR, real

time PCR, and the like; Gene expression microarrays, such as Affymetrix, Agilent, and Illumina microarray platforms; NanoString technology, including nCounter miRNA expression assays, which employs unique fluorescent–tagging of individual miRNA species followed by two-dimensional display and optical scanning and counting of miRNA molecules; Sequencing, such as, next Generation Sequencing (NGS) using specific adaptors and/or probes; and the like, or combinations thereof.

In some embodiments, the method may further include evaluating the tumor status by clinical parameters. In some embodiments, there is a correlation between circulating levels of the miRNAs and the clinical parameters. In some embodiments, the correlation is inverse correlation.

In some embodiments, the level of the circulating miRNA mir-10B and/or miR-21 is determined at one or more time points after initiation of treatment with the antiangiogenic drug. In some embodiments, the level of the circulating miRNA mir-10B and/or miR-21 is determined at various time points after initiation of treatment with the antiangiogenic drug. In some embodiments, the levels of circulating miRNA mir-10b and/or miR-21 is compared between consecutive measurements (i.e., measurements obtained between two samples obtained at consecutive time points) and treatment efficacy is determined based on the comparison.

According to some embodiments, the method of "determining severity" of cancer refers to determining cancer progression. In accordance with this embodiment, the control value is a value of the subject to which cancer progression is assessed. According to some embodiments, the method of "determining severity" of cancer refers to determining prognosis of the cancer.

According to some embodiments, the methods disclosed herein are applicable for determining a cancer state, cancer severity as well as treatment efficiency at any stage of cancer as well as for determining recurrent cancer.

As used herein the term "Cancer recurrence" is interchangeable with "cancer relapse" and refers to the return of a sign, symptom or disease after a remission. The cancer cells may re-appear in the same site of the primary tumor or in another location, such as in secondary cancer.

The methods of the invention are useful for "managing subject treatment" by the clinician or physician subsequent to the determination of treatment efficacy or severity of the cancerous state (e.g., cancer status). For example, if the severity of the cancerous state indicates that surgery

is appropriate, the physician may schedule the patient for surgery. Alternatively, if the severity of the cancerous state is acute or a late stage cancer, no further action may be warranted. Furthermore, if the results show that treatment has been successful, no further management may be necessary. Alternatively, if the result of the methods of the present invention is inconclusive or there is reason that confirmation of status is necessary, the physician may order more tests.

According to some embodiments, based on the determination of the treatment efficacy, treatment regime may be adjusted or modulated. Adjustment of treatment regime may include, for example, but not limited to: adjustment of dosage (reducing or increasing dosage), ceasing treatment, replacing or adding a drug, and the like

According to some embodiments, there is provided a kit for determining efficacy of an antiangiogenic treatment for brain cancer, comprising means for determining the levels of miR-21 and/or miR-10b in a serum sample of a subject, and instructions for using the kit in the determining efficacy of the antiangiogenic treatment for the brain cancer.

In some embodiments, the serum sample is obtained before and after the antiangiogenic treatment.

In some embodiments, the antiangiogenic treatment comprises administration of Bevacizumab to the subject.

In some embodiments, the means for determining the levels of miR-21 and/or miR-10b may include specific nucleic acid molecules (such as specific primers or probes) for identification of miR-21 and/or miR-10b by a suitable method, such as, for example, amplification reaction or sequencing reaction performed on RNA isolated from the serum sample.

According to some embodiments aspect, the present invention provide kits for the determining efficacy of an antiangiogenic treatment for brain cancer, comprising means for determining the levels of circulating miR-21 and/or miR-10b, in a serum sample of a subject afflicted with brain tumor and instructions for using said kits.

In some embodiments, it is disclosed herein that miR-21 and/or miR10b which are differentially quantified before and after an antiangiogenic therapy can serve as predictors for treatment response to the antiangiogenic therapy.

According to some embodiments, it is disclosed herein that miRNA-10b and mir-21 negatively and significantly correlate with enhancing and FLAIR tumor measurements in patients treated with bevacizumab.

According to some embodiments, it is disclosed herein that only two miRNAs (miR-10b and miR-21) that show differential quantification following bevacizumab treatment correlated significantly with enhancing and FLAIR tumor measurements either alone or the sum of both miRNAs.

According to some embodiments, it is disclosed herein that the quantification of both miR-10b and miR-21 is markedly altered by bevacizumab treatment and that the increased quantification tends to persist throughout the treatment period.

According to some embodiments, it is disclosed herein that miR-10b and miR-21, which are hypoxia-related, are significantly and highly quantified in the bloodstream of our high-grade glioma patients and in glial tumors.

According to some embodiments, it is disclosed herein that a significant negative correlation of miR-10b and miR-21 with cross sectional enhancing tumor diameters exists.

According to some embodiments, it is disclosed herein that there is lack of significant association between circulating miR-10b and miR-21, tumor measurements and RANO assessment in the patients treated with temozolomide (non antiangiogenic drug).

According to some embodiments, it is disclosed herein that the increased quantification of the circulating miR-10b and miR-21 under bevacizumab treatment marks the antiangiogenic effect of the treatment.

The terms "comprises", "comprising", "includes", "including", “having” and their conjugates mean "including but not limited to”. The terms "comprises" and "comprising" are limited in some embodiments to "consists" and "consisting", respectively. The term “consisting of" means “including and limited to“. The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein the term “about” in reference to a numerical value stated herein is to be understood as the stated value +/- 10%.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

The following examples are presented in order to more fully illustrate some embodiments of the invention. They should, in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLES

Materials and methods

Patient samples

One hundred and twenty serum samples were prospectively obtained for total RNA extraction from 28 adult patients diagnosed with either glioblastoma (n=23) or anaplastic astrocytoma (Table 1). Eligible patients were treated according to standard or experimental protocols and received first line temozolomide treatment (standard treatment, TMZ group) or bevacizumab (10 mg/kg every 14 days), either as second line treatment at tumor progression or as a first line therapy as part of the Avaglio study (ClinicalTrials.gov number NCT00943826) (bevacizumab group, Table 1). The first blood sample (n=28) was obtained prior to the initial diagnostic procedure (surgery or biopsy) and prior to any treatment. All subsequent samples (n=92) were attained within 14 days of a routine follow-up MRI evaluation. In the bevacizumab group a single sample was taken prior to initiation of bevacizumab treatment and all subsequent samples were taken under active bevacizumab therapy. At each point patients were assessed by physical and neurological exam and clinical tumor responses were evaluated by the RANO criteria (36). Routine MRI evaluation included gadolinium enhanced T1 weighted images and FLAIR sequences that served for tumor measurements. The sums of product of the perpendicular diameters of enhancing lesions and of FLAIR changes were recorded at each clinic visit.

RNA isolation

Total RNA was extracted from serum using MasterPure™ RNA Purification Kit (epicentre) according to the manufacturer's instructions with the following modifications: prior to RNA extraction the serum was centrifuged at RT for 1 min at 14K RPM in order to precipitate fat which might interfere with the reaction and 1µl of glycogen was added as a carrier at the start of the reaction.

cDNA

cDNA was produced using the qScript™ microRNA cDNA Synthesis Kit (Quanta Biosciences) according to the manufacturer's instructions. Briefly, RNA was polyadenylated with ATP by poly(A) polymerase at 37°C for 1 hr and then reverse transcribed.

RT real time PCR amplification and relative quantification

The miRNA amplification was carried out using the suitable primers of the relevant miRNAs (5-3):

RNU6-1 –

GUGCUCGCUUCGGCAGCACAUAUACUAAAAUUGGAACGAUACAGAGAAGAUUAG

CAUGGCCCCUGCGCAAGGAUGACACGCAAAUUCGUGAAGCGUUCCAUAUUUU

(SEQ ID NO:3)

hsa-miR-210-3p: CUGUGCGUGUGACAGCGGCUGA (SEQ ID NO:4)

hsa-miR-196b-5p :UAGGUAGUUUCCUGUUGUUGGG (SEQ ID NO:5)

hsa-miR-10b-5p :UACCCUGUAGAACCGAAUUUGUG (SEQ ID NO:2)

hsa-miR-21-5p: UAGCUUAUCAGACUGAUGUUGA (SEQ ID NO:1).

The reaction was performed using PerfeCTa SYBR Green FastMix ROX (Quanta Biosciences). qPCR reactions were performed using Step One Plus Real Time PCR (Applied Biosystems) in 96 well plates. PCR was initiated at 95°C for 2 min, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. The specificity of the reaction was verified by melt curve analysis. The relative quantification (RQ) of each miRNA was calculated based on its average quantification in the serum of 10 healthy volunteers and normalized to RNU6 using the comparative CT method. To analyze the differential quantification between healthy controls and patients with gliomas, relative quantification has been performed using CT values normalized to RNU-6 expressed as $2^{-[Ct(miRNA)-Ct(RNU-6]}$ ($2^{-\Delta CT}$).

Statistical analysis

Average fold changes of miRNA levels were used to evaluate the quantification level. Quantification levels were compared between the groups of healthy controls and tumor bearing patients using the student's t-test. To analyze the association between two selected variables, namely tumor measurements and miRNA expression or RANO assessment, Pearson product moment correlation coefficients were used based on a two-tailed test. For analysis of the correlation with RANO evaluation the latter was categorized numerically as progressive disease, stable disease partial response or complete response. Correlation tests were performed using binary variable of the fold changes. P-value< 0.05, was considered statistically significant.

Example 1 – Expression of circulating miRNAs in patients with glioma and in healthy subjects

Previous study (19) have shown that several miRNA molecules, including, miR-302b-3p, miR-21, miR-210, miR-10b, miR-196b, and miR-1271 are up-regulated in glial tumors as compared to normal brain, with a demonstrated fold expression of 3.56, 9.77, 57.02, 107.6, 548 and 1138 for miR-302b-3p, miR-21, miR-210, miR-10b, miR-196b and miR-1271, respectively.

The presence of the miRNAs was tested for detection in the serum of healthy individuals and the serum of patients harboring high-grade gliomas. The quantification of the miRNAs was evaluated in the serum samples of both 10 healthy controls and 30 high grade glioma patients. Serum was obtained from brain tumor patients on the day of hospitalization, prior to the first diagnostic surgical procedure. The results shown in **Fig. 1**, demonstrate that, surprisingly, significantly increased quantification level of only miR-10b and miR-21 was detected in the serum of the affected patients whereas miR-210, miR-196b and miR-1271 levels did not differ from healthy controls (figure 1). There was no evident detection of miR-302b-3p in the serum samples of either healthy controls or brain tumor patients.

Example 2 – Determining association between various types of treatment and changing dynamics of the circulating miRNA

To evaluate whether bevacizumab treatment is associated with changing dynamics of the circulating miRNA, the findings in the bevacizumab treated group were compared to those obtained for patients treated with a non-antiangiogenic drug, temozolomide. Temozolomide is not an antiangiogenic drug, but rather an alkylating agent, affecting the DNA methylation. The two treatment groups did not differ significantly regarding patients' characteristics and tumor sizes that were measured on initial evaluation and at last follow up (**Table 1**).

Table 1: Patients Characteristics

Characteristic	TMZ group (n=13)	Bevacizumab group (n=15)
Age, years Median (Range)	60 (32-78)	54 (23-72)
Gender Male/Female	8/ 5	8/ 7
Surgical procedure Resection/ Biopsy	12/ 1	14/ 1
Histology Anaplastic Astrocytoma Glioblastoma	2 11	3 12
Antiepileptic drugs Yes / No	10/ 3	11/ 4
Contrast enhancement Prior to surgery Prior to bevacizumab	12 -	13 14
Sum products of perpendicular diameters of enhancing lesion(s), median, mm Prior to surgery Prior to bevacizumab Last F/U	68 (0-152) - 75 (25-182)	48 (0-111) 87 (0-123) 82 (10-157)
Initial therapy Radiotherapy plus TMZ No Radiotherapy	13 -	14 1
Number of F/U serum samples Median (range)	4 (2-6)	3.5 (2-6)
Bevacizumab First line (Avaglio study) Second line (at progression) No. of treatments, median	-	4 11 12(2-30)
Second line chemotherapy (with bevacizumab) Carboplatin	-	3

PCV None		1 11

TMZ=Temozolomide; PCV=procarbazine, CCNU and vincristine; F/U=follow up

In order to evaluate the dynamics of measurements at each time point, the figures were converted to fold changes from previous measurement. Each treatment group was assessed separately.

The results of the longitudinal evaluations and its association with tumor measurements and RANO evaluation of response to treatment are presented in Table 2. As shown in Table 2, which presents the correlation between fold changes of miRNAs, the radiological evaluation of tumor dimensions and changes in the response assessment by RANO criteria, a significant negative correlation was found only in the bevacizumab group between enhancing tumor diameters and fold changes of either one of the miRNAs and for the average change of both miRNAs. Changes in FLAIR measurements in the bevacizumab group were found to correlate significantly and negatively mainly with fold changes of miR-10b and, and no significant correlation was found between changes of RANO evaluation and the expression of miRNAs. In the temozolomide group no significant correlation was found between fold changes of either the individual miRNAs or their combination or the measurements of tumor dimension and clinical response.

The results demonstrate that the circulating levels of miR-10b and/or miR-21 do not correlate with the Temozolomide treatment, whereas such correlation is identified for the Bevacizumab treatment.

Table 2. Association between fold changes of variables of tumor measurements, RANO assessment and miRNAs quantification under Temozolomide or Bevacizumab treatment.

	miR-10b		miR-21		Sum of miR-10b and miR-21	
	TMZ	Bev.	TMZ	Bev.	TMZ	Bev.
Sum of enhancing diameters	r= -0.023 p=0.906	r= -0.51 p<0.002	r= -0.106 p=0.532	r= -0.568 p<0.0001	r= 0.071 p=0.676	r= -0.648 p<0.0001
Sum of FLAIR diameters	r= -0.249 p=0.137	r= -0.365 p=0.031	r= -0.173 p=0.314	r= -0.248 p=0.151	r= -0.094 p=0.580	r= -0.322 p=0.059
RANO	r= -0.08 p=0.703	r= -0.309 p=0.068	r= -0.32 p=0.118	r= -0.189 p=0.270	r= -0.161 p=0.441	r=-0.116 p=0.523

TMZ = Temozolomide group, Bev. =Bevacizumab group

Example 3– Determining association between antiangiogenic treatment and changing dynamics of the circulating miRNAs

The results presented in Figs. 2A-B, 3-4, illustrate fold changes from pre-bevacizumab values and present only patients who had at least two follow up evaluations on bevacizumab treatment. Both miR-10b and miR-21 display high quantification levels when compared to pre bevacizumab levels as demonstrated on **Fig. 2A** where the sum of the relative quantification of the two miRNAs at each time point is shown. In 9 of the 15 patients miR-10b and miR-21 were highly induced (with fold induction ranging between 1.5–18 and 1.5 – 5, respectively), following the first bevacizumab treatment. **Fig. 2B** shows the longitudinal changes in the enhancing tumor measurements on imaging. In almost all the patients, the sum products of the perpendicular enhancing diameters was clearly reduced on bevacizumab treatment. The results presented in **Fig. 3**, show longitudinal evaluations of circulating miRNAs quantification and enhancing tumor diameters during bevacizumab treatment in 10 individual patients. The line graphs show the Percentage fold changes over time in the sum quantification of circulating miR-10b and miR-21 (light gray line) and in sum products of cross sectional enhancing diameters of the tumor measured on MRI (dark gray line) in 10 individual patients that had more than 2 measurements. Shown is

fold change from pre-bevacizumab values for the sum of both miRNA quantification and for the sum of products of perpendicular diameters of the enhancing tumor. At each time point, response to treatment was also assessed by RANO criteria and the longitudinal results are shown in **Fig. 4**. The combined clinical-radiological assessment categorized tumor response as stable disease in the majority of patients prior to the point that a progressive disease was notified. Some patients maintained the response for a prolonged period of time. In order to assess whether the dynamic of circulating miRNAs quantification reflects tumor response to bevacizumab therapy or to chemotherapy, fold changes of the variables at each time point were used for the correlation analysis which was performed similarly for the temozolomide treated group and for bevacizumab treated patients.

All in all, the results presented decorate the dynamic effects of antiangiogenic therapy on the level of circulating miR-10b and miR-21 in patients with high-grade gliomas.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without undue experimentation and without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation. The means, materials, and steps for carrying out various disclosed functions may take a variety of alternative forms without departing from the invention.

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CLAIMS:

1. A method for determining efficacy of antiangiogenic treatment for a brain cancer in a subject in need thereof, said method comprising:

   a) determining the levels of circulating miR-21 and/or miR-10b in a first serum sample of said subject;

   b) treating said subject with an antiangiogenic treatment;

   c) determining the levels of circulating miR-21 and/or miR-10b in a second serum sample of said subject; and

   d) comparing the levels of circulating miR-21 and/or miR-10b in said first and second serum samples,

   wherein modulation in the levels of circulating miR-21 and/or miR-10b from the first to the second serum sample is indicative of said treatment efficacy.

2. The method of claim 1, wherein, step a) is conducted at a time point prior to step b).
3. The method of claim 1, wherein steps a) and c) are conducted at distinct time points during step b).
4. The method of claim 1 further comprising repeating step c) for one or more times to determine the levels of circulating miR-21 and/or miR-10b in consecutive serum samples, obtained at designated time intervals; and comparing the levels of circulating miR-21 and/or miR-10b between said consecutive serum samples, wherein a modulation in the levels of circulating miR-21 and/or miR-10b between consecutive serum samples is indicative of said treatment efficacy.

5. The method of claim 4, wherein the time intervals between consecutive serum samples is in the range of 1-10 weeks.
6. The method of claim 1, wherein an increase in the levels of circulating miR-21 and/or miR-10b from the first to the second serum sample is indicative of the treatment being efficacious.
7. The method of claim 4, wherein an increase in the levels of circulating miR-21 and/or miR-10b from an earlier second serum sample and a later consecutive second serum sample is indicative of the treatment being efficacious.
8. The method of claim 4, wherein a decrease in the levels of circulating miR-21 and/or miR-10b from an earlier second serum sample and a later consecutive second serum sample is indicative of a reduction in treatment efficacy.
9. The method of claim 1, wherein the antiangiogenic treatment comprises an antiangiogenic drug selected from: Bevacizumab, itraconazole, carboxyamidotriazole, TNP-470, CM101, IFN-α, IL-12, platelet factor-4, suramin, SU5416, thrombospondin, VEGFR antagonists, angiostatic steroids and heparin, Cartilage-Derived Angiogenesis Inhibitory Factor, matrix metalloproteinase inhibitors, angiostatin, endostatin, -2methoxyestradiol, tecogalan, tetrathiomolybdate, thalidomide, thrombospondin and prolactin.
10. The method of claim 9, wherein the antiangiogenic drug is Bevacizumab.
11. The method of claim 1, wherein the brain tumor is glioma.
12. The method of claim 11, wherein the glioma is high grade glioma.
13. The method of claim 1, further comprising a step of isolating RNA from the first serum sample and/or the second serum prior to identification of the miRNA.

14. The method of claim 1, wherein the level of miRNA in the first serum sample and/or the second serum sample is determined by a method selected from: amplification reaction, sequencing reaction, microarray, or combinations thereof.

15. The method of claim 14, wherein the amplification reaction comprises PCR using specific nucleic acid primers for miR-21 and/or miR-10b.

16. The method of claim 14, wherein the sequencing reaction is next-generation sequencing, using specific nucleic-acid probes.

17. The method of claim 1, wherein the antiangiogenic treatment regime is adjusted based on the determined treatment efficacy.

18. A method for assessing treatment efficacy of antiangiogenic drug in a cancer subject having brain tumor, the method comprising determining the level of circulating miRNA molecules in serum samples of said subjects obtained before and after treatment with the antiangiogenic drug, and comparing the levels of the circulating miRNA molecules between the serum samples, wherein an increased level of expression of the miRNA between the serum samples obtained before and after treatment is indicative of increased treatment efficacy, wherein the miRNA molecules are selected from miR-10b, miR-21 or both.

19. The method of claim 18, wherein the level of the circulating miRNA mir-10B and/or miR-21 is determined at one or more time points after initiation of treatment with the antiangiogenic drug on consecutive serum samples.

20. The method of claim 19, further comprising comparing the circulating miRNA molecules between the consecutive serum samples, wherein an increased level of expression of the miRNA between consecutive serum samples is indicative of increased treatment efficacy.

21. The method of claim 19, wherein the consecutive serum samples are obtained at time intervals in the range of 1-10 weeks.

22. The method of claim 18, wherein the antiangiogenic drug is selected from Bevacizumab, itraconazole, carboxyamidotriazole, TNP-470, CM101, IFN-α, IL-12, platelet factor-4, suramin, SU5416, thrombospondin, VEGFR antagonists, angiostatic steroids +heparin, Cartilage-Derived Angiogenesis Inhibitory Factor, matrix metalloproteinase inhibitors, angiostatin, endostatin, -2methoxyestradiol, tecogalan, tetrathiomolybdate, thalidomide, thrombospondin and prolactin.

23. The method of claim 18, wherein the antiangiogenic drug is Bevacizumab.

24. The method of claim 18, wherein the brain tumor is glioma.

25. The method of claim 24, wherein the glioma is glioblastoma (GBM).

26. The method of claim 24, wherein the glioma is high grade glioma.

27. The method of claim 18, wherein RNA is isolated from the serum samples prior to identification of the miRNA.

28. The method of claim 18, wherein the level of miRNA in the serum is determined by a method selected from: amplification reaction, sequencing reaction, microarray, or combinations thereof.

29. The method of claim 28, wherein the amplification reaction comprises PCR using specific nucleic acid primers for miR-21 and/or miR-10b.

30. The method of claim 28, wherein the sequencing reaction is selected from next-generation sequencing, using specific nucleic-acid probes.

31. The method of claim 18, further comprising evaluating the tumor status by clinical parameters.

32. A kit for determining efficacy of an antiangiogenic treatment for brain cancer, comprising means for determining the levels of miR-21 and/or miR-10b in serum samples of a subject obtained before and after the antiangiogenic treatment, and instructions for using the kit in the determining efficacy of the antiangiogenic treatment for the brain cancer.
33. The kit of claim 32, wherein increase in the levels of miR-21 and/or miR-10b between the serum samples obtained before and after treatment in indicative of the treatment being efficacious.
34. The kit of claim 32, wherein the antiangiogenic treatment comprises administration of Bevacizumab to the subject.
35. The kit of claim 32, wherein the cancer is glioma.
36. The kit of claim 32, wherein the means comprises specific nucleic acid molecules for identification of miR-21 and/or miR-10b in the serum samples.
37. The kit of claim 36, wherein the nucleic acid molecules comprise specific primers for identification of miR-21 and/or miR-10b in an amplification reaction performed on RNA isolated from the serum sample.
38. The kit of claim 37, wherein the amplification reaction is selected from, PCR, RT-PCR, real-time PCR, or combinations thereof.
39. The kit of claim 36, wherein the nucleic acid molecules comprise specific probes for identification of miR-21 and/or miR-10b in a sequencing reaction performed on RNA or DNA isolated from the serum sample.

Fig. 1

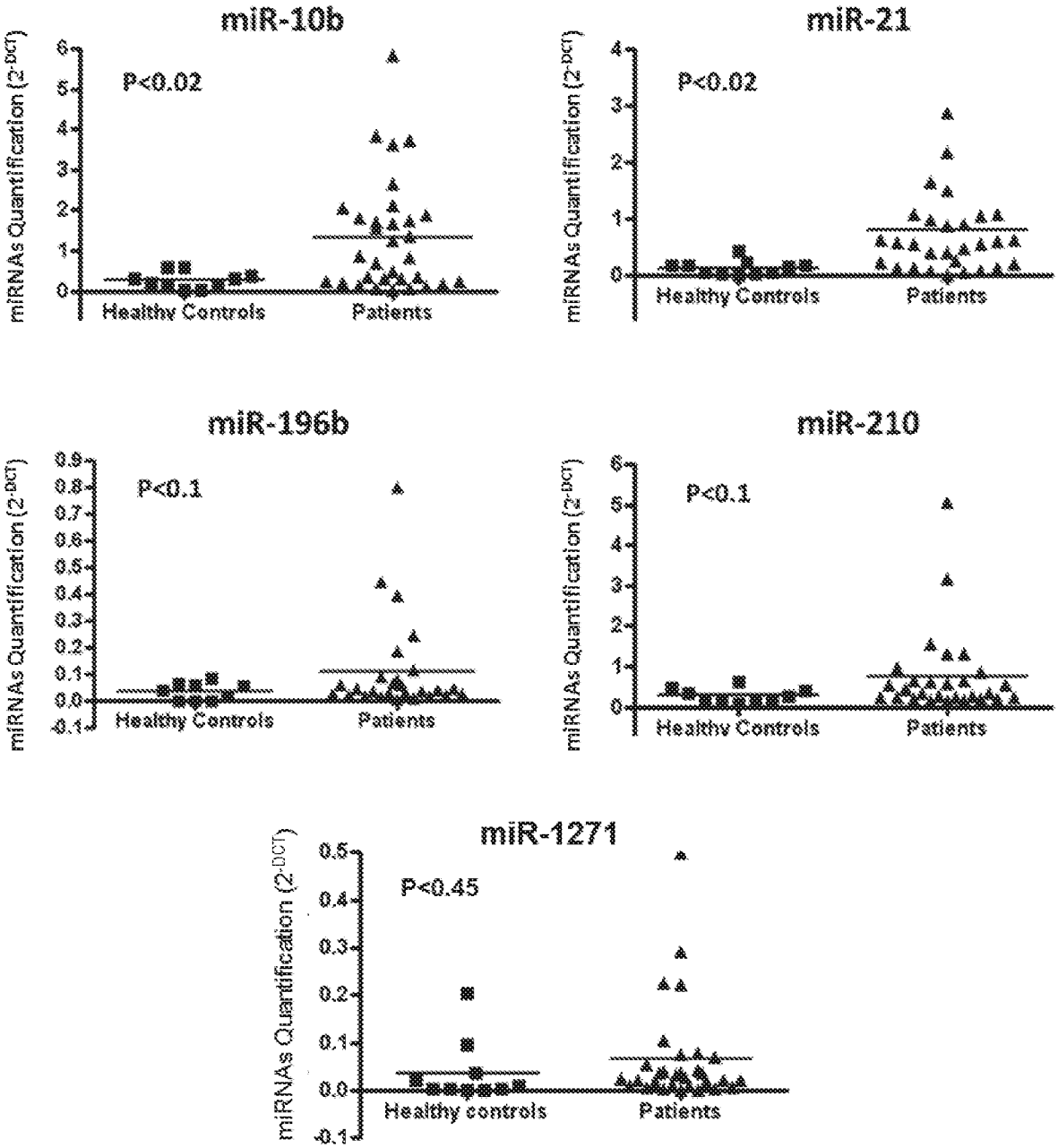

miR-10b
P<0.02
miRNAs Quantification (2-DCT)
Healthy Controls
Patients
miR-21
P<0.02
miRNAs Quantification (2-DCT)
Healthy Controls
Patients
miR-196b
P<0.1
miRNAs Quantification (2-DCT)
Healthy Controls
Patients
miR-210
P<0.1
miRNAs Quantification (2-DCT)
Healthy Controls
Patients
miR-1271
P<0.45
miRNAs Quantification (2-DCT)
Healthy controls
Patients

Fig. 2A

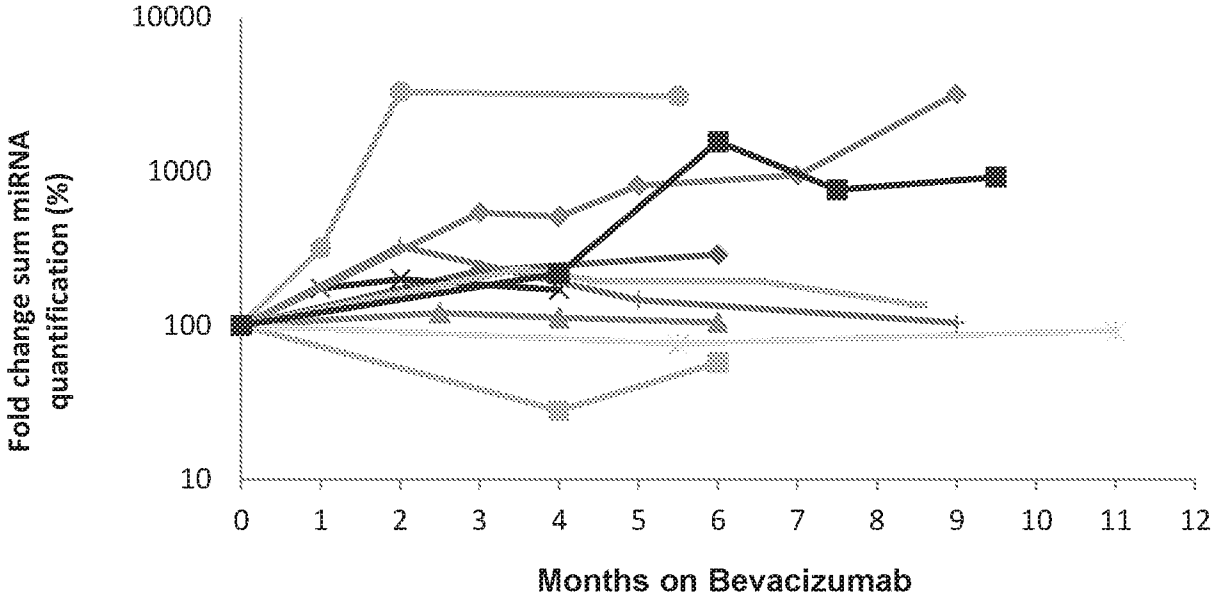

10000
1000
100
10
Fold change sum miRNA quantification (%)
0 1 2 3 4 5 6 7 8 9 10 11 12
Months on Bevacizumab

Fig. 2B

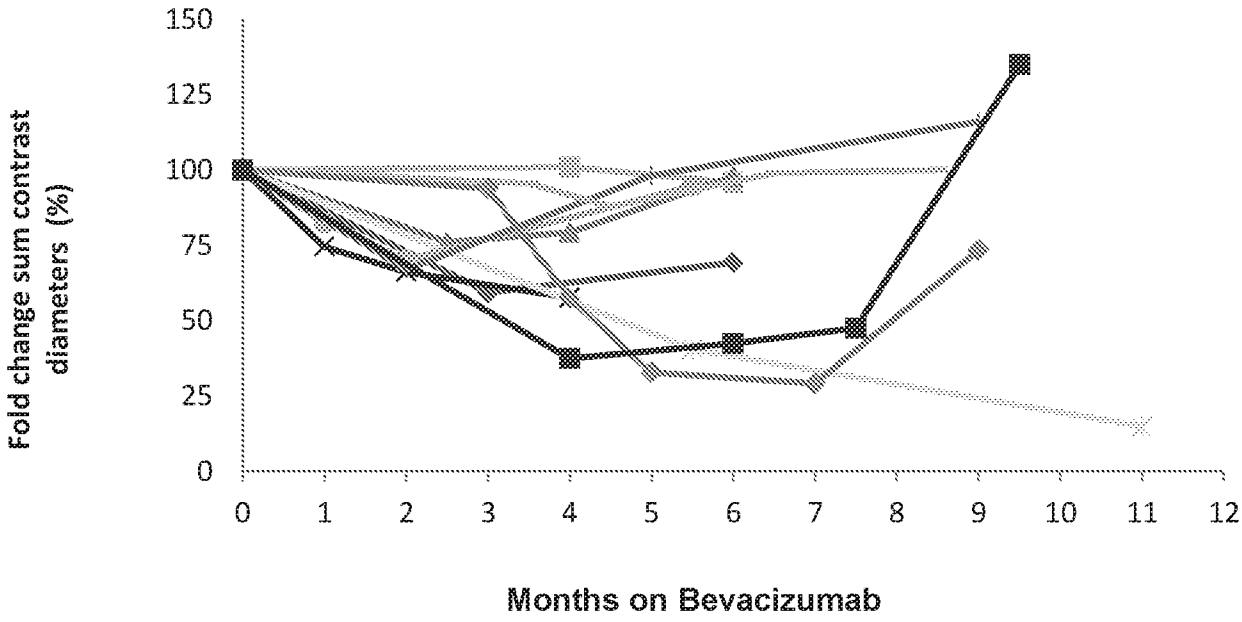

150
125
100
75
50
25
0
Fold change sum contrast diameters (%)
0 1 2 3 4 5 6 7 8 9 10 11 12
Months on Bevacizumab

Fig. 3

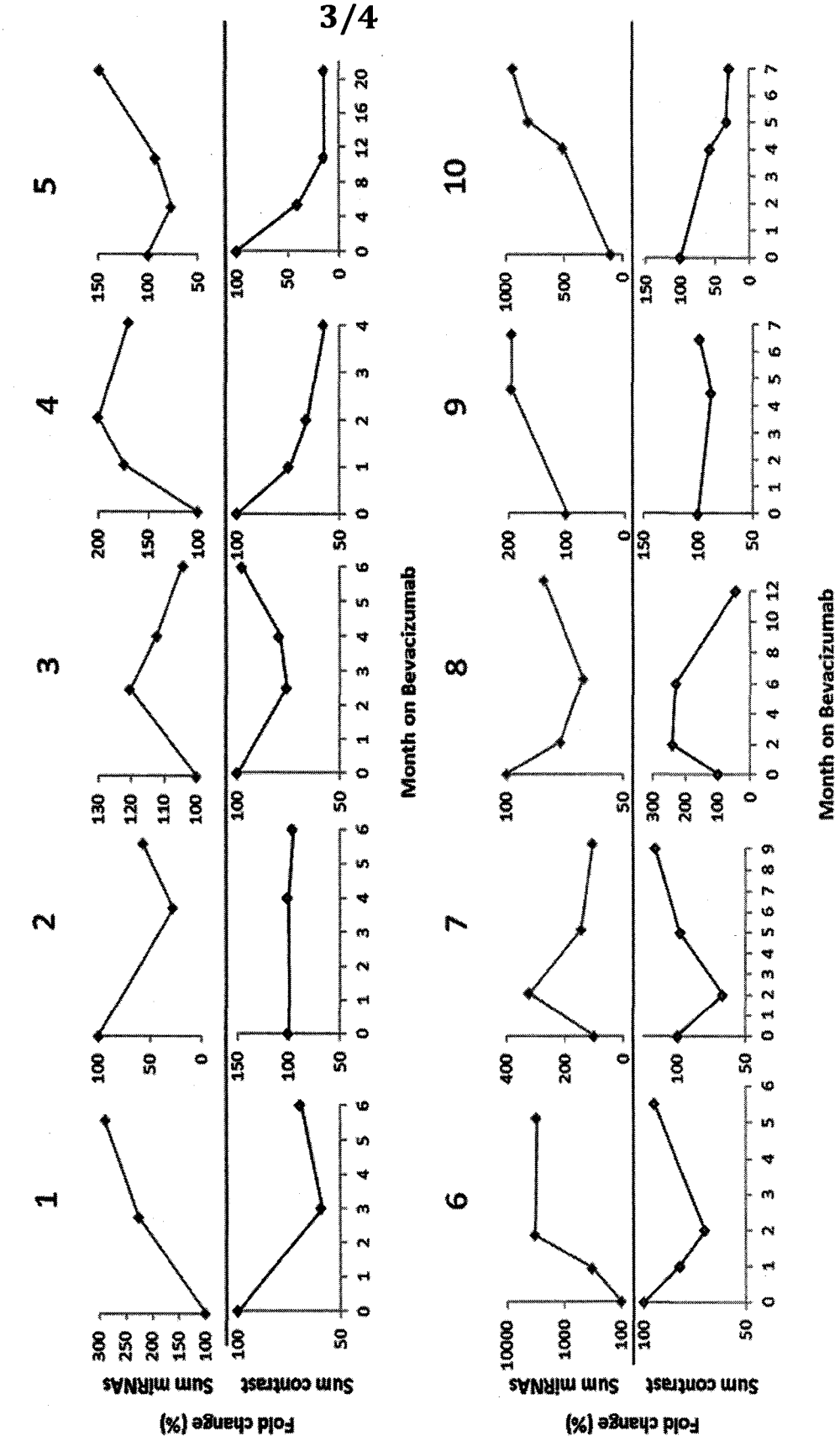
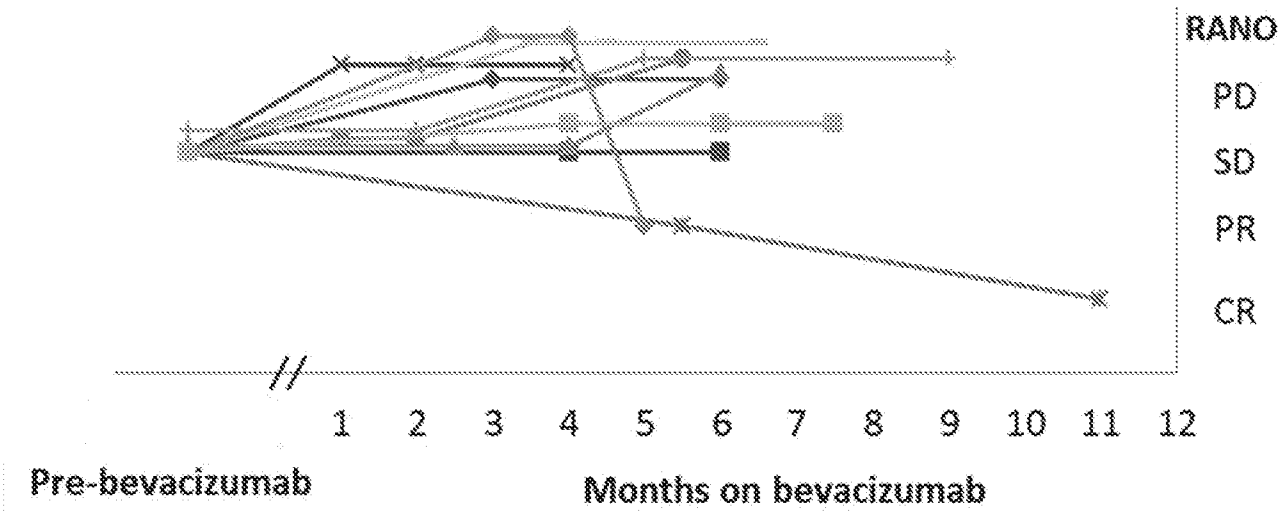

1
2
3
4
5
6
7
8
9
10
Fold change (%)
Sum miRNAs
Sum contrast
Month on Bevacizumab

Fig. 4


RANO
PD
SD
PR
CR
1
2
3
4
5
6
7
8
9
10
11
12
Pre-bevacizumab
Months on bevacizumab

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL2016/050048

A. CLASSIFICATION OF SUBJECT MATTER
IPC (2016.01) C12Q 1/68, G01N 33/574

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)
IPC (2016.01) C12Q 1/68

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)
Databases consulted: NCBI, PATENTSCOPE, THOMSON INNOVATION, Esp@cenet, Google Patents, Google Scholar, PatBase
Search terms used: glioblastoma, brain, miR-10b, miR-21, miRNA, bevacizumab, antiangiogenic, treatment efficacy

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011109440 A1 CARIS LIFE SCIENCES LUXEMBOURG HOLDINGS (LU) 09 Sep 2011 (2011/09/09) para 0006, 0009, 00292, 00356, 00486, 00919-00921, Fig. 22, claims 3,7,19, 22, 24-25, 58	1-5,9-17,32-39
A	Teplyuk et al:"MicroRNAs in cerebrospinal fluid identify glioblastoma and metastatic brain cancers and reflect disease activity". Neuro-Oncology Advance Access April 4, 2012, pages 1-12, doi:10.1093/neuonc/nos074 TEPLYUK ET AL 04 Apr 2012 (2012/04/04) Page 8, left column, last paragraph- right column first paragraph	1-39
A	US 2013/0344063 A1 KRICHEVSKY ANNA M; TEPLYUK NADIYA 26 Dec 2013 (2013/12/26) example 5	1-39
P,X	Siegal et al: Dynamics of circulating hypoxia mediated miRNAs and tumor response in high-grade glioma patients treated with bevacizumab. Neuro-Oncology 17:v69-v72, 2015. doi:10.1093/neuonc/nov211.15 SIEGAL ET AL 30 Nov 2015 (2015/11/30) the whole document	1-39

[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
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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
"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
13 Mar 2016	16 Mar 2016
Name and mailing address of the ISA: Israel Patent Office Technology Park, Bldg.5, Malcha, Jerusalem, 9695101, Israel Facsimile No. 972-2-5651616	Authorized officer KEDAR Laura Telephone No. 972-5651743

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL2016/050048

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

a. [X] forming part of the international application as filed:

[X] in the form of an Annex C/ST.25 text file.

[ ] on paper or in the form of an image file.

b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

[ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

[ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL2016/050048

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	Siegal et al:Dynamics of circulating hypoxia-mediated miRNAs and tumor response in patients with high-grade glioma treated with bevacizumab. Journal of Neurosurgery, January 22, 2016 (Summary) SIEGAL ET AL 22 Jan 2016 (2016/01/22) the whole document	1-39

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IL2016/050048

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PCT/IL2016/050048

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PCT/IL2016/050048

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PCT/IL2016/050048

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