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(54) Title: METHOD FOR PREDICTING SURVIVAL OF GLIOBLASTOMA PATIENT USING A TEN-MIRNA SIGNATURE

(57) Abstract: The present invention provides a ten miRNA signature that distinguishes high risk Glioblastoma patient from low risk Glioblastoma patient, wherein the miRNAs are hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p, hsa-miR-31, hsa-miR-222, hsa-miR-148a, hsa-miR-221, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a. The invention also provides processes and kit for determining survival prognosis in a subject with glioblastoma.



“METHOD FOR PREDICTING SURVIVAL OF GLIOBLASTOMA PATIENT USING A TEN-miRNA SIGNATURE”

FIELD OF INVENTION

5 The present invention relates to the field of cancer in particular to the method of predicting survival of glioblastoma patient using a ten-miRNA signature.

BACKGROUND OF THE INVENTION

 The grade IV astrocytoma, Glioblastoma, is the most common and malignant primary adult brain cancer (Furnari FB, Fenton T, Bachoo RM, Mukasa A, Stommel JM, et al. (2007) Malignant astrocytic glioma: genetics, biology, and paths to treatment. Genes Dev 21: 2683-2710). Despite advances in treatment modalities, the median survival is very poor. Since postoperative radiotherapy alone did not provide great benefit to GBM patients, several attempts have been made to find suitable adjuvant chemotherapy. The present standard treatment appears to be maximal safe resection of the tumor followed by irradiation and temozolomide adjuvant chemotherapy. However, it was found that not all patients were benefited from the addition of temozolomide. Further analysis revealed that methylation of MGMT promoter to be the strongest predictor for outcome and benefit from temozolomide chemotherapy (Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, et al. (2009) Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol 10: 459-466). In addition, recent molecular and genetic profiling studies have identified several markers and unique signatures as prognostic and predictive factors of GBM (Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. Cancer Cell 17: 98-110; Noushmehr H, Weisenberger DJ, Diefes K, Phillips HS, Pujara K, et al. Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. Cancer Cell 17: 510-522).

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MicroRNAs (miRNAs) are endogenous non-coding small RNAs, which negatively regulate gene expression either by binding to the 3'UTR leading to inhibition of translation or degradation of specific mRNA. Since miRNAs can act as Oncogenes or tumor suppressor genes, they have been linked to a variety of cancers (Yue J, Tigy G (2006) MicroRNA trafficking and human cancer. *Cancer Biol Ther* 5: 573-578). It has been shown that classification of multiple cancers based on miRNA expression signatures is more accurate than mRNA based signatures (Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, et al. (2005) MicroRNA expression profiles classify human cancers. *Nature* 435: 834-838). There have been a few attempts to profile miRNA expression either by microarray or RT-PCR in different grades of glioma (Ciafre SA, Galardi S, Mangiola A, Ferracin M, Liu CG, et al. (2005) Extensive modulation of a set of microRNAs in primary glioblastoma. *Biochem Biophys Res Commun* 334: 1351-1358; Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, et al. (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res* 33: e179; Silber J, Lim DA, Petritsch C, Persson AI, Maunakea AK, et al. (2008) miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells. *BMC Med* 6: 14; Godlewski J, Nowicki MO, Bronisz A, Williams S, Otsuki A, et al. (2008) Targeting of the Bmi-1 oncogene/stem cell renewal factor by microRNA-128 inhibits glioma proliferation and self-renewal. *Cancer Res* 68: 9125-9130; Rao SA, Santosh V, Somasundaram K Genome-wide expression profiling identifies deregulated miRNAs in malignant astrocytoma. *Mod Pathol*). Rao et al., profiled the expression of 756 miRNAs using 39 malignant astrocytoma and 7 normal brain samples and identified a 23-miRNA expression signatures which can discriminate anaplastic astrocytoma from glioblastoma. Other studies investigated the target identification and functional characterization of specific miRNAs (Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, et al. (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res* 33: e179; Godlewski J, Nowicki MO, Bronisz A, Williams S, Otsuki A, et al. (2008) Targeting of the Bmi-1 oncogene/stem cell renewal factor by microRNA-128 inhibits glioma proliferation and self-renewal. *Cancer Res* 68: 9125-9130; Gabriely G, Wurdinger T, Kesari S, Esau CC, Burchard J, et al. (2008) MicroRNA 21 promotes glioma invasion by targeting

matrix metalloproteinase regulators. *Mol Cell Biol* 28: 5369-5380; Huse JT, Brennan C, Hambardzumyan D, Wee B, Pena J, et al. (2009) The PTEN-regulating microRNA miR-26a is amplified in high-grade glioma and facilitates gliomagenesis in vivo. *Genes Dev* 23: 1327-1337; Kefas B, Godlewski J, Comeau L, Li Y, Abounader R, et al. (2008) microRNA-7
5 inhibits the epidermal growth factor receptor and the Akt pathway and is down-regulated in glioblastoma. *Cancer Res* 68: 3566-3572; Li Y, Guessous F, Zhang Y, Dipierro C, Kefas B, et al. (2009) MicroRNA-34a inhibits glioblastoma growth by targeting multiple oncogenes. *Cancer Res* 69: 7569-7576; Papagiannakopoulos T, Shapiro A, Kosik KS (2008) MicroRNA-21 targets a network of key tumor-suppressive pathways in glioblastoma cells. *Cancer Res* 68:
10 8164-8172; Zhang Y, Chao T, Li R, Liu W, Chen Y, et al. (2009) MicroRNA-128 inhibits glioma cells proliferation by targeting transcription factor E2F3a. *J Mol Med* 87: 43-51).

Many studies identifying miRNA expression signatures predicting patient survival have been done in several cancers like lung cancer, lymphocytic leukemia; lung adenocarcinoma, breast and pancreas cancers (Yu SL, Chen HY, Chang GC, Chen CY, Chen
15 HW, et al. (2008) MicroRNA signature predicts survival and relapse in lung cancer. *Cancer Cell* 13: 48-57; Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, et al. (2005) A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med* 353: 1793-1801; Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, et al. (2004) Reduced expression of the let-7 microRNAs in human lung cancers
20 in association with shortened postoperative survival. *Cancer Res* 64: 3753-3756; Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, et al. (2006) Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell* 9: 189-198; Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, et al. (2005) MicroRNA gene expression deregulation in human breast cancer. *Cancer Res* 65: 7065-7070; Roldo C, Missiaglia E, Hagan JP, Falconi
25 M, Capelli P, et al. (2006) MicroRNA expression abnormalities in pancreatic endocrine and acinar tumors are associated with distinctive pathologic features and clinical behavior. *J Clin Oncol* 24: 4677-4684). However, a miRNA signature that can predict the clinical outcome in GBM patients has not been found so far.

One of the major problems associated with the management and treatment of patients with glioblastoma is the inability to predict the survival of a patient. Identification of patients at an early stage of disease would be a very useful tool to determine which patients represent a higher risk subset, and thus, which patients may benefit maximally from advanced treatment.

A diagnostic assay that would allow identifying high-risk patients at an earlier time point and thus improving the outcomes in patients treated with advanced glioblastoma therapeutics would be of great utility. Thus, not only is there the need for a greater understanding of factors involved in the development and progression of glioblastoma, but there is also a need to be able to accurately predict prognosis in patients with newly diagnosed glioblastoma.

SUMMARY OF THE INVENTION

One aspect of the present invention relates to a process of determining survival prognosis in a subject with glioblastoma, wherein the process comprises measuring the level of miRNAs hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p, hsa-miR-31, hsa-miR-222, hsa-miR-148a, hsa-miR-221, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a in a biological sample, calculating a risk score of the biological sample using, wherein the risk score is calculated as $(-0.39 \times \text{expression of hsa-mir-20a}) + (-0.41 \times \text{expression of hsa-mir-106a}) + (-0.39 \times \text{expression of hsa-mir-17-5p}) + (0.28 \times \text{expression of hsa-mir-31}) + (0.23 \times \text{expression of hsa-mir-222}) + (0.19 \times \text{expression of hsa-mir-148a}) + (0.24 \times \text{expression of hsa-mir-221}) + (0.22 \times \text{expression of hsa-mir-146b}) + (0.19 \times \text{expression of hsa-mir-200b}) + (0.29 \times \text{expression of hsa-mir-193a})$, comparing the risk score of the biological sample with the risk score of a reference set of samples obtained from subjects with glioblastoma, and determining survival of the subject based on the value of the risk score, wherein the risk score equal to or more than 60th percentile of the reference set samples is indicative of lesser chances of survival compared to the risk score less than 60th percentile of the reference set samples.

Another aspect of the present invention provides a process of determining survival prognosis in a subject with glioblastoma, wherein the process comprises determining expression level of miRNAs hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p, hsa-miR-31, hsa-miR-222, hsa-miR-148a, hsa-miR-221, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a in a sample from a subject with glioblastoma; and comparing the expression level of the miRNAs

to the expression level of the miRNAs in a reference set of samples obtained from subjects with glioblastoma to determine prognosis, wherein increased expression level of hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p relative to the expression level of hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p in a reference set of samples obtained from subjects with glioblastoma is indicative of glioblastoma that is not associated with high risk; and increased expression level of hsa-miR-148a, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a relative to the expression level of hsa-miR-148a, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a in a reference set of samples obtained from subjects with glioblastoma and reduced expression level of hsa-miR-31, hsa-miR-222 and hsa-miR-221 relative to the expression level of hsa-miR-31, hsa-miR-222 and hsa-miR-221 in a reference set of samples obtained from subjects with glioblastoma is indicative of glioblastoma that is associated with high risk;

Another aspect of the present invention relates to a kit for determining survival prognosis in a subject with glioblastoma, wherein said kit comprises polynucleotides for analysis of miRNAs hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p, hsa-miR-31, hsa-miR-222, hsa-miR-148a, hsa-miR-221, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a.

Yet another aspect of the present invention relates to a microarray card for determining survival prognosis in a subject with glioblastoma, wherein the microarray comprises hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p, hsa-miR-31, hsa-miR-222, hsa-miR-148a, hsa-miR-221, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a specific probe oligonucleotides.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

Figure 1 shows Kaplan-Meier survival estimates overall survival of glioblastoma patients according to the 10 miRNA expression signature

A) 111 GBM patients in the training data set

B) 111 GBM patients in the testing data set

Figure 2 shows ten miRNA Risk-Score Analysis of 111 GBM patients (training set)

A) Heat map of ten miRNA expression profiles of GBM patients; rows represent risky and protective miRNAs, and columns represent patients. The blue line represents the miRNA signature cutoff dividing patients into low-risk and high-risk groups

B) Patient survival status along with risk score

C) miRNA risk-score distribution of the GBM patients

Figure 3 shows ten miRNA Risk-Score Analysis of 111 GBM patients (test set)

A) Heat map of ten miRNA expression profiles of GBM patients; rows represent risky and protective miRNAs, and columns represent patients. The blue line represents the miRNA signature cutoff dividing patients into low-risk and high-risk groups.

B) Patient survival status along with risk score

C) miRNA risk-score distribution of the GBM patients

Figure 4 shows comparison of overall survival of low risk group vs. high risk group throughout the study period in the training, the testing, and the entire patient sets

DETAILED DESCRIPTION OF THE INVENTION

As used herein the specification, "a" or "an" may mean one or more, unless clearly indicated otherwise. As used herein in the claim(s), when used in conjunction with the word "comprising," the words "a" or "an" may mean one or more than one. As used herein "another" may mean at least a second or more.

The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternative are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or."

A "sample" or "biological sample" is any biological material obtained from an individual. For example, it may be a blood sample or ant tissue sample obtained by biopsy.

The terms "microRNA" and "miRNA" used herein can be used interchangeably.

The terms "reference set of samples" and "training set" used herein can be used interchangeably.

Furthermore, any composition of the invention may be used in any method of the invention, and any method of the invention may be used to produce or to utilize any composition of the invention.

Other objects, features, and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications

within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

The present invention provides an assay or method to assess the prognosis in individual patients suffering from Glioblastoma (GBM), wherein the patient is under treatment selected from a group consisting of radiotherapy, chemotherapy, surgery, and combination thereof. The assay disclosed in the present invention is based on analyzing expression of specific microRNA by performing microRNA profiling of the patient. The present invention in particular discloses an assay for predicting the survival of patients suffering from Glioblastoma (GBM) using a ten mi-RNA signature.

10 **miRNA Sequence Information**

The ten microRNA signature (Table 6) associated with Glioblastoma (GBM) as disclosed in the present invention are hsa-miR-20a (SEQ ID NO: 1), hsa-miR-106a (SEQ ID NO: 2), hsa-miR-17-5p (SEQ ID NO: 3), hsa-miR-31 (SEQ ID NO: 4), hsa-miR-222 (SEQ ID NO: 5), hsa-miR-148a (SEQ ID NO: 6), hsa-miR-221 (SEQ ID NO: 7), hsa-miR-146b (SEQ ID NO: 8), hsa-miR-200b (SEQ ID NO: 9), and hsa-miR-193a (SEQ ID NO: 10)

The ten microRNA signature as disclosed in the present invention can also be used to identify potential targets for GBM treatment, wherein the genes targeted by these micro-RNA can be identified using the methods known in the art. Further, the genes and its products can be used as potential target for treatment of GBM.

In this study, we subjected the miRNA expression data from a total of 222 GBM patients derived from The Cancer Genome Atlas (TCGA) data set to Cox proportional regression analysis to identify the miRNAs that can predict patient survival. By using a sample-splitting approach, a 10 miRNA expression signature that can predict survival both in training and testing sets was identified. More importantly, using multivariate analysis along with patient age, the 10 miRNA expression signature was found to be an independent predictor of patient survival.

The expression level of the miRNAs can be determined using any process known in the art. For example, the process may involve any of a variety of techniques known to those of ordinary skill in the art. Examples of such techniques include reverse transcriptase (RT) PCR,

PCR, allele specific oligonucleotide hybridization, size analysis, sequencing, hybridization, 5' nuclease digestion, single-stranded conformation polymorphism analysis, allele specific hybridization, primer specific extension, and oligonucleotide ligation assays.

The sample can be any tissue sample obtained from the subject, such as but not limited to tumor sample obtained during surgery or biopsy. For example, the sample may be any tissue obtained by biopsy.

The expression level of the reference sample set can be obtained from a single subject or from a group of subjects. The expression level of miRNA expression can be determined using any method known to those of ordinary skill in the art, such as any of the methods discussed above and elsewhere in this description.

In some embodiments, the expression level is an average level of expression of hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p, hsa-miR-31, hsa-miR-222, hsa-miR-148a, hsa-miR-221, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a obtained from a cohort of subjects with glioblastoma with a known poor outcome following a therapeutic intervention.

In other embodiments, the reference level is an average level of expression of hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p, hsa-miR-31, hsa-miR-222, hsa-miR-148a, hsa-miR-221, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a obtained from a cohort of subjects with glioblastoma with a known good outcome following a therapeutic intervention. Good outcome can be measured by any method known to those of ordinary skill in the art. For example, good outcome can be assessed as improvement in signs or symptoms of glioblastoma or prolonged survival compared to another cohort of subjects.

The expression level may be a single value of miRNA expression level, or it may be a range of values of miRNA expression level. The expression level may also be depicted graphically as an area on a graph.

The formula for calculating the risk score is as provided below.

Risk score = $(-0.39 \times \text{expression of hsa-mir-20a}) + (-0.41 \times \text{expression of hsa-mir-106a}) + (-0.39 \times \text{expression of hsa-mir-17-5p}) + (0.28 \times \text{expression of hsa-mir-31}) + (0.23 \times \text{expression of hsa-mir-222}) + (0.19 \times \text{expression of hsa-mir-148a}) + (0.24 \times \text{expression of hsa-mir-221})$

mir-221) + (0.22 x expression of hsa-mir-146b) + (0.19 x expression of hsa-mir-200b) + (0.29 x expression of hsa-mir-193a).

In accordance with the present invention in one embodiment there is provided a process to assess the prognosis in individual patients suffering form Glioblastoma (GBM), said method comprises detecting expression levels of miRNAs hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p, hsa-miR-31, hsa-miR-222, hsa-miR-148a, hsa-miR-221, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a in a sample obtained from the patient; calculating the risk score based on the expression of the said miRNAs and predicting survival of the patient based on the value of risk score, wherein the patient showing low risk score has greater chances of survival compared to the patient showing high risk score.

In another embodiment of the present invention there is provided a process to assess the prognosis in individual patients suffering form Glioblastoma (GBM), said method comprises detecting expression levels of miRNAs hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p, hsa-miR-31, hsa-miR-222, hsa-miR-148a, hsa-miR-221, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a in a sample obtained from the patient; calculating the risk score based on the expression of the said miRNAs using the formula- Risk score = (-0.39 x expression of hsa-mir-20a) + (-0.41 x expression of hsa-mir-106a) + (-0.39 x expression of hsa-mir-17-5p) + (0.28 x expression of hsa-mir-31) + (0.23 x expression of hsa-mir-222) + (0.19 x expression of hsa-mir-148a) + (0.24 x expression of hsa-mir-221) + (0.22 x expression of hsa-mir-146b) + (0.19 x expression of hsa-mir-200b) + (0.29 x expression of hsa-mir-193a); and predicting survival of the patient based on the value of risk score, wherein the patient showing low risk score has greater chances of survival compared to the patient showing high risk score.

The process set forth herein further comprises obtaining a sample from the subject, the sample may be any sample as disclosed above, but in particular embodiment the sample is tumor samples obtained after surgery of glioblastoma patients.

In another embodiment of the present invention there is provided a range of risk score, wherein the risk score equal to or less than -0.7 of training set (reference samples set) indicates that the patient has a fair chance of survival.

In yet another embodiment of the present invention there is provided a range of cut off value for risk score which is -11.38 to 2.33 for training set (reference samples set) and -6.45 to 1.82 for test set.

Further embodiment of the present invention provides that the ten micro-RNA signature as disclosed in the present invention can predict if the patient belongs to the high risk or low risk group.

Still another embodiment of the present invention provides that the ten microRNA signature as disclosed in the present invention can predict GBM patient survival and other grades of glioma.

In one embodiment of the present invention there is provided a process of determining survival prognosis in a subject with glioblastoma, wherein the process comprises measuring the level of miRNAs hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p, hsa-miR-31, hsa-miR-222, hsa-miR-148a, hsa-miR-221, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a in a biological sample, calculating a risk score of the biological sample using, wherein the risk score is calculated as $(-0.39 \times \text{expression of hsa-mir-20a}) + (-0.41 \times \text{expression of hsa-mir-106a}) + (-0.39 \times \text{expression of hsa-mir-17-5p}) + (0.28 \times \text{expression of hsa-mir-31}) + (0.23 \times \text{expression of hsa-mir-222}) + (0.19 \times \text{expression of hsa-mir-148a}) + (0.24 \times \text{expression of hsa-mir-221}) + (0.22 \times \text{expression of hsa-mir-146b}) + (0.19 \times \text{expression of hsa-mir-200b}) + (0.29 \times \text{expression of hsa-mir-193a})$, comparing the risk score of the biological sample with the risk score of a reference set of samples obtained from subjects with glioblastoma, and determining survival of the subject based on the value of the risk score, wherein the risk score equal to or more than 60th percentile of the reference set samples is indicative of lesser chances of survival compared to the risk score less than 60th percentile of the reference set samples.

Another embodiment of the present invention there is provided a process of determining survival prognosis in a subject with glioblastoma, wherein the process comprises determining expression level of miRNAs hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p, hsa-miR-31, hsa-miR-222, hsa-miR-148a, hsa-miR-221, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a in a sample from a subject with glioblastoma; and comparing the expression level of the miRNAs to the expression level of the miRNAs in a reference set of samples obtained from subjects

with glioblastoma to determine prognosis, wherein increased expression level of hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p relative to the expression level of hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p in a reference set of samples obtained from subjects with glioblastoma is indicative of glioblastoma that is not associated with high risk; and increased expression level
5 of hsa-miR-148a, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a relative to the expression level of hsa-miR-148a, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a in a reference set of samples obtained from subjects with glioblastoma and reduced expression level of hsa-miR-31, hsa-miR-222 and hsa-miR-221 relative to the expression level of hsa-miR-31, hsa-miR-222 and hsa-miR-221 in a reference set of samples obtained from subjects with glioblastoma
10 is indicative of glioblastoma that is associated with high risk.

Yet another embodiment of the present invention provides a kit for determining survival prognosis in a subject with glioblastoma, wherein said kit comprises polynucleotides for analysis of microRNAs hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p, hsa-miR-31, hsa-miR-222, hsa-miR-148a, hsa-miR-221, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a.

15 The kit as disclosed in the present invention further comprises a set of primers specific for transcription or reverse transcription of miRNAs hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p, hsa-miR-31, hsa-miR-222, hsa-miR-148a, hsa-miR-221, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a.

The kit as disclosed in the present invention further comprises a miRNA array card,
20 wherein the one or more polynucleotides are arrayed on said card.

Another embodiment of the present invention provides a microarray card for determining survival prognosis in a subject with glioblastoma, wherein the microarray comprises hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p, hsa-miR-31, hsa-miR-222, hsa-miR-148a, hsa-miR-221, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a specific probe oligonucleotides.

25 **Methods for Analyzing Expression Level of miRNA**

Some embodiments of the process of the present invention involve analysis of miRNA expression level or gene expression level. Methods for analyzing gene expression or expression of miRNA include, but are not limited to, methods based on hybridization analysis of polynucleotides, sequencing of polynucleotides, and analysis of protein expression such as

proteomics-based methods. Commonly used methods for the quantification of mRNA expression level in a sample include northern blotting and in situ hybridization, RNase protection assays, and PCR-based methods, such as reverse transcription polymerase chain reaction (RT-PCR). In some embodiments, antibodies may be employed that can recognize
5 specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes.

Representative methods for sequencing-based gene expression analysis include Serial Analysis of Gene Expression (SAGE), and gene expression analysis by massively parallel signature sequencing (MPSS).

10 **PCR-Based Methods**

Gene expression level or miRNA expression level can be analyzed using techniques that employ PCR. PCR is useful to amplify and detect transcripts from a sample. Examples of PCT methodologies are as below.

RT-PCR is a sensitive quantitative method that can be used to compare mRNA levels
15 in different samples (e.g., endomyocardial biopsy samples) to examine gene expression signatures.

To perform RT-PCR, mRNA is isolated from a sample. For example, total RNA may be isolated from a sample of heart tissue. mRNA may also be extracted, for example, from frozen or archived paraffin-embedded and fixed tissue samples. Methods for mRNA extraction are
20 known in the art. RNA is then reverse transcribed into cDNA. The cDNA is amplified in a PCR reaction. A variety of reverse transcriptases are known in the art. The reverse transcription step is typically primed using specific primers, random hexamers, or oligo-dT primers, depending on the conditions and desired readout. For example, extracted RNA can be reverse-transcribed using a GeneAmp RNA PCR kit, following the manufacturer's
25 instructions. The derived cDNA can then be used as a template in the subsequent PCR reaction. The PCR reaction may employ the Taq DNA polymerase, which has a 5'-3' nuclease activity but lacks a 3'-5' proofreading endonuclease-activity. Two oligonucleotide primers are used to generate an amplicon in the PCR reaction.

For quantitative PCR, a third oligonucleotide, or probe, is used to detect nucleotide sequence located between the two PCR primers. The probe is non-extendible by Taq DNA polymerase enzyme, and typically is labeled with a reporter fluorescent dye and a quencher fluorescent dye. Any laser-induced emission from the reporter dye is quenched by the quenching dye when the two dyes are located close together as they are on the probe. During the amplification reaction, the Taq DNA polymerase enzyme cleaves the probe in a template-dependent manner. The resultant probe fragments disassociate in solution, and signal from the released reporter dye is free from the quenching effect of the second fluorophore. One molecule of reporter dye is liberated for each new molecule synthesized, and detection of the unquenched reporter dye provides the basis for quantitative analysis. RT-PCR can be performed using commercially available equipment.

To minimize errors and the effect of sample-to-sample variation, RT-PCR is usually performed using an internal standard. A suitable internal standard is expressed at a constant level among different tissues, and is unaffected by the experimental variable.

Microarrays

Evaluating gene expression level in a sample can also be performed with microarrays. Microarrays permit simultaneous analysis of a large number of gene expression products. Typically, polynucleotides of interest are plated, or arrayed, on a microchip substrate. The arrayed sequences are then hybridized with nucleic acids (e.g., DNA or RNA) from cells or tissues of interest. The source of mRNA typically is total RNA. If the source of mRNA is endomyocardial tissue, mRNA can be extracted. In various embodiments of the microarray technique, probes to at least 10, 25, 50, 100, 200, 500, 1000, 1250, 1500, or 1600 polynucleotides are immobilized on an array substrate (for example, a porous or nonporous solid support, such as a glass, plastic, or gel surface). The probes can include DNA, RNA, copolymer sequences of DNA and RNA, DNA and/or RNA analogues, or combinations thereof.

In some embodiments, a microarray includes a support with an ordered array of binding (e.g., hybridization) sites for each individual polynucleotide of interest. The microarrays can be addressable arrays, such as positionally addressable arrays where each

probe of the array is located at a known, predetermined position on the solid support such that the identity of each probe can be determined from its position in the array. Each probe on the microarray can be between about 10-50,000 nucleotides in length. The probes of the microarray can consist of nucleotide sequences with lengths: less than 1,000 nucleotides, such as sequences 10-1,000, or 10-500, or 10-200 nucleotides in length.

An array can include positive control probes, such as probes known to be complementary and hybridizable to sequences in the test sample, and negative control probes such as probes known to not be complementary and hybridizable to sequences in the test sample.

Glioblastoma (GBM)

Glioblastoma (GBM) is the most common and aggressive primary brain tumor with very poor patient median survival. To identify a microRNA (miRNA) expression signature that can predict GBM patient survival, we analyzed the miRNA expression data of GBM patients (n=222) derived from The Cancer Genome Atlas (TCGA) dataset. We divided the patients randomly into training and testing sets with equal number in each group. We identified 10 significant miRNAs using Cox regression analysis on the training set and formulated a risk score based on the expression signature of these miRNAs that segregated the patients into high and low risk groups with significantly different survival times (hazard ratio 2.4; 95% CI 1.4-3.8; $p < 0.0001$). Of these 10 miRNAs, 7 were found to be risky miRNAs and 3 were found to be protective. This signature was independently validated in the testing set (hazard ratio 1.7; 95% CI 1.1-2.8; $p = 0.002$). GBM patients with high risk scores had overall poor survival compared to the patients with low risk scores. Overall survival among the entire patient set was 35.0% at 2 years, 21.5% at 3 years, 18.5% at 4 years and 11.8% at 5 years in the low risk group, versus 11.0%, 5.5%, 0.0 and 0.0% respectively in the high risk group (hazard ratio 2.0; 95% CI 1.4-2.8; $p < 0.0001$). Cox multivariate analysis with patient age as a covariate on the entire patient set identified risk score based on the 10 miRNA expression signature to be an independent predictor of patient survival (hazard ratio 1.120; 95% CI 1.04-1.20; $p = 0.003$). Thus we have identified a miRNA expression signature that can predict GBM patient survival. These findings may have implications in the understanding of

gliomagenesis, development of targeted therapy and selection of high risk cancer patients for adjuvant therapy.

Multivariate regression analysis shows that the 10 miRNA expression signature is independent of age

5 In order to ascertain whether the 10 miRNA expression signature is an independent predictor of GBM patient's survival, we carried out Cox multivariate analysis. As has been shown before, patient age also predicted the GBM patients survival in univariate analysis ($p < 0.0001$; HR = 1.027; B = 0.027) (Table 4 and Table 5). The effect of the 10 miRNA expression signature and age on GBM patient survival was further evaluated by multivariate
10 Cox proportional hazard model. We found that both 10 miRNA expression signature ($p = 0.003$; HR = 1.120; B = 0.113) and age ($p = 0.004$; HR = 1.020; B = 0.019) are independent predictors of GBM patient survival (Table 4 and Table 5).

 In this study, we have identified a ten miRNA signature that is associated with survival of GBM patients. We confirmed these findings in a testing set. Patients with a high risk score
15 had shorter survival even after including patient age as a variable in a multivariate Cox model. These results suggest that miRNAs play an important role in molecular pathogenesis, progression and prognosis of GBMs.

 Predicting the benefit of various cancer therapies to patients is very important and forms the foundation of personalized cancer therapy. While the clinical features like age and
20 Karnofsky performance status are known prognostic markers among GBM patients, MGMT gene promoter methylation status is of great interest in recent times because it predicted response of GBM patients receiving temozolomide chemotherapy in addition to irradiation (Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, et al. (2009) Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on
25 survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol* 10: 459-466). Several other molecular markers with prognostic and predictive significance in GBMs have been identified (Palanichamy K, Erkkinen M, Chakravarti A (2006) Predictive and prognostic markers in human glioblastomas. *Curr Treat Options Oncol* 7: 490-504). Except for a few recent reports on the role of miRNAs in GBM

prognosis, the possibility of prognostic miRNA signatures have not been investigated (Zhi F, Chen X, Wang S, Xia X, Shi Y, et al. The use of hsa-miR-21, hsa-miR-181b and hsa-miR-106a as prognostic indicators of astrocytoma. *Eur J Cancer* 46: 1640-1649). To our knowledge, this is the first report of a miRNA expression signature predicting GBM patient survival.

The ten miRNA signature identified in this study included three miRNAs (miR-20a, miR-106a and miR-17-5p) that were protective and seven miRNAs (hsa-miR-31, hsa-miR-222, hsa-miR-148a, hsa-miR-221, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a) that were risky with respect to their association between their expression and patient survival. The protective miRNAs were expressed at a higher level in the low risk group compared to the high risk group and the risky miRNAs were expressed at a higher level in the high risk group than in the low risk group. The protective and risky nature of these miRNAs is suggestive of their functions being either inhibitory or promoting, respectively of various properties of cancer cells like proliferation, migration and invasion etc.

miR-31 has been shown to be an inhibitor of breast cancer metastasis by targeting RhoA, RDX and ITGA which are involved in tumor motility, invasion and resistance to anoikis (Valastyan S, Reinhardt F, Benaich N, Calogrias D, Szasz AM, et al. (2009) A pleiotropically acting microRNA, miR-31, inhibits breast cancer metastasis. *Cell* 137: 1032-1046). However, miR-31 has also been shown to be an oncogenic miRNA in lung cancer by targeting tumor suppressor genes and in head and neck cancer by targeting factor-inhibiting hypoxia-inducible factor (FIH) (Liu CJ, Tsai MM, Hung PS, Kao SY, Liu TY, et al. miR-31 ablates expression of the HIF regulatory factor FIH to activate the HIF pathway in head and neck carcinoma. *Cancer Res* 70: 1635-1644; Liu X, Sempere LF, Ouyang H, Memoli VA, Andrew AS, et al. MicroRNA-31 functions as an oncogenic microRNA in mouse and human lung cancer cells by repressing specific tumor suppressors. *J Clin Invest* 120: 1298-1309). Both miR-221 and 222 have shown to be upregulated in multiple cancers including glioblastoma, linked to promoting proliferation and radioresistance by targeting PTEN, p27 and p57 (Chun-Zhi Z, Lei H, An-Ling Z, Yan-Chao F, Xiao Y, et al. MicroRNA-221 and microRNA-222 regulate gastric carcinoma cell proliferation and radioresistance by targeting

PTEN. BMC Cancer 10: 367; Gillies JK, Lorimer IA (2007) Regulation of p27Kip1 by miRNA 221/222 in glioblastoma. Cell Cycle 6: 2005-2009; Medina R, Zaidi SK, Liu CG, Stein JL, van Wijnen AJ, et al. (2008) MicroRNAs 221 and 222 bypass quiescence and compromise cell survival. Cancer Res 68: 2773-2780).

5 Overexpression of miR-221 and miR222 has been shown to be associated with poor survival in hepatocellular carcinoma, pancreatic cancer and cervical cancer (Wong QW, Ching AK, Chan AW, Choy KW, To KF, et al. MiR-222 overexpression confers cell migratory advantages in hepatocellular carcinoma through enhancing AKT signaling. Clin Cancer Res 16: 867-875; Greither T, Grochola LF, Udelnow A, Lautenschlager C, Wurl P, et al. Elevated
10 expression of microRNAs 155, 203, 210 and 222 in pancreatic tumors is associated with poorer survival. Int J Cancer 126: 73-80; Pu XX, Huang GL, Guo HQ, Guo CC, Li H, et al. Circulating miR-221 directly amplified from plasma is a potential diagnostic and prognostic marker of colorectal cancer and is correlated with p53 expression. J Gastroenterol Hepatol 25: 1674-1680). Further, a low expression of p27Kip1, a target of miR-221 and 222, has been
15 correlated to poor prognosis in astrocytoms (Kirla RM, Haapasalo HK, Kalimo H, Salminen EK (2003) Low expression of p27 indicates a poor prognosis in patients with high-grade astrocytomas. Cancer 97: 644-648; Tamiya T, Mizumatsu S, Ono Y, Abe T, Matsumoto K, et al. (2001) High cyclin E/low p27Kip1 expression is associated with poor prognosis in astrocytomas. Acta Neuropathol 101: 334-340; Mizumatsu S, Tamiya T, Ono Y, Abe T,
20 Matsumoto K, et al. (1999) Expression of cell cycle regulator p27Kip1 is correlated with survival of patients with astrocytoma. Clin Cancer Res 5: 551-557).

miR-148a has been shown to regulate DNA methylation by targeting DNA methyltransferase 1 (DNMT1) (Pan W, Zhu S, Yuan M, Cui H, Wang L, et al. MicroRNA-21 and microRNA-148a contribute to DNA hypomethylation in lupus CD4+ T cells by directly
25 and indirectly targeting DNA methyltransferase 1. J Immunol 184: 6773-6781).

While Chou et al.(Chou CK, Chen RF, Chou FF, Chang HW, Chen YJ, et al. miR-146b is highly expressed in adult papillary thyroid carcinomas with high risk features including extrathyroidal invasion and the BRAF(V600E) mutation. Thyroid 20: 489-494) reported higher expression of miR-146b in high risk adult papillary thyroid carcinoma, other

reports indicate miR-146b is a metastasis suppressor by targeting matrix metalloproteases (Chou et al; Xia H, Qi Y, Ng SS, Chen X, Li D, et al. (2009) microRNA-146b inhibits glioma cell migration and invasion by targeting MMPs. Brain Res 1269: 158-165; Hurst DR, Edmonds MD, Scott GK, Benz CC, Vaidya KS, et al. (2009) Breast cancer metastasis suppressor 1 up-regulates miR-146, which suppresses breast cancer metastasis. Cancer Res 69: 1279-1283). Similar to our results, higher miR-146b levels is correlated to poor prognosis in squamous cell lung cancer (Raponi M, Dossey L, Jatkoe T, Wu X, Chen G, et al. (2009) MicroRNA classifiers for predicting prognosis of squamous cell lung cancer. Cancer Res 69: 5776-5783).

With respect to miR-200b, while Xia et al identified it to promote S-phase entry by targeting RND3 and increasing cyclin D1 expression, other reports suggest miR-200 to be an inhibitor of epithelial-to mesenchymal transition with decreased cell migration and increased sensitivity to EGFR-blocking agents (Xia H, Qi Y, Ng SS, Chen X, Li D, et al. (2009) microRNA-146b inhibits glioma cell migration and invasion by targeting MMPs. Brain Res 1269: 158-165; Adam L, Zhong M, Choi W, Qi W, Nicoloso M, et al. (2009) miR-200 expression regulates epithelial-to-mesenchymal transition in bladder cancer cells and reverses resistance to epidermal growth factor receptor therapy. Clin Cancer Res 15: 5060-5072).

In malignant cutaneous melanoma, while increased expression of miR-193a is associated with poor survival, miR-193a has been identified as epigenetically silenced tumor suppressor miRNA in oral cancer (Caramuta S, Egyhazi S, Rodolfo M, Witten D, Hansson J, et al. MicroRNA expression profiles associated with mutational status and survival in malignant melanoma. J Invest Dermatol 130: 2062-2070; Kozaki K, Imoto I, Mogi S, Omura K, Inazawa J (2008) Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in oral cancer. Cancer Res 68: 2094-2105).

miR-106a, one of the protective miRNAs was found to be over-expressed and oncogenic in human T-Cell leukemia (Landais S, Landry S, Legault P, Rassart E (2007) Oncogenic potential of the miR-106-363 cluster and its implication in human T-cell leukemia. Cancer Res 67: 5699-5707). However, in good correlation with our data, low expression of miR-106a was found to be associated with poor patient survival in glioma and colon cancer

(Zhi F, Chen X, Wang S, Xia X, Shi Y, et al. The use of hsa-miR-21, hsa-miR-181b and hsa-miR-106a as prognostic indicators of astrocytoma. *Eur J Cancer* 46: 1640-1649; Diaz R, Silva J, Garcia JM, Lorenzo Y, Garcia V, et al. (2008) Deregulated expression of miR-106a predicts survival in human colon cancer patients. *Genes Chromosomes Cancer* 47: 794-802).

5 The miR-17~92 cluster, which contains two protective miRNAs, miR-17-5p and miR-20a, has been found to accelerate the disease onset of Eμ-myc-induced B-cell lymphoma, promote lung cancer growth *in vitro*, activated by c-myc and promote tumor angiogenesis. Interestingly, in breast cancer, ovarian cancer and melanoma, miR-17~92 has been shown to be deleted and found to inhibit cell proliferation upon overexpression suggesting miR-17~92
10 cluster may have context dependent functions (Bonauer A, Dimmeler S (2009) The microRNA-17-92 cluster: still a miRacle? *Cell Cycle* 8: 3866-3873). Our results show that both miR-17-5p and miR-20a are upregulated in GBMs with higher expression correlating with increased survival. In good correlation with our data, lower expression of E2F1, a target of miR-20a and cyclin D1, a target of both miR-20a and miR-17 was found to predict longer
15 survival in gliomas (Alonso MM, Fueyo J, Shay JW, Aldape KD, Jiang H, et al. (2005) Expression of transcription factor E2F1 and telomerase in glioblastomas: mechanistic linkage and prognostic significance. *J Natl Cancer Inst* 97: 1589-1600; Liu C, Tu Y, Sun X, Jiang J, Jin X, et al. Wnt/beta-Catenin pathway in human glioma: expression pattern and clinical/prognostic correlations. *Clin Exp Med*; Sallinen SL, Sallinen PK, Kononen JT, Syrjakoski KM, Nupponen NN, et al. (1999) Cyclin D1 expression in astrocytomas is
20 associated with cell proliferation activity and patient prognosis. *J Pathol* 188: 289-293).

Even though the median survival time remains in the 12-15 months range, the prognosis of individual patients is variable and approximately 10% of the patients are known to survive for more than 2 years (Adamson C, Kanu OO, Mehta AI, Di C, Lin N, et al. (2009)
25 Glioblastoma multiforme: a review of where we have been and where we are going. *Expert Opin Investig Drugs* 18: 1061-1083).

At present, several molecular markers, including MGMT promoter methylation status for GBM patient prognosis, have been identified and many needs further validation before

their use in clinical settings (Palanichamy K, Erkkinen M, Chakravarti A (2006) Predictive and prognostic markers in human glioblastomas. *Curr Treat Options Oncol* 7: 490-504).

The ten miRNA signature, identified in this study, classifies patients successfully into low risk and high risk groups in both training and testing sets. This may help clinicians to identify patients belonging to high risk for more effective adjuvant therapy in addition to the standard treatment protocol. We also found that the ten miRNA signature is an independent predictor of GBM patient survival.

Our finding that ten miRNA signature can predict GBM patient's survival also likely to generate potential molecular targets for the development of anticancer therapy. Since miRNAs can target multiple genes, more thorough studies are needed to understand the mechanism of action of these miRNAs which is likely to result in better understanding of glioma.

In conclusion, the present invention discloses a ten miRNA signature that can predict GBM patient survival with a lot of potential prognostic and therapeutic implications for the GBM patient management.

EXAMPLES

It should be understood that the following examples described herein are for illustrative purposes only and that various modifications or changes in light will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

Example 1

Identification of a 10 miRNA expression signature from training set (Reference set samples)

The 222 GBM samples were divided randomly into a training set (n=111) or a testing set (n=111). Table 1 gives the age and gender distribution of the patients in both sets and the entire set. miRNA expression data corresponding to 305 miRNAs derived from the training set was subjected to Cox proportional hazard regression analysis to identify miRNAs, whose expression profile could be significantly correlated to patient survival. We identified a set of 10 miRNAs that were significantly correlated with patient survival (Table 2). These 10

miRNAs were then used to create a signature by calculating a risk score for each patient. A risk score formula was obtained for predicting the patient survival. Using the risk score formula, the 10 miRNA expression signature risk score was calculated for all patients in the training set. The patients were then ranked in the training set according to their risk score.

5 Using the 60th percentile risk score as cutoff in the training set, the patients were divided into high and low risk groups. Patients belonging to high risk group had a shorter median survival than patients with low risk score (12.6 months versus 19.3 months, hazard ratio 2.4; 95% CI 1.4-3.8; $p=0.0006$) (Figure 1 A; Table 3). Survival was greater in the low risk group than in the high risk group throughout the follow-up. Overall survival in the training set was 41.8% at
10 2 years, 25.6% at 3 years, 23.6% at 4 years and 14.8% at 5 years in the low risk group, versus 9.0%, 3.0%, 0.0 and 0.0% respectively in the high risk group (hazard ratio 2.4; 95% CI 1.4-3.8; $p = 0.0006$) (Table 3; Figure 4).

Example 2

15 Validation of the 10 miRNA expression signature for survival prediction in the testing set

To validate our finding, we calculated the risk score for the 111 patients from the testing set. Using the same cut-off value that was used for training set, the patients from the testing set were classified into low risk and high risk groups and subjected to survival
20 comparison. Similar to the results obtained in the training set, patients in the high risk group had shorter median survival than patients in the low risk group (12.1 months versus 18.0 months; hazard ratio 1.7; 95% CI 1.1-2.8; $p=0.0207$) (Figure 1 B; Table 3). As was seen in the training set, patient survival in the low risk group was better than that in the high-risk group throughout the 5 year follow-up time (Table 3). Risk score based classification of the entire
25 patient set also gave a similar result with the high risk group having a shorter median survival than the low risk group (12.6 months versus 18.3 months, hazard ratio 2.0; 95% CI 1.4-2.8; $p < 0.0001$) (Table 3). The higher overall survival of the low risk group throughout the study period in training, testing, and entire patient sets compared to the high risk group is shown (Figure 4).

Nature of 10 miRNA expression signature

Additional investigation of 10 miRNA set yielded several interesting observations. The distribution of miRNA expression values, patient risk scores and survival status of patients were analyzed independently for both training and testing set (Figure 2 and 3). There were 3
5 miRNAs that were protective and 7 miRNAs that were risky based on correlation of their expression and association with patient survival (Table 2). Tumors from patients belonging to high risk group tend to express higher levels of risky miRNAs, whereas tumors from patients with low risk group tend to express higher levels of protective miRNAs (Figures 2A). Similar results were obtained in the testing set as well (Figure 3A). A comparison of risk score with
10 patient survival status and risk score distribution among GBM patients of the training set and test set are shown (Figure 2 B, C and 3 B, C). It is interesting to note that the three protective miRNAs are up regulated in GBMs compared to normal samples (Table 2). However, among risky miRNAs, three were down regulated and four were up regulated in GBMs (Table 2).

We further carefully analyzed the 10 miRNA set to determine whether a subset of
15 miRNAs can be used to predict GBM patient survival. The four most significant miRNAs from the set of 10, all with $p \leq 0.0005$ with (miR-20a, miR-106A, miR-31 and miR-222) were chosen and their expression signature derived risk score was used to predict GBM patient survival. The results showed that unlike the ten miRNA signature, the four miRNA did not consistently correlate with patient survival in training and testing set.

Example 3

Statistical analysis

The 222 samples were randomly assigned to a training data set ($n=111$) or a testing data set ($n=111$). The expression level of each miRNA was assessed by Cox regression analysis using the BRB array tools (Simon R, Lam A, Li MC, Ngan M, Menenzes S, et al.
25 (2007) Analysis of Gene Expression Data Using BRB-Array Tools. Cancer Inform 3: 11-17) package in the training set, and the most significant miRNAs were identified, using the permutation test method, with 10,000 permutations. We found 10 out of the 305 miRNAs to be significantly correlated with survival ($p \leq 0.001$).

Using these 10 significant miRNAs, we constructed a formula that would predict survival. This formula was devised using the Cox regression coefficients derived from the Cox proportional hazard analysis. Specifically, we assigned each patient a risk score that is a linear combination of the expression levels of the significant miRNAs weighted by their respective Cox regression coefficients (Lossos IS, Czerwinski DK, Alizadeh AA, Wechser MA, Tibshirani R, et al. (2004) Prediction of survival in diffuse large-B-cell lymphoma based on the expression of six genes. *N Engl J Med* 350: 1828-1837). According to this model, patients having high risk scores are expected to have poor survival outcomes as compared to patients having low risk scores. The risk scores are calculated as follows:

Risk score = $(-0.39 \times \text{expression of hsa-mir-20a}) + (-0.41 \times \text{expression of hsa-mir-106a}) + (-0.39 \times \text{expression of hsa-mir-17-5p}) + (0.28 \times \text{expression of hsa-mir-31}) + (0.23 \times \text{expression of hsa-mir-222}) + (0.19 \times \text{expression of hsa-mir-148a}) + (0.24 \times \text{expression of hsa-mir-221}) + (0.22 \times \text{expression of hsa-mir-146b}) + (0.19 \times \text{expression of hsa-mir-200b}) + (0.29 \times \text{expression of hsa-mir-193a})$.

The significant miRNAs that formed the signature were of two types-risky and protective. Risky miRNAs were defined as those that had hazard ratio for death greater than 1. Protective miRNAs were defined as those that had hazard ratio for death less than 1. Using this definition, we found 3 protective miRNAs and 7 risky miRNAs.

We divided patients in the training data set into high-risk and low-risk groups by risk score. We used the 60th percentile risk score as the cut-off, since this divided the training set patients into two groups having different survival times with highest significance. The Kaplan-Meier method was used to estimate overall survival time for the two groups. Differences in survival times were analyzed using the two-sided log rank test. We followed the strategy of splitting patients into training and testing sets, as we had no independent cohort that we could verify our signature with. We used the splitting strategy as opposed to cross-validation, since this has been found to be a better strategy [Simon R, Radmacher MD, Dobbin K, McShane LM (2003) Pitfalls in the use of DNA microarray data for diagnostic and prognostic classification. *J Natl Cancer Inst* 95: 14-18]. We also used Cox multivariate

analysis to evaluate the contribution of patient age as an independent prognostic factor. The miRNA risk score and age were used in the multivariate analysis.

Table 1: The age and gender distribution of the patients

Patient characteristic	Training set		<i>p</i> -value*	Test set		<i>p</i> -value*	Overall patient set		<i>p</i> -value*
	Low risk (N=69)	High risk (N=42)		Low risk (N=68)	High risk (N=43)		Low risk (N=137)	High risk (N=85)	
Age (Mean±SD)	51.5 ± 16.6	54.4 ± 11.9	0.5	54 ± 12.7	58.3 ± 10.2	0.1	52.8 ± 14.8	56.4 ± 11.2	0.1
Gender									
Female (%)	26 (37.7)	13 (31)	0.5	27 (39.7)	12 (27.9)	0.2	53 (38.7)	25 (29.4)	0.2
Male (%)	43 (62.3)	29 (69)		41 (60.3)	31 (72.1)		84 (61.3)	60 (70.6)	

*Two-tailed *p*-value obtained from Mann-Whitney test

5 Table 2: Ten miRNA signature that predicts survival in glioblastoma patients

Type	S. No.	miRNA	Hazard Ratio	Coefficient	Permutation <i>p</i> -value	Median log2 expression value (ratio of GBM vs. normal)		GBMs Vs Normal	
						Low risk	High risk	regulation	P value (two-tailed)
Protective	1	hsa-miR-20a	0.68	-0.39	0.0004	1.83	0.86	upregulated	<0.0001
	2	hsa-miR-106a	0.66	-0.41	0.0005	1.67	0.65	upregulated	<0.0001
	3	hsa-miR-17-5p	0.68	-0.39	0.0008	1.60	0.69	upregulated	<0.0001
Risky	4	hsa-miR-31	1.32	0.28	0.0001	-1.83	-0.82	downregulated	0.0022
	5	hsa-miR-222	1.26	0.23	0.0004	-2.15	-0.70	downregulated	0.0002
	6	hsa-miR-148a	1.21	0.19	0.0010	1.16	2.83	upregulated	<0.0001
	7	hsa-miR-221	1.27	0.24	0.0014	-1.93	-0.53	downregulated	0.0003
	8	hsa-miR-146b	1.25	0.22	0.0025	0.10	1.22	upregulated	<0.0001
	9	hsa-miR-200b	1.21	0.19	0.0029	-0.02	0.83	upregulated	<0.0001
	10	hsa-miR-193a	1.34	0.29	0.0045	1.05	2.10	upregulated	<0.0001

**p*-value obtained from Mann-Whitney test

Table 3: Validation of the 10 miRNA expression signature for survival prediction in the testing set

Patient set	Deaths/Patient	Hazard Ratio	Median survival	Survival (95% CI)				
				1 year (%)	2 years (%)	3 years	4 years	5 years

	(%)	(95% CI)	(months, 95% CI)			(%)	(%)	(%)
Training set								
Low risk (n = 69)	57/69 (82.6)	1	19.3 (12.7- 25.9)	66.5 (54.9- 78.1)	41.8 (29.5- 54.1)	25.6 (14.5- 36.7)	23.6 (12.6- 34.6)	14.8 (5- 24.6)
High risk (n = 42)	38/42 (90.5)	2.4 (1.4- 3.8)	12.6 (10.1- 15.1)	50.6 (35.1- 66.1)	9.0 (0.0- 18.4)	3 (0.0- 8.7)	0.0	0.0
Test set								
Low risk (n = 68)	55/68 (80.9)	1	18.0 (14.5- 21.5)	64.8 (52.8- 76.8)	28(16.4- 39.6)	17.3 (7.5- 27.1)	13.5 (5.1- 21.9)	6.2 (0- 13.5)
High risk (n = 43)	36/43 (83.7)	1.7 (1.1- 2.8)	12.1(6.8- 17.4)	49.8 (34.5- 64.1)	13.3(2.2- 24.4)	8.9 (0.0- 19.3)	0.0	0.0
Overall patient set								
Low risk (n = 137)	112/137 (81.8)	1	18.3 (15- 21.6)	65.6 (57.4- 72.8)	35.0 (26.4- 43.2)	21.5 (14.1- 28.9)	18.5 (11.2- 25.8)	11.8 (5.5- 18.1)
High risk (n = 85)	74/85 (87.1)	2.0 (1.4- 2.8)	12.6 (10.3- 14.9)	51.4 (40.6- 62.2)	11.0 (3.5- 18.5)	5.5 (0- 11.2)	0.0	0.0

5 Table 4: Univariate Cox regression analysis of risk score and age and multivariate cox analysis with age and risk score (based on 10 miRNAs) in the entire patient set (n=222)

Variable	Hazard ratio	p-value*
Univariate analysis		
Risk score	1.165	<0.0001
Age	1.027	<0.0001
Multivariate analysis		
Risk score	1.120	0.003
Age	0.019	0.004

*Two-tailed p-value obtained from Mann-Whitney test

Table 5: Univariate Cox regression analysis of risk score for the various training and test sets using risk score based on four miRNA expression signature.

Variable	Hazard ratio	<i>p</i> -value*
Univariate analysis		
Risk score-10 miRNA training	2.107	<0.0001
Risk score-10 miRNA test	1.971	0.002
Risk score-4 miRNA training	1.540	<0.0001
Risk score-4 miRNA test	1.306	0.052

*Two-tailed *p*-value obtained from Mann-Whitney test

5 Tables 6: miRNAs

miRNA	Sequences	SEQUENCE ID NOs.
hsa-miR-20a	UAAAGUGCUUAUAGUGCAGGUAG	SEQ ID NO: 1
hsa-miR-106a	AAAAGUGCUUACAGUGCAGGUAG	SEQ ID NO: 2
hsa-miR-17-5p	CAAAGUGCUUACAGUGCAGGUAG	SEQ ID NO: 3
hsa-miR-31	AGGCAAGAUGCUGGCAUAGCU	SEQ ID NO: 4
hsa-miR-222	AGCUACAUCUGGCUACUGGGU	SEQ ID NO: 5
hsa-miR-148a	UCAGUGCACUACAGAACUUUGU	SEQ ID NO: 6
hsa-miR-221	AGCUACAUUGUCUGCUGGGUUUC	SEQ ID NO: 7
hsa-miR-146b	UGAGAACUGAAUCCAUAGGCU	SEQ ID NO: 8
hsa-miR-200b	UAAUACUGCCUGGUAUAUGAUGA	SEQ ID NO: 9
hsa-miR-193a	AACUGGCCUACAAAGUCCAGU	SEQ ID NO: 10

The foregoing embodiments and examples are merely exemplary and are not to be construed as limiting the present invention. The description of the present invention is intended to be illustrative, and not to limit the scope of the claims. Many alternatives, modifications, and variations will be apparent to those skilled in the art.

We Claim:

1. A process of determining survival prognosis in a subject with glioblastoma, wherein said process comprises

- a. measuring the level of microRNAs hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p, hsa-miR-31, hsa-miR-222, hsa-miR-148a, hsa-miR-221, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a in a biological sample,
- b. calculating a risk score of the biological sample using, wherein the risk score is calculated as $(-0.39 \times \text{expression of hsa-mir-20a}) + (-0.41 \times \text{expression of hsa-mir-106a}) + (-0.39 \times \text{expression of hsa-mir-17-5p}) + (0.28 \times \text{expression of hsa-mir-31}) + (0.23 \times \text{expression of hsa-mir-222}) + (0.19 \times \text{expression of hsa-mir-148a}) + (0.24 \times \text{expression of hsa-mir-221}) + (0.22 \times \text{expression of hsa-mir-146b}) + (0.19 \times \text{expression of hsa-mir-200b}) + (0.29 \times \text{expression of hsa-mir-193a})$,
- c. comparing the risk score of the biological sample with the risk score of a reference set of samples obtained from subjects with glioblastoma, and
- d. determining survival of the subject based on the value of the risk score

wherein the risk score equal to or more than 60th percentile of the reference set samples is indicative of lesser chances of survival compared to the risk score less than 60th percentile of the reference set samples.

2. A process of determining survival prognosis in a subject with glioblastoma, wherein said process comprises

- a. determining expression level of microRNAs hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p, hsa-miR-31, hsa-miR-222, hsa-miR-148a, hsa-miR-221, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a in a sample from a subject with glioblastoma; and
- b. comparing the expression level of the miRNAs to the expression level of the miRNAs in a reference set of samples obtained from subjects with glioblastoma to determine prognosis,

wherein

- (i) increased expression level of hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p relative to the expression level of hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p in a reference set of samples obtained from subjects with glioblastoma is indicative of glioblastoma that is not associated with high risk; and

- (ii) increased expression level of hsa-miR-148a, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a relative to the expression level of hsa-miR-148a, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a in a reference set of samples obtained from subjects with glioblastoma and reduced expression level of hsa-miR-31, hsa-miR-222 and hsa-miR-221 relative to the expression level of hsa-miR-31, hsa-miR-222 and hsa-miR-221 in a reference set of samples obtained from subjects with glioblastoma is indicative of glioblastoma that is associated with high risk.
- 5
3. A kit for determining survival prognosis in a subject with glioblastoma, wherein said kit comprises polynucleotides for analysis of miRNAs hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p, hsa-miR-31, hsa-miR-222, hsa-miR-148a, hsa-miR-221, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a.
- 10
4. The kit as claimed in claim 3, wherein the kit further comprises a set of primers specific for transcription or reverse transcription of miRNAs hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p, hsa-miR-31, hsa-miR-222, hsa-miR-148a, hsa-miR-221, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a.
- 15
5. The kit as claimed in claim 3, wherein the kit further comprises a miRNA array card, wherein the one or more polynucleotides are arrayed on said card.
6. A microarray card for determining survival prognosis in a subject with glioblastoma, wherein the microarray comprises hsa-miR-20a, hsa-miR-106a, hsa-miR-17-5p, hsa-miR-31, hsa-miR-222, hsa-miR-148a, hsa-miR-221, hsa-miR-146b, hsa-miR-200b and hsa-miR-193a specific probe oligonucleotides.
- 20

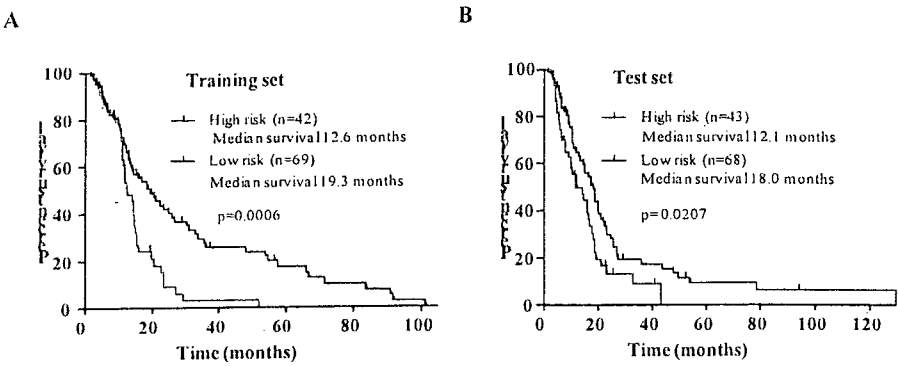


Figure 1

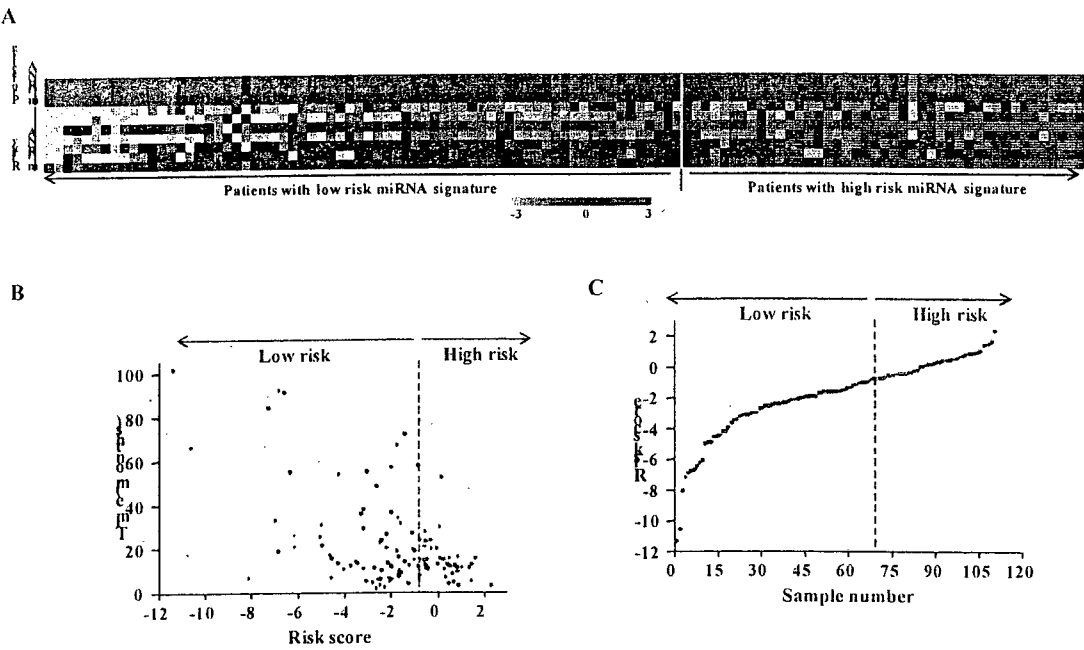


Figure 2

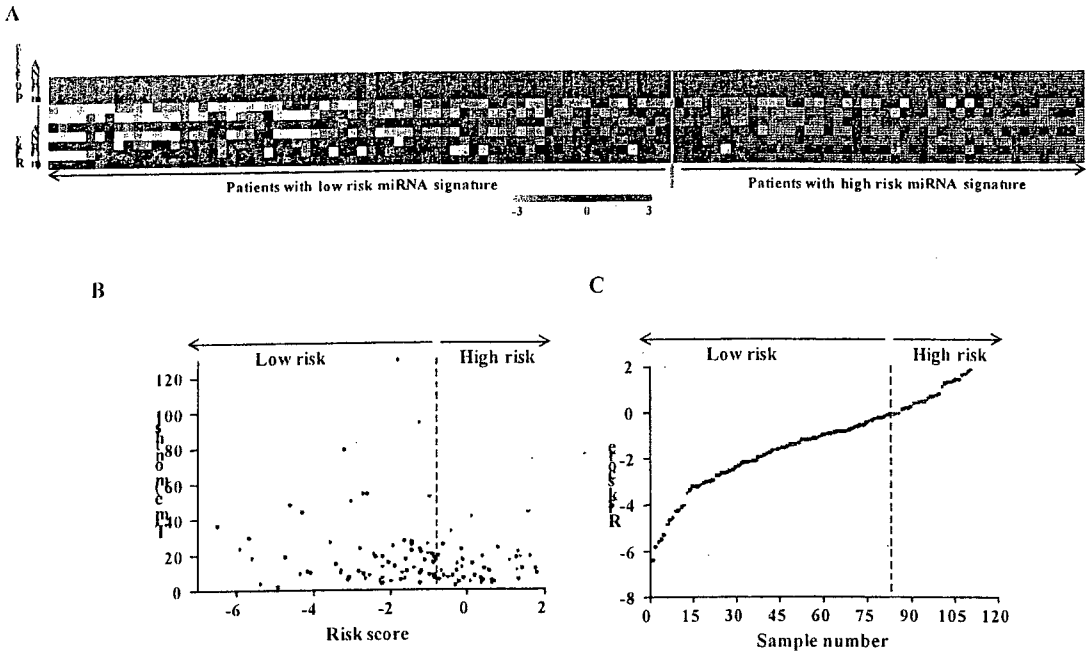


Figure 3

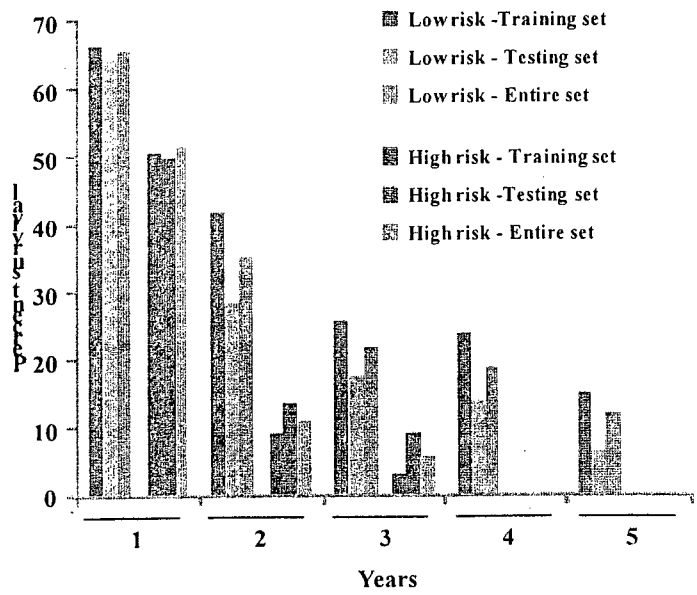


Figure 4

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB2012/000312

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C12Q 1/68 (2006.01)

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)

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: EPODOC, WPI, MEDLINE, HCAPLUS, BIOSIS, EMBASE, BIOTECHABS. Keywords: micro RNA, glioblastoma and like terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	The Cancer Genome Atlas (TCGA) archive unc.edu_GBM.H-miRNA_8x15K.Level_1.1.7.0, 2 May 2007 [retrieved on 12 April 2012] Retrieved from internet <URL: https://tcga-data.nci.nih.gov/tcgafiles/ftp_auth/distro_ftpusers/anonymous/tumor/gbm/cgcc/unc.edu/h-mirna_8x15k/mirna/unc.edu_GBM.H-miRNA_8x15K.Level_1.1.7.0/US14702406_251643612132_S01_miRNA-v1_95_May07_1_1.txt & README for archive unc.edu_GBM.H-miRNA_8x15K.Level_1.1.7.0, 12 August 2010 [retrieved on 12 April 2012] Retrieved from internet <URL: https://tcga-data.nci.nih.gov/tcgafiles/ftp_auth/distro_ftpusers/anonymous/tumor/gbm/cgcc/unc.edu/h-mirna_8x15k/mirna/unc.edu_GBM.H-miRNA_8x15K.Level_1.1.7.0/README_DCC.txt	
X	See Whole Document	3-6

☒ Further documents are listed in the continuation of Box C☒ See patent family annex

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

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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

"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)

"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

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Date of the actual completion of the international search

19 April 2012

Date of mailing of the international search report

19 April 2012

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB2012/000312

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RAO, S. A. M., et al., 'Genome-wide expression profiling identifies deregulated miRNAs in malignant astrocytoma', Modern Pathology, 2010, vol. 23, pages 1404-1417 Figure 1; Supplementary Tables S2; S4, S5 and S7 WO 2010/066384 A1 (MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E. V. et al.) 17 June 2010	1-6
A	Page 9, para 3; page 26, last para SRINIVASAN, S., et al., 'A ten-microRNA expression signature predicts survival in glioblastoma', PLoS ONE, March 2011, vol. 6, issue 3, pages e17438 1-7	
P, X	See Whole Document	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/IB2012/000312

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
WO	2010066384	EP	2199412	EP	2358902	US	2012053224
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.							
END OF ANNEX							