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CA 2667617 A1 2008/05/08

(21) 2 667 617

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2007/11/01
(87) Date publication PCT/PCT Publication Date: 2008/05/08
(85) Entrée phase nationale/National Entry: 2009/04/24
(86) N° demande PCT/PCT Application No.: US 2007/023660
(87) N° publication PCT/PCT Publication No.: 2008/054828
(30) Priorité/Priority: 2006/11/01 (US60/855,895)

(51) Cl.Int./Int.Cl. *C40B 40/06* (2006.01), *C07H 21/02* (2006.01), *C12N 15/11* (2006.01), *C12Q 1/68* (2006.01), *C40B 30/04* (2006.01)

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(54) Titre : SIGNATURE DE L'EXPRESSION DE MICROARN POUR LA PREDICTION DE LA SURVIE ET DES METASTASES DANS LE CARCINOME HEPATO-CELLULAIRE

(54) Title: MICRORNA EXPRESSION SIGNATURE FOR PREDICTING SURVIVAL AND METASTASES IN HEPATOCELLULAR CARCINOMA

(57) Abrégé/Abstract:
Provided herein are methods and compositions for the diagnosis, prognosis and treatment of Hepatocellular carcinoma (HCC). Also provided are methods of identifying anti-HCC agents.

CA 2667617 A1 2008/05/08

(21) 2 667 617

(13) A1

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 May 2008 (08.05.2008)

PCT

(10) International Publication Number
WO 2008/054828 A3

(51) International Patent Classification:
C12Q 1/68 (2006.01) *C07H 21/04* (2006.01)
C07H 21/02 (2006.01)

(21) International Application Number:
PCT/US2007/023660

(22) International Filing Date:
1 November 2007 (01.11.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/855,895 1 November 2006 (01.11.2006) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

(88) Date of publication of the international search report:
11 December 2008

(15) Information about Correction:
Previous Correction:
see Notice of 19 June 2008

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WO 2008/054828 A3

TITLE

MICRORNA EXPRESSION SIGNATURE FOR PREDICTING SURVIVAL AND METASTASES IN HEPATOCELLULAR CARCINOMA

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CROSS-REFERENCE TO RELATED APPLICATIONS AND STATEMENT REGARDING SPONSORED RESEARCH

[0001] The present invention claims the benefit of the provisional patent application Ser. No. 60/855,895 filed November 1, 2007. This invention was made with government support under NCI Grant No. RO1 CA128609. The government has certain rights in this invention.

BACKGROUND OF THE INVENTION

[0002] Hepatocellular carcinoma (HCC) represents an extremely poor prognostic cancer that remains one of the most common and aggressive human malignancies worldwide (1; 2). The dismal outcome has been attributed to the major hallmarks of HCC, intra-hepatic metastases or post-surgical recurrence. New tumor colonies frequently invade into the major branches of the portal vein and possibly other parts of the liver (3-6). Resection or liver transplantation, are the best options for a potential cure however, only about 20 percent of HCC patients, defined by parameters of relatively normal liver function and a manageable tumor lesion as determined by the available clinical staging systems, are currently eligible for surgical intervention. Moreover, resected patients often have a high frequency of metastasis/recurrence, and post-operative 5 year survival is only 30-40 percent.

[0003] Liver transplantation for HCC patients remains controversial due to a shortage of organ donors and the poor performance of current staging systems in selecting appropriate candidates, especially at early disease stages. These systems are essential, particularly in malignant diseases, to provide advice to patients and guidance for assessment and treatment. Clinical evaluation and therapeutic decisions in HCC is complex because they depend on both the grade of cancer spread (tumor staging) and residual liver function (chronic liver disease stage). Although well-defined and generally accepted staging systems are available for almost all cancers, HCC is an exception, with

many different staging systems globally introduced to accommodate each stratum of the disease and a current lack of consensus on which one is best (7-12). Thus, an accurate prognostic predictor and a sensible selection criterion that can be applied to HCC patients for rational treatment decisions remains a challenging task.

[0004] The recent identification of prognostic molecular biomarkers offers hope for advance diagnosis of HCC. Using cDNA microarray technology, the inventors developed a unique gene expression signature to predict prognosis and metastasis of HCC patients (13). The presence of a molecular prognostic signature in primary HCC clinical specimens was confirmed by several recent studies (14; 15). Since HCC is usually present in inflamed liver, the inventors also developed a unique predictor based on the expression of genes in the liver microenvironment of HCC patients, which was principally different from that of the tumor (16). Like many other prognostic signatures based on cDNA gene expression profiling, both the tumor and microenvironment signatures contain several hundred cellular coding genes. Therefore, it would be a challenging task to identify relevant biomarkers or potential pharmacological targets and interrogate scores of genes in clinical practice.

[0005] Recent studies indicate that expression profiling with small non-coding RNA gene products (-22nt) known as microRNAs (miRNAs or miRs) is a superior method for cancer subtype classification and prognostication (17-19). miRNAs exist in many organisms and play key regulatory roles in mRNA translation and degradation by base pairing to partially complementary sites of the mRNA, predominantly in the 3' untranslated region (20-22). miRNAs are expressed as long precursor RNAs that are processed by Drosha, a cellular nuclease, and subsequently transported to the cytoplasm by an Exportin-5-dependent mechanism (23; 24). miRNAs are then cleaved by the DICER enzyme, resulting in ---17-24 nt miRNAs that associate with a RNA-induced silencing-like complex (25; 26). The expression patterns, function and regulation of miRNAs in normal and neoplastic human cells are largely unknown but emerging data and their frequent location at fragile sites, common break-points or regions of amplification or loss of heterozygosity reveal that they may play significant roles in human carcinogenesis.

[0006] The enhanced expression of precursor miR-155 in Burkitt's lymphomas and the frequent deletion or downregulation of several miRNAs have been observed in B cell chronic lymphocytic leukemia (CLL) and in many cancer types, including breast, lung,

ovarian, cervical, colorectal, prostate, and lymphoid (17;18;27-34). Functional analysis has also revealed the downregulation of PTEN by miR-21, the tumor suppressor function of the let-7 family and the oncogenic function of the miRl 7-92 cluster (35-37). The biological and clinical relevance of miRNA expression patterns have been shown in human B cell CLL and solid tumors, including breast cancers (18; 30; 38). Each miRNA has the unique capability to potentially regulate the expression of hundreds of coding genes and thereby modulate several cellular pathways including proliferation, apoptosis and stress response (39). This phenomenon makes miRNAs superior molecular markers and targets for interrogation and as such, miRNA expression profiling can be utilized as a tool for cancer diagnosis (17; 40).

SUMMARY OF THE INVENTION

[0007] In a broad aspect, there is provided herein a unique miRNA signature that can significantly distinguish HCC venous metastasis from metastasis-free HCC. In contrast to HCC staging systems, this signature is capable of predicting survival and recurrence of HCC patients with multinodular or solitary tumors, including those with early-stage disease. Moreover, this signature is an independent and significant predictor of patient prognosis and relapse when compared to other available clinical parameters. This miRNA signature is useful to enable HCC prognosis and has clinical utility for the advance identification of HCC patients with a propensity towards metastasis/recurrence.

[0008] There is provided herein a system of the identification of a chronic hepatocellular carcinoma (HCC), cancer-specific signature of miRNAs that are differentially expressed relative to normal control cells.

[0009] Accordingly, provided herein are methods of diagnosing whether a subject has, or is at risk for developing, HCC comprising measuring the level of at least one miR gene product in a test sample from the subject, wherein an alteration in the level of the miR gene product in the test sample, relative to the level of a corresponding miR gene product in a control sample, is indicative of the subject either having, or being at risk for devèloping, HCC.

[0010] The level of the at least one miR gene product can be measured using a variety of techniques that are well-known to those of skill in the art. In one embodiment, the level of the at least one miR gene product is measured using Northern blot analysis. In

another embodiment, the level of the at least one miR gene product in the test sample is less than the level of the corresponding miR gene product in the control sample. Also, in another embodiment, the level of the at least one miR gene product in the test sample can be greater than the level of the corresponding miR gene product in the control sample.

[0011] Also provided herein are methods of diagnosing a HCC associated with one or more prognostic markers in a subject, comprising measuring the level of at least one miR gene product in a HCC sample from the subject, wherein an alteration in the level of the at least one miR gene product in the test sample, relative to the level of a corresponding miR gene product in a control sample, is indicative of the subject having a HCC associated with the one or more prognostic markers.

[0012] In one embodiment, the level of the at least one miR gene product is measured by reverse transcribing RNA from a test sample obtained from the subject to provide a set of target oligodeoxynucleotides; hybridizing the target oligodeoxynucleotides to a microarray comprising miRNA-specific probe oligonucleotides to provide a hybridization profile for the test sample; and, comparing the test sample hybridization profile to a hybridization profile generated from a control sample. An alteration in the signal of at least one miRNA is indicative of the subject either having, or being at risk for developing, HCC.

[0013] Also provided herein are methods of treating HCC in a subject, wherein the signal of at least one miRNA, relative to the signal generated from the control sample, is de-regulated (e.g., down-regulated and/or up-regulated).

[0014] In certain embodiments, a microarray comprises miRNA-specific probe oligonucleotides for one or more miRNAs selected from one or more of the SEQ ID NOS: 1 - 22, as shown in Figure 11, and, in particular certain embodiments, one miR gene product comprises one or more of: miR-219 [SEQ ID NO: 20], miR-207 [SEQ ID NO: 18], miR-30c [SEQ ID NO: 6], and miR124A [SEQ ID NO: 4].

[0015] Also provided herein are methods of diagnosing whether a subject has, or is at risk for developing, a HCC associated with one or more adverse prognostic markers in a subject, by reverse transcribing RNA from a test sample obtained from the subject to provide a set of target oligodeoxynucleotides; hybridizing the target oligodeoxynucleotides to a microarray comprising miRNA-specific probe oligonucleotides to provide a hybridization profile for the test sample; and, comparing the test sample

hybridization profile to a hybridization profile generated from a control sample. An alteration in the signal is indicative of the subject either having, or being at risk for developing, the cancer.

[0016] Also provided herein are methods of treating HCC in a subject who has HCC in which at least one miR gene product is down-regulated or up-regulated in the cancer cells of the subject relative to control cells. When the one or more miR gene product is down-regulated in the cancer cells, the method comprises administering to the subject an effective amount of at least one isolated miR gene product, such that proliferation of cancer cells in the subject is inhibited. When one or more miR gene product is up-regulated in the cancer cells, the method comprises administering to the subject an effective amount of at least one compound for inhibiting expression of at least one miR gene product, such that proliferation of cancer cells in the subject is inhibited. In certain embodiments, the at least one isolated miR gene product is selected miR-219 [SEQ ID NO: 20], miR-207 [SEQ ID NO: 18], miR-30c [SEQ ID NO: 6] and miR124A and combinations thereof.

[0017] Also provided herein are methods of treating HCC in a subject, comprising: determining the amount of at least one miR gene product in HCC cells, relative to control cells; and, altering the amount of miR gene product expressed in the HCC cells by: administering to the subject an effective amount of at least one isolated miR gene product, if the amount of the miR gene product expressed in the cancer cells is less than the amount of the miR gene product expressed in control cells; or administering to the subject an effective amount of at least one compound for inhibiting expression of the at least one miR gene product, if the amount of the miR gene product expressed in the cancer cells is greater than the amount of the miR gene product expressed in control cells, such that proliferation of cancer cells in the subject is inhibited. In certain embodiments, at least one isolated miR gene product is selected from the group consisting of miR-219 [SEQ ID NO: 20], miR-207 [SEQ ID NO: 18], miR-30c [SEQ ID NO: 6] and miR124A, and combinations thereof.

[0018] Also provided herein are pharmaceutical compositions for treating HCC, comprising at least one isolated miR gene product and a pharmaceutically-acceptable carrier. In a particular embodiment, the pharmaceutical compositions comprise at least one isolated miR gene product corresponds to a miR gene product that is down-regulated

in HCC cells relative to suitable control cells.

[0019] In another particular embodiment, the pharmaceutical composition comprises at least one miR expression regulator (for example, an inhibitor) compound and a pharmaceutically-acceptable carrier.

[0020] Also provided herein are pharmaceutical compositions that include at least one miR expression regulator compound that is specific for a miR gene product that is up- or down-regulated in HCC cells relative to suitable control cells.

[0021] Also provided herein are methods of identifying an anti-HCC agent, comprising providing a test agent to a cell and measuring the level of at least one miR gene product associated with decreased expression levels in HCC cells, wherein an increase in the level of the miR gene product in the cell, relative to a suitable control cell, is indicative of the test agent being an anti-HCC agent. In certain embodiments, the miR gene product comprises one or more of the SEQ ID NOS: 1 - 22, as shown in Figure 11. In particular certain embodiments, one miR gene product comprises one or more of: miR-219 [SEQ ID NO: 20], miR-207 [SEQ ID NO: 18], miR-30c [SEQ ID NO: 6], and miR124A [SEQ ID NO: 4].

[0022] Also provided herein are methods of identifying an anti-HCC agent, comprising providing a test agent to a cell and measuring the level of at least one miR gene product associated with increased expression levels in HCC cells, wherein a decrease in the level of the miR gene product in the cell, relative to a suitable control cell, is indicative of the test agent being an anti-HCC agent.

[0023] In certain embodiments, the miR gene product comprises one or more of the SEQ ID NOS: 1 - 22, as shown in Figure 11. In particular certain embodiments, one miR gene product comprises one or more of: miR-219 [SEQ ID NO: 20], miR-207 [SEQ ID NO: 18], miR-30c [SEQ ID NO: 6], and miR124A [SEQ ID NO: 4].

[0024] Various objects and advantages of this invention will become apparent to those skilled in the art from the following detailed description of the preferred embodiment, when read in light of the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawings will be

provided by the Office upon request and payment of the necessary fee.

[0026] **FIGURE 1**: Schematic of the search for a miRNA signature that can predict HCC prognosis.

[0027] **FIGURE 2**: Significant differentially expressed miRNAs in metastatic vs non-metastatic liver tissues from HCC patients.

[0028] (FIG. 2A) Hierarchical clustering of 20 miRNA genes whose expression was significantly ($p<0.001$) altered in metastasis (M; blue bars; n=30) and non-metastasis samples (NM; yellow bars; n=104) from class prediction analysis using 4 different algorithms (compound covariate predictor, linear discriminant analysis, nearest neighbor and support vector machines) employing leave-one-out cross validation to establish prediction accuracy. Each row represents an individual gene and each column represents an individual tissue sample. Genes were ordered by center correlation and complete linkage according to the ratios of abundance in each tissue sample compared to a normal liver tissue pool (n=8), which were normalized to the mean abundance of genes. Pseudocolors indicate transcript levels below, equal, or above the mean (green, black and red, respectively). The scale represents the gene expression ratios from -4 to 4 in log 2 scale.

[0029] (Fig. 2B) Kaplan-Meier survival analysis of metastasis and non-metastasis samples based on prediction outcome of the 20 miRNAs.

[0030] **FIGURE 3:** Analysis of the classification capacity of the 20-miRNA or 4-miRNA signature in the testing cohort or early-stage HCC. Kaplan-Meier overall survival analysis of 110 HCC patients based on predicted classification by the (FIG. 3A) 20-miRNA predictor; (FIG. 3B) 4-miRNA predictor. Kaplan-Meier overall survival analysis of 89 early-stage HCC patients based on predicted classification by the (FIG. 3C) 20-miRNA predictor (FIG. 3D) 4-miRNA predictor. Kaplan-Meier relapse-free survival analysis of 89 early-stage HCC patients based on predicted classification by the (FIG. 3E) 20-miRNA predictor (FIG. 3F) 4-miRNA predictor.

[0031] **FIGURE 4.** Table 1 showing the clinical characteristics of patients for Example I.

[0032] **FIGURE 5.** Table 2 showing univariate and multivariate analyses of factors associated with survival and recurrences (TMM stage I and II).

[0033] **FIGURE 6.** Table 3 - Summary of 20 micro RNAs with a prognostic value

to predict HCC survival/

[0034] **FIGURE 7.** Table 4 - Clinical staging of the poorly-defined set.

[0035] **FIGURE 8**. Table 5 - Univariate and multivariate analyses of factors associated with survival and recurrence (BCLC Stage 0 and A).

[0036] **FIGURE 9.** Table 6 - Univariate and multivariate analyses of factors associated with survival and recurrence.

[0037] **FIGURE 10**. Analysis of the classification capacity of staging systems in the testing cohort. Kaplan-Meier survival analysis of 110 HCC patients based on predicted classification by (FIG. 10A) TNM staging (FIGL 10B) OKUDA staging (FIG. 10C) CLIP staging or (FIG. 10D) BCLC staging.

[0038] **FIGURE 11**. A table containing a set of 22 miRNAs useful for predicting HCC, [SEQ ID NOS: 1-22].

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

[0039] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not intended to limit the scope of the current teachings. In this application, the use of the singular includes the plural unless specifically stated otherwise.

[0040] The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

[0041] Also, the use of "comprise", "contain", and "include", or modifications of those root words, for example but not limited to, "comprises", "contained", and "including", are not intended to be limiting. The term "and/or" means that the terms before and after can be taken together or separately. For illustration purposes, but not as a limitation, "X and/or Y" can mean "X" or "Y" or "X and Y".

[0042] It is understood that an miRNA is derived from genomic sequences or a gene. In this respect, the term "gene" is used for simplicity to refer to the genomic sequence encoding the precursor miRNA for a given miRNA. However, embodiments of the invention may involve genomic sequences of a miRNA that are involved in its expression, such as a promoter or other regulatory sequences.

[0043] The term "miRNA" generally refers to a single-stranded molecule, but in

specific embodiments, molecules implemented in the invention will also encompass a region or an additional strand that is partially (between 10 and 50% complementary across length of strand), substantially (greater than 50% but less than 100% complementary across length of strand) or fully complementary to another region of the same single-stranded molecule or to another nucleic acid. Thus, nucleic acids may encompass a molecule that comprises one or more complementary or self-complementary strand(s) or "complement(s)" of a particular sequence comprising a molecule. For example, precursor miRNA may have a self-complementary region, which is up to 100% complementary miRNA probes of the invention can be or be at least 60, 65, 70, 75, 80, 85, 90, 95, or 100% complementary to their target.

[0044] The term "combinations thereof" as used herein refers to all permutations and combinations of the listed items preceding the term. For example, "A, B, C, or combinations thereof" is intended to include at least one of: A, B, C, AB, AC, BC, or ABC, and if order is important in a particular context, also BA, CA, CB, ACB, CBA, BCA, BAC, or CAB.

[0045] The section headings used herein are for organizational purposes only and are not to be construed as limiting the described subject matter in any way. All literature and similar materials cited in this application, including patents, patent applications, articles, books, treatises, and internet web pages are expressly incorporated by reference in their entirety for any purpose. In the event that one or more of the incorporated literature and similar materials defines or uses a term in such a way that it contradicts that term's definition in this application, this application controls.

[0046] MicroRNAs (miRNAs) are transcripts of a new class of small noncoding RNA genes that are able to distinguish several types of aggressive cancers, including hepatocellular carcinoma (HCC), from their normal counterparts. HCC patients have a very poor prognosis due to high rate of metastasis, and current staging systems are not capable of accurately determining patient prognosis, especially at early stages of this disease. The inventors investigated whether unique miRNAs are associated with prognosis and metastases in HCC.

[0047] The inventors examined the miRNA expression profiles of 490 specimens from radical resection of 244 HCC patients. The inventors discovered a unique miRNA signature based on 134 clinically well-defined metastatic and non-metastatic HCC

specimens. The unique signature was used to predict the prognostic outcomes of a 110 independent HCC specimens.

[0048] The miRNA signature composed of 20 unique oligonucleotides can significantly discriminate ($p<0.001$) 30 primary HCC tissues with venous metastases from 104 metastasis-free solitary HCC with cross validation in a training cohort. However, significant miRNAs could not be identified from the corresponding non-cancerous hepatic tissues.

[0049] The tumor metastasis miRNA signature was a significant predictor of patient survival ($p<0.0023$) and recurrence ($p=0.002$) is 89 early stage HCC. A refined signature composed of 4 selected miRNAs had a similar prediction power. Notably, high miR-219 [SEQ ID NO: 20] and miR-207 [SEQ ID NO: 18] and low miR-30c [SEQ ID NO: 6] and miR-124a [SEQ ID NO: 4] expression correlated with venous metastases and poor survival. Cox proportional hazards modeling also revealed that this signature was superior to other clinical variables, including the known staging systems, for predicting patient survival.

[0050] The unique miRNA signature is useful for HCC prognosis, particularly in patients whose outcome is hard to predict by conventional staging systems. The examples herein show that measurement of certain miRNA levels in HCC have clinical utility for the advance identification of patients who are likely to develop metastases and subsequently classify them for appropriate treatment.

[0051] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

[0052] **EXAMPLE I**

[0053] **HCC and Associated Conditions**:

[0054] Hepatic tissues were obtained with informed consent from patients who underwent radical resection between 2002 and 2003 at the Liver Cancer Institute and

Zhongshan Hospital (Fudan University, Shanghai, China). The study was approved by the Institutional Review Board of the Liver Cancer Institute and NIH. Gene expression profiles were conducted in primary HCC and corresponding noncancerous hepatic tissues from 244 Chinese HCC patients. Among them, 93% had underlying cirrhosis and 68% had a serum alpha-fetoprotein (AFP) level > 20 ng/ml (Figure 4 - Table 1).

[0055] The general strategy for partitioning cases and testing the miRNA signature is outlined in Figure 1. A total of 134 well-defined cases were used as the training group. Among them, 30 had primary HCC lesions accompanied by tumor emboli found in the major branches of the portal vein (n=25), inferior vena cava (n=2) or common bile duct (n=4; one also with tumor thrombi in inferior vena cava) and 104 had solitary HCC with no metastasis/recurrence found at follow-up (3yr).

[0056] In the validation analysis, the inventors used a testing group of 110 independent cases (Figure 1: poorly-defined set) whose prognosis could not be accurately determined at the time of resection by several HCC staging systems. The testing cases included 43 multinodular and 67 solitary HCC. Of the 43 multinodular HCC cases, 18 developed intrahepatic recurrence and one developed extrahepatic metastasis in addition to an intrahepatic recurrence. Of the 67 solitary HCC cases, 4 patients had a solitary tumor with an appearance of aggregated nodules, 10 developed intra- and/or extrahepatic metastases while 49 developed intrahepatic recurrence confirmed at follow-up (3yr). In addition, eight normal liver tissues from disease-free patients [described in (16)] were included as normal controls.

[0057] **RNA isolation and miRNA arrays**:

[0058] The RNA isolation and miRNA array methodology were essentially as previously described (13; 17). In the analysis of the 244 HCC cases, RNA was isolated in a pairwise fashion from tumor or non-tumor tissue and samples were selected in random order for miRNA analysis to avoid grouping bias. A total of 488 microarrays were performed (see Example II).

[0059] **Statistical analyses**:

[0060] Unsupervised hierarchical clustering analysis was performed by the GENESIS software version 1.5 developed by Alexander Sturn (IBMT-TUG, Graz, Austria). The BRB ArrayTools software V3.3 was used for supervised analysis, as previously described (13; 16). The Kaplan-Meier survival analysis was used to compare

patient survival based on prediction results, using Excel-based WinSTAT software (http://www.winstat.com). The statistical p value was generated by the Cox-Mantel log-rank test. Cox proportional hazards regression was used to analyze the effect of sixteen clinical variables on patient survival or recurrence using STATA 9.2 (College Station, TX) (see Example II). The statistical significance was defined as $p<0.05$. TargetScan analysis was based on a website tool developed by Ben Lewis (http://genes.mit.edu/targetscan/index.html) (see Example II) (41).

[0061] **RESULTS:**

[0062] **The search for a miRNA metastasis signature in HCC tissues.**

[0063] In a cohort of 244 HCC cases, the inventors compared primary HCC or noncancerous tissues from 30 cases with venous metastases (M) and 104 non-metastasis cases (NM) by a supervised class comparison approach (see methods in Example II) (Figure 1, and Figure 4 - Table 1). The inventors identified 20 miRNAs that can discriminate the tumor tissues of M from NM cases (Figure 2A and Figure 6 - Table 3).

[0064] When the non-cancerous tissue miRNA expression data were used, the inventors could not identify any miRNA capable of distinguishing M from NM at the same statistical significance level (data not shown). Thus, there are more measurable changes in miRNA expression in tumor cells compared to that of the hepatic microenvironment, which suggests that analysis of miRNA expression in tumor tissues may be better suited for differentiating HCC patient groups.

[0065] Moreover, significant miRNAs could not be identified when a comparison of these tissues was made with other clinical variables including multinodular status, microvascular invasion and 4 clinical staging systems (data not shown). Therefore, the expression of certain miRNAs appeared to correlate with metastasis only when macrovascular invasion was evident. Of the 20 miRNAs, 4 were overexpressed in M while 16 were overexpressed in NM.

[0066] **Composition and predictive value of a refined miRNA metastasis signature.**

[0067] To determine if the 20-miRNA signature was related to patient prognosis, the inventors first performed multivariate nearest neighbor class prediction with 10% cross-validation and 1000 permutations. This analysis resulted in a statistically significant prediction of metastases with an overall accuracy of 76% ($p=0.001$). Kaplan Meier

survival analysis based on the 20-miRNA prediction results revealed that the predicted metastasis group had a significantly shorter survival period when compared to the non-metastasis group ($p<0.042$) (Figure 2B). Thus, this signature is associated with patient prognosis.

[0068] To further test the robustness of the miRNA signature, the inventors tested its ability to predict an independent set of HCC cases based on the results of the cross-validated training set (Figure 1). The inventors found that the predicted M group had a significantly worse survival rate than the NM group ($p=0.009$) (Figure 3A).

[0069] Using a gene reduction approach with various miRNA combinations, the inventors found that significant prediction of survival can still be achieved with only 4 miRNAs miR-219 [SEQ ID NO: 20], miR-207 [SEQ ID NO: 18], miR-30c [SEQ ID NO: 6], and miR124A [SEQ ID NO: 4] ($p=0.003$) (Figure 3B).

[0070] It appeared that an increased expression of miR-219 [SEQ ID NO: 20] and miR-207 [SEQ ID NO: 18] and decreased expression of miR-30c [SEQ ID NO: 6] and miR-124a [SEQ ID NO: 4] are associated with HCC venous metastases and prognosis (Figure 6 - Table 3).

[0071] In contrast, 4 HCC prognostic staging systems (i.e., TNM, OKUDA, CLIP or BCLC) were incapable of predicting patient survival in this testing cohort (Figure 6 - Figure 3, Figure 7 - Table 4, and Figure 8 - Table 5).

[0072] Since the ability to predict risk of cancer spread at early stages of HCC can have a significant clinical impact, the inventors also assayed the prognostic capacity of the 20- or 4miRNA signature for early stage HCC patients (TNM stage I or II; $n=89$). Similar to the entire testing set, a significantly worse survival was observed for the predicted M patients versus NM by both the 20- or 4-miRNA signature in the early stage cohort ($p=0.022$ or $p=0.027$) (Figure 3C and D).

[0073] In addition, the inventors also tested the capacity of the signatures to predict recurrence in the early stage cohort and found that the predicted M group based on the 20 or 4 miRNA signature had a higher recurrence rate ($p=0.002$ or $p=0.020$) than the NM group (Figure 3E and F, Figure 5 - Table 2).

[0074] Meanwhile, the clinical staging systems were incapable of predicting overall or disease-free survival in this cohort (Figure 5 - Table 2). Thus, the miRNA signature identified is a superior predictor of HCC patient outcome, particularly for early

stage disease.

[0075] **Comparison of the miRNA predictor and known clinical staging systems.**

[0076] Next, the inventors performed Cox proportional hazards regression analysis to determine whether the miRNA predictor was confounded by underlying clinical conditions within the early stage cohort. A univariate analysis revealed that the miRNA signature was a significant predictor of survival and recurrence ($p=0.027$ and $p=0.002$, respectively) (Figure 5 - Table 2).

[0077] The multivariate parsimonious survival model, which controlled for potential confounding covariates demonstrated that the miRNA predictor was associated with a significant 3.0 fold increased risk of death for patients with the M versus the NM expression profile (Figure 5 - Table 2).

[0078] The multivariate parsimonious recurrence model, demonstrated that the miRNA predictor was associated with a significant 2.8 fold increased risk of recurrence for those with the M expression profile compared with that of NM (Figure 5 - Table 2) when controlling for potential confounders. The inventors also performed Cox regression analysis on an early stage cohort determined by BCLC staging (Stage 0 and A) and on the entire testing cohort and found similar results (Figure 8 Table 5 and Figure 9 - Table 6).

[0079] In contrast, the clinical HCC staging systems were not capable of predicting patient prognosis and relapse within the testing cohort (Figure 5 - Table 2 and Figure 8 - Table 5). Thus, the miRNA signature is an independent predictor for both survival and relapse.

[0080] **DISCUSSION of EXAMPLE I:**

[0081] A majority of HCC patients are diagnosed at a late stage and only a small percentage fit resection or transplantation criteria. The outcome of HCC patients has been less than satisfactory, largely due to the lack of a simple, validated and universal clinical staging system with robust predictive power, especially for early stage patients and for those with solitary or multinodular HCC that eventually metastasize or recur. Thus, a key challenge to improving HCC patient outcome is early detection and classification.

[0082] The inventors have shown that the expression of 20 miRNAs, or even 4 miRNAs, can significantly predict the survival of HCC patients with solitary or multinodular tumors who develop metastasis/recurrence and can effectively do so in HCC

patients with relatively small tumors who were at an early stage of this disease. In contrast, the clinical HCC staging systems were unable to distinguish the outcome of these patients.

[0083] The 4 miRNAs with the most significant weight in the signature have not been associated with the progression of any human malignancies reported and may therefore be uniquely associated with metastatic HCC. The inventors note that since multinodular HCC patients had a better survival and recurrence rate than solitary HCC patients within this cohort, the association of these outcomes was inversely associated with nodular type.

[0084] Isolation, amplification and expression analysis techniques for miRNA are rapidly progressing, increasing the likelihood of feasible miRNA profiling in clinical tissue. Since miRNAs can be used to provide a higher accuracy in subtype classification and the examples herein show a superior ability to distinguish classically poor-to-predict HCC patient cohorts, grouping patients according to their miRNA signature expression may have clinical utility. The advance identification of poor prognosis patients (M) by the miRNA signature may allow for more personalized, directed or aggressive treatment regimens than patients classified in the good prognosis group (NM).

[0085] The miRNAs and/or the miRNA signature may also be used for prioritizing HCC patients to receive liver transplantation because of the limited supply of available donors and the lack of an adequate allocation system.

[0086] Another advantage is that, for optimum clinical use and potentially more efficient diagnosis, it would be appropriate to have a minimum number of genes that can discriminate patients who are likely to develop more aggressive forms of the disease. The inventors have demonstrated that as few as 4 miRNAs are capable of significantly discriminating HCC patients who have a poor outcome. Thus, these miRNAs are promising tools that may facilitate HCC diagnosis, particularly for early stage patients, and allow for appropriate clinical counsel and treatment.

[0087] The miRNAs and/or Mir signature can be also useful to identify candidate miRNA targets that are differentially expressed in patients who develop metastases/recurrence.

[0088] Also, these miRNAs are useful to provide insight into the biological consequence of miRNA alteration in HCC. The miRNAs and/or miR signature is also

useful to develop and/ or serve as therapeutic targets to reverse the potential outcome of patients with a poor prognostic signature defined by miRNA classification.

[0089] Another advantage is the miRNAs and/or miR signature is useful in developing methods and/or compositions to reverse the course of the disease. Such reversion possibilities may occur, for example, through gene therapy options to alter the expression of miRNAs or their targets. Other non-limiting examples include inactivation of oncogenic phenotypes by synthetic antisense oligonucleotides, generation of specific inhibitors to abrogate miRNA/target gene interaction or overexpression of tumor suppressive phenotypes using viral or liposomal delivery.

[0090] The miRNAs and/or miR signature are useful for the early diagnosis and associated interventional treatment and can be used to change the rather fatalistic approach to HCC. The miRNA signature disclosed herein can thus be used to classify HCC patients at an early stage, enabling their diagnosis and improving clinical outcome.

[0091] **EXAMPLE II**

[0092] **HBV and Associated Hepatic Conditions**

[0093] The sample enrollment criteria included those with a history of hepatitis B virus HBV infection or HBV-related liver cirrhosis, HCC diagnosed by two independent pathologists, detailed information on clinical presentation and pathological characteristics; and detailed follow-up data for at least 3 years, which included intrahepatic recurrence, intrahepatic venous metastasis, lymph node involvement, extrahepatic metastases, disease-free and overall survival, as well as the cause of death.

[0094] The updated TNM classification is superior to other staging systems, including CLIP and Okuda for HCC patients who undergo resection and was therefore chosen to stratify early stage patients (TNM stage I and II) for analysis of miRNA prediction capacity (1; 2). Since a prospective study revealed that the BCLC system was superior to the new TNM classification system updated in 2002, the inventors also performed Cox proportional hazards modeling based on early stage patients categorized by BCLC (Stage 0 and A).

[0095] **miRNA arrays**:

[0096] The miRNA microarray platform (V 2.0) was composed of 250 non-redundant human and 200 mouse miRNAs and arrays were performed at the Microarray Shared Resource, Comprehensive Cancer Center at the Ohio State University. To

examine the robustness of the miRNA array platform, the inventors first analyzed whether miRNA expression can differentiate 244 HCC tissues from their paired surrounding noncancerous hepatic tissues (Figure 4 - Table 6).

[0097] Using a supervised class comparison method with a univariate paired t-test and a multivariate test with 1000 permutations of the class label with the false discovery rate set to <1 with 99% confidence, the inventors identified 209 non-redundant miRNAs that can significantly discriminate HCC tumor tissues (T) from their paired nontumor tissue (NT) (data not shown)

[0098] These significant miRNAs clearly separate T and NT samples, illustrated by hierarchical clustering analysis (data not shown). Multivariate class prediction algorithm analyses with 10% cross-validation and 100 random permutations indicated that these miRNAs can provide a statistically significant prediction of T and NT samples ($p<0.01$) with a >97% accuracy by the nearest neighbor predictor (data not shown). These initial analyses indicated that the miRNA arrays were robust and can identify a significant difference between tumor and noncancerous hepatic tissues. The same method was used to compare metastasis (M) and non-metastasis (NM) cases.

[0099] **Statistical analyses**:

[00100] Cox proportional hazards regression was used to analyze the effect of clinical variables on patient overall and relapse-free survival, including age, sex, HBV active status, pre-resection alphafetoprotein (AFP), cirrhosis, alanine transferase (ALT), Child-Pugh score, tumor size, tumor encapsulation, nodular type, the status of microvascular invasion, Edmondson grade and several HCC prognosis staging systems including BCLC staging (3), CLIP classification (4), Okuda staging (5), or TNM classification (AJCC/UICC, 6th edition) (6). A univariate test was used to examine the influence of the miRNA predictor or each clinical variable on patient survival or recurrence for the entire testing set (n=110; Figure 8 - Table 5 and Figure 9 - Table 6) or early stage HCC (n=89; Figure 5 - Table 2).

[00101] A multivariate analysis was performed to estimate the hazards ratio of the miRNA predictor while controlling for clinical variables identified from a stepwise selection process using both forward addition and backwards selection routines with significance set at $p<0.05$. Furthermore, the hazards ratio for the miRNA predictor alone was compared to the hazards ratio for the miRNA predictor with each of the clinical

variables. If a 10% change in the hazards ratio of the predictor was observed with the addition of a single covariate, this variable was controlled for in the final Cox proportional hazards model.

[00102] For the entire testing set, the most parsimonious survival model included the 20 miRNA predictor, tumor size, multinodular status and TNM staging while the most parsimonious recurrence model included the 20 miRNA predictor, multinodular status, TNM staging, BCLC staging and Okuda staging. For the early stage HCC set, the most parsimonious survival model included the 20 miRNA predictor, AFP, cirrhosis, tumor size, multinodular status, microvascular invasion and TNM staging while the most parsimonious recurrence model included the 20 miRNA predictor, tumor size, multinodular status and TNM staging.

[00103] Multi-collinearity of the covariates was assessed and was not found to be present and it was determined that all final models met the proportional hazards assumption. The statistical significance was defined as $p<0.05$. The inventors do note that in the univariate analyses Child-Pugh class could not be accurately analyzed due to the small sample size within this covariate compared to the other assessed clinical variables in this cohort.

[00104] To provide a sense of confidence in the potential miRNA target list output generated from the TargetScan bioinformatics approach, the inventors restricted the search by focusing on potential miRNA targets that were part of the 153-gene HCC tumor signature of venous metastases identified recently (7) and had a low FDR score (<0.3).

[00105] The inventors further limited output to only those potential cellular targets whose expression in metastatic HCC was inversely correlated with that of the corresponding miRNA. A summary of these host targets based on the search criteria described above is included in Figure 6 - Table 3.

[00106] Figure 10 shows an analysis of the classification capacity of staging systems in the testing cohort. Kaplan-Meier survival analysis of 110 HCC patients based on predicted classification by (A) TNM staging (B) OKUDA staging (C) CLIP staging or (D) BCLC staging.

[00107] **EXAMPLE III**

[00108] In one particular aspect, there is provided herein a method of diagnosing whether a subject has, or is at risk for developing, hepatocellular carcinoma (HCC). The

method generally includes measuring the level of at least one miR gene product in a test sample from the subject and determining whether an alteration in the level of the miR gene product in the test sample, relative to the level of a corresponding miR gene product in a control sample, is indicative of the subject either having, or being at risk for developing, HCC. In certain embodiments, the level of the at least one miR gene product is measured using Northern blot analysis. Also, in certain embodiments, the level of the at least one miR gene product in the test sample is less than the level of the corresponding miR gene product in the control sample, and/or the level of the at least one miR gene product in the test sample is greater than the level of the corresponding miR gene product in the control sample.

[00109] In certain embodiments, the miR gene product comprises one or more of the SEQ ID NOS: 1 - 22, as shown in Figure 11. In particular certain embodiments, one miR gene product comprises one or more of: miR-219 [SEQ ID NO: 20], miR-207 [SEQ ID NO: 18], miR-30c [SEQ ID NO: 6], and miR124A [SEQ ID NO: 4].

[00110] **EXAMPLE IV**

[00111] **Measuring miR Gene Products**

[00112] The level of the at least one miR gene product can be measured by reverse transcribing RNA from a test sample obtained from the subject to provide a set of target oligodeoxynucleotides; hybridizing the target oligodeoxynucleotides to a microarray comprising miRNA-specific probe oligonucleotides to provide a hybridization profile for the test sample; and, comparing the test sample hybridization profile to a hybridization profile generated from a control sample. An alteration in the signal of at least one miRNA is indicative of the subject either having, or being at risk for developing, HCC.

[00113] **EXAMPLE V**

[00114] **Diagnostic and Therapeutic Applications**

[00115] In another aspect, there is provided herein are methods of treating HCC in a subject, where the signal of at least one miRNA, relative to the signal generated from the control sample, is de-regulated (e.g., down-regulated and/or up-regulated).

[00116] In certain embodiments, the miR gene product comprises one or more of the SEQ ID NOS: 1 - 22, as shown in Figure 11. In particular certain embodiments, one miR gene product comprises one or more of: miR-219 [SEQ ID NO: 20], miR-207[SEQ ID NO: 18], miR-30c [SEQ ID NO: 6], and miR124A [SEQ ID NO: 4] and combinations

thereof.

[00117] Also provided herein are methods of diagnosing whether a subject has, or is at risk for developing, a HCC associated with one or more adverse prognostic markers in a subject, by reverse transcribing RNA from a test sample obtained from the subject to provide a set of target oligodeoxynucleotides; hybridizing the target oligodeoxynucleotides to a microarray comprising miRNA-specific probe oligonucleotides to provide a hybridization profile for the test sample; and, comparing the test sample hybridization profile to a hybridization profile generated from a control sample. An alteration in the signal is indicative of the subject either having, or being at risk for developing, the cancer.

[00118] Also provided herein are methods of treating HCC in a subject who has HCC in which at least one miR gene product is down-regulated or up-regulated in the cancer cells of the subject relative to control cells. When the one or more miR gene product is down-regulated in the cancer cells, the method comprises administering to the subject an effective amount of at least one isolated miR gene product, such that proliferation of cancer cells in the subject is inhibited. When one or more miR gene product is up-regulated in the cancer cells, the method comprises administering to the subject an effective amount of at least one compound for inhibiting expression of at least one miR gene product, such that proliferation of cancer cells in the subject is inhibited. In certain embodiments, the at least one isolated miR gene product is selected miR-219 [SEQ ID NO: 20], miR-207 [SEQ ID NO: 18], miR-30c [SEQ ID NO: 6] and miR124A and combinations thereof.

[00119] Also provided herein are methods of treating HCC in a subject, comprising: determining the amount of at least one miR gene product in HCC cells, relative to control cells; and, altering the amount of miR gene product expressed in the HCC cells by: administering to the subject an effective amount of at least one isolated miR gene product, if the amount of the miR gene product expressed in the cancer cells is less than the amount of the miR gene product expressed in control cells; or administering to the subject an effective amount of at least one compound for inhibiting expression of the at least one miR gene product, if the amount of the miR gene product expressed in the cancer cells is greater than the amount of the miR gene product expressed in control cells, such that proliferation of cancer cells in the subject is inhibited. In certain embodiments, the miR

gene product comprises one or more of the SEQ ID NOS: 1 - 22, as shown in Figure 11. In particular certain embodiments, one miR gene product comprises one or more of: miR-219 [SEQ ID NO: 20], miR-207[SEQ ID NO: 18], miR-30c [SEQ ID NO: 6], and miR124A [SEQ ID NO: 4] and combinations thereof.

[00120] **EXAMPLE VI**

[00121] **Compositions**

[00122] Also provided herein are pharmaceutical compositions for treating HCC, comprising at least one isolated miR gene product and a pharmaceutically-acceptable carrier. In a particular embodiment, the pharmaceutical compositions comprise at least one isolated miR gene product corresponds to a miR gene product that is down-regulated in HCC cells relative to suitable control cells. In certain embodiments, the miR gene product comprises one or more of the SEQ ID NOS: 1 - 22, as shown in Figure 11. In particular certain embodiments, one miR gene product comprises one or more of: miR-219 [SEQ ID NO: 20], miR-207 [SEQ ID NO: 18], miR-30c [SEQ ID NO: 6], and miR124A [SEQ ID NO: 4].

[00123] In another particular embodiment, the pharmaceutical composition comprises at least one miR expression regulator (for example, an inhibitor) compound and a pharmaceutically-acceptable carrier.

[00124] Also provided herein are pharmaceutical compositions that include at least one miR expression regulator compound that is specific for a miR gene product that is up- or down-regulated in HCC cells relative to suitable control cells.

[00125] Also provided herein are methods of identifying an anti-HCC agent, comprising providing a test agent to a cell and measuring the level of at least one miR gene product associated with decreased expression levels in HCC cells, wherein an increase in the level of the miR gene product in the cell, relative to a suitable control cell, is indicative of the test agent being an anti-HCC agent. In certain embodiments, the miR gene product comprises one or more of the SEQ ID NOS: 1 - 22, as shown in Figure 11. In particular certain embodiments, one miR gene product comprises one or more of: miR-219 [SEQ ID NO: 20], miR-207 [SEQ ID NO: 18], miR-30c [SEQ ID NO: 6], and miR124A [SEQ ID NO: 4] and combinations thereof.

[00126] Also provided herein are methods of identifying an anti-HCC agent, comprising providing a test agent to a cell and measuring the level of at least one miR

gene product associated with increased expression levels in HCC cells, wherein a decrease in the level of the miR gene product in the cell, relative to a suitable control cell, is indicative of the test agent being an anti-HCC agent. In a particular embodiment, the miR gene product is selected from the group consisting of miR-219 [SEQ ID NO: 20], miR-207 [SEQ ID NO: 18], miR-30c [SEQ ID NO: 6] and miR124A and combinations thereof.

[00127] **EXAMPLE VII**

[00128] **Kits**

[00129] Any of the compositions described herein may be comprised in a kit. In a non-limiting example, reagents for isolating miRNA, labeling miRNA, and/or evaluating an miRNA population using an array are included in a kit. The kit may further include reagents for creating or synthesizing miRNA probes. The kits will thus comprise, in suitable container means, an enzyme for labeling the miRNA by incorporating labeled nucleotide or unlabeled nucleotides that are subsequently labeled. It may also include one or more buffers, such as reaction buffer, labeling buffer, washing buffer, or a hybridization buffer, compounds for preparing the miRNA probes, and components for isolating miRNA. Other kits may include components for making a nucleic acid array comprising oligonucleotides complementary to miRNAs, and thus, may include, for example, a solid support.

[00130] For any kit embodiment, including an array, there can be nucleic acid molecules that contain a sequence that is identical or complementary to all or part of any of SEQ ID NOS: 1- 22.

[00131] The components of the kits may be packaged either in aqueous media or in lyophilized form. The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which a component may be placed, and preferably, suitably aliquoted. Where there is more than one component in the kit (labeling reagent and label may be packaged together), the kit also will generally contain a second, third or other additional container into which the additional components may be separately placed. However, various combinations of components may be comprised in a vial. The kits of the present invention also will typically include a means for containing the nucleic acids, and any other reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained.

[00132] When the components of the kit are provided in one and/or more liquid solutions, the liquid solution is an aqueous solution, with a sterile aqueous solution being one preferred solution. Other solutions that may be included in a kit are those solutions involved in isolating and/or enriching miRNA from a mixed sample.

[00133] However, the components of the kit may be provided as dried powder(s). When reagents and/or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means. The kits may also include components that facilitate isolation of the labeled miRNA. It may also include components that preserve or maintain the miRNA or that protect against its degradation. The components may be RNAse-free or protect against RNAses.

[00134] Also, the kits can generally comprise, in suitable means, distinct containers for each individual reagent or solution. The kit can also include instructions for employing the kit components as well the use of any other reagent not included in the kit. Instructions may include variations that can be implemented. It is contemplated that such reagents are embodiments of kits of the invention. Also, the kits are not limited to the particular items identified above and may include any reagent used for the manipulation or characterization of miRNA.

[00135] It is also contemplated that any embodiment discussed in the context of an miRNA array may be employed more generally in screening or profiling methods or kits of the invention. In other words, any embodiments describing what may be included in a particular array can be practiced in the context of miRNA profiling more generally and need not involve an array per se.

[00136] It is also contemplated that any kit, array or other detection technique or tool, or any method can involve profiling for any of these miRNAs. Also, it is contemplated that any embodiment discussed in the context of an miRNA array can be implemented with or without the array format in methods of the invention; in other words, any miRNA in an miRNA array may be screened or evaluated in any method of the invention according to any techniques known to those of skill in the art. The array format is not required for the screening and diagnostic methods to be implemented.

[00137] The kits for using miRNA arrays for therapeutic, prognostic, or diagnostic applications and such uses are contemplated by the inventors herein. The kits can include

an miRNA array, as well as information regarding a standard or normalized miRNA profile for the miRNAs on the array. Also, in certain embodiments, control RNA or DNA can be included in the kit. The control RNA can be miRNA that can be used as a positive control for labeling and/or array analysis.

[00138] The methods and kits of the current teachings have been described broadly and generically herein. Each of the narrower species and sub-generic groupings falling within the generic disclosure also form part of the current teachings. This includes the generic description of the current teachings with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[00139] **EXAMPLE VIII**

[00140] **Array Preparation and Screening**

[00141] Also provided herein are the preparation and use of miRNA arrays, which are ordered macroarrays or microarrays of nucleic acid molecules (probes) that are fully or nearly complementary or identical to a plurality of miRNA molecules or precursor miRNA molecules and that are positioned on a support material in a spatially separated organization. Macroarrays are typically sheets of nitrocellulose or nylon upon which probes have been spotted. Microarrays position the nucleic acid probes more densely such that up to 10,000 nucleic acid molecules can be fit into a region typically 1 to 4 square centimeters. Microarrays can be fabricated by spotting nucleic acid molecules, e.g., genes, oligonucleotides, etc., onto substrates or fabricating oligonucleotide sequences in situ on a substrate. Spotted or fabricated nucleic acid molecules can be applied in a high density matrix pattern of up to about 30 non-identical nucleic acid molecules per square centimeter or higher, e.g. up to about 100 or even 1000 per square centimeter. Microarrays typically use coated glass as the solid support, in contrast to the nitrocellulose-based material of filter arrays. By having an ordered array of miRNA-complementing nucleic acid samples, the position of each sample can be tracked and linked to the original sample. A variety of different array devices in which a plurality of distinct nucleic acid probes are stably associated with the surface of a solid support are known to those of skill in the art. Useful substrates for arrays include nylon, glass and silicon. The arrays may vary in a number of different ways, including average probe length, sequence or types of probes, nature of bond between the probe and the array

surface, e.g. covalent or non-covalent, and the like. The labeling and screening methods described herein and the arrays are not limited in its utility with respect to any parameter except that the probes detect miRNA; consequently, methods and compositions may be used with a variety of different types of miRNA arrays. In certain embodiments, the miR gene product comprises one or more of the SEQ ID NOS: 1 - 22, as shown in Figure 11. In particular certain embodiments, one miR gene product comprises one or more of: miR-219 [SEQ ID NO: 20], miR-207[SEQ ID NO: 18], miR-30c [SEQ ID NO: 6], and miR124A [SEQ ID NO: 4].

[00142] In accordance with the provisions of the patent statutes, the principle and mode of operation of this invention have been explained and illustrated in its preferred embodiment. However, it must be understood that this invention may be practiced otherwise than as specifically explained and illustrated without departing from its spirit or scope.

[00143] **References**

[00144] The references discussed above and the following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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CLAIMS

What is claimed is:

1. A method of diagnosing whether a subject has, or is at risk for developing, hepatocellular carcinoma (HCC), comprising measuring the level of at least one miR gene product in a test sample from the subject, wherein an alteration in the level of the miR gene product in the test sample, relative to the level of a corresponding miR gene product in a control sample, is indicative of the subject either having, or being at risk for developing, HCC.

2. The method of claim 1, wherein the level of the at least one miR gene product is measured using Northern blot analysis.

3. The method of claim 1, wherein the level of the at least one miR gene product in the test sample is less than the level of the corresponding miR gene product in the control sample.

4. The method of claim 1, wherein the level of the at least one miR gene product in the test sample is greater than the level of the corresponding miR gene product in the control sample.

5. The method of claim 1, wherein the at least one miR gene product is one or more of SEQ ID NOs. 1-22.

6. The method of claim 1, wherein the at least one miR gene product is one or more of: miR-30c [SEQ ID NO: 6], mir-124A [SEQ ID NO: 4], miR-207 [SEQ ID NO: 18] and miR-219 [SEQ ID NO: 20], and combinations thereof.

7. The method of claim 6, wherein the at least one of the levels of miR-219 [SEQ ID NO: 20] and mir-207 [SEQ ID NO: 18] are above a predetermined standard level.

8. The method of claim 6, wherein the at least one of the levels of miR-30c [SEQ ID NO: 6] and miR-124A [SEQ ID NO: 4] are below a predetermined standard level.

9. A method of diagnosing a HCC associated with one or more prognostic markers in a subject, comprising measuring the level of at least one miR gene product in a HCC sample from the subject, wherein an alteration in the level of the at least one miR gene product in the test sample, relative to the level of a corresponding miR gene product in a control sample, is indicative of the subject having a HCC associated with the one or more prognostic markers.

10. A method of diagnosing whether a subject has, or is at risk for developing, HCC, comprising:

(1) reverse transcribing RNA from a test sample obtained from the subject to provide a set of target oligodeoxynucleotides;

(2) hybridizing the target oligodeoxynucleotides to a microarray comprising miRNA-specific probe oligonucleotides to provide a hybridization profile for the test sample; and,

(3) comparing the test sample hybridization profile to a hybridization profile generated from a control sample,

wherein an alteration in the signal of at least one miRNA is indicative of the subject either having, or being at risk for developing, HCC.

11. The method of claim 10, wherein the signal of at least one miRNA, relative to the signal generated from the control sample, is down-regulated.

12. The method of claim 10, wherein the signal of at least one miRNA, relative to the signal generated from the control sample, is up-regulated.

13. The method of claim 10, wherein the microarray comprises miRNA-specific probe oligonucleotides for one or more miRNAs selected from SEQ ID NOs. 1-22.

14. The method of claim 10, wherein the microarray comprises miRNA-specific probe oligonucleotides for one or more of: miR-30c [SEQ ID NO: 6], mir-124A [SEQ ID NO: 4], miR-207 [SEQ ID NO: 18] and miR-219 [SEQ ID NO: 20], and combinations thereof.

15. A method of diagnosing whether a subject has, or is at risk for developing, a HCC associated with one or more adverse prognostic markers in a subject, comprising:

(1) reverse transcribing RNA from a test sample obtained from the subject to provide a set of target oligodeoxynucleotides;

(2) hybridizing the target oligodeoxynucleotides to a microarray comprising miRNA-specific probe oligonucleotides to provide a hybridization profile for the test sample; and,

(3) comparing the test sample hybridization profile to a hybridization profile generated from a control sample,

wherein an alteration in the signal is indicative of the subject either having, or being at risk for developing, HCC.

16. The method of claim 15, wherein the microarray comprises at least one miRNA-specific probe oligonucleotide for a miRNA selected one or more of SEQ ID NOs. 1-22.

17. The method of claim 15, wherein the microarray comprises at least one miRNA-specific probe oligonucleotide for a miRNA selected from one or more of: miR-30c [SEQ ID NO: 6], mir-124A [SEQ ID NO: 4], miR-207 [SEQ ID NO: 18] and miR-219 [SEQ ID NO: 20], and combinations thereof.

18. A method of treating HCC in a subject who has a HCC in which at least one miR gene product is down-regulated or up-regulated in the cancer cells of the subject relative to control cells, comprising:

(1) when the at least one miR gene product is down-regulated in the cancer cells, administering to the subject an effective amount of at least one isolated miR gene product, such that proliferation of cancer cells in the subject is inhibited; or

(2) when the at least one miR gene product is up-regulated in the cancer cells, administering to the subject an effective amount of at least one compound for inhibiting expression of the at least one miR gene product, such that proliferation of cancer cells in the subject is inhibited.

19. The method of claim 18, wherein the at least one isolated miR gene product in step (1) is selected from one or more of SEQ ID NOs. 1-22.

20. The method of claim 18, wherein the at least one isolated miR gene product in step (1) is selected from miR-30c [SEQ ID NO: 6], mir-124A [SEQ ID NO: 4], miR-207 [SEQ ID NO: 18] and miR-219 [SEQ ID NO: 20], and combinations thereof.

21. The method of claim 18, wherein the at least one miR gene product in step (2) is selected from: one or more of SEQ ID NOs. 1-22.

22. The method of claim 18, wherein the at least one miR gene product in step (2) is selected from: miR-30c [SEQ ID NO: 6], mir-124A [SEQ ID NO: 4], miR-207 [SEQ ID NO: 18] and miR-219 [SEQ ID NO: 20] and combinations thereof.

23. A method of treating HCC in a subject, comprising:

(1) determining the amount of at least one miR gene product in HCC cells, relative to control cells; and

(2) altering the amount of miR gene product expressed in the HCC cells by:

i) administering to the subject an effective amount of at least one isolated miR gene product, if the amount of the miR gene product expressed in the cancer cells is less than the amount of the miR gene product expressed in control cells; or

ii) administering to the subject an effective amount of at least one compound for inhibiting expression of the at least one miR gene product, if the amount of the miR gene product expressed in the cancer cells is greater than the amount of the miR gene product expressed in control cells, such that proliferation of cancer cells in the subject is inhibited.

24. The method of claim 23, wherein the at least one isolated miR gene product in step (i) is selected from one or more of SEQ ID NOs. 1-22.

25. The method of claim 23, wherein the at least one isolated miR gene product in step (i) is selected from one or more of: miR-219 [SEQ ID NO: 20], miR-207 [SEQ ID NO: 18], miR-30c [SEQ ID NO: 6] and miR124A, and combinations thereof.

26. The method of claim 23, wherein the at least one miR gene product in step (ii) is selected from one or more of SEQ ID NOs. 1-22.

27. The method of claim 23, wherein the at least one miR gene product in step (ii) is selected from one or more of: miR-30c [SEQ ID NO: 6], mir-124A [SEQ ID NO: 4], miR-207 [SEQ ID NO: 18] and miR-219 [SEQ ID NO: 20], and combinations thereof.

28. A pharmaceutical composition for treating HCC, comprising at least one isolated miR gene product and a pharmaceutically-acceptable carrier.

29. The pharmaceutical composition of claim 28, wherein the at least one isolated miR gene product corresponds to a miR gene product that is up- or down-regulated in HCC cells relative to suitable control cells.

30. The pharmaceutical composition of claim 29, wherein the isolated miR gene product is selected from one or more of SEQ ID NOs. 1-22.

31. The pharmaceutical composition of claim 29, wherein the isolated miR gene product is selected from one or more of: miR-30c [SEQ ID NO: 6], mir-124A [SEQ ID NO: 4], miR-207 [SEQ ID NO: 18] and miR-219 [SEQ ID NO: 20], and combinations thereof.

32. A pharmaceutical composition for treating HCC, comprising at least one miR expression inhibitor compound and a pharmaceutically-acceptable carrier.

33. The pharmaceutical composition of claim 32, wherein the at least one miR expression inhibitor compound is specific for a miR gene product that is up-regulated in HCC cells relative to suitable control cells.

34. The pharmaceutical composition of claim 25, wherein the at least one miR expression inhibitor compound is specific for a miR gene product selected from one or more of SEQ ID NOs. 1-22.

35. The pharmaceutical composition of claim 25, wherein the at least one miR expression inhibitor compound is specific for a miR gene product selected from one or more of: miR-30c [SEQ ID NO: 6], mir-124A [SEQ ID NO: 4], miR-207 [SEQ ID NO: 18] and miR-219 [SEQ ID NO: 20], and combinations thereof.

36. A method of identifying an anti-HCC agent, comprising providing a test agent to a cell and measuring the level of at least one miR gene product associated with decreased expression levels in HCC cells, wherein an increase in the level of the miR gene product in the cell, relative to a suitable control cell, is indicative of the test agent being an anti-HCC agent.

37. The method of claim 36, wherein the miR gene product is selected from one or more of SEQ ID NOs. 1-22.

38. The method of claim 36, wherein the miR gene product is selected from one or more of miR-30c [SEQ ID NO: 6], mir-124A [SEQ ID NO: 4], miR-207 [SEQ ID NO: 18] and miR-219 [SEQ ID NO: 20], and combinations thereof.

39. A method of identifying an anti-HCC agent, comprising providing a test agent to a cell and measuring the level of at least one miR gene product associated with increased expression levels in HCC cells, wherein a decrease in the level of the miR gene product in the cell, relative to a suitable control cell, is indicative of the test agent being an anti-HCC agent.

40. The method of claim 39, wherein the miR gene product is selected from one or more of SEQ ID NOs. 1-22.

41. The method of claim 39, wherein the miR gene product is selected from one or more of: miR-30c [SEQ ID NO: 6], mir-124A [SEQ ID NO: 4], miR-207 [SEQ ID NO: 18] and miR-219 [SEQ ID NO: 20] and combinations thereof.

42. A method for identifying a correlation between miRNA expression and a disease or condition comprising identifying miRNA differentially expressed in a sample representative of the disease or condition compared to a normal sample, wherein the disease comprises one or more of a hepatocellular carcinoma (HCC) associated disease or a hepatitis B associated disease.

43. The method of claim 42, wherein identifying involves: a) labeling miRNA in the sample; and b) hybridizing the labeled miRNA to an miRNA array.

44. The method of claim 43, wherein the miRNA in the sample is isolated before or after labeling.

45. The method of claim 42, wherein the sample is a biological sample.

46. The method of claim 42, wherein the biological sample is from a patient.

47. The method of claim 42, wherein the miRNA profile is generated by a process comprising: a) labeling miRNA in the sample; b) hybridizing the miRNA to an miRNA array; and, c) determining miRNA hybridization to the array, wherein an miRNA profile is generated.

48. An miRNA array comprising one or more miRNA probes immobilized on a solid support, wherein the probes comprise: a) an miRNA coding sequence; b) an amine attached to the 5' or 3' end of the probe, wherein the probes have one or more miRNA coding sequences of one or more of: SEQ ID NOs:1-22.

49. An miRNA array comprising one or more miRNA probes immobilized on a solid support, wherein the probes comprise: a) an miRNA coding sequence; b) an amine attached to the 5' or 3' end of the probe, wherein the probes have one or more miRNA coding sequences of one or more of: miR-30c [SEQ ID NO: 6], mir-124A [SEQ ID NO: 4], miR-207 [SEQ ID NO: 18] and miR-219 [SEQ ID NO: 20] and combinations thereof.

50. The miRNA array of claim 49, wherein the miRNA array comprises miRNA probes having miRNA coding sequence that includes the entire processed miRNA sequence.

51. The miRNA array of claim 50, wherein the miRNA coding sequence also includes at least 2-5 nucleotides of coding sequence upstream and/or downstream of the processed miRNA sequence.

52. The miRNA array of claim 52, wherein the miRNA coding sequence includes 4 nucleotides of coding sequence upstream and downstream of the processed miRNA sequence.

53. The miRNA array of claim 53, wherein the miRNA array comprises miRNA probes further comprising: c) at least a first linker sequence flanking the miRNA coding sequence.

54. A kit for generating an miRNA profile for a sample comprising, in suitable container means, two or more miRNA probes wherein the probes have one or more miRNA coding sequences of SEQ ID NOs: 1-22.

55. A kit for generating an miRNA profile for a sample comprising, in suitable container means, two or more miRNA probes wherein the probes have one or more miRNA coding sequences of: miR-30c [SEQ ID NO: 6], mir-124A [SEQ ID NO: 4], miR-207 [SEQ ID NO: 18] and miR-219 [SEQ ID NO: 20] and combinations thereof.

56. The kit of claim 54, further comprising reagents for labeling miRNA in the sample.

57. The kit of claim 55, further comprising reagents for labeling miRNA in the sample.

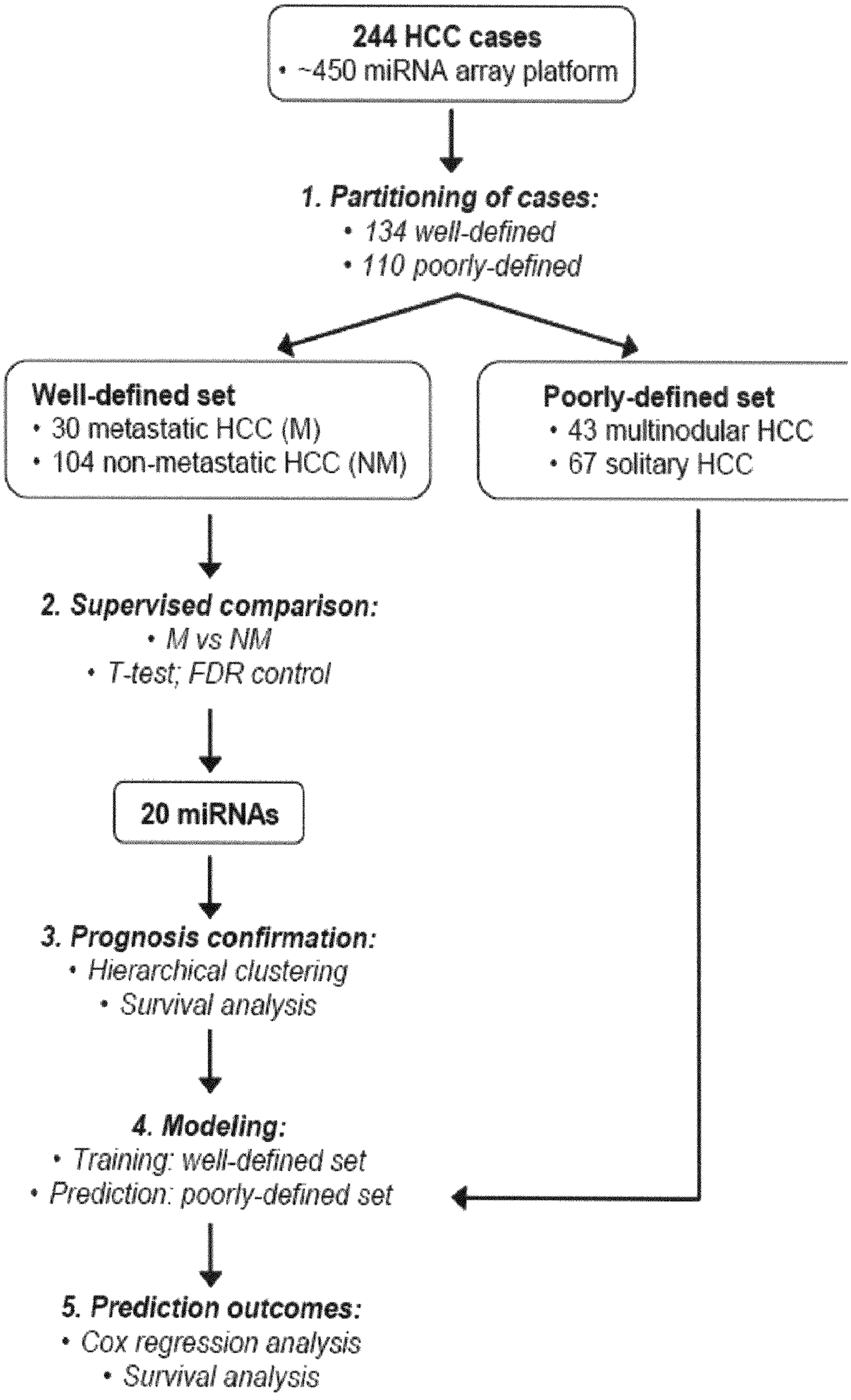
244 HCC cases
• ~450 miRNA array platform
1. Partitioning of cases:
• 134 well-defined
• 110 poorly-defined
Well-defined set
• 30 metastatic HCC (M)
• 104 non-metastatic HCC (NM)
Poorly-defined set
• 43 multinodular HCC
• 67 solitary HCC
2. Supervised comparison:
• M vs NM
• T-test; FDR control
20 miRNAs
3. Prognosis confirmation:
• Hierarchical clustering
• Survival analysis
4. Modeling:
• Training: well-defined set
• Prediction: poorly-defined set
5. Prediction outcomes:
• Cox regression analysis
• Survival analysis

Fig. 1

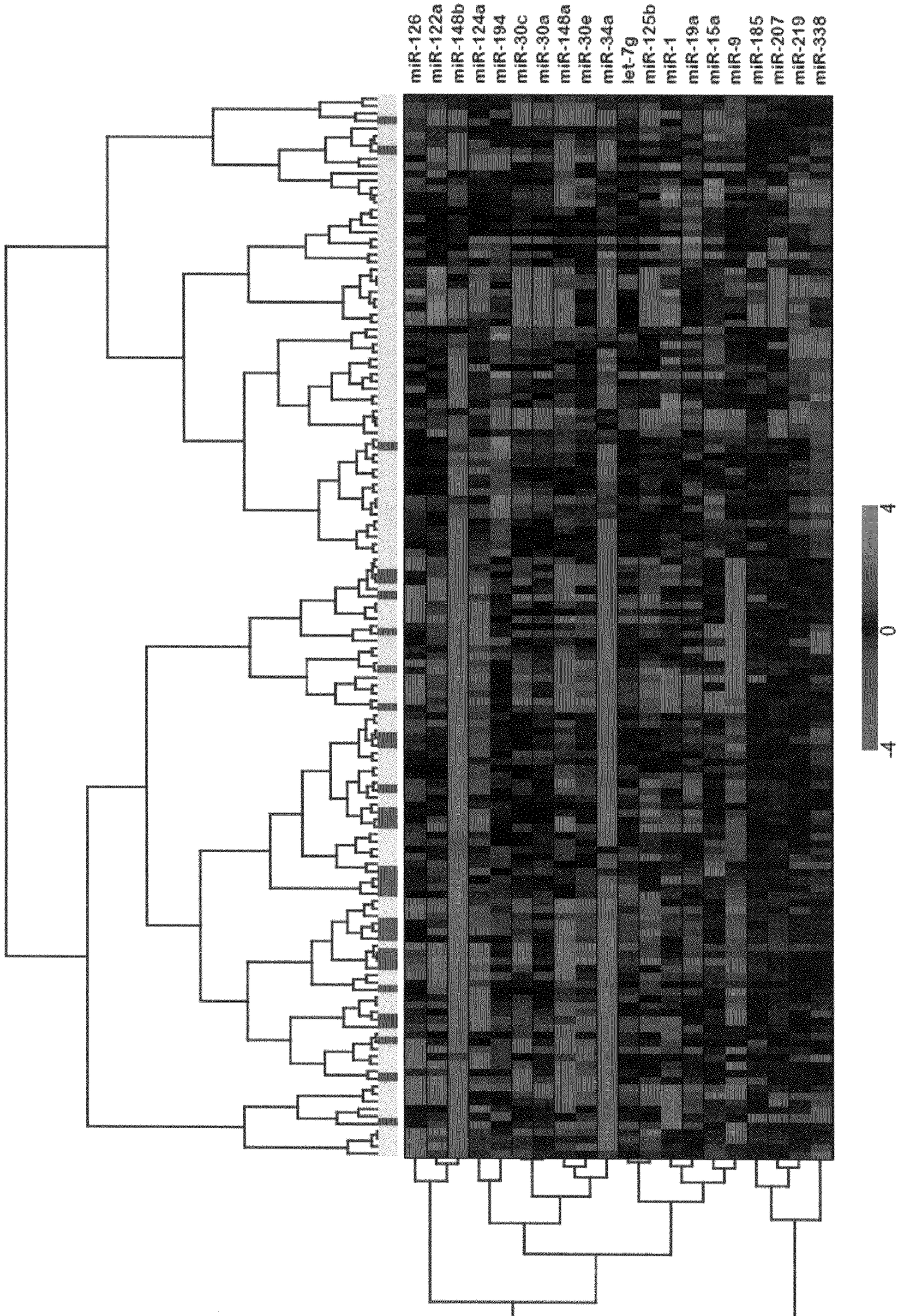
miR-126
miR-122a
miR-148b
miR-124a
miR-194
miR-30c
miR-30a
miR-148a
miR-30e
miR-34a
let-7g
miR-125b
miR-1
miR-19a
miR-15a
miR-9
miR-185
miR-207
miR-219
miR-338
4
0
-4

Fig. 2A

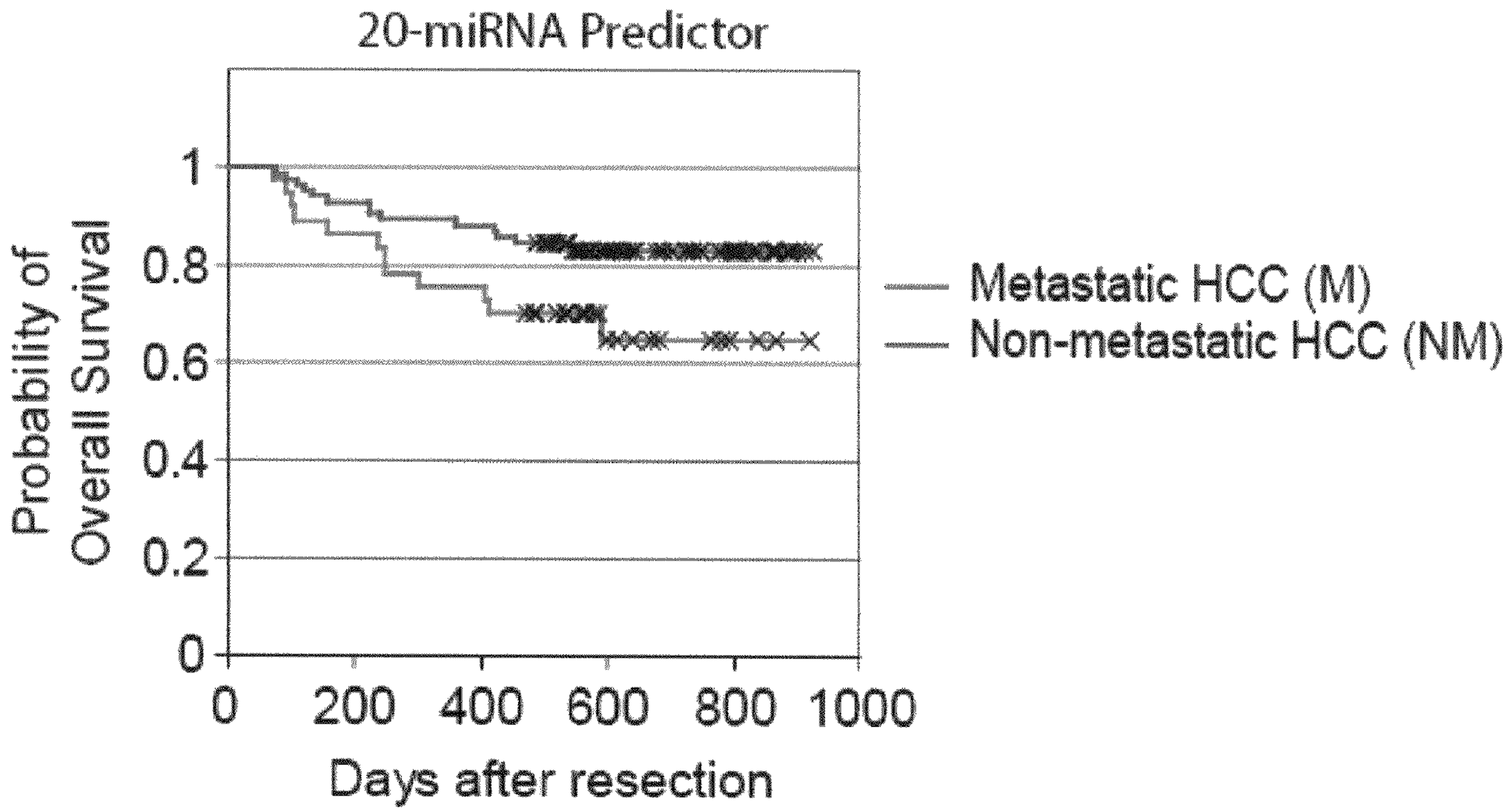

Fig. 2B

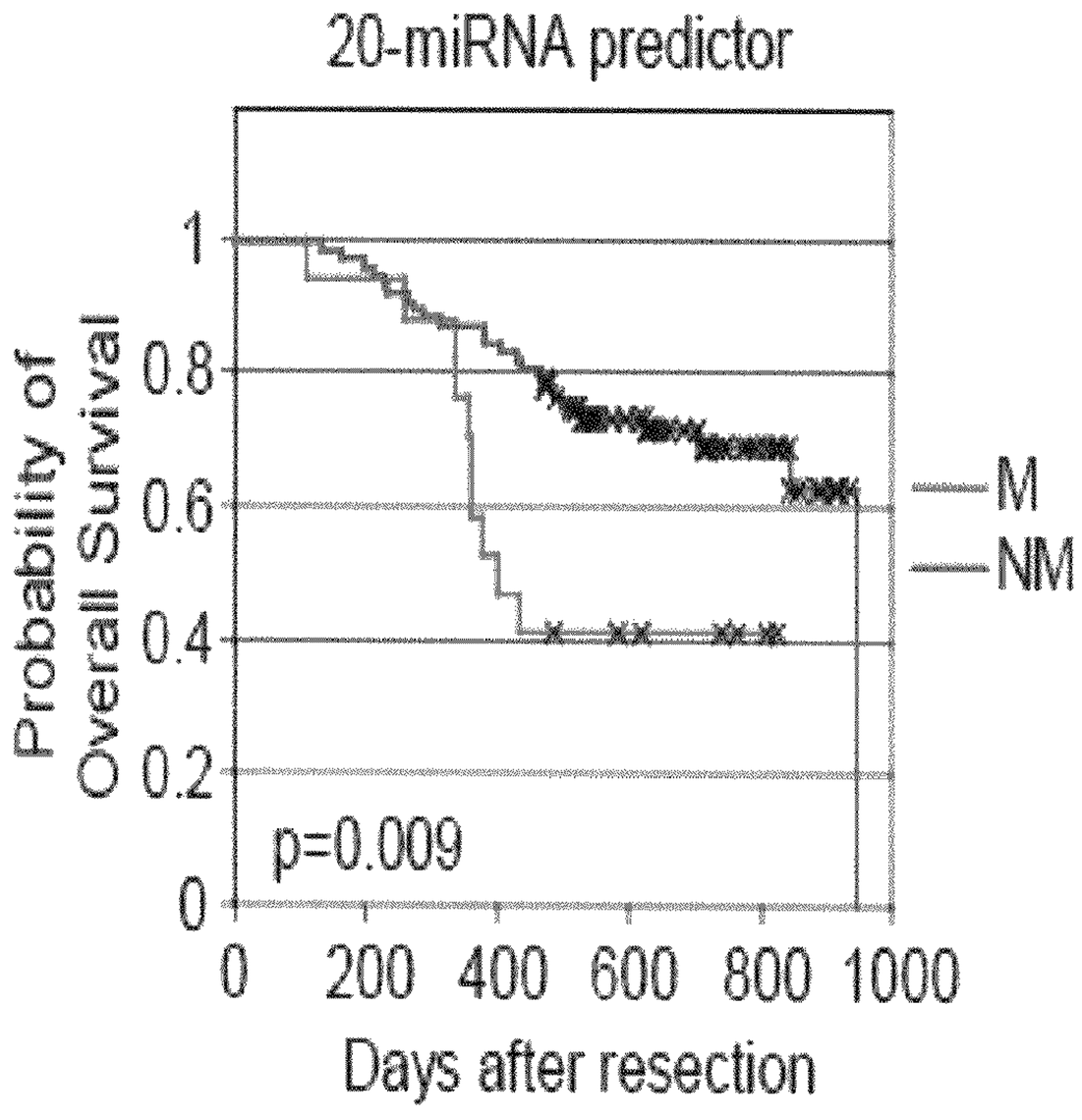

Fig. 3A

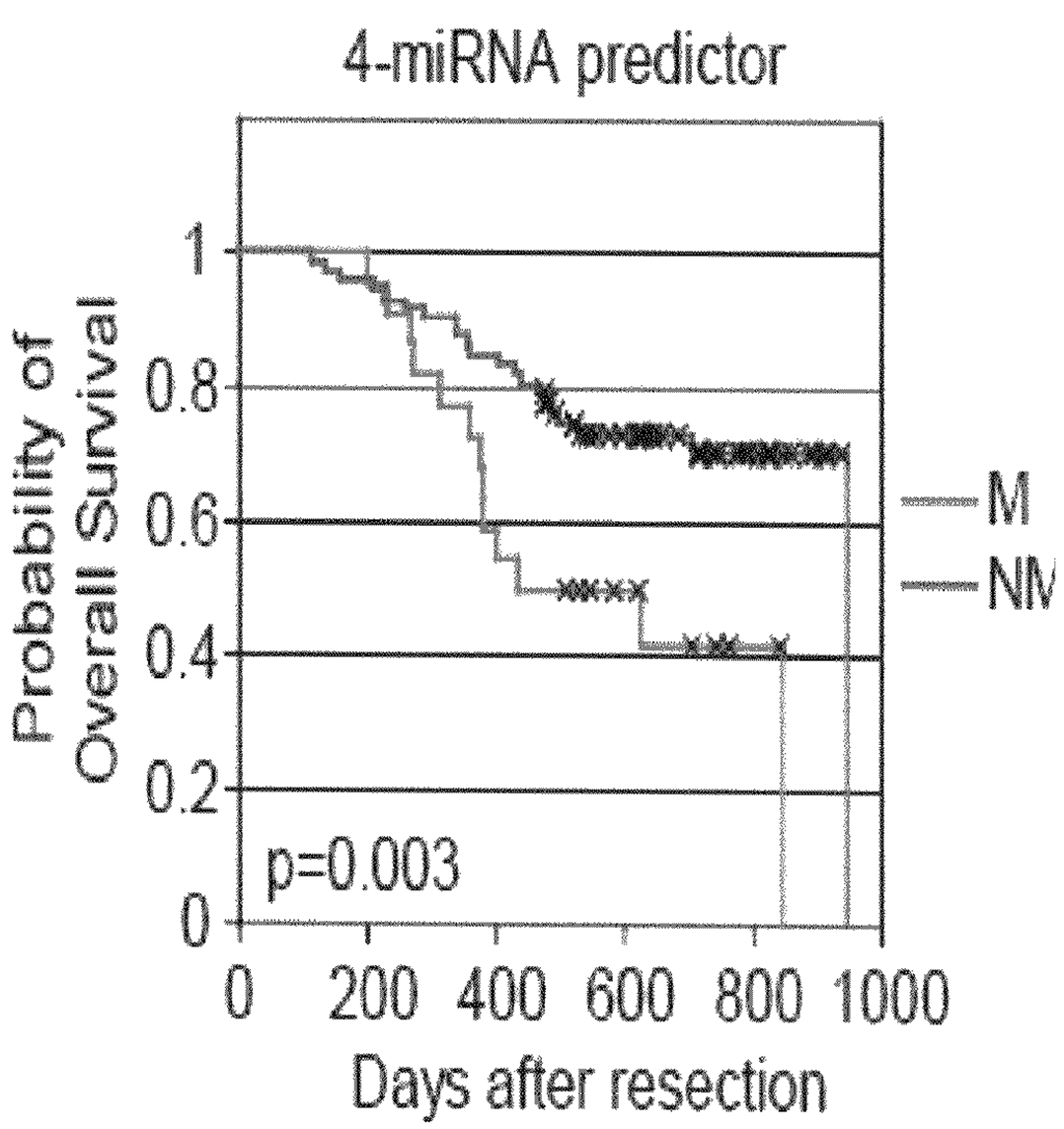

Fig. 3B

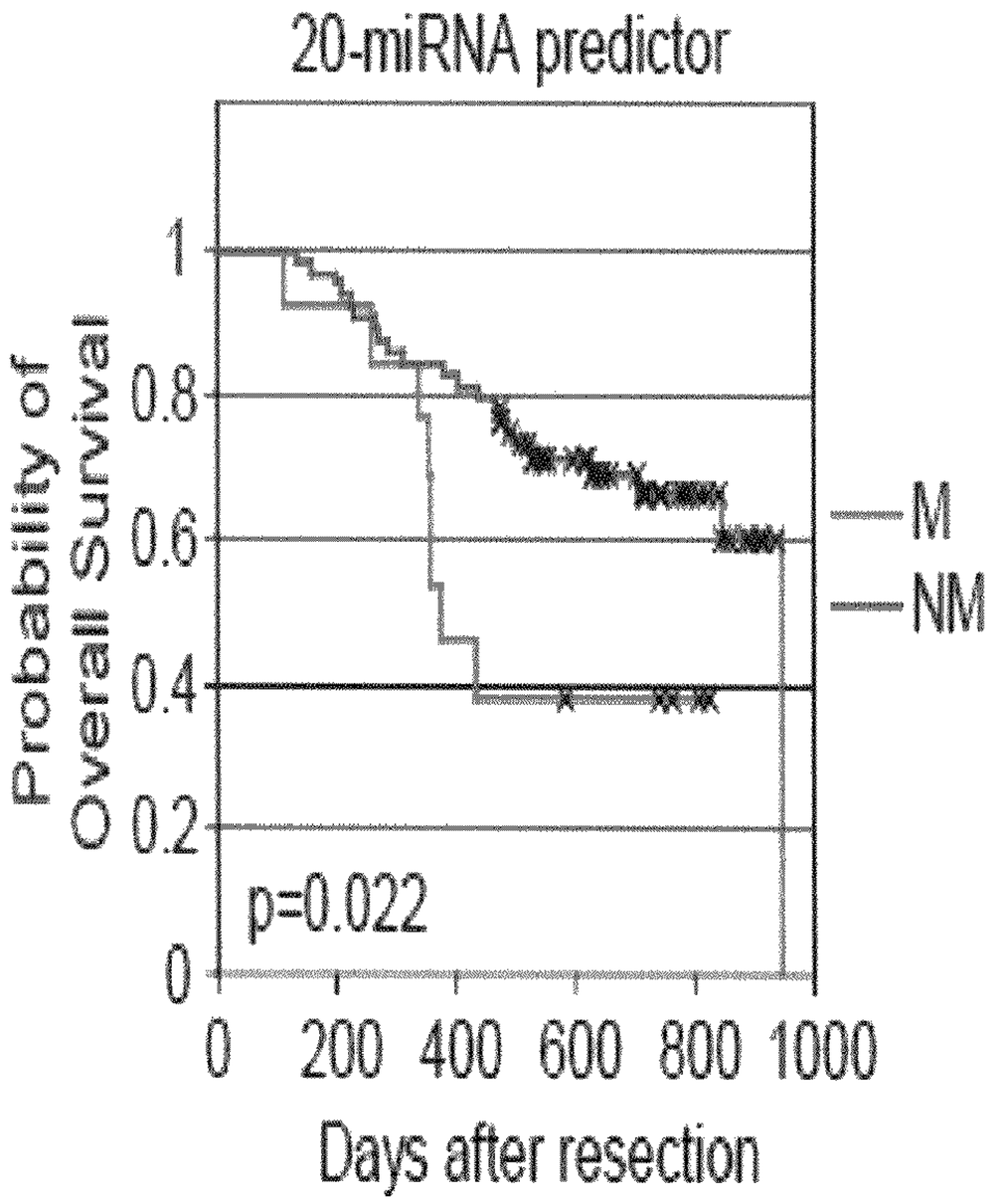

Fig. 3C

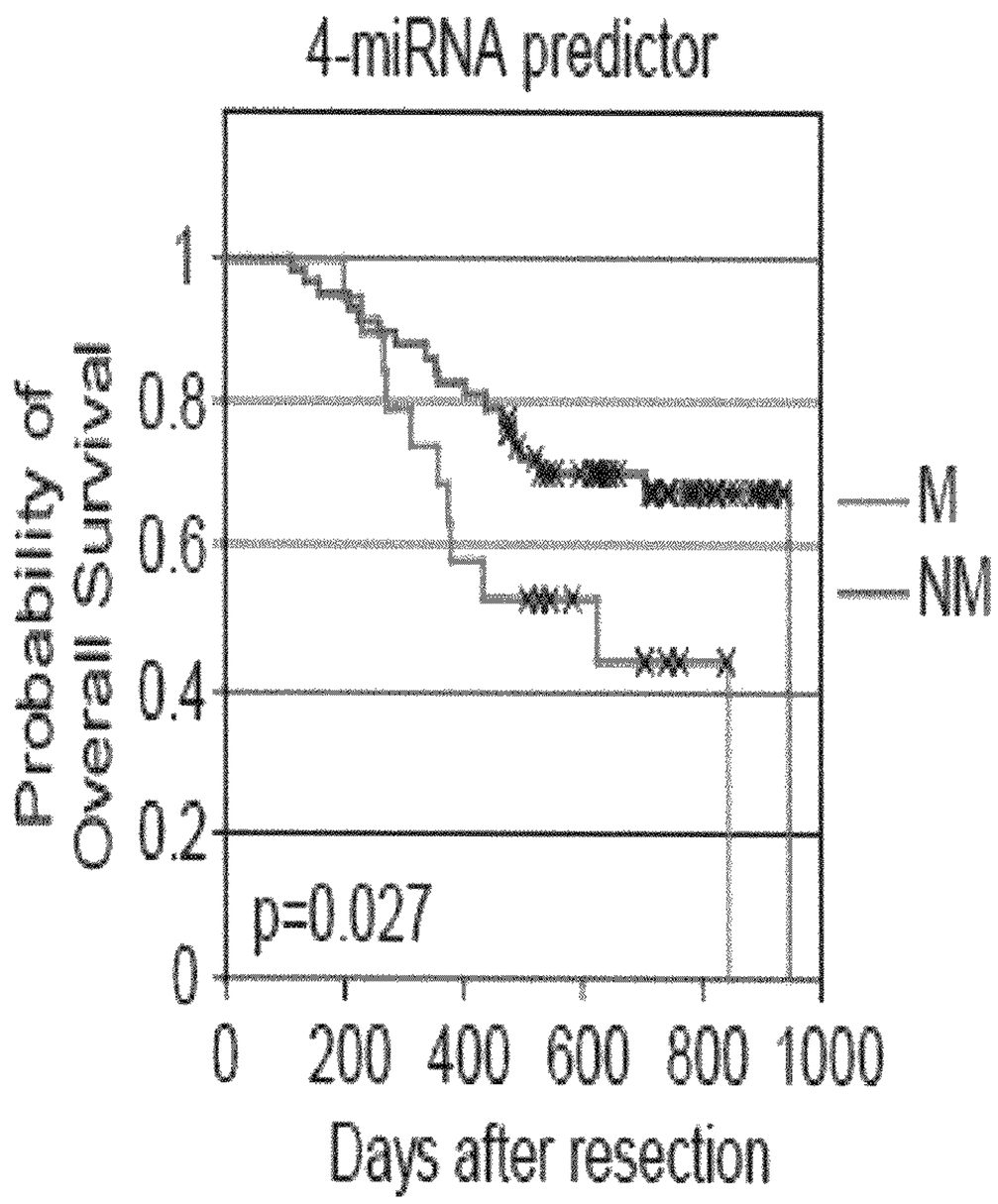

Fig. 3D

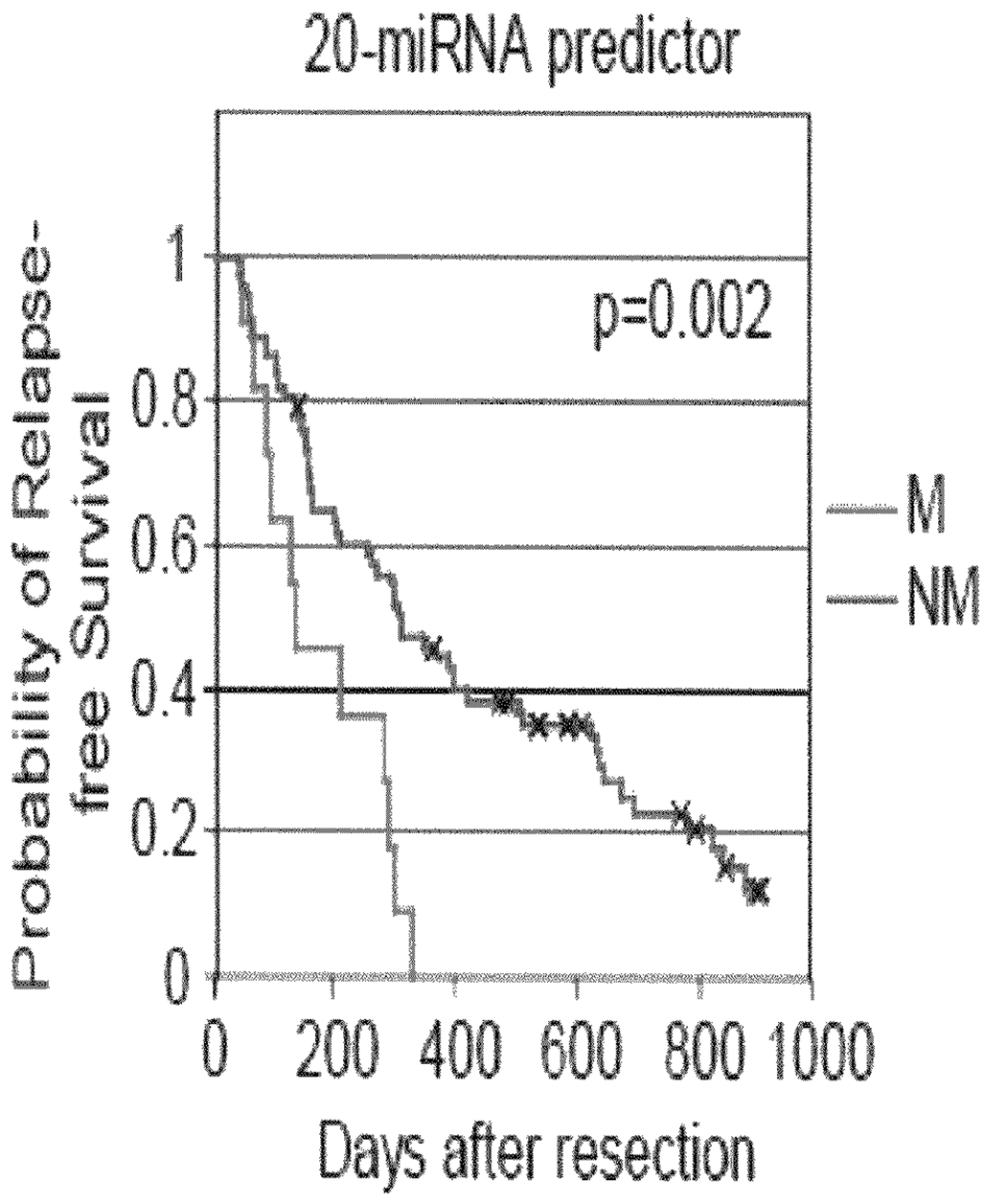

Fig. 3E

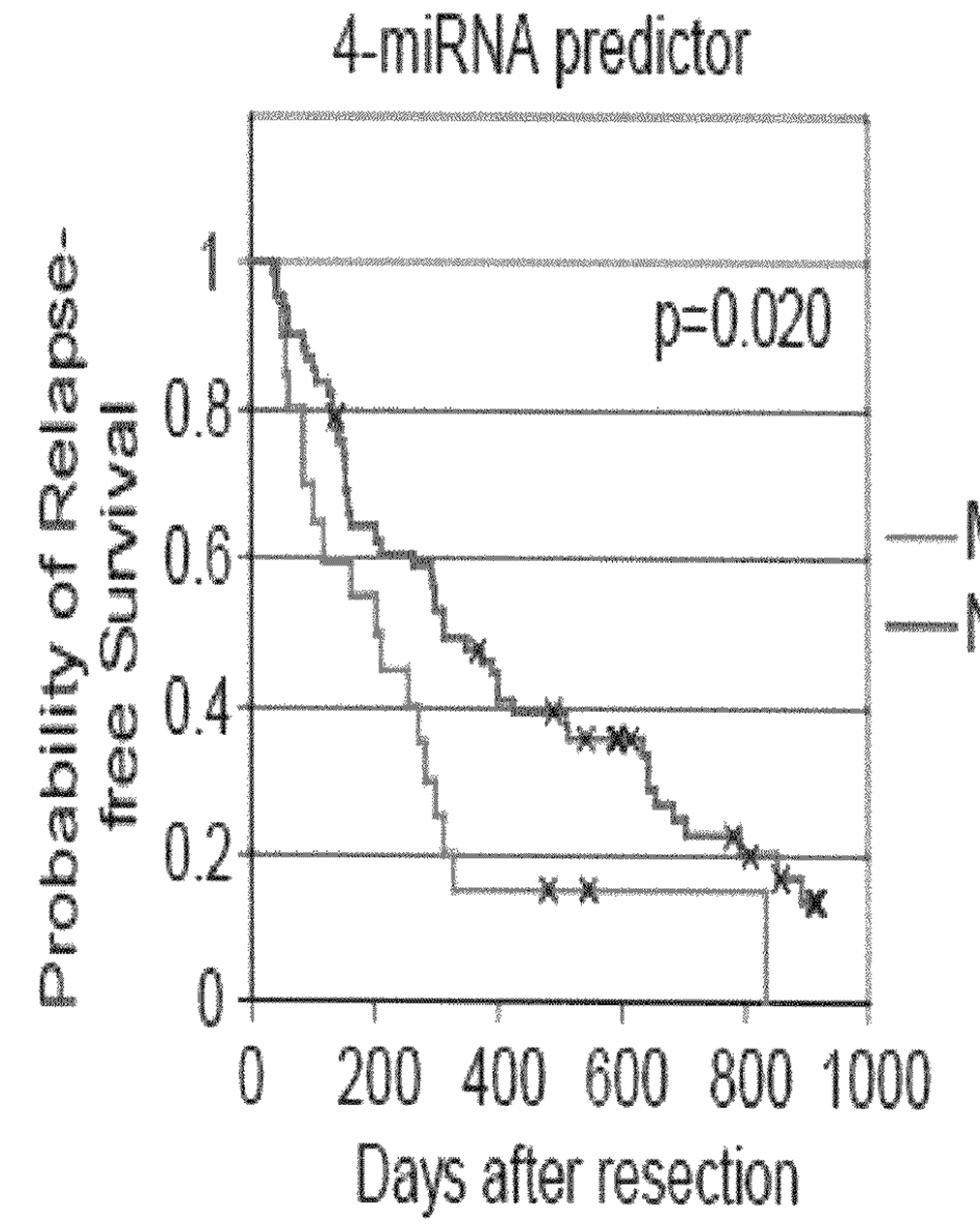

Fig. 3F

Clinical Variable	Value[a]
Male	213 (87)
Age at diagnosis--yr	
Median	50
Range	13-83
Viral Hepatitis Status[b]	
CC	172 (70)
AVR-CC	66 (27)
No Data	6 (2)
Tumor size--cm	
≤ 3	88 (36)
>3	156 (64)
Multinodular	
No	191 (78)
Yes	53 (22)
Child-Pugh Class	
A	233 (96)
B	11 (4)
Metastasis/Recurrence	
No	146 (60)
Yes	98 (40)
TNM Stage	
I	99 (40)
II	90 (37)
III	53 (21)
IV	2 (<1)

[a] Each value represents: the number of patients (the % of patients)
[b] CC, Chronic carrier; AVR-CC, active viral replication chronic carrier

Fig. 4 - Table 1

Clinical Variable	Value[a]
BCLC Staging	
0	6 (5)
A	74 (67)
B	27 (25)
C	3 (3)
CLIP Staging	
0	45 (41)
1	40 (36)
2	25 (23)
OKUDA Staging	
0	99 (90)
1	11 (10)
TNM Staging	
I	32 (29)
II	56 (51)
III	22 (19)

[a] Each value represents: the number of patients (the % of patients)

Fig. 7 - Table 4

Clinical variable	Survival				Recurrence			
	Univariate analysis[a]		Multivariate analysis[b]		Univariate analysis		Multivariate analysis	
	Hazard Ratio (95% CI[c])	p value	Hazard Ratio (95% CI)	p value	Hazard Ratio (95% CI)	p value	Hazard Ratio (95% CI)	p value
miRNA predictor (M vs NM)[d]	**2.5 (1.1-5.8)**	**0.027**	**3.0 (1.2-7.8)**	**0.023**	**2.9 (1.5-5.7)**	**0.002**	**2.8 (1.4-5.7)**	**0.004**
Age (≥50yr vs <50yr)	1.3 (0.6-2.8)	0.463	n.a[f]		1.4 (0.8-2.3)	0.205	n.a.	
Sex (M vs F)	1.8 (0.4-7.7)	0.415	n.a.		1.4 (0.6-3.3)	0.417	n.a.	
HBV (AVR-CC vs CC)[e]	1.1 (0.5-2.5)	0.726	n.a.		1.3 (0.8-2.2)	0.276	n.a.	
AFP (>20ng/ml vs ≤20 ng/ml)	1.3 (0.6-2.9)	0.470	1.7 (0.7-3.8)	0.215	1.1 (0.6-1.8)	0.825	n.a.	
Cirrhosis (Yes vs No)	1.0 (0.2-4.1)	0.961	1.3 (0.3-6.3)	0.704	1.4 (0.4-4.5)	0.558	n.a.	
ALT (≥50U/L vs <50U/L)	0.6 (0.3-1.4)	0.257	n.a.		1.3 (0.8-2.1)	0.296	n.a.	
Child-Pugh score (B vs A)	n.a	n.a	n.a.		n.a.	n.a.	n.a.	
Tumor size (>3cm vs ≤3cm)	3.3 (1.4-7.7)	**0.006**	3.0 (1.2-7.4)	**0.015**	2.2 (1.3-3.6)	**0.002**	1.9 (1.1-3.1)	**0.015**
Tumor encapsulation (None vs Complete)	1.1 (0.5-2.3)	0.864	n.a.		0.6 (0.3-1.0)	**0.041**	n.a.	
Multinodular (Yes vs No)	0.2 (0.1-0.7)	**0.009**	0.1 (0.0-0.5)	**0.003**	0.4 (0.2-0.7)	**0.003**	0.3 (0.2-0.7)	**0.002**
Microvascular invasion (Yes vs No)	1.7 (0.8-3.7)	0.146	0.7 (0.2-3.4)	0.698	1.1 (0.7-1.8)	0.683	n.a.	
Edmondson Grade (III+IV vs I+II)	1.0 (0.5-2.1)	0.994	n.a.		1.2 (0.7-2.0)	0.542	n.a.	
TNM stage (II vs I)	1.2 (0.6-2.7)	0.596	3.6 (0.7-18.6)	0.127	0.9 (0.5-1.4)	0.587	1.7 (0.9-3.0)	**0.081**
CLIP stage (1 vs 0)	1.0 (0.5-2.2)	0.993	n.a.		1.0 (0.6-1.7)	0.920	n.a.	
CLIP stage (2 vs 0)	0.5 (0.1-1.8)	0.304	n.a.		0.4 (0.2-1.0)	**0.047**	n.a.	
BCLC stage (B+C vs 0+A)	0.7 (0.2-2.2)	0.508	n.a.		1.1 (0.5-2.1)	0.867	n.a.	

[a] Univariate analysis, Cox proportional hazards regression
[b] Multivariate analysis, Cox proportional hazards regression
[c] 95% CI, 95% confidence interval
[d] miR predictor, predictor composed of 20 miRs
[e] CC, Chronic carrier; AVR-CC, active viral replication chronic carrier

Fig. 5 - Table 2

No	miRNA	Genomic Location	Parametric p-value	% CV support	Mean Intensities in M	Mean Intensities in NM	Ratio (M/NM)	Expressed in metastatic HCC	Potential host targets*	Estimated false discovery rate (FDR)*
1	**mir-219-1*** **	6p21.32	0.0002	100	578	391	1.48	**up**	**PCDH17; **EPHA4	0.11; 0.14
2	**mir-207**	n.a.	0.0002	90	3676	2432	1.51	**up**	n.a.	
3	**mir-338**	17q25.3	0.0001	90	356	250	1.42	up	n.a.	
4	**mir-185**	22q11.21	0.0009	40	461	346	1.33	up	n.a.	
5	**mir-30c-1**	1p34.2	0.0001	90	813	1618	0.50	**down**	KIAA0063	0.13
6	**mir-124a-2**	8q12.3	0.0004	90	236	448	0.53	**down**	G3BP2; GYS1; VAMP3	0.12; 0.14; 0.002
7	**mir-1-2**	18q11.2	0.0002	80	294	571	0.51	down	G3BP2; GCLC	0.23; 0.15
8	**mir-19a**	13q31.3	0.0004	60	535	947	0.56	down	n.a.	
9	**mir-34a**	1p36.2	0.0004	50	261	539	0.48	down	SPTBN2	0.3
10	**mir-9-2**	5q14.3	0.0005	50	197	347	0.57	down	RAB8A; SLC20A2; VAMP3	0.18; 0.071; 0.18
11	**mir-122a**	18q21.31	0.0005	50	466	781	0.60	down	GYS1	0.12
12	**mir-148a**	7p15.2	0.0004	40	539	1084	0.50	down	GTF2H1; PSCD3	0.27; 0.3
13	**mir-125b-2**	21q21.1	0.0007	40	1346	2337	0.58	down	ITGA9; YES1	0.25; 0.2
14	**mir-15a**	13q14.2	0.0010	40	294	461	0.64	down	ASPH; SLC20A2; SPTBN2	0.26; 0.036; 0.24
15	**mir-30e**	1p34.2	0.0010	40	960	1512	0.63	down	n.a.	
16	**mir-148b**	12q13.13	0.0005	30	578	1063	0.54	down	GTF2H1; PSCD3; **CSF1	0.27; 0.23; 0.24
17	**mir-194**	1q41	0.0008	30	406	689	0.59	down	HBEGF	0.15
18	**mir-30a**	6q13	0.0008	30	2915	4572	0.64	down	n.a.	
19	**mir-126**	9q34.3	0.0009	30	226	395	0.57	down	n.a.	
20	**let-7g**	3p21.2	0.0009	30	582	838	0.69	down	PSCD3	0.14

*Potential host target genes with estimated FDR are based on TARGETSCAN. These genes are a part of the 153-gene metastasis signature described in Ye et al, Nat Med, 9, 416-423, 2003, except the three genes marked with ** that are associated with HCC venous metastases recently identified (Budhu et al, Cancer Cell 2006, 10(2):99-111).

***The 4-miRNA predictor consists of miR-219-1, miR-207, miR-30c-1 and miR-124a-2.

Fig. 6 - Table 3

Clinical variable	Survival				Recurrence			
	Univariate analysis[a]		Multivariate analysis[b]		Univariate analysis		Multivariate analysis	
	Hazard Ratio (95% CI[c])	p value	Hazard Ratio (95% CI)	p value	Hazard Ratio (95% CI)	p value	Hazard Ratio (95% CI)	p value
miR predictor (M vs NM)[d]	**2.5 (1.1-6.2)**	**0.033**	**3.4 (1.3-9.0)**	**0.012**	**3.0 (1.4-6.4)**	**0.004**	**2.9 (1.3-6.3)**	**0.009**
Age (≥50yr vs <50yr)	1.5 (0.7-3.2)	0.330	n.a[f]		1.3 (0.7-2.2)	0.387	n.a[f]	
Sex (M vs F)	1.4 (0.3-5.9)	0.653	n.a.		1.6 (0.6-4.3)	0.398	n.a.	
HBV (AVR-CC vs CC)[e]	1.4 (0.6-3.1)	0.393	n.a.		1.3 (0.8-2.2)	0.356	n.a.	
AFP (>20ng/ml vs ≤20 ng/ml)	1.4 (0.6-3.2)	0.365	1.7 (0.8-3.9)	0.190	1.0 (0.6-1.7)	0.953	n.a.	
Cirrhosis (Yes vs No)	0.8 (0.2-3.4)	0.753	n.a.		2.0 (0.5-8.4)	0.321	n.a.	
ALT (≥50U/L vs <50U/L)	0.9 (0.4-2.0)	0.818	n.a.		1.3 (0.8-2.2)	0.324	n.a.	
Child-Pugh score (B vs A)	n.a	n.a	n.a.		n.a	n.a	n.a.	
Tumor size (>3cm vs ≤3cm)	3.6 (1.5-8.5)	**0.004**	4.0 (1.6-10.2)	**0.004**	2.9 (1.6-4.9)	**0.000**	1.8 (1.0-3.3)	**0.044**
Tumor encapsulation (None vs Complete)	1.1 (0.5-2.5)	0.750	n.a.		0.6 (0.4-1.1)	0.118	n.a.	
Multinodular (Yes vs No)[g]	n.a.	n.a.	n.a.		0.3 (0.1-0.6)	**0.001**	0.3 (0.1-0.7)	**0.007**
Microvascular invasion (Yes vs No)	1.8 (0.8-4.0)	0.136	2.3 (0.3-19.2)	0450	1.1 (0.7-1.9)	0.662	n.a.	
Edmondson Grade (III+IV vs I+II)	1.0 (0.5-2.2)	0.998	n.a.		1.1 (0.6-1.9)	0.786	n.a.	
TNM stage (II vs I)	1.3 (0.6-2.9)	0.561	1.0 (0.1-10.0)	0.970	0.9 (0.5-1.5)	0.667	1.8 (1.0-3.2)	0.970
TNM stage (III vs I)	1.0 (0.1-7.6)	0.974	0.4 (0.0-8.1)	0.555	n.a.	n.a.	n.a.	
CLIP stage (1 vs 0)	1.0 (0.4-2.1)	0.925	n.a.		1.0 (0.6-1.8)	0.988	n.a.	
CLIP stage (2 vs 0)	0.3 (0.0-2.4)	0.258	n.a.		0.3 (0.0-1.0)	**0.042**	n.a.	
BCLC stage (A vs 0)	2.2 (0.3-16.3)	0.438	n.a.		1.4 (0.5-3.8)	0.550	n.a.	
Okuda stage (1 vs 0)	1.2 (0.4-3.6)	0.706	n.a.		0.8 (0.3-2.1)	0.722	n.a.	

[a] Univariate analysis, Cox proportional hazards regression
[b] Multivariate analysis, Cox proportional hazards regression
[c] 95% CI, 95% confidence interval
[d] miR predictor, predictor composed of 20 miRs
[e] CC, Chronic carrier; AVR-CC, active viral replication chronic carrier
[f] n.a., not applicable

Fig. 8 - Table 5

Clinical variable	Survival				Recurrence			
	Univariate analysis[a]		Multivariate analysis[b]		Univariate analysis		Multivariate analysis	
	Hazard Ratio (95% CI[c])	p value	Hazard Ratio (95% CI)	p value	Hazard Ratio (95% CI)	p value	Hazard Ratio (95% CI)	p value
miRNA predictor (M vs NM)[d]	**2.7 (1.3-5.8)**	**0.009**	**3.3 (1.5-7.4)**	**0.003**	**1.9 (1.0-3.5)**	**0.057**	**2.3 (1.2-4.6)**	**0.013**
Age (≥50yr vs <50yr)	1.3 (0.7-2.6)	0.452	n.a.[f]		1.4 (0.9-2.3)	0.122	n.a.	
Sex (M vs F)	1.7 (0.4-7.0)	0.486	n.a.		1.2 (0.5-2.8)	0.672	n.a.	
HBV (AVR-CC vs CC)[e]	1.5 (0.7-3.0)	0.250	n.a.		1.3 (0.8-2.2)	0.234	n.a.	
AFP (>20ng/ml vs ≤20 ng/ml)	1.3 (0.6-2.8)	0.478	n.a.		1.0 (0.6-1.7)	0.956	n.a.	
Cirrhosis (Yes vs No)	1.3 (0.3-5.6)	0.669	n.a.		1.9 (0.6-6.0)	0.280	n.a.	
ALT (≥50U/L vs <50U/L)	0.8 (0.4-1.6)	0.503	n.a.		1.1 (0.7-1.8)	0.561	n.a.	
Child-Pugh score (B vs A)	n.a	n.a	n.a.		n.a.	n.a.	n.a.	
Tumor size (>3cm vs ≤3cm)	2.7 (1.2-6.2)	**0.021**	2.8 (1.2-6.7)	**0.017**	1.5 (0.9-2.4)	0.117	n.a.	
Tumor encapsulation (None vs Complete)	1.2 (0.6-2.5)	0.648	n.a.		0.7 (0.4-1.2)	0.205	n.a.	
Multinodular (Yes vs No)	0.3 (0.1-0.7)	**0.006**	0.2 (0.1-0.6)	**0.002**	0.3 (0.2-0.5)	**<0.001**	0.2 (0.1-0.4)	**<0.001**
Microvascular invasion (Yes vs No)	1.9 (0.9-3.9)	0.075	n.a.		1.1 (0.7-1.8)	0.562	n.a.	
Edmondson Grade (III+IV vs I+II)	1.1 (0.5-2.1)	0.948	n.a.		1.2 (0.7-1.9)	0.564	n.a.	
TNM stage (II vs I)	1.2 (0.6-2.7)	0.588	3.1 (1.3-7.2)	**0.010**	0.9 (0.5-1.4)	0.567	1.7 (1.0-3.1)	0.053
TNM stage (III vs I)	0.7 (0.2-2.2)	0.519	1.6 (0.4-5.8)	0.506	0.3 (0.1-0.8)	**0.010**	0.6 (0.2-1.7)	0.309
CLIP stage (1 vs 0)	1.0 (0.6-2.7)	0.602	n.a.		0.8 (0.5-1.3)	0.410	n.a.	
CLIP stage (2 vs 0)	0.7 (0.2-2.3)	0.544	n.a.		0.4 (0.2-0.7)	**0.006**	n.a.	
BCLC stage (B+C vs 0+A)	0.6 (0.2-1.4)	0.242	n.a.		0.5 (0.3-0.9)	**0.028**	2.2 (0.9-5.7)	0.097
Okuda stage (1 vs 0)	1.7 (0.7-4.1)	0.236	n.a.		1.3 (0.6-2.7)	0.506	2.0 (0.9-4.6)	0.085

[a] Univariate analysis, Cox proportional hazards regression
[b] Multivariate analysis, Cox proportional hazards regression
[c] 95% CI, 95% confidence interval
[d] miR predictor, predictor composed of 20 miRs
[e] CC, Chronic carrier; AVR-CC, active viral replication chronic carrier
[f] n.a., not applicable

Fig. 9 - Table 6

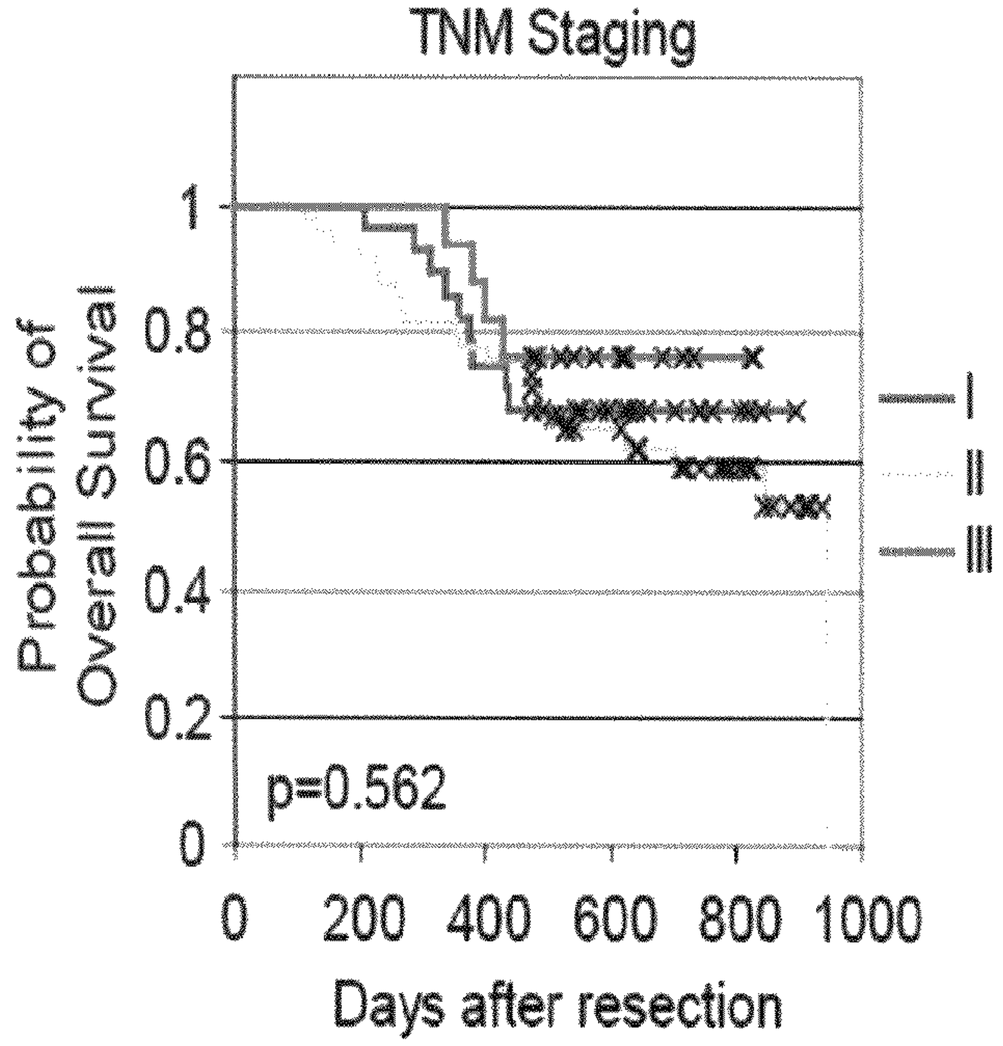

Fig. 10A

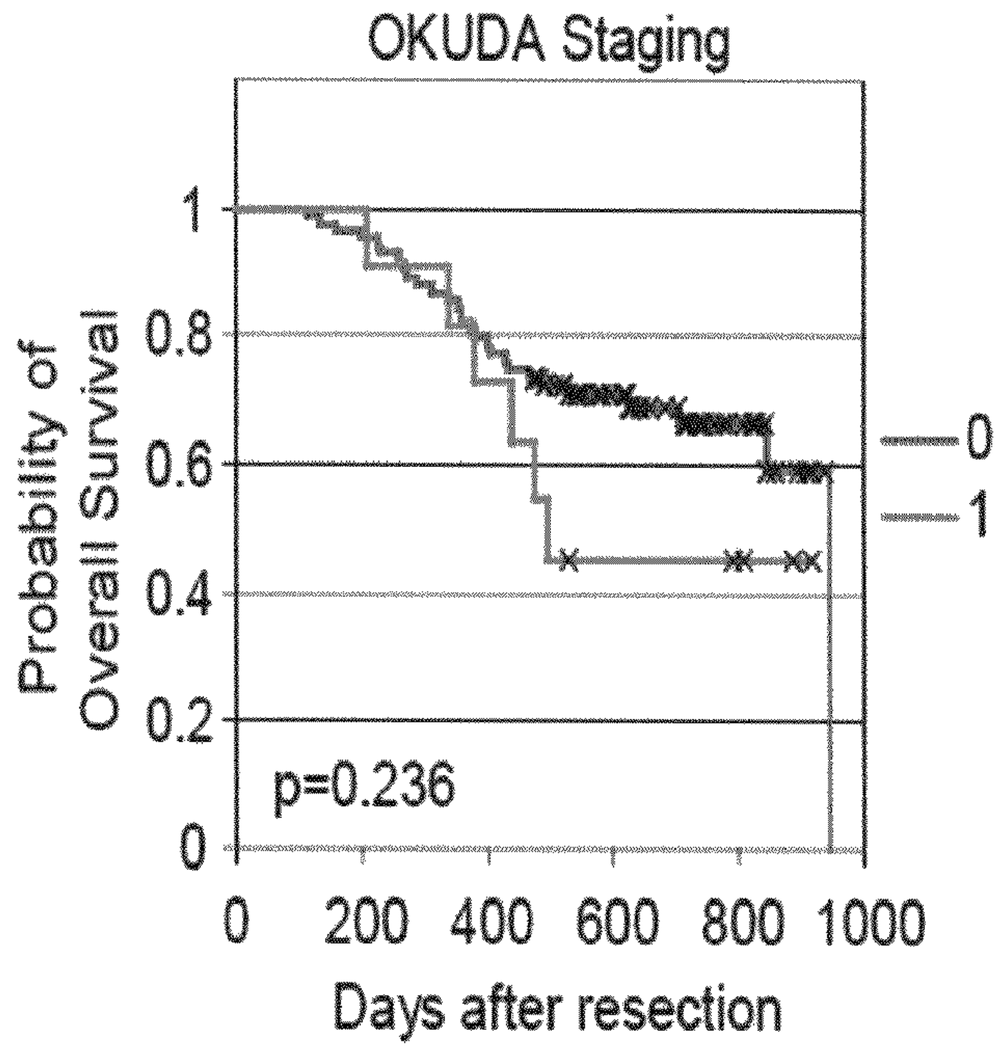

Fig. 10B

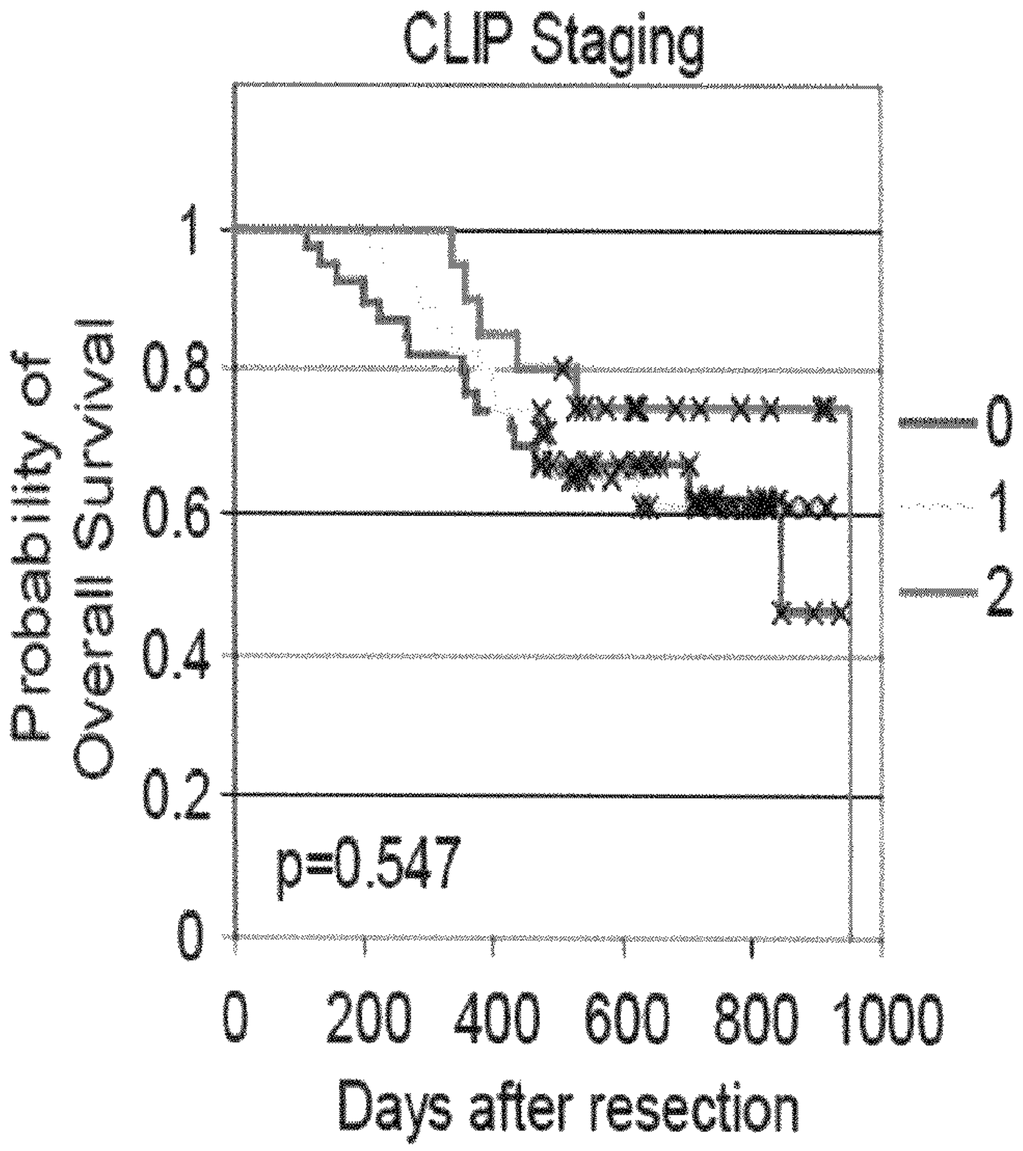

Fig. 10C

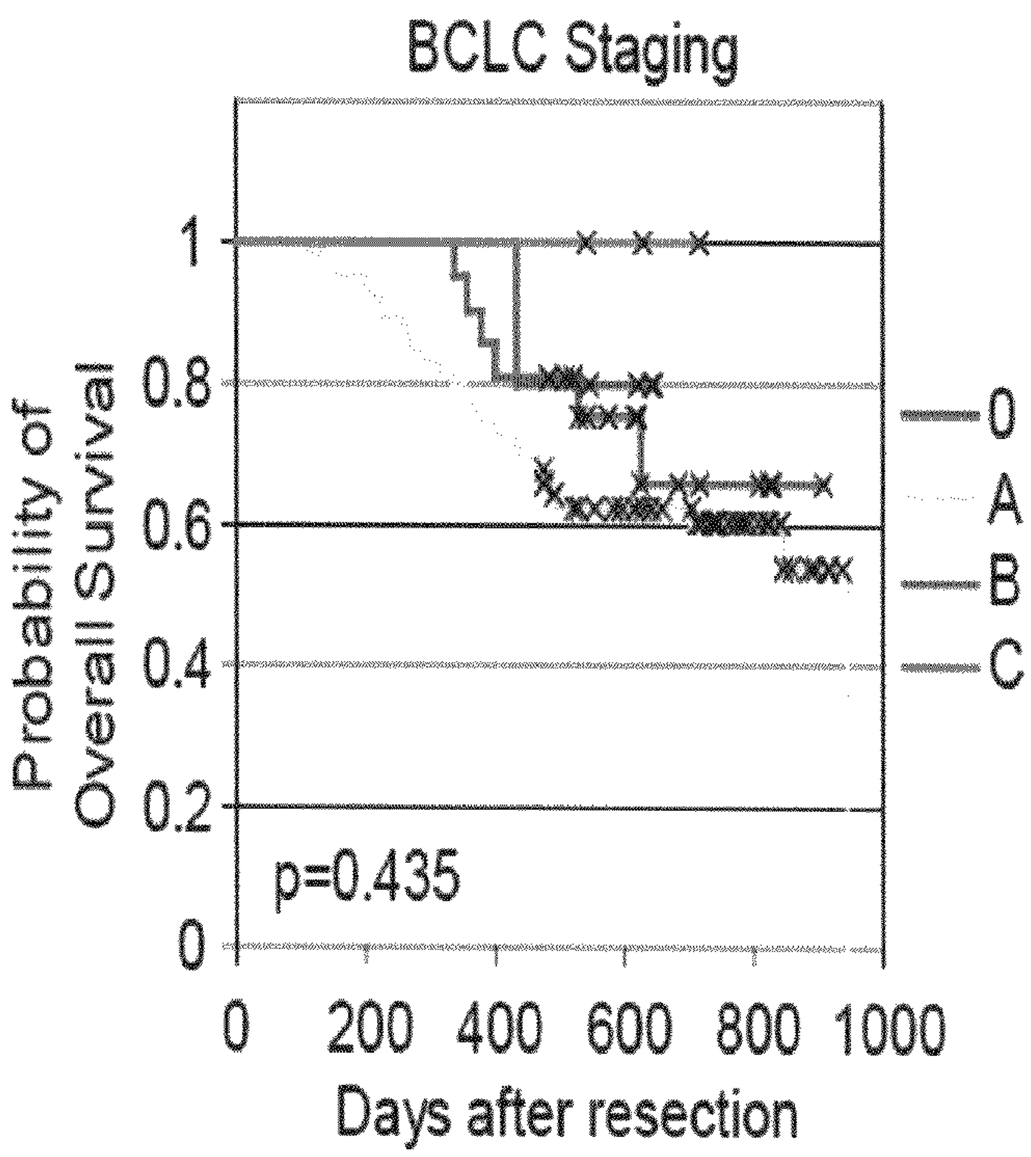

Fig. 10D

SEQ ID NO	microRNA	Sequence (5'-...-3')
1	miR-126	UCGUACCGUGAGUAAUAAUGCG
2	miR-122	UGGAGUGUGACAAUGGUGUUUG
3	miR-148b	UCAGUGCAUCACAGAACUUUGU
4	miR-124a	UAAGGCACGCGGUGAAUGCC
5	miR-194	UGUAACAGCAACUCCAUGUGGA
6	miR-30c	UGUAAACAUCCUACACUCUCAGC
7	miR-30a	UGUAAACAUCCUCGACUGGAAG
8	miR-148a	UCAGUGCACUACAGAACUUUGU
9	miR-30e	UGUAAACAUCCUUGACUGGAAG
10	miR-34a	UGGCAGUGUCUUAGCUGGUUGU
11	Let-7g	UGAGGUAGUAGUUUGUACAGUU
12	miR-125b	UCCCUGAGACCCUAACUUGUGA
13	miR-1	UGGAAUGUAAAGAAGUAUGUAU
14	miR-19a	UGUGCAAAUCUAUGCAAAACUGA
15	miR-15a	UAGCAGCACAUAAUGGUUUGUG
16	miR-9	UCUUUGGUUAUCUAGCUGUAUGA
17	miR-185	UGGAGAGAAAGGCAGUUCCUGA
18	miR-207	GCUUCUCCUGGCUCUCCUCCCUC
19	miR-219-5p	UGAUUGUCCAAACGCAAUUCU
20	miR-219-3p	AGAGUUGAGUCUGGACGUCCCG
21	miR-338-5p	AACAAUAUCCUGGUGCUGAGUG
22	miR-338-3p	UCCAGCAUCAGUGAUUUUGUUG

Fig. 11