



(51) International Patent Classification:

C12Q 1/6886 (2018.01)

(21) International Application Number:

PCT/US2019/067942

(22) International Filing Date:

20 December 2019 (20.12.2019)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/784,240 21 December 2018 (21.12.2018) US

62/811,080 27 February 2019 (27.02.2019) US

(71) Applicant: BAYLOR RESEARCH INSTITUTE

[US/US]; 2001 Bryan St., Dallas, Texas 75201 (US).

(72) Inventors: GOEL, Ajay; c/o Baylor Research Institute,

2001 Bryan St., Dallas, Texas 75201 (US). SHIMURA,

Tadanobu; c/o Baylor Research Institute, 2001 Bryan St.,
Dallas, Texas 75201 (US).

(74) Agent: STELLMAN, Laurie B.F.; 98 San Jacinto Blvd.,

Suite 1100, Austin, Texas 78701 (US).

(81) Designated States (unless otherwise indicated, for every

kind of national protection available): AE, AG, AL, AM,

AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ,
CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO,
DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,
HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP,
KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,
OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,
SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, 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, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ,
UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,
TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,
EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— as to applicant's entitlement to apply for and be granted a
patent (Rule 4.17(ii))

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

(54) Title: METHODS FOR PROGNOSING, DIAGNOSING, AND TREATING GASTRIC CANCER

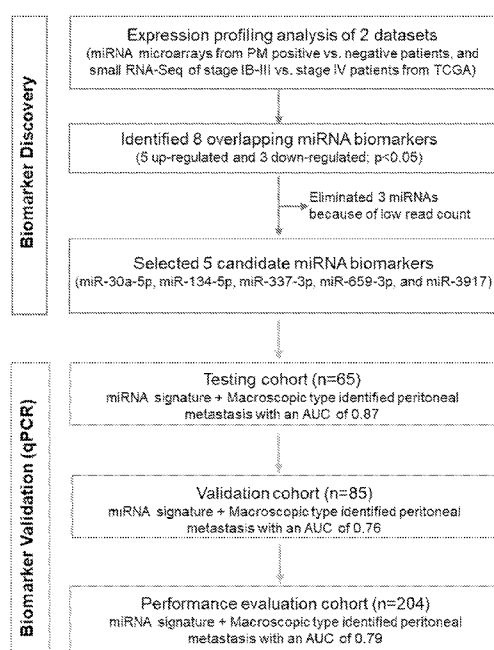


FIG. 1A

(57) Abstract: The current disclosure provides an miRNA signature for detecting gastric cancer. Aspects of the disclosure relate to a method for treating, evaluating, prognosing, and/or diagnosing a patient for treating a patient with gastric cancer, the method comprising treating the patient for gastric cancer after the expression level of miR-30a-5p, miR-134-5p, miR-337-3p, miR-659-3p, and miR-3917 has been determined in a sample from the patient.

Published:

- *without international search report and to be republished
upon receipt of that report (Rule 48.2(g))*

METHODS FOR PROGNOSING, DIAGNOSING, AND TREATING GASTRIC CANCER

DESCRIPTION

[0001] This application claims the benefit of priority to U.S. Provisional Application Serial
5 No. 62/784,240, filed December 21, 2018 and U.S. Provisional Application Serial No. 62/811,080,
filed February 27, 2019, each of which are hereby incorporated by reference in their entirety.

BACKGROUND OF THE INVENTION

I. Field of the Invention

[0002] The present invention relates generally to the fields of molecular biology and oncology.
10 More particularly, it concerns methods and compositions involving cancer prognosis, diagnosis
and treatment.

II. Background

[0003] Gastric cancer is the fourth most common cause of cancer related deaths worldwide. In
particular, peritoneal metastasis is the most frequently occurring type of gastric cancer. The
15 aggressiveness of gastric cancer peritoneal metastasis is well-recognized, and early detection can
significantly improve the outcome of patients. Currently, computed tomography (CT) or positron
emission tomography (PET) are commonly used to diagnose peritoneal metastasis. However, the
sensitivity of CT and PET is inadequate for identifying peritoneal metastatic lesions, and though
staging laparoscopy can be used to identify peritoneal metastasis at a much higher rate than CT or
20 PET-CT, this radical procedure is invasive and requires general anesthesia which increases the risk
of complications.

[0004] It has been demonstrated that several tumor markers are upregulated in advanced
gastric cancers and overexpressed in the serum of patients with gastric cancer peritoneal
metastasis. However, individually or in combination the sensitivity of these tumor markers for
25 detecting peritoneal metastasis is poor. A robust molecular marker which could accurately identify
patients with gastric cancer peritoneal metastasis by a marker-based signature prior to performing
invasive surgery is expected to be clinically transformative. Identification of a predictive marker

will alter treatment strategies and improve care for these patients, leading to a reduction in the morbidity and mortality associated with this malignancy.

SUMMARY OF THE INVENTION

[0005] The current disclosure fulfills a need in the art by providing a miRNA-based signature for diagnosing gastric cancer patients with peritoneal metastasis. Aspects of the disclosure relate to a method for treating a patient with gastric cancer peritoneal metastasis, the method comprising treating the patient for gastric cancer after the expression level of 1, 2, 3, 4 or 5 of the biomarkers selected from miR-30a-5p, miR-134-5p, miR-337-3p, miR-659-3p, and miR-3917 has been measured or determined in a sample from the patient.

[0006] Further aspects relate to method for evaluating a gastric cancer patient comprising measuring the level of expression of 1, 2, 3, 4, or 5 of the biomarkers selected from miR-30a-5p, miR-134-5p, miR-337-3p, miR-659-3p, and miR-3917 in a sample from the patient.

[0007] Further aspects of the disclosure relate to a method of prognosing and/or diagnosing a patient with gastric cancer comprising a) measuring the level of expression of one or more of miR-30a-5p, miR-134-5p, miR-337-3p, miR-659-3p, and miR-3917 in a sample from the patient; b) comparing the level(s) of expression to a control sample(s) or control level(s) of expression; and, c) prognosing and/or diagnosing the patient based on the levels of measured expression.

[0008] In some embodiments, at least miR-30a-5p, miR-659-3p and miR-3917 were determined or measured in a sample from the patient. In some embodiments, at least miR-30a-5p and miR-659-3p were determined or measured in a sample from the patient. In some embodiments, at least miR-30a-5p was determined or measured in a sample from the patient. In some embodiments, at least miR-134-5p was determined or measured in a sample from the patient. In some embodiments, at least miR-337-3p was determined or measured in a sample from the patient. In some embodiments, at least miR-659-3p was determined or measured in a sample from the patient. In some embodiments, at least miR-3917 was determined or measured in a sample from the patient.

[0009] Some embodiments further involve isolating nucleic acids such as ribonucleic or RNA from a biological sample or in a sample of the patient. Other steps may or may not include amplifying a nucleic acid in a sample and/or hybridizing one or more probes to an amplified or non-amplified nucleic acid. The methods may further comprise assaying nucleic acids in a sample.

Further embodiments include isolating or analyzing protein expression in a biological sample for the expression of the biomarker. In certain embodiments, a microarray may be used to measure or assay the level of the biomarkers in a sample. The methods may further comprise recording the biomarker expression or activity level in a tangible medium or reporting the expression or activity level to the patient, a health care payer, a physician, an insurance agent, or an electronic system.

[0010] In some embodiments, the method further comprises determining the macroscopic Borrmann type of the gastric tumor. In some embodiments, the patient has been diagnosed with gastric cancer. In some embodiments, the patient has not been diagnosed with distant metastasis. In some embodiments, the patient has not been diagnosed with peritoneal metastasis. In some embodiments, the patient has not been diagnosed with or has not been treated for peritoneal metastasis or Stage IV gastric cancer.

[0011] In some embodiments, the method further comprises treating the patient for cancer after measuring the level of expression of one or more listed biomarkers. In some embodiments, the biomarker is measured prior to surgical resection of the tumor or prior to total or subtotal gastrectomy. In some embodiments, the biomarker is measured after surgical resection of the tumor or after total or subtotal gastrectomy. In some embodiments, the patient has undergone surgery to resect all or part of the cancer. In some embodiments, the patient has not undergone surgical resection of the tumor.

[0012] In some embodiments, the level of expression of miR-30a-5p was determined or measured pre-operative and/or post-operative. In some embodiments, the level of expression of miR-134-5p was determined or measured pre-operative and/or post-operative. In some embodiments, the level of expression of miR-337-3p was determined or measured pre-operative and/or post-operative. In some embodiments, the level of expression of miR-659-3p was determined or measured pre-operative and/or post-operative. In some embodiments, the level of expression of miR-3917 was determined or measured pre-operative and/or post-operative. In some embodiments, the patient has not undergone laparoscopy of gastric cancer tissues.

[0013] In some embodiments, the expression levels of the one or more biomarkers in the patient was determined to be i) increased compared to the levels of expression in samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk. In some embodiments, the expression levels of at least one of

the biomarkers in the patient was determined to be i) increased compared to the levels of expression in samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk. In some embodiments, the expression levels of at least two of the biomarkers in the patient was determined to be i) increased compared to the levels of expression in samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk. In some embodiments, the expression levels of at least three of the biomarkers in the patient was determined to be i) increased compared to the levels of expression in samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk. the expression levels of at least four of the biomarkers in the patient was determined to be i) increased compared to the levels of expression in samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk. In some embodiments, the expression levels of at least five of the biomarkers in the patient was determined to be i) increased compared to the levels of expression in samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk.

[0014] In some embodiments, the patient was determined to have a macroscopic Borrmann type III or IV gastric tumor. In some embodiments, the patient is treated for peritoneal metastasis. In some embodiments, the treatment comprises one or more of cytoreductive surgery, hyperthermic intraperitoneal chemotherapy (HIPEC), chemotherapy, neo-adjuvant chemotherapy, adjuvant chemotherapy, subtotal or total gastrectomy, tumor resection, and endoscopic resection. In some embodiments, the chemotherapy comprises paclitaxel. In some embodiments, the chemotherapy is administered by intraperitoneal administration. In some embodiments, the chemotherapy comprises one or more chemotherapeutic agents described herein. In some embodiments, the chemotherapy excludes one or more chemotherapeutic agents described herein.

[0015] In some embodiments, the expression levels of the one or more biomarkers in the patient was determined to be i) decreased compared to the levels of expression in samples from patients identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal tissues. In some embodiments, the expression levels of at least one of the biomarkers in the patient was determined to be i) decreased compared to the levels of expression in samples from patients identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal tissues. In some embodiments, the expression levels of at least two of the biomarkers in the patient was determined to be i) decreased compared to the levels of expression in samples from patients identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal tissues. In some embodiments, the expression levels of at least three of the biomarkers in the patient was determined to be i) decreased compared to the levels of expression in samples from patients identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal tissues. In some embodiments, the expression levels of at least four of the biomarkers in the patient was determined to be i) decreased compared to the levels of expression in samples from patients identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal tissues. In some embodiments, the expression levels of at least five of the biomarkers in the patient was determined to be i) decreased compared to the levels of expression in samples from patients identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal tissues.

[0016] In some embodiments, the patient was determined to have a macroscopic Borrmann type I or II gastric tumor. In some embodiments, the treatment comprises one or more of surgery with either subtotal or total gastrectomy, tumor resection, endoscopic resection. In some embodiments, the treatment excludes one or more of cytoreductive surgery, hyperthermic intraperitoneal chemotherapy (HIPEC), chemotherapy, neo-adjuvant chemotherapy, and adjuvant chemotherapy.

[0017] In some embodiments, low risk is indicative of a patient with a low risk for distant metastasis and/or peritoneal metastasis and good overall survival (OS) rate, and high risk is indicative of a patient with a high risk for distant metastasis and/or peritoneal metastasis and poor overall survival (OS) rate.

5 **[0018]** In some embodiments, the method further comprises comparing the level(s) of expression to a control sample(s) or control level(s) of expression. In some embodiments, the control sample(s) have expression levels that are representative of expression levels in samples from patients identified as low risk, of patients not having gastric cancer, or of patients having gastric cancer but not having peritoneal metastasis. In some embodiments, the control levels(s)
10 comprise the levels of expression of the one or more biomarkers in non-cancerous gastric tissues. In some embodiments, the control sample(s) have expression levels that are representative of expression levels in samples from patients identified as high risk or of patients having peritoneal metastasis.

[0019] The expression level or activity level from a control sample may be an average value,
15 a normalized value, a cut-off value, or an average normalized value. The expression level or activity level may be an average or mean obtained from a significant proportion of patient samples. The expression or activity level may also be an average or mean from one or more samples from the patient.

[0020] In some embodiments, the elevated level/increased expression or reduced
20 level/decreased expression is at least 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 50, 100, 150, 200, 250, 500, or 1000 fold (or any derivable range therein) or at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, or 900% different than the control, or any derivable range therein. In some embodiments, a level of expression may be qualified as “low” or “high,” which indicates the patient expresses a certain gene at a level relative
25 to a reference level or a level with a range of reference levels that are determined from multiple samples meeting particular criteria. The level or range of levels in multiple control samples is an example of this. In some embodiments, that certain level or a predetermined threshold value is at, below, or above 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25,
26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51,
30 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 percentile,

or any range derivable therein. Moreover, the threshold level may be derived from a cohort of individuals meeting a particular criteria. The number in the cohort may be, be at least, or be at most 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 441, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000 or more (or any range derivable therein).

[0021] In some embodiments, the control may be the average level of expression of the miRNA in a biological sample from a subject having gastric cancer or determined to be at risk for gastric cancer. The control may be the level of expression of the miRNA in a biological sample from a subject with stage I, II, III, or IV gastric cancer (or any TMN stage defined herein). One skilled in the art would understand that, when comparing the expression level of the miRNA in a biological sample from a test subject to the expression level from a subject with gastric cancer, the decision to treat the subject for gastric cancer or diagnose or provide a prognosis that the subject has or is likely to get gastric cancer is based on the a level of expression that is similar to the control or within 1, 2, 3, 4, or 5 deviations or differs by less than 1, 3, 5, 10, 15, 20, 30, or 40% (or any derivable range therein).

[0022] In some embodiments, the patient has been diagnosed with gastric cancer. In some embodiments, the method further comprises treating the patient for cancer after measuring the level of expression of one or more listed biomarkers. In some embodiments, the biomarker is measured prior to surgical resection of the tumor or prior to total or subtotal gastrectomy. In some embodiments, the biomarker is measured after surgical resection of the tumor or after total or subtotal gastrectomy. In some embodiments, the level of expression of miR-30a-5p was measured pre-operative and/or post-operative. In some embodiments, the level of expression of miR-134-5p was measured pre-operative and/or post-operative. In some embodiments, the level of expression of miR-337-3p was measured pre-operative and/or post-operative. In some embodiments, the level of expression of miR-659-3p was measured pre-operative and/or post-operative. In some embodiments, the level of expression of miR-3917 was measured pre-operative and/or post-operative.

[0023] In some embodiments, the method further comprises determining the macroscopic Borrmann type of the gastric tumor. In some embodiments, the patient has not been diagnosed with or has not been treated for peritoneal metastasis or Stage IV gastric cancer.

[0024] In some embodiments, the patient is prognosed as high risk and/or treated when the
5 expression levels of the one or more biomarkers in the patient was i) increased compared to the levels of expression in samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk. In some
10 embodiments, the patient is prognosed as high risk and/or treated when the expression levels of at least one of the biomarkers in the patient was i) increased compared to the levels of expression in samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk. In some embodiments, the patient is prognosed as
15 high risk and/or treated when the expression levels of at least two of the biomarkers in the patient was i) increased compared to the levels of expression in samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk. In some embodiments, the patient is prognosed as high risk and/or treated when the expression levels of at least three of the biomarkers in the patient was i) increased compared to the
20 levels of expression in samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk. In some embodiments, the patient is prognosed as high risk and/or treated when the expression levels of at least four of the biomarkers in the patient was i) increased compared to the levels of expression in
25 samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk. In some embodiments, the patient is prognosed as high risk and/or treated when the expression levels of at least five of the biomarkers in the patient was i) increased compared to the levels of expression in samples from patients identified as not
30 having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of

expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk.

[0025] In some embodiments, the patient is prognosed as high risk and/or treated when the patient was determined to have a macroscopic Borrmann type III or IV gastric tumor. In some
5 embodiments, the patient is diagnosed as having peritoneal metastasis. In some embodiments, the treatment comprises one or more of cytoreductive surgery, hyperthermic intraperitoneal chemotherapy (HIPEC), chemotherapy, neo-adjuvant chemotherapy, adjuvant chemotherapy, subtotal or total gastrectomy, tumor resection, and endoscopic resection. In some embodiments, the chemotherapy comprises paclitaxel. In some embodiments, the chemotherapy is administered
10 by intraperitoneal administration.

[0026] In some embodiments, the patient is prognosed as low risk and/or treated when the expression levels of the one or more biomarkers in the patient is i) decreased compared to the levels of expression in samples from patients identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified
15 as not having peritoneal metastasis or in normal tissues. In some embodiments, the patient is prognosed as low risk and/or treated when the expression levels of at least one of the biomarkers in the patient is i) decreased compared to the levels of expression in samples from patients identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal
20 tissues. In some embodiments, the patient is prognosed as low risk and/or treated when the expression levels of at least two of the biomarkers in the patient is i) decreased compared to the levels of expression in samples from patients identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal tissues. In some embodiments, the patient is
25 prognosed as low risk and/or treated when the expression levels of at least three of the biomarkers in the patient is i) decreased compared to the levels of expression in samples from patients identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal tissues. In some embodiments, the patient is prognosed as low risk and/or treated when the
30 expression levels of at least four of the biomarkers in the patient is i) decreased compared to the levels of expression in samples from patients identified as having peritoneal metastasis or

identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal tissues. In some embodiments, the patient is prognosed as low risk and/or treated when the expression levels of at least five of the biomarkers in the patient is i) decreased compared to the levels of expression in samples from patients
 5 identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal tissues.

[0027] In some embodiments, the patient is prognosed as low risk and/or treated when the patient was determined to have a macroscopic Borrmann type I or II gastric tumor. In some
 10 embodiments, the treatment comprises one or more of surgery with either subtotal or total gastrectomy, tumor resection, endoscopic resection. In some embodiments, the treatment excludes one or more of cytoreductive surgery, hyperthermic intraperitoneal chemotherapy (HIPEC), chemotherapy, neo-adjuvant chemotherapy, and adjuvant chemotherapy.

[0028] In some embodiments, methods will involve determining or calculating a prognosis
 15 score based on data concerning the expression or activity level of one or more of the biomarkers, meaning that the expression or activity level of one or more of the biomarkers is at least one of the factors on which the score is based. A prognosis score will provide information about the patient, such as the general probability whether the patient is sensitive to a particular therapy or has poor survival or high chances of recurrence. In certain embodiments, a prognosis value is expressed as
 20 a numerical integer or number that represents a probability of 0% likelihood to 100% likelihood that a patient has a chance of poor survival or cancer recurrence or poor response to a particular treatment.

[0029] In some embodiments, the prognosis score is expressed as a number that represents a probability of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25,
 25 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 5 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% likelihood (or any range derivable therein) that a patient has a chance of poor survival or cancer recurrence or poor response to a particular treatment. Alternatively, the probability may be
 30 expressed generally in percentiles, quartiles, or deciles.

[0030] A difference between or among weighted coefficients or expression or activity levels or between or among the weighted comparisons may be, be at least or be at most about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11.0, 11.5, 12.0, 12.5, 13.0, 13.5, 14.0, 14.5, 15.0, 15.5, 16.0, 16.5, 17.0, 17.5, 18.0, 18.5, 19.0, 19.5, 20.0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, 350, 355, 360, 365, 370, 375, 380, 385, 390, 395, 400, 410, 420, 425, 430, 440, 441, 450, 460, 470, 475, 480, 490, 500, 510, 520, 525, 530, 540, 550, 560, 570, 575, 580, 590, 600, 610, 620, 625, 630, 640, 650, 660, 670, 675, 680, 690, 700, 710, 720, 725, 730, 740, 750, 760, 770, 775, 780, 790, 800, 810, 820, 825, 830, 840, 850, 860, 870, 875, 880, 890, 900, 910, 920, 925, 930, 940, 950, 960, 970, 975, 980, 990, 1000 times or –fold (or any range derivable therein).

[0031] In some embodiments, determination of calculation of a diagnostic, prognostic, or risk score is performed by applying classification algorithms based on the expression values of biomarkers with differential expression p values of about, between about, or at most about 0.005, 0.006, 0.007, 0.008, 0.009, 0.01, 0.011, 0.012, 0.013, 0.014, 0.015, 0.016, 0.017, 0.018, 0.019, 0.020, 0.021, 0.022, 0.023, 0.024, 0.025, 0.026, 0.027, 0.028, 0.029, 0.03, 0.031, 0.032, 0.033, 0.034, 0.035, 0.036, 0.037, 0.038, 0.039, 0.040, 0.041, 0.042, 0.043, 0.044, 0.045, 0.046, 0.047, 0.048, 0.049, 0.050, 0.051, 0.052, 0.053, 0.054, 0.055, 0.056, 0.057, 0.058, 0.059, 0.060, 0.061, 0.062, 0.063, 0.064, 0.065, 0.066, 0.067, 0.068, 0.069, 0.070, 0.071, 0.072, 0.073, 0.074, 0.075, 0.076, 0.077, 0.078, 0.079, 0.080, 0.081, 0.082, 0.083, 0.084, 0.085, 0.086, 0.087, 0.088, 0.089, 0.090, 0.091, 0.092, 0.093, 0.094, 0.095, 0.096, 0.097, 0.098, 0.099, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or higher (or any range derivable therein). In certain embodiments, the prognosis score is calculated using one or more statistically significantly differentially expressed biomarkers (either individually or as difference pairs), including expression or activity levels in a biomarker, gene, or protein.

[0032] In some embodiments, the sample from the patient comprises gastric cancer tissue. In some embodiments, the normal tissues comprises non-cancerous gastric tissues. In some embodiments, the sample from the patient comprises a serum sample. In some embodiments, the sample from the patient comprises nucleic acids. In some embodiments, the sample from the patient comprises a fractionated serum sample comprising nucleic acids. In some embodiments, the samples from patients identified as not having peritoneal metastasis or identified as low risk comprises the level of expression of the one or more biomarkers in a serum sample or samples from patients without peritoneal metastasis. In some embodiments, the expression level of no other biomarker in the biological sample was determined or measured.

[0033] In some embodiments, the biological sample from the patient is a sample from a primary gastric cancer tumor. In some embodiments, the biological sample is from a tissue or organ as described herein. In still further embodiments, the method may comprise obtaining a sample of the subject or patient. Non-limiting examples of the sample include a tissue sample, a whole blood sample, a urine sample, a saliva sample, a serum sample, a plasma sample, or a fecal sample. In particular embodiments, the sample is a serum sample, a plasma sample or a whole blood sample.

[0034] The methods of obtaining a sample of the subject or patient provided herein include methods of biopsy such as fine needle aspiration, core needle biopsy, vacuum assisted biopsy, incisional biopsy, excisional biopsy, punch biopsy, shave biopsy or skin biopsy.

[0035] In certain embodiments the sample is obtained from a biopsy from intestinal, stomach, or other associated gastric tissues. In other embodiments the sample may be obtained from any of the tissues provided herein that include but are not limited to gall bladder, skin, heart, lung, breast, pancreas, liver, muscle, kidney, smooth muscle, bladder, intestine, brain, prostate, esophagus, or thyroid tissue.

[0036] In certain aspects the sample is obtained from cystic fluid or fluid derived from a tumor or neoplasm. In yet other embodiments the cyst, tumor or neoplasm is in the digestive system. In certain aspects of the current methods, any medical professional such as a doctor, nurse or medical technician may obtain a biological sample for testing. In further aspects of the current methods, the patient or subject may obtain a biological sample for testing without the assistance of a medical professional, such as obtaining a whole blood sample, a urine sample, a fecal sample, a buccal sample, or a saliva sample.

[0037] In further embodiments, the sample may be a fresh, frozen or preserved sample or a fine needle aspirate. In particular embodiments, the sample is a formalin-fixed, paraffin embedded (FFPE) sample. An acquired sample may be placed in short term or long term storage by placing in a suitable medium, excipient, solution, or container. In certain cases storage may require keeping the sample in a refrigerated, or frozen environment. The sample may be quickly frozen prior to storage in a frozen environment. In certain instances the frozen sample may be contacted with a suitable cryopreservation medium or compound. Examples of cryopreservation mediums or compounds include but are not limited to: glycerol, ethylene glycol, sucrose, or glucose.

[0038] Further aspects relate to a kit comprising 1, 2, 3, 4, or 5 detection agents for determining expression levels of biomarkers for gastric cancer, wherein the biomarkers comprise one or more of miR-30a-5p, miR-134-5p, miR-337-3p, miR-659-3p, and miR-3917.

[0039] In some embodiments, the kit further comprises one or more negative or positive control samples and/or control detection agents. In some embodiments, the detection agents comprise nucleic acids. In some embodiments, the detection agents comprise nucleic acid probes that hybridize to a biomarker gene or fragment thereof. In some embodiments, the detection agents comprise a pair of nucleic acid primers that are capable of amplifying a biomarker gene or a fragment thereof. In some embodiments, the detection agent comprises an antibody that specifically binds to a biomarker protein. In some embodiments, the probes are labeled. In some embodiments, the kit further comprises nucleic acid probes for detecting a control. In some embodiments, the control comprises a RNA, miRNA, or a biomarker protein or gene not differentially expressed in liver cancer or in fast or slow DT liver cancer or HCC. In some embodiments, the probe comprises nucleic acid primers that are capable of amplifying the RNA or a cDNA made from the RNA by PCR. In some embodiments, the kit further comprises reagents for performing one or more of reverse transcriptase PCR, DNA amplification by PCR, and real-time PCR. In some embodiments, the kit further comprises instructions for use.

[0040] Any of the methods described herein may be implemented on tangible computer-readable medium comprising computer-readable code that, when executed by a computer, causes the computer to perform one or more operations. In some embodiments, there is a tangible computer-readable medium comprising computer-readable code that, when executed by a computer, causes the computer to perform operations comprising: a) receiving information corresponding to an expression or activity level of a gene, biomarker or protein in a sample from

a patient; and b) determining a difference value in the expression or activity levels using the information corresponding to the expression or activity levels in the sample compared to a control or reference expression or activity level for the gene.

[0041] In other aspects, tangible computer-readable medium further comprise computer-readable code that, when executed by a computer, causes the computer to perform one or more additional operations comprising making recommendations comprising: wherein the patient in the step a) is under or after a first treatment for gastric cancer, administering the same treatment as the first treatment to the patient if the patient does not have increased expression or activity level; administering a different treatment from the first treatment to the patient if the patient has increased expression or activity level.

[0042] In some embodiments, receiving information comprises receiving from a tangible data storage device information corresponding to the expression or activity levels from a tangible storage device. In additional embodiments the medium further comprises computer-readable code that, when executed by a computer, causes the computer to perform one or more additional operations comprising: sending information corresponding to the difference value to a tangible data storage device, calculating a prognosis score for the patient, treating the patient with a traditional gastric therapy if the patient does not have expression or activity levels, and/or or treating the patient with an alternative gastric therapy if the patient has increased expression or activity levels.

[0043] The tangible, computer-readable medium further comprise computer-readable code that, when executed by a computer, causes the computer to perform one or more additional operations comprising calculating a prognosis score for the patient. The operations may further comprise making recommendations comprising: administering a treatment comprising a thymidylate synthase inhibitor to a patient that is determined to have a decreased expression or activity level.

[0044] The terms “subject,” “mammal,” and “patient” are used interchangeably. In some embodiments, the subject is a mammal. In some embodiments, the subject is a human. In some embodiments, the subject is a mouse, rat, rabbit, dog, donkey, or a laboratory test animal such as fruit fly, zebrafish, etc.

[0045] The biomarkers described herein, such as the gene or RNA biomarkers may correspond to the human gene or RNA. In some embodiments, the biomarkers corresponds to a human, mammalian, mouse, dog, cat, or a homolog of a human gene or RNA.

[0046] It is contemplated that the methods and compositions include exclusion of any of the
5 embodiments described herein.

[0047] Throughout this application, the term “about” is used according to its plain and ordinary meaning in the area of cell and molecular biology to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value.

[0048] The use of the word “a” or “an” when used in conjunction with the term “comprising”
10 may mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.”

[0049] As used herein, the terms “or” and “and/or” are utilized to describe multiple components in combination or exclusive of one another. For example, “x, y, and/or z” can refer to “x” alone, “y” alone, “z” alone, “x, y, and z,” “(x and y) or z,” “x or (y and z),” or “x or y or z.” It
15 is specifically contemplated that x, y, or z may be specifically excluded from an embodiment.

[0050] The words “comprising” (and any form of comprising, such as “comprise” and “comprises”), “having” (and any form of having, such as “have” and “has”), “including” (and any form of including, such as “includes” and “include”), “characterized by” (and any form of including, such as “characterized as”), or “containing” (and any form of containing, such as
20 “contains” and “contain”) are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

[0051] The compositions and methods for their use can “comprise,” “consist essentially of,” or “consist of” any of the ingredients or steps disclosed throughout the specification. The phrase “consisting of” excludes any element, step, or ingredient not specified. The phrase “consisting
25 essentially of” limits the scope of described subject matter to the specified materials or steps and those that do not materially affect its basic and novel characteristics. It is contemplated that embodiments described in the context of the term “comprising” may also be implemented in the context of the term “consisting of” or “consisting essentially of.”

[0052] It is specifically contemplated that any limitation discussed with respect to one
30 embodiment of the invention may apply to any other embodiment of the invention. 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. Aspects of an embodiment set forth in the Examples are also embodiments that may be implemented in the context of embodiments discussed elsewhere in a different Example or elsewhere in the application, such as in the Summary of Invention, Detailed Description of the Embodiments, Claims, and description of Figure Legends.

[0053] 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 specific 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.

BRIEF DESCRIPTION OF THE DRAWINGS

[0054] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0055] FIG. 1A-C. Identification of candidate miRNAs in primary tumors from gastric cancer patients with peritoneal metastasis. (A) Schematic of the study design for biomarker discovery and validation in various patient cohorts. (B) Heatmap illustrating differentially expressed miRNAs between peritoneal metastasis positive and negative samples identified from the miRNA-microarray dataset. (C) Differentially expressed candidate miRNAs between stage IV and stage IB-III GC patients in the TCGA dataset. Abbreviations; PM, peritoneal metastasis. * $p < 0.05$, by Mann-Whitney U test.

[0056] FIG. 2A-C. Candidate three miRNAs were differentially expressed in peritoneal metastasis positive patients in the testing cohort. (A) The expression of candidate five miRNAs between PM positive and negative samples. (B) Differential expression of candidate miRNAs in the sub-group analysis of stage IV patients between PM positive and negative patients. (C) Kaplan meier analysis for overall survival (OS) between two groups dichotomized by Youden's index for PM in combined miRNA signature. Abbreviations; PM, peritoneal metastasis. * $p < 0.05$, ** $p < 0.01$, by Mann-Whitney U test.

[0057] FIG. 3A-D. Candidate miRNAs were differentially expressed in peritoneal metastasis positive patients in the testing cohort. (A) Diagnostic robustness of miRNA candidates represented by receiver operating characteristic (ROC) curves. (B) The waterfall plot representing risk score of PM positive and negative patients based on the combined miRNAs signature. (C) ROC curve of the combined miRNA signature for the detection of PM. Abbreviations; Sen, sensitivity; Spe, specificity. (D) Kaplan-Meier analysis for overall survival (OS) between two groups dichotomized by Youden's index for PM in individual overexpressing miRNAs.

[0058] FIG. 4A-D. Candidate three miRNAs were differentially expressed in peritoneal metastasis positive patients in the validation cohort. (A) The expression of candidate three miRNAs between PM positive and negative samples. (B) Diagnostic robustness of miRNA candidates represented by ROC curve. (C) Differential expression of candidate three miRNAs in the sub-group analysis of stage IV patients between PM positive and negative patients. (D) Kaplan meier analysis for OS in individual three miRNAs. Abbreviations; PM, peritoneal metastasis; Sen, sensitivity; Spe, specificity. * $p < 0.05$, by Mann-Whitney U test.

[0059] FIG. 5A-F. Diagnostic accuracy of peritoneal metastasis detection and prognostic significance of combined miRNA signature in validation cohort and performance evaluation cohort. The box plot representing risk scores of PM positive and negative patients in the validation (A), and the performance evaluation cohort (D). ROC curves for the detection of PM in validation (B), and performance evaluation cohort (E). Kaplan-Meier analysis for OS between two groups dichotomized by Youden's index for PM based on the combined miRNA signature in validation cohort (C), and the performance evaluation cohort (F). Abbreviations; PM, peritoneal metastasis; Sen, sensitivity; Spe, specificity. ** $p < 0.01$, *** $p < 0.01$ by Mann-Whitney U test.

[0060] FIG. 6A-D. Candidate three miRNAs were differentially expressed in peritoneal metastasis positive patients in the performance evaluation cohort. (A) The expression of candidate three miRNAs between PM positive and negative samples. (B) Diagnostic robustness of miRNA candidates represented by ROC curve. (C) Differential expression of candidate three miRNAs in the sub-group analysis of stage IV patients between PM positive and negative patients. (D) Kaplan meier analysis for OS in individual three miRNAs. Abbreviations; PM, peritoneal metastasis; Sen, sensitivity; Spe, specificity. ** $p < 0.01$, *** $p < 0.001$, by Mann-Whitney U test.

[0061] FIG. 7A-D. Diagnostic robustness of peritoneal metastasis detection of combined miRNA signature and tumor macroscopic type in testing cohort (A) ROC curve of the combined

miRNA signature, tumor macroscopic type, and their combination for detection of PM. (B) Nomogram, (C) Predicted probability plot, and (D) Cost-benefit curve for PM detection.

[0062] FIG. 8A-H. Diagnostic accuracy of peritoneal metastasis detection of combined miRNA signature and tumor macroscopic type in validation cohort and performance evaluation cohort. ROC curves derived from the combination miRNA signature, tumor macroscopic type, and their combination for detection of PM in the validation (A), and performance evaluation cohort (B). Nomogram and validation of their predicted probability plots in the validation (C and E) and performance evaluation (D and F) cohorts. Cost-benefit curves for PM detection in the validation (G), and performance evaluation cohort (H).

[0063] FIG. 9A-C. Identification of candidate miRNAs in primary tumors from peritoneal metastasis positive patients. (A) Schematics of target miRNA identification using miRNA-microarray and TCGA datasets. (B) Heatmap of differentially expressed miRNAs between peritoneal metastasis positive and negative samples identified from miRNA-microarray dataset. (C) Differentially expressed candidate miRNAs between stage IV and stage IB - III GC patients in TCGA dataset. Abbreviations; GC, gastric cancer; PM, peritoneal metastasis. * $p < 0.05$.

[0064] FIG. 10A-D. Candidate miRNAs were differentially expressed in peritoneal metastasis positive patients in cohort 1. (A) The expression of candidate miRNAs between peritoneal metastasis positive and negative samples. (B) Diagnostic robustness of miRNA candidates represented by receiver operating characteristic (ROC) curve. (C) ROC curve of the combined miRNAs signature for detect peritoneal metastasis (left). The waterfall plot representing risk score of peritoneal metastasis positive and negative patients based on the combined miRNAs signature. (D) Kaplan meier analysis for overall survival between two groups dichotomized by Youden index for peritoneal metastasis in individual overexpressing miRNAs and in combined miRNA signature. * $p < 0.05$, by Mann-Whitney U test.

[0065] FIG. 11A-C. Differential expression of candidate miRNAs between peritoneal metastasis positive and negative patients in cohorts (A) 1, (B) 2, and (C) 3.

[0066] FIG. 12A-D. Candidate miRNAs were differentially expressed in peritoneal metastasis positive patients in cohort 2. (A) The expression of candidate three miRNAs between peritoneal metastasis positive and negative samples. (B) Diagnostic robustness of miRNA candidates represented by receiver operating characteristic (ROC) curve. (C) ROC curve of the combined miRNAs signature for detect peritoneal metastasis (left). The waterfall plot representing risk score

of peritoneal metastasis positive and negative patients based on the combined miRNAs signature. (D) Kaplan meier analysis for overall survival in individual three miRNAs and combined miRNA signature. * $p < 0.05$, ** $p < 0.01$, by Mann-Whitney U test.

[0067] FIG. 13A-D. Differential expression of candidate miRNAs between peritoneal metastasis positive and negative samples in fresh frozen cohort. (A) The expression of candidate three miRNAs between peritoneal metastasis positive and negative samples. (B) Diagnostic robustness of miRNA candidates represented by receiver operating characteristic (ROC) curve (right). (C) ROC curve of the combined miRNAs signature for detect peritoneal metastasis (left). The waterfall plot representing risk score of peritoneal metastasis positive and negative patients based on the combined miRNAs signature. (D) Kaplan meier analysis for overall survival in individual three miRNAs and combined miRNA signature. * $p < 0.05$, ** $p < 0.01$, by Mann-Whitney U test.

[0068] FIG. 14A-C. Diagnostic capacity of the miRNA signature upon combination of tumor macroscopic type with the miRNA signature in patient cohorts (A) 1, (B) 2, and (C) 3.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0069] Described herein are improved methods for treating gastric cancer patients. The inventors aimed to do a genomewide transcriptomic profiling to develop a miRNA-based signature for the identification of peritoneal metastasis (PM) in patients with gastric cancer (GC). Even though PM in patients with GC has long been recognized to associate with poor survival, currently there is lack of availability of molecular biomarkers for its robust diagnosis. Systematic biomarker discovery was performed by analyzing miRNA expression profiles in primary tumors from GC patients with and without PM, and subsequently validated the expression of candidate miRNA biomarkers in three independent clinical cohorts of 354 patients with advanced GC. Five miRNAs (miR-30a-5p, -134-5p, -337-3p, -659-3p, and -3917) were identified during the initial discovery phase; three of which (miR-30a-5p, -659-3p, and -3917) were significantly overexpressed in the primary tumors from PM-positive patients in the testing cohort ($p=0.002$, 0.04 and 0.007 respectively), and robustly distinguished patients with versus without peritoneal metastasis ($AUC=0.82$). Furthermore, high expression of these miRNAs was also associated with poor prognosis ($HR=2.18$, $p=0.04$). The efficacy of the combination miRNA-signature was subsequently validated in an independent validation cohort ($AUC=0.74$). Finally, the miRNA

signature when combined together with the macroscopic Borrmann's type score offered a superior diagnostic in all three cohorts (AUC=0.87, 0.76, 0.79, respectively), and led to establishment of a risk-prediction nomogram for the diagnosis of PM in GC patients. Thus, the novel miRNA-based signature is a robust diagnostic tool for identifying peritoneal metastasis in GC patients, which could lead to improved survival outcomes in gastric cancer and more efficient and effective treatment of gastric cancer patients..

I. Definitions

[0070] As used herein, the term "antibody" encompasses antibodies and antibody fragments thereof, derived from any antibody-producing mammal (e.g., mouse, rat, rabbit, and primate including human), that specifically bind to an antigenic polypeptide. Exemplary antibodies include polyclonal, monoclonal and recombinant antibodies; multispecific antibodies (e.g., bispecific antibodies); humanized antibodies; murine antibodies; chimeric, mouse-human, mouse-primate, primate-human monoclonal antibodies; and anti-idiotypic antibodies, and may be any intact molecule or fragment thereof.

[0071] "Prognosis" refers to as a prediction of how a patient will progress, and whether there is a chance of recovery. "Cancer prognosis" generally refers to a forecast or prediction of the probable course or outcome of the cancer, with or without a treatment. As used herein, cancer prognosis includes the forecast or prediction of any one or more of the following: duration of survival of a patient susceptible to or diagnosed with a cancer, duration of recurrence-free survival, duration of progression free survival of a patient susceptible to or diagnosed with a cancer, response rate in a group of patients susceptible to or diagnosed with a cancer, duration of response in a patient or a group of patients susceptible to or diagnosed with a cancer, and/or likelihood of metastasis in a patient susceptible to or diagnosed with a cancer. Prognosis also includes prediction of favorable responses to cancer treatments, such as a conventional cancer therapy. A response may be either a therapeutic response (sensitivity or recurrence-free survival) or a lack of therapeutic response (residual disease, which may indicate resistance or recurrence).

[0072] The terms "substantially the same," "not significantly different," or "within the range" refers to a level of expression that is not significantly different than what it is compared to. Alternatively, or in conjunction, the terms refer to a level of expression that is less than 2, 1.5, or

1.25 fold different or less than 2, 1, or 0.5 standard deviations than the expression or activity level it is compared to.

[0073] By “subject” or “patient” is meant any single subject for which therapy is desired, including humans, cattle, dogs, guinea pigs, rabbits, chickens, and so on. Also intended to be included as a subject are any subjects involved in clinical research trials not showing any clinical sign of disease, or subjects involved in epidemiological studies, or subjects used as controls.

[0074] The term “primer” or “probe” as used herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty and/or thirty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded and/or single-stranded form, although the single-stranded form is preferred. A probe may also refer to a nucleic acid that is capable of hybridizing by base complementarity to a nucleic acid of a gene of interest or a fragment thereof.

[0075] As used herein, “increased expression” or “elevated expression” or “decreased expression” refers to an expression level of a biomarker in the subject's sample as compared to a reference level representing the same biomarker or a different biomarker. In certain aspects, the reference level may be a reference level of expression from a non-cancerous tissue from the same subject. Alternatively, the reference level may be a reference level of expression from a different subject or group of subjects. For example, the reference level of expression may be an expression level obtained from a sample (e.g., a tissue, fluid or cell sample) of a subject or group of subjects without cancer, with fast doubling time HCC, or with slow doubling time HCC, or an expression level obtained from a non-cancerous tissue of a subject or group of subjects with cancer. The reference level may be a single value or may be a range of values. The reference level of expression can be determined using any method known to those of ordinary skill in the art. The reference level may also be depicted graphically as an area on a graph. In certain embodiments, a reference level is a normalized level.

[0076] The term “determining” or “evaluating” as used herein may refer to measuring, quantitating, or quantifying (either qualitatively or quantitatively).

II. Gastric Cancer Staging and Treatments

[0077] Methods and compositions may be provided for treating gastric cancer with particular applications of biomarker expression or activity levels. Based on a profile of biomarker expression or activity levels, different treatments may be prescribed or recommended for different cancer patients. In some embodiments, the gastric cancer comprises a cancer stage, TNM, and/or is further characterized as having features described below.

A. Cancer staging

[0078] Gastric cancer, also known as stomach cancer tends to develop slowly over many years. Before a true cancer develops, pre-cancerous changes often occur in the inner lining (mucosa) of the stomach. These early changes rarely cause symptoms and therefore often go undetected.

[0079] Cancers starting in different sections of the stomach may cause different symptoms and tend to have different outcomes. The cancer's location can also affect the treatment options. For example, cancers that start at the gastroesophageal (GE) junction are staged and treated the same as cancers of the esophagus. A cancer that starts in the cardia of the stomach but then grows into the GE junction is also staged and treated like a cancer of the esophagus.

[0080] Stomach cancers can spread (metastasize) in different ways. They can grow through the wall of the stomach and invade nearby organs. They can also spread to the lymph vessels and nearby lymph nodes. Lymph nodes are bean-sized structures that help fight infections. The stomach has a very rich network of lymph vessels and nodes. As the stomach cancer becomes more advanced, it can travel through the bloodstream and spread to organs such as the liver, lungs, and bones. If cancer has spread to the lymph nodes or to other organs, the patient's outlook is not as good.

[0081] Different types of stomach cancer include: adenocarcinomas, lymphomas, gastrointestinal stromal tumor (GIST), and carcinoid tumor. Squamous cell carcinoma, small cell carcinoma, and leiomyosarcoma, can also start in the stomach, but these cancers are very rare.

[0082] The most common staging system is the TNM (for tumors/nodes/metastases) system, from the American Joint Committee on Cancer (AJCC). The TNM system assigns a number based on three categories. "T" denotes the degree of invasion of the intestinal wall, "N" the degree of lymphatic node involvement, and "M" the degree of metastasis. The broader stage of a cancer is

usually quoted as a number I, II, III, IV derived from the TNM value grouped by prognosis; a higher number indicates a more advanced cancer and likely a worse outcome. Details of this system are in the tables below:

T categories of stomach cancer	T0: No signs of a main tumor can be found.
	Tis: Cancer cells are only in the top layer of cells of the mucosa (innermost layer of the stomach) and have not grown into deeper layers of tissue such as the lamina propria or muscularis mucosa. This stage is also known as carcinoma in situ.
	T1: The tumor has grown from the top layer of cells of the mucosa into the next layers below such as the lamina propria, the muscularis mucosa, or submucosa.
	T1a: The tumor is growing into the lamina propria or muscularis mucosa.
	T1b: The tumor has grown through the lamina propria and muscularis mucosa and into the submucosa.
	T2: The tumor is growing into the muscularis propria layer.
	T3: The tumor is growing into the subserosa layer.
	T4: The tumor has grown into the serosa and may be growing into a nearby organ (spleen, intestines, pancreas, kidney, etc.) or other structures such as major blood vessels.
N categories of stomach cancer	T4a: The tumor has grown through the stomach wall into the serosa, but the cancer hasn't grown into any of the nearby organs or structures.
	T4b: The tumor has grown through the stomach wall and into nearby organs or structures.
	N0: No spread to nearby lymph nodes.
	N1: The cancer has spread to 1 to 2 nearby lymph nodes.
	N2: The cancer has spread to 3 to 6 nearby lymph nodes.

	N3: The cancer has spread 7 or more nearby lymph nodes. N3a: The cancer has spread to 7 to 15 nearby lymph nodes. N3b: The cancer has spread to 16 or more nearby lymph nodes.
M categories of stomach cancer	M0: No distant metastasis (the cancer has not spread to distant organs or sites, such as the liver, lungs, or brain). M1: Distant metastasis (the cancer has spread to organs or lymph nodes far away from the stomach).

AJCC stage	TNM stage
Stage 0	Tis N0 M0
Stage I-A	T1 N0 M0
Stage I-B	T1, N1, M0; or T2, N0, M0
Stage II-A	T1, N2, M0; or T2, N1, M0; or T3, N0, M0
Stage II-B	T1, N3, M0; or T2, N2, M0; or T3, N1, M0; or T4a, N0, M0
Stage III-A	T2, N3, M0; or T3, N2, M0; or T4a, N1, M0
Stage III-B	T3, N3, M0; or T4a, N2, M0; or T4b, N0 or N1, M0
Stage III-C	T4a, N3, M0; or T4b, N2 or N3, M0
Stage IV	Any T, any N, M1

[0083] Another commonly used classification method for gastric cancer is the Borrmann classification system. Borchard FJH, *Classification of gastric carcinoma*, Hepato-Gastroenterology, 1990;37(2):223–232. The Borrmann system stratifies gastric cancer patients based on tumor location and macroscopic appearance and growth state of the tumor. Based on the Borrmann criteria, gastric cancers are divided into four Types: Borrmann Type I, primarily exogenous growth, usually broad-based polypoid carcinomas with protrusions; Borrmann Type II, with a central, bowl-shaped ulcer and elevated margins and a relatively distinct boundary between the cancer and surrounding tissue; Borrmann Type III, centrally ulcerating carcinoma without a ridge, elevated margins and indistinct borders; Borrmann Type IV, diffuse tumor with indistinct borders and infiltration of the gastric wall. Type I and II are localized types of tumors, whereas Types III and IV are infiltrative type tumors.

[0084] The “cancer” referred to in the methods described herein may include or exclude any of the above stages or TNM categories. The “cancer” referred to in the methods described herein may include or exclude any of the above stages or TNM categories. For example the cancer may be or may exclude Stage 0, Stage I-A, Stage I-B, Stage II-A, Stage II-B, Stage III-A, Stage III-B,

Stage III-C, or Stage IV cancer. The patient may be one that has and/or has been determined to have Stage 0, Stage I-A, Stage I-B, Stage II-A, Stage II-B, Stage III-A, Stage III-B, Stage III-C, or Stage IV cancer. Furthermore, the cancer may be stage N0 and/or M0; T1, N0, and/or M0; T1, N1, and/or M0; T2, N0, and/or M0; T1, N2, and/or M0; T2, N1, and/or M0; T3, N0, and/or M0; T1, N3, and/or M0; T2, N2, and/or M0; T3, N1, and/or M0; T4a, N0, and/or M0; T2, N3, and/or M0; T3, N2, and/or M0; T4a, N1, and/or M0; T3, N3, and/or M0; T4a, N2, and/or M0; T4b and/or N0; N1 and/or M0; T4a, N3, and/or M0; T4b and/or N2; N3 and/or M0; Any T, any N, and/or M1.

B. Therapy

[0085] Methods of the disclosure relate to treating subjects and patients with a cancer therapy.

The cancer therapy may be one described below and may be given with respect to a patient having been determined to have a certain biomarker profile. For example, in some embodiments, the therapy described below is given to a patient with a poor prognosis, unfavorable prognosis, or to a patient determined to be high risk. In some embodiments, the therapy described below is given to a patient with a favorable prognosis, or to a patient determined to be low risk. Also contemplated are combinations of the therapies described below.

[0086] For a very early stage (T1a) cancer, some doctors may recommend a non-surgical treatment called endoscopic mucosal resection. This is the removal of the tumor with an endoscope. In early stages (stages 0 or I), when the cancer is still only in the stomach, surgery is used to remove the part of the stomach with cancer and nearby lymph nodes. This is called a subtotal or partial gastrectomy. In a partial gastrectomy, the surgeon connects the remaining part of the stomach to the esophagus or small intestine.

[0087] If the cancer has spread to the outer stomach wall with or without having spread to the lymph nodes, surgery plus chemotherapy or chemotherapy and radiation therapy may be used. The surgeon can perform a subtotal gastrectomy or a total gastrectomy, which is the removal of all of the stomach. During a total gastrectomy, the surgeon attaches the esophagus directly to the small intestine. Regional lymph nodes are often removed during surgery because the cancer may have spread to those lymph nodes. This is called a lymphadenectomy.

[0088] Cytoreductive surgery with hyperthermic intraperitoneal chemotherapy (HIPEC) is also used to treat cancers that have originated in or spread to the abdominal cavity, such as gastric cancer. During the first cytoreductive portion of the procedure, all visible tumors are surgically

removed. Other organs may also be partially or completely removed if tumors cannot be separated from the organ's surface. The cytoreductive procedure is followed by hyperthermic intraperitoneal chemotherapy (HIPEC) to destroy any remaining microscopic cancer cells. A high dose chemotherapy solution heated to between 107.6°F – 109.4°F (42-43°C) is delivered directly into the abdominal cavity through catheters for approximately 90 minutes to eliminate remaining cancer cells while preserving healthy cells.

[0089] Radiation therapy is the use of high-energy x-rays or other particles to destroy cancer cells. A radiation therapy regimen may comprise a specific number of treatments given over a set period of time. Patients with stomach cancer usually receive external-beam radiation therapy, which is radiation given from a machine outside the body. Radiation therapy may be used before surgery to shrink the size of the tumor or after surgery to destroy any remaining cancer cells.

[0090] Chemotherapy is the use of drugs to destroy cancer cells, usually by stopping the cancer cells' ability to grow and divide. Chemotherapy is given by a medical oncologist. Systemic chemotherapy gets into the bloodstream to reach cancer cells throughout the body. Common ways to give chemotherapy include an intravenous (IV) tube placed into a vein using a needle or in a pill or capsule that is swallowed (orally). A chemotherapy regimen usually comprises a specific number of cycles given over a set period of time. A patient may receive 1 drug at a time or combinations of different drugs at the same time.

[0091] The goal of chemotherapy can be to destroy cancer remaining after surgery, slow the tumor's growth, or reduce cancer-related symptoms. It also may be combined with radiation therapy. Exemplary chemotherapeutic regimens include, for example, the combination of fluorouracil (5-FU, Adrucil) and cisplatin (Platinol). Newer drugs similar to 5-FU, such as capecitabine (Xeloda), and similar to cisplatin, such as oxaliplatin (Eloxatin), appear to work equally well. Other drugs commonly used include docetaxel (Docefrez, Taxotere), epirubicin (Ellence), irinotecan (Camptosar), and paclitaxel (Taxol).

[0092] Antimetabolites can be used in cancer treatment, as they interfere with DNA production and therefore cell division and the growth of tumors. Because cancer cells spend more time dividing than other cells, inhibiting cell division harms tumor cells more than other cells. Antimetabolites masquerade as a purine (azathioprine, mercaptopurine) or a pyrimidine, chemicals that become the building-blocks of DNA. They prevent these substances becoming incorporated in to DNA during the S phase (of the cell cycle), stopping normal development and division. They also

affect RNA synthesis. However, because thymidine is used in DNA but not in RNA (where uracil is used instead), inhibition of thymidine synthesis via thymidylate synthase selectively inhibits DNA synthesis over RNA synthesis. Due to their efficiency, these drugs are the most widely used cytostatics. In the ATC system, they are classified under L01B.

5 [0093] Thymidylate synthase inhibitors are chemical agents which inhibit the enzyme thymidylate synthase and have potential as an anticancer chemotherapy. As an anti-cancer chemotherapy target, thymidylate synthetase can be inhibited by the thymidylate synthase inhibitors such as fluorinated pyrimidine fluorouracil, or certain folate analogues, the most notable one being raltitrexed (trade name Tomudex). Additional agents include pemetrexed, nolatrexed,
10 ZD9331, and GS7904L.

[0094] In further embodiments, there may be involved prodrugs that can be converted to thymidylate synthase inhibitors in the body, such as Capecitabine (INN), an orally administered chemotherapeutic agent used in the treatment of numerous cancers. Capecitabine is a prodrug, that is enzymatically converted to 5-fluorouracil in the body.

15 [0095] If cancer has entered the lymph nodes, adding the chemotherapy agents fluorouracil or capecitabine increases life expectancy. Chemotherapy agents for this condition may include capecitabine, fluorouracil, irinotecan, leucovorin, oxaliplatin and UFT. Another type of agent that is sometimes used are the epidermal growth factor receptor inhibitors.

[0096] In certain embodiments, alternative treatments may be prescribed or recommended
20 based on the biomarker profile. In addition to traditional chemotherapy for gastric cancer patients, cancer therapies also include a variety of combination therapies with both chemical and radiation based treatments. Combination chemotherapies include, for example, cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, busulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin,
25 bleomycin, plicomycin, mitomycin, etoposide (VP16), tamoxifen, raloxifene, estrogen receptor binding agents, taxol, gemcitabine, navelbine, farnesyl-protein transferase inhibitors, transplatin, 5-fluorouracil, vincristin, vinblastin and methotrexate, or any analog or derivative variant of the foregoing.

[0097] Just as for chemotherapy, radiotherapy can be used in the neoadjuvant and adjuvant
30 setting for some stages of gastric cancer.

[0098] Targeted therapy may also be used in the methods described herein. Targeted therapy is a treatment that targets the cancer's specific genes, proteins, or the tissue environment that contributes to cancer growth and survival. This type of treatment blocks the growth and spread of cancer cells while limiting damage to healthy cells. In some embodiments, the doctor may run tests
5 to identify the genes, proteins, and other factors in a tumor. This helps doctors better match each patient with the most effective treatment whenever possible.

[0099] In some embodiments, the methods further comprise testing a biological sample from the patient for HER2 expression. In some embodiments, the patients with HER2- positive stomach cancer are treated with trastuzumab (Herceptin) In some embodiments, this is in combination with
10 chemotherapy. Herceptin is one type of HER2-targeted therapy. For patients with metastatic or recurrent gastroesophageal cancer that is HER2 positive, ASCO, ASCP, and CAP recommend a combination of chemotherapy and HER2-targeted therapy. If the cancer is HER2 negative, HER2-targeted therapy is not a treatment option, and a doctor will give other options for treating the cancer.

[0100] For patients whose tumor has grown while receiving initial chemotherapy, the drug called ramucirumab (Cyramza) may be used as an additional treatment. Ramucirumab is a type of targeted therapy called an anti-angiogenic. It is focused on stopping angiogenesis,
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[0101] which is the process of making new blood vessels. Because a tumor needs the nutrients delivered by blood vessels to grow and spread, the goal of anti-angiogenesis therapies is to "starve"
20 the tumor.

[0102] Immunotherapies that are designed to boost the body's natural defenses to fight the cancer may also be used. Immunotherapeutics, generally, rely on the use of immune effector cells and molecules to target and destroy cancer cells. The immune effector may be, for example, an antibody specific for some marker on the surface of a tumor cell. The antibody alone may serve as
25 an effector of therapy or it may recruit other cells to actually effect cell killing. The antibody also may be conjugated to a drug or toxin (chemotherapeutic, radionuclide, ricin A chain, cholera toxin, pertussis toxin, etc.) and serve merely as a targeting agent. Alternatively, the effector may be a lymphocyte carrying a surface molecule that interacts, either directly or indirectly, with a tumor cell target. Various effector cells include cytotoxic T cells and NK cells.

[0103] Generally, the tumor cell must bear some marker that is amenable to targeting, i.e., is not present on the majority of other cells. Many tumor markers exist and any of these may be suitable for targeting.

[0104] In yet another embodiment, the treatment is a gene therapy. In certain embodiments, the therapeutic gene is a tumor suppressor gene. A tumor suppressor gene is a gene that, when present in a cell, reduces the tumorigenicity, malignancy, or hyperproliferative phenotype of the cell. This definition includes both the full length nucleic acid sequence of the tumor suppressor gene, as well as non-full length sequences of any length derived from the full length sequences. It being further understood that the sequence includes the degenerate codons of the native sequence or sequences which may be introduced to provide codon preference in a specific host cell. Examples of tumor suppressor nucleic acids within this definition include, but are not limited to APC, CYLD, HIN-1, KRAS2b, p16, p19, p21, p27, p27mt, p53, p57, p73, PTEN, Rb, Uteroglobin, Skp2, BRCA-1, BRCA-2, CHK2, CDKN2A, DCC, DPC4, MADR2/JV18, MEN1, MEN2, MTS1, NF1, NF2, VHL, WRN, WT1, CFTR, C-CAM, CTS-1, zacl, scFV, 5 MMAC1, FCC, MCC, Gene 26 (CACNA2D2), PL6, Beta* (BLU), Luca-1 (HYAL1), Luca-2 (HYAL2), 123F2 (RASSF1), 101F6, Gene 21 (NPRL2), or a gene encoding a SEM A3 polypeptide and FUS1. Other exemplary tumor suppressor genes are described in a database of tumor suppressor genes at www.cise.ufl.edu/~yyl/HTML-TSGDB/Homepage.litml. This database is herein specifically incorporated by reference into this and all other sections of the present application. Nucleic acids encoding tumor suppressor genes, as discussed above, include tumor suppressor genes, or nucleic acids derived therefrom (e.g., cDNAs, cRNAs, mRNAs, and subsequences thereof encoding active fragments of the respective tumor suppressor amino acid sequences), as well as vectors comprising these sequences. One of ordinary skill in the art would be familiar with tumor suppressor genes that can be applied.

[0105] The methods described herein may include or exclude any of the cancer therapies described in the disclosure.

C. Monitoring

[0106] In certain aspects, the biomarker-based method may be combined with one or more other gastric cancer diagnosis or screening tests at increased frequency if the patient is determined to be at high risk for recurrence or have a poor prognosis based on the miRNA described above.

[0107] In some embodiments, the methods of the disclosure further include one or more monitoring tests. The monitoring protocol may include any methods known in the art. In particular, the monitoring include obtaining a sample and testing the sample for diagnosis. For example, the monitoring may include endoscopy, biopsy, endoscopic ultrasound, X-ray, barium swallow, a Ct scan, a MRI, a PET scan, laparoscopy, or HER2 testing. In some embodiments, the monitoring test comprises radiographic imaging. Examples of radiographic imaging this is useful in the methods of the disclosure includes hepatic ultrasound, computed tomographic (CT) abdominal scan, liver magnetic resonance imaging (MRI), body CT scan, and body MRI.

D. ROC analysis

[0108] In statistics, a receiver operating characteristic (ROC), or ROC curve, is a graphical plot that illustrates the performance of a binary classifier system as its discrimination threshold is varied. The curve is created by plotting the true positive rate against the false positive rate at various threshold settings. (The true-positive rate is also known as sensitivity in biomedical informatics, or recall in machine learning. The false-positive rate is also known as the fall-out and can be calculated as 1 - specificity). The ROC curve is thus the sensitivity as a function of fall-out. In general, if the probability distributions for both detection and false alarm are known, the ROC curve can be generated by plotting the cumulative distribution function (area under the probability distribution from $-\infty$ to $+\infty$) of the detection probability in the y-axis versus the cumulative distribution function of the false-alarm probability in x-axis.

[0109] ROC analysis provides tools to select possibly optimal models and to discard suboptimal ones independently from (and prior to specifying) the cost context or the class distribution. ROC analysis is related in a direct and natural way to cost/benefit analysis of diagnostic decision making.

[0110] The ROC curve was first developed by electrical engineers and radar engineers during World War II for detecting enemy objects in battlefields and was soon introduced to psychology to account for perceptual detection of stimuli. ROC analysis since then has been used in medicine, radiology, biometrics, and other areas for many decades and is increasingly used in machine learning and data mining research.

[0111] The ROC is also known as a relative operating characteristic curve, because it is a comparison of two operating characteristics (TPR and FPR) as the criterion changes. ROC

analysis curves are known in the art and described in Metz CE (1978) Basic principles of ROC analysis. *Seminars in Nuclear Medicine* 8:283-298; Youden WJ (1950) An index for rating diagnostic tests. *Cancer* 3:32-35; Zweig MH, Campbell G (1993) Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clinical Chemistry* 39:561-577; and Greiner M, Pfeiffer D, Smith RD (2000) Principles and practical application of the receiver-operating characteristic analysis for diagnostic tests. *Preventive Veterinary Medicine* 45:23-41, which are herein incorporated by reference in their entirety. A ROC analysis may be used to create cut-off values for prognosis and/or diagnosis purposes.

III. miRNAs

[0112] Methods of the disclosure relate to the detection of one or more of miR-30a-5p, miR-134-5p, miR-337-3p, miR-659-3p, and miR-3917 for treating gastric cancer. The known sequences of the micro RNAs are exemplified by the following:

[0113] hsa-miR-30a-5p: uguaaacauccucgacuggaag (SEQ ID NO:1); hsa-miR-134-5p: ugugacugguugaccagagggg (SEQ ID NO:2); hsa-miR-337-3p : cuccuauaugaugccuuuc (SEQ ID NO:3); hsa-miR-659-3p: cuugguucagggagggucccca (SEQ ID NO:4); and hsa-miR-3917: gcucggacugagcagguggg (SEQ ID NO:5).

IV. Sample Preparation

[0114] In certain aspects, methods involve obtaining a sample from a subject. The methods of obtaining provided herein may include methods of biopsy such as fine needle aspiration, core needle biopsy, vacuum assisted biopsy, incisional biopsy, excisional biopsy, punch biopsy, shave biopsy or skin biopsy. In certain embodiments the sample is obtained from a biopsy from intestinal or mucosal tissue by any of the biopsy methods previously mentioned. In other embodiments the sample may be obtained from any of the tissues provided herein that include but are not limited to non-cancerous or cancerous tissue and non-cancerous or cancerous tissue from the serum, gall bladder, mucosal, skin, heart, lung, breast, pancreas, blood, liver, muscle, kidney, smooth muscle, bladder, colon, intestine, brain, prostate, esophagus, or thyroid tissue. Alternatively, the sample may be obtained from any other source including but not limited to blood, sweat, hair follicle, buccal tissue, tears, menses, feces, or saliva. In certain aspects the sample is obtained from cystic fluid or fluid derived from a tumor or neoplasm. In yet other embodiments the cyst, tumor, or

neoplasm is gastric or in the digestive system. In certain aspects of the current methods, any medical professional such as a doctor, nurse or medical technician may obtain a biological sample for testing. Yet further, the biological sample can be obtained without the assistance of a medical professional.

5 **[0115]** A sample may include but is not limited to, tissue, cells, or biological material from cells or derived from cells of a subject. The biological sample may be a heterogeneous or homogeneous population of cells or tissues. The biological sample may be obtained using any method known to the art that can provide a sample suitable for the analytical methods described herein. The sample may be obtained by non-invasive methods including but not limited to:
10 scraping of the skin or cervix, swabbing of the cheek, saliva collection, urine collection, feces collection, collection of menses, tears, or semen.

[0116] The sample may be obtained by methods known in the art. In certain embodiments the samples are obtained by biopsy. In other embodiments the sample is obtained by swabbing, endoscopy, scraping, phlebotomy, or any other methods known in the art. In some cases, the
15 sample may be obtained, stored, or transported using components of a kit of the present methods. In some cases, multiple samples, such as multiple gastric samples may be obtained for diagnosis by the methods described herein. In other cases, multiple samples, such as one or more samples from one tissue type (for example gastric) and one or more samples from another specimen (for example serum) may be obtained for diagnosis by the methods. In some cases, multiple samples
20 such as one or more samples from one tissue type (e.g. gastric) and one or more samples from another specimen (e.g. serum) may be obtained at the same or different times. Samples may be obtained at different times are stored and/or analyzed by different methods. For example, a sample may be obtained and analyzed by routine staining methods or any other cytological analysis methods.

25 **[0117]** In some embodiments the biological sample may be obtained by a physician, nurse, or other medical professional such as a medical technician, endocrinologist, cytologist, phlebotomist, radiologist, or a pulmonologist. The medical professional may indicate the appropriate test or assay to perform on the sample. In certain aspects a molecular profiling business may consult on which assays or tests are most appropriately indicated. In further aspects of the current methods, the
30 patient or subject may obtain a biological sample for testing without the assistance of a medical

professional, such as obtaining a whole blood sample, a urine sample, a fecal sample, a buccal sample, or a saliva sample.

[0118] In other cases, the sample is obtained by an invasive procedure including but not limited to: biopsy, needle aspiration, endoscopy, or phlebotomy. The method of needle aspiration may further include fine needle aspiration, core needle biopsy, vacuum assisted biopsy, or large core biopsy. In some embodiments, multiple samples may be obtained by the methods herein to ensure a sufficient amount of biological material.

[0119] General methods for obtaining biological samples are also known in the art. Publications such as Ramzy, Ibrahim Clinical Cytopathology and Aspiration Biopsy 2001, which is herein incorporated by reference in its entirety, describes general methods for biopsy and cytological methods. In one embodiment, the sample is a fine needle aspirate of a esophageal or a suspected esophageal tumor or neoplasm. In some cases, the fine needle aspirate sampling procedure may be guided by the use of an ultrasound, X-ray, or other imaging device.

[0120] In some embodiments of the present methods, the molecular profiling business may obtain the biological sample from a subject directly, from a medical professional, from a third party, or from a kit provided by a molecular profiling business or a third party. In some cases, the biological sample may be obtained by the molecular profiling business after the subject, a medical professional, or a third party acquires and sends the biological sample to the molecular profiling business. In some cases, the molecular profiling business may provide suitable containers, and excipients for storage and transport of the biological sample to the molecular profiling business.

[0121] In some embodiments of the methods described herein, a medical professional need not be involved in the initial diagnosis or sample acquisition. An individual may alternatively obtain a sample through the use of an over the counter (OTC) kit. An OTC kit may contain a means for obtaining said sample as described herein, a means for storing said sample for inspection, and instructions for proper use of the kit. In some cases, molecular profiling services are included in the price for purchase of the kit. In other cases, the molecular profiling services are billed separately. A sample suitable for use by the molecular profiling business may be any material containing tissues, cells, nucleic acids, genes, gene fragments, expression products, gene expression products, or gene expression product fragments of an individual to be tested. Methods for determining sample suitability and/or adequacy are provided.

[0122] In some embodiments, the subject may be referred to a specialist such as an oncologist, surgeon, or endocrinologist. The specialist may likewise obtain a biological sample for testing or refer the individual to a testing center or laboratory for submission of the biological sample. In some cases the medical professional may refer the subject to a testing center or laboratory for submission of the biological sample. In other cases, the subject may provide the sample. In some cases, a molecular profiling business may obtain the sample.

V. Nucleic Acid Assays

[0123] Aspects of the methods include assaying nucleic acids to determine expression or activity levels. Arrays can be used to detect differences between two samples. Specifically contemplated applications include identifying and/or quantifying differences between RNA from a sample that is normal and from a sample that is not normal, between a cancerous condition and a non-cancerous condition, between one cancerous condition, such as fast doubling time cells and another cancer condition, such as slow doubling time cells, or between two differently treated samples. Also, RNA may be compared between a sample believed to be susceptible to a particular disease or condition and one believed to be not susceptible or resistant to that disease or condition. A sample that is not normal is one exhibiting phenotypic trait(s) of a disease or condition or one believed to be not normal with respect to that disease or condition. It may be compared to a cell that is normal with respect to that disease or condition. Phenotypic traits include symptoms of, or susceptibility to, a disease or condition of which a component is or may or may not be genetic or caused by a hyperproliferative or neoplastic cell or cells.

[0124] To determine expression levels of a biomarker, an array may be used. An array comprises a solid support with nucleic acid probes attached to the support. Arrays typically comprise a plurality of different nucleic acid probes that are coupled to a surface of a substrate in different, known locations. These arrays, also described as “microarrays” or colloquially “chips” have been generally described in the art, for example, U.S. Pat. Nos. 5,143,854, 5,445,934, 5,744,305, 5,677,195, 6,040,193, 5,424,186 and Fodor et al., 1991), each of which is incorporated by reference in its entirety for all purposes. Techniques for the synthesis of these arrays using mechanical synthesis methods are described in, e.g., U.S. Pat. No. 5,384,261, incorporated herein by reference in its entirety for all purposes. Although a planar array surface is used in certain aspects, the array may be fabricated on a surface of virtually any shape or even a multiplicity of

surfaces. Arrays may be nucleic acids on beads, gels, polymeric surfaces, fibers such as fiber optics, glass or any other appropriate substrate, see U.S. Pat. Nos. 5,770,358, 5,789, 162, 5,708,153, 6,040,193 and 5,800,992, which are hereby incorporated in their entirety for all purposes.

5 [0125] Further assays useful for determining biomarker expression include, but are not limited to, nucleic amplification, polymerase chain reaction, quantitative PCR, RT-PCR, in situ hybridization, Northern hybridization, hybridization protection assay (HP A)(GenProbe), branched DNA (bDNA) assay (Chiron), rolling circle amplification (RCA), single molecule hybridization detection (US Genomics), Invader assay (ThirdWave Technologies), and/or Bridge
10 Litigation Assay (Genaco).

[0126] A further assay useful for quantifying and/or identifying nucleic acids, such as nucleic acids comprising biomarker genes, is RNAseq. RNA-seq (RNA sequencing), also called whole transcriptome shotgun sequencing, uses next-generation sequencing (NGS) to reveal the presence and quantity of RNA in a biological sample at a given moment in time. RNA-Seq is used to analyze
15 the continually changing cellular transcriptome. Specifically, RNA-Seq facilitates the ability to look at alternative gene spliced transcripts, post-transcriptional modifications, gene fusion, mutations/SNPs and changes in gene expression. In addition to mRNA transcripts, RNASeq can look at different populations of RNA to include total RNA, small RNA, such as miRNA, tRNA, and ribosomal profiling. RNA-Seq can also be used to determine exon/intron boundaries and verify
20 or amend previously annotated 5' and 3' gene boundaries.

VI. Administration of Therapeutic Compositions

[0127] The therapy provided herein may comprise administration of a combination of therapeutic agents, such as a first cancer therapy and a second cancer therapy. The therapies may be administered in any suitable manner known in the art. For example, the first and second cancer
25 treatment may be administered sequentially (at different times) or concurrently (at the same time). In some embodiments, the first and second cancer treatments are administered in a separate composition. In some embodiments, the first and second cancer treatments are in the same composition.

[0128] Embodiments of the disclosure relate to compositions and methods comprising
30 therapeutic compositions. The different therapies may be administered in one composition or in

more than one composition, such as 2 compositions, 3 compositions, or 4 compositions. Various combinations of the agents may be employed.

[0129] The therapeutic agents of the disclosure may be administered by the same route of administration or by different routes of administration. In some embodiments, the cancer therapy is administered intravenously, intramuscularly, subcutaneously, topically, orally, transdermally, intraperitoneally, intraorbitally, by implantation, by inhalation, intrathecally, intraventricularly, or intranasally. In some embodiments, the antibiotic is administered intravenously, intramuscularly, subcutaneously, topically, orally, transdermally, intraperitoneally, intraorbitally, by implantation, by inhalation, intrathecally, intraventricularly, or intranasally. The appropriate dosage may be determined based on the type of disease to be treated, severity and course of the disease, the clinical condition of the individual, the individual's clinical history and response to the treatment, and the discretion of the attending physician.

[0130] The treatments may include various "unit doses." Unit dose is defined as containing a predetermined-quantity of the therapeutic composition. The quantity to be administered, and the particular route and formulation, is within the skill of determination of those in the clinical arts. A unit dose need not be administered as a single injection but may comprise continuous infusion over a set period of time. In some embodiments, a unit dose comprises a single administrable dose.

[0131] The quantity to be administered, both according to number of treatments and unit dose, depends on the treatment effect desired. An effective dose is understood to refer to an amount necessary to achieve a particular effect. In the practice in certain embodiments, it is contemplated that doses in the range from 10 mg/kg to 200 mg/kg can affect the protective capability of these agents. Thus, it is contemplated that doses include doses of about 0.1, 0.5, 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, and 200, 300, 400, 500, 1000 $\mu\text{g/kg}$, mg/kg , $\mu\text{g/day}$, or mg/day or any range derivable therein. Furthermore, such doses can be administered at multiple times during a day, and/or on multiple days, weeks, or months.

[0132] In certain embodiments, the effective dose of the pharmaceutical composition is one which can provide a blood level of about 1 μM to 150 μM . In another embodiment, the effective dose provides a blood level of about 4 μM to 100 μM ; or about 1 μM to 100 μM ; or about 1 μM to 50 μM ; or about 1 μM to 40 μM ; or about 1 μM to 30 μM ; or about 1 μM to 20 μM ; or about 1 μM to 10 μM ; or about 10 μM to 150 μM ; or about 10 μM to 100 μM ; or about 10 μM to 50 μM ;

or about 25 μM to 150 μM ; or about 25 μM to 100 μM ; or about 25 μM to 50 μM ; or about 50 μM to 150 μM ; or about 50 μM to 100 μM (or any range derivable therein). In other embodiments, the dose can provide the following blood level of the agent that results from a therapeutic agent being administered to a subject: about, at least about, or at most about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 μM or any range derivable therein. In certain embodiments, the therapeutic agent that is administered to a subject is metabolized in the body to a metabolized therapeutic agent, in which case the blood levels may refer to the amount of that agent. Alternatively, to the extent the therapeutic agent is not metabolized by a subject, the blood levels discussed herein may refer to the unmetabolized therapeutic agent.

[0133] Precise amounts of the therapeutic composition also depend on the judgment of the practitioner and are peculiar to each individual. Factors affecting dose include physical and clinical state of the patient, the route of administration, the intended goal of treatment (alleviation of symptoms versus cure) and the potency, stability and toxicity of the particular therapeutic substance or other therapies a subject may be undergoing.

[0134] It will be understood by those skilled in the art and made aware that dosage units of $\mu\text{g}/\text{kg}$ or mg/kg of body weight can be converted and expressed in comparable concentration units of $\mu\text{g}/\text{ml}$ or mM (blood levels), such as 4 μM to 100 μM . It is also understood that uptake is species and organ/tissue dependent. The applicable conversion factors and physiological assumptions to be made concerning uptake and concentration measurement are well-known and would permit those of skill in the art to convert one concentration measurement to another and make reasonable comparisons and conclusions regarding the doses, efficacies and results described herein.

VII. Pharmaceutical Compositions

[0135] In certain aspects, the compositions or agents for use in the methods, such as chemotherapeutic agents or biomarker modulators, are suitably contained in a pharmaceutically acceptable carrier. The carrier is non-toxic, biocompatible and is selected so as not to detrimentally affect the biological activity of the agent. The agents in some aspects of the disclosure may be

formulated into preparations for local delivery (i.e. to a specific location of the body, such as skeletal muscle or other tissue) or systemic delivery, in solid, semi-solid, gel, liquid or gaseous forms such as tablets, capsules, powders, granules, ointments, solutions, depositories, inhalants and injections allowing for oral, parenteral or surgical administration. Certain aspects of the disclosure also contemplate local administration of the compositions by coating medical devices and the like.

[0136] Suitable carriers for parenteral delivery via injectable, infusion or irrigation and topical delivery include distilled water, physiological phosphate-buffered saline, normal or lactated Ringer's solutions, dextrose solution, Hank's solution, or propanediol. In addition, sterile, fixed oils may be employed as a solvent or suspending medium. For this purpose any biocompatible oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables. The carrier and agent may be compounded as a liquid, suspension, polymerizable or non-polymerizable gel, paste or salve.

[0137] The carrier may also comprise a delivery vehicle to sustain (i.e., extend, delay or regulate) the delivery of the agent(s) or to enhance the delivery, uptake, stability or pharmacokinetics of the therapeutic agent(s). Such a delivery vehicle may include, by way of non-limiting examples, microparticles, microspheres, nanospheres or nanoparticles composed of proteins, liposomes, carbohydrates, synthetic organic compounds, inorganic compounds, polymeric or copolymeric hydrogels and polymeric micelles.

[0138] In certain aspects, the actual dosage amount of a composition administered to a patient or subject can be determined by physical and physiological factors such as body weight, severity of condition, the type of disease being treated, previous or concurrent therapeutic interventions, idiopathy of the patient and on the route of administration. The practitioner responsible for administration will, in any event, determine the concentration of active ingredient(s) in a composition and appropriate dose(s) for the individual subject.

[0139] In certain embodiments, pharmaceutical compositions may comprise, for example, at least about 0.1 % of an active agent, such as an isolated exosome, a related lipid nanovesicle, or an exosome or nanovesicle loaded with therapeutic agents or diagnostic agents. In other embodiments, the active agent may comprise between about 2% to about 75% of the weight of the unit, or between about 25% to about 60%, for example, and any range derivable therein. In other non-limiting examples, a dose may also comprise from about 1 microgram/kg/body weight, about

5 microgram/kg/body weight, about 10 microgram/kg/body weight, about 50 microgram/kg/body weight, about 100 microgram/kg/body weight, about 200 microgram/kg/body weight, about 350 microgram/kg/body weight, about 500 microgram/kg/body weight, about 1 milligram/kg/body weight, about 5 milligram/kg/body weight, about 10 milligram/kg/body weight, about 50 milligram/kg/body weight, about 100 milligram/kg/body weight, about 200 milligram/kg/body weight, about 350 milligram/kg/body weight, about 500 milligram/kg/body weight, to about 1000 mg/kg/body weight or more per administration, and any range derivable therein. In non-limiting examples of a derivable range from the numbers listed herein, a range of about 5 microgram/kg/body weight to about 100 mg/kg/body weight, about 5 microgram/kg/body weight to about 500 milligram/kg/body weight, etc., can be administered.

[0140] Solutions of pharmaceutical compositions can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

[0141] In certain aspects, the pharmaceutical compositions are advantageously administered in the form of injectable compositions either as liquid solutions or suspensions; solid forms suitable or solution in, or suspension in, liquid prior to injection may also be prepared. These preparations also may be emulsified. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. For instance, the composition may contain 10 mg or less, 25 mg, 50 mg or up to about 100 mg of human serum albumin per milliliter of phosphate buffered saline. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like.

[0142] Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethylolate. Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, etc. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial agents, antifungal agents, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components the pharmaceutical composition are adjusted according to well-known parameters.

[0143] Additional formulations are suitable for oral administration. Oral formulations include such typical excipients as, for example, pharmaceutical grades of mannitol, lactose, starch,

magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. The compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders.

[0144] In further aspects, the pharmaceutical compositions may include classic pharmaceutical preparations. Administration of pharmaceutical compositions according to certain aspects may be via any common route so long as the target tissue is available via that route. This may include oral, nasal, buccal, rectal, vaginal or topical. Topical administration may be particularly advantageous for the treatment of skin cancers, to prevent chemotherapy-induced alopecia or other dermal hyperproliferative disorder. Alternatively, administration may be by orthotopic, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions that include physiologically acceptable carriers, buffers or other excipients. For treatment of conditions of the lungs, aerosol delivery can be used. Volume of the aerosol is between about 0.01 ml and 0.5 ml.

[0145] An effective amount of the pharmaceutical composition is determined based on the intended goal. The term “unit dose” or “dosage” refers to physically discrete units suitable for use in a subject, each unit containing a predetermined-quantity of the pharmaceutical composition calculated to produce the desired responses discussed above in association with its administration, i.e., the appropriate route and treatment regimen. The quantity to be administered, both according to number of treatments and unit dose, depends on the protection or effect desired.

[0146] Precise amounts of the pharmaceutical composition also depend on the judgment of the practitioner and are peculiar to each individual. Factors affecting the dose include the physical and clinical state of the patient, the route of administration, the intended goal of treatment (e.g., alleviation of symptoms versus cure) and the potency, stability and toxicity of the particular therapeutic substance.

VIII. Kits

[0147] Certain aspects of the present invention also concern kits containing compositions of the invention or compositions to implement methods of the invention. In some embodiments, kits can be used to evaluate one or more biomarkers. In certain embodiments, a kit contains, contains at least or contains at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48,

49, 50, 100, 500, 1,000 or more probes, primers or primer sets, synthetic molecules or inhibitors, or any value or range and combination derivable therein. In some embodiments, there are kits for evaluating biomarker activity in a cell.

[0148] Kits may comprise components, which may be individually packaged or placed in a container, such as a tube, bottle, vial, syringe, or other suitable container means.

[0149] Individual components may also be provided in a kit in concentrated amounts; in some embodiments, a component is provided individually in the same concentration as it would be in a solution with other components. Concentrations of components may be provided as 1x, 2x, 5x, 10x, or 20x or more.

[0150] Kits for using probes, primers, synthetic nucleic acids, nonsynthetic nucleic acids, biomarker binding polypeptides, antibodies, and/or inhibitors of the disclosure for prognostic or diagnostic applications are included as part of the disclosure. Specifically contemplated are any such molecules corresponding to any biomarker identified herein, which includes nucleic acid primers/primer sets and probes that are identical to or complementary to all or part of a biomarker, which may include noncoding sequences of the biomarker, as well as coding sequences of the biomarker, and any such molecules that hybridize to a biomarker nucleic acid.

[0151] In certain aspects, negative and/or positive control nucleic acids, probes, and inhibitors are included in some kit embodiments. The control molecules can be used to verify efficiency and/or control for sample quality or to normalize expression. In addition, a kit may include a sample that is a negative or positive control for methylation of one or more biomarkers. In some embodiments, a control includes a nucleic acid that contains at least one CpG or is capable of identifying a CpG methylation site.

[0152] It is contemplated that any method or composition described herein can be implemented with respect to any other method or composition described herein and that different embodiments may be combined. The claims originally filed are contemplated to cover claims that are multiply dependent on any filed claim or combination of filed claims.

[0153] Any embodiment of the disclosure involving a specific biomarker is contemplated also to cover embodiments involving biomarkers whose sequences are at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% identical to the mature sequence of the specified nucleic acid.

[0154] Embodiments of the disclosure include kits for analysis of a pathological sample by assessing biomarker profile for a sample comprising, in suitable container means, two or more biomarker probes, wherein the biomarker probes detect one or more of the biomarkers identified herein. The kit can further comprise reagents for labeling nucleic acids in the sample and/or probes and detecting agents. The kit may also include labeling reagents, including at least one of amine-modified nucleotide, poly(A) polymerase, and poly(A) polymerase buffer. Labeling reagents can include an amine-reactive dye.

IX. Examples

[0155] 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 inventor 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.

Example 1 – Genomewide expression profiling identifies a novel miRNA-based signature for the detection of peritoneal metastasis in patients with gastric cancer

[0156] This study aimed to do a genomewide transcriptomic profiling to develop a miRNA-based signature for the identification of peritoneal metastasis (PM) in patients with gastric cancer (GC). Even though PM in patients with GC has long been recognized to associate with poor survival, currently there is lack of availability of molecular biomarkers for its robust diagnosis. Systematic biomarker discovery was performed by analyzing miRNA expression profiles in primary tumors from GC patients with and without PM, and subsequently validated the expression of candidate miRNA biomarkers in three independent clinical cohorts of 354 patients with advanced GC. Five miRNAs (miR-30a-5p, -134-5p, -337-3p, -659-3p, and -3917) were identified during the initial discovery phase; three of which (miR-30a-5p, -659-3p, and -3917) were significantly overexpressed in the primary tumors from PM-positive patients in the testing cohort ($p=0.002$, 0.04 and 0.007 respectively), and robustly distinguished patients with versus without peritoneal metastasis ($AUC=0.82$). Furthermore, high expression of these miRNAs also associated with poor prognosis ($HR=2.18$, $p=0.04$). The efficacy of the combination miRNA-signature was

subsequently validated in an independent validation cohort (AUC=0.74). Finally, the miRNA signature when combined together with the macroscopic Borrmann's type score offered a superior diagnostic in all three cohorts (AUC=0.87, 0.76, 0.79, respectively), and led to establishment of a risk-prediction nomogram for the diagnosis of PM in GC patients. Thus, the novel miRNA-based signature is a robust diagnostic tool for identifying peritoneal metastasis in GC patients, which could lead to improved survival outcomes in gastric cancer.

A. INTRODUCTION

[0157] Gastric cancer (GC) is the fourth most common cause of cancer-related deaths worldwide [1]. In particular, GC patients with peritoneal metastasis (PM) have an exceptionally poor survival outcomes, which is further compounded by the lack of availability of effective treatments [2-5]. Currently, computed tomography (CT) or positron emission tomography (PET) are commonly used to diagnose presence of PM in GC patients, but the diagnostic sensitivity of these imaging modalities for identifying such metastatic lesions is quite inadequate [6]. Furthermore, even though staging laparoscopy can detect PM at much higher rates compared to CT or PET-CT, this radical procedure is invasive, requires general anesthesia for patients, and hence increases the risk of diagnostic complications [7].

[0158] Considering that PM positive GC patients have a more aggressive disease, and it is well-recognized that its early detection can significantly improve patient outcomes; currently there are no molecular biomarkers used clinically to detect such metastases. Tumor markers, including CA125 and CA72-4 are frequently upregulated in patients with advanced GCs, and are also shown to be overexpressed in sera of patients with PM [8-10]. However, the challenge remains that individually or even as a combination, the diagnostic accuracy of these biomarkers for detecting PM remains very poor [11]. Furthermore, if PM can be identified prior to the surgery, it can allow potentially more effective treatment approaches including hyperthermic intraperitoneal chemotherapy (HIPEC) with cytoreductive surgery. Although this procedure is only performed by select institutions, HIPEC has been shown to be a very effective treatment for GC patients with PM [12-15]. Therefore, availability of robust molecular biomarkers which could help facilitate identification of definitive PM in GC patients, could be clinically transformative as it will permit a timely intervention leading to reduction in the morbidity and mortality associated with this malignancy.

[0159] Herein, the inventors conducted a comprehensive miRNA expression profiling of primary GC tissues, with and without presence of PM. Subsequently, rigorous bioinformatic approaches were utilized to identify and prioritize key miRNAs as potential biomarkers for detecting PM, followed by their validation in multiple, independent cohorts of GC patients with advanced disease. The inventors' efforts led to the identification and establishment of a novel miRNA-based signature that could be used to diagnose PM in GC patients.

B. MATERIALS AND METHODS

1. Study Design and patient specimens

[0160] The current study consisted of a systematic and comprehensive biomarker discovery and a validation phases. In the discovery phase, the inventors generated microarray-based miRNA expression profiling results, followed by additional comparison in The Cancer Genome Atlas (TCGA) dataset, for the identification of candidate miRNAs that can detect PM in GC patients. Initially the inventors identified differentially expressed miRNAs in the primary tumors from PM positive vs. negative patients, with a p value of <0.05 as an initial cut-off criteria in the miRNA-profiling data. Next, the inventors analyzed TCGA dataset to identify miRNAs that were differentially expressed in the stage IV vs. other advanced GC patients. Subsequently, the inventors selected miRNAs that were commonly dysregulated in both datasets of GC patients.

[0161] To evaluate the diagnostic accuracy of the miRNA biomarkers identified in the discovery phase, the inventors examined their expression by TaqMan-based qRT-PCR assays in three independent cohorts of GC patients. The three clinical validation cohorts enrolled 354 gastric cancer patients, comprising of a testing cohort of 65 patients enrolled at the Mie University, a validation cohort of 85 patients from the Kumamoto University, and a performance evaluation cohort of 204 patients from the Nagoya University, Japan. The testing and validation cohorts included paraffin embedded tissues, while the performance evaluation cohort comprised of frozen tissues. Further information on patient demographics and clinicopathological characteristics are provided in the Table 1. A written informed consent was obtained from all patients, and the Institutional Review Boards of all participating institutions approved the study.

2. MiRNA microarray expression profiling

[0162] Custom miRNA microarray expression profiling was performed using the Agilent SurePrint G3 Human miRNA microarray 8X60K v3 (Agilent Technologies, Santa Clara, CA), in primary tumor tissue specimens from GC patients with PM (n = 6) and without PM (n = 6). Total RNA was extracted according to the manufacturer's protocols. Quantitative evaluation of total RNA was performed by Nanodrop. Extracted RNA was labeled using the miRNA Complete Labeling Reagent and Hyb Kit, followed by hybridization of the labeled RNAs onto the Human miRNA Microarray 8x60K Rel.21.0. Arrays were scanned and images analyzed by the Feature Extraction Software (v.10.7.3.1) from Agilent Technologies.

[0163] The raw data of each spot was normalized by substitution with a mean intensity of the background signal determined by signal intensities of all blank spots with 95% confidence intervals. Measurements of spots with the signal intensities greater than 2 standard deviations (SD) of the background signal intensity were considered to be valid. The relative expression level of a given miRNA was calculated by comparing the signal intensities of the valid spots throughout the microarray experiments. In order to normalize the microarray data, "LIMMA" package (R studio, Boston, MA) was used for background corrections, followed by quantile normalization of the array results.

3. RNA isolation and quantitative real time PCR

[0164] Total RNA was extracted from FFPE and fresh frozen tissue samples stored in RNAlater using the miRNeasy FFPE Kit (Qiagen, Valencia, CA) and miRNeasy Mini Kit (Qiagen), respectively. The expression of miRNAs was analyzed by TaqMan miRNA real-time qRT-PCR assays (Applied Biosystems, Foster City, CA) using the QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems). Following primers were used for qRT-PCR: miR-30a-5p, miR-134-5p, miR-337-3p, miR-659-3p (assay no: 000417, 001186, 002157, 001514 Catalog no: 4427975, Thermo Fisher Scientific) and miR-3917 (Assay no: 464692_mat, Cat no: 440886, Thermo Fisher Scientific), and U6 (Assay no: 001973, Cat no: 4427975 Thermo Fisher Scientific). U6 was used as an endogenous control for data normalization. The expression levels of miRNAs were calculated using the $2^{-\Delta CT}$ method.

4. Statistical analysis

[0165] Comparison of miRNA expression between two independent groups were analyzed using the Two tailed Mann-Whitney U test (D'Agostino-Pearson omnibus normality test was used to determine Gaussian distribution of miRNA expression). A receiver operating characteristic (ROC) curve was generated and the area under the ROC (AUROC) curve was established for discriminating patients with and without PM. Youden's index for PM was used in each clinical cohort to determine the optimal cutoff thresholds for individual miRNAs, as well as the combined miRNA signature risk score. To analyze correlations between miRNAs biomarkers and various clinicopathological features, as well as in multivariate analysis, Youden's index was used to dichotomize patients into high and low expression groups. The differences between groups were analyzed by the Fisher's exact test. The ROC curve for PM using the combined miRNA signature and various clinical risk factors which remained in multivariate analysis were also generated by logistic regression analysis. Overall survival (OS) was defined as the period from the date of GC diagnosis to the date of last follow up, and OS analysis was performed by Log-rank test by dichotomizing patients using the Youden's index of individual miRNAs and combined miRNA signature for PM. A p-value of <0.05 was considered statistically significant. All statistical analyses were performed using the Medcalc statistical software V.16.2.0 (Medcalc Software bvba, Ostend, Belgium), JMP software 10.0.2 (SAS Institute, Cary, NC), and GraphPad Prism V7.0 (GraphPad Software, San Diego, CA), and R (version 3.5.0, Vienna, Austria). Cost-Benefit curves were created using rmda packages, nomograms and predicted probability plot were created using rms packages in R (version 3.5.0, Vienna, Austria).

C. RESULTS

1. Identification of candidate miRNAs overexpressed in gastric cancer patients with peritoneal metastasis

[0166] In order to identify miRNAs specifically dysregulated in GC patients with PM, the inventors performed miRNA expression profiling in six patients each, with and without PM. The inventors used a p-value of <0.05 as the initial criteria to identify differentially expressed miRNAs (Figure 1A). Subsequently, the inventors identified 513 candidate miRNAs, of which 364 were upregulated in the primary tumors of GC patients with PM (Figure 1B). To further narrow down the list of miRNA candidates for developing a clinically relevant diagnostic signature, the

inventors next analyzed the TCGA dataset to identify miRNAs that were specifically differentially expressed in stage IV vs. other stages of GC patients. The rationale was to ensure that miRNAs identified in the microarray dataset were associated with metastatic GCs, and not dysregulated in other GC stages. A comparison of stage IV vs. stage IB-III tumors identified 104 differentially expressed miRNAs, of which 46 were upregulated and 58 were downregulated. The inventors thereafter overlapped miRNAs that were dysregulated both in PM and stage IV GC patients, and identified eight miRNAs, of which five were upregulated (miR-30a-5p, miR-134-5p, miR-337-3p, miR-659-3p, and miR-3917) and three were downregulated (miR-718, miR-1281, and miR-3162). Since the read count of the three downregulated miRNAs were extremely low, the inventors excluded these and were able to successfully validate the remaining five miRNAs in the TCGA dataset (Figure 1C).

2. The candidate miRNA biomarkers were overexpressed gastric cancer patients with peritoneal metastasis

[0167] To evaluate the clinical significance of the five miRNA candidates that the inventors identified during the discovery phase, the inventors examined their expression in tissue specimens from a clinical testing cohort of advanced GC patients (n=65; Table 1). Correlation between clinicopathological factors and expression of individual miRNAs (dichotomized into high and low groups) are shown in Supplementary Table 1. In brief, four of the miRNAs were significantly overexpressed in patients with Stage IV disease (Supplementary Table 1). Next, to directly address the clinical relevance of these candidate miRNAs for the detection of PM in GC patients, the inventors interrogated their expression in patients with and without such metastases. Intriguingly, three of the five miRNAs, miR-30a-5p, miR-659-3p and miR-3917, were significantly overexpressed in the primary tumors of PM positive vs. negative patients ($p=0.002$, 0.04 and 0.007 , respectively; Figure 2A). Furthermore, these miRNAs were able to robustly distinguish patients with vs. those without PM (AUC values for miR-30a-5p, miR-659-3p, and miR-3917 were 0.77 , 0.68 , and 0.74 , respectively; Figure 3A). A sub-group analysis of stage IV patients revealed that the expression of these miRNAs in patients with PM tended to be higher compared to patients without such metastases (Figure 2B). Next, the inventors evaluated whether a combination signature of these three miRNA candidates might further improve the overall robustness; which was indeed the case as the risk scores and the AUC value of this signature significantly improved to 0.82 (sensitivity= 85.7% , specificity= 68.6% ; Figure 3B and C) vs. individual markers in

distinguishing PM positive GC patients. Collectively these data indicate that the miRNAs discovery phase from the expression profiling of PM patients was a success, and that these biomarkers were dysregulated and were able to diagnose GC patients harboring peritoneal metastases.

5 [0168] Next, the inventors evaluated the prognostic significance of these miRNAs by examining the overall survival (OS) using Kaplan-Meier analysis. While the patients with high expression of miR-659-3p did not demonstrate significant differences in survival, the ones with overexpression of miR-30a-5p and miR-3917 exhibited significantly inferior OS ($p=0.001$, and $p=0.0009$ respectively; Figure 3D). Patients with high risk score derived from the combination of
10 three miRNA signature also revealed worse OS vis-à-vis those with low risk scores ($p=0.04$; Figure 2C).

3. Successful validation of miRNA biomarkers for a robust identification of gastric cancer patients with peritoneal metastasis

[0169] Next, to further validate the efficacy of the three miRNAs identified in the testing
15 cohort, the inventors assessed their expression in an independent validation cohort ($n=85$; Table 1). The correlation between various clinicopathological factors and relative expression of the three miRNAs are shown in Supplementary Table 2. Consistent with the findings from the testing cohort, all three biomarkers demonstrated a significant upregulation in patients with stage IV disease (Supplementary Table 2). Furthermore, the expression of all three miRNAs, miR-30a-5p,
20 miR-659-3p and miR-3917, was significantly upregulated in patients with PM vs. those without ($p=0.02$, 0.02 , and 0.03 respectively; Figure 4A). Furthermore, these three miRNAs robustly discriminated patients with vs. without PM as evidenced from the ROC curves and corresponding high AUC values (AUC for miR-30a-5p= 0.69 ; miR-659-3p= 0.73 ; and miR-3917= 0.68 ; Figure 4B). Next, the inventors evaluated the efficacy of this combination signature in identifying patients
25 with PM. The risk scores derived from the combination signature in patients with PM were significantly higher than those without PM (Figure 5A), and this signature was significantly superior vs. individual markers in discriminating GC patients with from those without PM (AUC= 0.74 , sensitivity= 92.9% , specificity= 52.1% ; Figure 5B). Likewise, the inventors successfully validated the findings from the testing cohort that patients with high expression of
30 these three individual miRNAs, as well as the combination signature, resulted in significantly

worse OS (miR-30a-5p, miR-659-3p, miR-3917, and combination signature: $p=0.02$, 0.0001, 0.006 and 0.02 respectively; Figure 5C and Figure 4D).

4. Performance evaluation of miRNA biomarkers for the identification of peritoneal metastasis in gastric cancer patients

5 [0170] While the inventors were able to successfully identify and validate the biomarker potential of the miRNA biomarkers for the diagnosis of PM in GC patients, the inventors believe an ideal scenario for their clinical translation would be evaluation in the pre-surgical biopsy specimens obtained during diagnostic upper gastro-endoscopy. Considering that such tiny biopsy specimens are generally preserved in a fresh frozen state, the inventors next examined the performance of the miRNA biomarkers in fresh frozen specimens for the identification of PM in GC patients. The correlation between clinicopathological factors and relative expression of the three miRNAs is summarized in Supplementary Table 3. Interestingly, the inventors observed that miR-30a-5p and miR-659-3p was significantly upregulated in patients with PM vs. those without ($p=0.0007$ and 0.004 respectively; Figure 6A). Furthermore, these miRNAs were also able to robustly distinguish GC patients with PM (AUC values for miR-30a-5p, miR-659-3p, and miR-3917 were 0.66, 0.64, and 0.58, respectively; Figure 6B). As was the case with the testing and validation cohorts, in the sub-group analysis of stage IV patients, the expression of miR-30a-5p and miR-659-3p in patients with PM was significantly higher as well (Figure 6C).

[0171] The inventors next evaluated the performance of these miRNA biomarkers as a combination signature, and consistent with the results from the previous two cohorts, the risk scores derived from the combination signature in patients with PM were significantly higher than without PM ($p<0.001$; Figure 5D), and this signature robustly discriminated GC with PM (AUC=0.67, sensitivity=59.6%, specificity=70.7%; Figure 5E); highlighting its translational potential in the clinic. In addition, the inventors noted that the patients with high expression of miR-30a-5p showed poorer OS ($p = 0.007$, Figure 6D), and patients with high risk scores derived from the combination signature showed significantly worse OS ($p=0.04$; Figure 5F).

5. Tumor macroscopic type together with the miRNA signature further improved the diagnostic accuracy for identifying peritoneal metastasis in gastric cancer patients

[0172] Next, using a logistic regression model, the inventors assessed the performance of various clinicopathological factors associated with PM, in conjunction with the miRNA signature, to determine whether the miRNA signature might serve as an independent factor for diagnosing PM in GC patients. The inventors also aimed to identify if any of the clinical factor(s) could be used in conjunction with the miRNA signature in further improving its diagnostic accuracy. The inventors focused on important clinical factors that can be identified prior to surgery for a more optimal translation of this model in clinical settings. In the initial univariate analysis, tumor macroscopic Borrmann's type III or IV ($p=0.003$), and the miRNA combination signature ($p=0.0002$) were associated with detection of PM in the testing cohort (Table 2). Subsequent multivariate analysis revealed that both tumor macroscopic Borrmann's type III or IV ($p=0.01$), identification in GC patients. Similarly, tumor macroscopic Borrmann's type III or IV, larger tumor size and high expression of the combination miRNA signature were significant in the validation cohort, both in univariate and multivariate analysis (macroscopic Borrmann's type III or IV, $p=0.03$; larger tumor size, $p=0.007$; and high expression of combined miRNA signature, $p=0.0007$; Table 2). More importantly, even in the final performance evaluation cohort, the univariate analysis revealed that tumor macroscopic type III or IV ($p<0.0001$), larger tumor size ($p=0.0006$), diffused histology ($p=0.02$) and the miRNA combination signature ($p=0.0002$) were associated with detection of PM (Table 2), and majority of these features remained significant as independent factors for PM detection in GC patients in subsequent multivariate analysis (Table 2). Collectively, the risk-diagnosis model indicated that along with the miRNA signature, tumor macroscopic type appears to be an important factor for identifying GC patients with PM.

6. Establishment of a prediction probability nomogram for the identification of high-risk gastric cancer patients with peritoneal metastasis

[0173] Next, the inventors assessed whether combining tumor macroscopic type with the miRNA signature might further improve the overall diagnostic potential for PM in GC patients. The ROC curves revealed that indeed combination of miRNA signature and macroscopic type significantly improved the diagnostic performance in all three clinical cohorts (Testing cohort; AUC=0.87, sensitivity=78.6%, specificity=84.3%, Validation cohort, Figure 7A; AUC=0.76, sensitivity=71.4%, specificity=70.4%, Figure 8A; Performance evaluation cohort; AUC=0.79, sensitivity=68.1%, specificity=79%; Figure 8B).

[0174] Using the statistically significant features, the inventors established a nomogram for predicting the diagnostic probability for the presence of PM in GC patients. It was very exciting to witness that the nomogram was extremely robust, where the miRNA signature and the macroscopic type were significant predictors for the presence of PM in GC patients in the testing (Figure 7B), validation (Figure 8C) and performance evaluation (Figure 8D) cohorts. Subsequent logistic calibration analyses revealed that expected vs. observed predictability of the nomograms in all three cohorts was significantly congruent, as evidenced from these plots in Figure 7C and Figures 8E and 8F. Finally, the inventors undertook cost:benefit analysis for the use of the biomarkers in diagnosing PM in clinical settings, and analysis revealed that the diagnostic model was significantly cost-effective as a diagnostic approach for identifying high-risk GC patients with peritoneal metastasis in all three cohorts (testing cohort, Figure 7D; validation cohort, Figure 8G; Performance evaluation cohort, Figure 8H). Collectively, these data suggest that the miRNA signature in combination with the tumor macroscopic type allows robust diagnosis of GC patients with peritoneal metastasis.

D. DISCUSSION

[0175] Currently, there is lack of availability of accurate biomarkers for the detection of peritoneal metastasis (PM) in gastric cancer (GC) patients. While computed tomography allows detection of large(r) foci with such metastasis, it lacks adequate diagnostic significance for microscopically smaller metastatic lesions. Therefore, in the current study the inventors undertook a systematic and comprehensive effort to develop miRNA-based molecular biomarkers for the identification of PM in GC patients. The inventors first performed computational analysis in two, genomewide expression profiling datasets to discover miRNAs dysregulated in GC patients with PM. The inventors thereafter assessed the efficacy of a five miRNA panel in the two independent clinical cohorts, which led to the identification of three miRNAs which were consistently overexpressed in both cohorts (testing and validation), and the combination miRNA signature was significantly more robust in discriminating GC patients with vs. without PM. In order to translate the findings into the clinic, the inventors evaluated the robustness of the miRNA biomarkers in a performance evaluation cohort of fresh frozen specimens that would mimic biopsies in pre-surgical endoscopy settings, and the miRNA signature performed equally robustly even in this cohort. Furthermore, high expression of all three miRNAs individually and their combination was

associated with inferior overall survival. Finally, using linear regression analysis the inventors established a diagnostic probability nomogram by combining risk scores from the miRNA signature together with the macroscopic Borrmann's type, which were significantly superior and offered a positive cost:benefit for their clinical application in diagnosing peritoneal metastasis in gastric cancer patients.

[0176] MiRNAs are short, single-stranded, noncoding RNAs which are frequently dysregulated in cancers. Previous studies have demonstrated that several miRNAs appear to have functional associations with PM in GC patients [23-25]. Using a peritoneal metastatic GC derived cell line, miR-136 was identified as a tumor-suppressive miRNA which attenuated metastatic potential of GC cells [23]. Similarly, a series of *in vitro* and *in vivo* experiments demonstrated miR-3978 as a potential tumor suppressor-miRNA, which inhibited legumain, a lysosomal cysteine endopeptidase [24, 25]. Collectively, these studies supported functional relevance of miRNAs in GC and suggested their potential clinical significance for the diagnosis of patients with PM.

[0177] Among the initially identified panel of miRNAs overexpressed in primary tumors of PM-positive patients, miR-30a, miR-659 and miR-3917 were consistently overexpressed and successfully validated in three independent patient cohorts. In pancreatic cancer, high expression of miR-30a was shown to promote migratory and invasive potential through upregulation of epithelial-to-mesenchymal-transition related genes [27]. Furthermore, overexpression of miR-30, including miR-30a, resulted in suppression of SOCS3, a key regulator of Jak/STAT3 pathway, and subsequently enhanced glioma stem cell growth [28]. Similarly, miR-30 is overexpressed in both GC tissues and its overexpression enhanced cellular proliferation and suppressed apoptosis through inhibition of p53 [29]. Collectively, these data indicate that miR-30a acts as an oncogene. In contrast, functional role of miR-659 and miR-3917 is unclear and remains to be elucidated.

[0178] The current study demonstrates that a combination of the miRNA signature together with the tumor macroscopic type (Borrmann's type III and IV) was significantly superior in identifying GC patients with PM. The Borrmann's classification was developed in 1926, and is widely used to classify GCs based on endoscopic characteristics. Several studies have shown that Borrmann's type correlates with other clinicopathological factors including depth of invasion, tumor stage, lymph node metastasis, distant metastasis and PM [30-32]. Considering that GCs with macroscopic Borrmann's type III and IV are consistently associated with PM, it was not surprising

that macroscopic type was identified as one of the key factors for PM diagnosis. Since, macroscopic stages for GC can be determined during endoscopy prior to the surgery, the risk probability nomogram, which combines macroscopic type with the miRNA signature offers a potentially attractive choice for the early diagnosis of PM in gastric cancer patients.

[0179] Currently GC patients with PM are typically treated by chemotherapy or palliative surgery. However, there is no standardized treatment regimen for such patients, which can easily vary between institutions [7]. Nevertheless, recent studies have proposed various treatment strategies for these patients with PM. For example, intraperitoneal administration of paclitaxel significantly improved the outcome of GC patients with PM [33]. Although paclitaxel is a commonly used drug for ovarian, breast and lung cancers [34-36], it was recently recognized that its intraperitoneal administration was also effective for the treatment of PM in GC patients [37-40]. Furthermore, recently the use of cytoreductive surgery combined with hyperthermic intraperitoneal chemotherapy (HIPEC) has been shown to be effective for GC patients with PM [13-15, 41], which could improve the overall outcome of these patients.

[0180] While the inventors have shown that a miRNA signature could be utilized to robustly identify presence of PM in GC patients, these findings must be tested in a larger cohorts to evaluate their true clinical potential. Moreover, due to limited sample size, in the present study the inventors were unable to evaluate whether this miRNA signature could identify metachronous PM.

[0181] In conclusion, the inventors have developed a novel miRNA-based signature for the detection of PM in GC patients, and validated its robustness in multiple independent clinical cohorts. The inventors have established a risk-diagnosis nomogram that offers a potentially attractive approach for the diagnosis of PM patients in gastric cancer patients, providing a more personalized approach for improving the overall survival and mortality in this patient population.

E. TABLES

Table 1: Clinicopathological characteristics of patients in each of the clinical cohorts

		Testing cohort	Validation cohort	Performance evaluation cohort
Variable		number	number	number
Gender	Male	40	62	158
	Female	25	23	46
Age at operation	median (range)	69 (18-90)	70 (25-92)	69 (20-88)

Macroscopical type	type1, 2	31	43	100
	type3,4	34	42	104
Tumor size (mm)	median (range)	55 (12-170)	55 (10-120)	55 (15-180)
Histological type	intestinal	27	48	76
	Diffuse	38	37	128
T factor	T2	12	20	40
	T3	14	35	59
	T4	39	30	105
Lymph node metastasis	Negative	21	29	59
	Positive	44	56	145
UICC Stage	1B	8	16	23
	2	15	26	47
	3	18	17	69
	4	24	26	65
Lymphatic invasion	Negative	5	36	20
	Positive	60	49	184
Venous invasion	Negative	26	14	67
	Positive	39	71	137

Table 2: Multivariate logistic regression analysis for the diagnosis of peritoneal metastasis in gastric cancer patients in each cohort

Variable	Univariate analysis			Multivariate analysis		
	OR	95%CI	P-value	OR	95%CI	P-value
Testing cohort						
Age ≥ 69	1.87	0.57-6.82	0.31			
Male	1.16	0.35-4.24	0.81			
Macroscopical type 3 or 4	7.91	1.91-54.27	0.003	6.83	1.45-50.61	0.01
Tumor size >55mm	2.19	0.66-7.99	0.2			
Poorly differentiated histology	2.05	0.60-8.28	0.26			
MiRNA combined signature high risk	13.12	3.12-90.96	0.0002	11.74	2.61-84.97	0.0008
Validation cohort						
Age ≥ 70	0.54	0.15-1.72	0.3			
Male	1.44	0.40-6.84	0.6			
Macroscopical type 3 or 4	4.73	1.34-22.26	0.01	4.97	1.20-26.99	0.03
Tumor size >55mm	4.73	1.34-22.26	0.01	6.63	1.62-35.88	0.007
Poorly differentiated histology	2.76	0.86-9.80	0.09			
MiRNA combined signature high risk	14.15	2.60-263.78	0.0007	17.52	2.89-343.90	0.0007
Performance evaluation cohort						
Age ≥ 69	1.48	0.77-2.88	0.24			
Male	0.81	0.38-1.77	0.58			
Macroscopical type 3 or 4	5.82	2.74-13.59	<0.0001	6.44	2.76-16.51	<0.0001
Tumor size >55mm	3.25	1.64-6.72	0.0006	2.24	1.03-5.01	0.04
Poorly differentiated histology	2.31	1.13-5.07	0.02	1.73	0.76-4.17	0.19
MiRNA combined signature high risk	3.56	1.82-7.09	0.0002	5.22	2.42-11.80	<0.0001
OR, Odds Ratio; CI, Confidence Interval. Bold indicates a statistically significant.						

Supplementary Table 1. Correlation between clinicopathological factors and expression of individual miRNAs in the testing cohort

Variable	number	miR-30a relative expression			p value	miR-134 relative expression			p value	miR-337 relative expression			p value	miR-659 relative expression			p value	miR-3917 relative expression			p value
		High (n=22)	Low (n=43)			High (n=39)	Low (n=26)			High (n=41)	Low (n=24)			High (n=22)	Low (n=43)			High (n=12)	Low (n=53)		
Gender	male	16	24			24	16			27	13			17	23			9	31		
	female	6	19		0.28	15	10	1		14	11		0.43	5	20		0.1	3	22		0.34
Age at operation	≥69	13	21			20	14			18	16			14	20			9	25		
	<69	9	22		0.6	19	12	1		23	8		0.12	8	23		0.29	3	28		0.11
Macro-scopical type	type1, 2	8	23			16	15			19	12			9	22			4	27		
	type3,4	14	20		0.29	23	11	0.2		22	12		0.8	13	21		0.6	8	26		0.35
Tumor size (mm)	>55	16	16			19	13			19	13			14	18			3	30		
	<55	6	27		0.009	20	13	1		22	11		0.61	8	25		0.12	9	23		0.06
Histological type	intestinal	12	15			13	14			13	14			10	17			6	21		
	diffuse	10	28		0.18	26	12	0.13		28	10		0.04	12	26		0.79	6	32		0.53
T factor	T2, T3	3	23			11	15			13	13			8	18			3	23		
	T4	19	20		0.003	28	11	0.02		28	11		0.12	14	25		0.79	9	30		0.33
Lymph node metastasis	negative	1	20			11	10			12	9			3	18			1	20		
	positive	21	23		6E-04	28	16	0.43		29	15		0.59	19	25		0.03	11	33		0.08
UICC Stage	1B,2,3	7	34			20	21			22	19			9	32			3	38		
	4	15	9		3E-04	19	5	0.02		19	5		0.06	13	11		0.01	9	15		0.006
Lymphatic invasion	negative	0	5			2	2			3	2			1	4			0	5		
	positive	22	38		0.16	37	23	0.38		38	22		1	21	39		0.65	12	48		0.57
Venous invasion	negative	5	21			18	8			20	6			5	21			0	26		
	positive	17	22		0.06	21	18	0.3		21	18		0.07	17	22		0.06	12	27		0.006

Supplementary Table 2: Correlation between clinicopathological factors and expression of individual miRNAs in the validation cohort

Variable		number	miR-30a relative expression		p value	miR-659 relative expression		p value	miR-3917 relative expression		p value
			High (n=21)	Low (n=64)		High (n=24)	Low (n=61)		High (n=26)	Low (n=59)	
Gender	male	62	16	46		19	43		18	44	
	female	23	5	18	0.78	5	18	0.59	8	15	0.61
Age at operation	≥70	41	11	30		10	31		12	29	
	<70	44	10	34	0.8	14	30	0.48	14	30	0.82
Macroscopical type	type1, 2	43	7	36		7	36		8	35	
	type3,4	42	14	28	0.08	17	25	0.02	18	24	0.02
Tumor size (mm)	>55	42	9	33		11	32		16	26	
	<55	43	12	31	0.62	13	29	0.64	10	33	0.16
Histological type	intestinal	48	12	36		12	36		14	34	
	diffuse	37	9	28	1	12	25	0.48	12	25	0.81
T factor	T2, T3	55	10	45		9	46		13	42	
	T4	30	11	19	0.07	15	15	0.002	13	17	0.08
Lymph node metastasis	negative	29	6	23		5	24		8	21	
	positive	56	15	41	0.6	19	37	0.13	18	38	0.81
UICC Stage	1B,2,3	59	11	48		9	50		12	47	
	4	26	10	16	0.06	15	11	1E-04	14	12	0.004
Lymphatic invasion	negative	36	9	27		7	29		8	28	
	positive	49	12	37	1	17	32	0.15	18	31	0.16
Venous invasion	negative	14	3	11		1	13		2	12	
	positive	71	18	53	1	23	48	0.1	24	47	0.21

Supplementary Table 3: Correlation between clinicopathological factors and expression of individual miRNAs in the performance evaluation cohort

Variable	number	miR-30a relative expression		p value	miR-659 relative expression		p value	miR-3917 relative expression		p value
		High (n=35)	Low (n=169)		High (n=56)	Low (n=148)		High (n=35)	Low (n=169)	
Gender	male	26	132		43	115		27	131	
	female	9	37	0.66	13	33	1	8	38	1
Age at operation	≥69	18	84		28	74		17	85	
	<69	17	85	1	28	74	1	18	84	1
Macro-scopical type	type1, 2	15	85		34	66		20	80	
	type3,4	20	84	0.46	22	82	0.04	15	89	0.35
Tumor size (mm)	>55	19	80		31	68		23	76	
	<55	16	89	0.46	25	80	0.27	12	93	0.03
Histological type	intestinal	12	64		24	52		15	61	
	diffuse	23	105	0.85	32	96	0.33	20	108	0.45
T factor	T2, T3	12	87		23	76		16	83	
	T4	23	82	0.09	33	72	0.21	19	86	0.85
Lymph node metastasis	negative	11	48		17	42		13	46	
	positive	24	121	0.69	39	106	0.86	22	123	0.31
UICC Stage	1B,2,3	16	123		30	109		19	120	
	4	19	46	0.003	26	39	0.007	16	49	0.07
Lymphatic invasion	negative	1	19		5	15		4	16	
	positive	34	150	0.21	51	133	1	31	153	0.76
Venous invasion	negative	12	55		19	48		12	55	
	positive	23	114	0.85	37	100	0.87	23	114	0.85

Example 2 – A miRNA-based signature for detection of gastric cancer peritoneal metastasis

[0182] Despite prognosis associated with peritoneal metastasis from gastric cancer is particularly poor, currently there is no robust molecular biomarker to diagnose gastric cancer peritoneal metastasis. Herein, the inventors have comprehensively characterized microRNA (miRNA) profile of primary tumors from patients with peritoneal metastasis and compared to those without by microarray. Subsequently the inventors examined biomarker potential of candidate miRNAs found to be dysregulated in tumors from patients with peritoneal metastasis in three independent cohorts, 354 in total, comprised of advanced gastric cancers (tumors with T stage greater than T2) by qRT-PCR. Five miRNAs (miR-30a-5p, miR-134-5p, miR-337-3p, miR-659-3p, and miR-3917) identified from the initial discovery phase were evaluated in a formalin-fixed paraffin-embedded advanced gastric cancer specimens. Three out of five miRNAs were significantly overexpressed in the primary tumors of peritoneal metastasis positive patients compared to those without peritoneal metastasis and the combination of these miRNAs robustly distinguished peritoneal metastasis positive patients (area under the curve of receiver operating characteristic (AUC) = 0.82). Furthermore, high expression of these miRNAs associated with poor prognosis. The efficacy of this miRNA signature was subsequently validated in an independent cohort (AUC = 0.74). In addition, the inventors confirmed that the combined miRNA signature identified peritoneal metastasis positive patients in an independent fresh frozen tissue cohort (AUC = 0.67) and the combination with macroscopic Borrmann type improved diagnostic capability of the miRNA signature. Collectively, the inventors have demonstrated that the expression of specific miRNAs from primary tumors can be used to identify gastric cancer patients with peritoneal metastasis.

A. INTRODUCTION

[0183] Gastric cancer is the fourth most common cause of cancer related deaths worldwide [1]. In particular, peritoneal metastasis is the most frequently occurring type of gastric cancer metastasis and has exceptionally poor prognosis which is further compounded by the lack of effective treatment regimen [2-5]. Currently, computed tomography (CT) or positron emission tomography (PET) is commonly used to diagnose peritoneal metastasis, but the sensitivity of them are inadequate for identifying peritoneal metastatic lesions [6]. Furthermore, despite staging laparoscopy can identify peritoneal metastasis at a much higher rate than CT or PET-CT, this

radical procedure is invasive and requires general anesthesia which increases the risk of complications [7].

[0184] Although aggressiveness of gastric cancer peritoneal metastasis is well-recognized and early detection can significantly improve the outcome of patients, currently there is no molecular biomarker used clinically to detect peritoneal metastasis. CA125 and CA72-4 are tumor markers typically upregulated in advanced gastric cancers and also shown to be overexpressed in serum of patients with gastric cancer peritoneal metastasis [8-10]. However, individually or even as a combination the sensitivity of these tumor markers for detecting peritoneal metastasis is poor [11]. Furthermore, if peritoneal metastasis can be detected prior to the surgery, it is possible to perform alternative treatments such as hyperthermic intraperitoneal chemotherapy (HIPEC) with cytoreductive surgery. Although this procedure is only performed by limited institutions, HIPEC has been shown to be an effective treatment for gastric cancer patients with peritoneal metastasis [12-15]. Therefore, if there is a robust molecular marker which could identify patients with gastric cancer peritoneal metastasis, alternative treatment strategies can be implemented for patients with peritoneal metastasis.

[0185] Herein, the inventors conducted a comprehensive miRNA profiling of primary tumors derived from peritoneal metastasis patients and compared to that of those without peritoneal metastasis using miRNA-microarray. Subsequently clinical significance of key miRNAs dysregulated in the primary tumors from gastric cancer patients with peritoneal metastasis identified from microarray profiling were evaluated in multiple independent clinical patient cohorts comprised of advanced gastric cancers. The data indicates that miRNA-based signature could be used to diagnose gastric cancer patients with peritoneal metastasis.

B. MATERIALS AND METHODS

1. Samples and Study Design

[0186] This study analyzed 354 tissue specimens, which comprised of 150 formalin-fixed paraffin-embedded (FFPE) primary gastric cancer tissues and 204 fresh primary gastric cancer tissues. These tissues were collected from patients enrolled at Mie University Hospital, Kumamoto University Hospital, and Nagoya University Hospital in Japan. Further information on patient demographics and clinicopathological characteristics are provided in the Supplementary Table 1.

[0187] The current study consists of discovery phase and validation phase. In the discovery phase, the inventors used miRNA-microarray data that the inventors have generated as well as the Cancer Genome Atlas (TCGA) dataset to identify miRNA candidates for further validation. Initially the inventors identified differentially expressed miRNAs in the primary tumors of peritoneal metastasis positive patients compared to those without peritoneal metastasis using miRNA-microarray data with $p < 0.05$ as the initial cutoff criteria. Furthermore, the inventors used TCGA dataset to identify miRNAs differentially expressed in the stage IV patients compared to other gastric cancer stages. Subsequently, the inventors have selected miRNAs identified in both datasets as candidate miRNAs. To evaluate the efficacy of the miRNA candidates identified in the discovery phase, the inventors examined the expression of the candidate miRNAs by TaqMan-based qRT-PCR in three independent tissue validation cohorts. Written informed consent was obtained from all patients, and the Institutional Review Boards of all participating institutions approved the study.

2. MiRNA microarray

[0188] Custom miRNA expression microarray was performed using the Agilent SurePrint G3 Human miRNA microarray 8X60K v3 (Agilent Technologies, Santa Clara, CA), for expression profiling of primary tumor samples from gastric cancer patients with peritoneal metastasis ($n = 6$), and without peritoneal metastasis ($n = 6$). RNA was extracted according to the manufacturer's protocols. Quantitative evaluation of total RNA was performed by Nanodrop. Extracted total RNA were labeled using the miRNA Complete Labeling Kit, Labeled RNAs were hybridized onto Human miRNA Microarray Kit Release, 8x60K. Arrays were scanned and images analyzed by the Feature Extraction Software from Agilent Technologies.

[0189] The raw data of each spot was normalized by substitution with a mean intensity of the background signal determined by all blank spots' signal intensities of 95% confidence intervals. Measurements of spots with the signal intensities greater than 2 standard deviations (SD) of the background signal intensity were considered to be valid. A relative expression level of a given miRNA was calculated by comparing the signal intensities of the valid spots throughout the microarray experiments. In order to normalize the microarray data, "limma" package in R (R studio, Boston, MA) was used to normalize the data by performing background correction and quantile normalization methodology was used to normalize the array data.

3. RNA isolation and quantitative real time PCR

[0190] Total RNA extraction from FFPE tissue samples and fresh frozen tissue samples stored in RNA later were carried out with miRNeasy FFPE Kit (Qiagen, Valencia, CA) and miRNeasy Mini Kit (Qiagen) respectively according to the manufacturer's instructions. The expression of miRNAs was analyzed by TaqMan miRNA real-time qRT-PCR assays (Applied Biosystems, Foster City, CA) using QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems). Following primers were used for qRT-PCR: miR-30a-5p, miR-134-5p, miR-337-3p, miR-669-3p (assay no: 000417, 001186, 002157, 001514 Catalog no: 4427975, Thermo Fisher Scientific) and miR-3917 (Assay no: 464692_mat, Cat no: 440886, Thermo Fisher Scientific). In addition, U6 (Assay no: 001973, Cat no: 4427975 Thermo Fisher Scientific) was used as an endogenous control for data normalization. The expression levels of miRNAs were calculated using the $2^{-\Delta CT}$ method.

4. Statistical analysis

[0191] Comparison of miRNA expression between two independent groups were analyzed using two tailed Mann-Whitney U test (D'Agostino-Pearson omnibus normality test was used to determine Gaussian distribution of miRNA expression). A receiver operating characteristic (ROC) curve was generated and the area under the ROC curve was established for discriminating patients with and without peritoneal metastasis. Youden index for peritoneal metastasis was used in each clinical cohort to determine the optimal cutoff value of individual miRNAs and combined miRNA signature risk score. To analyze correlation between clinicopathological factors and individual miRNA expression in each cohort, Youden index was also used to dichotomize patients into high and low expression group, and difference between groups was analyzed by fisher's exact test. In multivariate logistic regression analysis for peritoneal metastasis, patients were dichotomized into high and low risk groups with combined miRNA signature by Youden index for peritoneal metastasis, were dichotomized into two groups with age and tumor size by their respective median values, and were dichotomized into two groups with macroscopic tumor type by Borrmann criteria. ROC curve for peritoneal metastasis using combined miRNA signature and clinical risk factors which remained in multivariate analysis are also generated by logistic regression analysis. Overall survival (OS) was defined as the period from the date of GC diagnosis to the date of last follow up, and OS analysis was performed by Log-rank test by dichotomizing patients using the Youden

index of individual miRNAs and combined miRNA signature for peritoneal metastasis. p-value of < 0.05 was considered statistically significant. All statistical analyses were performed using the Medcalc statistical software V.16.2.0 (Medcalc Software bvba, Ostend, Belgium), JMP software 10.0.2 (SAS Institute, Cary, NC), and GraphPad Prism V7.0 (GraphPad Software, San Diego, CA).

C. RESULTS

1. Identification of miRNA candidates which are overexpressed in gastric cancer patients with peritoneal metastasis

[0192] In order to identify miRNAs dysregulated in peritoneal metastasis, the inventors utilized miRNA microarray to profile primary tumors obtained from six patients with peritoneal metastasis and compare to that of six without peritoneal metastasis. The inventors used p-value < 0.05 as the initial criteria to identify differentially expressed miRNAs (Figure 9A). Subsequently, the inventors identified 513 differentially expressed miRNAs, of which 364 were upregulated in the primary tumor of peritoneal metastasis positive patients (Figure 9B). To further narrow down the miRNA candidates, the inventors next interrogated TCGA dataset to determine miRNAs that are specifically differentially expressed in stage IV gastric cancer patients. While TCGA does not contain specific information on the type of gastric cancer metastasis, the inventors wanted to ensure that miRNAs the inventors identified in miRNA-microarray dataset are specific to metastatic gastric cancers and not dysregulated in other gastric cancer stages. Accordingly, the inventors compared miRNA expression between Stage IV against Stage IB to III tumors using the following criteria of p-value < 0.05 and identified 104 differentially expressed miRNAs, of which 46 were upregulated and 58 were downregulated. The inventors then overlapped miRNAs which the inventors identified in the microarray dataset and miRNAs overexpressed specifically in stage IV tumors in the TCGA dataset and identified eight miRNAs, of which five were upregulated (miR-30a-5p, miR-134-5p, miR-337-3p, miR-659-3p, and miR-3917) and three were downregulated (miR-718, miR-1281, and miR-3162). Considering that the read count of downregulated miRNAs were extremely low, the inventors have decided to use five miRNAs upregulated miRNAs as the initial candidate (Figure 9C).

2. The candidate miRNAs were overexpressed in the primary tumors of gastric cancer peritoneal metastasis patients in the clinical cohort 1

[0193] To evaluate clinical significance of the five miRNA candidates that the inventors have identified in the discovery phase, the inventors examined the expression of these five miRNAs in an advanced gastric cancer clinical cohort by qRT-PCR. Correlation between clinicopathological factors and relative expression of individual miRNAs (dichotomized into high and low groups) are shown in supplementary Table 2. In brief, 4 out of 5 miRNAs were significantly overexpressed in patients with Stage IV (Supplementary Table 2). The inventors then compared the expression of the candidate miRNAs between patients with or without peritoneal metastasis (Figure 10A). Intriguingly, the expression of three out of five miRNAs, miR-30a-5p, miR-659-3p and miR-3917 were overexpressed in the primary tumors of peritoneal metastasis positive patients compared to those with negative peritoneal metastasis ($p = 0.002$, 0.04 and 0.007 respectively). These miRNAs were able to distinguish peritoneal positive patients from those without peritoneal metastasis (AUC values for miR-30a-5p, miR-659-3p, and miR-3917 were 0.77 , 0.68 , and 0.74 respectively) (Figure 10B). Furthermore the sub-group analysis among Stage IV patients showed that the expression of these miRNAs in patients with peritoneal metastasis tended to be higher than in patients without peritoneal metastasis, especially the expression of miR-3917 showed significance between two groups (Figure 11A). Next, the inventors evaluated whether combining these three miRNA candidates will improve the overall robustness for identifying peritoneal metastasis patients. Intriguingly, three miRNA combined signature was able to distinguish peritoneal metastasis positive patients robustly with the AUC value of 0.82 (Figure 10C). Collectively these data indicate that miRNAs identified from miRNA profiling of peritoneal metastasis were consistently dysregulated in clinical specimens and were able to diagnose peritoneal metastasis patients. Next, the inventors evaluated the prognostic significance of these miRNAs by examining overall survival (OS) using Kaplan-Meier analysis. Although the patients with high expression of miR-659-3p was not significant for OS ($p = 0.13$), the patients with high expression of miR-30a-5p and miR-3917 showed poorer OS than those with low expression ($p = 0.001$, and $p = 0.0009$ respectively) (Figure 10D). Patients with high risk score of combined three miRNA signature also showed poorer OS than those with low risk score ($p = 0.04$) (Figure 10D).

3. Combined miRNA signature robustly identifies patients with peritoneal metastasis in the validation cohort 2

[0194] Next, to validate the efficacy of the three miRNAs identified in cohort 1, the inventors assessed the expression of these miRNAs in an independent validation cohort (n=85). Correlation between clinicopathological factors and relative expression of three miRNAs are shown in Supplementary Table 3. Consistent with cohort 1, all miRNAs showed overexpression or upregulating trend in patients with Stage IV (Supplementary Table 3). The expression of all three miRNAs, miR-30a-5p, miR-659-3p and miR-3917, were overexpressed in primary tumors of peritoneal metastasis positive patients compared to those without peritoneal metastasis ($p = 0.02$, 0.02 , and 0.03 respectively) (Figure 12A). Furthermore, these three miRNAs discriminated peritoneal metastasis patients from those without peritoneal metastasis (AUC values for miR-30a-5p, miR-659-3p, and miR-3917 were 0.69, 0.73, and 0.68 respectively) (Figure 12B). Sub-group analysis among Stage IV patients also showed that the expression of these miRNAs in patients with peritoneal metastasis tended to be higher (but no significance) than in patients without peritoneal metastasis (Figure 11B). Next, the inventors evaluated the efficacy of the combined three miRNA signature to identify patients with peritoneal metastasis. Consistent with the first cohort, the combined miRNA signature was able to discriminate peritoneal metastasis patients from those without peritoneal metastasis (AUC = 0.74) (Figure 12C). In addition, the inventors demonstrated that the patients with high expression of these three individual miRNAs as well as the combined miRNA signature resulted in poorer OS than those with low expression (miR-30a-5p, miR-659-3p, miR-3917, and combined signature: $p = 0.02$, 0.0001 , 0.006 and 0.02 respectively) (Figure 12D).

4. Combined miRNA signature robustly identifies patients with peritoneal metastasis in a clinical cohort with fresh frozen samples

[0195] If the miRNAs identified in this study were to be used clinically, the expression of these miRNAs should be assessed as biopsy samples harvested at the time of diagnostic upper-gastroendoscopy. Typically these biopsy samples are stored as fresh frozen samples. Therefore, the inventors examined whether the miRNAs that the inventors have identified can be used to identify peritoneal metastatic patients in fresh frozen samples. Correlation between clinicopathological factors and relative expression of three miRNAs are summarized in Supplementary Table 4. The expression of two miRNAs, miR-30a-5p and miR-659-3p ($p = 0.0007$, and 0.004 respectively)

were overexpressed, and the expression of miR-3917 showed upregulating trend ($p = 0.08$) in peritoneal metastasis positive patients compared to those with negative peritoneal metastasis (Figure 13A). These miRNAs were able to distinguish peritoneal metastasis positive patients from those without peritoneal metastasis (AUC values for miR-30a-5p, miR-659-3p, and miR-3917 were 0.66, 0.64, and 0.58 respectively) (Figure 13B). As for the sub-group analysis among Stage IV patients, the expression of miR-30a-5p and miR-659-3p in patients with peritoneal metastasis were significantly higher than in patients without peritoneal metastasis (Figure 11C). The inventors then evaluated the efficacy of these miRNAs as a combined miRNAs signature. Consistent with FFPE-based clinical cohorts, the combined miRNA signature was able to discriminate peritoneal metastasis patients from those without peritoneal metastasis (AUC = 0.67) (Figure 13C). In addition, the inventors showed that the patients with high expression of miR-30a-5p showed poorer OS than those with low expression ($p = 0.007$), while miR-659-3p and miR-3917 were not significant for OS ($p = 0.15$, and $p = 0.17$ respectively) (Figure 13D). Patients with high risk score of combined three miRNA signature showed poorer OS than those with low risk score ($p = 0.04$) (Figure 13D).

5. Tumor macroscopic type with combined miRNAs signature robustly identifies peritoneal metastasis patients

[0196] The inventors next assessed clinicopathological factors associated with peritoneal metastasis in all three cohorts to determine whether the miRNA signature is an independent factor for peritoneal metastasis diagnosis using logistic regression model. Furthermore, the inventors aimed to identify whether any other clinical factor could be combined with the miRNA signature to improve the detection of peritoneal metastasis in gastric cancer patients. The inventors specifically focused on the clinical factors which can be identified prior to the surgery, so that the clinical factors can be combined with miRNA signature to identify patients with peritoneal metastasis prior to surgery. In the initial univariate analysis, tumor macroscopic Borrmann type III or IV ($p = 0.003$), and the miRNA combined signature ($p = 0.0002$) were associated with detection of peritoneal metastasis in cohort 1 (Table 1). Subsequent multivariate analysis showed that both tumor macroscopic Borrmann type III or IV ($p = 0.01$), and the miRNA combined signature ($p = 0.0008$) were independent factors for peritoneal metastasis detection (Table 1). Similarly, univariate analysis revealed that tumor macroscopic Borrmann type III or IV ($p = 0.01$), and larger tumor size ($p = 0.01$) were parameters associated with peritoneal metastasis as well as high

expression of combined miRNA signature ($p = 0.0007$) in cohort 2 (Table 2). In multivariate analysis, macroscopic Borrmann type III or IV ($p = 0.03$), larger tumor size ($p = 0.007$) and high expression of combined miRNA signature ($p = 0.0007$) remained as independent factors for peritoneal metastasis detection (Table 2). Consistently, in the third cohort comprised from fresh frozen samples, the univariate analysis showed tumor macroscopic type III or IV ($p < 0.0001$), larger tumor size ($p = 0.0006$), diffuse histological type ($p = 0.02$) and the miRNA combined signature ($p = 0.0002$) were associated with detection of peritoneal metastasis (Table 3). Subsequent multivariate analysis showed that tumor macroscopic type III or IV ($p < 0.0001$), larger tumor size ($p = 0.04$), and the miRNA combined signature ($p < 0.0001$) remained as independent factors for peritoneal metastasis detection (Table 3). Collectively the inventors have identified that along with the miRNA signature, tumor macroscopic type appears to be an important factor for identifying patients with peritoneal metastasis.

[0197] Next, the inventors assessed whether combining tumor macroscopic type with the miRNA signature could improve the overall diagnostic capacity of the miRNA signature. In cohort 1, combination of the miRNA signature with macroscopic type improve the diagnostic robustness ($AUC = 0.87$) (Figure 14A). Consistently, in cohort 2, an addition of tumor macroscopic type was able to effectively identify peritoneal metastasis patients ($AUC = 0.76$) (Figure 14B). In fresh frozen cohort, as the inventors expected, combination of the miRNA signature with macroscopic type also improve the diagnostic robustness ($AUC = 0.79$) (Figure 14C). Collectively, these data suggest that the miRNA signature when combined with tumor macroscopic type can diagnose gastric cancer patients with peritoneal metastasis robustly.

D. DISCUSSION

[0198] Currently, there is no accurate marker to detect gastric cancer peritoneal metastasis and staging laparoscopy is invasive and can only diagnose visible peritoneal metastasis. Therefore, in the current study the inventors investigated whether the expression of miRNAs in the primary tumors could be used to identify gastric cancer peritoneal metastasis. The inventors first used two high throughput datasets to comprehensively examined miRNAs dysregulated in peritoneal metastasis. The inventors then assessed the efficacy of five miRNA candidates in the two independent clinical cohorts comprised of advanced gastric cancer specimens. In particular, the inventors identified that three miRNAs that were consistently overexpressed in both cohorts and

the combined miRNA signature robustly discriminated peritoneal metastasis patients from those without peritoneal metastasis and high expression of these miRNAs were associated with poor overall survival. An addition of macroscopic type signature improved the diagnostic robustness of the miRNA signature in both cohorts. In addition, the inventors further evaluated the robustness of these miRNAs in a clinical cohort with fresh frozen samples to mimic typical biopsy samples and showed that the miRNA signature identified patients with peritoneal metastasis effectively.

[0199] MiRNAs are a short single-stranded non-coding RNAs which are dysregulated in cancers. In this study, the inventors identified five miRNAs overexpressed in primary tumors of patients with gastric cancer peritoneal metastasis from comprehensive miRNA profiling using two high throughput datasets in the discovery phase and validated these candidate miRNAs in multiple clinical cohorts. The data indicates that miRNAs could be used to identify patients with peritoneal metastasis. Previous studies have demonstrated that several miRNAs appear to have functional association with gastric cancer peritoneal metastasis [23-25]. Using a peritoneal metastatic gastric cancer derived cell line, miR-136 was identified as a tumor-suppressive miRNA which attenuated metastatic potential of gastric cancer cells when overexpressed [23]. Similarly, a series of in vitro and in vivo experiments demonstrated miR-3978 as a potential tumor suppressor-miR which inhibits legumain, a lysosomal cysteine endopeptidase of the asparaginyl endopeptidase family [24, 25]. Collectively, these previous studies support functional relevance of miRNAs in gastric cancer peritoneal metastasis and miRNAs could be used to diagnose patients with gastric cancer peritoneal metastasis.

[0200] In this study, the inventors identified five miRNAs overexpressed in primary tumors of patients with gastric cancer peritoneal metastasis. In particular, miR-30a, miR-659 and miR-3917 were consistently overexpressed in peritoneal metastasis positive patients in all three cohorts. In pancreatic cancer, high expression of miR-30a was shown to promote migratory and invasive capability through enhancement of epithelial-to-mesenchymal-transition related genes including fibronectin, vimentin, and N-cadherin [27]. Furthermore, overexpression of miR-30, including miR-30a, resulted in suppression of SOCS3, a key regulator of Jak/STAT3 pathway, and subsequently enhanced glioma stem cell growth [28]. Similarly, miR-30 was shown to be overexpressed in both gastric cancer tissues and overexpression of miR-30 enhanced cellular proliferation and suppressed apoptosis through inhibition of p53 [29]. Collectively, these data

indicate that miR-30a act as an oncogene. In contrast, functional role of miR-659 and miR-3917 is unclear and remains to be elucidated.

[0201] The current study demonstrated that combination of the miRNA signature with tumor macroscopic type (Borrmann type III and IV) was able to efficiently identify gastric cancer with peritoneal metastasis. Borrmann classification was developed in 1926 and is widely used to classify gastric cancers based on endoscopic characteristics. Several studies have shown that Borrmann type correlates with other clinicopathological factors including depth of invasion, tumor stage, lymph node metastasis, distant metastasis and peritoneal metastasis [30-32]. Considering that gastric cancers with macroscopic Borrmann type III and IV have been consistently associated with peritoneal metastasis, it was not surprising that macroscopic type was identified as one of the key factors for peritoneal metastasis diagnosis. Gastric cancer macroscopic stages can be determined during endoscopy prior to the surgery. Therefore, it is feasible to combine macroscopic type with the miRNA signature to determine whether a patient has peritoneal metastasis.

[0202] Currently gastric cancer patient with peritoneal metastasis is typically treated by chemotherapy or palliative surgery. However, there is no standardized treatment regimen for gastric cancer patients with peritoneal metastasis and treatments can vary between institutions [7]. Nevertheless, recent study have shown the development of the treatment strategy for peritoneal metastasis. For example, Intraperitoneal administration of paclitaxel significantly improves the outcome of patients with gastric cancer peritoneal metastasis [33]. Although paclitaxel is well used conventional drug for ovarian, breast and lung cancers and acts by interfering with the function of microtubules during cell division [34-36], it was recently recognized that intraperitoneal administration instead of conventional systemic administration of paclitaxel was effective for treatment of gastric cancer peritoneal metastasis [37-40]. Furthermore, recently the use of cytoreductive surgery combined with hyperthermic intraperitoneal chemotherapy (HIPEC) has been shown to be effective for gastric cancer patients with peritoneal metastasis [13-15, 41]. Collectively, with advancements in treatment strategies, identification of gastric cancer peritoneal metastasis patients could improve the overall outcome of these patients.

[0203] While the inventors have shown that miRNAs could be utilized to identify peritoneal metastasis patients, the miRNA signature must be tested in a larger cohort to evaluate their true potential as a diagnostic marker. However, considering that the number of patients with peritoneal metastasis is relatively scarce, it would require several institutions over an extended period of time

to collect samples prospectively and evaluate the efficacy of the signature. Moreover, due to limited sample number, the inventors were unable to evaluate whether this miRNA signature could identify metachronous peritoneal metastasis.

[0204] In conclusion, the inventors have developed miRNA-based signature for detection of gastric cancer peritoneal metastasis, and validated the robustness of the signature in multiple independent clinical cohorts. The study demonstrated that miRNA expression in the primary tumors can be used to identify peritoneal metastasis patients and may lead to improvement of individualized treatment strategies for gastric cancer patients with peritoneal metastasis.

E. TABLES

Table 1: Multivariate logistic regression analysis for peritoneal metastasis diagnosis in cohort 1

variable	Univariate analysis			Multivariate analysis		
	OR	95%CI	P-value	OR	95%CI	P-value
MiRNA combined signature high risk	13.12	3.12-90.96	0.0002	11.74	2.61-84.97	0.0008
Age $\geq$ 69	1.87	0.57-6.82	0.31			
Male	1.16	0.35-4.24	0.81			
Macroscopical type 3 or 4	7.91	1.91-54.27	0.003	6.83	1.45-50.61	0.01
Tumor size >55mm	2.19	0.66-7.99	0.2			
Poorly differentiated histology	2.05	0.60-8.28	0.26			
OR, Odds Ratio; CI, Confidence Interval. Bold indicates a statistically significant.						

Table 2: Multivariate logistic regression analysis for peritoneal metastasis diagnosis in cohort 2

variable	Univariate analysis			Multivariate analysis		
	OR	95%CI	P-value	OR	95%CI	P-value
MiRNA combined signature high risk	14.15	2.60-263.78	0.0007	17.52	2.89-343.90	0.0007
Age $\geq$ 70	0.54	0.15-1.72	0.3			
Male	1.44	0.40-6.84	0.6			
Macroscopical type 3 or 4	4.73	1.34-22.26	0.01	4.97	1.20-26.99	0.03
Tumor size >55mm	4.73	1.34-22.26	0.01	6.63	1.62-35.88	0.007
Poorly differentiated histology	2.76	0.86-9.80	0.09			
OR, Odds Ratio; CI, Confidence Interval. Bold indicates a statistically significant.						

Table 3: Multivariate logistic regression analysis for peritoneal metastasis diagnosis in fresh frozen cohort

variable	Univariate analysis			Multivariate analysis		
	OR	95%CI	P-value	OR	95%CI	P-value
MiRNA combined signature high risk	3.56	1.82-7.09	0.0002	5.22	2.42-11.80	<0.0001
Age $\geq$ 69	1.48	0.77-2.88	0.24			
Male	0.81	0.38-1.77	0.58			
Macroscopical type 3 or 4	5.82	2.74-13.59	<0.0001	6.44	2.76-16.51	<0.0001
Tumor size >55mm	3.25	1.64-6.72	0.0006	2.24	1.03-5.01	0.04
Poorly differentiated histology	2.31	1.13-5.07	0.02	1.73	0.76-4.17	0.19
OR, Odds Ratio; CI, Confidence Interval. Bold indicates a statistically significant.						

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Supplementary Table 1: Clinicopathological characteristics of patients

		cohort 1	cohort 2	cohort 3
Variable		number	number	number
Gender	male	40	62	158
	female	25	23	46
Age at operation	median (range)	69 (18-90)	70 (25-92)	69 (20-88)
Macroscopical type	type1, 2	31	43	100
	type3,4	34	42	104
Tumor size (mm)	median (range)	55 (12-170)	55 (10-120)	55 (15-180)
Histological type	intestinal	27	48	76
	diffuse	38	37	128
T factor	T2	12	20	40
	T3	14	35	59
	T4	39	30	105
Lymph node metastasis	negative	21	29	59
	positive	44	56	145
UICC Stage	1B	8	16	23
	2	15	26	47
	3	18	17	69
	4	24	26	65
Lymphatic invasion	negative	5	36	20
	positive	60	49	184
Venous invasion	negative	26	14	67
	positive	39	71	137

Supplementary Table 2: Correlation between clinicopathological factors and expression of individual miRNAs in cohort 1

Variable		#	miR-30a relative expression		p value	miR-134 relative expression		p value	miR-337 relative expression		p value	miR-659 relative expression		p value	miR-3917 relative expression		p value
			High (n=22)	Low (n=43)		High (n=39)	Low (n=26)		High (n=41)	Low (n=24)		High (n=22)	Low (n=43)		High (n=12)	Low (n=53)	
Gender	male	40	16	24		24	16		27	13		17	23		9	31	
	female	25	6	19	0.28	15	10	1	14	11	0.43	5	20	0.1	3	22	0.34
Age at operation	≥69	34	13	21		20	14		18	16		14	20		9	25	
	<69	31	9	22	0.6	19	12	1	23	8	0.12	8	23	0.29	3	28	0.11
Macroscopical type	type1, 2	31	8	23		16	15		19	12		9	22		4	27	
	type3,4	34	14	20	0.29	23	11	0.2	22	12	0.8	13	21	0.6	8	26	0.35
Tumor size (mm)	>55	32	16	16		19	13		19	13		14	18		3	30	
	<55	33	6	27	0.009	20	13	1	22	11	0.61	8	25	0.12	9	23	0.06
Histological type	intestinal	27	12	15		13	14		13	14		10	17		6	21	
	diffuse	38	10	28	0.18	26	12	0.13	28	10	0.04	12	26	0.79	6	32	0.53
T factor	T2, T3	26	3	23		11	15		13	13		8	18		3	23	
	T4	39	19	20	0.003	28	11	0.02	28	11	0.12	14	25	0.79	9	30	0.33
Lymph node metastasis	negative	21	1	20		11	10		12	9		3	18		1	20	
	positive	44	21	23	6E-04	28	16	0.43	29	15	0.59	19	25	0.03	11	33	0.08
UICC Stage	1B,2,3	41	7	34		20	21		22	19		9	32		3	38	
	4	24	15	9	3E-04	19	5	0.02	19	5	0.06	13	11	0.01	9	15	0.006
Lymphatic invasion	negative	5	0	5		2	2		3	2		1	4		0	5	
	positive	60	22	38	0.16	37	23	0.38	38	22	1	21	39	0.65	12	48	0.57
Venous invasion	negative	26	5	21		18	8		20	6		5	21		0	26	
	positive	39	17	22	0.06	21	18	0.3	21	18	0.07	17	22	0.06	12	27	0.006

Supplementary Table 3: Correlation between clinicopathological factors and expression of individual miRNAs in cohort 2

			miR-30a relative expression		p value	miR-659 relative expression		p value	miR-3917 relative expression		p value
Variable		#	High (n=21)	Low (n=64)		High (n=24)	Low (n=61)		High (n=26)	Low (n=59)	
Gender	male	62	16	46		19	43		18	44	
	female	23	5	18	0.78	5	18	0.59	8	15	0.61
Age at operation	≥70	41	11	30		10	31		12	29	
	<70	44	10	34	0.8	14	30	0.48	14	30	0.82
Macroscopical type	type1, 2	43	7	36		7	36		8	35	
	type3,4	42	14	28	0.08	17	25	0.02	18	24	0.02
Tumor size (mm)	>55	42	9	33		11	32		16	26	
	<55	43	12	31	0.62	13	29	0.64	10	33	0.16
Histological type	intestinal	48	12	36		12	36		14	34	
	diffuse	37	9	28	1	12	25	0.48	12	25	0.81
T factor	T2, T3	55	10	45		9	46		13	42	
	T4	30	11	19	0.07	15	15	0.002	13	17	0.08
Lymph node metastasis	negative	29	6	23		5	24		8	21	
	positive	56	15	41	0.6	19	37	0.13	18	38	0.81
UICC Stage	1B,2,3	59	11	48		9	50		12	47	
	4	26	10	16	0.06	15	11	1E-04	14	12	0.004
Lymphatic invasion	negative	36	9	27		7	29		8	28	
	positive	49	12	37	1	17	32	0.15	18	31	0.16
Venous invasion	negative	14	3	11		1	13		2	12	
	positive	71	18	53	1	23	48	0.1	24	47	0.21

Supplementary Table 4: Correlation between clinicopathological factors and expression of individual miRNAs in fresh frozen cohort

			miR-30a relative expression		p value	miR-659 relative expression		p value	miR-3917 relative expression		p value
Variable		#	High (n=35)	Low (n=169)		High (n=56)	Low (n=148)		High (n=35)	Low (n=169)	
Gender	male	158	26	132		43	115		27	131	
	female	46	9	37	0.66	13	33	1	8	38	1
Age at operation	≥69	102	18	84		28	74		17	85	
	<69	102	17	85	1	28	74	1	18	84	1
Macroscopical type	type1, 2	100	15	85		34	66		20	80	
	type3,4	104	20	84	0.46	22	82	0.04	15	89	0.35
Tumor size (mm)	>55	99	19	80		31	68		23	76	
	<55	105	16	89	0.46	25	80	0.27	12	93	0.03
Histological type	intestinal	76	12	64		24	52		15	61	
	diffuse	128	23	105	0.85	32	96	0.33	20	108	0.45
T factor	T2, T3	99	12	87		23	76		16	83	
	T4	105	23	82	0.09	33	72	0.21	19	86	0.85
Lymph node metastasis	negative	59	11	48		17	42		13	46	
	positive	145	24	121	0.69	39	106	0.86	22	123	0.31
UICC Stage	1B,2,3	139	16	123		30	109		19	120	
	4	65	19	46	0.003	26	39	0.007	16	49	0.07
Lymphatic invasion	negative	20	1	19		5	15		4	16	
	positive	184	34	150	0.21	51	133	1	31	153	0.76
Venous invasion	negative	67	12	55		19	48		12	55	
	positive	137	23	114	0.85	37	100	0.87	23	114	0.85

* * *

- 5 [0205] All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and
- 10 physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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25 Hyperthermic Intraperitoneal Chemotherapy and Neoadjuvant Intraperitoneal/Systemic Chemotherapy on Peritoneal Metastases from Gastric Cancer. *Annals of surgical oncology* 2017; 24: 478-485.

WHAT IS CLAIMED IS:

1. A method for treating a patient with gastric cancer, the method comprising treating the patient for gastric cancer after the expression level of one or more biomarkers selected from miR-30a-5p, miR-134-5p, miR-337-3p, miR-659-3p, and miR-3917 has been determined in a sample from the patient.
2. The method of claim 1, wherein at least miR-30a-5p, miR-659-3p and miR-3917 were determined in a sample from the patient.
3. The method of claim 1 or 2, wherein at least miR-30a-5p and miR-659-3p were determined in a sample from the patient.
4. The method of claim 1, wherein at least miR-30a-5p was determined in a sample from the patient.
5. The method of claim 1, wherein at least miR-134-5p was determined in a sample from the patient.
6. The method of claim 1, wherein at least miR-337-3p was determined in a sample from the patient.
7. The method of claim 1, wherein at least miR-659-3p was determined in a sample from the patient.
8. The method of claim 1, wherein at least miR-3917 was determined in a sample from the patient.
9. The method of any one of claims 1-8, wherein the patient has not been diagnosed with or has not been treated for peritoneal metastasis or Stage IV gastric cancer.
10. The method of any one of claims 1-9, wherein the expression levels of the one or more biomarkers in the patient was determined to be i) increased compared to the levels of expression in samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk.
11. The method of any one of claims 1-10, wherein the expression levels of at least one of the biomarkers in the patient was determined to be i) increased compared to the levels of expression in samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk.

12. The method of any one of claims 1-10, wherein the expression levels of at least two of the biomarkers in the patient was determined to be i) increased compared to the levels of expression in samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk.

13. The method of any one of claims 1-10, wherein the expression levels of at least three of the biomarkers in the patient was determined to be i) increased compared to the levels of expression in samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk.

14. The method of any one of claims 1-10, wherein the expression levels of at least four of the biomarkers in the patient was determined to be i) increased compared to the levels of expression in samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk.

15. The method of any one of claims 1-10, wherein the expression levels of at least five of the biomarkers in the patient was determined to be i) increased compared to the levels of expression in samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk.

16. The method of any one of claims 1-15, wherein the patient was determined to have a macroscopic Borrmann type III or IV gastric tumor.

17. The method of any one of claims 1-16, wherein the patient is treated for peritoneal metastasis.

18. The method of any one of claim 10-17, wherein the treatment comprises one or more of cytoreductive surgery, hyperthermic intraperitoneal chemotherapy (HIPEC), chemotherapy, neo-adjuvant chemotherapy, adjuvant chemotherapy, subtotal or total gastrectomy, tumor resection, and endoscopic resection.

19. The method of claim 18, wherein the chemotherapy comprises paclitaxel.

20. The method of claim 19, wherein the chemotherapy is administered by intraperitoneal administration.

21. The method of any one of claims 1-9, wherein the expression levels of the one or more biomarkers in the patient was determined to be i) decreased compared to the levels of expression in samples from patients identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal tissues.

22. The method of claim 21, wherein the expression levels of at least one of the biomarkers in the patient was determined to be i) decreased compared to the levels of expression in samples from patients identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal tissues.

23. The method of claim 21, wherein the expression levels of at least two of the biomarkers in the patient was determined to be i) decreased compared to the levels of expression in samples from patients identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal tissues.

24. The method of claim 21, wherein the expression levels of at least three of the biomarkers in the patient was determined to be i) decreased compared to the levels of expression in samples from patients identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal tissues.

25. The method of claim 21, wherein the expression levels of at least four of the biomarkers in the patient was determined to be i) decreased compared to the levels of expression in samples from patients identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal tissues.

26. The method of claim 21, wherein the expression levels of at least five of the biomarkers in the patient was determined to be i) decreased compared to the levels of expression in samples from patients identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal tissues.

27. The method of any one of claims 21-26, wherein the patient was determined to have a macroscopic Borrmann type I or II gastric tumor.
28. The method of any one of claims 21-27, wherein the treatment comprises one or more of surgery with either subtotal or total gastrectomy, tumor resection, endoscopic resection.
29. The method of any one of claims 21-28, wherein the treatment excludes one or more of cytoreductive surgery, hyperthermic intraperitoneal chemotherapy (HIPEC), chemotherapy, neo-adjuvant chemotherapy, and adjuvant chemotherapy.
30. The method of any one of claim 1-29, wherein the wherein the sample from the patient comprises gastric cancer tissue.
31. The method of any one of claims 10-30, wherein the normal tissues comprises non-cancerous gastric tissues.
32. The method of any one of claims 1-26, wherein the sample from the patient comprises a serum sample.
33. The method of any one of claims 1-32, wherein the sample from the patient comprises nucleic acids.
34. The method of claim 32 or 33, wherein the sample from the patient comprises a fractionated serum sample comprising nucleic acids.
35. The method of any one of claims 1-34, wherein the samples from patients identified as not having peritoneal metastasis or identified as low risk comprises the level of expression of the one or more biomarkers in a serum sample or samples from patients without peritoneal metastasis.
36. The method of any of claims 1-35, wherein the expression level of no other biomarker in the biological sample was determined.
37. The method of any of claims 1-36, wherein the patient has undergone surgery to resect all or part of the cancer.
38. The method of any one of claims 1-36, wherein the patient has not undergone surgical resection of the tumor.
39. The method of any of claims 1-38, wherein the level of expression of miR-30a-5p was determined pre-operative and/or post-operative.
40. The method of any of claims 1-37 or 39, wherein the level of expression of miR-134-5p was determined pre-operative and/or post-operative.

41. The method of any of claims 1-37 or 39-40, wherein the level of expression of miR-337-3p was determined pre-operative and/or post-operative.
42. The method of any of claims 1-37 or 39-41, wherein the level of expression of miR-659-3p was determined pre-operative and/or post-operative.
43. The method of any of claims 1-37 or 39-42, wherein the level of expression of miR-3917 was determined pre-operative and/or post-operative.
44. The method of any one of claims 1-43, wherein the patient has not undergone laparoscopy of gastric cancer tissues.
45. The method of any one of claims 10-44, wherein low risk is indicative of a patient with a low risk for distant metastasis and/or peritoneal metastasis and good overall survival (OS) rate, and high risk is indicative of a patient with a high risk for distant metastasis and/or peritoneal metastasis and poor overall survival (OS) rate.
46. A method for evaluating a gastric cancer patient comprising measuring the level of expression of one or more biomarkers selected from miR-30a-5p, miR-134-5p, miR-337-3p, miR-659-3p, and miR-3917 in a sample from the patient.
47. The method of claim 46, wherein at least miR-30a-5p, miR-659-3p and miR-3917 were measured in a sample from the patient.
48. The method of claim 46 or 47, wherein at least miR-30a-5p and miR-659-3p were measured in a sample from the patient.
49. The method of any one of claims 46-48, wherein the method further comprises determining the macroscopic Borrmann type of the gastric tumor.
50. The method of any one of claims 46-49, wherein the patient has not been diagnosed with distant metastasis.
51. The method of any one of claims 46-50, wherein the patient has not been diagnosed with peritoneal metastasis.
52. The method of any one of claims 46-51, wherein the sample from the patient comprises serum.
53. The method of any one of claims 46-52, wherein the sample from the patient comprises nucleic acids.
54. The method of claim 52 or 53, wherein the sample from the patient comprises a fractionated serum sample comprising nucleic acids.

55. The method of any one of claims 46-51, wherein the sample from the patient comprises gastric cancer tissues.
56. The method of any one of claims 46-55, wherein at least miR-30a-5p was measured.
57. The method of any one of claims 46-55, wherein at least miR-134-5p was measured.
58. The method of any one of claims 46-55, wherein at least miR-337-3p was measured.
59. The method of any one of claims 46-55, wherein at least miR-659-3p was measured.
60. The method of any one of claims 46-55, wherein at least miR-3917 was measured.
61. The method of any of claims 46-60, wherein the expression level of no other biomarker in the biological sample is measured.
62. The method of any of claims 46-61, further comprising comparing the level(s) of expression to a control sample(s) or control level(s) of expression.
63. The method of claim 62, wherein the control sample(s) have expression levels that are representative of expression levels in samples from patients identified as low risk, of patients not having gastric cancer, or of patients having gastric cancer but not having peritoneal metastasis.
64. The method of claim 62, wherein the control levels(s) comprise the levels of expression of the one or more biomarkers in non-cancerous gastric tissues.
65. The method of claim 62, wherein the control sample(s) have expression levels that are representative of expression levels in samples from patients identified as high risk or of patients having peritoneal metastasis.
66. The method of any of claims 46-65, wherein the patient has been diagnosed with gastric cancer.
67. The method of any of claims 46-66, further comprising treating the patient for cancer after measuring the level of expression of one or more listed biomarkers.
68. The method of any one of claims 46-67, wherein the biomarker is measured prior to surgical resection of the tumor or prior to total or subtotal gastrectomy.
69. The method of any one of claims 46-67, wherein the biomarker is measured after surgical resection of the tumor or after total or subtotal gastrectomy.
70. The method of any of claims 46-69, wherein the level of expression of miR-30a-5p was measured pre-operative and/or post-operative.
71. The method of any of claims 46-70, wherein the level of expression of miR-134-5p was measured pre-operative and/or post-operative.

72. The method of any of claims 46-71, wherein the level of expression of miR-337-3p was measured pre-operative and/or post-operative.
73. The method of any of claims 46-72, wherein the level of expression of miR-659-3p was measured pre-operative and/or post-operative.
74. The method of any of claims 46-73, wherein the level of expression of miR-3917 was measured pre-operative and/or post-operative.
75. A method of prognosing and/or diagnosing a patient with gastric cancer comprising
- a) measuring the level of expression of one or more of miR-30a-5p, miR-134-5p, miR-337-3p, miR-659-3p, and miR-3917 in a sample from the patient;
 - b) comparing the level(s) of expression to a control sample(s) or control level(s) of expression; and,
 - c) prognosing and/or diagnosing the patient based on the levels of measured expression.
76. The method of claim 75, wherein at least miR-30a-5p, miR-659-3p and miR-3917 were measured in a sample from the patient.
77. The method of claim 75 or 76, wherein at least miR-30a-5p and miR-659-3p were measured in a sample from the patient.
78. The method of any one of claims 75-77, wherein at least miR-30a-5p was measured in a sample from the patient.
79. The method of claim 75, wherein at least miR-134-5p was measured in a sample from the patient.
80. The method of claim 75, wherein at least miR-337-3p was measured in a sample from the patient.
81. The method of claim 75, wherein at least miR-659-3p was measured in a sample from the patient.
82. The method of claim 75, wherein at least miR-3917 was measured in a sample from the patient.
83. The method of any one of claims 75-82, wherein the method further comprises determining the macroscopic Borrmann type of the gastric tumor.
84. The method of any one of claims 75-83, wherein the patient has not been diagnosed with or has not been treated for peritoneal metastasis or Stage IV gastric cancer.
85. The method of any one of claims 75-84, wherein the patient is prognosed as high risk and/or treated when the expression levels of the one or more biomarkers in the patient was i) increased

compared to the levels of expression in samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk.

86. The method of any one of claims 75-85, wherein the patient is prognosed as high risk and/or treated when the expression levels of at least one of the biomarkers in the patient was i) increased compared to the levels of expression in samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk.

87. The method of any one of claims 75-85, wherein the patient is prognosed as high risk and/or treated when the expression levels of at least two of the biomarkers in the patient was i) increased compared to the levels of expression in samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk.

88. The method of any one of claims 75-85, wherein the patient is prognosed as high risk and/or treated when the expression levels of at least three of the biomarkers in the patient was i) increased compared to the levels of expression in samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk.

89. The method of any one of claims 75-85, wherein the patient is prognosed as high risk and/or treated when the expression levels of at least four of the biomarkers in the patient was i) increased compared to the levels of expression in samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk.

90. The method of any one of claims 75-85, wherein the patient is prognosed as high risk and/or treated when the expression levels of at least five of the biomarkers in the patient was i) increased compared to the levels of expression in samples from patients identified as not having peritoneal metastasis, identified as low risk, or in normal tissues or ii) within the range of expression levels in samples of patients identified as having peritoneal metastasis or identified as high risk.

91. The method of any one of claims 83-90, wherein the patient is prognosed as high risk and/or treated when the patient was determined to have a macroscopic Borrmann type III or IV gastric tumor.

92. The method of any one of claims 85-91, wherein the patient is diagnosed as having peritoneal metastasis.
93. The method of any one of claim 85-92, wherein the treatment comprises one or more of cytoreductive surgery, hyperthermic intraperitoneal chemotherapy (HIPEC), chemotherapy, neo-adjuvant chemotherapy, adjuvant chemotherapy, subtotal or total gastrectomy, tumor resection, and endoscopic resection.
94. The method of claim 93, wherein the chemotherapy comprises paclitaxel.
95. The method of claim 94, wherein the chemotherapy is administered by intraperitoneal administration.
96. The method of any one of claims 75-84, wherein the patient is prognosed as low risk and/or treated when the expression levels of the one or more biomarkers in the patient is i) decreased compared to the levels of expression in samples from patients identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal tissues.
97. The method of claim 96, wherein the patient is prognosed as low risk and/or treated when the expression levels of at least one of the biomarkers in the patient is i) decreased compared to the levels of expression in samples from patients identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal tissues.
98. The method of claim 96, wherein the patient is prognosed as low risk and/or treated when the expression levels of at least two of the biomarkers in the patient is i) decreased compared to the levels of expression in samples from patients identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal tissues.
99. The method of claim 96, wherein the patient is prognosed as low risk and/or treated when the expression levels of at least three of the biomarkers in the patient is i) decreased compared to the levels of expression in samples from patients identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal tissues.
100. The method of claim 96, wherein the patient is prognosed as low risk and/or treated when the expression levels of at least four of the biomarkers in the patient is i) decreased compared to the

levels of expression in samples from patients identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal tissues.

101. The method of claim 96, wherein the patient is prognosed as low risk and/or treated when the expression levels of at least five of the biomarkers in the patient is i) decreased compared to the levels of expression in samples from patients identified as having peritoneal metastasis or identified as low risk or ii) within the range of expression levels in samples of patients identified as not having peritoneal metastasis or in normal tissues.

102. The method of any one of claims 96-101, wherein the patient is prognosed as low risk and/or treated when the patient was determined to have a macroscopic Borrmann type I or II gastric tumor.

103. The method of any one of claims 96-102, wherein the treatment comprises one or more of surgery with either subtotal or total gastrectomy, tumor resection, endoscopic resection.

104. The method of any one of claims 96-103, wherein the treatment excludes one or more of cytoreductive surgery, hyperthermic intraperitoneal chemotherapy (HIPEC), chemotherapy, neo-adjuvant chemotherapy, and adjuvant chemotherapy.

105. The method of any one of claim 75-104, wherein the wherein the sample from the patient comprises gastric cancer tissue.

106. The method of any one of claims 85-105, wherein the normal tissues comprises non-cancerous gastric tissues.

107. The method of any one of claims 75-104, wherein the sample from the patient comprises a serum sample.

108. The method of any one of claims 75-107, wherein the sample from the patient comprises nucleic acids.

109. The method of claim 107 or 108, wherein the sample from the patient comprises a fractionated serum sample comprising nucleic acids.

110. The method of any one of claims 75-107, wherein the samples from patients identified as not having peritoneal metastasis or identified as low risk comprises the level of expression of the one or more biomarkers in a serum sample or samples from patients without peritoneal metastasis.

111. The method of any of claims 75-110, wherein the expression level of no other biomarker in the biological sample was measured.

112. The method of any of claims 75-111, wherein the patient has undergone surgery to resect all or part of the cancer.
113. The method of any one of claims 75-111, wherein the patient has not undergone surgical resection of the tumor.
114. The method of any of claims 75-113, wherein the level of expression of miR-30a-5p was measured pre-operative and/or post-operative.
115. The method of any of claims 75-114, wherein the level of expression of miR-134-5p was measured pre-operative and/or post-operative.
116. The method of any of claims 75-115, wherein the level of expression of miR-337-3p was measured pre-operative and/or post-operative.
117. The method of any of claims 75-116, wherein the level of expression of miR-659-3p was measured pre-operative and/or post-operative.
118. The method of any of claims 75-117, wherein the level of expression of miR-3917 was measured pre-operative and/or post-operative.
119. The method of any one of claims 75-118, wherein the patient has not undergone laparoscopy of gastric cancer tissues.
120. The method of any one of claims 85-119, wherein low risk is indicative of a patient with a low risk for distant metastasis and/or peritoneal metastasis and good overall survival (OS) rate, and high risk is indicative of a patient with a high risk for distant metastasis and/or peritoneal metastasis and poor overall survival (OS) rate.
121. A kit comprising 1, 2, 3, 4, or 5 detection agents for determining expression levels of biomarkers for gastric cancer, wherein the biomarkers comprise one or more of miR-30a-5p, miR-134-5p, miR-337-3p, miR-659-3p, and miR-3917.
122. The kit of claim 121, wherein the kit further comprises one or more negative or positive control samples and/or control detection agents.

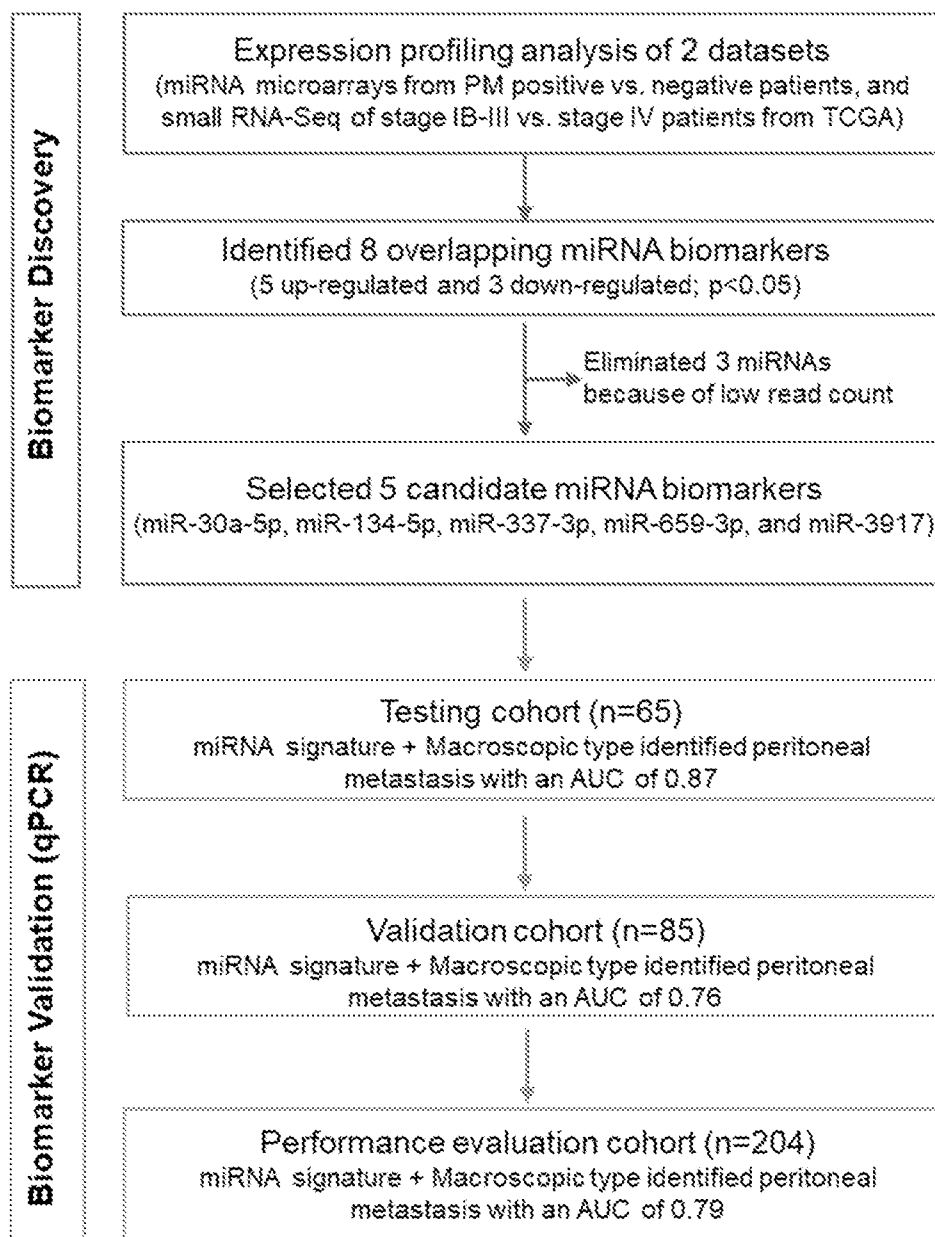


FIG. 1A

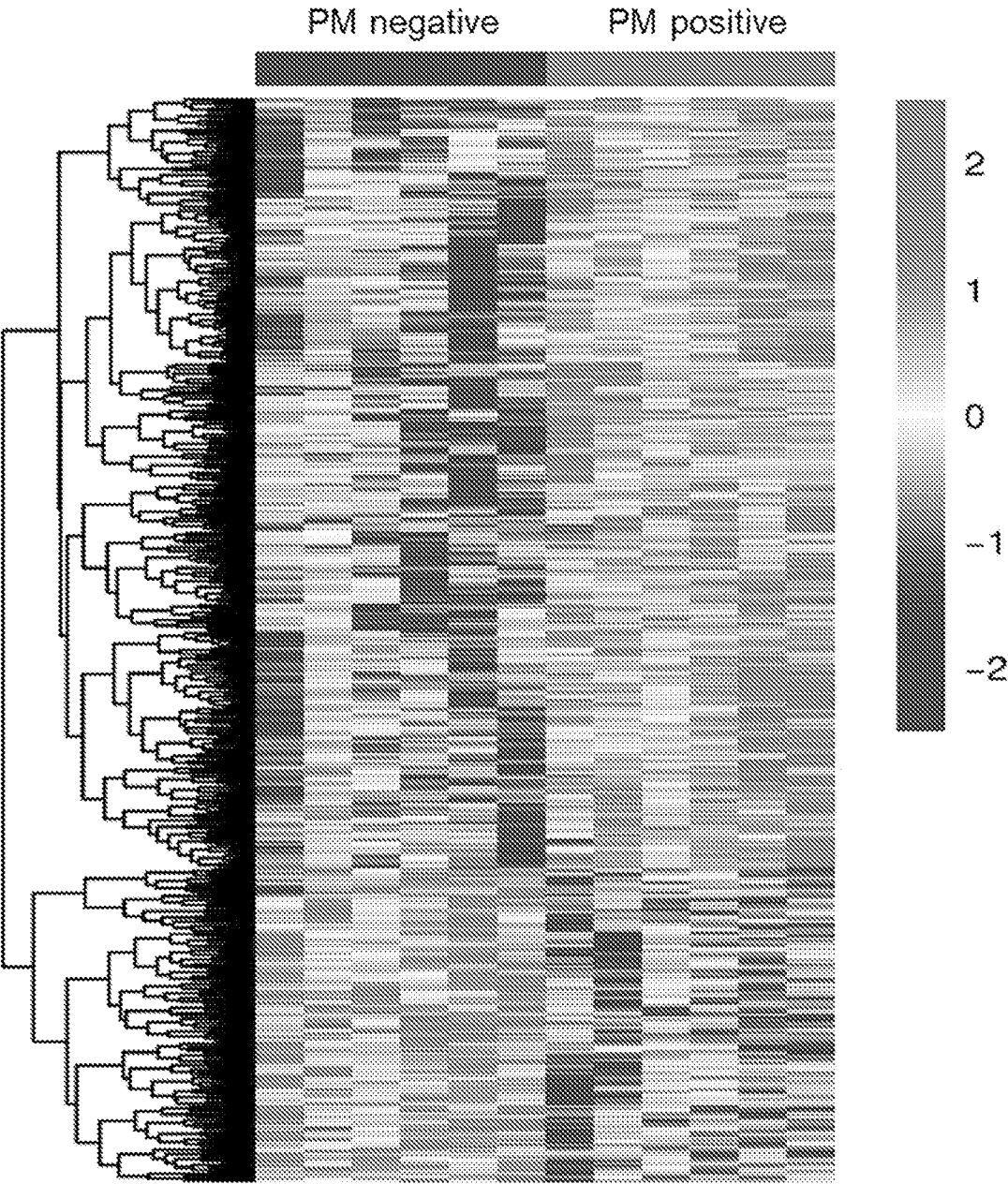


FIG. 1B

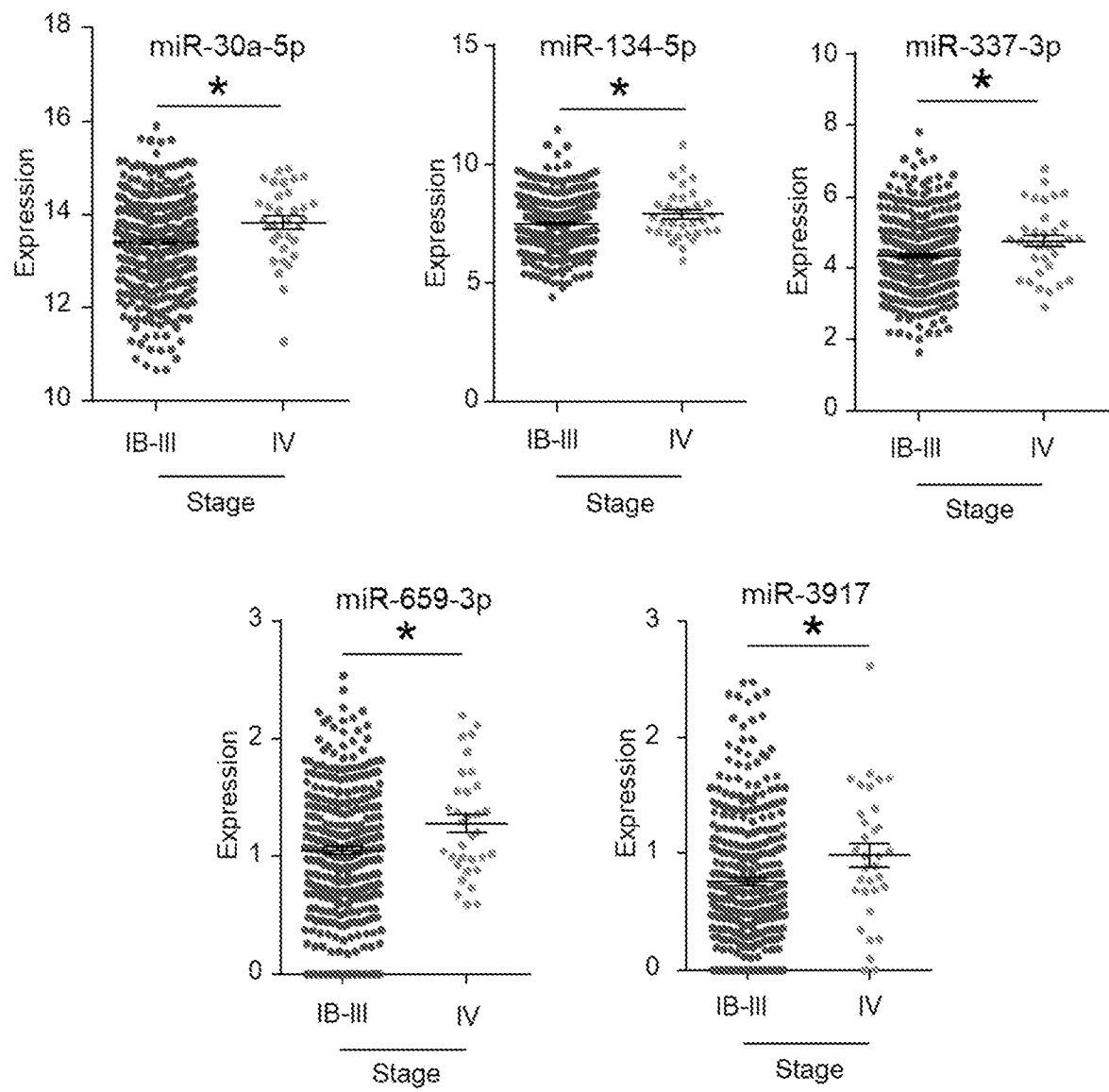


FIG. 1C

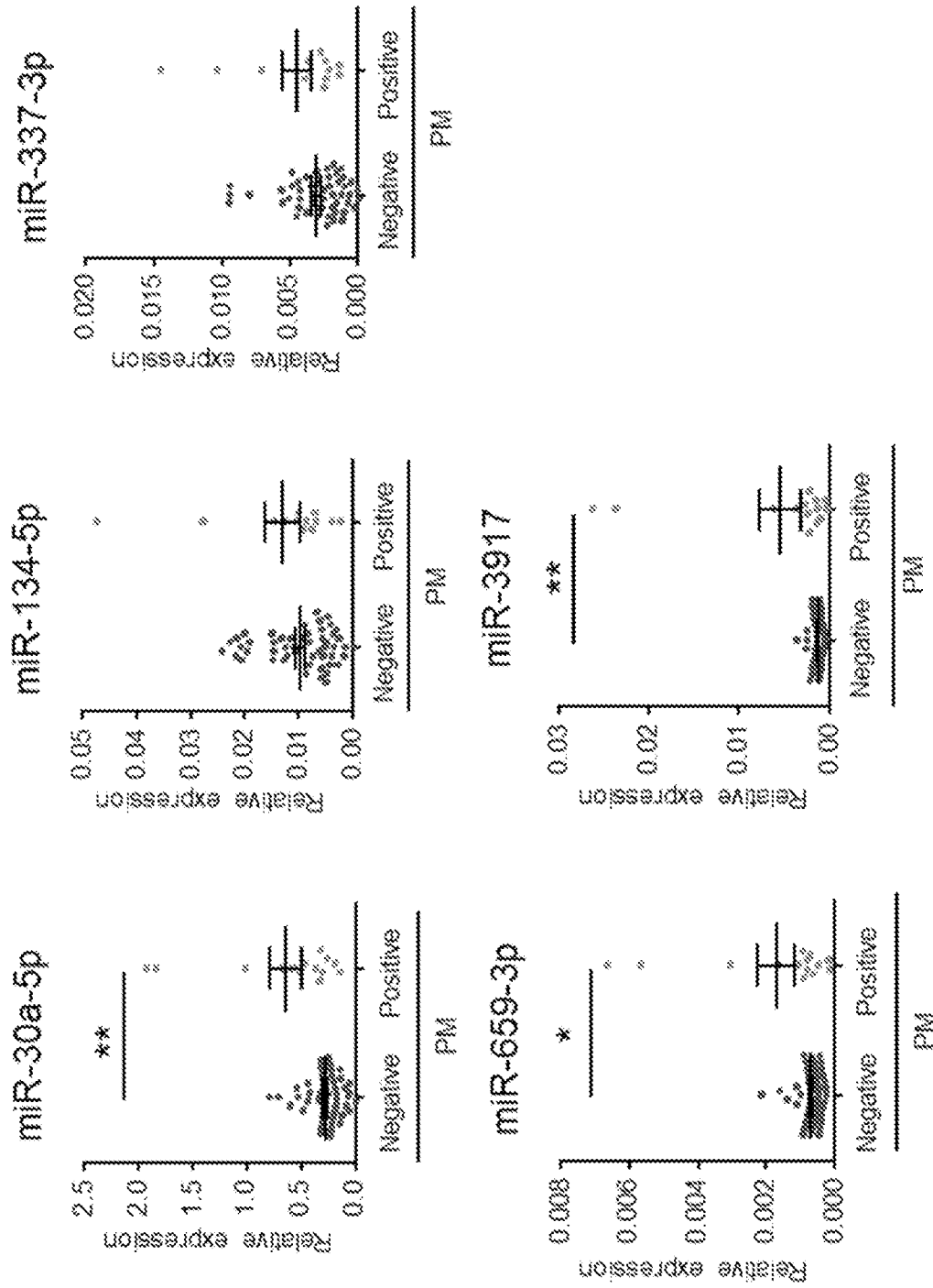


FIG. 2A

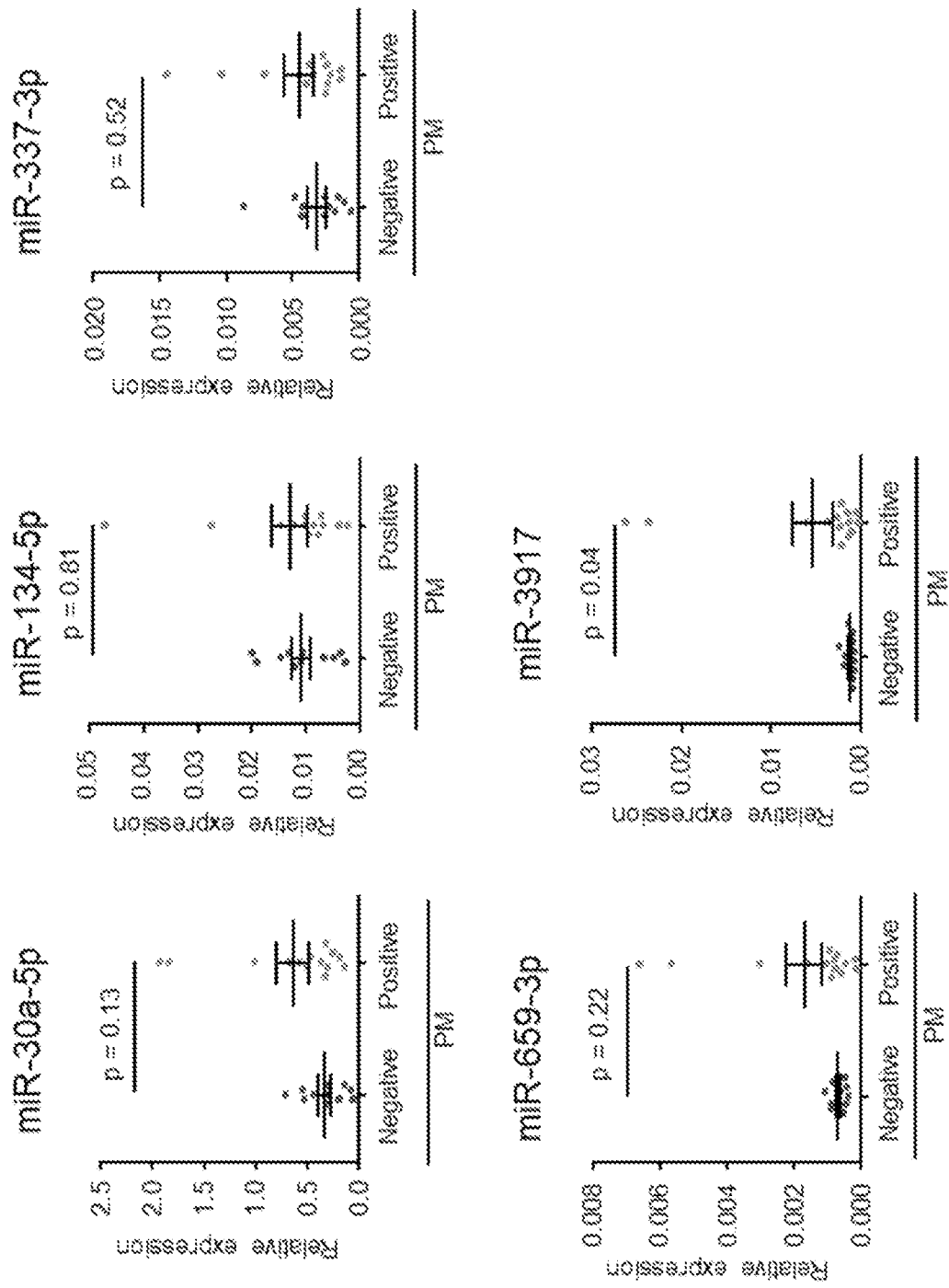
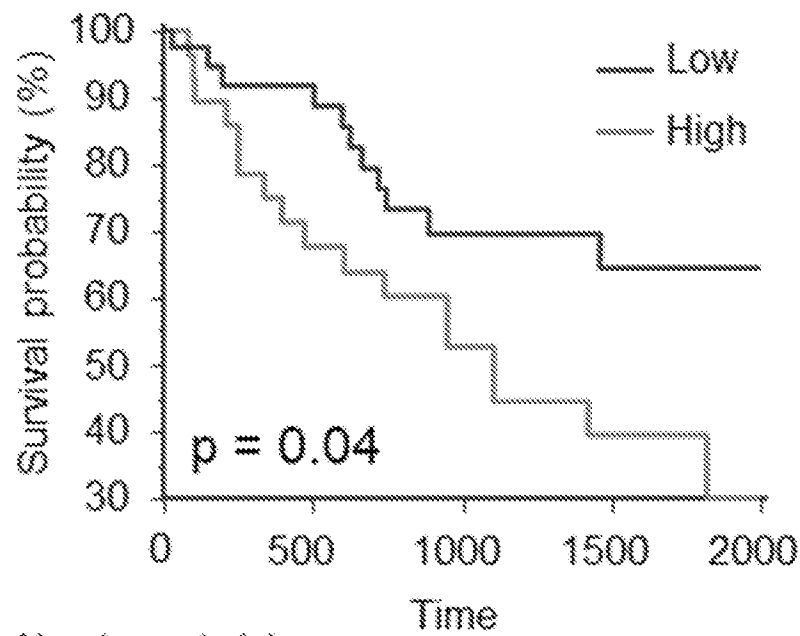


FIG. 2B

Combination signature



Number at risk

High risk score

28 18 14 6 3

Low risk score

37 30 19 12 2

FIG. 2C

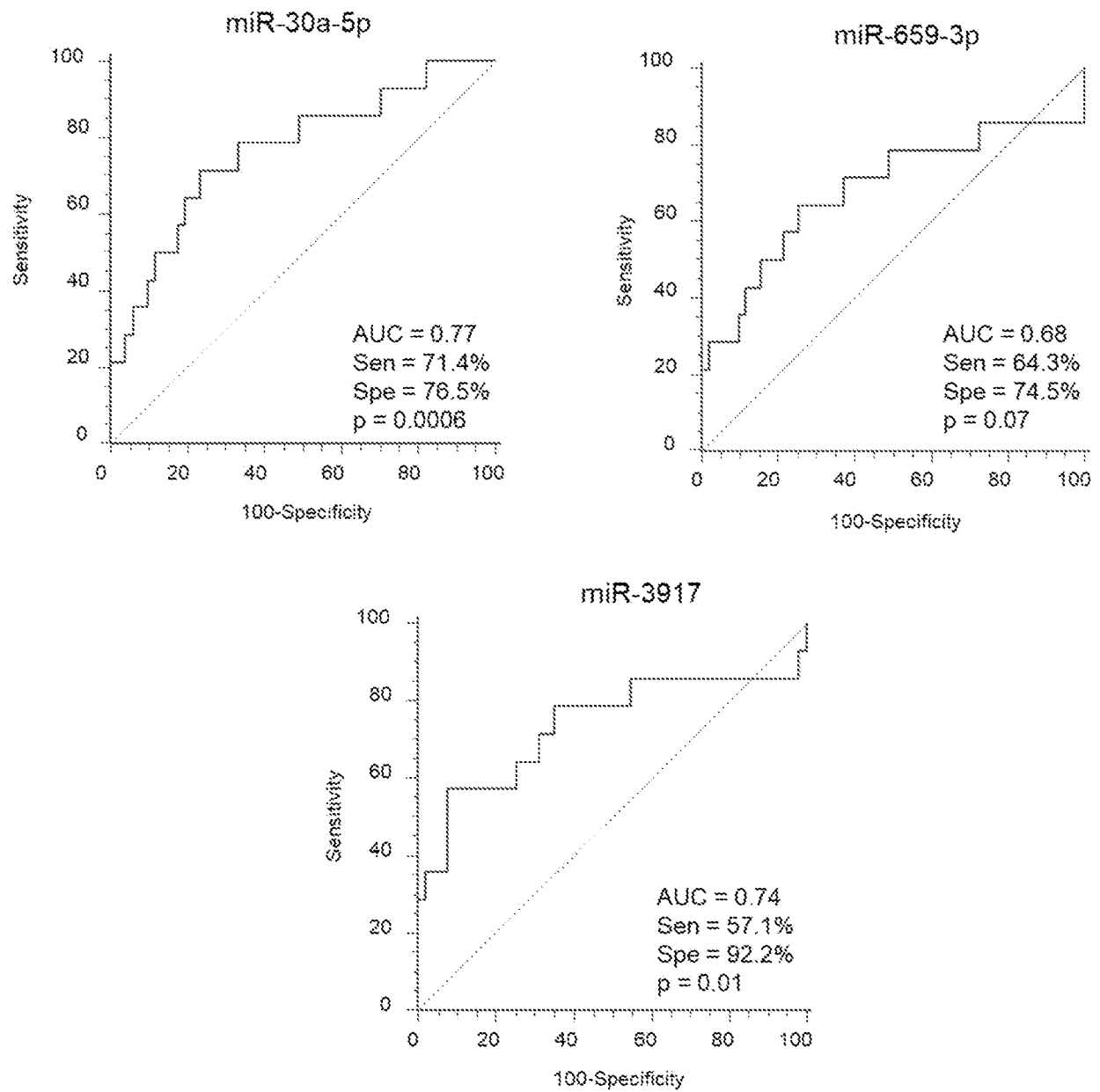
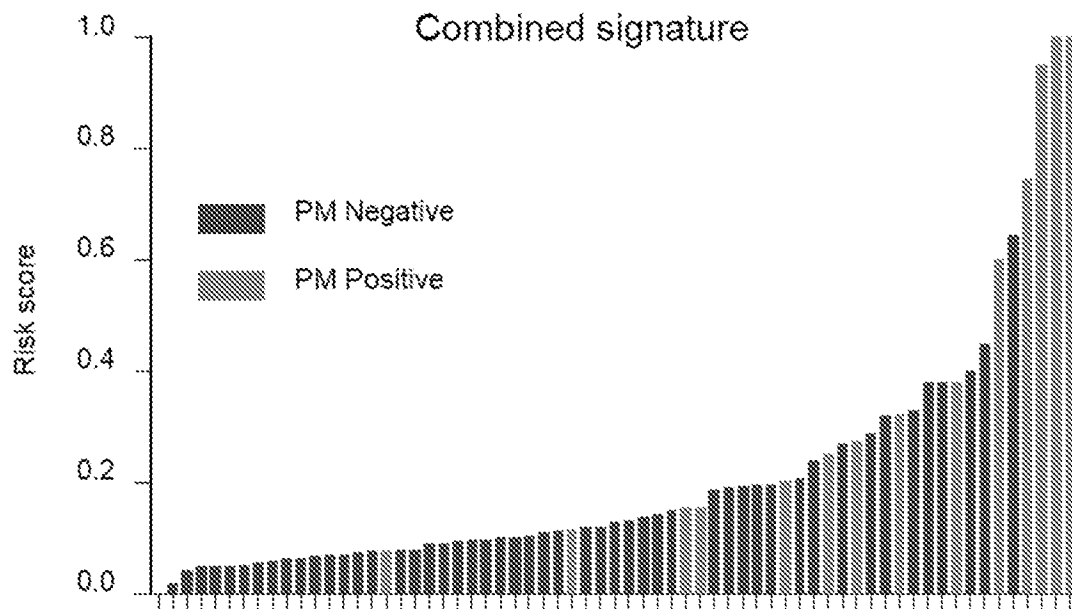


FIG. 3A

B.



C.

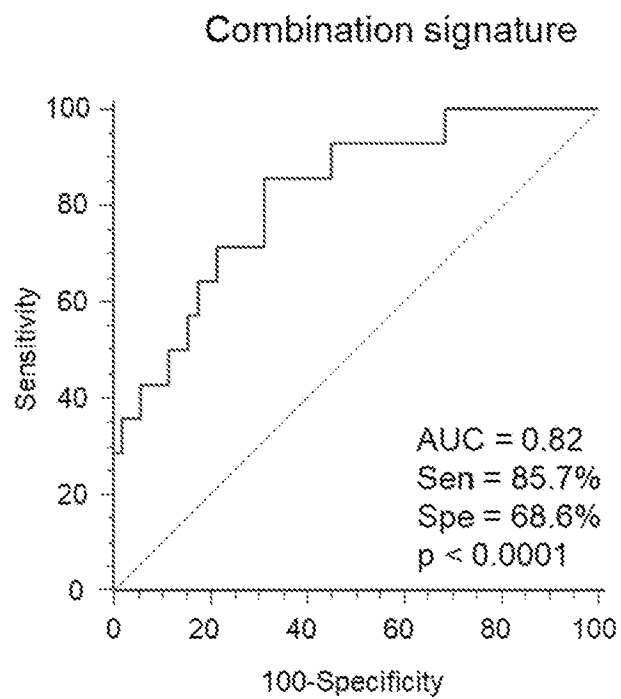


FIG. 3B-C

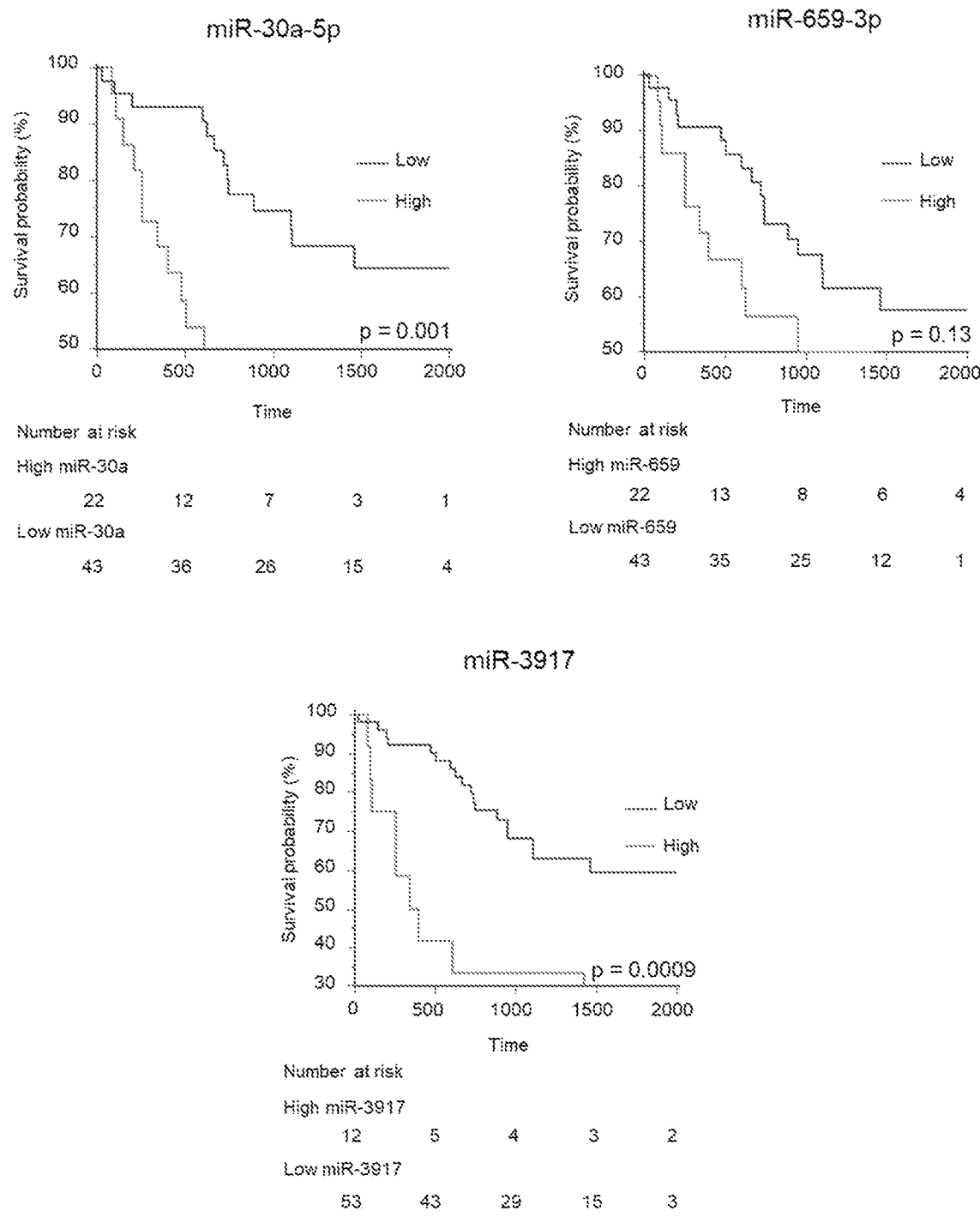


FIG. 3D

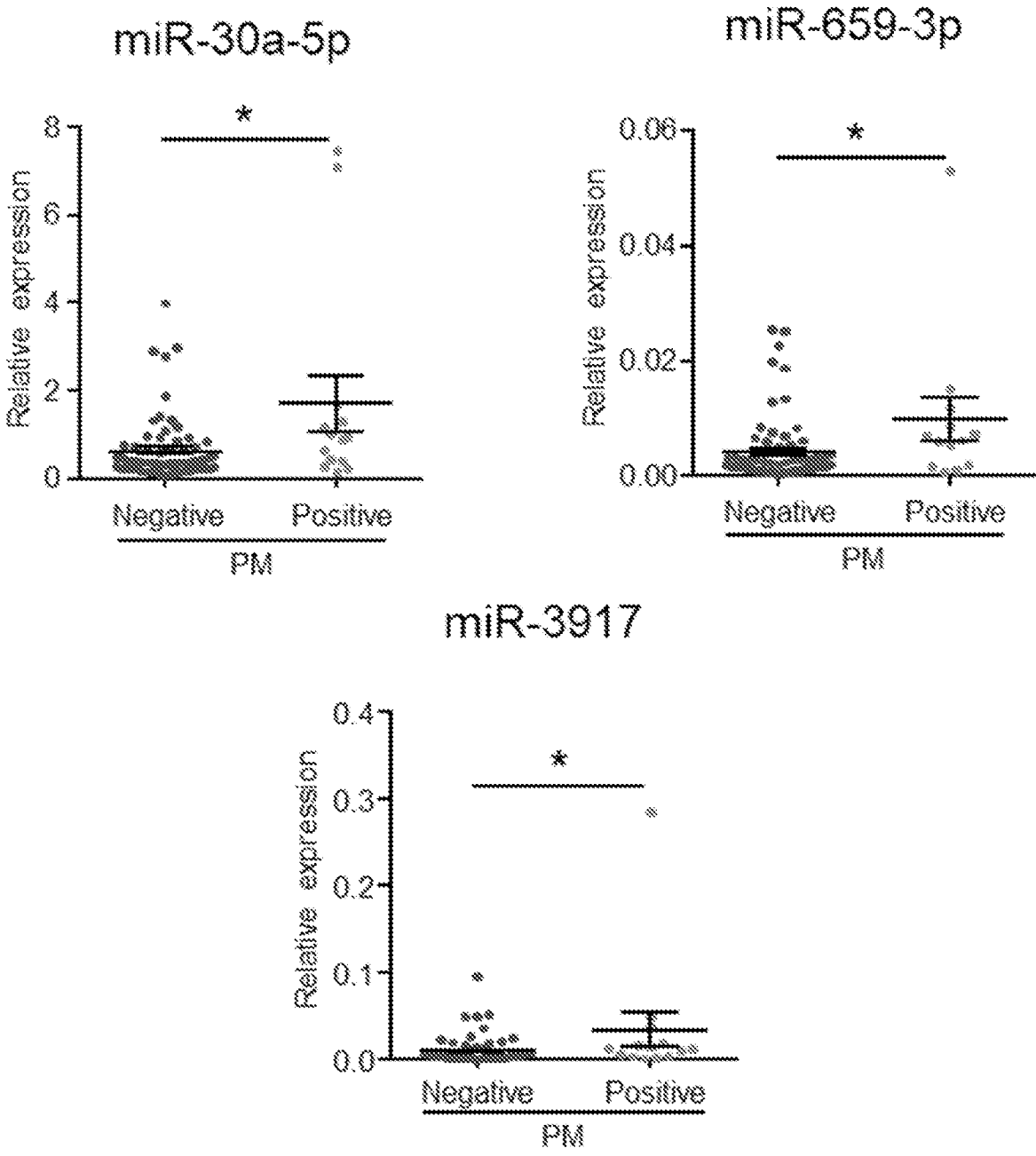


FIG. 4A

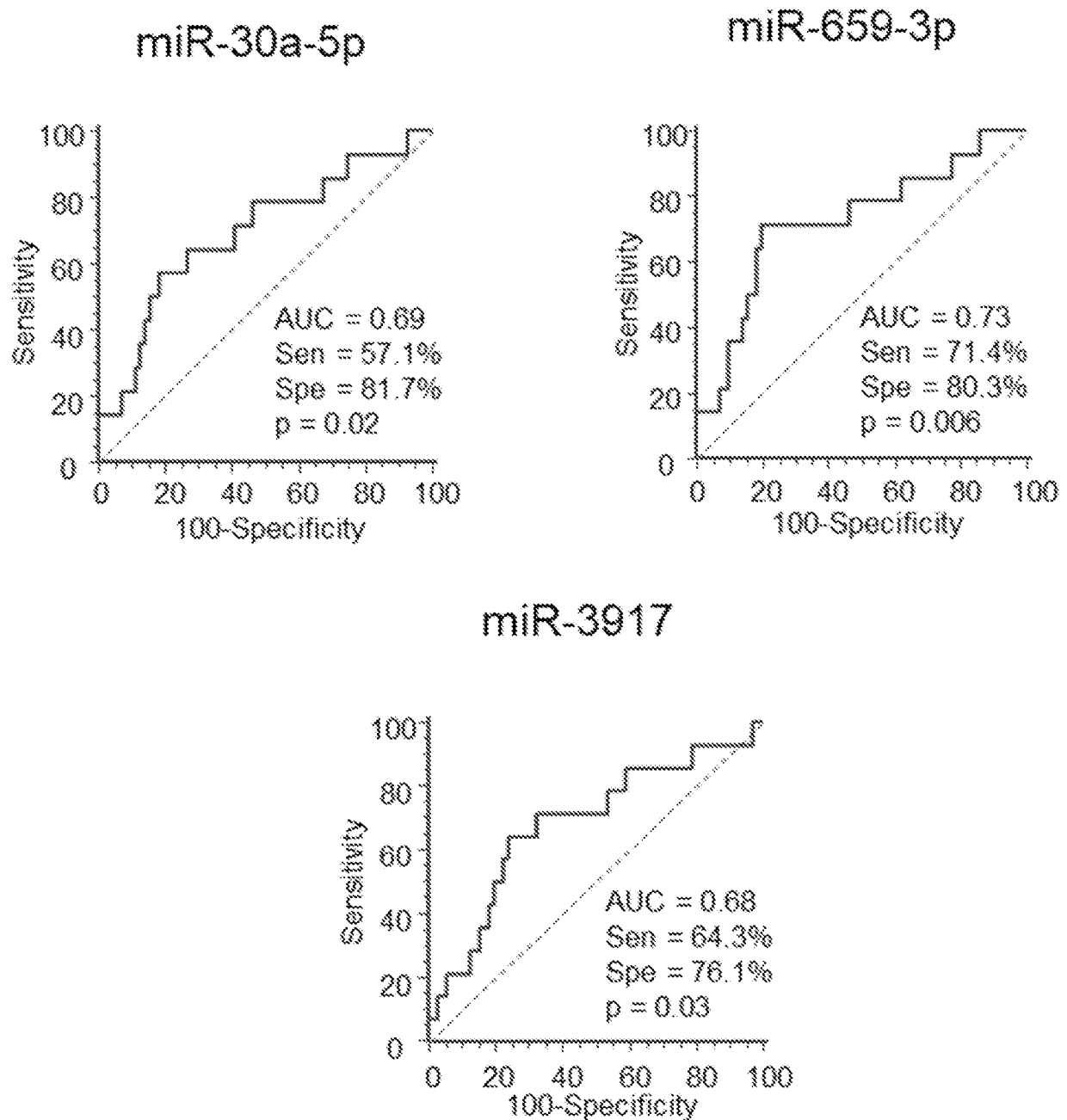


FIG. 4B

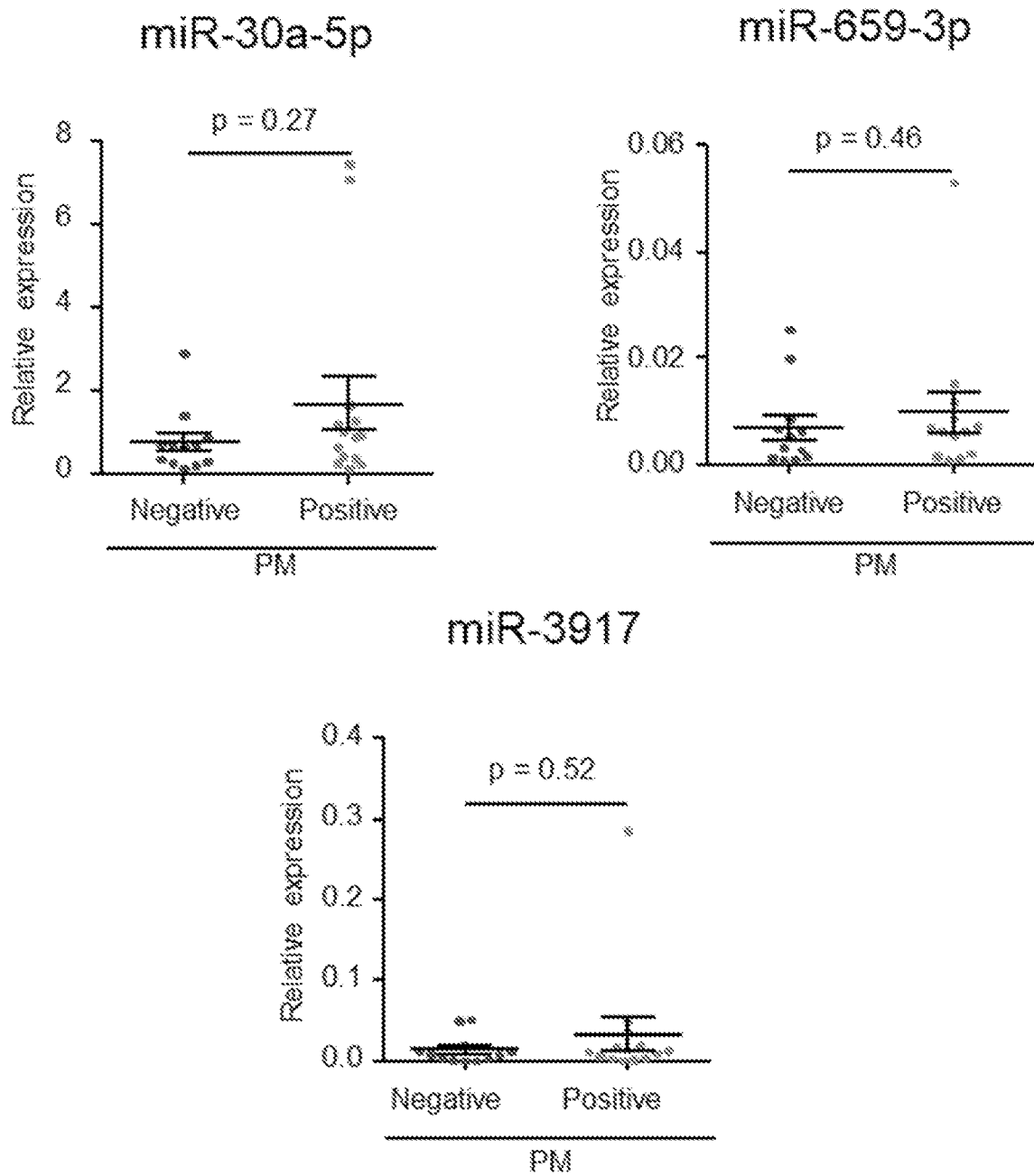
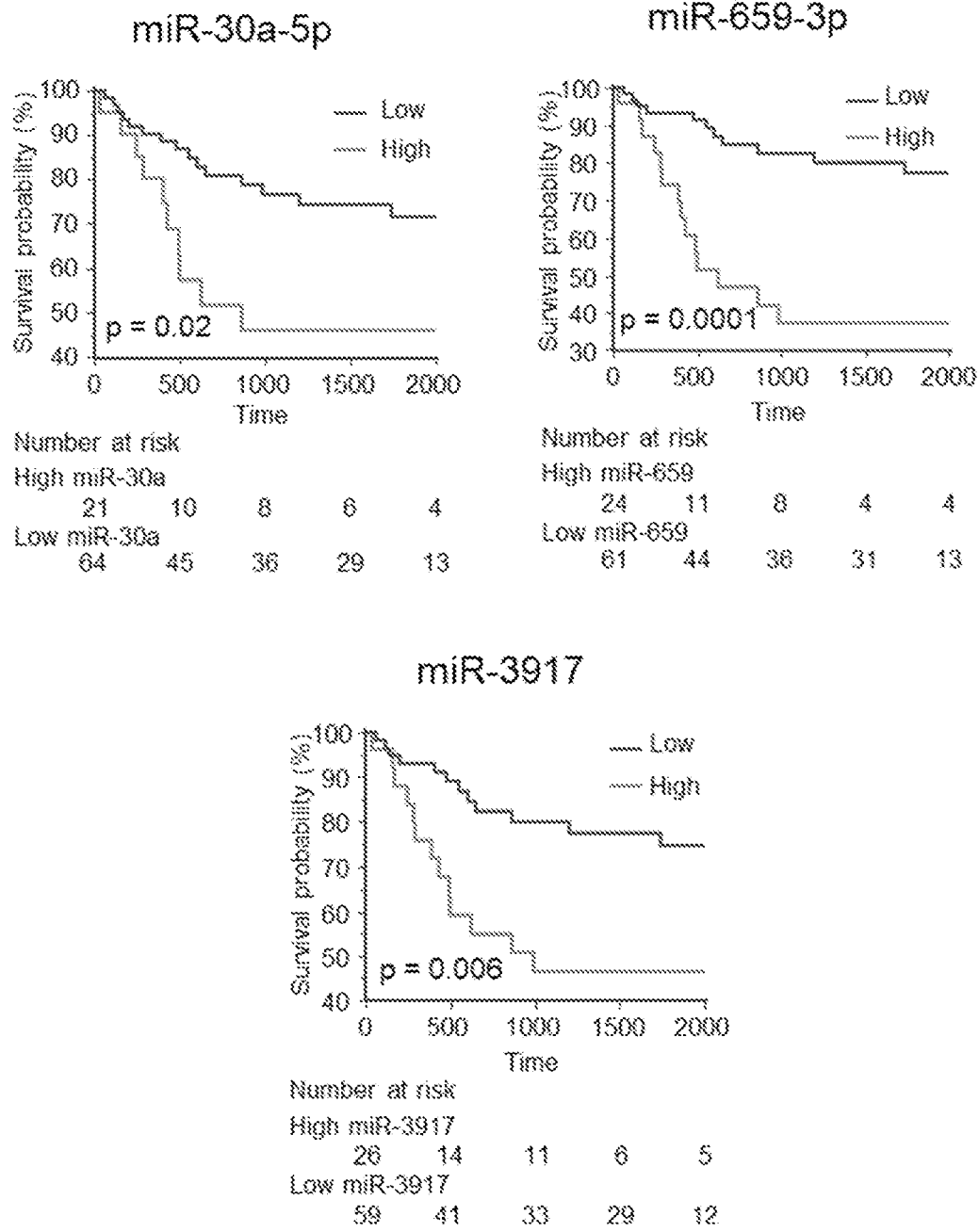


FIG. 4C



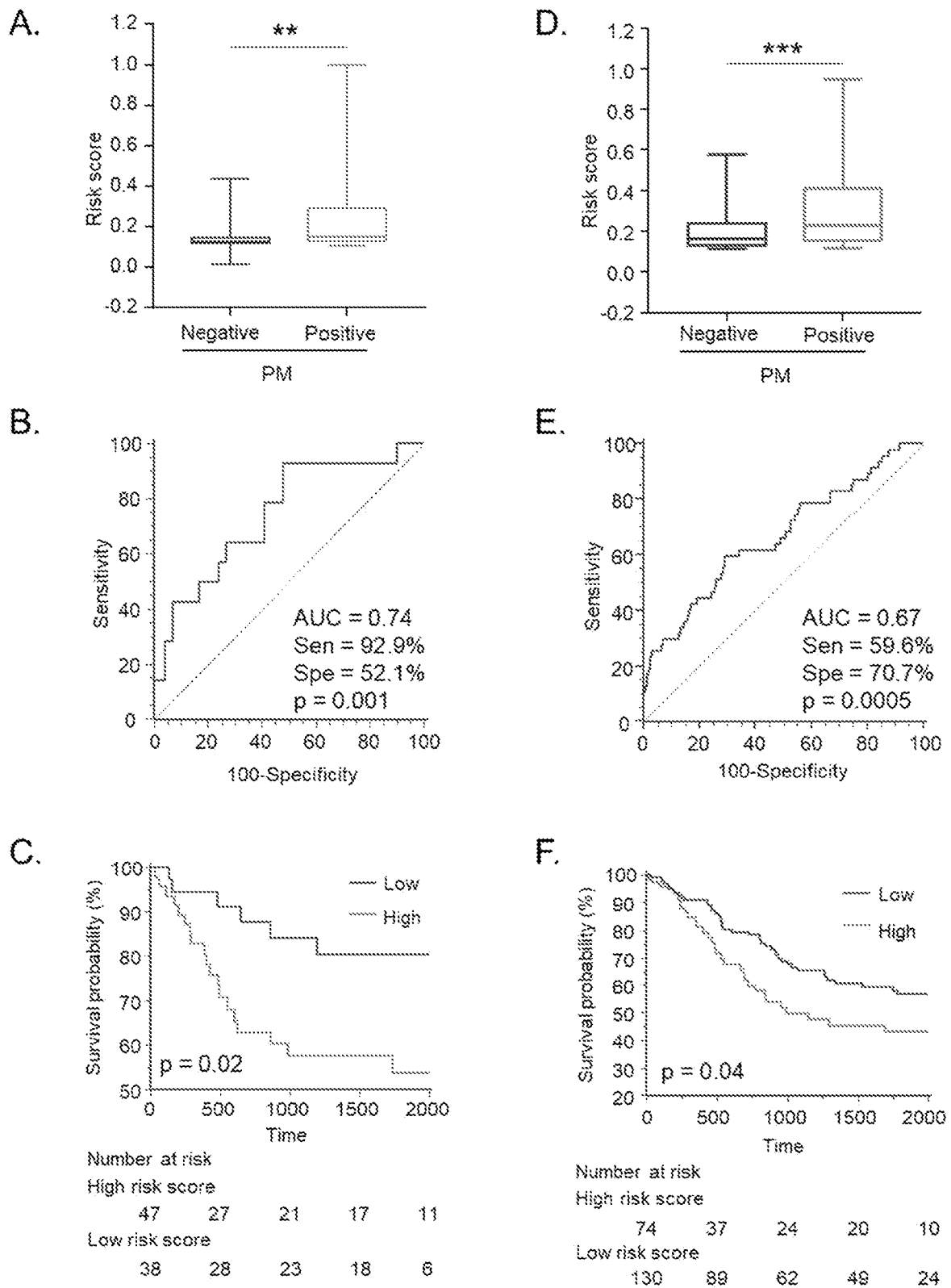


FIG. 5A-F

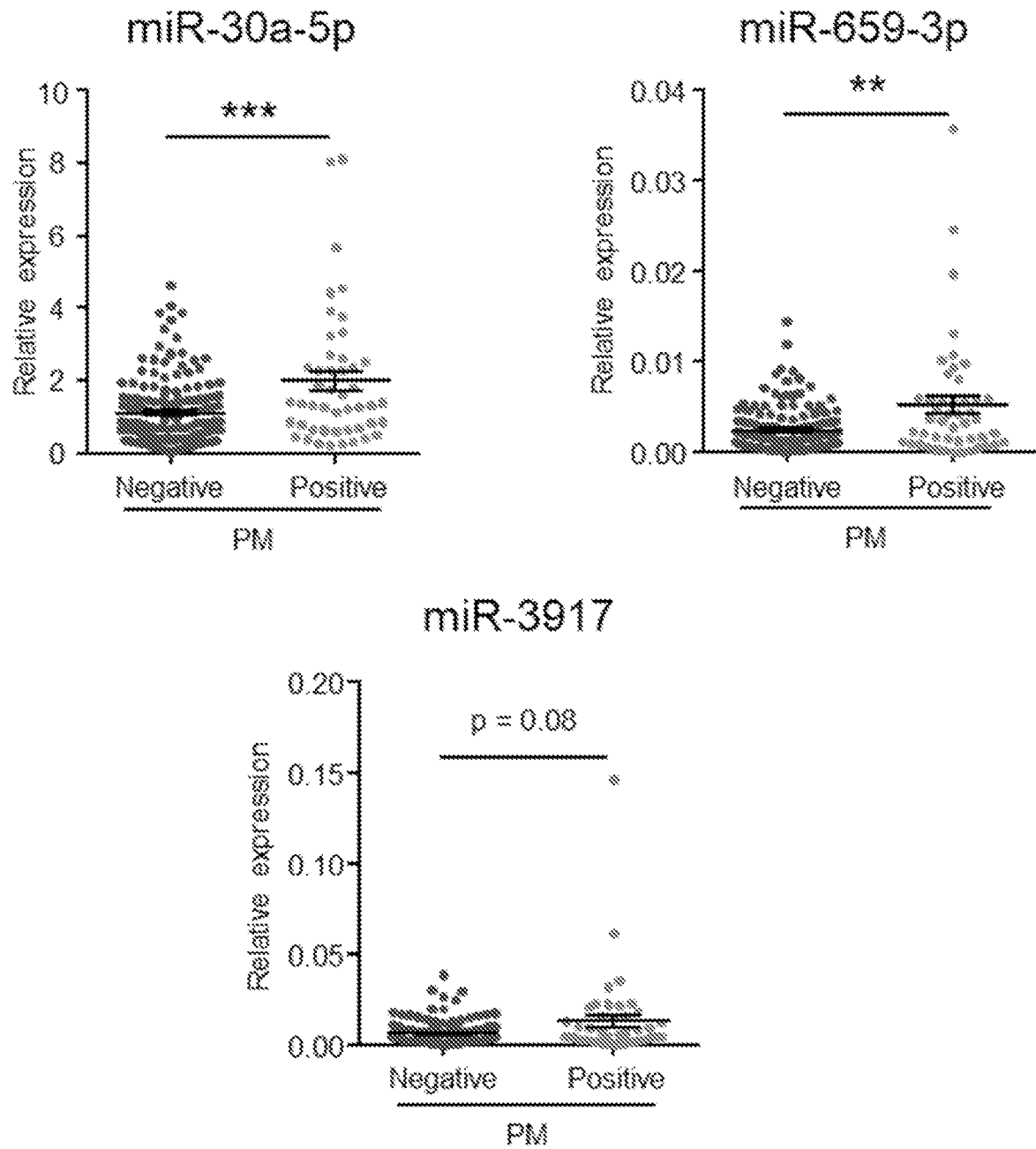


FIG. 6A

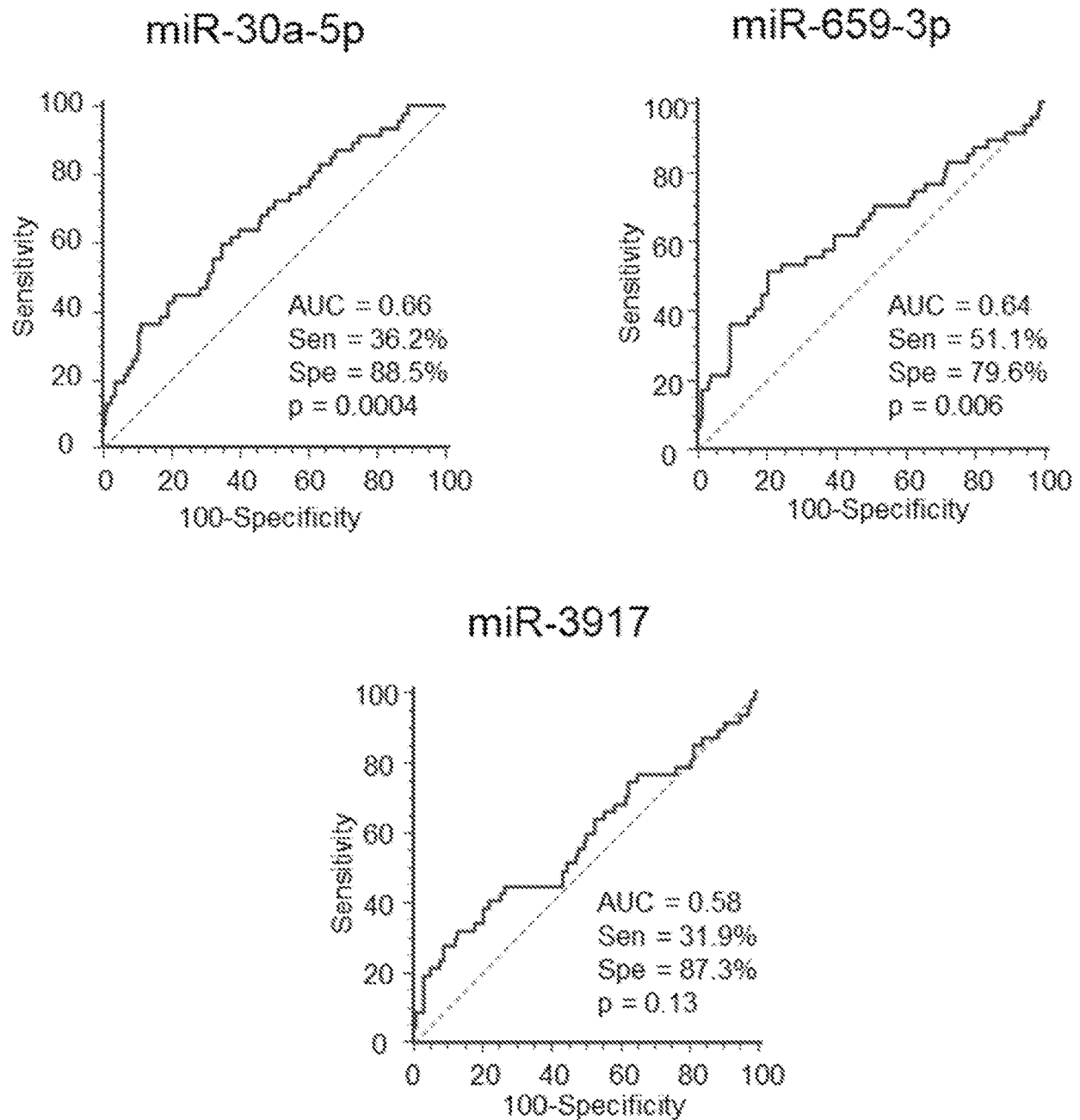


FIG. 6B

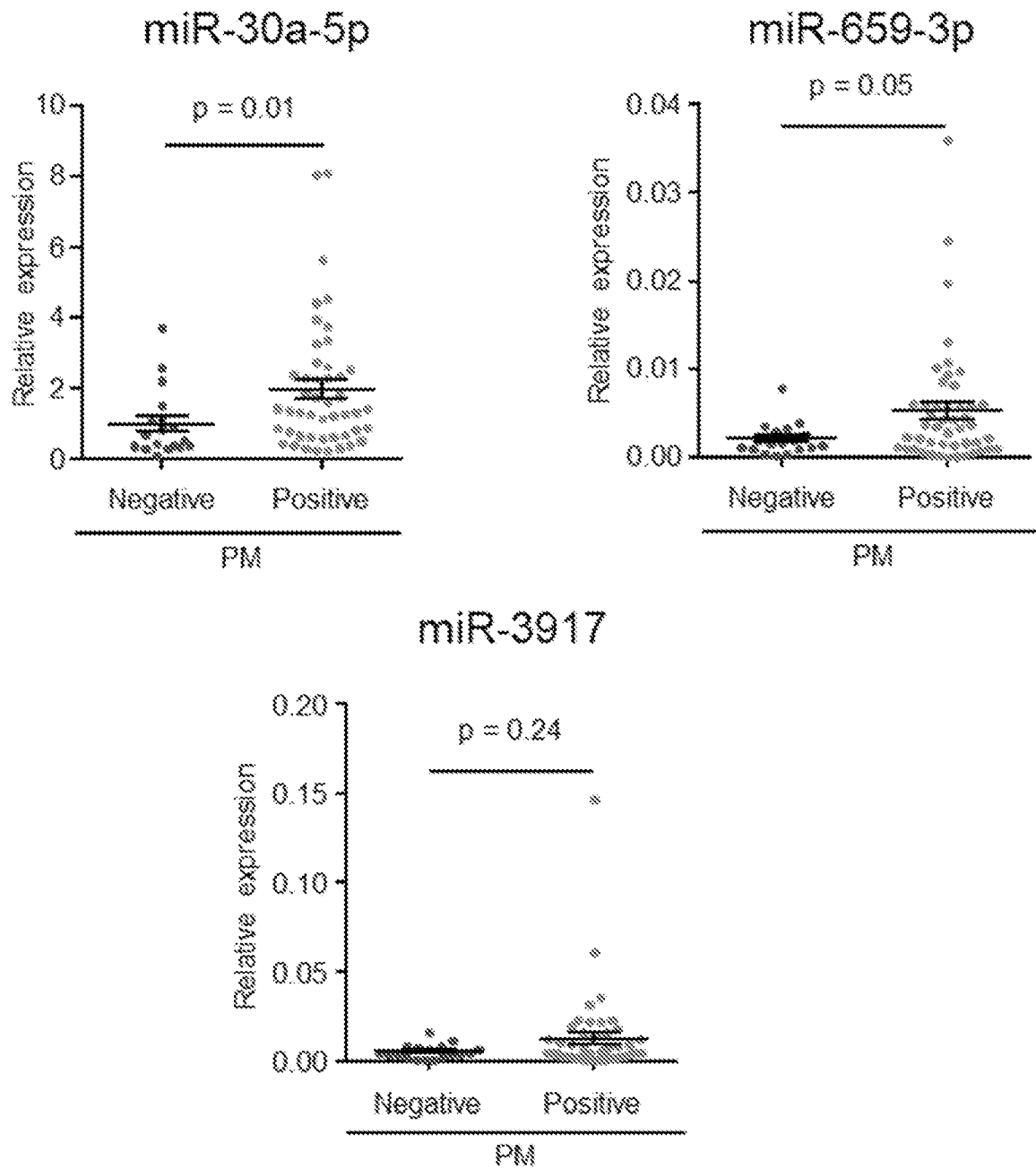


FIG. 6C

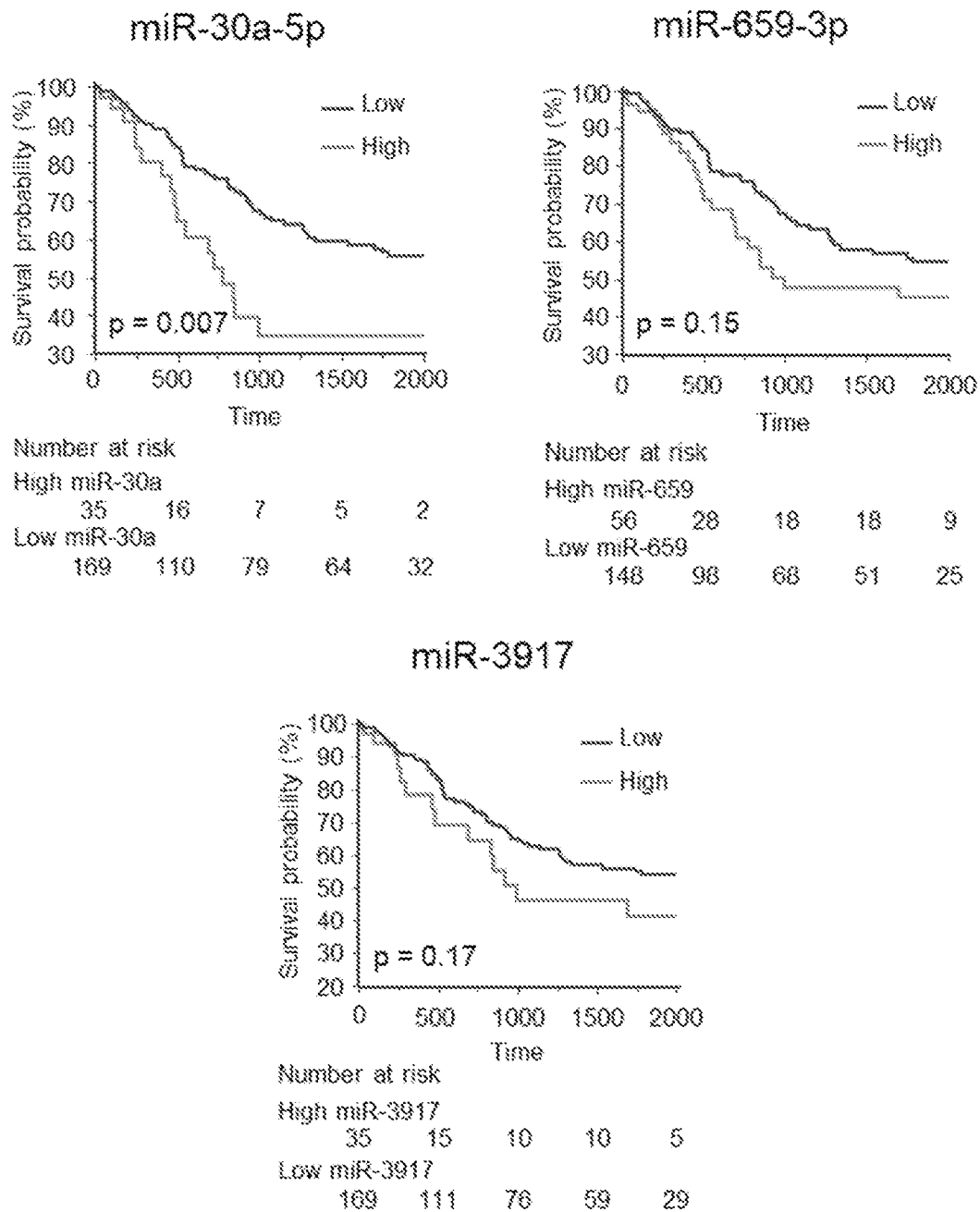
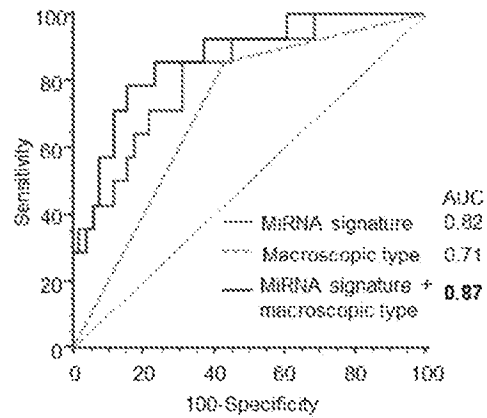
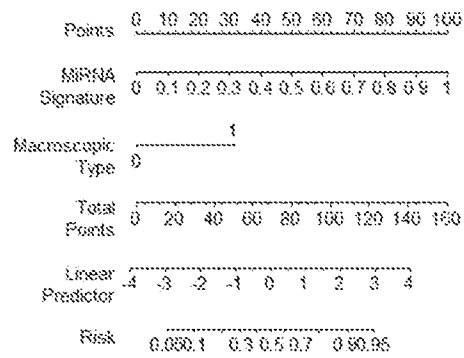


FIG. 6D

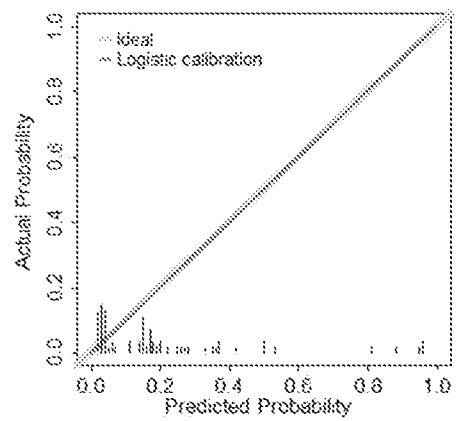
A.



B.



C.



D.

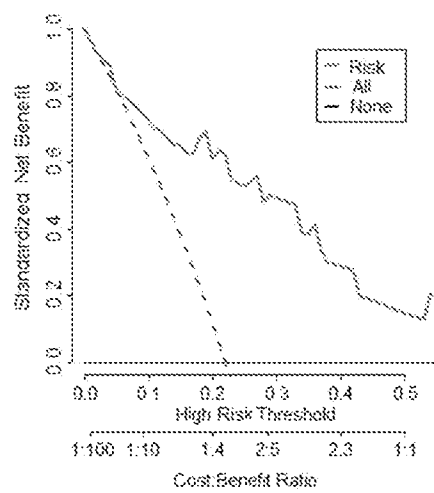


FIG. 7A-D

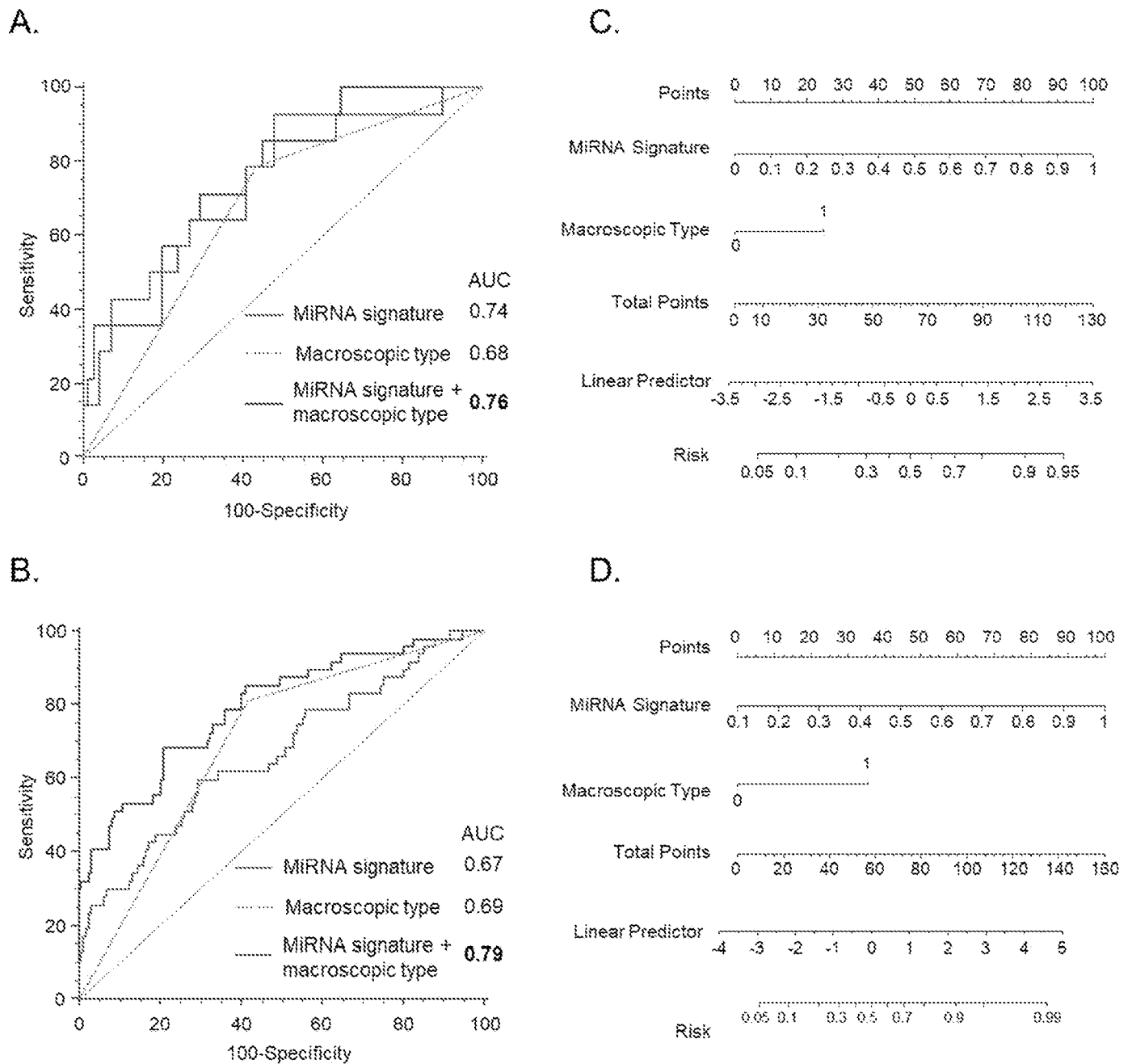


FIG. 8A-D

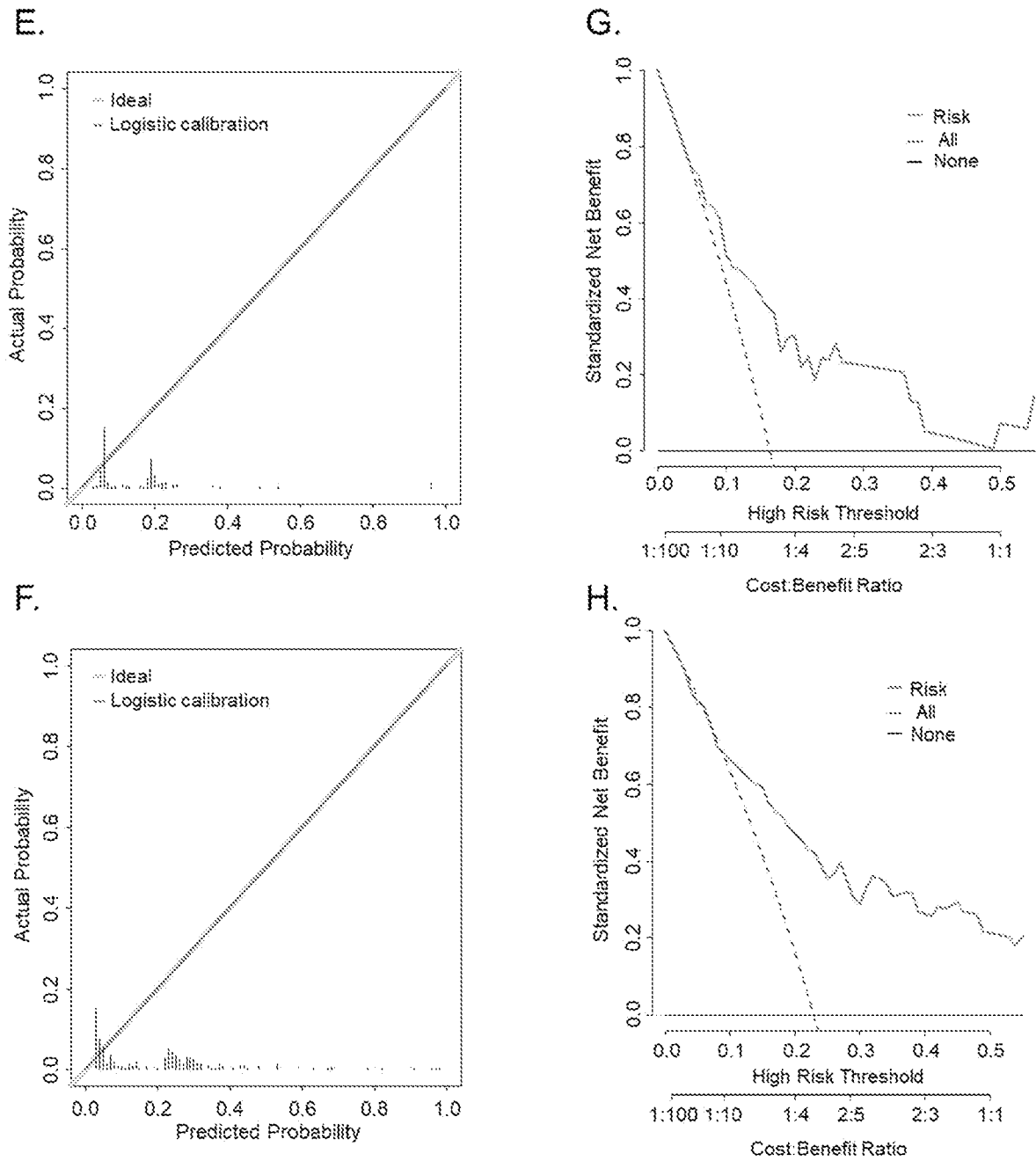


FIG. 8E-H

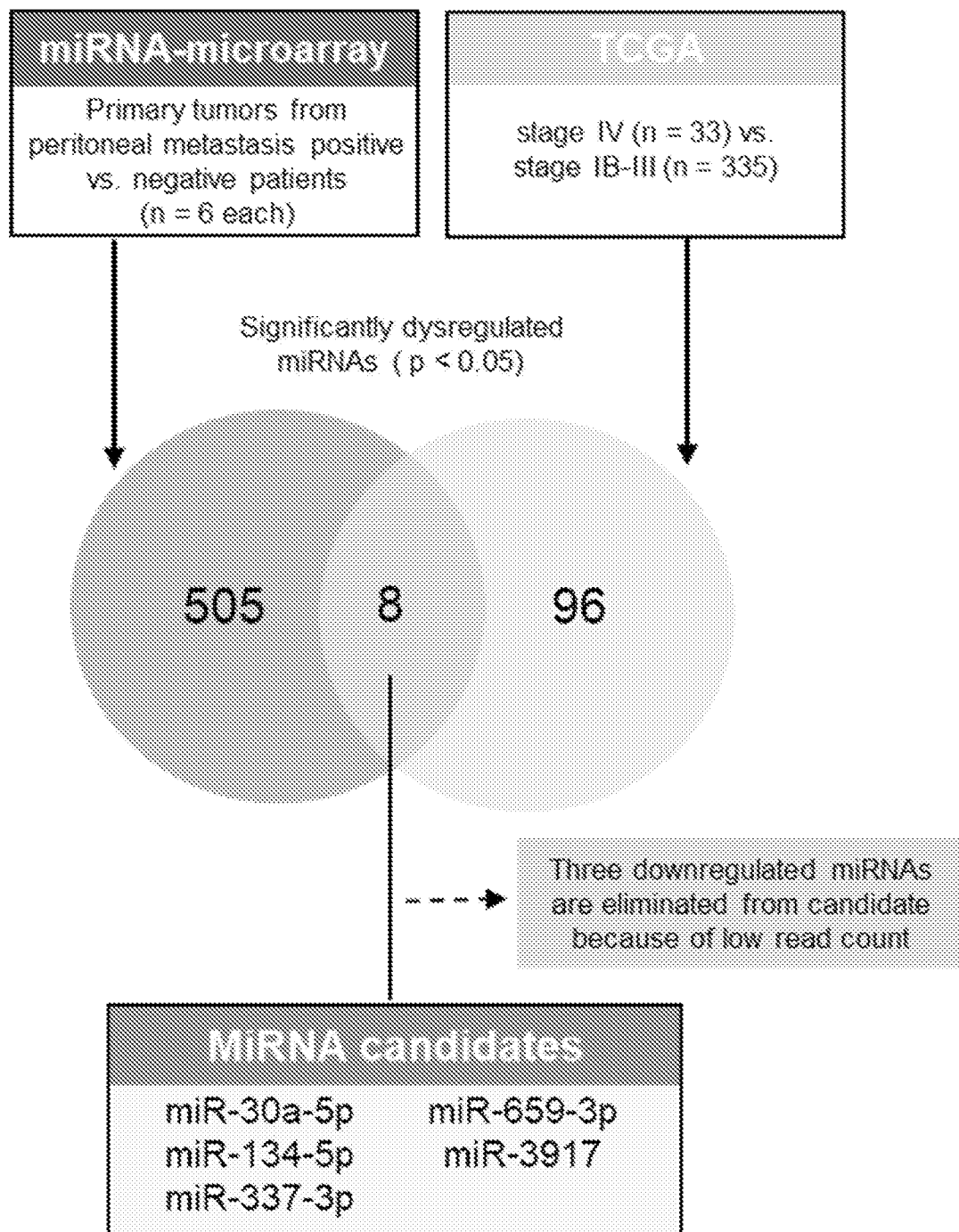


FIG. 9A

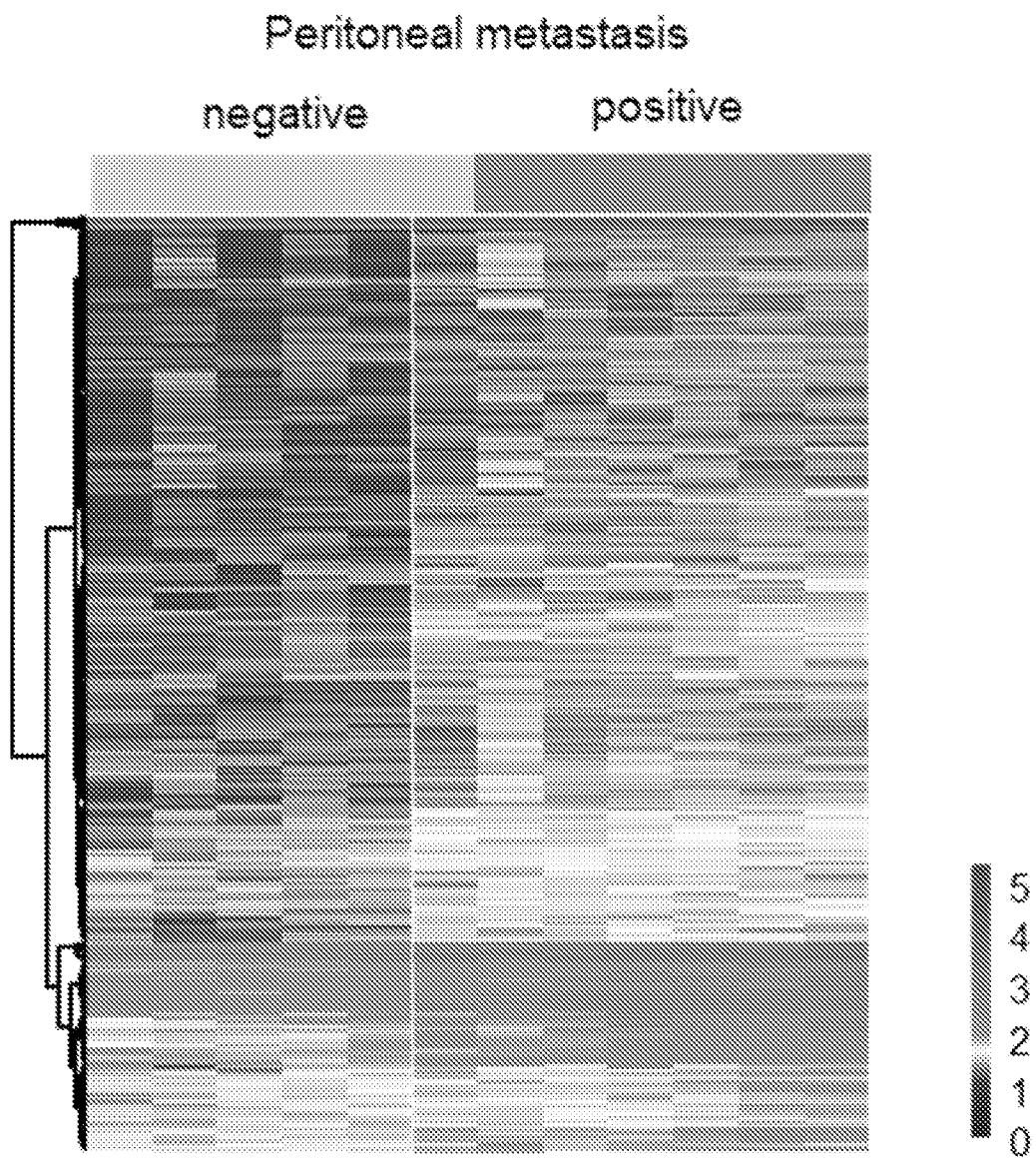


FIG. 9B

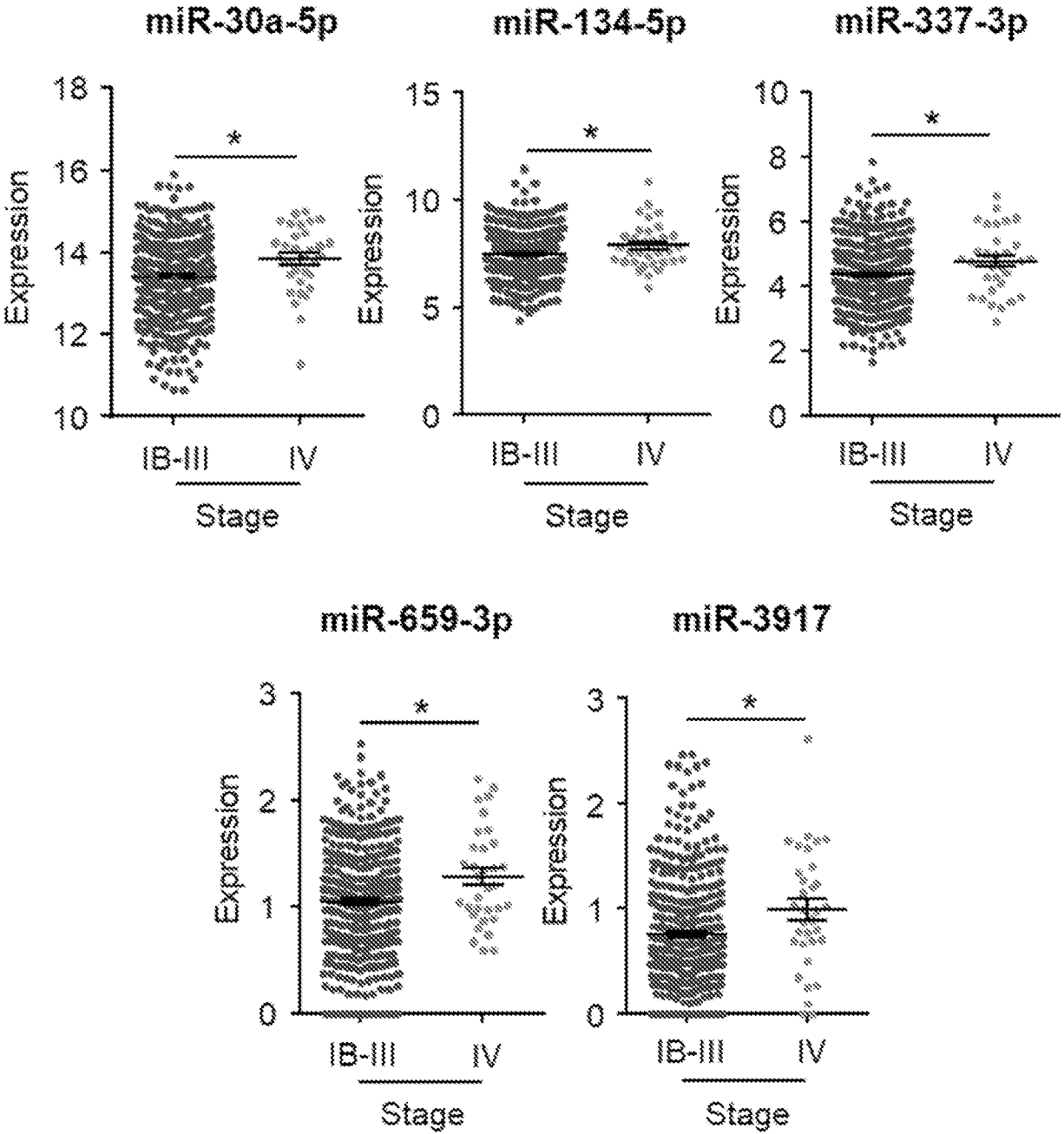


FIG. 9C

Cohort 1

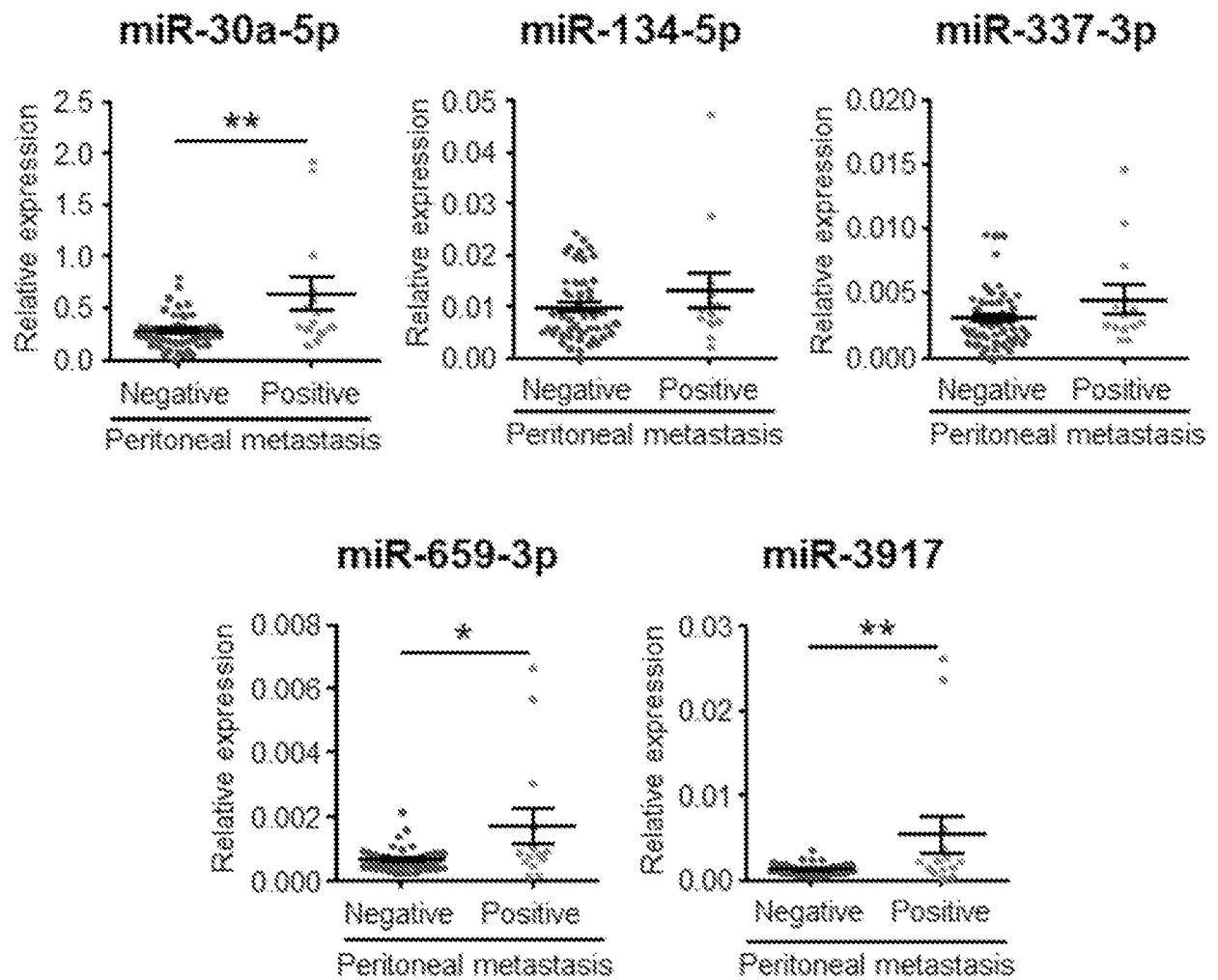


FIG. 10A

Cohort 1

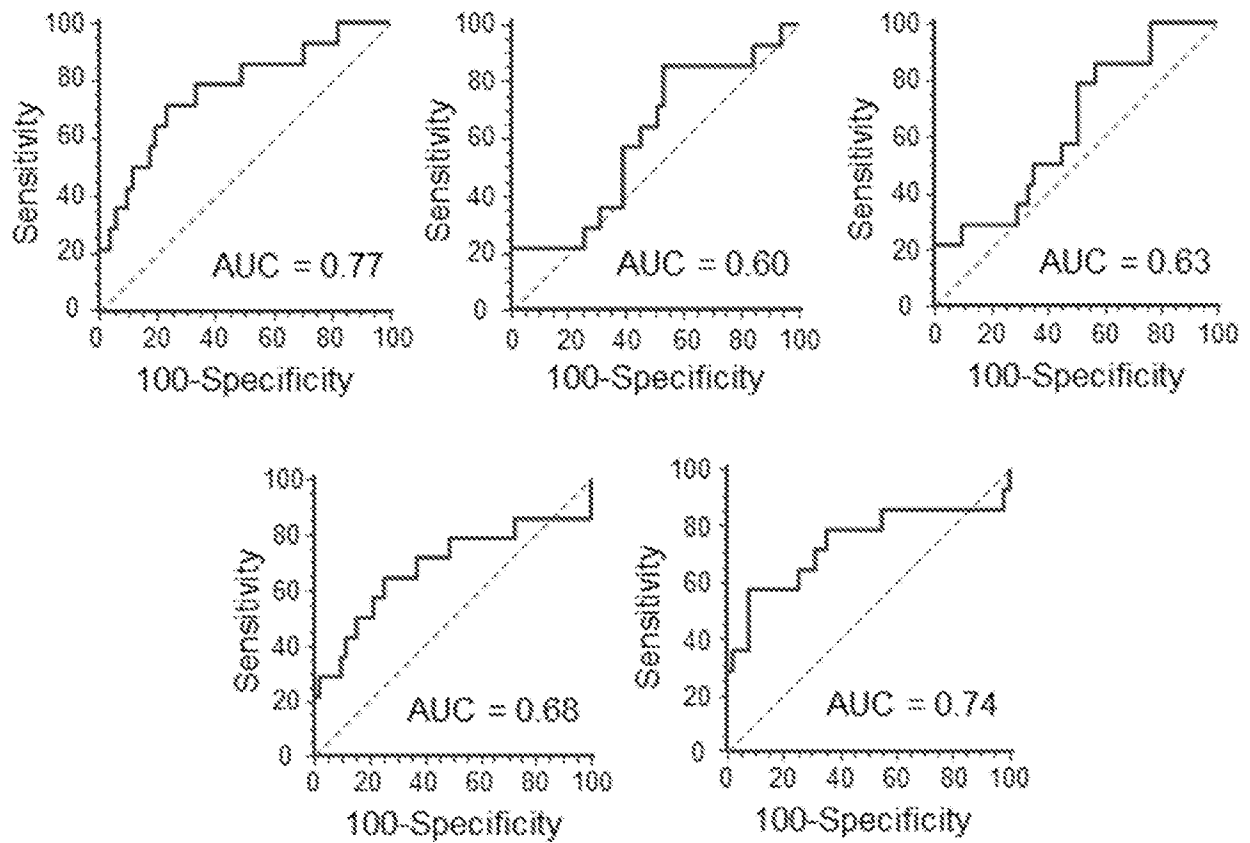


FIG. 10B

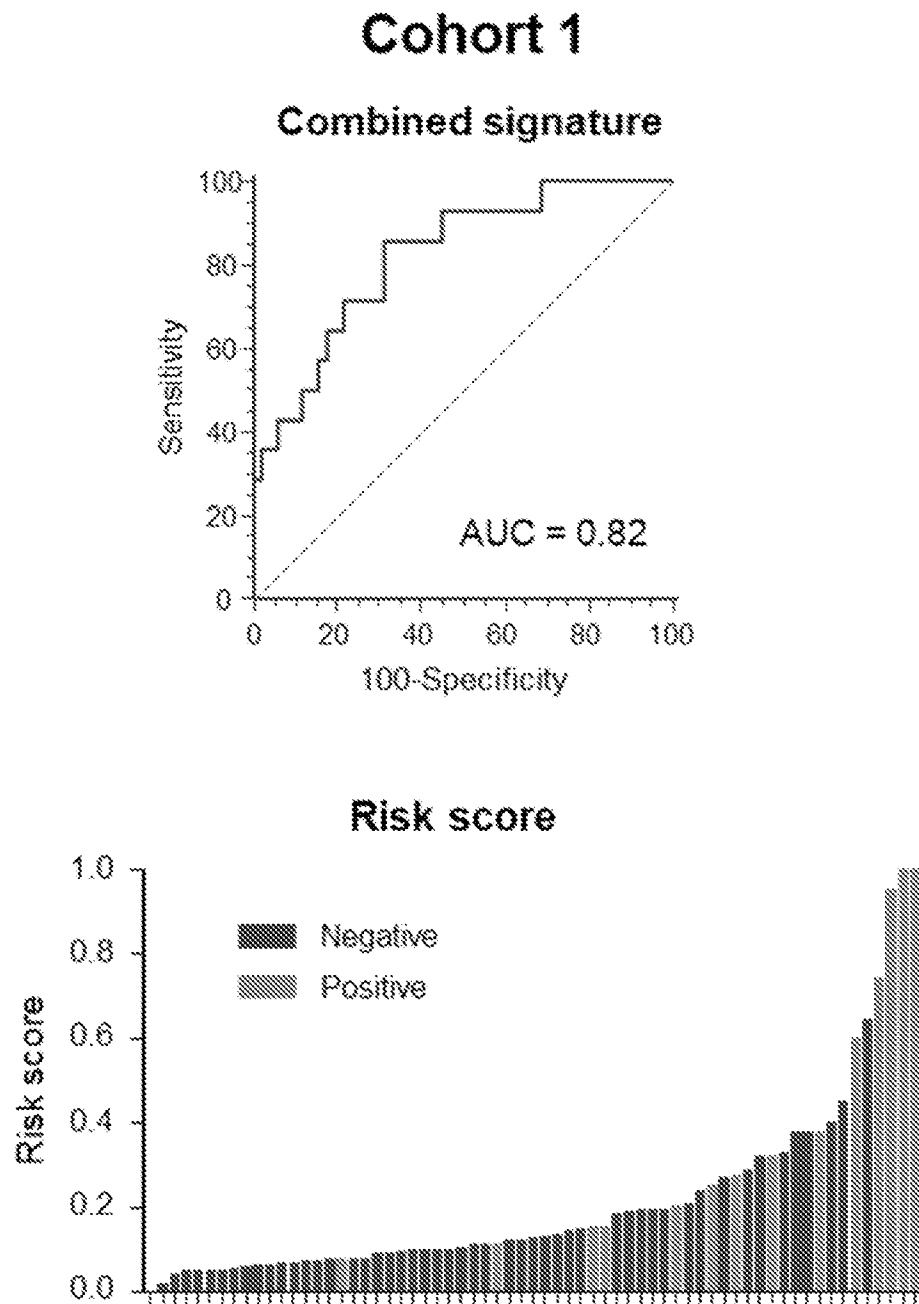


FIG. 10C

Cohort 1

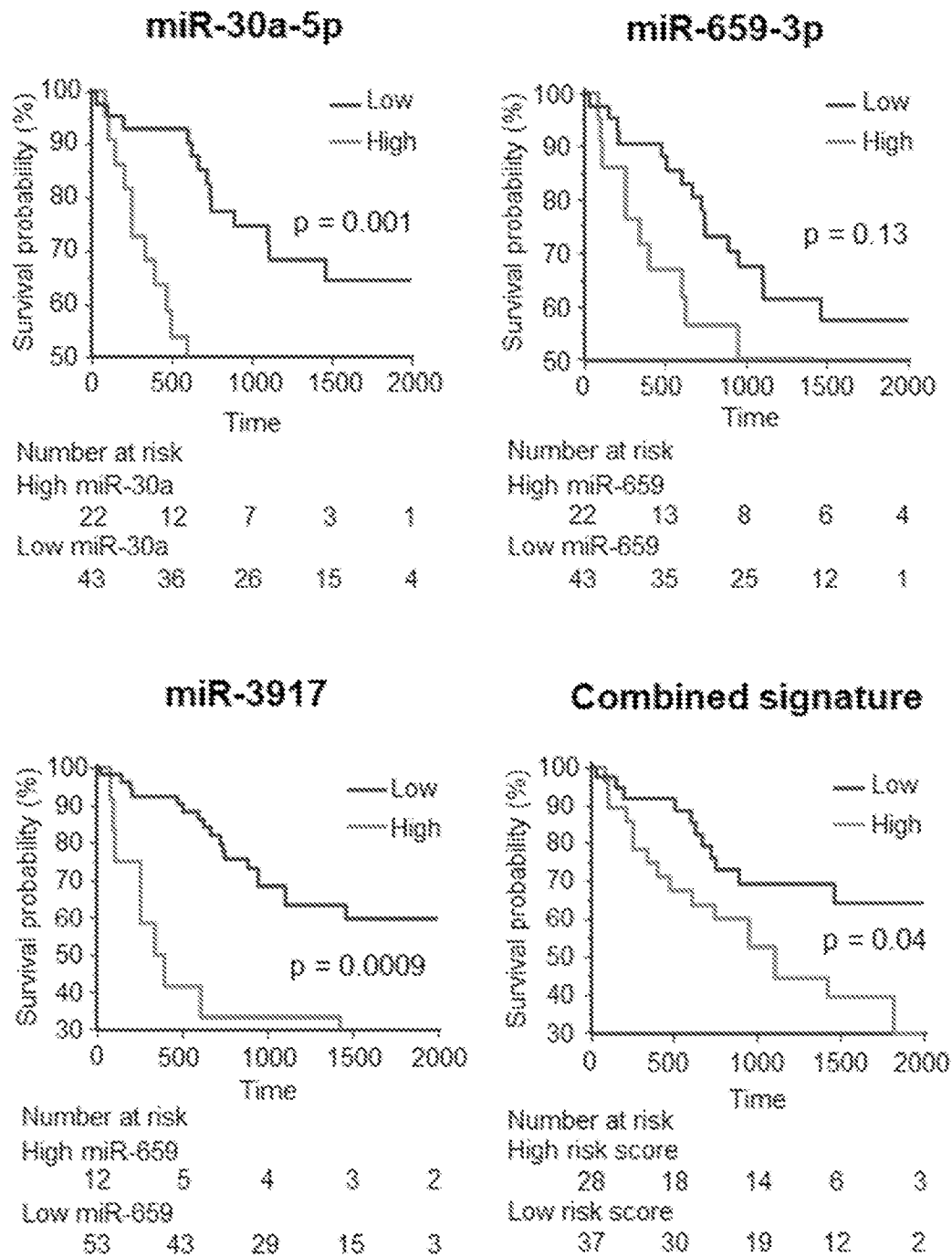


FIG. 10D

Cohort 1

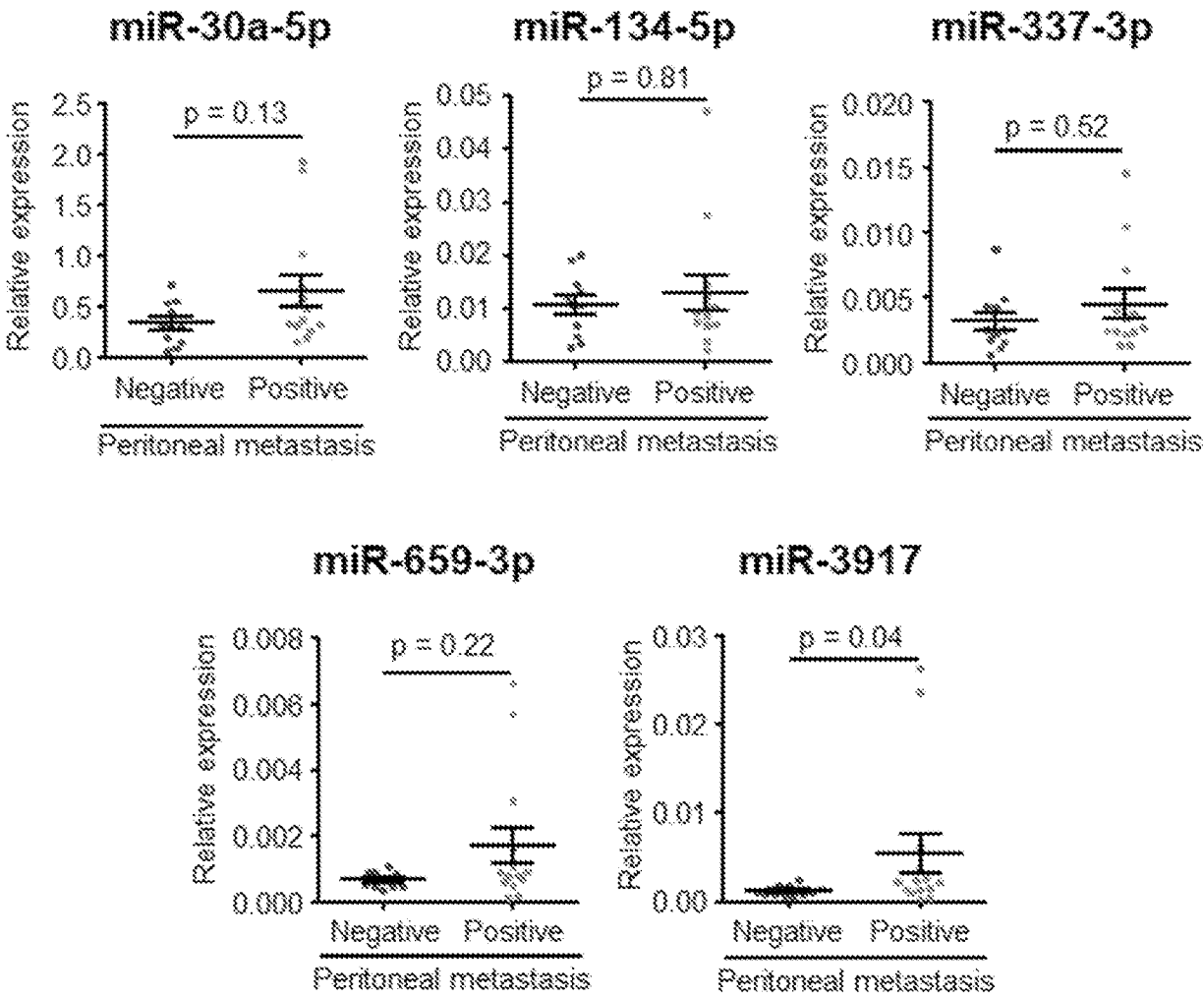
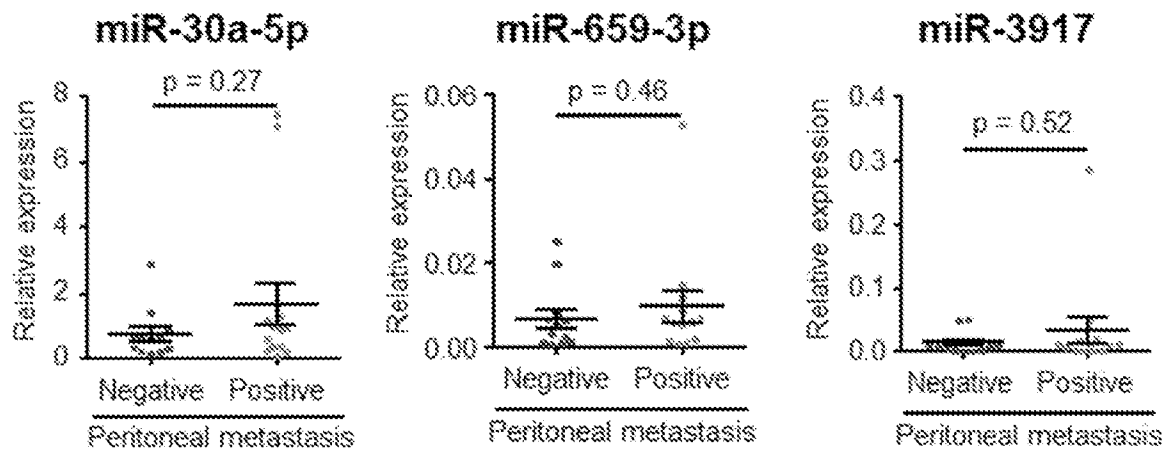


FIG. 11A

B

Cohort 2



C

Cohort 3

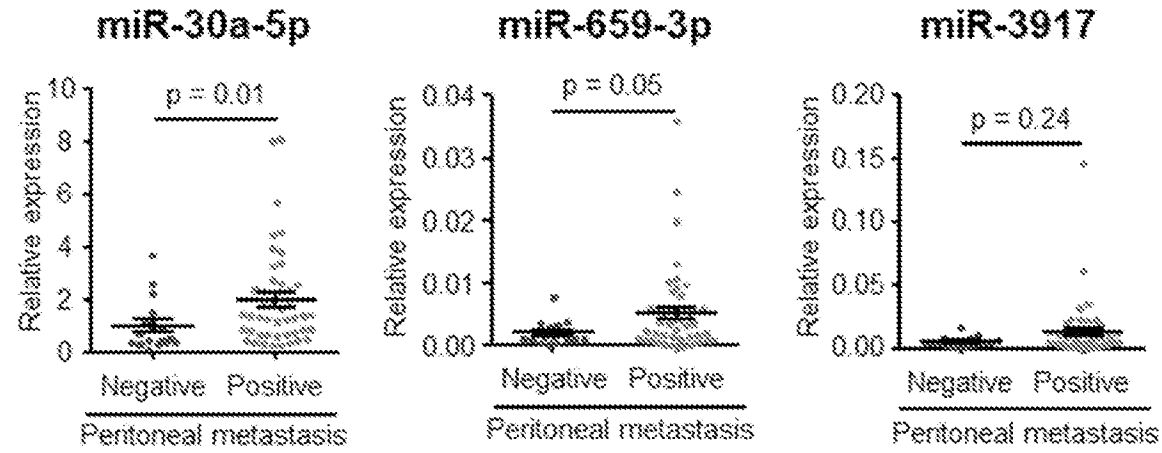
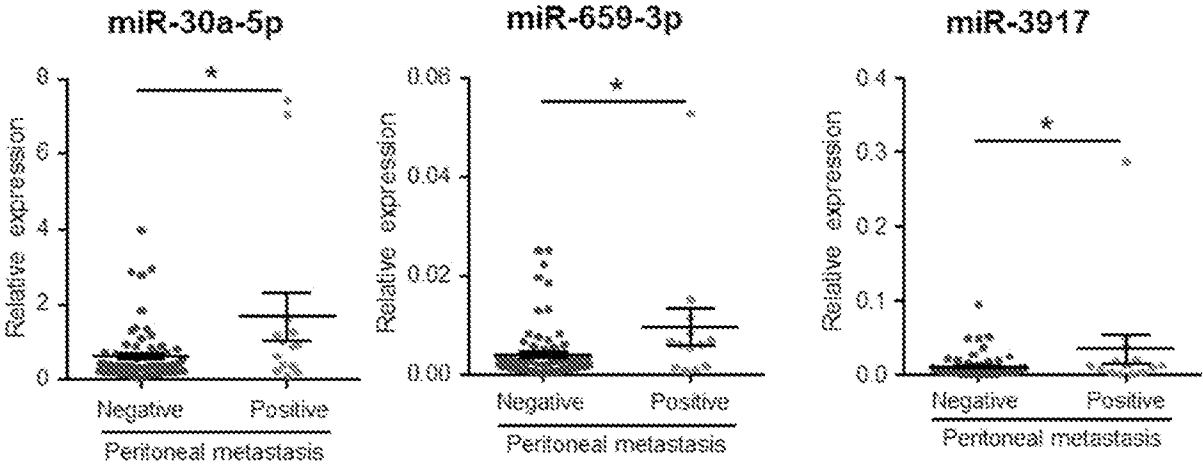


FIG. 11B-C

Cohort 2

A



B

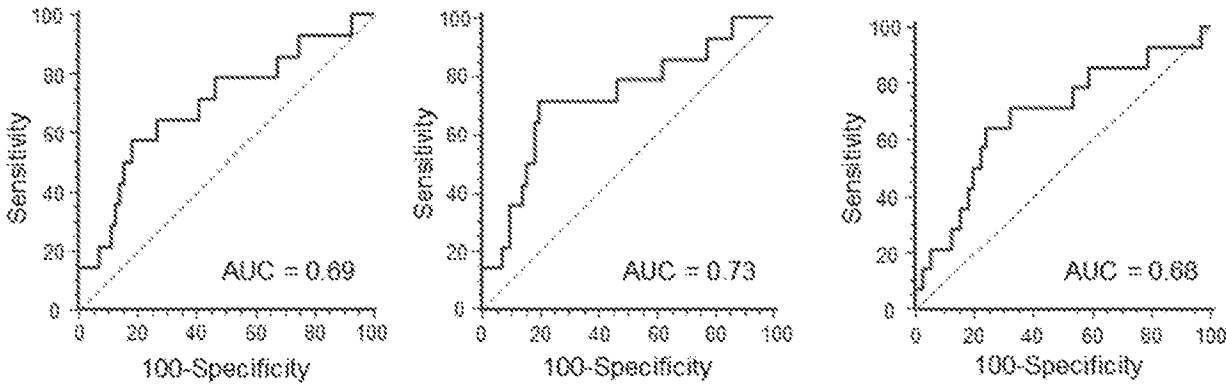


FIG. 12A-B

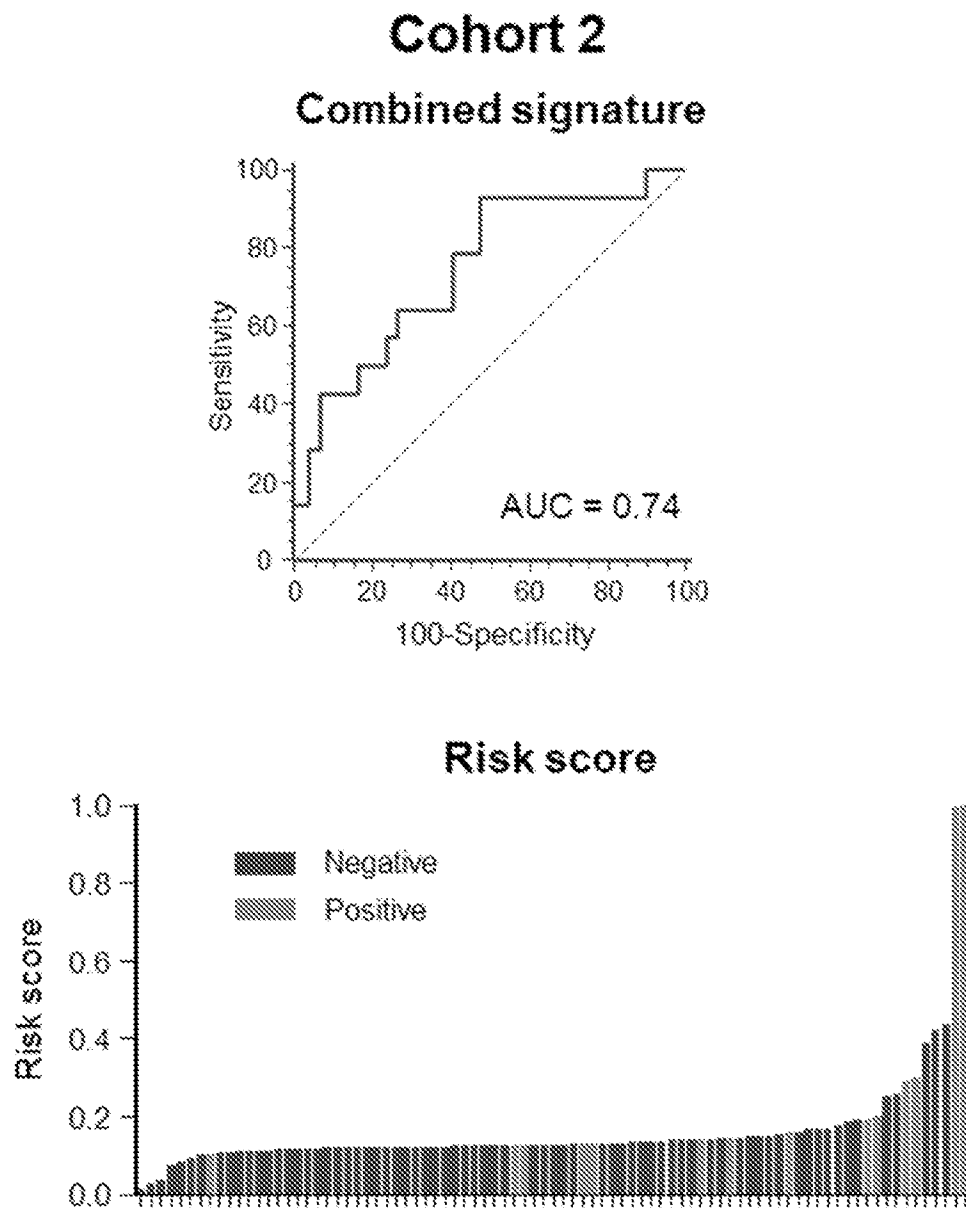


FIG. 12C

Cohort 2

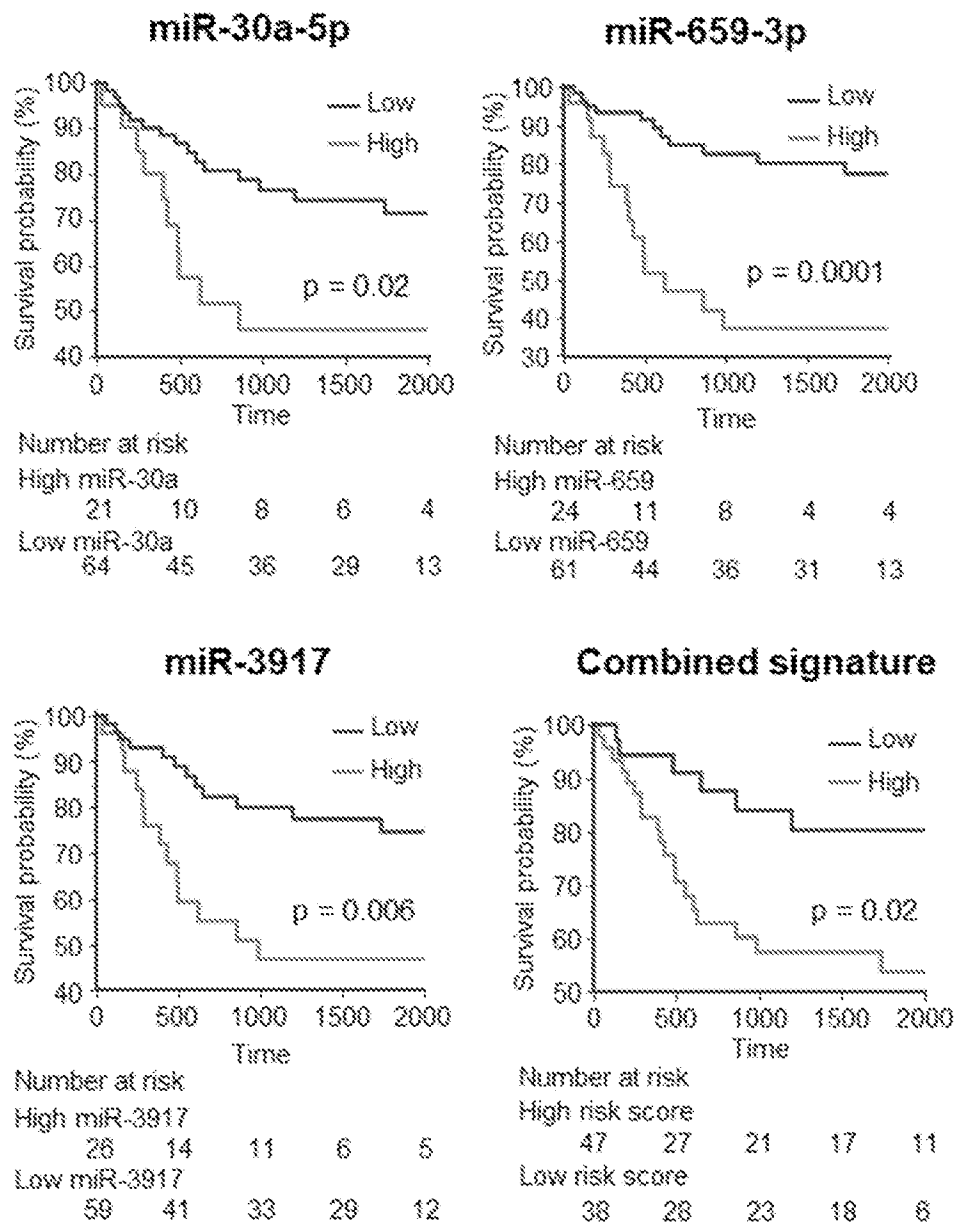


FIG. 12D

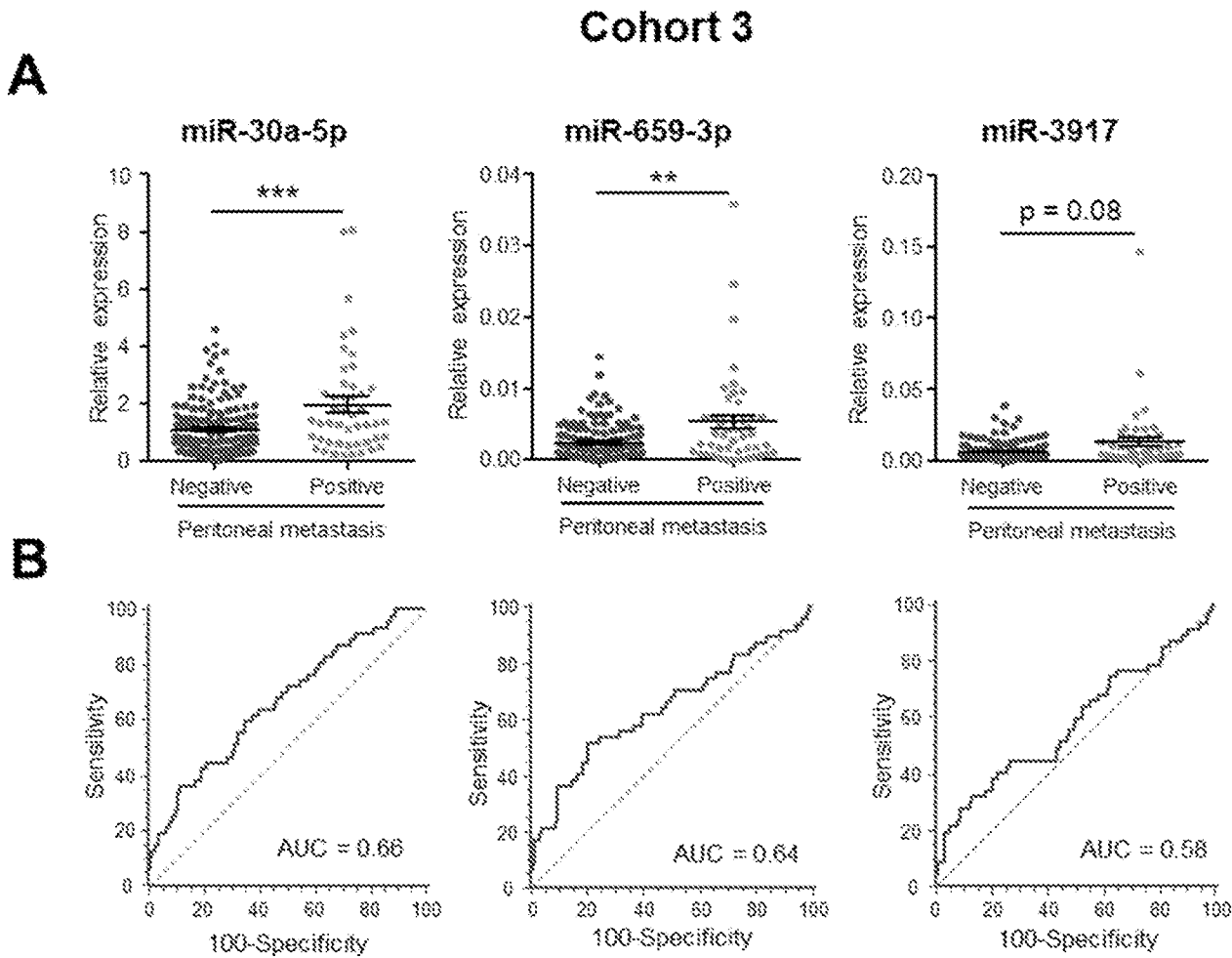


FIG. 13A-B

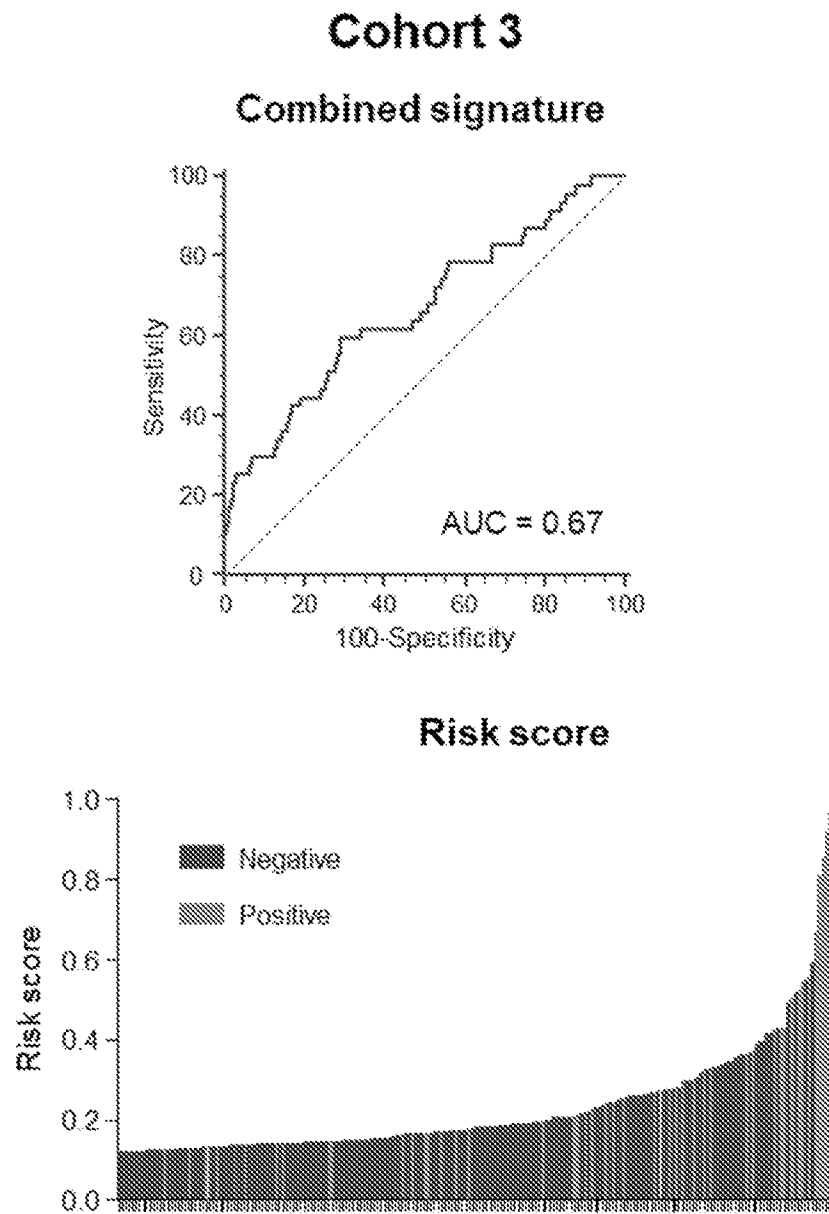


FIG. 13C

Cohort 3

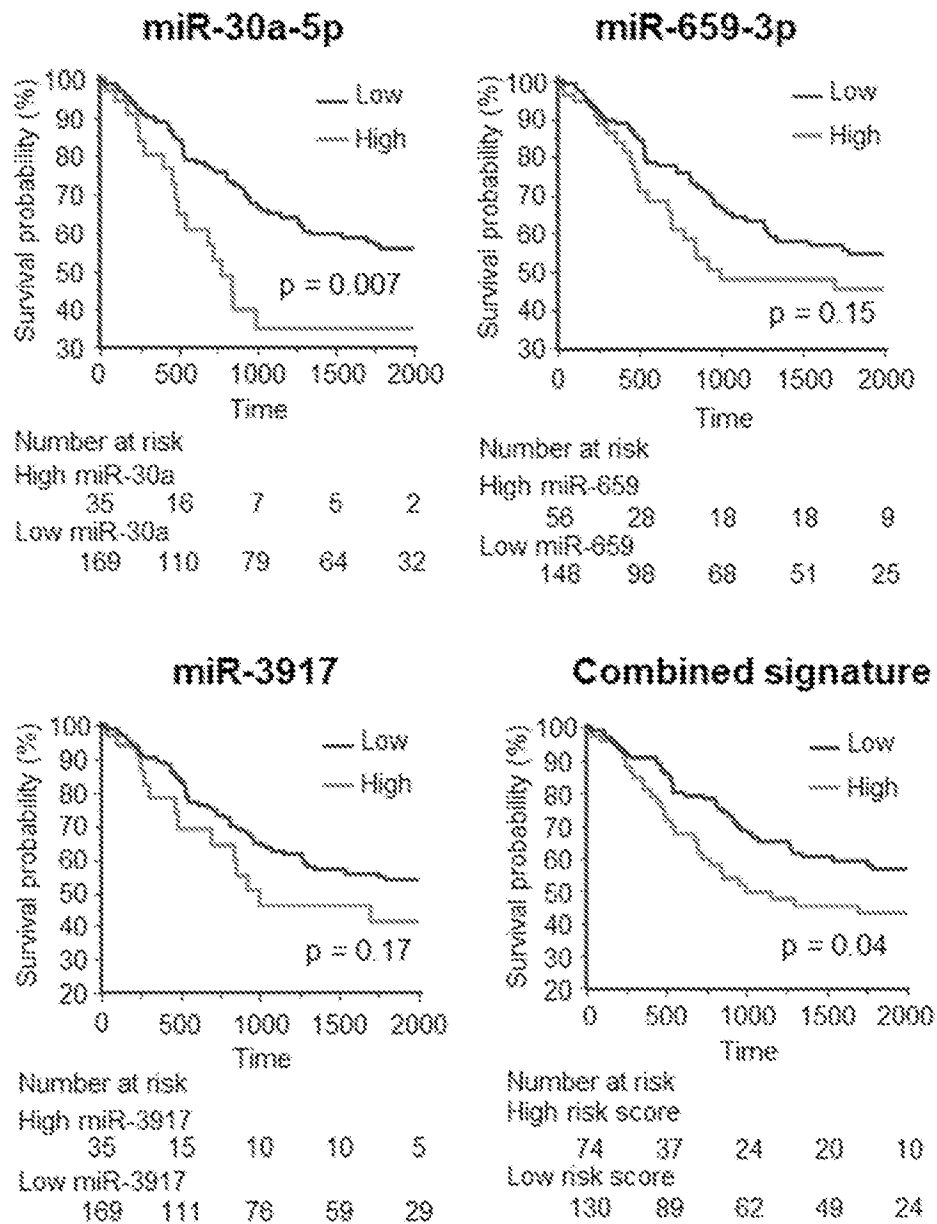


FIG. 13D

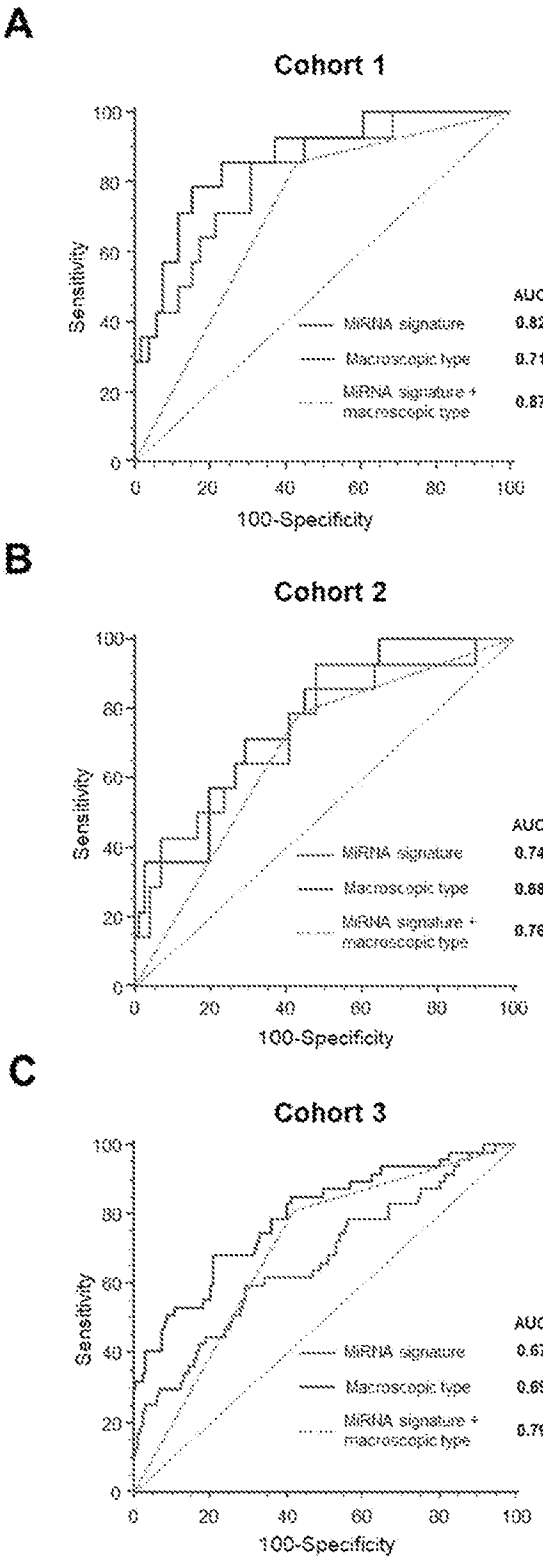


FIG. 14A-C