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(54) Title: PROGNOSTIC MICRO-RNA SIGNATURE FOR SARCOMA

(57) Abstract: There is provided a prognostic micro-RNA signature for sarcoma comprising at least one of Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132.

PROGNOSTIC MICRO-RNA SIGNATURE FOR SARCOMA

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Patent Application No. 61/835,743 filed June 17, 2013, incorporated herein by reference.

FIELD OF THE INVENTION

The invention relates to a micro-RNA signature for sarcoma.

BACKGROUND

Sarcomas are cancers of mesenchymal origin which represent 2% of human malignancies[1]. One of the most common STS subtype is the undifferentiated pleomorphic sarcoma (UPS) which is amongst the most aggressive STS with a high propensity for metastasis; associated with a dismal 5-year overall survival of 30-50%[2-4]. The prognostic determinants in STS are grade, tumor size and surgical margin[5], which are however not useful in determining who may benefit from chemotherapy[6,7]. Thus, there is a need to develop novel biomarkers, which will provide both insights into the complex biology of UPS, and facilitate individualization of cancer therapy.

MicroRNAs (miRNA) are small non-coding RNA molecules of ~22-nucleotides that form one of the largest class of gene regulators by targeting up to 60% of the mRNAs to translational repression or degradation. There have been three studies describing miRNA expression patterns for a diverse group of sarcomas. These studies, which ranged from 27 to 270 samples of 5-22 different STS histologies, demonstrated that miRNA expression profiling can differentiate some STS histologies[8-10]. Hisaoka *et al.* further functionally characterized miRNAs specific to synovial sarcomas, demonstrating that modulating miR-let-7e and miR-99b levels affected downstream gene targets and suppressed cell proliferation in vitro, suggesting a potential role for these miRNAs in STS[9]. To date however, there have not been reports of miRNA profiling of STS in relation to clinical outcome.

SUMMARY OF THE INVENTION

In an aspect, there is provided a method of prognosing or classifying a subject with undifferentiated pleomorphic sarcoma (UPS) comprising: (a) determining the expression of at least one biomarker in a test sample from the subject selected from Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132; and (b) comparing expression of the at least one biomarker in the test sample with expression of the at least one biomarker in a control sample; wherein a difference or similarity in the expression of the at least one biomarker between the control and the test sample is used to prognose or classify the subject with UPS into a low risk group or a high risk group of developing metastasis.

In an aspect, there is provided a method of selecting a therapy for a subject with UPS, comprising the steps: (a) classifying the subject with UPS into a high risk group or a low risk group according to the method described herein; and (b) selecting a more aggressive therapy, preferably adjuvant chemotherapy or radiation therapy, for the high risk group or a less aggressive therapy, preferably no adjuvant chemotherapy or no radiation therapy, for the low risk group.

In an aspect, there is provided a method of selecting a therapy for a subject with UPS, comprising the steps: (a) determining the expression of at least one biomarker in a test sample from the subject selected from Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132; (b) comparing expression of the at least one biomarker in the test sample with expression of the at least one biomarker in a control sample; (c) classifying the subject in a high risk group or a low risk group, wherein a difference or a similarity in the expression of the at least one biomarker between the control sample and the test sample is used to classify the subject into a high risk group or a low risk group; (d) selecting a more aggressive therapy, preferably adjuvant chemotherapy or radiation therapy, for the high risk group or a less aggressive therapy, preferably no adjuvant chemotherapy or no radiation therapy, for the low risk group.

In an aspect, there is provided a composition comprising a plurality of isolated nucleic acid sequences, wherein each isolated nucleic acid sequence hybridizes to: (a) Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132; and/or (b)a nucleic acid complementary to a), wherein the composition is used to measure the level of Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132 expression.

In an aspect, there is provided an array comprising, for each of Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132, one or more polynucleotide probes complementary and hybridizable thereto.

In an aspect, there is provided a computer program product for use in conjunction with a computer having a processor and a memory connected to the processor, the computer program product comprising a computer readable storage medium having a computer mechanism encoded thereon, wherein the computer program mechanism may be loaded into the memory of the computer and cause the computer to carry out the method described herein.

In an aspect, there is provided a computer implemented product for predicting a prognosis or classifying a subject with UPS comprising: (a) a means for receiving values corresponding to a subject expression profile in a subject sample; and (b) a database comprising a reference expression profile associated with a prognosis, wherein the subject biomarker expression profile and the biomarker reference profile each have at least one value representing the expression level of at least one biomarker selected from Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132; wherein the computer implemented product selects the biomarker reference expression profile most similar to the subject biomarker expression profile, to thereby predict a prognosis or classify the subject.

In an aspect, there is provided a computer implemented product for determining therapy for a subject with UPS comprising: (a) a means for receiving values corresponding to a subject expression profile in a subject sample; and (b) a database comprising a reference expression profile associated with a prognosis, wherein the subject biomarker expression profile and the biomarker reference profile each have at least one value representing the expression level of at least one biomarker selected from Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132; wherein the computer implemented product selects the biomarker reference expression profile most similar to the subject biomarker expression profile, to thereby predict the therapy.

In an aspect, there is provided a computer readable medium having stored thereon a data structure for storing the computer implemented product described herein. In an embodiment, the data structure is capable of configuring a computer to respond to queries based on records belonging to the data structure, each of the records comprising: (a) a value that identifies a biomarker reference expression profile of at

least one of Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132; (b) a value that identifies the probability of a prognosis associated with the biomarker reference expression profile.

In an aspect, there is provided a computer system comprising (a) a database including records comprising a biomarker reference expression profile of at least one of Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132 associated with a prognosis or therapy; (b) a user interface capable of receiving a selection of expression levels of the at least one biomarker for use in comparing to the biomarker reference expression profile in the database; (c) an output that displays a prediction of prognosis or therapy according to the biomarker reference expression profile most similar to the expression levels of the at least one biomarker.

In an aspect, there is provided a kit comprising reagents for detecting the expression of any or all of Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132 in a sample.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows application of the 6-miRNA prognostic signature on a) the "Validation Set" and analyzed for Distant Metastasis Free Survival (DMFS) based on their risk group "High" and "Low" and b) metastasis samples.

Figure 2 shows association of a) individual miRNAs within the 6-miR signature with distant metastasis free survival (DMFS) and disease free survival (DFS) of the combined datasets (Training + Validation) on univariate analysis (log-rank) and multivariate analysis (Cox PH regression) and b) miRNA-138 expression with DFS in the UPS samples. Values presented as p-value and hazard ratios (HR).

Figure 3 shows summary of assays from different dataset and findings related to the 6-miR prognostic signature and RhoA gene expression.

Figure 4 shows overall survival of patients from the "Training Set"(Black) and "Validation Set"(Red)

Figure 5 shows unsupervised hierarchical clustering using the Ward method of miRNA expression from 4 Normal tissues (Adipose, Carotid, Vein and Smooth Muscle), 4 primary UPS cell lines (STS48, STS93, STS109 and STS117) and 42 UPS samples from the "Training Set" (metastatic in red, non-metastatic in white).

Figure 6 shows that for migration and invasion assays, 1.5×10^5 cells were seeded inside the insert with medium containing 1% serum. High serum (20%) medium was then added to the bottom chamber of 24-well plates to serve as a chemo-attractant. Invasion index is calculated as (% Invasion of Test Cell)/(% Invasion of Control Cell) and depicted in a) following transfection with 10nM of pre-miR-138 or 50nM of Locked Nucleic Acid (LNA) of miR-138 and in b) for the morphological changes of the cells on the migration and invasion chambers.

Figure 7 shows clonogenic assay of STS117 cells following transfection with 10nM or 50nM of Locked Nucleic Acid (Control-Scrambled and mir-138).

Figure 8 shows selection of genes affected by the transfection of STS 117 cells by LNA-antimiR-138, LNA-antimiR-224 and pre-miR-375 while excluding genes modulated by LNA-antimiR-130a. Global mRNA profiling of STS117 cells 24-hours post-transfection were done using the Affymetrix Human Genome U133 plus 2.0 array that was processed with Affymetrix's WT Express protocol and 100 ng of starting material. The arrays were hybridized for 17 hrs at 45oC and washed and stained in fluidic station P450. Images were acquired with GeneChip scanner 3000 and preliminary analysis was carried out with Affymetrix gene expression console.

Figure 9 shows the quantification of a) RhoA mRNA expression in primary samples from non-metastatic (n=14) and metastatic patients (n=14), and metastatic samples (n=10). Significant differences in RhoA mRNA expression were observed between each group (Student t-test $p \leq 0.006$). Data plotted as delta Ct normalized to the average values from the No-DM group (Lower number = lower expression), b) Protein level and activity downstream of miR-138 and RhoA.

Figure 10 shows miR-138-Rho-ROCK pathway schema (a), (b) proposed changes secondary to increased expression of miR-138 in combination with reduced RhoA and (c) in the potential convergence of targets from the other miRNA within the prognostic signature.

Figure 11 shows distant metastasis free survival of 1056 breast cancer patients from 7 datasets dichotomized by the median expression of RhoA. Median follow-up was 238 months.

DESCRIPTION

In the following description, numerous specific details are set forth to provide a thorough understanding of the invention. However, it is understood that the invention may be practiced without these specific details.

A common and aggressive subtype of soft-tissue sarcoma, undifferentiated pleomorphic sarcoma (UPS) was examined to develop and validate a prognostic signature for distant metastasis-free survival (DMFS) composed of micro-RNAs (miRNA).

Following central pathology review, 110 fresh frozen UPS samples annotated prospectively with clinical data were split into independent training and validation cohorts. Following global miRNA profiling of the training set, multivariate regression model was fit on the miRNA expression values to yield a 6-miRNA signature model associated with DMFS. The expression of the 6 miRNAs were then measured in the validation set and metastatic samples to test the signature using Kaplan Meier and multivariate analysis adjusted for: patient age, gender, tumor grade, size, depth and radiotherapy use. Following cellular miRNA level modulation, in-vitro assays were used to derive biological understanding of miRNAs in promoting metastasis. Public breast cancer datasets (n=1912) were analyzed in silico to determine the prognostic value of the signature and RhoA in breast cancers.

Using the 6-miRNA training signature, patients from the validation set were successfully categorized into "High" and "Low" risk groups for DMFS (HR:2.2; p=0.05) and classified all metastatic samples as “High” risk. The signature is capable of predicting patient DMFS (HR:3.5; p=0.0001) after adjusting for other prognostic markers. In-vitro experiments suggest the involvement of RhoA/C-ROCK1/2-LIMK1/2 as downstream signature miRNA targets. The prognostic ability of the 6-miRNA signature (HR:1.8; p=0.01) and RhoA (HR:0.6; p=0.013) were demonstrated in the breast datasets.

A prognostic 6-miRNA signature has been successfully developed and validated for UPS. This signature could help identify patients at "High" risk for distant metastasis, who might benefit from more aggressive systemic therapies. Common pathways that promote the development of metastasis in sarcomas and breast cancers may be present and are potential targets for future therapeutic investigations.

In an aspect, there is provided a method of prognosing or classifying a subject with undifferentiated pleomorphic sarcoma (UPS) comprising: (a) determining the expression of at least one biomarker in a test sample from the subject selected from Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132; and (b) comparing expression of the at least one biomarker in the test sample with expression of the at least one biomarker in a control sample; wherein a difference or similarity in the expression of the at least one biomarker between the control and the test sample is used to prognose or classify the subject with UPS into a low risk group or a high risk group of developing metastasis.

The term "level of expression" or "expression level" as used herein refers to a measurable level of expression of the products of biomarkers, such as, without limitation, the level of micro-RNA, messenger RNA transcript expressed or of a specific exon or other portion of a transcript, the level of proteins or portions thereof expressed of the biomarkers, the number or presence of DNA polymorphisms of the biomarkers, the enzymatic or other activities of the biomarkers, and the level of specific metabolites.

As used herein, the term "control" refers to a specific value or dataset that can be used to prognose or classify the value e.g. expression level or reference expression profile obtained from the test sample associated with an outcome class. A person skilled in the art will appreciate that the comparison between the expression of the biomarkers in the test sample and the expression of the biomarkers in the control will depend on the control used.

The term "differentially expressed" or "differential expression" as used herein refers to a difference in the level of expression of the biomarkers that can be assayed by measuring the level of expression of the products of the biomarkers, such as the difference in level of micro-RNA or a portion thereof expressed . In a preferred embodiment, the difference is statistically significant. The term "difference in the level of expression" refers to an increase or decrease in the measurable expression level of

a given biomarker, for example as measured by the amount of micro-RNA as compared with the measurable expression level of a given biomarker in a control.

The term "low risk" as used herein in respect of UPS refers to a lower risk of UPS r as compared to a general or control population.

The term "sample" as used herein refers to any fluid, cell or tissue sample from a subject that can be assayed for biomarker expression products and/or a reference expression profile, e.g. genes differentially expressed in subjects.

In some embodiments, the level of gene expression is determined and compared.

A person skilled in the art will appreciate that a number of methods can be used to detect or quantify the level of micro-RNA within a sample, including arrays, such as microarrays, RT-PCR (including quantitative RT-PCR), nanostring and various sequencing technologies. By way of non-limiting example, the RNA was quantified as follows in the disclosed exampels: Training Set: Total RNA from all tumor samples were extracted using the RNeasy kits (Qiagen). Global profiling of miRNA expression on the "Training Set" was performed using the TaqMan® Human Micro-RNA Array A (Applied Biosystems, Inc. CA, USA). Total RNA (300ng) was first reverse-transcribed with the Multiplex RT pool set, then quantitated using an Applied Biosystems 7900 HT Real-Time PCR system as previously described1. Data were normalized using endogenous controls (RNU6B, RNU44 and RNU48) that were simultaneously quantified. Validation Set: Single well quantification of miRNA expressions was assessed by initially reverse-transcribing 200ng of total RNA with multiscribe reverse transcriptase and miR-specific primers (50nM), followed by qRT-PCR analysis using TaqMan microRNA Assays (Applied Biosystems). [Hui AB, Shi W, Boutros PC, et al: Robust global micro-RNA profiling with formalin-fixed paraffin-embedded breast cancer tissues. Lab Invest 89:597-606, 2009].

In various embodiments, the at least one biomarkers is two biomarkers, three biomarkers, four biomarkers, five biomarkers, or six biomarkers.

In some embodiments, overexpression of Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132 and underexpression of Mir-221 is indicative of high risk.

In some embodiments, classification of the subject into a low or high risk group is based on a score = -0.15*'miR-132'-0.299*'miRNA-138'-0.217*'miR-143'+0.427*'miR-221'-0.334*'miR-224'-0.35*'miR-491-5p'.

In some embodiments, determining the biomarker expression level comprises use of quantitative PCR or an array, preferably sequencing technologies or nanostring.

In some embodiments, the sample comprises a tissue sample.

In an aspect, there is provided a method of selecting a therapy for a subject with UPS, comprising the steps: (a) classifying the subject with UPS into a high risk group or a low risk group according to the method described herein; and (b) selecting a more aggressive therapy, preferably adjuvant chemotherapy or radiation therapy, for the high risk group or a less aggressive therapy, preferably no adjuvant chemotherapy or no radiation therapy, for the low risk group.

In an aspect, there is provided a method of selecting a therapy for a subject with UPS, comprising the steps: (a) determining the expression of at least one biomarker in a test sample from the subject selected from Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132; (b) comparing expression of the at least one biomarker in the test sample with expression of the at least one biomarker in a control sample; (c) classifying the subject in a high risk group or a low risk group, wherein a difference or a similarity in the expression of the at least one biomarker between the control sample and the test sample is used to classify the subject into a high risk group or a low risk group; (d) selecting a more aggressive therapy, preferably adjuvant chemotherapy or radiation therapy, for the high risk group or a less aggressive therapy, preferably no adjuvant chemotherapy or no radiation therapy, for the low risk group.

In an aspect, there is provided a composition comprising a plurality of isolated nucleic acid sequences, wherein each isolated nucleic acid sequence hybridizes to: (a) Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132; and/or (b)a nucleic acid complementary to a), wherein the composition is used to measure the level of Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132 expression.

The term “nucleic acid” includes DNA and RNA and can be either double stranded or single stranded.

The term "hybridize" or "hybridizable" refers to the sequence specific non-covalent binding interaction with a complementary nucleic acid. In a preferred embodiment, the hybridization is under high stringency conditions. Appropriate stringency conditions which promote hybridization are known to those skilled in the art, or can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1 6.3.6. For example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C may be employed.

In an aspect, there is provided an array comprising, for each of Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132, one or more polynucleotide probes complementary and hybridizable thereto.

The term "probe" as used herein refers to a nucleic acid sequence that will hybridize to a nucleic acid target sequence. In one example, the probe hybridizes to the RNA biomarker or a nucleic acid sequence complementary thereof. The length of probe depends on the hybridization conditions and the sequences of the probe and nucleic acid target sequence. In one embodiment, the probe is at least 8, 10, 15, 20, 25, 50, 75, 100, 150, 200, 250, 400, 500 or more nucleotides in length.

The term "primer" as used herein refers to a nucleic acid sequence, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand is induced (e.g. in the presence of nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH). The primer must be sufficiently long to prime the synthesis of the desired extension product in the presence of the inducing agent. The exact length of the primer will depend upon factors, including temperature, sequences of the primer and the methods used. A primer typically contains 15-25 or more nucleotides, although it can contain less or more. The factors involved in determining the appropriate length of primer are readily known to one of ordinary skill in the art.

In an aspect, there is provided a computer program product for use in conjunction with a computer having a processor and a memory connected to the processor, the computer program product comprising a computer readable storage medium having a computer mechanism encoded thereon, wherein the computer program mechanism

may be loaded into the memory of the computer and cause the computer to carry out the method described herein.

In an aspect, there is provided a computer implemented product for predicting a prognosis or classifying a subject with UPS comprising: (a) a means for receiving values corresponding to a subject expression profile in a subject sample; and (b) a database comprising a reference expression profile associated with a prognosis, wherein the subject biomarker expression profile and the biomarker reference profile each have at least one value representing the expression level of at least one biomarker selected from Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132; wherein the computer implemented product selects the biomarker reference expression profile most similar to the subject biomarker expression profile, to thereby predict a prognosis or classify the subject. Preferably, computer implemented product is for use with the method described herein.

In an aspect, there is provided a computer implemented product for determining therapy for a subject with UPS comprising: (a) a means for receiving values corresponding to a subject expression profile in a subject sample; and (b) a database comprising a reference expression profile associated with a prognosis, wherein the subject biomarker expression profile and the biomarker reference profile each have at least one value representing the expression level of at least one biomarker selected from Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132; wherein the computer implemented product selects the biomarker reference expression profile most similar to the subject biomarker expression profile, to thereby predict the therapy. Preferably, computer implemented product is for use with the method described herein.

In an aspect, there is provided a computer readable medium having stored thereon a data structure for storing the computer implemented product described herein. In an embodiment, the data structure is capable of configuring a computer to respond to queries based on records belonging to the data structure, each of the records comprising: (a) a value that identifies a biomarker reference expression profile of at least one of Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132; (b) a value that identifies the probability of a prognosis associated with the biomarker reference expression profile.

In an aspect, there is provided a computer system comprising (a) a database including records comprising a biomarker reference expression profile of at least one of Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132 associated with a prognosis or therapy; (b) a user interface capable of receiving a selection of expression levels of the at least one biomarker for use in comparing to the biomarker reference expression profile in the database; (c) an output that displays a prediction of prognosis or therapy according to the biomarker reference expression profile most similar to the expression levels of the at least one biomarker.

In an aspect, there is provided a kit comprising reagents for detecting the expression of any or all of Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132 in a sample.

The advantages of the present invention are further illustrated by the following examples. The examples and their particular details set forth herein are presented for illustration only and should not be construed as a limitation on the claims of the present invention.

EXAMPLES

Methods and Materials

Patient information and tissues

Institutional Research Ethics Board approval was obtained from the University Health Network (UHN) and Mount-Sinai Hospital in Toronto for this study. Samples are collected from multiple Canadian institutions and stored as fresh frozen samples within the Clinical Core and Tumor Bank at the Mount-Sinai Hospital, which also prospectively annotate the samples with clinical data. The "Training Set" of UPS was comprised of 42 fresh frozen samples that had undergone central pathology review at the Mount-Sinai Hospital and were obtained from Stage I-III patients diagnosed from 1988-1999. Following central pathology review of 100 other sarcoma samples from patients diagnosed from 2000-2010, 68 fresh frozen samples were confirmed to be UPS from Stage I-III patients and formed the "Validation Set". Samples were collected from patients prior to any chemotherapy or radiotherapy. Corresponding fresh frozen metastatic samples (n=10) from lung metastatectomies of 6 patients from the

"Validation Set" were obtained from the UHN biobank. Pathology review of the metastasis corresponded with the original diagnosis of the primary tumors.

Four RNA samples originating from non-cancerous human tissues of mesenchymal origin were purchased from Clontech Laboratories, Inc. (Smooth muscle), Applied Biosystem, Inc. (Adipose tissue) and Agilent Technologies, Inc. (Carotid Artery and Vein).

RNA purification from UPS samples

Total RNA from the tumors were extracted using the RNeasy kits (Qiagen). Samples were assayed randomly, with clinical outcome unknown, to avoid experimental bias.

Cell lines and reagents

Three primary cell lines (STS48, STS93, STS117) from patients diagnosed with UPS were used for in-vitro experiments. The cells were incubated at 37°C under 5% CO_2 in DMEM:F12 1:1 media with 10% bovine serum. Lipofectamine 2000 (Invitrogen) was used to transfect cells with pre-miRs (Invitrogen), Locked-Nucleic-Acid (LNA) antimiRs (Exiqon) and siRNAs (Qiagen). Total RNA was isolated using the Total RNA Purification Kit (Norgen, Inc.), according the manufacturer's protocol. Recovered RNA concentrations and quality were measured using the Nanodrop 1000A spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The cytotoxic effects of transfections were investigated in cells using clonogenic assays[11]. Cell-cycle analysis of pre-miRNA-138 and LNA-antimiRNA-138 transfected cells were done using propidium iodide staining as previously described[11]. Analyses were made using the BD FACScalibur using the FL-2 channel. The flow cytometry data were analyzed using FlowJo software (Tree Star, Inc.). Invasion and migration of cells were assayed using the BD BioCoat Matrigel Invasion Chambers and Control Inserts (BD Bioscience) as per the manufacturer's instructions.

Quantification of miRNA and mRNA

MiRNA profiling

Global profiling of miRNA expression on the "Training Set" was performed using the TaqMan® Human Micro-RNA Array A (Applied Biosystems, Inc. CA, USA). Total RNA (300ng) was first reverse-transcribed with the Multiplex RT pool set, then quantitated

using an Applied Biosystems 7900 HT Real-Time PCR system as previously described[12]. Data was normalized using endogenous controls (RNU6B, RNU44 and RNU48) that were simultaneously quantified. The resulting ΔCt values were used for hierarchical clustering and signature derivation. Clustering was done using JMP 10 (SAS institute, Cary, NC, USA).

Real-time Quantification of RNAs

Single well quantification of miRNA expressions was assessed by initially reverse-transcribing 200ng of total RNA with multiscribe reverse transcriptase and miRNA-specific primers (50nM), followed by qRT-PCR analysis using TaqMan microRNA Assays (Applied Biosystems)[12]. Quantitative RT-PCR was also utilized to analyze mRNA expression of: DICER, RHOA, RHOC, ROCK1, ROCK2, LEPR, LIMK1 and GAPDH. Following reverse transcription of 200ng of total RNA using SuperScript III Reverse Transcriptase (Invitrogen), qRT-PCR was done using SYBR Green PCR Master Mix (Applied Biosystems). Primers for PCR amplifications were designed using Primer 3 Input (version 0.4.0) (Table 1). Relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method[13].

Table 1: Primer sequences used for quantitative RT-PCR:

SEQ ID NO:	Gene	(F) 5' => 3'	(R) 5' => 3'
1, 2	**DICER1**	AAGGAAGCTGGCAAACAAGA	AAAACGAACCACCAAGTTGC
3, 4	**RHOA**	AAGGACCAGTTCCCAGAGGT	TTCTGGGGTCCACTTTTCTG
5, 6	**RHOC**	GAGAGCTGGCCAAGATGAAG	TTGGGGATCTCAGAGAATGG
7, 8	**ROCK1**	ACGGGACAAAATGGGAGAGT	ACAAGGGAGGGAGAAGAGGA
9, 10	**ROCK2**	AGAACCTGTCAAGCGTGGTA	CAAGGCTTGGAGTTGTGACC
11, 12	**LEPR**	AGGACGAAAGCCAGAGACAA	AAATGCCTGGGCCTCTATCT
13, 14	**LIMK1**	TGTAGCCACAGAGGATGCTG	TGAGGCAGATGAAACACTCG
15, 16	**GAPDH**	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG

Affymetrix Human Genome U133 plus 2.0

Global gene expression was profiled using the Affymetrix Human Genome U133 plus 2.0 array (Affymetrix, Inc). The arrays were run at Ontario Cancer Institute Genomics Centre using 100ng of total RNA. Data were pre-processed using the RMA method and 3X median for background removal.

Western blot analysis

Total protein extracts were harvested from cell lines 48 hours post-transfection and prepared for immunoblotting as previously described[14]. Membranes were probed with anti-RhoA, anti-RhoC, anti-ROCK1, anti-ROCK2, anti-LIMK1, anti-LIMK2, anti-phosphoLIMK1, anti-phosphoLIMK2 (Cell Signaling Technology, Inc.) and anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mAbs (1:15,000 dilution; Abcam, Inc.), followed by secondary antibodies conjugated to horseradish peroxidase (1:2,000 dilution; Abcam, Inc.) GAPDH protein levels were used as loading controls. Western blots were quantified with the Adobe Photoshop Pixel Quantification Plug-In (Richard Rosenman Advertising & Design.

Statistical analyses

Development and validation of miRNA signature:

Univariate analyses were conducted on the "Training Set" using Cox proportional hazard (PH) regression model. Potentially associated miRNAs (p-value <0.05) were applied into multivariate models while adjusting for multiple clinical factors. Cox PH regression models were built for time to event outcomes: Distant-metastasis-free-survival (DMFS), OS, disease-free-survival (DFS), and local-recurrence-free-survival. Stepwise-selection algorithm was implemented for model selection to select the 6 most-significantly associated miRNAs for the DFMS signature. Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated for significant predictors. Statistical significance level for multivariate analysis was <0.05. The signature score was based on the weighted combination of the miRNAs with the estimated regression coefficient of the Cox PH regression model as the weight[15,16]. Statistics were performed using SAS version 9.1 (SAS institute, Cary, NC, USA) and the R package (http://CRAN.R-project.org, R Foundation, Vienna, Austria).

All in-vitro experiments were conducted at least 3 independent times, with the data presented as the mean ± SEM. The statistical differences between treatment groups were determined using a Student *t*-test when comparing 2 treatment groups.

Breast cancer dataset – TCGA breast cancer (BRCA) dataset: for the 856 samples in which corresponding miRNA and mRNA were profiled, miRNA counts-per-million-reads and mRNA RPKM from the dataset were converted into z-scores. The sarcoma signature scoring was then applied onto the z-scores to dichotomize patients into risk groups (High and Low). MRNA expressions were dichotomized by the median Z-score for univariate and multivariate analysis. The Desmedt (n=198)[17], Kao (n=327)[18], Loi1 (n=87)[19], Loi3 (n=77)[20], Minn2 (n=99)[21], Schmidt (n=200)[22], Symmans1 (n=195)[23] and Symmans2 (n=103)[23] breast cancer datasets were used for assessment of mRNA (RhoA, LIMK1 and Dicer) expressions and outcomes. These datasets were selected for their use of Affymetrix mRNA profiling platforms with annotated clinical outcome. All mRNA expressions were converted into z-scores within each dataset, and then combined together for analysis.

RESULTS AND DISCUSSION

Sample and Patient Characteristics:

Descriptive statistics of the patients in the "Training Set" and "Validation Set" are provided in Table 2. As patients from the "Validation Set" were treated more recently than patients in the "Training Set", the "Validation Set" had shorter follow up ($p<0.0001$), larger tumors ($p=0.03$) and older patient age ($p=0.0004$). Despite the aforementioned differences, the 5-year OS probabilities of the 2 cohorts were similar (54% vs. 58%; Log-rank $p=0.83$) (Figure 4).

Table 2: Patient, disease and treatment characteristics of the UPS "Training Set" and "Validation Set"

Factors		Training set N=42	Validation set N=68	P-value
Gender	M:F	23:18	41:27	0.67
Stage	I/II III	40% 60%	38% 62%	0.82
Grade	2 3	26% 74%	21% 79%	0.73
Adjuvant Chemo	No Yes	98% 2%	98% 2%	0.72
Adjuvant RT	No Yes	29% 71%	32% 68%	0.54
Median age (range)		64 (35-95)	68 (32-90)	0.03
Median size (range)		6.25 (1.3-28)	11.5(2.2-28)	0.0004
Median follow-up mos (range)		123 (18-225)	28.5(0-116)	<0.0001

Development and validation of the miRNA signature:

Global miRNA profiling of the "Training Set" showed that 179 (47.3%) of the mean miRNA expressions were significantly (t-test $p<0.00013$) reduced in UPS in comparison to normal tissues. None of the miRNAs were significantly over-expressed in UPS in comparison to normal tissues. Based on the univariate and multivariate modeling of the miRNA expressions from the "Training Set" (Table 3) and adjusting for clinical factors, a prognostic signature score for DMFS consisting of 6 miRNAs was developed: Score = -0.15*'miR-132'-0.299*'miRNA-138'-0.217*'miR-143'+0.427*'miR-221'-0.334*'miR-224'-0.35*'miR-491-5p'. The patients were stratified into "Low risk" and "High risk" categories according to their signature score value: low risk (score <median); high risk (risk score >median). The hazard ratio (HR) for DMFS was 16.0 in the training set ($p<0.0001$).

Table 3: List of miRNAs associated with clinical outcomes of patients in the "Training Set". MiRNAs within the final signature prognostic of Distant Metastasis Free survival are in red. Distant Metastasis (DM), Local Recurrence (LR), Disease Free Survival (DFS) and Overall Survival (OS)

DM	LR	DFS	OS
hsa-miR-10a	hsa-let-7g	hsa-miR-125b	hsa-miR-224
hsa-miR-93	hsa-miR-139-5p	hsa-miR-128	hsa-miR-491-5p
hsa-miR-106b	hsa-miR-211	hsa-miR-130a	
hsa-miR-128	hsa-miR-449a	hsa-miR-138	
hsa-miR-130a	hsa-miR-512-3p	hsa-miR-139-5p	
hsa-miR-132		hsa-miR-181a	
hsa-miR-143		hsa-miR-197	
hsa-miR-221		hsa-miR-224	
hsa-miR-224		hsa-miR-331-3p	
hsa-miR-34a		hsa-miR-339-3p	
hsa-miR-374a		hsa-miR-433	
hsa-miR-455-3p		hsa-miR-886-5p	
hsa-miR-491-5p			
hsa-miR-744			

To verify the signature, the score for each sample from the "Validation Set" (n=68) was calculated using the miRNA expression measurements from single-well qRT-PCR and the above formula. In the validation set, "High-Risk" patients were 2.2 times more likely to develop metastasis (p = 0.05) (Figure 1a) than "Low Risk" patients on univariate analysis. After adjusting for patient age, gender, tumor grade, maximum size, depth, and use of adjuvant radiotherapy, the signature remained capable of discriminating patients at "High" and "Low" risk of developing DM (HR: 3.5; p=0.0001)

in the combined groups of patients (Training + Validation set) (Table 4a). Only tumor depth persisted to be significantly associated with DMFS on multivariate analysis (HR: 0.3; p=0.04).

Table 4a: Multivariate analysis of the 6-miRNA signature score for its ability to predict distant metastasis free survival in the combined UPS cohort of "Training Set" and "Validation Set".

Parameter		**p-value**	**Hazard ratio**
6-miR signature risk	**High vs. Low**	**0.0001**	**3.5**
Age at diagnosis	Continuous	0.1	1.02
Gender	F vs. M	0.07	0.6
Tumor Grade	2 vs. 3	0.36	0.7
Tumor Size	Continuous	0.11	1.04
Tumor Depth	**Sup. Vs. Deep**	**0.04**	**0.3**
Radiotherapy	No vs. Yes	0.77	1.1

Application of the signature scoring on the metastatic samples classified all metastatic samples as "High" risk, with 9 of the 10 metastasis having higher risk scores than their corresponding primaries (Figure 1b).

MiRNA-138 promotes invasion in sarcoma cells

Three primary cell lines (STS48, STS93, and STS117) derived from patients diagnosed with UPS were used to study the biologic functions of miRNAs in UPS. Global miRNA profiling of the "Training Set", 4 primary UPS cell lines (STS48, STS93, STS109 and STS117) and 4 RNA samples from normal mesenchymal tissues (Adipose, Carotid, Vein and Smooth Muscle) demonstrated that the expression of

miRNAs differed between normal and sarcoma samples (Figure 5). To select candidate miRNAs for more in depth studies, screening migration/invasion and clonogenic assays were performed following modulation of cellular miRNA levels (Table 5). These experiments suggested that increased expression of miRNA-138 promoted cell invasion without affecting survival (Figures 6a and 7) or cell cycle (data not shown). Of the 6 miRNAs within the signature, elevated expression of miRNA-138 was best associated with shorter DMFS (log-rank p=0.60) and DFS (p=0.017) on Kaplan-Meier univariate analysis and Cox PH multivariate analysis (DMFS: HR=2.3; p=0.008) (Figure 2a and b) of the combined UPS datasets.

Table 5: List of miRNAs selected for functional screening using data from the global profiling of the "Training Set" and 4 normal mesenchymal tissue RNAs. STS117 cells were transfected with 50nM of Locked Nucleic Acid antimir-128, 130a, 138, 139-5p and 224, and pre-miR-375 to evaluate the effect of miRNA modulation on the migration, invasion and clonogenic survival of the cells.

miR	**UPS vs Normal (fold less)**	**DMFS P-value**	**Met vs. No-Met (fold more)**	**Migration %**	**Invasion %**	**Invasion index**	**Clonogenic Survival %**
miR 128	1.32E+01	0.003	2.16	55	66	0.44	70
miR 130a	7.07E+02	0.004	2.94	28	28	0.5	25
miR 138	9.82E+01	0.09	2.66	162	46	0.3	106
miR 139-5p	1.05E+03	0.08	2.03	64	66	3	50
miR 224	1.13E+05	0.02	4.71	40	12	1	100
miR 375	4.85E+06	0.065	2.48	17	18	1.1	34

To determine potential pathways related to increased metastatic potentials in UPS, global mRNA expression analysis was performed on STS117 cells, which spontaneously develop lung metastasis in NOD-SCID mice xenografts, following their transfections with LNA – antimiR-130a, antimiRNA-138, antimiR-224 and pre-miR-375.

As transfection with LNA-miR-130a was cytotoxic and had no effect on cellular invasion, while LNA-antimiRNA-138, 224 and pre-miR-375 reduced the migration and invasion of cells, we identified common genes affected by the transfection of LNA-antimiRNA-138, 224 and pre-miR-375, but not by LNA-antimiRNA-130a (Figure 8). In addition to validated targets of miRNA-138 such RhoC and ROCK2[24,25], these genes are potentially important to the observed increase in invasiveness of UPS cells secondary miRNA-138 over-expression.

In addition, global mRNA profiling of the "Training Set" (unpublished data from JW and IA) identified the under-expression of RhoA to be associated with increased odds of developing metastasis. To validate this finding, RhoA mRNA expression was measured in 28 samples from the "Validation Set" (14 patients who developed metastasis and 14 patients without metastasis) and the 10 metastatic samples. The association between metastasis and reduced expression of RhoA mRNA was validated in both the "Validation Set" and the metastatic samples ($p \leq 0.006$) (Figure 9a).

Pathway analysis (DAVID, g-profiler) of validated miRNA-138 target genes and the above identified genes suggested that the cofilin metastatic pathway is modulated by the expression of miRNA-138 and potentially other miRNA within the signature. QRT-PCR experiments confirmed findings from prior publication that the overexpression of miRNA-138 reduced the mRNA level of RhoC and ROCK2 by a mean of 2.6 (SEM:0.08) and 3.1 (SEM:0.22) folds respectively. Western blot analysis demonstrated the predicted down-regulation of RhoC with the transfection of miRNA-138 (Figure 9b). However, paradoxically increased ROCK1 and ROCK2 levels were observed in miRNA-138 transfected cells. The activities of ROCKs were also increased as observed by the phosphorylation of their downstream target, LIMK1 and LIMK2 (Figure 9b, 10a and b).

In silico analysis of the prognostic value of signature in other cancers:

Application of the signature was explored in the breast cancers dataset (n=856) due to 1) the similarity between the observed morphological changes in UPS cells and previously described changes in breast cancer cells following miRNA-138 and Rho modulation[26], 2) prior success in cross-validating the "cinsarc" sarcoma-derived mRNA signature[27] and 3) the breast dataset being the largest dataset containing miRNA, mRNA and clinical data. The 6-miR signature demonstrated a trend (HR: 1.5,

95%Confidence Interval: 0.9-2.5; p=0.13) for its ability to predict for overall survival after adjusting for patient age, gender and disease stage in melanoma (Table 4b).

Table 4b: Cox-regression proportional hazard analysis of the 6-miR signature adjusting for clinical factors in Melanoma TCGA database

	Sig.	HR
Age (Old vs. Young)	.174	1.426
Gender (F vs. M)	.720	.907
Stage	.102	
I vs II	.823	1.077
I vs III	.045	1.885
I vs IV	.163	2.451
Signature Risk (High vs. Low)	.134	1.484

N=129; 67 deaths

In breast cancers, the signature was prognostic for patient overall survival (HR: 1.8, 95% Confidence Interval: 1.2-2.7; p=0.005) after adjusting for patient age, disease stage and ER status. We then postulated that the expressions of the genes (RhoA, RhoC, ROCK1, ROCK2, LIMK1, LIMK2, CFL1 and CFL2) within the cofilin pathway (Table 4c; Figure 10) and DICER (miRNA processing) may also be correlated with patient survival.

Table 4c: Cox-regression proportional hazard analysis of the 6-miR signature adjusting for clinical factors in Breast cancers from the TCGA database.

	Sig.	Exp(B)
Signature Risk (High vs. Low)	.010	1.837
Age (Old vs. Young)	.101	1.418
Stage	.000	
I vs II	.873	1.045
I vs III	.002	2.563
I vs IV	.000	7.820
Estrogen receptor: Negative vs. Positive	.006	1.873
LIMK1 (High vs. Low)	.012	.572
RHOA (High vs. Low)	.013	.598
DICER (High vs. Low)	.006	1.782

N=856; 105 deaths

Indeed, they were all significantly ($p<0.042$) associated with overall survival on univariate analysis. Following multivariate analysis and backward selection modeling, 6-miR signature score (HR:1.8; p=0.01), LIMK1(HR:0.6; p=0.012), RhoA (HR:0.6, p=0.013) and DICER (HR:1.8; p=0.01) remain associated with overall survival after adjusting for patient age, disease stage and ER status (Table 6). The associations of the 3 genes (RhoA, LIMK1 and DICER) with breast cancer patient DMFS were further tested using 1056 previously profiled breast samples from 7 datasets. Low RhoA expression was significantly (univariate Log-rank p=0.01) associated with shorter DMFS (Figure 11). No multivariate analysis was performed due to lack of clinical annotations.

Table 6: Cox proportional hazard regression analysis of the 6-miR signature adjusting for clinical factors in Breast cancers from the TCGA database.

	p-value	HR
Signature Risk (High vs. Low)	**.010**	**1.837**
Age (Old vs. Young)	.101	1.418
Stage	.000	
I vs II	.873	1.045
I vs III	.002	2.563
I vs IV	.000	7.820
Estrogen receptor: Negative vs. Positive	.006	1.873
LIMK1 (High vs. Low)	.012	.572
RHOA (High vs. Low)	.013	.598
DICER (High vs. Low)	.006	1.782

N=856; 105 deaths; median f/u: 17.7 months

Two studies had previously described prognostic molecular signatures for STS composed of 3 hypoxia related mRNA expressions and a 177 mRNA gene signature. Yet, neither of these signatures has been validated[28,29]. Chibon et al. published the "cinsarc" signature composed of 67 differentially expressed mRNAs related to cell cycle progression and chromosomal stability[27] that is able to dichotomize patients with STS, breast cancers and lymphoma into high and low risk groups for the development of metastasis. While the "cinsarc" signature demonstrated that increasing genomic complexity of tumors is associated with higher risk of metastasis, the genes involved in the signature were selected from pathways related to mitosis control and chromosomal integrity and are thus not specific to STS or likely to be druggable targets[30,31].

Our current study used a homogeneous subtype of STS, the UPS to 1) investigate the use of miRNAs to prognosticate patients and 2) derive biological understanding of the mechanisms by which STS metastasize. The current study developed and validated a

6-miR signature that predicts for DMFS (HR: 3.5) in UPS independently from other prognostic factors such as patient age, gender, tumor size, grade, depth and use of adjuvant treatment (Table 4a). The validity of the signature and the potential biological roles of the 6-miRNAs were further supported by the higher risk scores observed in metastatic samples when compared to the risk scores of the corresponding primaries (Figure 1b). The increasing risk score from "Low-risk" primaries to "High-risk" primaries to metastasis supports a biological selection for cells with a pattern of miRNA expression that promotes UPS metastasis.

Phenotypic screening of candidate miRNAs (Table 5 and Figure 3a) and the correlation between higher expression of miRNA-138 with increased risk of metastasis (Figure 4) encouraged further investigation on the biological functions of miRNA-138. Examination of potential downstream targets of miRNA-138 combined with the validation that reduced RhoA (Figure 2a) is associated with metastasis suggests that the cofilin pathway[32,33] is involved in the promotion of UPS metastasis (Figure 10a). Functional assays in-vitro confirmed the inhibitory effect of miRNA-138 on RhoC, disinhibiting RhoA to activate downstream effector ROCKs and LIMKs (Figure 2b). These molecular changes resulted in observed losses in the spindled cell shape of pre-miRNA-138 transfected sarcoma cells (Figure 6b), corroborating with previously observed morphological changes secondary to increased RhoA-ROCK activity in prostate and breast cancer cells[26].

Given the similarity between the phenotypic changes secondary to miRNA-138 modulation in UPS (Figure 6b) and breast cancer cells[26], we postulated that the 6-miR signature may be prognostic in breast cancer and explored the signature's value using the TCGA breast cancer dataset. Remarkably, the 6-miR signature along with 3 genes from the cofilin pathway (RhoA and LIMK1) and miRNA machinery (DICER) were prognostic for breast cancer patient OS after adjusting for patient age, disease stage and ER status (Table 4c). The association between the 3 genes and breast cancer patient outcome was further tested using 1056 publically available mRNA profiles from breast cancers annotated with patient DMFS from 7 studies[17,19-23]. Indeed, RhoA low-expression was significantly (log-rank p=0.01) associated with worsened DMFS (Figure 11), as found in UPS (Figure 9a). These results suggest a common mechanism involving miRNA and RhoA may exist in UPS and breast cancer in promoting metastasis. Contrary to prior findings in which RhoA and RhoC were thought to be prometastatic and involved in epithelial-mesenchymal transition

(EMT)[24,34-39], the current results derived from sarcoma and breast cancer clinical and in-vitro data suggest that reduced expression of RhoA and RhoC is associated with higher metastatic rates. Our unanticipated finding is supported by 2 recent publications that demonstrated the importance of cellular plasticity in cancer cells to undergo EMT followed by a reversion through mesenchymal-epithelial transition to colonize the target metastatic environment and grow[40,41]. Furthermore, given the complexity of the metastatic process, miRNA-138 probably works in partnership with other molecular changes, such as RhoA and the other 5-miRNAs within the UPS prognostic signature to induce and/or promote biological changes in patients (Figure 10c).

Nair et al.'s systematically reviewed publications dedicated to study the role of miRNAs in predicting clinical outcomes in cancers and identified 41 studies on 20 human cancers with at least 10 samples[42]. Among these studies, the median study size was 65 samples, and only 6 (13%) of the studies proceeded to validate their findings using independent cohorts, with 3(7%) of the studies using multivariate adjustments in their analysis. While the current study's sample size (n=110) is at par with other published miRNA biomarker studies, the robustness of our investigation was optimized through the use of central pathology review to ensure a homogeneous subtype (UPS) of a rare cancer (STS) was profiled than validated using an independent sets of UPS with prospectively annotated clinical data. After adjusting for other potential prognostic factors, the signature remains significantly associated with DMFS. The findings were further corroborated using metastatic samples, functional assays and independent breast cancer datasets from 1912 samples.

Although preferred embodiments of the invention have been described herein, it will be understood by those skilled in the art that variations may be made thereto without departing from the spirit of the invention or the scope of the appended claims. All documents disclosed herein are incorporated by reference.

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CLAIMS:

1. A method of prognosing or classifying a subject with undifferentiated pleomorphic sarcoma (UPS) comprising:

(a) determining the expression of at least one biomarker in a test sample from the subject selected from Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132; and

(b) comparing expression of the at least one biomarker in the test sample with expression of the at least one biomarker in a control sample;

wherein a difference or similarity in the expression of the at least one biomarker between the control and the test sample is used to prognose or classify the subject with UPS into a low risk group or a high risk group of developing metastasis.

2. The method of claim 1, wherein the at least one biomarkers is two biomarkers.

3. The method of any one of claims 1-2, wherein the at least one biomarkers is three biomarkers.

4. The method of any one of claims 1-2, wherein the at least one biomarkers is four biomarkers.

5. The method of any one of claims 1-2, wherein the at least one biomarkers is five biomarkers.

6. The method of any one of claims 1-2, wherein the at least one biomarkers is six biomarkers.

7. The method of any one of claims 1-6, wherein overexpression of Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132 and underexpression of Mir-221 is indicative of high risk.

8. The method of any one of claims 1-7, wherein classification of the subject into a low or high risk group is based on a score = -0.15*'miR-132'-0.299*'miRNA-138'-0.217*'miR-143'+0.427*'miR-221'-0.334*'miR-224'-0.35*'miR-491-5p'.

9. The method of any one of claims 1-8, wherein determining the biomarker expression level comprises use of quantitative PCR or an array, preferably sequencing technologies or nanostring.

10. The method of claim 1, wherein the sample comprises a tissue sample.

11. A method of selecting a therapy for a subject with UPS, comprising the steps:

(a) classifying the subject with UPS into a high risk group or a low risk group according to the method of any one of claims 1-10; and

(b) selecting a more aggressive therapy, preferably adjuvant chemotherapy or radiation therapy, for the high risk group or a less aggressive therapy, preferably no adjuvant chemotherapy or no radiation therapy, for the low risk group.

12. A method of selecting a therapy for a subject with UPS, comprising the steps:

(a) determining the expression of at least one biomarker in a test sample from the subject selected from Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132;

(b) comparing expression of the at least one biomarker in the test sample with expression of the at least one biomarker in a control sample;

(c) classifying the subject in a high risk group or a low risk group, wherein a difference or a similarity in the expression of the at least one biomarker between the control sample and the test sample is used to classify the subject into a high risk group or a low risk group;

(d) selecting a more aggressive therapy, preferably adjuvant chemotherapy or radiation therapy, for the high risk group or a less aggressive therapy, preferably no adjuvant chemotherapy or no radiation therapy, for the low risk group.

13. A composition comprising a plurality of isolated nucleic acid sequences, wherein each isolated nucleic acid sequence hybridizes to:

(a) Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132; and/or

(b) a nucleic acid complementary to a),

wherein the composition is used to measure the level of Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132 expression.

14. An array comprising, for each of Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132, one or more polynucleotide probes complementary and hybridizable thereto.

15. A computer program product for use in conjunction with a computer having a processor and a memory connected to the processor, the computer program product comprising a computer readable storage medium having a computer mechanism encoded thereon, wherein the computer program mechanism may be loaded into the memory of the computer and cause the computer to carry out the method of any one of claims 1-12.

16. A computer implemented product for predicting a prognosis or classifying a subject with UPS comprising:

(a) a means for receiving values corresponding to a subject expression profile in a subject sample; and

(b) a database comprising a reference expression profile associated with a prognosis, wherein the subject biomarker expression profile and the biomarker reference profile each have at least one value representing the expression level of at least one biomarker selected from Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132;

wherein the computer implemented product selects the biomarker reference expression profile most similar to the subject biomarker expression profile, to thereby predict a prognosis or classify the subject.

17. A computer implemented product of claim 16 for use with the method of any one of claims 1-12.

18. A computer implemented product for determining therapy for a subject with UPS comprising:

(a) a means for receiving values corresponding to a subject expression profile in a subject sample; and

(b) a database comprising a reference expression profile associated with a prognosis, wherein the subject biomarker expression profile and the biomarker

reference profile each have at least one value representing the expression level of at least one biomarker selected from Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132;

wherein the computer implemented product selects the biomarker reference expression profile most similar to the subject biomarker expression profile, to thereby predict the therapy.

19. The computer implemented product of claim 18 for use with the method of claim 12.

20. A computer readable medium having stored thereon a data structure for storing the computer implemented product of any one of claims 16-19.

21. The computer readable medium according to claim 20, wherein the data structure is capable of configuring a computer to respond to queries based on records belonging to the data structure, each of the records comprising:

(a) a value that identifies a biomarker reference expression profile of at least one of Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132;

(b) a value that identifies the probability of a prognosis associated with the biomarker reference expression profile.

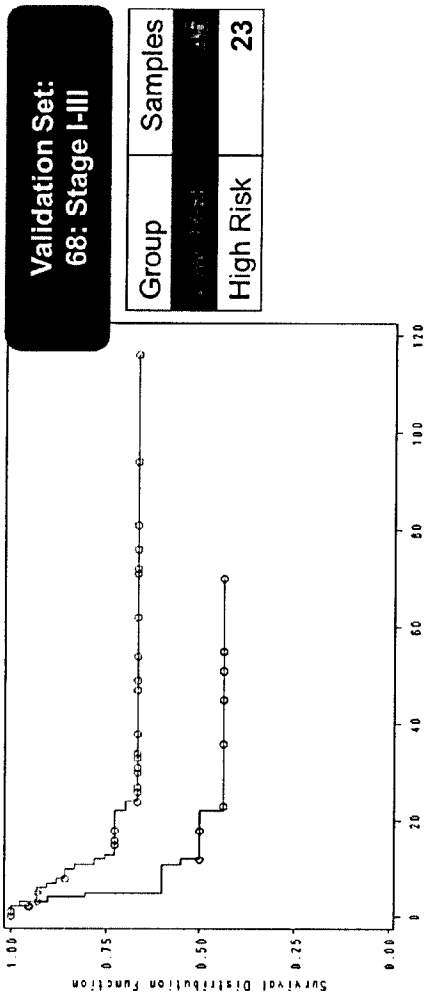
22. A computer system comprising

(a) a database including records comprising a biomarker reference expression profile of at least one of Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132 associated with a prognosis or therapy;

(b) a user interface capable of receiving a selection of expression levels of the at least one biomarker for use in comparing to the biomarker reference expression profile in the database;

(c) an output that displays a prediction of prognosis or therapy according to the biomarker reference expression profile most similar to the expression levels of the at least one biomarker.

23. A kit comprising reagents for detecting the expression of any or all of Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132 in a sample.



	P-value	Hazard Ratio	95% HR Confidence Limits	
High vs. Low	0.05	2.2	1.0	4.9

Figure 1A

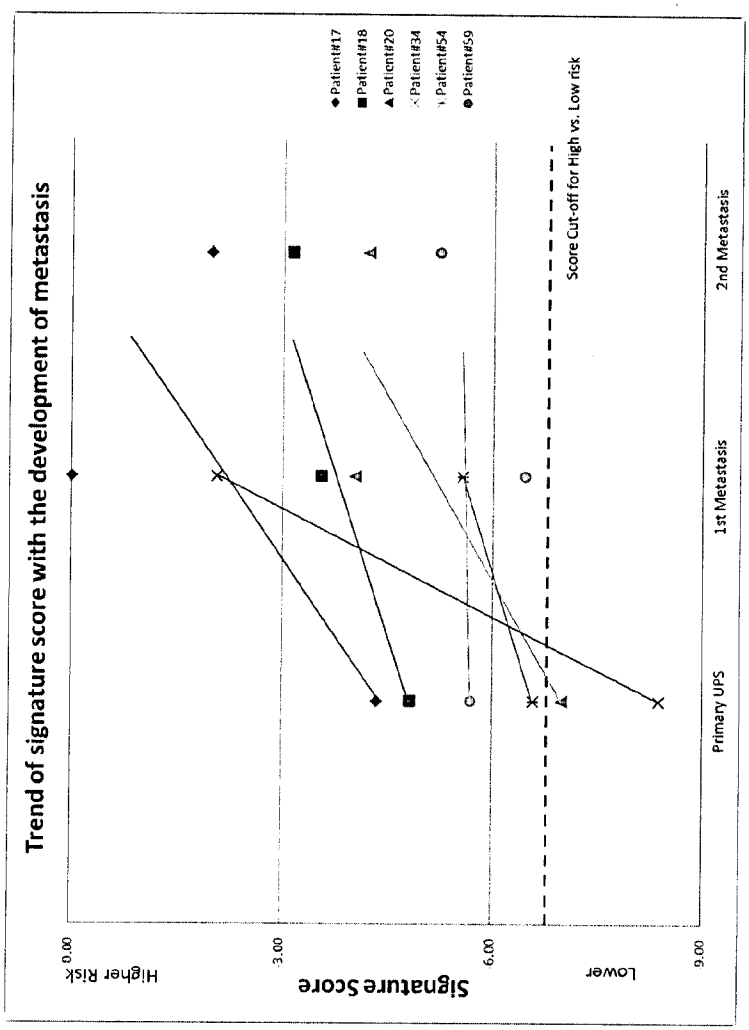

Trend of signature score with the development of metastasis
Patient#17
Patient#18
Patient#20
Patient#34
Patient#54
Patient#59
Score Cut-off for High vs. Low risk
Signature Score
0.00
3.00
6.00
9.00
Higher Risk
Lower
Primary UPS
1st Metastasis
2nd Metastasis

Figure 1B

	Univariate Analysis – p-value		Multivariate Analysis for DMFS*	
	DMFS	DFS	p-value	HR
Mir-132	0.24	0.44	0.90	
Mir-138	0.06	0.017	0.008	2.3
Mir-143	0.039	0.26	0.16	
Mir-221	0.19	0.4	0.40	
Mir-224	0.004	0.027	0.040	1.4
Mir-491-5p	0.022	0.15	0.28	

a) *Multivariate analysis adjusted for patient age, gender, tumor grade, size and disease stage.

Figure 2A

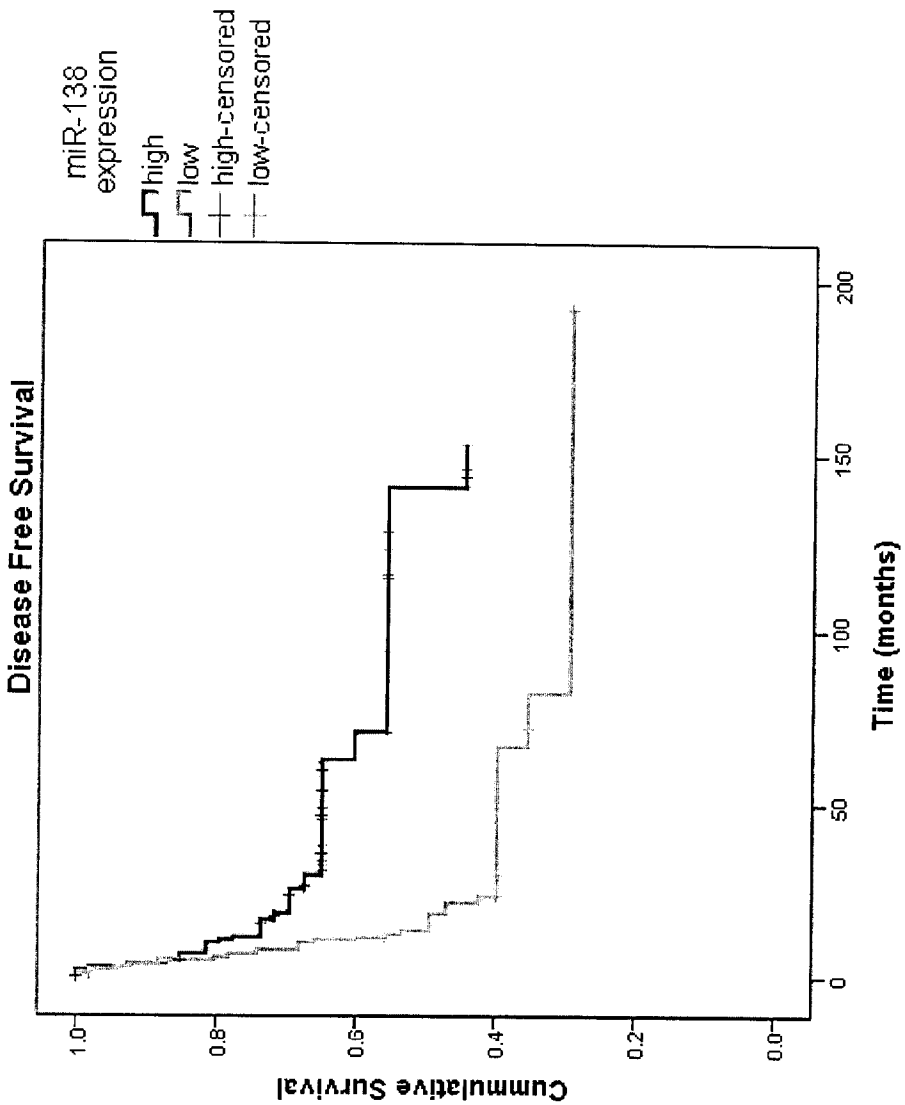

Disease Free Survival
miR-138 expression
high
low
high-censored
low-censored
Cummulative Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
Time (months)

Figure 2B

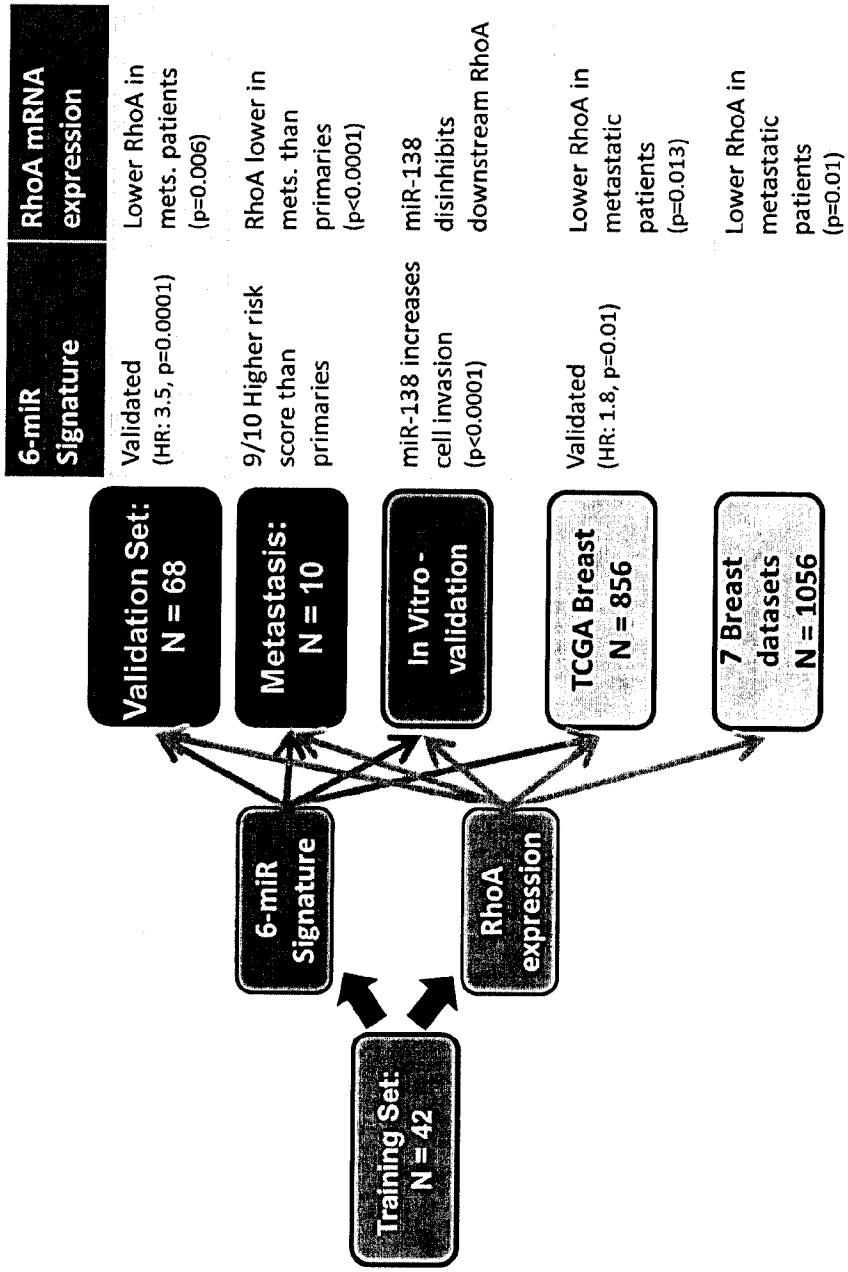

6-miR Signature
RhoA mRNA expression
Validation Set: N = 68
Validated (HR: 3.5, p=0.0001)
Lower RhoA in mets. patients (p=0.006)
Metastasis: N = 10
9/10 Higher risk score than primaries
RhoA lower in mets. than primaries (p<0.0001)
In Vitro - validation
miR-138 increases cell invasion (p<0.0001)
miR-138 disinhibits downstream RhoA
TCGA Breast N = 856
Validated (HR: 1.8, p=0.01)
Lower RhoA in metastatic patients (p=0.013)
7 Breast datasets N = 1056
Lower RhoA in metastatic patients (p=0.01)
6-miR Signature
RhoA expression
Training Set: N = 42

Figure 3

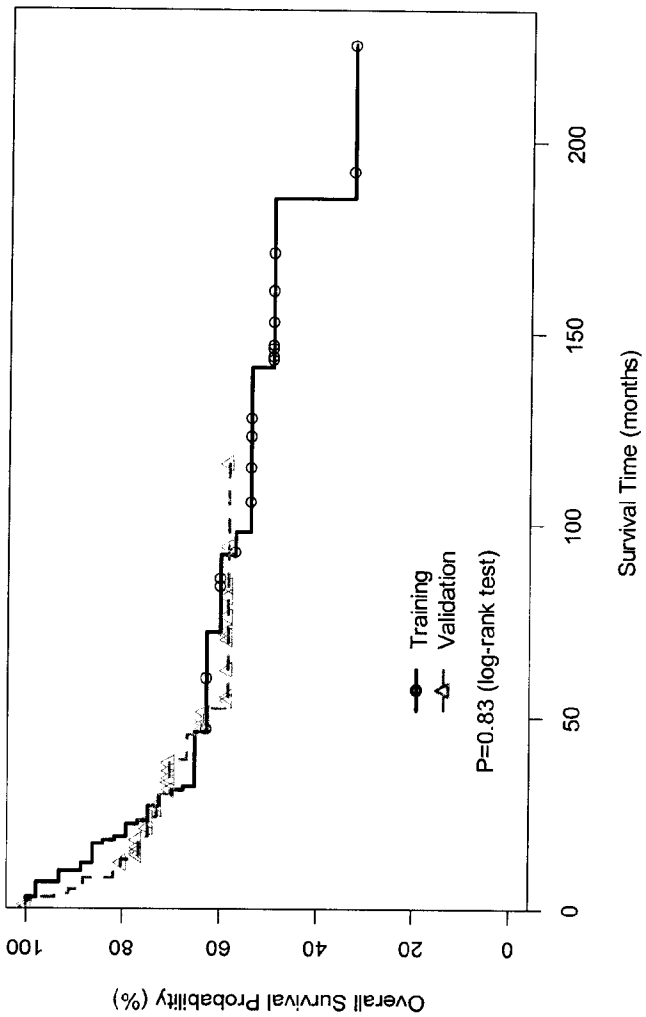

Overall Survival Probability (%)
0
20
40
60
80
100
0
50
100
150
200
Survival Time (months)
Training
Validation
P=0.83 (log-rank test)

Figure 4

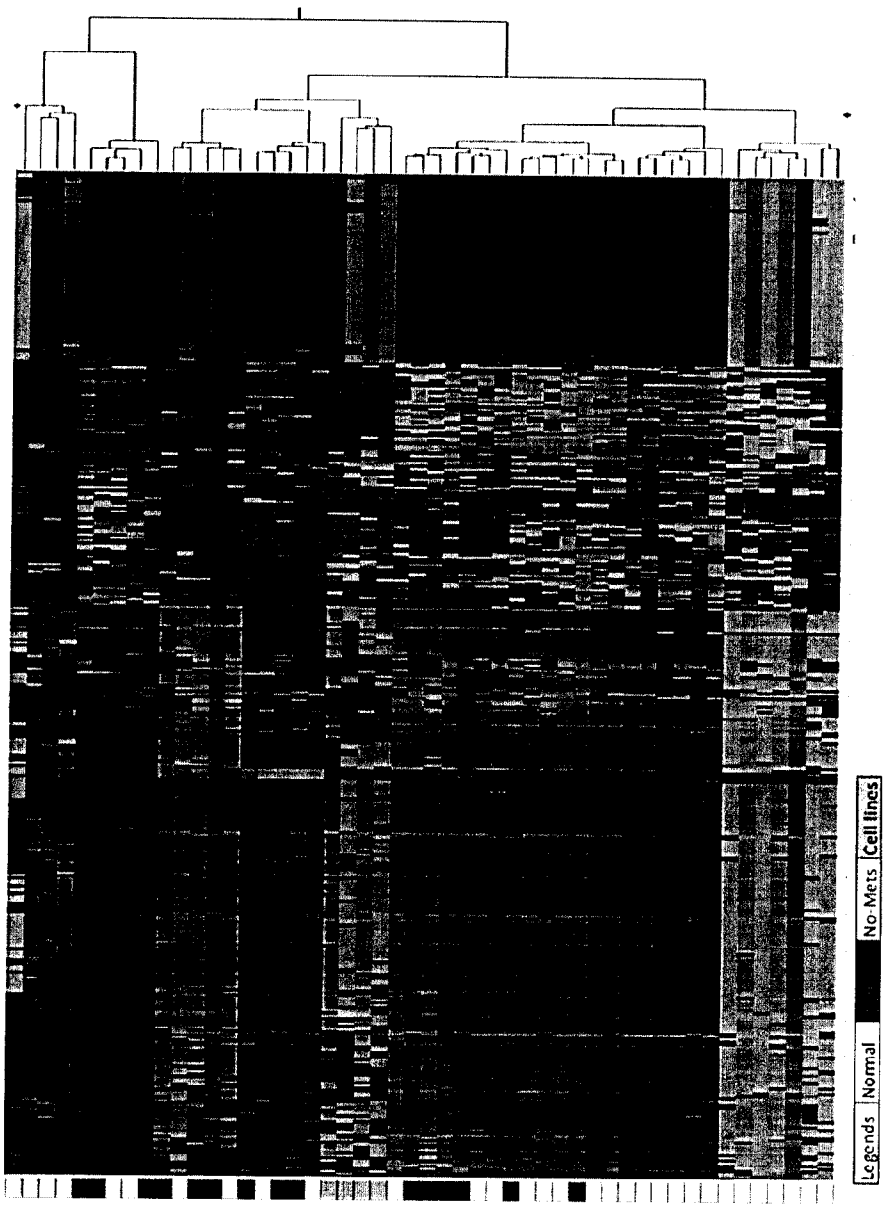


Figure 5

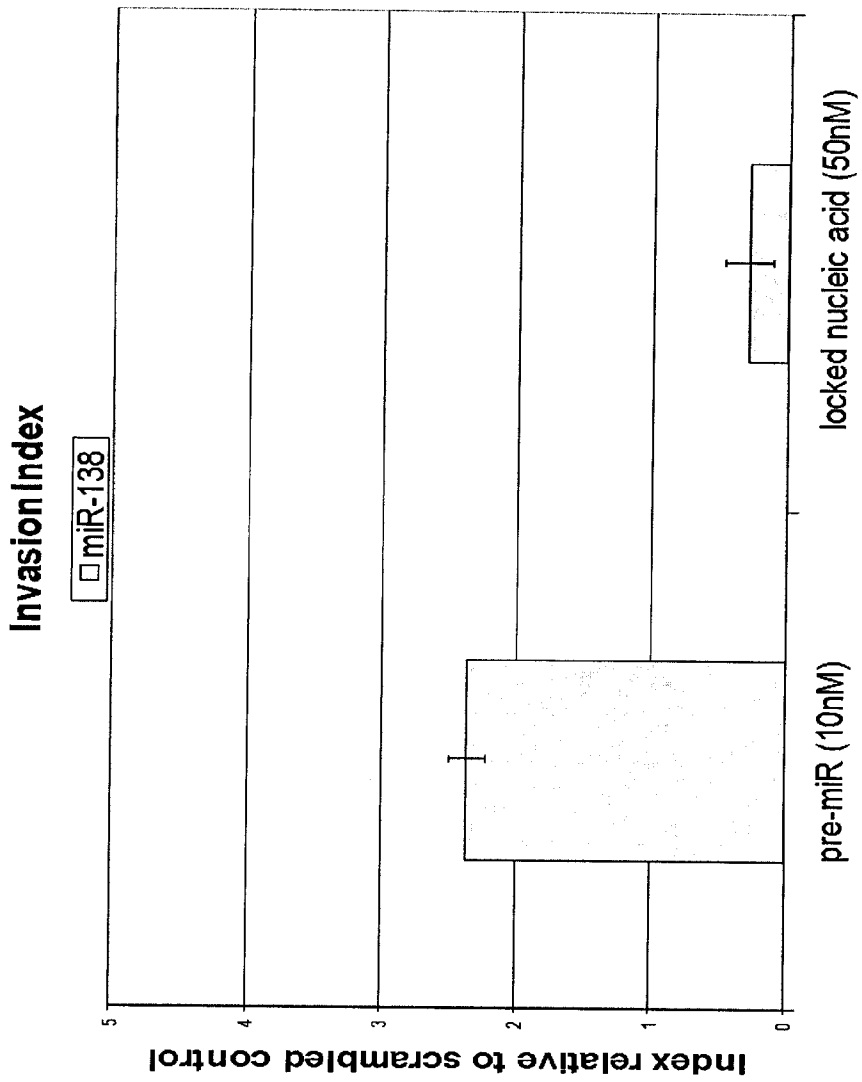
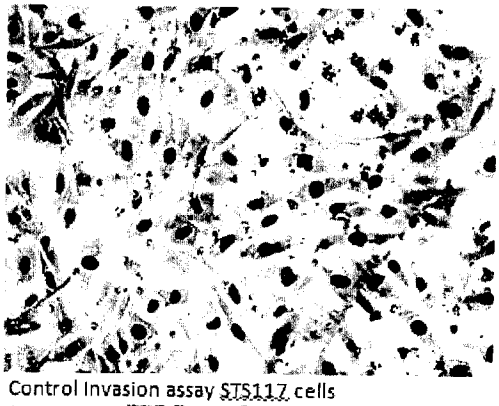
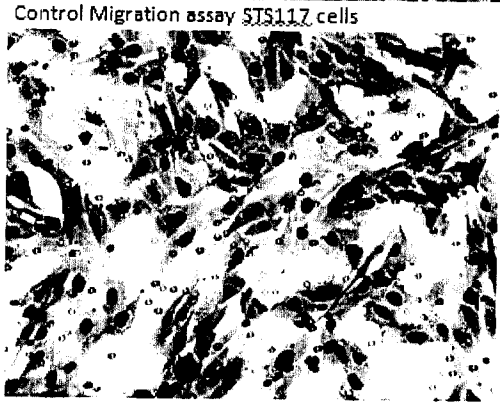

Invasion Index
miR-138
Index relative to scrambled control
0
1
2
3
4
5
pre-miR (10nM)
locked nucleic acid (50nM)

Figure 6A

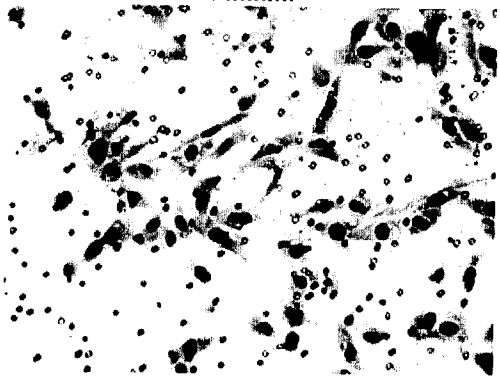
Control Migration assay STS117 cells



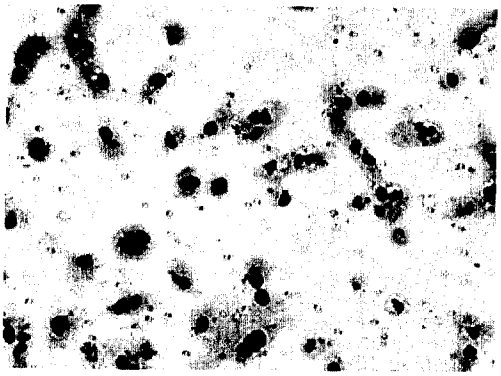
Control Invasion assay STS117 cells



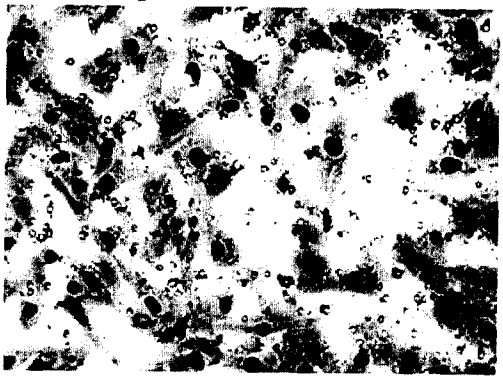
LNA—miR-138 Migration assay STS117 cells



LNA – miR-138 Invasion assay STS117 cells – spindle looking cells



pre-miR-138 Migration assay STS117 cells



pre-miR-138 Invasion assay STS117 cells – Loss of spindle shape

Figure 6B

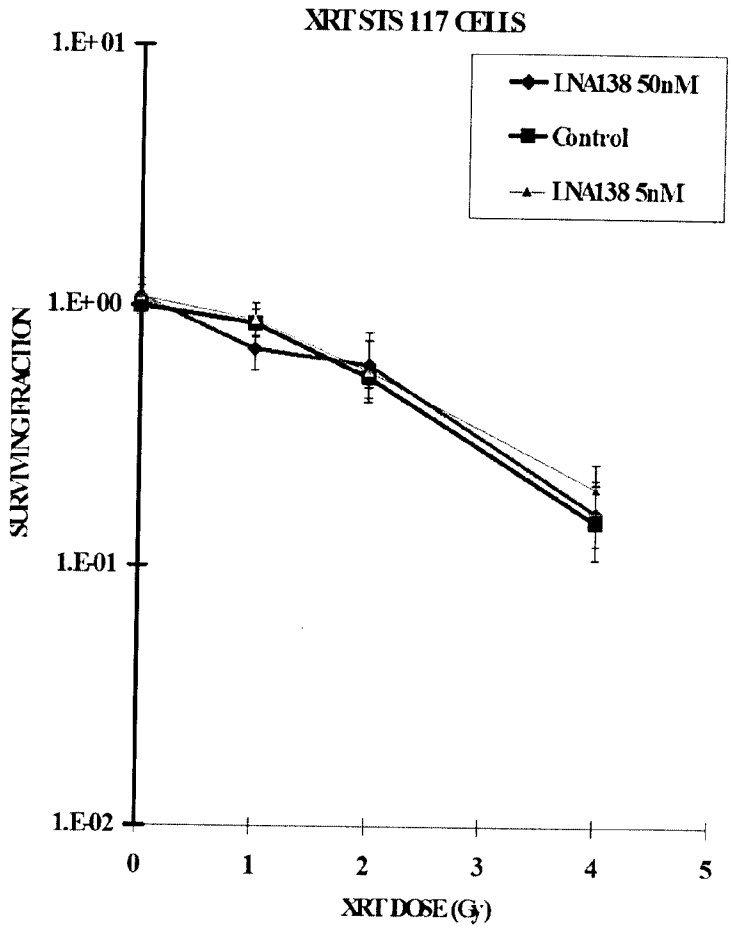
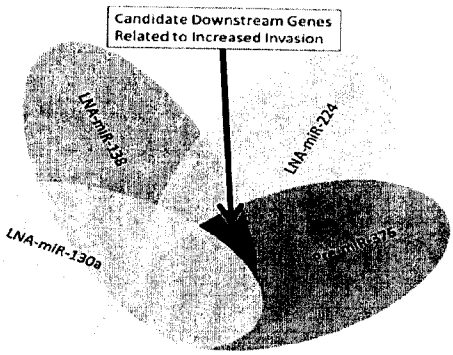

XRT SIS 117 CELLS
1.E+01
1.E+00
1.E-01
1.E-02
SURVIVING FRACTION
0
1
2
3
4
5
XRT DOSE (Gy)
LNA138 50nM
Control
LNA138 5nM

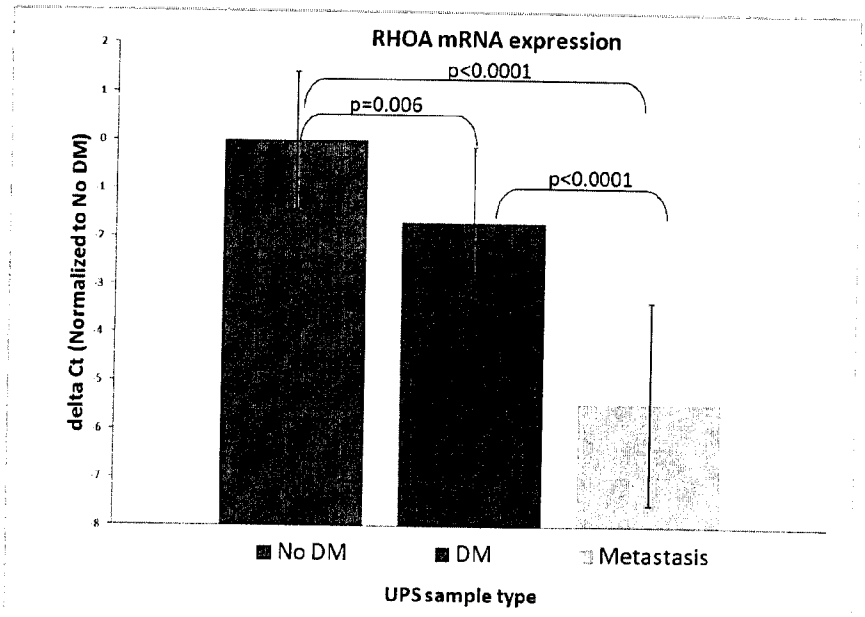
Figure 7



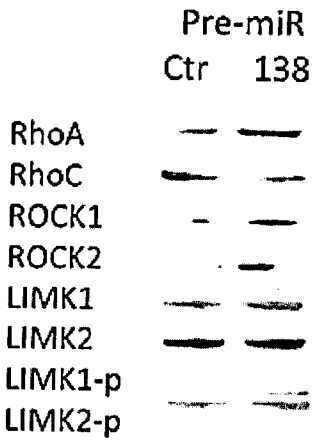
miR-138 + miR-224 intersect	miR-138 + miR-224 intersect Exclude miR-130a	miR-138 + miR-224 + miR-375 intersect
ALDH8A1	LEPR	LEPR
SLC22A1	SCAPER	STAU2
LEPR	MRC1	ITGBL1
ZDHHC11	PLCB1	SGMS1
STAU2	CCDC91	STX8
SCAPER	SGMS1	LIMCH1
CCDC88A	SCN9A	GPR126
TOM1L1		BCN2
MRC1		
BRD8		
ITGBL1		
POLA1		
RBMS3		
PLCB1		
MAD1L1		
AGBL2		
PLCB4		
CCDC91		
LPP		
ITGB1BP2		
GAS6		
AVEN		
SGMS1		
EPHX3		
STX8		
FGF18		
LIMCH1		
GPR126		
TEAD1		
BNC2		
SCN9A		
SMYD3		
C5		

Figure 8

a)



b)



Protein Level as assessed using Adobe Photoshop Pixel Quantification

	pre-miR Control	pre-miR 138
RhoA	1	1.34
RhoC	1	0.48
ROCK1	1	4.29
ROCK2	1	26.9
P-LIMK1:LIMK1	1	12
P-LIMK2:LIMK2	1	2.5

Figure 9

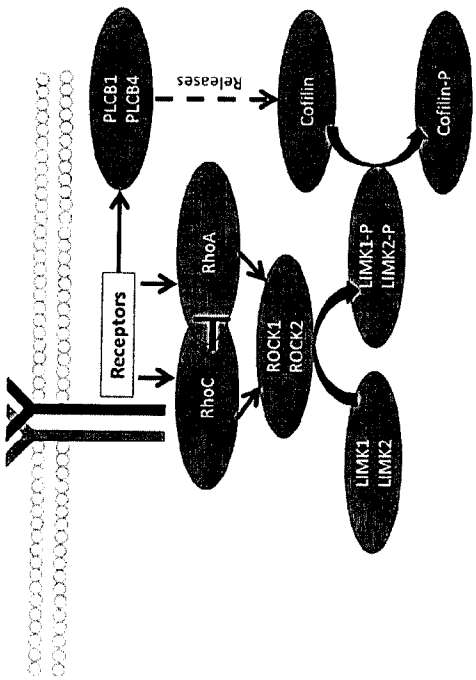

PLCB1
PLCB4
Releases
Cofilin
Cofilin-P
RhoA
Receptors
RhoC
ROCK1
ROCK2
LIMK1-P
LIMK2-P
LIMK1
LIMK2

Figure 10A

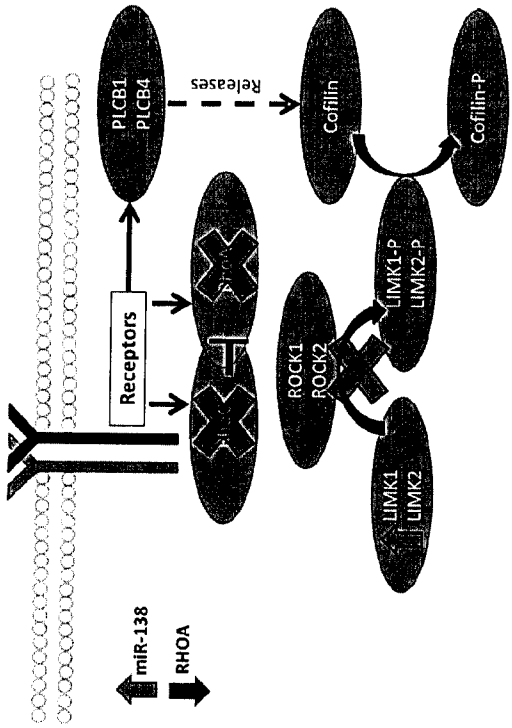

PLCB1
PLCB4
Releases
Cofilin
Cofilin-P
Receptors
LIMK1-P
LIMK2-P
ROCK1
ROCK2
LIMK1
LIMK2
miR-138
RHOA

Figure 10B

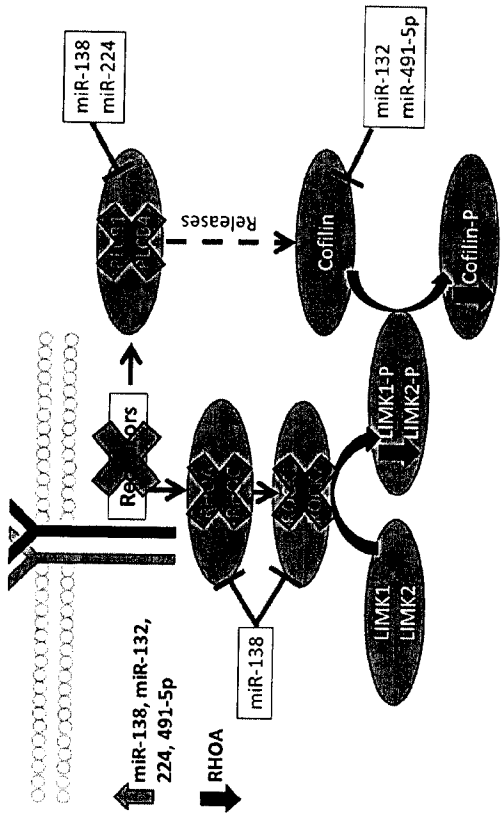

miR-138
miR-224
miR-132
miR-491-5p
Releases
Cofilin
Cofilin-P
LIMK1-P
LIMK2-P
LIMK1
LIMK2
miR-138
miR-138, miR-132,
224, 491-5p
RHOA

Figure 10C

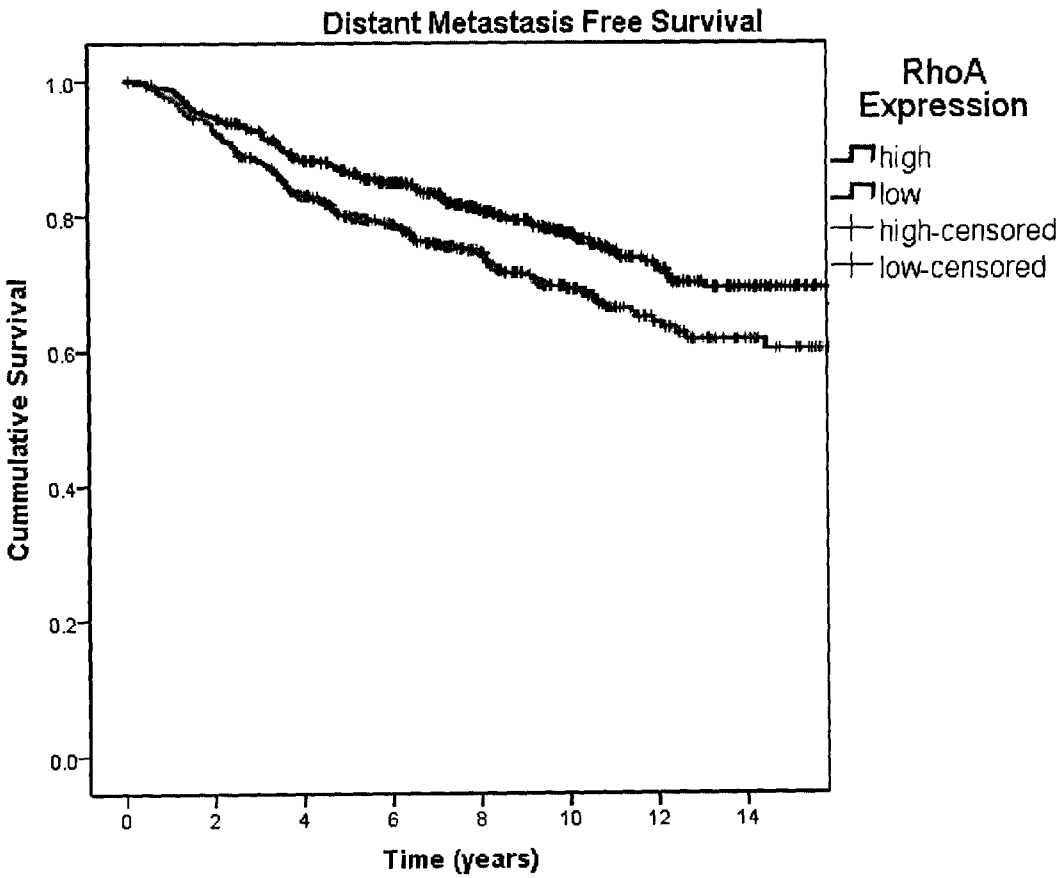

Distant Metastasis Free Survival
Cummulative Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
14
Time (years)
RhoA Expression
high
low
high-censored
low-censored

Figure 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2014/000501

A. CLASSIFICATION OF SUBJECT MATTER
IPC: *C40B 40/06* (2006.01) , *C12Q 1/68* (2006.01) , *C40B 30/00* (2006.01) , *G01N 33/48* (2006.01) , *G01N 33/50* (2006.01) , *G06F 19/20* (2011.01), *C07H 21/00* (2006.01) , *C12N 15/113* (2010.01)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC (2006.01): C40B 40/06, C12Q 1/68, C40B 30/00, G01N 33/48, G01N 33/50 C07H 21/00; IPC (2011.01): G06F 19/20; IPC (2010.01): C12N 15/113

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
DATABASES: QUESTEL ORBIT, SCOPUS, PUBMED, CANADIAN PATENT DATABASE
KW: fibrosarcoma, malignant fibrous histiosarcoma, undifferentiated pleomorphic sarcoma, pleomorphic undifferentiated sarcoma, cancer, Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143, Mir-132

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US2008/0076674 (LITMAN, T. et al) 27 March 2008 (27-03-2008) *abstract; Claims 1-6 and 18*	1, 6-14 and 23
A	US2009/0239815 (LITMAN, T. et al) 24 September 2009 (24-09-2009) *abstract; paragraphs [0422-0429] and [0434-0435]*	1, 6-14 and 23
X	APPLIED BIOSYSTEMS. TaqMan® Human MicroRNA Arrays. Technical Bulletin. 2008. Downloaded on 03 September 2014 (03-09-2014) at internet address: *http://tools.lifetechnologies.com/content/sfs/manuals/cms_054742.pdf* *whole document*	13-14 and 23

☐ Further documents are listed in the continuation of Box C.

☑ See patent family annex.

*	Special categories of cited documents:
"A"	document defining the general state of the art which is not considered to be of particular relevance
"E"	earlier application or patent but published on or after the international filing date
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed
"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search 03 September 2014 (03-09-2014)	Date of mailing of the international search report
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Authorized officer Pascal Lachance (819) 994-8889

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2014/000501

Patent Document Cited in Search Report	Publication Date	Patent Family Members(s)	Publication Date
US2008076674A1	27 March 2008 (27-03-2008)	None	
US2009239815A1	24 September 2009 (24-09-2009)	US2009239815A1	24 September 2009 (24-09-2009)
		US8188255B2	29 May 2012 (29-05-2012)
		EP2090665A2	19 August 2009 (19-08-2009)
		US2012184603A1	19 July 2012 (19-07-2012)
		WO2008046911A2	24 April 2008 (24-04-2008)
		WO2008046911A3	03 July 2008 (03-07-2008)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2014/000501

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☑ Claim Nos.: 15-22 (wholly)
because they relate to subject matter not required to be searched by this Authority, namely:

See extra sheet.

2. ☑ Claim Nos.: 1 and 6-23 (partially) and 2-5 (wholly)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

See extra sheet.

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
See extra sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☑ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant=s protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Continued from Box No. II

The combinatorially large number of possible sets of one or more miRNA biomarkers defined by claims 1-23, for use in prognosing or classifying a subject with undifferentiated pleomorphic sarcoma (UPS), are not supported by the description and fail to comply with the requirements of **Article 5 of the PCT** and **Article 6 of the PCT** to such an extent that a meaningful search over the entire claimed scope is not possible.

An effort was nevertheless made by the ISA to identify subject matter on which a meaningful search could be performed. In view of the description, the subject matter apparently most important for the applicant is a prognostic signature for Distant Metastasis Free Survival (DMFS) relating to UPS consisting of six miRNAs, wherein said six miRNAs are Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132, wherein the expression levels of said miRNAs can be used to classify patients into "low risk" and "high risk" groups of developing metastasis. The scope of the search and examination was therefore limited to the subject matter of claims 1 and 6-23 insofar as they relate to the 6-miRNA signature consisting of Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132, for use in prognosing or classifying a subject with UPS. The subject matter of claims 2-5 was not subject to search as said claims relate to biomarker signatures consisting of 2, 3, 4 and 5 biomarkers, respectively.

Claims 15-19 and 22 are directed to a computer program, computer implemented products and a computer system that are not adequately described in the application beyond a statement of their desired result, namely to carry out the diagnostic methods defined in the claims or to be used in conjunction with the methods defined in the claims, and which therefore fail to comply with the requirements of **Article 5 of the PCT** and **Article 6 of the PCT** to such an extent that a meaningful search of said claims is not possible. Further, said claims are directed to computer algorithms which output a prognosis, determine a therapy or are used in conjunction with the methods of claims 1-12. These claims are thus directed to schemes, rules, methods of doing business or of performing purely mental acts, which the international authority is not required to search according to **Article 17** and **Rule 39 (iii) of the PCT**.

Claims 20-21 are directed to a computer readable medium which has stored thereon a data structure or database. These claims are directed to the mere presentation of information, which the international authority is not required to search according to **Article 17** and **Rule 39 (v) of the PCT**.

Continued from Box No. III

The application is directed to methods, compositions, computer programs, computer implemented products, a computer readable medium, a computer system and a kit for prognosing or classifying a subject with undifferentiated pleomorphic sarcoma (UPS) using combinations of one or more of the miRNA biomarkers selected from Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132. As each of the miRNA biomarkers is structurally distinct, the only feature which might serve to unify the subject matter of the claims is the general concept of using combinations of one or more miRNA biomarkers in expression-based diagnosis of UPS.

However, the general concept of using combinations of one or more miRNA biomarkers in expression-based diagnosis of UPS is known in the prior art, as disclosed for example in each of US2008/0076674 and US2009/0239815, and thus the claims do not relate to a single inventive concept *a posteriori* under **Rule 13.1 of the PCT**. Therefore the claims are directed to a plurality of alleged inventions wherein each specific combination of one or more of the miRNA biomarkers selected from Mir-221, Mir-491-5p, Mir-224, Mir-138, Mir-143 and Mir-132 represents a separate invention.

The claims must be limited to one inventive concept as set out in **Rule 13 of the PCT**.