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- (71) **Applicant:** SYDDANSK UNIVERSITET [DK/DK];
Campusvej 55, DK-5230 Odense M (DK).
- (72) **Inventors:** DITZEL, Henrik; Soegade 4, DK-8660
Skanderborg (DK). KODAH, Annette Raskov; J.B.
Winsloews Vej 25, DK-5000 Odense C (DK).
- (74) **Agent:** ORSNES, Henrik; Forskerparken 10, DK-5230
Odense M (DK).
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(54) **Title:** CIRCULATING MICRORNA BASED CANCER BIOMARKERS

(57) **Abstract:** There is provided methods of diagnosing whether a subject has, or is at risk for developing, breast cancer, comprising measuring the level of miR-145, miR-107, and miR-39-5p in a test sample from the subject and comparing the level of the miRNA in the test sample to the level of a corresponding miRNA in a control sample.



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Circulating microRNA based Cancer Biomarkers

FIELD OF THE INVENTION

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The present invention relates to a breast cancer-specific signature of miRNAs that are differentially expressed in cancer cells, such as breast cancer and colorectal cancer, relative to normal control cells. Specifically the invention relates to these miRNAs in the circulation of women with breast cancer relative to healthy controls.

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BACKGROUND OF THE INVENTION

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The breast cancer (BC) incidence worldwide is steadily increasing and BC is among the most frequent cancers in women in Europe and North America. Early diagnosis is essential when it comes to saving lives as early stage tumors are more likely to be cured. Currently mammography screening is the gold standard diagnostic tool in many countries; however it is not without limitations as it is associated with a substantial over diagnosis of breast cancer[1]. Therefore, there is a need for developing new methods for earlier detection of breast cancer - alone or combined with mammography, as well as markers that can provide an early indication of whether a given treatment is effective. To date, there are no highly sensitive and specific minimally invasive biomarkers for detection of breast cancer at an early stage.

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Major and intensive research has been focused on early detection, treatment and prevention. This has included an emphasis on determining the presence of precancerous or cancerous ductal epithelial cells. These cells are analyzed, for example, for cell morphology, for protein markers, for nucleic acid markers, for chromosomal abnormalities, for biochemical markers, and for other characteristic changes that would signal the presence of cancerous or precancerous cells. This has led to various molecular alterations that have been reported in breast cancer, few of which have been well characterized in human clinical breast specimens. Molecular alterations include presence/absence of estrogen and progesterone steroid receptors, HER-2 expression/amplification (Mark HE, et al. HER-2/neu gene amplification in

stages I-IV breast cancer detected by fluorescent in situ hybridization. Genet Med; 1 (3):98-103 1999), Ki-67 (an antigen that is present in all stages of the cell cycle except G0 and used as a marker for tumor cell proliferation, and prognostic markers (including oncogenes, tumor suppressor genes, and angiogenesis markers).

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MicroRNAs (miRNAs) are small, 18-25 nucleotide long, non-coding RNA molecules that down-regulates the translation of messenger RNA (mRNA) in both animal and plant cells. MicroRNAs control a wide array of physiological and pathological processes, including development, differentiation, cellular proliferation, programmed cell death, oncogenesis, and metastasis by modulating the expression of their target genes through cleaving mRNA molecules or inhibiting their translation. A tumor suppressor miRNA typically blocks the expression of a "true" oncogene. Conversely, miRNA oncogenes block the expression of "true" tumor suppressor genes with increased risk of tumor formation [2-5]. That miRNAs represent a promising new class of diagnostic biomarkers was demonstrated in 2005 by Lu et al. who showed that a molecular signature consisting of approx. 200 miRNAs was superior to a corresponding mRNA signature based on over 20,000 genes when it came to classifying poorly differentiated tumors [6]. When it comes to detection of miRNAs in body fluids, Lawrie et al [7] were among the first to describe the detection of circulating microRNAs in the serum of patients with diffuse large cell B-cell lymphoma. Since then, circulating miRNAs have been proposed as promising novel biomarkers for cancer and several other diseases. One of the most important advantages of miRNAs, apart from being easily measured in blood components, is their remarkable stability in plasma and serum, where they most likely are protected from RNase degradation due to their binding to Argonaute proteins [8, 9]. These characteristics make circulating miRNAs very promising biomarkers for disease detection and in the last couple of years an increasing interest has been seen in this field.

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WO07016548A2 describes the identification of a breast cancer-specific signature of microRNAs (miRNAs) that are differentially expressed in breast cancer cells, relative to normal control cells. An alteration (e.g., an increase, a decrease) in the level of the miRNA in the test sample, relative to the level of a corresponding miRNA in a control sample, is indicative of the subject either having, or being at risk for developing,

breast cancer. miR-145 and miR-143 are down-regulated in the studied breast cancer patients.

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But to date it still remains unclear whether a certain combination of circulating miRNAs can serve as a diagnostic test in early breast cancer detection. While previous studies on circulating miRNAs in breast cancer have mostly focused on alterations of single miRNAs it was decided to search for a miRNA signature with an ability to discriminate between breast cancer and healthy controls.

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MAR-AGUILAR, F. et al. [14] disclose measuring the level of specific miRNAs for use as biomarkers for breast cancer (the abstract). The authors disclose measuring the level of the miRNAs miR-145, miR-155, and miR-382 as biomarker for breast cancer compared to measurement of the level of any of said biomarkers alone. All said three miRNAs were up-regulated in breast cancer patients.

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NG, E.K.O. et al. [15] disclose measuring the level of specific miRNAs for use as biomarkers for determination of breast cancer. The authors disclose measuring the level of the miRNAs miR-145 and miR-451 as biomarkers for breast cancer compared to measurement of the level of the biomarkers alone. miR-145 was down-regulated and miR-451 up-regulated in the studied breast cancer patients.

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WO 2011/110644 discloses measuring the level of specific miRNAs for use as biomarkers for use in the diagnosis and prognosis of a number of cancers, in particular breast cancer. WO 2011/110644 specifically discloses measuring the level of the miRNAs miR-195, miR-181c, miR-342, and let-7a as a biomarker for breast cancer. The results demonstrated a high sensitivity to specificity ratio, and a resulting area under the curve (AUC) of receiver operating characteristics (ROC) at 0.932. Funher, WO 2011/110644 discloses the use of at least one of miR-145 or miR-143 as a biomarker for breast cancer. All four miRNAs were upregulated in the studied breast cancer patients.

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WO 2011/127219 discloses the identification of specific biomarkers that are used for the diagnosis or prognosis of e.g. breast cancer. The breast cancer specific

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biomarkers can include one or more (overexpressed miRNAs, underexpressed miRNAs, or any combination thereof, such as miR-145 or miR-143.

5 US 2008/076674 discloses a method for the characterization of breast cancer by use of miRNA, and the microRNA may be selected from miR-145, miR-143, miR-365 or miR-15a.

10 WO2007/081740 discloses a method of diagnosing whether a subject has or is at risk for developing breast cancer, comprising measuring the level of at least one miRNA gene product in a test sample from the subject, where the least one miRNA gene product may be miR-145.

15 WO 2011/109940 discloses the identification of specific biomarkers that are used for the diagnosis or prognosis of e.g. breast cancer. The inventors report that miR-145 and miR-143 are downregulated in breast cancer patients.

20 There is a need for new biomarkers in serum for accurate and early diagnosis of BC as well as other cancers, such as colorectal cancer.

SUMMARY OF THE INVENTION

25 The present invention is based on the identification of a cancer-specific signature of circulating miRNAs.

30 Accordingly, the invention encompasses methods of diagnosing whether a subject has, or is at risk for developing cancer, in particular breast cancer and colorectal cancer, comprising measuring the level of at least one miRNA of miR-145, miR-107, and miR-139-5p, preferably the combination of miR-145, miR-107, and miR-139-5p, in a test sample, such as serum, plasma, or full blood from the subject and comparing the level of the miRNA in the test sample to the level of a corresponding miRNA in a control sample. The inventors have surprisingly found that a more reliable diagnosis
35 is obtained with miR-145, miR-107, and miR-139-5p than other known combinations.

An alteration (e.g., an increase, a decrease) in the level of the miRNA in the test sample, relative to the level of a corresponding miRNA in a control sample, is indicative of the subject either having, or being at risk for developing cancer, such as breast cancer. In certain embodiments, the at least one miRNA is selected from the group consisting of miR-145, miR-107, and miR-139-5p, and combinations thereof. The level of the at least one miRNA can be measured using a variety of techniques that are well known to those of skill in the art. In a particularly preferred embodiment the level of miR-15a, miR-18a, miR-107, miR-133a, miR-139-5p, miR-143, miR-145, miR-365, and miR-425 is used to perform the diagnostic method of the present invention.

In one embodiment, the level of the at least one miRNA from miR-145, miR-107, and miR-139-5p is measured using a microarray that comprises miRNA-specific probe oligonucleotides to provide a hybridization profile for the test sample, and comparing the test sample hybridization profile to a hybridization profile generated from a control sample. An alteration in the signal of at least one miRNA in the test sample, such as serum, relative to the control sample is indicative of the subject either having, or being at risk for developing cancer, such as breast cancer or colorectal cancer.

In a further embodiment, the microarray comprises miRNA-specific probe oligonucleotides for one or more miRNAs selected from the group consisting of miR-365, miR-425, miR-143, miR-133a, miR-15a, and miR-18a, in addition to the at least one miRNA from miR-365, miR-425, and miR-139-5p and combinations thereof.

The invention also provides methods of diagnosing cancer, such as breast cancer and colorectal cancer, associated with one or more prognostic markers, comprising measuring the level of at least one miRNA from miR-145, miR-107, and miR-139-5p in a breast cancer test sample from a subject and comparing the level of the at least one miRNA in the breast cancer test sample to the level of a corresponding miRNA in a control sample. The cancer can be associated with one or more adverse prognostic markers associated with cancer, such as, but not limited to, estrogen receptor expression, progesterone receptor expression, positive lymph node metastasis, high proliferative index, detectable p53 expression, advanced tumor stage, and high vascular invasion.

The invention also provides methods of monitoring cancer treatment, such as breast cancer and colorectal cancer treatment, wherein at least one de-regulated (e.g., down-regulated, up-regulated) miRNA in the cancer cells of the subject is normalized
5 indicating that the treatment has been effective.

The invention also provides methods of monitoring cancer recurrence, such as breast cancer and colorectal cancer recurrence, wherein at least one miRNA is de-regulated (e.g., down-regulated, up-regulated) in the cancer cells of the subject after a period of
10 miRNA normalization.

The invention also encompasses methods of treating cancer, such as breast cancer and colorectal cancer, in a subject, wherein at least one miRNA is de-regulated (e.g., down-regulated, up-regulated) in the cancer cells of the subject. When the at least
15 one isolated miRNA is down-regulated in the breast cancer cells, the method comprises administering an effective amount of the at least one isolated miRNA, such that proliferation of cancer cells in the subject is inhibited. In one embodiment, the method comprises administering an effective amount of the at least one isolated miRNA, such that proliferation of cancer cells in the subject is inhibited. When the at
20 least one isolated miRNA is up-regulated in the cancer cells, the method comprises administering to the subject an effective amount of at least one compound for inhibiting expression of the at least one miR gene, such that proliferation of cancer cells is inhibited.

In related embodiments, the invention provides methods of treating cancer, such as breast cancer and colorectal cancer, in a subject, comprising determining the amount of at least one miRNA in cancer cells from the subject, relative to control cells. If expression of the miRNA is deregulated in cancer cells, the methods further comprise altering the amount of the at least one miRNA expressed in the cancer cells. If the
25 amount of the miRNA expressed in the cancer cells is less than the amount of the miRNA expressed in control cells, the method comprises administering an effective amount of at least one isolated miRNA.
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BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1 shows the workflow of the diagnostic method of the present invention.

Figure 2 shows a Principal Component Analysis (PCA) plot with unsupervised clustering analysis of miRNA profiles of the samples analyzed in the global test set.

10 Figure 3 shows ROC curve analysis using the 9 miRNA profile (miR-15a, miR-18a, miR-107, miR-133a, miR-139-5p, miR-143, miR-145, miR-365, and miR-425) for discriminating breast cancer cases from healthy controls.

15 DETAILED DESCRIPTION OF THE INVENTION

The present invention is based, in part, on the identification of particular miRNAs whose expression is altered in breast cancer cells relative to normal control cells, and microRNAs whose expression is altered in breast cancer cells associated with
20 particular prognostic features, relative to breast cancer cells lacking such features.

As used herein interchangeably, a "miRNA," "microRNA," "miR," or "miRNA" refers to the unprocessed or processed RNA transcript from an miR gene. As the miRNAs are not translated into protein, the term "miRNAs" does not include proteins. The
25 unprocessed miR gene transcript is also called an "miR precursor," and typically comprises an RNA transcript of about 70-100 nucleotides in length. The miR precursor can be processed by digestion with an RNase (for example, Dicer, Argonaut, or RNase III, e.g., E. coli RNase III) into an active 19-25 nucleotide RNA molecule. This active 19-25 nucleotide RNA molecule is also called the "processed"
30 miR gene transcript or "mature" miRNA.

The level of at least one miRNA can be measured in cells of a biological sample obtained from the subject. For example, a blood sample can be removed from a subject suspected of having breast cancer associated with by conventional biopsy
35 techniques. In another example, a tissue sample can be removed from the subject.

An alteration (i.e., an increase or decrease) in the level of a miRNA in the sample obtained from the subject, relative to the level of a corresponding miRNA in a control sample, is indicative of the presence of breast cancer in the subject. In one embodiment, the level of the at least one miRNA in the test sample is greater than the level of the corresponding miRNA in the control sample (i.e., expression of the miRNA is "up-regulated"). As used herein, expression of an miRNA is "up-regulated" when the amount of miRNA in a cell or blood sample from a subject is greater than the amount the same miRNA in a control cell or blood sample. In another embodiment, the level of the at least one miRNA in the test sample is less than the level of the corresponding miRNA in the control sample (i.e., expression of the miRNA is "down-regulated"). As used herein, expression of an miRNA is "down-regulated" when the amount of miRNA in a cell or blood sample from a subject is less than the amount produced from the same miRNA in a control cell or tissue sample. The relative miRNA expression in the control and normal samples can be determined with respect to one or more RNA expression standards. The standards can comprise, for example, a zero miRNA expression level, the miRNA expression level in a standard cell line, or the average level of miRNA expression previously obtained for a population of healthy human controls.

The level of a miRNA in a sample can be measured using any technique that is suitable for detecting miRNA expression levels in a biological sample. Suitable techniques for determining miRNA expression levels in cells from a biological sample (e.g., Northern blot analysis, RT-PCR, quantitative RT-PCR, microarray, in situ hybridization) are well known to those of skill in the art.

Accordingly, the invention provides methods of diagnosing whether a subject has, or is at risk for developing, breast cancer, comprising reverse transcribing RNA from a test sample obtained from the subject to provide a set of target oligo-deoxynucleotides, hybridizing the target oligo-deoxynucleotides to a microarray comprising miRNA-specific probe oligonucleotides to provide a hybridization profile for the test sample, and comparing the test sample hybridization profile to a hybridization profile generated from a control sample, wherein an alteration in the signal of at least one miRNA is indicative of the subject either having, or being at risk for developing, breast cancer. In one embodiment, the microarray comprises miRNA-

specific probe oligonucleotides for a substantial portion of the human miRNome. In a particular embodiment, the microarray comprises miRNA-specific probe oligonucleotides for one or more miRNAs selected from the group consisting of miR-145, miR-107, and miR-139-5p. In a preferred embodiment the microarray also
5 comprises miRNA-specific probe oligonucleotides for one or more miRNAs selected from the group consisting of miR-365, miR-425, miR-143, miR-133a, miR-15a, and miR-18a and combinations thereof.

The microarray can be prepared from gene-specific oligonucleotide probes generated
10 from known miRNA sequences. The array may contain two different oligonucleotide probes for each miRNA, one containing the active, mature sequence and the other being specific for the precursor of the miRNA. The array may also contain controls, such as one or more mouse sequences differing from human orthologs by only a few bases, which can serve as controls for hybridization stringency conditions. tRNAs
15 from both species may also be printed on the microchip, providing an internal, relatively stable, positive control for specific hybridization. One or more appropriate controls for non-specific hybridization may also be included on the microchip. For this purpose, sequences are selected based upon the absence of any homology with any known miRNAs.

The microarray may be fabricated using techniques known in the art. For example, probe oligonucleotides of an appropriate length, e.g., 40 nucleotides, are 5'-amine modified at position C6 and printed using commercially available microarray systems, e.g., the GeneMachine OmniGrid(TM) 100 Microarrayer and Amersham
25 CodeLink(TM) activated slides. Labeled cDNA oligomer corresponding to the target RNAs is prepared by reverse transcribing the target RNA with labeled primer. Following first strand synthesis, the RNA/DNA hybrids are denatured to degrade the RNA templates. The labeled target cDNAs thus prepared are then hybridized to the microarray chip under hybridizing conditions, e.g., 6X SSPE/30% formamide at 25 C
30 for 18 hours, followed by washing in 0.75X TNT at 37 C for 40 minutes. At positions on the array where the immobilized probe DNA recognizes a complementary target cDNA in the sample, hybridization occurs. The labeled target cDNA marks the exact position on the array where binding occurs, allowing automatic detection and quantification. The output consists of a list of hybridization events, indicating the
35 relative abundance of specific cDNA sequences, and therefore the relative

abundance of the corresponding complementary miRs, in the patient sample. According to one embodiment, the labeled cDNA oligomer is a biotin-labeled cDNA, prepared from a biotin-labeled primer. The microarray is then processed by direct detection of the biotin-containing transcripts using, e.g., Streptavidin-Alexa647 conjugate, and scanned utilizing conventional scanning methods. Image intensities of each spot on the array are proportional to the abundance of the corresponding miR in the patient sample.

Other techniques for measuring miR gene expression are also within the skill in the art, and include various techniques for measuring rates of RNA transcription and degradation. The invention also provides methods of diagnosing a breast cancer associated with one or more prognostic markers, comprising measuring the level of at least one miRNA in a breast cancer test sample from a subject and comparing the level of the at least one miRNA in the breast cancer test sample to the level of a corresponding miRNA in a control sample. An alteration (e.g., an increase, a decrease) in the signal of at least one miRNA in the test sample relative to the control sample is indicative of the subject either having, or being at risk for developing, breast cancer associated with the one or more prognostic markers.

The breast cancer can be associated with one or more prognostic markers or features, including, a marker associated with an adverse (i.e., negative) prognosis, or a marker associated with a good (i.e., positive) prognosis. In certain embodiments, the breast cancer that is diagnosed using the methods described herein is associated with one or more adverse prognostic features selected from the group consisting of estrogen receptor expression, progesterone receptor expression, positive lymph node metastasis, high proliferative index, detectable p53 expression, advanced tumor stage, and high vascular invasion. Particular microRNAs whose expression is altered in breast cancer cells associated with each of these prognostic markers are described herein. In one embodiment, the level of the at least one miRNA is measured by reverse transcribing RNA from a test sample obtained from the subject to provide a set of target oligodeoxynucleotides, hybridizing the target oligodeoxynucleotides to a microarray that comprises miRNA-specific probe oligonucleotides to provide a hybridization profile for the test sample, and comparing the test sample hybridization profile to a hybridization profile generated from a control sample.

The invention will now be illustrated by the following non-limiting examples.

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EXAMPLE

Serum samples (total 183, 108 cases and 75 healthy controls) were selected from a larger cross sectional cohort collected at the Department of Oncology, Odense University Hospital, Denmark, in the period 1994-1997 [11]. Samples were collected at time of diagnosis, prior to surgery, and immediately stored at -80° C. Histopathological diagnosis was confirmed after surgical resection of the tumors. The control serum samples were collected from healthy women with no history of malignant diseases and no inflammatory conditions as well as no cancer diagnosis or diagnosis of any inflammatory diseases within 5 years of sample collection (by journal reviews). The age-matched healthy women were randomly selected from the Danish CPR-registry for a given case with a birthday of +/- one day. All cases and controls were Caucasian.

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Study Design

The study consists of three stages (Fig. 1): Marker discovery, Marker selection and Marker validation. *Marker discovery*: Global miRNA analysis was performed on serum from 48 postmenopausal patients with ER-positive early stage breast cancer (24 with lymph node metastasis and 24 without lymph node metastasis) and 24 age-matched and disease-free healthy controls using LNA-based quantitative PCR (qRT-PCR).

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Marker selection: based on their resampling inclusion frequencies 9 microRNAs were selected for further validation.

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Marker validation: Selected miRNAs were validated in an independent cohort of 111 serum samples from 60 postmenopausal patients with ER-positive early stage breast cancer and 51 healthy controls. The study was approved by the regional ethics committee.

The selected miRNAs were also validated in a data set from a Swedish study of mucosal Lichten Planus carried out by Nylander et al [12]. This study was chosen because the same LNA-based platform was used for their serum analysis.

5 *miRNA-isolation from serum*

Total RNA was extracted from serum using the Qiagen miRNeasy® Mini Kit. Serum was thawed on ice and centrifuged at 3000 x g for 5 min at 4°C. An aliquot of 200 µL of serum per sample was transferred to a new tube, 750 µL of Qiazol mixture containing 0.94 µg/µL of MS2 bacteriophage RNA was added, mixed and incubated for 5 min before 200 µL chloroform was added. The content was again mixed, 10 incubated for 2 min and centrifuged at 12,000 x g for 15 min at 4°C. The upper aqueous phase was transferred to a new tube and 1.5x volume of 100% ethanol was added. The contents were mixed thoroughly and 750 µL of the sample was transferred to a Qiagen RNeasy® Mini spin column in a collection tube followed by 15 centrifugation at 15,000 x g for 30 sec at room temperature (RT). The process was repeated until all remaining sample had been loaded. The spin column was rinsed with 700 µL Qiagen RWT buffer and centrifuged at 15,000 x g for 1 min at RT followed by another rinse with 500 µL Qiagen RPE buffer and centrifuged at 15,000 x g for 1 min at RT. A rinse step (500 µL RPE buffer) was repeated 2X. The spin 20 column was transferred to a new collection tube and centrifuged at 15,000 x g for 2 min at RT. The spin column was transferred to a new tube and the lid was left uncapped for 1 min to allow the content to dry. Total RNA was eluted by adding 50 µL of RNase-free water to the membrane of the spin column and incubating for 1 min before centrifugation at 15,000 x g for 1 min at room temperature. The RNA was 25 stored in a -80°C freezer.

Sample quality

To obtain a rough estimate of the data quality for each sample the level of microRNAs (number of microRNAs detected) and average miRNA content in all samples were 30 compared. Very similar data was obtained, suggesting that the samples were of similar quality and have been processed reproducibly.

microRNA real time PCR

RNA (4 µl) was reverse transcribed in 20 µl reactions using the miRCURY LNA™ 35 Universal RT microRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon).

cDNA was diluted 50 x and assayed in 10 µl PCR reactions according to the manufacturer's protocol for miRCURY LNA™ Universal RT microRNA PCR; each microRNA was assayed once by qPCR on the microRNA serum focus panel. Negative controls excluding template from the reverse transcription reaction was performed and profiled like the samples. The amplification was performed in a LightCycler® 480 Real-Time PCR System (Roche) in 384 well plates. The amplification curves were analyzed using the Roche LC software, both for determination of Cp (by the 2nd derivative method) and for melting curve analysis.

Normalization and data analysis

Normalization was performed based on the mean of the assays detected in all samples as this is shown to be the best normalization for qPCR studies involving numerous assays[13] For the present study, this included 65 assays. The formula used to calculate the normalized Cp values was: Normalized Cp = mean (n=65) – assay Cp

Based on the most stably expressed miRNAs in the discovery set, miR-10b and miR-30a were chosen for normalization in the validation set.

All assays were inspected for distinct melting curves and the Tm was checked to be within known specifications for the assay. Furthermore assays had to be detected with 5 Cp's less than the negative control, and with Cp<37 to be included in the data analysis. Data that did not pass these criteria were omitted from any further analysis.

Principal Component Analysis (PCA), a method used to reduce the dimension of large data sets, was performed on the top 50 microRNAs that had the largest variation across all samples. An overview of how the samples clustered based on this variance was obtained (Fig. 2). In Figure 2 the following notion is used: A = cases with lymph node metastasis, B = healthy controls, and C = cases without lymph node metastasis.

Assessment of hemolysis

To assess hemolysis two microRNAs were used. One that is expressed in red blood cells (miRNA-451), and one that is relatively stable in serum and plasma and not

affected by hemolysis (miRNA-23a). The ratio between these two microRNAs correlates to the degree of hemolysis.

Statistical analysis

To compare the 3 experimental groups a one factor anova was performed. Multivariate logistic regression analysis and resampling was established in order to decide which miRNAs to proceed with in the validation project. Resampling means that data is randomly split into training and test data several times. A signature is developed in each training data set. The resampling inclusion frequency then is the relative frequency of signatures where a certain miRNA is contained. MicroRNAs with a resampling inclusion frequency $\geq 0,3$ were selected for further validation. Receiver operating characteristics (ROC) curves were calculated to discriminate samples from women with or without breast cancer. The optimal sensitivity and specificity from ROC-curve was determined.

Marker Discovery and Marker Selection

Global miRNA analysis was performed on serum from 48 postmenopausal patients with ER-positive early stage breast cancer (24 with lymph node metastasis and 24 without lymph node metastasis) and 24 age-matched and disease-free healthy controls using LNA-based quantitative PCR (qRT-PCR). Table 1 summarize the clinical and histopathological features of breast cancer patients and healthy controls.

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Table 1

Patient characteristics	Ln+ (N=24)	Ln- (N=24)	Controls (N=24)
Median age(range)	58 (47-71)	58 (50-69)	57 (45-69)
BMI, median(range)	26(19-35)	25 (20-37)	24(19-39)
Smoking status			
yes (%)	6 (25)	4 (16)	6 (25)
no (%)	13 (54)	10 (42)	18 (75)
unknown (%)	5 (21)	10 (42)	0
Post-menopausal (%)	23 (96)	22 (92)	
Pre-menopausal (%)	1 (4)	2 (8)	
ER+ (%)	24 (100)	24(100)	
Tumor size(mm,range), median	16,5 (5-36)	16,5 (2-35)	
Node positive (%)	24 (100)	0	
1-4 (%)	19 (79)		
5-9 (%)	2 (8)		
10+ (%)	3 (13)		
Ductal carcinoma (%)	24 (100)	21(88)	

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Among the 175 miRNAs analyzed, 67 miRNAs were differentially expressed in serum of breast cancer patients vs. healthy controls. Comparing breast cancer patients with vs. without lymph node metastasis, no miRNAs were identified that could distinguish the two groups. As a result the 24 ER-positive early stage breast cancer with lymph node metastasis and 24 without lymph node metastasis were pooled in the further

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analysis. Based on the ratio between miR-451 and miR-23a, which have been shown to be associated with hemolysis under the blood drawing process, no signs of hemolysis were detected in the samples.

5 Based on multivariate logistic regression analysis and resampling inclusion frequencies a 9 miRNA signature including miR-15a, miR-18a, miR-107, miR-133a, miR-139-5p, miR-143, miR-145, miR-365, miR-425, was identified for further validation. . Based on an algorithm for the 9 miRNA profile was obtained. Odds ratios and resampling inclusion frequencies for each miRNA in the profile are summarized
10 in Table 2.

Table 2

microRNA	Odds ratio	Resampling inclusion frequencies
miR-15a	1.33	0,33
miR-107	1.23	0,42
miR-18a	1.12	0,31
miR-425	1.1	0,3
miR-143	0.88	0,4
miR-139-5p	0.72	0,4
miR-365	0.89	0,32
miR-133a	0.75	0,37
miR-145	0.52	0,87

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Multivariate analysis showed that there were no association between the 9 miRNA profile and BMI, smoking status, tumor grade, tumor size or lymph node status was observed.

20 *Profile validation*

To validate the discriminating power of the 9 miRNA signature, miRNA in serum from an independent group of 60 early stage was analyzed, ER-positive, breast cancer patients and 51 healthy age-matched controls for the expression levels of miR-15a, miR-18a, miR-107, miR-133a, miR-139-5p, miR-143, miR-145, miR-365, and miR-

425. In addition, the expression level of miR-451 and miR-23a were examined to evaluate the extent of hemolysis in the samples, however no signs of hemolysis was observed in the samples.

5 All samples were obtained from the previously mentioned cohort from Odense University Hospital, Denmark. Patient characteristics are summarized in Table 3. Among the breast cancer patients 63 % were post-menopausal and 37 % were pre-menopausal. The precise menopausal status for the healthy controls were not available, however 65 % were above the age of 50 where they would most likely be
10 post-menopausal.

Table 3

Patient characteristics	Cases	Controls
Median age(range)	57(32-69)	58 (33-70)
Post menopausal	38 (63)	(65% above
Pre menopausal	22 (37)	
ER+	60(100)	
Tumor size(mm,range),	15 (2-65)	
Lymph node status		
0	42 (70)	
1-4	15 (25)	
5-9	1 (2)	
10+	2 (3)	

15 To assess whether the 9 miRNA signature was specific to postmenopausal breast cancer patients, we compared the expression of the selected miRNAs in post-menopausal and pre-menopausal breast cancer patients and found no significant difference in the expression, suggesting that the signature is not related to
20 menopausal status.

When examining the 9 miRNA profile in data from a Swedish lichen planus study [12] no difference was found in the expression of the profile between patients with

multifocal mucosal lichen planus and healthy controls, indicating that the profile is not just simply a general profile of inflammation.

5 Based on the ratio between miR-451 and miR-23a no signs of hemolysis were detected in the samples that might have affected the miRNA expression levels.

Conclusion

10 While previous studies on circulating miRNAs in BC have focused on alterations of single miRNA we here present a 3-9 miRNA signature with an improved ability to discriminate between BC and healthy controls.

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CLAIMS

1. A method of diagnosing whether a subject has, or is at risk of developing cancer,
5 such as breast cancer or colorectal cancer, or monitoring the progression or regression of said cancer in a subject, comprising measuring the level of miR-145, miR-107, and miR-139-5p, in a test sample from said subject, wherein an alteration in the level of the miRNA in the test sample, such as serum, plasma, or full blood, relative to the level of a corresponding miRNA in a control sample, is
10 indicative of the subject either having, or being at risk for developing, cancer, or respond to any treatment of the cancer.
2. The method of claim 1, wherein the method does not comprise the invasive step of collecting the sample from said subject.
- 15 3. The method of claim 1 or 2, wherein the cancer is breast cancer.
4. The method of any one of the claims 1-3, wherein the method further comprises measuring the level of at least one miR-365, miR-425, miR-143, miR-133a, miR-15a, and miR-18a, such as the combination of miR-365, miR-425, miR-143, miR-133a, miR-15a, and miR-18a.
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5. The method of any one of the claims 1-4, wherein the level of the at least one miRNA is measured using Northern blot analysis.
- 25 6. The method of any one of the claims 1-5, wherein the level of the at least one miRNA in the test sample is less than the level of the corresponding miRNA in the control sample.
- 30 7. The method of any one of the claims 1-5, wherein the level of the at least one miRNA in the test sample is greater than the level of the corresponding miRNA in the control sample.
- 35 8. An assay for predicting whether a subject has, or is at risk of developing cancer, such as breast cancer or colorectal cancer, said assay comprising means for detecting the level of miRNA of miR-145, miR-107, and miR-139-5p in a test sample from said subject.

9. The assay of claim 8 further comprising means for measuring the level of at least one miRNA of miR-365, miR-425, miR-143, miR-133a, miR-15a, and miR-18a, such as the combination of miR-365, miR-425, miR-143, miR-133a, miR-15a, and
5 miR-18a.
10. The assay of claim 8 or 9, wherein the assay is based on microRNA microarray.

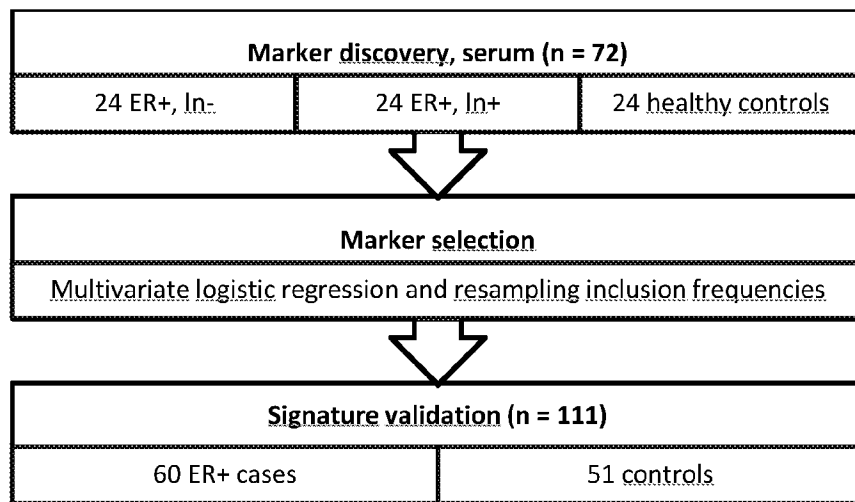


Figure 1

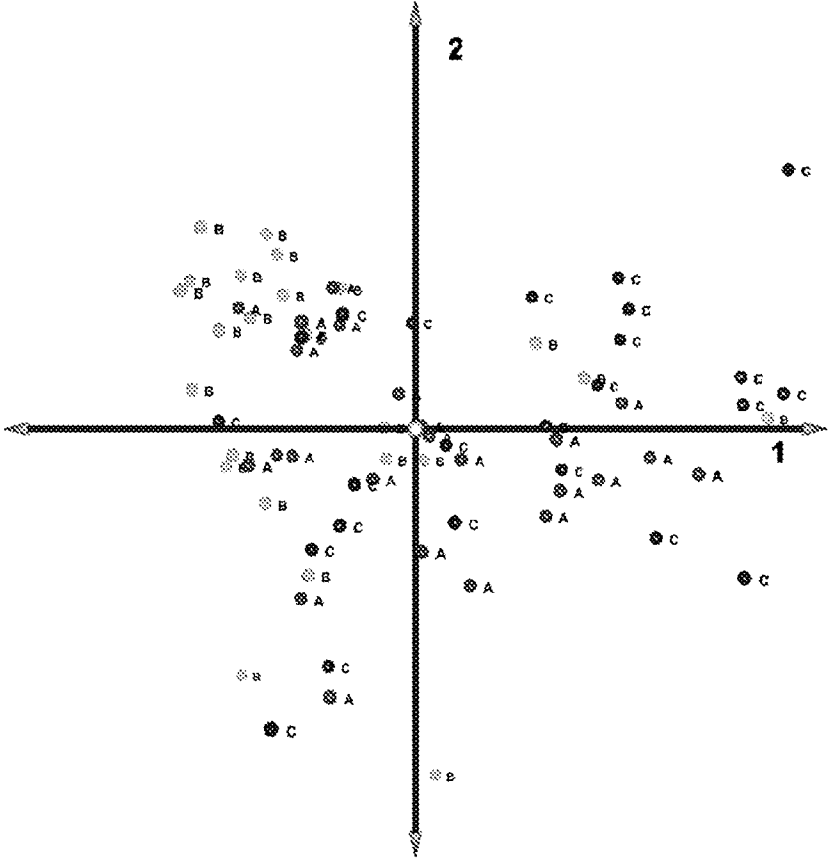


Figure 2

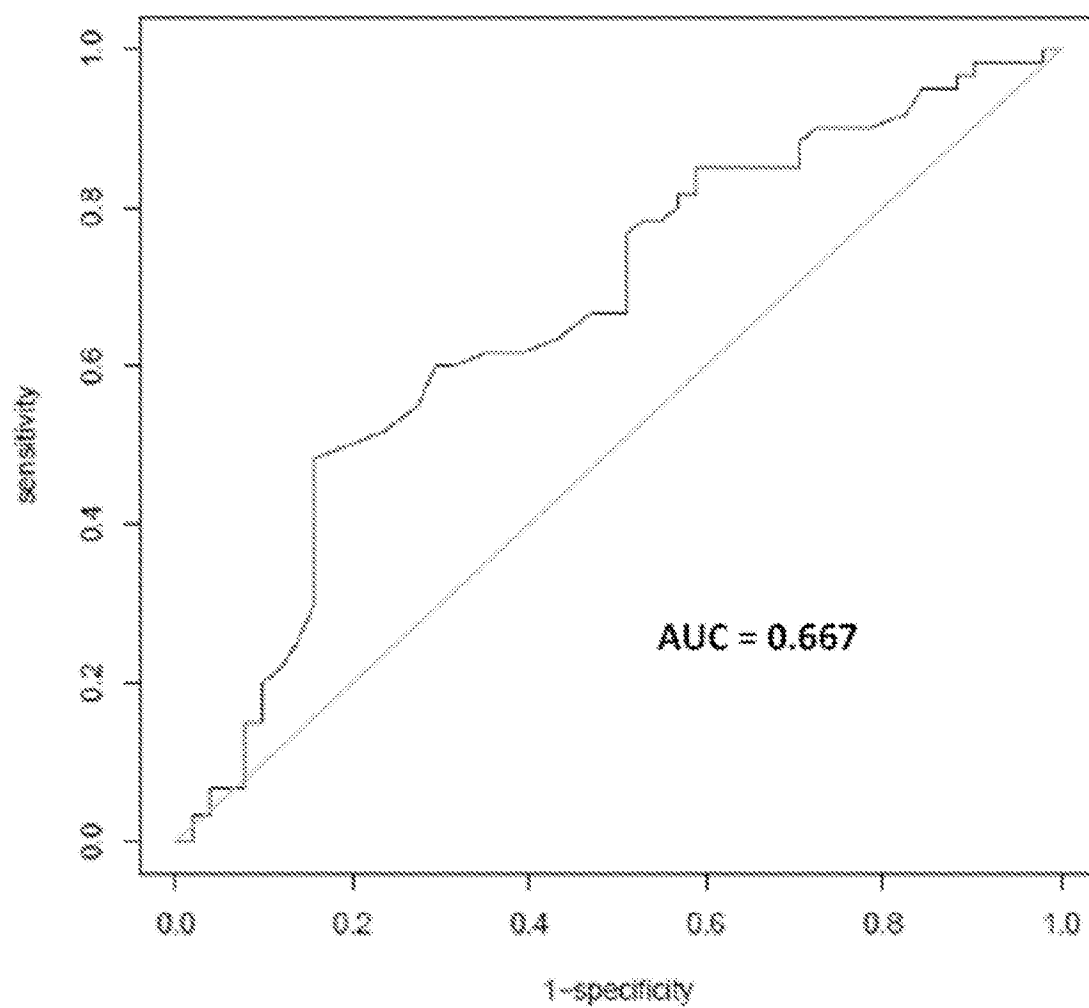


Figure 3

INTERNATIONAL SEARCH REPORT

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PCT/DK2014/050174

A. CLASSIFICATION OF SUBJECT MATTER C12Q 1/68 (2006.01), G01N 33/574 (2006.01) According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC & CPC: C12Q, G01N Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched DK, NO, SE, FI: Classes as above. Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) CHEMICAL ABSTRACTS, MEDLINE, BIOSIS, EMBASE, EPODOC, WPI, FULL TEXT: ENGLISH, ELSEVIER (XPESP)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CA 2811486 A1 (THE OHIO STATE UNIVERSITY RESEARCH FOUNDATION (US)) 2007.07.19. Page 4, lines 18-31; page 51, lines 4-29; page 54; table 8; claim 101.	1-10
X	MAR-AGUILAR, F. et al.: "Serum circulating microRNA profiling for identification of potential breast cancer biomarkers", DISEASE MARKERS, 2013.02.08, Vol. 34, No. 3, pages 163-169 The abstract; figures. 1-2; page 166, left column, paragraph 2 - right column, paragraph 2.	1-10
X	NG, E.K.O. et al.: "Circulating microRNAs as Specific Biomarkers for Breast Cancer Detection", PLOS ONE, 2013.01.03, Vol. 8, No. 1, e53141, pages 1-10 The abstract; "Marker Selection and Training"; "Marker Validation"; figures 3-5.	1-10
☒ 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		Date of mailing of the international search report
30/07/2014		07/08/2014
Name and mailing address of the ISA Nordic Patent Institute Helgeshøj Allé 81 DK - 2630 Taastrup, Denmark. Facsimile No. + 45 43 50 80 08		Authorized officer Louise Boisen Telephone No. +45 43 50 83 56

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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