



(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/BE2016/000006

(22) International Filing Date:
20 January 2016 (20.01.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/105.553 20 January 2015 (20.01.2015) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,

DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— of inventorship (Rule 4.17(iv))

Published:

— with international search report (Art. 21(3))

— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))

— with sequence listing part of description (Rule 5.2(a))

(54) Title: COLON CANCER

(57) Abstract: The present invention relates generally to a method of predicting clinical outcome in a subject diagnosed with colorectal cancer comprising determining evidence of a miRNA profile or the expression of one or more predictive RNA transcripts in a biological sample, in particular a biological sample of cancer cells obtained from the subject. It further concerns using the profile a prognostic miRNA signature for relapse-free survival (RFS) in prognosis of a patient for colorectal cancer, in particular stage III colon cancer, and recurrence or response to therapy for colon cancer, in particular stage III colon cancer.



COLON CANCER

Background and Summary

BACKGROUND OF THE INVENTION

5 A. Field of the Invention

The present invention relates generally to a method of predicting clinical outcome in a subject diagnosed with colorectal cancer comprising determining evidence of a miRNA profile or the expression of one or more predictive RNA transcripts in a biological sample, in particular a
10 biological sample of cancer cells or derived miRNA obtained from the subject. It further concerns using the profile a prognostic miRNA signature for relapse-free survival (RFS) in prognosis of a patient for colorectal cancer, in particular stage III colon cancer, and recurrence or response to therapy for colon cancer, in particular stage III colon cancer.

More particularly the present invention concerns a method to stratify patients into groups with
15 high and low risk of relapse in colorectal cancer, in particular stage III colon cancer by determining expression levels of one or more miRNA from Table 2 in a biological sample taken from the patient and determining a prognosis for colorectal cancer based on the miRNA expression levels, wherein said normalized expression levels of miRNA are positively correlated with an increased likelihood of a positive clinical outcome. Such miRNA-based
20 prognostic signature can be used for identifying the patients with an increased risk for relapse in stage III colon cancer. This may facilitate patient counselling and individualize management of patients with this disease.

Several documents are cited throughout the text of this specification. Each of the documents
25 herein (including any manufacturer's specifications, instructions etc.) are hereby incorporated by reference; however, there is no admission that any document cited is indeed prior art of the present invention.

30 B. Description of the Related Art

Current staging methods do not accurately predict the risk of disease recurrence for patients who have had surgery for stage III colon cancer. Consequently, some patients are over-treated while others may not receive the treatment they need.

Thus, there is a need in the art for additional colon cancer markers that are predictive of recurrence in early clinical stage disease.

- 5 We postulate that expression patterns of microRNAs (miRNAs) could, if combined into a single model, improve postoperative risk stratification for these patients.

Present invention provides a solution by a miRNA-based prognostic signature for relapse in stage III colon cancer. This facilitate patient counselling and individualize management of
10 patients with this disease and rule-in or rule-out for certain treatments.

SUMMARY OF THE INVENTION

15

The present invention solves the problems of the related art that current staging methods do not accurately predict the risk of disease recurrence for patients who have had surgery for stage III colon cancer and that consequently, some patients are over-treated while others may not receive the treatment they need, by identifying predictive miRNA's of which the
20 expression of one or more of the predictive RNA transcripts in a biological sample of a subject is prognostic for relapse-free survival (RFS) or for recurrence or response to therapy for colon cancer, in particular stage III colon cancer.

More particularly the present invention concerns a method to stratify patients into groups with
25 high and low risk of relapse in colorectal cancer, in particular stage III colon cancer

The present invention provides additional methods for diagnosis and prognosis of colorectal cancer by, in certain aspects, identifying miRNAs that are differentially expressed or mis-regulated in various states of diseased, normal, cancerous, and/or abnormal tissues, including
30 but not limited to normal colon and colorectal cancer. Further, the invention describes a method for diagnosing colorectal cancer that is based on determining levels (increased or decreased) of selected miRNAs in patient-derived samples. In certain aspects, RNA may or may not be isolated from a sample. In other aspects, sample RNA will be coupled to a label

and/or a probe. In a further aspect, data from RNA assessment will be transformed into a score or index indicative of expression levels.

MiRNA expression analysed profiled by qPCR from 521 stage III colon cancer tumour tissues (PETACC-3 (Pan European Trial in Adjuvant Colon Cancer) clinical trial) provided a miRNA-based prognostic signature for relapse-free survival (RFS) using a lasso-penalized Cox regression model, and assessed prognostic ability of the score using cross-validation. The estimated prognostic ability of the analytic tool was further compared with that of several previously derived prognostic mRNA expression signatures. Based on lasso-penalized Cox regression, a prognostic signature comprising 34 miRNAs, a prognostic signature consisting essentially of 34 miRNAs or a prognostic signature consisting of 34 miRNAs was identified. The signature stratified patients into groups with high and low risk of relapse, respectively. Furthermore, the prognostic ability is independent of traditionally used prognostic factors such as T-stage, N-stage, MSI status and previously derived mRNA expression signatures.

In certain aspects, a miRNA that is differentially expressed between colorectal cancer tissue and normal adjacent tissue is used to assess a patient having or suspected of having colorectal cancer, e.g. diagnosing and/or prognosing the patient's condition. A miRNA used to diagnose or prognose colorectal cancer can include one or more of hsa-miR-4532, hsa-miR-499a-5p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-135a-5p, hsa-miR-545-3p, hsa-miR-4802-5p, hsa-miR-4641, hsa-miR-3689f, hsa-miR-431-5p, hsa-miR-1266, hsa-miR-130b-3p, hsa-miR-16-1-3p and hsa-miR-155-5p.

In certain aspects one or more miRNA selected from the group consisting of hsa-miR-4532, hsa-miR-499a-5p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-135a-5p, hsa-miR-545-3p, hsa-miR-4802-5p, hsa-miR-4641, hsa-miR-3689f, hsa-miR-431-5p, hsa-miR-1266, hsa-miR-130b-3p, hsa-miR-16-1-3p and hsa-miR-155-5p, or combinations thereof, can be used in the diagnosis and prognosis of colorectal cancer.

In still a further aspect one or more miRNA can be used to assess the likelihood of colorectal cancer recurrence by evaluating the expression of one or more miRNA selected from hsa-miR-4532, hsa-miR-499a-5p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-135a-5p, hsa-miR-545-3p, hsa-miR-4802-5p, hsa-miR-4641, hsa-miR-3689f, hsa-miR-431-5p, hsa-miR-1266,

hsa-miR-130b-3p, hsa-miR-16-1-3p and hsa-miR-155-5p. In certain aspects the assessment is independent of the stage of cancer being assessed.

In still yet another aspect, a patient with stage III colorectal cancer can be assessed by evaluating the expression level of one or more miRNA that is selected from the group consisting of hsa-miR-4532, hsa-miR-499a-5p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-135a-5p, hsa-miR-545-3p, hsa-miR-4802-5p, hsa-miR-4641, hsa-miR-3689f, hsa-miR-431-5p, hsa-miR-1266, hsa-miR-130b-3p, hsa-miR-16-1-3p and hsa-miR-155-5p.

In still yet another aspect, a patient with stage III colorectal cancer can be assessed by evaluating the expression level of one or more miRNA selected from the group consisting of hsa-miR-4532, hsa-miR-499a-5p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-135a-5p, hsa-miR-545-3p, hsa-miR-4802-5p, hsa-miR-4641, hsa-miR-3689f, hsa-miR-431-5p, hsa-miR-1266, hsa-miR-130b-3p, hsa-miR-16-1-3p and hsa-miR-155-5p.

In certain aspects patients with stage III colorectal cancer can be assessed for recurrence and/or response to therapy by evaluating expression levels of one or more of miRNA selected from the group consisting of hsa-miR-4532, hsa-miR-499a-5p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-135a-5p, hsa-miR-545-3p, hsa-miR-4802-5p, hsa-miR-4641, hsa-miR-3689f, hsa-miR-431-5p, hsa-miR-1266, hsa-miR-130b-3p, hsa-miR-16-1-3p and/or hsa-miR-155-5p.

In yet another aspects patients with stage III colorectal cancer can be assessed for recurrence and/or response to therapy by evaluating expression levels of one or more of miRNA selected from the group consisting of hsa-miR-616-5p, hsa-miR-642a-5p, hsa-miR-342-5p, hsa-miR-199a-5p, hsa-miR-4532, hsa-miR-499a-5p, hsa-miR-922, hsa-miR-654-3p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-199b-5p, hsa-miR-30a-5p, hsa-miR-23a-5p, hsa-miR-874, hsa-miR-135a-5p, hsa-miR-148b-3p, hsa-miR-551b-5p, hsa-miR-181c-3p, hsa-miR-345-5p, hsa-miR-545-3p, hsa-miR-191-3p, hsa-miR-4802-5p, hsa-miR-4641, hsa-miR-3689f, hsa-miR-431-5p, hsa-miR-1266, hsa-miR-516a-3p, hsa-miR-548c-5p, hsa-miR-130b-3p, hsa-miR-219-5p, hsa-miR-502-3p, hsa-miR-33a-3p, hsa-miR-16-1-3p and/or hsa-miR-155-5p.

In yet another aspects patients with stage III colorectal cancer can be assessed for recurrence and/or response to therapy by evaluating expression of miRNA of the group consisting of hsa-miR-616-5p, hsa-miR-642a-5p, hsa-miR-342-5p, hsa-miR-199a-5p, hsa-miR-4532, hsa-

miR-499a-5p, hsa-miR-922, hsa-miR-654-3p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-199b-5p, hsa-miR-30a-5p, hsa-miR-23a-5p, hsa-miR-874, hsa-miR-135a-5p, hsa-miR-148b-3p, hsa-miR-551b-5p, hsa-miR-181c-3p, hsa-miR-345-5p, hsa-miR-545-3p, hsa-miR-191-3p, hsa-miR-4802-5p, hsa-miR-4641, hsa-miR-3689f, hsa-miR-431-5p, hsa-miR-1266, hsa-miR-516a-3p, hsa-miR-548c-5p, hsa-miR-130b-3p, hsa-miR-219-5p, hsa-miR-502-3p, hsa-miR-33a-3p, hsa-miR-16-1-3p and hsa-miR-155-5p.

Corresponding miRNA sequences that can be used in the context of the invention include, but are not limited to, all or a portion of those sequences in the sequence listing provided herein, as well as the miRNA precursor sequence, or complement of one or more of these miRNAs.

Further scope of applicability of the present invention will become apparent from the detailed description given hereinafter. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

Detailed Description

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

The following detailed description of the invention refers to the accompanying drawings. The same reference numbers in different drawings identify the same or similar elements. Also, the following detailed description does not limit the invention. Instead, the scope of the invention is defined by the appended claims and equivalents thereof.

Several documents are cited throughout the text of this specification. Each of the documents herein (including any manufacturer's specifications, instructions etc.) are hereby incorporated by reference; however, there is no admission that any document cited is indeed prior art of the present invention.

The present invention will be described with respect to particular embodiments and with reference to certain drawings but the invention is not limited thereto but only by the claims.

Furthermore, the terms first, second, third and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequential or chronological order. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein.

It is to be noticed that the term “comprising”, used in the description or claims, should not be interpreted as being restricted to the means listed thereafter; it does not exclude other elements or steps. It is thus to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more other features, integers, steps or components, or groups thereof.

Reference throughout this specification to “one embodiment” or “an embodiment” means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances of the phrases “in one embodiment” or “in an embodiment” in various places throughout this specification are not necessarily all referring to the same embodiment, but may. Furthermore, the particular features, structures or characteristics may be combined in any suitable manner, as would be apparent to one of ordinary skill in the art from this disclosure, in one or more embodiments.

Similarly it should be appreciated that in the description of exemplary embodiments of the invention, various features of the invention are sometimes grouped together in a single embodiment, figure, or description thereof for the purpose of streamlining the disclosure and aiding the understanding of one or more of the various inventive aspects. This method of disclosure, however, is not to be interpreted as reflecting an intention that the claimed invention requires more features than are expressly recited in each claim. Rather, as the following claims reflect, inventive aspects lie in less than all features of a single foregoing disclosed embodiment. Thus, the claims following the detailed description are hereby

expressly incorporated into this detailed description, with each claim standing on its own as a separate embodiment of this invention.

Furthermore, while some embodiments described herein include some but not other features included in other embodiments, combinations of features of different embodiments are meant to be within the scope of the invention, and form different embodiments, as would be understood by those in the art. For example, in the following claims, any of the claimed embodiments can be used in any combination.

In the description provided herein, numerous specific details are set forth. However, it is understood that embodiments of the invention may be practiced without these specific details. In other instances, well-known methods, structures and techniques have not been shown in detail in order not to obscure an understanding of this description.

Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein.

It is intended that the specification and examples be considered as exemplary only.

Each and every claim is incorporated into the specification as an embodiment of the present invention. Thus, the claims are part of the description and are a further description and are in addition to the preferred embodiments of the present invention.

Each of the claims set out a particular embodiment of the invention.

The following terms are provided solely to aid in the understanding of the invention.

Colorectal cancer is the third most common cancer form, with approximately 1.4 million new cases worldwide per year (International Agency for Research on Cancer (IARC) fact sheets, 2014) The European Journal of Cancer published a multi-group research study in 2013, estimating that, of the 3.45 million new cases of cancer diagnosed in 2012, nearly 450,000 were CRC. Furthermore, of the 1.75 million cancer-related deaths in 2012, almost 215,000 were due to CRC. All the figures from this study and similar statistics from the World Health Organization indicate that CRC is the second most common type of cancer among women, and third most cancer type among men. More critically, CRC is the second leading cause of cancer deaths in Europe. (Frost & Sullivan Technical Insights, Medical Device Technology October 2013).

Staging systems are used to characterize the severity of a cancer in terms of tumor size and degree of spread in the body, to estimate the prognosis of the patient and to guide decision concerning treatment. However, current staging systems typically do not predict the risk of disease recurrence accurately enough, with the consequence that some patients unnecessarily go through lengthy and painful treatment while the treatment need in other patients, potentially with lower-stage cancers, is not recognized.

It is extremely important, both from a patient-oriented and from a social-economic point of view, to systematically recognize these over- and under-treated patients. Molecular-level measurements of entities such as miRNA, mRNA or protein expression levels provide a deeper insight into the functional state of the cells and may be used in addition to the staging system with the aim of obtaining a more sensitive and accurate risk prediction for individual patients. Several gene expression signatures have been developed for prognostic use in various types of cancer (REFS).

The term “miRNA” or “miR” is used according to its ordinary and plain meaning and refers to a microRNA molecule found in eukaryotes that is involved in RNA-based gene regulation. See, e.g., Carrington et al., *Science*. 2003 Jul 18;301(5631):336-8., which is hereby incorporated by reference. The term will be used to refer to the single-stranded RNA molecule processed from a precursor. MicroRNAs (miRNAs) are small (19-25 nt), noncoding RNAs with important regulatory functions. MiRNAs are frequently altered in various cancers and it may therefore be hypothesized and play an important role in the cancer development and progression. miRNAs are also stable in FFPE tissues (*Bovell et al, Front Biosci 4:1937-1940 (2012); Jung et al, Clin Chemistry 56(6):998-1006 (2010); Xi et al, RNA 13:1668-1674 (2007)) as well as in body fluids (Chen, Cell Research 18:997-1006 (2008); Kosaka et al, Cancer Science 101(10):2087-2092 (2010)); Wang et al, Molecules 19:1912-1938 (2014)), which is an advantage in terms of obtaining reproducible and reliable measurements, and suggests that miRNAs may be suitable candidates for biomarkers.. Names of miRNAs and their sequences related to the present invention are provided herein.*

Exosome microRNA profiles can be determined according to conventional methods in the art (see e.g., total RNA is extracted using Trizol(R). Samples of exosomes are subjected to RT-PCR panel analysis for miRNA expression (RNA Universal RT microRNA PCR Services, Exiqon). The miRNA with greatest standard deviation in relative expression levels are

depicted in the heat map diagram and microRNA arrays identified dozens of miRNAs in the exosomes. Quantitatively, exosome miRNAs differed significantly from miRNAs expressed by their parent cells. MicroRNA profiles of epithelial exosomes and their parental cells are analyzed using microRNA array by mercury miRCURY™ RNA Isolation of Exiqon. Except
5 as otherwise noted herein, therefore, the process of the present disclosure can be carried out in accordance with such processes.

Microvesicles are small membranous vesicles that exist in or are secreted from various types of cells. Microvesicles secreted from cells include: (i) exosomes, which are vesicles having a
10 diameter of 30 to 100 nm that originate from cells; (ii) ectosomes (also called shedding microvesicles (SMVs)), which are vesicles that are released directly from plasma membranes and have a diameter of 50 to 1000 nm; and (iii) apoptotic blebs, which are vesicles secreted from dying cells that have a diameter of 50 to 5000 nm. It has been confirmed by using an
15 electron microscope that exosomes are not directly released from a plasma membrane, but rather originate from specific intracellular regions called multivesicular bodies (MVBs), and are then released into the extracellular environment as exosomes. Although it has not yet been clearly determined which molecular mechanisms are involved in the generation of exosomes, it is known that red blood cells, other various kinds of immune cells, including B-lymphocytes, T-lymphocytes, dendritic cells, blood platelets, and macrophages, and even
20 tumor cells are able to produce and secrete exosomes when in a normal live state. Exosomes are also known to be separated and excreted as different cell types depending on whether they are in a normal state, a pathological state, or an abnormal state. Microvesicles may contain microRNAs (miRNAs), which may be used for detection of the status of individual cells or organisms. The status may be a disease, for example, cancer, hereditary diseases, heart
25 diseases, or neuronal diseases, such as schizophrenia.

"Vesicle" refers to a membranous structure that is surrounded by a lipid bilayer. For example, the vesicle may be an exosome or a microvesicle. "Microvesicle" refers to a small vesicle with a membranous structure that originates from a cell. The term "microvesicle" may be
30 interchangeably used herein with the terms "circulating microvesicle" or "microparticle." Microvesicles may exist in cells or may be secreted from cells. Microvesicles secreted from cells may include exosomes, ectosomes (shedding microvesicles (SMVs)), or apoptotic blebs. Exosomes are membranous vesicles of about 30 to about 100 nm diameter that originate from phagocytes. Ectosomes (SMVs) are large membranous vesicles of about 50 to about 1000 nm

diameter that are directly released from plasma membranes. Apoptotic blebs are vesicles of about 50 to about 5000 nm diameter that are leaked from dying cells. In vivo microvesicles may contain microRNAs or messenger RNAs (mRNAs). Surface proteins of microvesicles may be disease-specific markers.

5

hsa-miR-616-5p corresponds to the nucleotide sequence of SEQ ID NO. 1

hsa-miR-642a-5p corresponds to the nucleotide sequence of SEQ ID NO. 2

hsa-miR-342-5p corresponds to the nucleotide sequence of SEQ ID NO. 3

hsa-miR-199a-5p corresponds to the nucleotide sequence of SEQ ID NO. 4

10 hsa-miR-4532 corresponds to the nucleotide sequence of SEQ ID NO. 5 or SEQ ID NO. 6

hsa-miR-499a-5p corresponds to the nucleotide sequence of SEQ ID NO. 7

hsa-miR-922 corresponds to the nucleotide sequence of SEQ ID NO. 8 or SEQ ID NO. 9

hsa-miR-654-3p corresponds to the nucleotide sequence of SEQ ID NO. 10

hsa-miR-23b-5p corresponds to the nucleotide sequence of SEQ ID NO. 11

15 hsa-miR-30d-5p corresponds to the nucleotide sequence of SEQ ID NO. 12

hsa-miR-199b-5p corresponds to the nucleotide sequence of SEQ ID NO. 13

hsa-miR-30a-5p corresponds to the nucleotide sequence of SEQ ID NO. 14

hsa-miR-23a-5p corresponds to the nucleotide sequence of SEQ ID NO. 15

hsa-miR-874 corresponds to the nucleotide sequence of SEQ ID NO. 16

20 hsa-miR-874-5p corresponds to the nucleotide sequence of SEQ ID NO. 17

hsa-miR-874-3p corresponds to the nucleotide sequence of SEQ ID NO. 18

hsa-miR-135a-5p corresponds to the nucleotide sequence of SEQ ID NO. 19

hsa-miR-148b-3p corresponds to the nucleotide sequence of SEQ ID NO. 20

hsa-miR-551b-5p corresponds to the nucleotide sequence of SEQ ID NO. 21

25 hsa-miR-181c-3p corresponds to the nucleotide sequence of SEQ ID NO. 22

hsa-miR-345-5p corresponds to the nucleotide sequence of SEQ ID NO. 23

hsa-miR-545-3p corresponds to the nucleotide sequence of SEQ ID NO. 24

hsa-miR-191-3p corresponds to the nucleotide sequence of SEQ ID NO. 25

hsa-miR-4802-5p corresponds to the nucleotide sequence of SEQ ID NO. 26

5 hsa-miR-46415p corresponds to the nucleotide sequence of SEQ ID NO. 27 or SEQ ID NO. 28

hsa-miR-3689f corresponds to the nucleotide sequence of SEQ ID NO. 29 or SEQ ID NO. 30

hsa-miR-431-5p corresponds to the nucleotide sequence of SEQ ID NO. 31

hsa-miR-1266 corresponds to the nucleotide sequence of SEQ ID NO. 32

hsa-miR-1266-5p corresponds to the nucleotide sequence of SEQ ID NO. 33

10 hsa-miR-1266-3p corresponds to the nucleotide sequence of SEQ ID NO. 34

hsa-miR-516a-3p corresponds to the nucleotide sequence of SEQ ID NO. 35

hsa-miR-548c-5p corresponds to the nucleotide sequence of SEQ ID NO. 36

hsa-miR-130b-3p corresponds to the nucleotide sequence of SEQ ID NO. 37

hsa-miR-219-5p corresponds to the nucleotide sequence of SEQ ID NO. 38

15 hsa-miR-502-3p corresponds to the nucleotide sequence of SEQ ID NO. 39

hsa-miR-33a-3p corresponds to the nucleotide sequence of SEQ ID NO. 40

hsa-miR-16-1-3p corresponds to the nucleotide sequence of SEQ ID NO. 41

hsa-miR-155-5p corresponds to the nucleotide sequence of SEQ ID NO. 42

20 In a particular embodiment present invention concerns the finding of miRNA signature that surprisingly very suitable to predict disease recurrence risk in stage III colon cancer patients, using data from the PETACC-3 (Pan European Trial in Adjuvant Colon Cancer) randomized clinical trial. We show that the signature robustly stratifies the patients into subsets with high and low risk of recurrence, respectively. Moreover, the prognostic ability is intact after
25 stratification of the samples by traditionally used prognostic factors such as T-stage, N-stage and MSI status, and the miRNA signature performs better than many prognostic mRNA expression signatures in the art.

EXAMPLES

Example 1 Tumor samples and data preparation

5 Within the PETACC-3 clinical trial, formalin-fixed paraffin-embedded tissue blocks were collected after cancer diagnosis and independently of future research plans. The mutation status of BRAF exon 15 was assessed by allele-specific real-time polymerase chain reaction (REF) and confirmed by a second analysis, using Sequenom. RNA of sufficient quantity and quality was extracted from 895 samples, and gene expressions were measured on the ALMAC
10 Colorectal Cancer DSA platform (Craigavon, Northern Ireland); a customized Affymetrix chip with 61,528 probe sets.

In total, 688 unique samples passed the final quality control. Of this series of tumors, 47 (6.8%) were BRAF mutated (BRAFM) (REF). The PETACC-3 gene expression data were
15 retrospectively analyzed to derive a "BRAF-likeness" gene signature discriminating between c.1799T<A (p.V600E) BRAFM and double-wild-type (WT2; BRAF and KRAS wild-type) tumors (Popovici et al, JCO 2012). The signature genes were split into two groups based on whether they were up- or downregulated in the BRAFM group. The signature score for a patient was then defined as the difference between the average (normalized) expression of the
20 signature genes upregulated in BRAFM and the average (normalized) expression of the signature genes downregulated in BRAFM. Patients with a positive BRAF signature are considered "BRAF positive", while patients with a negative BRAF signature are considered "BRAF negative". In addition to the BRAF-likeness score, available clinico-pathological and derived variables include KRAS mutation status, BRAF mutation status, MSI status, stage,
25 grade, age, gender, T-stage, N-stage and tumor site. Three survival end points are recorded: overall survival (OS), relapse-free survival (RFS) and survival after relapse (SAR). For the patients who relapse, we define a fourth end point (denoted "relapse3years"), encoding whether or not the relapse occurred within 3 years after surgery.

30 MiRNA expression were measured in a total of 683 patients. For the miRNA profiling, RNA isolated from FFPE samples was reverse transcribed using the stem-loop RT Megaplex pools A and B (Life Technologies). MiRNA cDNA was then pre-amplified and quantified using miRNA specific Taqman probes. Cq-values were determined based on a threshold of 0.1 and

filtered using a detection cut-off of 32 (LT), and using Qiagen miScript (QI) ((Qiagen[®] QuantiTect, Germany)) cycling program, which consists of an initial hold at 95 °C for 15 min followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 70 °C for 30 s. MiRNA expression values were normalized using the modified global mean method (*D'haene et al, Methods Mol Biol. 2012;822:261-72 and Pieter Mestdagh et al, Nature Methods 11, 809–815 (2014)*). Normalized expression values are represented on a log2 scale. In order to assess data quality, we calculated two quality control parameters: the number of detected miRNAs per sample and the mea miRNA expression per sample. In addition, technical data QC (based on U6 replicates) were performed to reveal possible aberrations. After excluding 11 patients that did not pass the quality control and 27 patients with local relapses, 645 patients remained. A total of 627 of the 940 measured miRNAs were detected in at least 20% of the patients and passed the quality control. MiRNA expression values below the detection thresholds were substituted with the lowest detected value of the respective miRNA across the patients, minus 1 (on the log2 scale), and ComBat (Biostatistics. 2007 Jan;8(1):118-27) was used to eliminate the batch effects resulting from processing the samples on different days. Finally, we excluded stage II patients as well as control probes and miRNAs whithout a valid MIMAT ID, which led to a final data set consisting of 610 miRNAs and 521 patients that was used for further analysis. **Table 1** shows the characteristics of the patient cohort used in this study.

		Stage III (N=521)
Age (range)		21-76
Sex	M	307 (58.9%)
	F	214 (41.1%)
N-stage	N0	0 (0.0%)
	N1	343 (65.8%)
	N2	178 (34.2%)
MSI status	MSI-H	49 (9.4%)
	MSS	424 (81.4%)
	N/A	48 (9.2%)
site	left	316 (60.7%)
	right	195 (37.4%)
T-stage	T1/2	47 (9.0%)
	T3	397 (76.2%)
	T4	77 (14.8%)
BRAF status	mut	31 (6.0%)
	wt	467 (89.6%)
	N/A	23 (4.4%)
BRAF-	BRAFpos	120 (23.0%)

likeness		
	BRAFneg	398 (76.4%)
	N/A	3 (0.6%)

Table 1. Patient characteristics**Example 2 Statistical analysis**

5 LASSO (Tibshirani, J Royal Stat Soc Series B 1996) penalized Cox proportional hazards regression (as implemented in the *glmnet* R package, version 1.9-8 (REF)) was used to extract a miRNA signature associated with RFS from the stage III cohort (N=521). The expression values for each miRNA were standardized before the signature extraction, by subtraction of the mean value and division by the standard deviation of the expression values across the 521

10 patients. Five-fold cross-validation was used to determine the optimal penalization parameter, and thereby implicitly the number of miRNAs in the final signature. We included N-stage, T-stage and MSI status as non-penalized predictors in the regression, since our aim was to extract a miRNA signature with prognostic ability independently of known prognostic factors. The signature score was defined as a linear combination of the selected miRNAs, with

15 weights given by the penalized coefficients from the Cox regression.

To obtain an estimate of the prognostic value of the signature we implemented a repeated three-fold cross-validation (CV) approach on the stage III cohort. More precisely, for each cross-validation fold we derived an RFS-related signature from the corresponding training set as described above, with the exception that the penalization parameter was chosen to obtain a

20 signature with approximately the same number of miRNAs as in the signature extracted from the entire cohort. After standardizing the corresponding test set using the mean and standard deviation derived from the training set, we estimated the signature scores for the test set as well as the training set samples. To ensure that the signature scores were comparable across

25 cross-validation folds, we standardized the test set scores by subtracting the mean and dividing by the standard deviation of the training set scores. We also defined a binary score ("high" or "low") by setting a cutoff at the median score among the samples in the training set. The scores were combined across all cross-validation folds, yielding a predicted signature score for each patient.

30 We evaluated the association between the signature and RFS and OS endpoints by including the signature score derived as described in the previous paragraph as a predictor in a

multivariable Cox regression together with T-stage, N-stage and MSI status. To evaluate the added value of the miRNA signature in the presence of other molecular signatures, we also included previously derived prognostic mRNA expression signatures as covariates. The Wald test p-value for the score was used as a proxy for the prognostic performance of the derived signature, that is, the level of association with the endpoint that we would expect when applying it to a new, unseen data set. We also calculated the improvement in the area under the time-dependent ROC curves (REF) at 3 years obtained by including the miRNA score as a predictor in the Cox model in addition to the clinical covariates (using the *survivalROC* R package, version 1.0.3). For the binary "relapse3years" variable, we used logistic regression in place of Cox regression to obtain a corresponding p-value and improvement in the (regular) AUC (calculated by the *ROCR* R package, version 1.0-5) achieved by including the miRNA score as a predictor.

The cross-validation procedure was repeated 100 times to minimize the influence of a specific split. This means that in the process, we created 300, potentially different, signatures (100 runs x 3 CV folds). In addition to the original signature, we used the inclusion rate for a miRNA in the CV signatures as an estimate of its importance for the prognostic ability. For each pair of miRNAs, we estimated the degree of co-occurrence across the 300 signatures, using the Kulczinski similarity:

where N_1 and N_2 are the number of CV signatures where miRNA₁ and miRNA₂ are included, respectively, and N_{12} is the number of co-occurrences of miRNA₁ and miRNA₂ across the 300 signatures. A high Kulczinski similarity implies that the two miRNAs often co-occur in the same signature.

To further evaluate the significance of the extracted signature, we compared its performance to that obtained after two different randomization procedures (applied throughout the cross-validation framework). In the first randomization procedure (denoted "permdata"), we permuted the patient identifiers in the miRNA expression data before extracting the signature. This procedure thus corresponds to applying a proper signature extraction procedure to a data set without any non-random signal. In the second permutation approach (denoted "permcoeff"), we extracted the signature from the original data, but permuted the derived signature weights (the penalized regression coefficients) afterwards, across all miRNAs. This approach thus corresponds to applying an improper ("random") signature extraction procedure

to a data set with the same biological signal as the original one. By comparing the results obtained by the three approaches, we could evaluate the estimated performance of the derived miRNA signature.

- 5 Linear models (as implemented in the *limma* R package, version 3.20.6) were used to evaluate the association between clinico-pathological variables and (combinations of) miRNAs. Cox proportional hazards models were used to evaluate the association between (combinations of) miRNAs and survival endpoints (using the *survival* R package, version 2.37-7). P-values were adjusted for multiple testing using the Benjamini-Hochberg method (*Benjamini, Y and*
 10 *Hochberg, Y. "Controlling the False Discovery Rate: A practical and powerful approach to multiple testing." Journal of the Royal Statistical Society Series B, 1995: 289-300).*

Example 3 Construction of the signature

Using the LASSO-penalized Cox regression with T-stage, N-stage and MSI status as
 15 unpenalized covariates, we extracted a miRNA signature consisting of 34 miRNAs (**Table 2, Figure 1**). Many of the signature miRNAs were also included in a large fraction of the signatures extracted during the cross-validation procedure (**Table 2**), indicating the robustness of the signature and the importance of the included miRNAs as prognostic biomarkers. This can be compared with the signatures derived from randomized data, where no indication was
 20 seen for preferential inclusion of certain miRNAs. Among the 34 miRNAs most frequently occurring in the CV signatures, 31 were present in the final signature, indicating that the latter provides a good representation of a reproducible pattern in the data set. The three miRNAs that were frequently included in the CV signatures but do not appear in the final signature were hsa-miR-3689b-5p (included in 97 /300 CV signatures, 32.3%), hsa-miR-1229-3p
 25 (92/300, 30.7%) and hsa-miR-21-3p (84/300, 28.0%). Moreover, the miRNAs in the final signature co-occurred more frequently than other combinations of miRNAs across the 300 CV signatures, suggesting stable associations among the selected miRNAs, and that they are not easily exchangeable with other miRNAs.

miRNA	weight	nbr.incl	miRNA	weight	nbr.incl
hsa-miR-616-5p	0.174	296 (98.7%)	hsa-miR-155-5p	-0.223	294 (98.0%)
hsa-miR-642a-5p	0.142	283 (94.3%)	hsa-miR-16-1-3p	-0.132	252 (84.0%)
hsa-miR-342-5p	0.065	189 (63.0%)	hsa-miR-33a-3p	-0.081	226 (75.3%)
hsa-miR-199a-	0.059	147 (49.0%)	hsa-miR-502-3p	-0.068	197 (65.7%)

5p					
hsa-miR-4532	0.055	60 (20.0%)	hsa-miR-219-5p	-0.052	157 (52.3%)
hsa-miR-499a-5p	0.053	235 (78.3%)	hsa-miR-130b-3p	-0.049	120 (40.0%)
hsa-miR-922	0.045	159 (53.0%)	hsa-miR-548c-5p	-0.047	171 (57.0%)
hsa-miR-654-3p	0.044	180 (60.0%)	hsa-miR-516a-3p	-0.039	175 (58.3%)
hsa-miR-23b-3p	0.036	153 (51.0%)	hsa-miR-1266	-0.032	141 (47.0%)
hsa-miR-30d-5p	0.031	135 (45.0%)	hsa-miR-431-5p	-0.028	126 (42.0%)
hsa-miR-199b-5p	0.029	142 (47.3%)	hsa-miR-3689f	-0.027	127 (42.3%)
hsa-miR-30a-5p	0.021	130 (43.3%)	hsa-miR-4641	-0.022	104 (34.7%)
hsa-miR-23a-5p	0.021	111 (37.0%)	hsa-miR-4802-5p	-0.017	88 (29.3%)
hsa-miR-874	0.009	119 (39.7%)	hsa-miR-191-3p	-0.014	84 (28.0%)
hsa-miR-135a-5p	0.001	127 (42.3%)	hsa-miR-545-3p	-0.005	72 (24.0%)
			hsa-miR-345-5p	-0.002	189 (63.0%)
			hsa-miR-181c-3p	-0.002	118 (39.3%)
			hsa-miR-551b-5p	-0.002	123 (41.0%)
			hsa-miR-148b-3p	-0.002	89 (29.7%)

Table 2. Contributions to the miRNA signature

The miRNAs included in the signature showed a relatively low correlation with each other, and rather appeared to be representative of several different, uncorrelated, patterns (**Figure 2**). This could suggest that the outcome that we were predicting (relapse-free survival) is complex and a reliable prediction relies on many different biological pathways. It is also a typical feature of LASSO penalization, which decreases redundancy in the signature in favor of sparsity. Indeed, among the 408 miRNAs included in at least one of the 300 cross-validation signatures, higher pairwise correlation typically tended to correspond to lower co-occurrence rate.

Only one signature miRNA (hsa-miR-499a-5p) was significantly (adjusted $p < 0.05$, $|\log FC| > 1$) associated with BRAF-likeness and, similarly, only one (hsa-miR-874) was significantly associated with MSI status, which suggests that the extracted signature indeed provides independent information in addition to these features. Thirteen of the 34 miRNAs were individually significantly (adjusted $p < 0.05$) associated with RFS in separate multivariable Cox regressions using T-stage, N-stage and MSI as covariates.

Example 4 Performance evaluation

The cross-validation based performance estimation showed a strong association between the derived miRNA signatures and relapse-free survival, as well as the overall survival (OS) and relapse3years endpoints, with p-value distributions centered around 10^{-6} for RFS and around 10^{-4} for the two other endpoints. The p-value distributions are markedly shifted compared to those derived with the two randomization protocols. No significant association was found with survival after relapse (SaR).

While the improvement in the AUC was modest (around 0.04 units, for a typical AUC close to 0.7), the results are consistent across CV splits and considerably better than those obtained for the randomly extracted signatures. Results from a representative of the 100 CV runs (the one with the median p-value) illustrated that higher signature scores were associated with worse RFS and OS, and higher probability to relapse within 3 years **Table 3**). Corresponding results for the CV runs resulting in the highest and lowest p-values are shown in **Tables 5-6**. Moreover, the signature provided an additional risk stratification within each T-stage and N-stage group, as well as for both BRAF-like and non-BRAF-like patients.

Univariable (only signature score as predictor)

RFS	HR	95% CI	p
score	1.75	(1.44, 2.12)	1.6e-8
OS	HR	95% CI	p
score	1.73	(1.39, 2.17)	1.5e-6

Multivariable

RFS	HR	95% CI	p
T-stage (T12 vs T3)	0.53	(0.25, 1.15)	0.11
T-stage (T4 vs T3)	1.41	(0.97, 2.05)	0.069
N-stage (N2 vs N1)	2.07	(1.52, 2.83)	4.0e-6
MSS	1.69	(0.90, 3.15)	0.10
BRAFpos	1.20	(0.81, 1.77)	0.36
score	1.63	(1.34, 1.99)	1.2e-6
OS	HR	95% CI	p
T-stage (T12 vs T3)	0.54	(0.22, 1.35)	0.19
T-stage (T4 vs T3)	1.67	(1.10, 2.54)	0.016
N-stage (N2 vs N1)	1.93	(1.35, 2.75)	0.00029
MSS	2.08	(1.03, 4.21)	0.041
BRAFpos	1.89	(1.25, 2.87)	0.0027
score	1.61	(1.28, 2.03)	5.9e-5

Table 3. Results from Cox model fits, for the CV iteration with the median association between the signature score and RFS

Example 5 Comparison with mRNA expression signatures

5 We evaluated the prognostic value of five mRNA expression signatures by including each of them, separately, as a predictor in a Cox proportional hazards model with RFS as endpoint and T-stage, N-stage and MSI status as covariates. As for the miRNA signature, the Wald p-value and the improvement in the AUC were used to evaluate their prognostic value (**Table 4**). Comparing these values to the distribution of values obtained for the miRNA signature
10 shows that the expected prognostic value of the miRNA signature exceeds that of the mRNA expression signatures.

Signature	p-value	Δ AUC
VDS	0.69	0.012
ALM	0.045	0.010
GHS	0.0027	0.026
MDA	0.044	0.019
EMT	0.015	0.016

Table 4. Wald p-values and Δ AUC for the five evaluated mRNA expression signatures, in Cox regression models with T-stage, N-stage and MSI status as covariates.

To further estimate the added value of the miRNA signature in the presence of mRNA
15 signatures, we included all five mRNA signatures as additional covariates in the Cox model evaluating the performance of the miRNA signature. Even after this modification, the miRNA signature was highly prognostic and significantly outperformed the randomly derived signatures.

20 Present invention concerns the development of a miRNA-based prognostic signature for relapse-free survival in a large set of stage III colon cancer patients. The signature comprises 34 miRNAs or it essentially consists thereof, preferably all the 34 miRNAs and a risk score is obtained as a linear combination of the expression levels of these miRNAs, with weights derived from a penalized Cox regression. A high value of the risk score is strongly associated
25 with worse relapse-free survival. This association holds even when the patient set is stratified by common prognostic factors such as T-stage, N-stage and MSI status as well as previously derived prognostic mRNA expression signatures.

Univariable (only signature score as predictor)			
RFS	HR	95% CI	p
score	2.08	(1.70, 2.53)	4.5e-13
OS	HR	95% CI	p
score	2.37	(1.88, 2.98)	1.8e-13
Multivariable			
RFS	HR	95% CI	p
T-stage (T12 vs T3)	0.57	(0.27, 1.24)	0.16
T-stage (T4 vs T3)	1.34	(0.92, 1.95)	0.12
N-stage (N2 vs N1)	2.22	(1.62, 3.04)	6.4e-7
MSS	1.55	(0.82, 2.91)	0.17
BRAFpos	1.03	(0.70, 1.53)	0.87
score	2.08	(1.68, 2.57)	1.6e-11
OS	HR	95% CI	p
T-stage (T12 vs T3)	0.63	(0.25, 1.57)	0.32
T-stage (T4 vs T3)	1.54	(1.01, 2.34)	0.045
N-stage (N2 vs N1)	2.11	(1.48, 3.03)	4.5e-5
MSS	1.88	(0.93, 3.80)	0.081
BRAFpos	1.68	(1.11, 2.54)	0.015
score	2.32	(1.82, 2.97)	1.9e-11

Table 5. Results from Cox model fits, for the CV iteration with the strongest association between the signature score and RFS

5

Univariable (only signature score as predictor)			
RFS	HR	95% CI	p
score	1.41	(1.17, 1.70)	3.5e-4
OS	HR	95% CI	p
score	1.37	(1.10, 1.71)	4.6e-3
Multivariable			
RFS	HR	95% CI	p
T-stage (T12 vs T3)	0.47	(0.22, 1.02)	0.056
T-stage (T4 vs T3)	1.49	(1.03, 2.17)	0.036
N-stage (N2 vs N1)	2.04	(1.50, 2.78)	7.0e-6
MSS	1.68	(0.90, 3.13)	0.10
BRAFpos	1.17	(0.80, 1.73)	0.42
score	1.32	(1.09, 1.60)	4.0e-3

OS	HR	95% CI	p
T-stage (T12 vs T3)	0.49	(0.20, 1.22)	0.13
T-stage (T4 vs T3)	1.77	(1.16, 2.69)	0.0081
N-stage (N2 vs N1)	1.93	(1.35, 2.76)	3.1e-4
MSS	2.07	(1.03, 4.19)	0.042
BRAFpos	1.85	(1.22, 2.80)	0.0036
score	1.27	(1.02, 1.59)	0.035

Table 6. Results from Cox model fits, for the CV iteration with the weakest association between the signature score and RFS

5

The cross-validation based performance estimation showed a strong association between the derived miRNA signatures and relapse-free survival, as well as the overall survival (OS) and relapse3years endpoints, with p-value distributions centered around 10⁻⁶ for RFS and around 10⁻⁴ for the two other endpoints. The p-value distributions are markedly shifted compared to those derived with the two randomization protocols. No significant association was found with survival after relapse (SaR).

10

Particular and preferred aspects of the invention are set out in the accompanying independent and dependent claims. Features from the dependent claims may be combined with features of the independent claims and with features of other dependent claims as appropriate and not merely as explicitly set out in the claims.

15

Thus, the claims following the detailed description are hereby expressly incorporated into this detailed description, with each claim standing on its own as a separate embodiment of this invention.

20

Drawing Description

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will become more fully understood from the detailed description given herein below and the accompanying drawings which are given by way of illustration only, and thus are not limitative of the present invention, and wherein:

25

Figure 1. is a schematic view illustrating of the contributions of individual miRNAs to the derived signature. Red bars (to the left) indicate negative contributions, and blue bars (to the

right) indicate positive contributions. Stars indicate miRNAs that are significantly associated⁵⁴ with RFS in separate Cox PH models with T-stage, N-stage and MSI status as covariates.

- 5 Figure 2. Left shows the Pairwise Pearson correlation among the miRNAs included in the final signature. Right: shows a heatmap and hierarchical clustering of all stage III patients and miRNAs, using Pearson correlation similarity and average linkage. The miRNAs included in the signature are indicated.
- 10 A particular embodiment of present invention concerns a method for evaluating a patient comprising the steps of: (a) determining expression levels of one or more miRNA from the group consisting of hsa-miR-4532, hsa-miR-499a-5p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-135a-5p, hsa-miR-545-3p, hsa-miR-4802-5p, hsa-miR-4641, hsa-miR-3689f, hsa-miR-431-5p, hsa-miR-1266, hsa-miR-130b-3p, hsa-miR-16-1-3p and hsa-miR-155-5p in a
- 15 biological sample comprising a portion of a suspect lesion taken from the patient using one or more oligonucleotides that specifically interact with the miRNA to detect the miRNA, and (b) determining a diagnosis or prognosis for colorectal cancer based on the miRNA expression levels. Such patient may have been suspected of having colorectal cancer or the patient is at risk of colorectal cancer recurrence. The cancer can be colorectal cancer. In a particular
- 20 embodiment determining a diagnosis concerns screening for a pathological condition, staging a pathological condition, or assessing response of a pathological condition to therapy or determining a diagnosis is determining if the patient has colorectal cancer. The method can further comprising normalizing the expression levels of miRNA. This normalizing can concern adjusting expression levels of miRNA relative to expression levels of one or more
- 25 nucleic acid in the sample. This method can further comprise comparing miRNA expression levels in the sample to miRNA expression levels in a normal tissue sample or reference, wherein the sample from the patient and the normal tissue sample are preferable colorectal samples or vesicles or miRNA release in the blood circulating by such colorectal tissues.
- In a particular embodiment the normal tissue sample is not from the patient being evaluated or
- 30 the normal tissue sample is taken from the patient being evaluated, or the normal tissue sample is normal adjacent tissue. Particular embodiments of the method are those wherein determining a prognosis involves estimating the likelihood of recurrence of colorectal cancer, wherein expression of the miRNA is determined by an amplification assay or a hybridization assay, wherein amplification assay is a quantitative amplification assay for instance wherein

the quantitative amplification assay is quantitative RT-PCR. Such hybridization assay can be an array hybridization assay or a solution hybridization assay. In a particular embodiment the method further comprising providing a report of the diagnosis or prognosis. One of the following features can create a particular variant of an embodied method : it further comprises one or more of the following obtaining a sample from the patient; the expression levels of miRNA are determined without extracting RNA from the sample; the expression levels of miRNA are determined after extracting RNA from the sample; it further comprises labelling miRNA to be detected; the sample is a tissue sample; the sample is fresh, frozen, fixed, or embedded or the sample is a formalin fixed, paraffin-embedded (FFPE) tissue.

Another embodiment of present invention is a method for assessing the likelihood of colorectal cancer recurrence in a patient comprising the steps of: (a) determining the expression levels of one or more miRNA from hsa-miR-4532, hsa-miR-499a-5p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-135a-5p, hsa-miR-545-3p, hsa-miR-4802-5p, hsa-miR-4641, hsa-miR-3689f, hsa-miR-431-5p, hsa-miR-1266, hsa-miR-130b-3p, hsa-miR-16-1-3p and hsa-miR-155-5p in a biological sample comprising colorectal cancer cells taken from the patient, and (b) determining a prognosis for colorectal cancer recurrence based on the miRNA expression levels.

This method is particularly useful for a patient is at risk of colorectal cancer recurrence. The method may further comprise normalizing the expression levels of the miRNA relative to at least a second nucleic acid in the sample. The cancer is preferably colon cancer. In this method recurrence can be a second instance of cancer within colon or rectal tissues of the patient, or in tissues adjacent to the colon or rectum of the patient after a first instance of colorectal cancer has been treated or recurrence is a second instance of cancer within non-colon or non-rectal tissues distant from a first instance of colorectal cancer, or the first instance of colorectal cancer is Stage III cancer.

The method can further comprise comparing miRNA expression levels to a reference, wherein the reference is a sample from a patient comprising cancer cells, wherein the patient has been diagnosed with colorectal cancer and has not had a recurrence of colorectal cancer or wherein the reference is a comparative dataset.

For this method a sample that is fresh, frozen, fixed, or embedded, for instance formalin fixed, paraffin-embedded (FFPE) tissue can be used.

The method can use frozen tissue or a blood sample to isolate circulating miRNA, isolated tumour cells or cancer vesicles or a sample is a plasma sample to isolate circulating miRNA or cancer vesicles. The method can further comprise comparing miRNA expression levels in

the biological sample to miRNA expression levels in a normal colorectal tissue or a cancerous colorectal tissue wherein one or more of the following : the sample from the patient and the normal sample are colorectal samples, the normal tissue is from a patient that has had a recurrence of colorectal cancer or the normal sample is normal adjacent tissue. This method of present invention can involve the following analytical steps: extracting RNA from the sample, labelling miRNA from the sample, determining expression of the mi by an amplification assay or a hybridization assay, amplification assay is a quantitative amplification assay, to use as quantitative amplification assay a quantitative RT-PCR, to use an hybridization assay whereby the array hybridization assay or a solution hybridization assay. This method can further comprise providing a report of the diagnosis and/or prognosis.

A particular embodiment of present invention is a kit for analysis of a sample by assessing miRNA profile for a sample comprising, in suitable container means, two or more miRNA hybridization or amplification reagents comprising one or more probe or amplification primer for one or more miRNA selected from Table 3, 4, 5, 6, 7, 10 and/or 11. This kit can in a particular embodiment comprise miRNA selected from a group consisting of hsa-miR-4532, hsa-miR-499a-5p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-135a-5p, hsa-miR-545-3p, hsa-miR-4802-5p, hsa-miR-4641, hsa-miR-3689f, hsa-miR-431-5p, hsa-miR-1266, hsa-miR-130b-3p, hsa-miR-16-1-3p and hsa-miR-155-5p. It further can comprise reagents for detecting a miRNA in the sample for instance a miRNA hybridization reagent which comprises hybridization probes. It further can comprise a miRNA amplification reagent comprises one or more of amplification primers or a probe for the detection of a miRNA sequence.

Particular embodiments of this kit are anyone of the following : the miRNA is hsa-miR-4532, the miRNA is hsa-miR-499a-5p, the miRNA is hsa-miR-23b-5p, the miRNA is hsa-miR-30d-5p, the miRNA is hsa-miR-135a-5p, the miRNA is hsa-miR-545-3p, the miRNA is hsa-miR-4802-5p, the miRNA is hsa-miR-4641, the miRNA is hsa-miR-3689f, the miRNA is hsa-miR-431-5p, the miRNA is hsa-miR-1266, the miRNA is hsa-miR-130b-3p, the miRNA is hsa-miR-16-1-3p and/or the miRNA is hsa-miR-155-5p.

Yet another embodiment of present invention concerns a method for predicting a subject's response to a treatment for colorectal cancer (CRC), wherein the subject has been diagnosed with CRC, the method comprising: obtaining a biological sample from the subject, wherein the biological sample is selected from the group consisting of a blood sample, a tumor, one or more exosomes and any combination thereof; preparing the biological sample for measurement of MiRNA expression therein; measuring expression of a miRNA of the group

consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p in the thus prepared biological sample, wherein expression comprises MiRNA expression; and comparing the expression of the miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p with a reference measurement; wherein differential expression the miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p in the biological sample as compared to the reference measurement indicates that the subject will not respond to the treatment, and whereby the differential expression of a miRNA of the group consisting of Hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p indicates that the subject will respond to the treatment.

Yet another method of present invention concerns method for predicting a subject's response to a treatment for colorectal cancer (CRC), wherein the subject has been diagnosed with CRC, the method comprising: obtaining a biological sample from the subject, wherein the biological sample is selected from the group consisting of a blood sample, a tumor, one or more exosomes and any combination thereof; preparing the biological sample for measurement of MiRNA expression therein; measuring expression of a miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p in the thus prepared biological sample, wherein expression comprises MiRNA expression; and comparing the expression of the miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p with a reference measurement; whereby the differential expression of a miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p correlates negative with relapse-free survival (RFS) whereby the differential expression of a miRNA of the group consisting of Hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p correlates positive with relapse-free survival (RFS).

Yet another embodiment of present invention concerns a method of analysing a biological sample of a subject, wherein the subject has been diagnosed as suffering from colorectal cancer (CRC), the method comprising: reacting the biological sample with a first compound to form a first complex, the first complex comprising a miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p

and the first compound, and measuring expression of miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p in the CRC subject. This method can further comprise: reacting the biological sample with a second compound to form a second complex, the second complex comprising a miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p and the second compound, and measuring expression of the miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p in the CRC subject. And this can further comprise reacting the biological sample with a third compound to form a third complex, the third complex comprising a miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p and the third compound, and measuring expression of the miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p in the CRC subject. Or such method can further comprise reacting the biological sample with a fourth compound to form a fourth complex, the fourth complex comprising a miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p expression product and the fourth compound, and measuring expression of the miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p in the CRC subject. Alternatively such method can comprise reacting the biological sample with a fifth compound to form a fifth complex, the fifth complex comprising a miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p expression product and the fifth compound, and measuring expression of the miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p in the CRC subject.

Yet another embodiment of present invention concerns a method of analysing a biological sample of a subject, wherein the subject has been diagnosed as suffering from colorectal cancer (CRC), the method comprising: reacting the biological sample with a first compound to form a first complex, the first complex comprising a miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p and the first compound, and measuring expression of the miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p in the CRC subject; reacting the biological sample with a second compound to form a second complex, the second complex comprising another miRNA of the group consisting of hsa-

miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p and the second compound, and measuring expression of *the other* miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p in the CRC subject; reacting the biological sample with a third compound to form a third complex, the third complex comprising yet another miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p expression product and the third compound, and measuring expression of this other miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p in the CRC subject; and reacting the biological sample with a fourth compound to form a fourth complex, the fourth complex comprising yet another miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p expression product and the fourth compound, and measuring expression of this other miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p in the CRC subject.

Yet another method of present invention concerns a biomarker panel comprising: a solid phase; a first compound bound to the solid phase, which first compound forms a first complex with a miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p. This biomarker panel can further comprise: a second compound bound to the solid phase, which second compound forms a second complex with another miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p. These biomarker panels can further comprising: a third compound bound to the solid phase, which third compound forms a third complex with yet another miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p; and a fourth compound bound to the solid phase, which fourth compound forms a fourth complex with yet another miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p. Moreover these biomarker panels can further comprise a biological sample of a subject diagnosed as suffering from CRC, said biological sample in contact with the first, second, third, and fourth compounds.

Moreover these biomarker panels can further comprise at least one further compound bound to the solid phase, which further compound forms a further complex with an expression product of a miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-

33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p. Moreover these biomarker panels can further comprise discrete means for detecting each said complex.

Yet another embodiment of present invention concerns a method for predicting a subject's response to a treatment for colorectal cancer (CRC), wherein the subject has been diagnosed with CRC, the method comprising: obtaining a biological sample from the subject, wherein the biological sample is selected from the group consisting of a blood sample, a tumor, one or more exosomes and any combination thereof; preparing the biological sample for measurement of MiRNA expression therein; measuring expression of a miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p in the thus prepared biological sample, wherein expression comprises MiRNA expression; and comparing the expression of the miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p with a reference measurement; wherein differential expression the miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p in the biological sample as compared to the reference measurement indicates that the subject will not respond to the treatment, whereby the differential expression of a miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p correlates positive with relapse-free survival (RFS) and whereby the differential expression of a miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p correlates positive with relapse-free survival (RFS).

Yet another embodiment of present invention concerns a method of analysing a biological sample of a subject, wherein the subject has been diagnosed as suffering from colorectal cancer (CRC), the method comprising: reacting the biological sample with a first compound to form a first complex, the first complex comprising a miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p and the first compound, and measuring expression of miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p in the CRC subject. This method can further comprise reacting the biological sample with a second compound to

form a second complex, the second complex comprising a miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p and the second compound, and measuring expression of the miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p in the CRC subject, or it can further comprise reacting the biological sample with a third compound to form a third complex, the third complex comprising a miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p and the third compound, and measuring expression of the miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p in the CRC subject or it can further comprising: reacting the biological sample with a fourth compound to form a fourth complex, the fourth complex comprising a miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p expression product and the fourth compound, and measuring expression of the miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p in the CRC subject or it can further comprise reacting the biological sample with a fifth compound to form a fifth complex, the fifth complex comprising a miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p expression product and the fifth compound, and measuring expression of the miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p in the CRC subject.

Yet another embodiment of present invention concerns a method of analysing a biological sample of a subject, wherein the subject has been diagnosed as suffering from colorectal cancer (CRC), the method comprising: reacting the biological sample with a first compound to form a first complex, the first complex comprising a miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p and the first compound, and measuring expression of the miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p in the CRC subject; reacting the biological sample with a second compound to form a second complex, the second

complex comprising another miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p and the second compound, and measuring expression of *the other* miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p in the CRC subject; reacting the biological sample with a third compound to form a third complex, the third complex comprising yet another miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p expression product and the third compound, and measuring expression of this other miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p in the CRC subject; and reacting the biological sample with a fourth compound to form a fourth complex, the fourth complex comprising yet another miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p expression product and the fourth compound, and measuring expression of this other miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p in the CRC subject.

Yet another embodiment of present invention concerns a biomarker panel comprising: a solid phase; a first compound bound to the solid phase, which first compound forms a first complex with a miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p. This biomarker panel can further comprise, a second compound bound to the solid phase, which second compound forms a second complex with another miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p and it can further comprise a third compound bound to the solid phase, which third compound forms a third complex with yet another miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p; and it can further comprise a fourth compound bound to the solid phase, which fourth compound forms a fourth complex with yet another miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p. This above biomarker panels can further comprise a biological sample of a subject diagnosed as suffering from CRC, said biological sample in contact with the first, second, third, and fourth

compounds or at least one further compound bound to the solid phase, which further compound forms a further complex with an expression product of a miRNA of the group consisting of hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p and this can further comprise discrete means

5 for detecting each said complex.

COLON CANCER

Claims

What is claimed is:

- 5 1. A microRNA gene product signature comprising or consisting essentially of at least one miR gene product of hsa-miR-4532, hsa-miR-499a-5p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-135a-5p, hsa-miR-545-3p, hsa-miR-4802-5p, hsa-miR-4641, hsa-miR-3689f, hsa-miR-431-5p, hsa-miR-1266, hsa-miR-130b-3p, hsa-miR-16-1-3p and hsa-miR-155-5p, including their corresponding homologues and complementary
10 sequences, that can distinguish low risk disease and high risk disease characterized by biochemical failure, aggressive disease, recurrence and/or relapse in colon cancer patients.
- 15 2. A microRNA gene product signature comprising or consisting essentially of at least one miR gene product of hsa-miR-4532, hsa-miR-499a-5p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-135a-5p, hsa-miR-545-3p, hsa-miR-4802-5p, hsa-miR-4641, hsa-miR-3689f, hsa-miR-431-5p, hsa-miR-1266, hsa-miR-130b-3p, hsa-miR-16-1-3p and hsa-miR-155-5p, including their corresponding homologues and complementary
20 sequences, and at least one gene product of hsa-miR-616-5p, hsa-miR-642a-5p, hsa-miR-499a-5p, hsa-miR-922, hsa-miR-654-3p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-199b-5p, hsa-miR-30a-5p, hsa-miR-23a-5p, hsa-miR-874, hsa-miR-148b-3p, hsa-miR-551b-5p, hsa-miR-181c-3p, hsa-miR-545-3p, hsa-miR-191-3p, hsa-miR-516a-3p, hsa-miR-548c-5p, hsa-miR-219-5p, hsa-miR-502-3p and hsa-miR-33a-3p, that can distinguish low risk disease and high risk disease characterized by biochemical failure, aggressive disease, recurrence and/or relapse in colon cancer
25 patients.
- 30 3. A microRNA gene product signature comprising or consisting essentially of all miR of hsa-miR-616-5p, hsa-miR-642a-5p, hsa-miR-342-5p, hsa-miR-199a-5p, hsa-miR-4532, hsa-miR-499a-5p, hsa-miR-922, hsa-miR-654-3p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-199b-5p, hsa-miR-30a-5p, hsa-miR-23a-5p, hsa-miR-874, hsa-miR-135a-5p, hsa-miR-148b-3p, hsa-miR-551b-5p, hsa-miR-181c-3p, hsa-miR-345-5p, hsa-miR-545-3p, hsa-miR-191-3p, hsa-miR-4802-5p, hsa-miR-4641, hsa-miR-3689f, hsa-miR-431-5p, hsa-miR-1266, hsa-miR-516a-3p, hsa-miR-548c-5p, hsa-miR-130b-3p, hsa-miR-219-5p, hsa-miR-502-3p, hsa-miR-33a-3p, hsa-miR-16-1-3p and/or hsa-

miR-155-5p, including their corresponding homologues and complementary sequences, that can distinguish low risk disease and high risk disease characterized by biochemical failure, aggressive disease, recurrence and/or relapse in colon cancer patients

- 5 4. The use of the microRNA gene product signature of any one of the previous claims 1 to 3, for prognosis for relapse-free survival (RFS) colorectal cancer in colon rectal cancer patients.
5. The use of the microRNA gene product signature of any one of the previous claims 1 to 3, for distinguishing recurrence or response to therapy for colon cancer.
- 10 6. The use of the microRNA gene product signature of any one of the previous claims 1 to 3, for distinguish low risk disease and high risk disease characterized by biochemical failure, aggressive disease, recurrence and/or relapse in particular stage III colon cancer.
- 15 7. A method of detecting, diagnosing, monitoring, classifying or screening a sample for high risk colon rectal cancer characterized by biochemical failure, aggressive disease, recurrence and/or relapse in a subject comprising detecting a microRNA signature of any one of claims 1 to 3.
- 20 8. The method according to claim 7 comprising: (a) subjecting the sample to a procedure to detect at least one miR gene product of the microRNA signature in the sample; and (b) diagnosing high risk colon rectal cancer by comparing the amount or status of the at least one miR gene product to the amount or status of the at least one miR gene product obtained from a sample from a control subject who does not suffer from high risk colon rectal cancer or from the subject taken at a different time.
- 25 9. The method according to claim 7 wherein post-surgical progression of colon rectal cancer in the subject is detected and the method comprises (a) assessing the level of at least one miR gene product of the microRNA signature in a sample from the subject; and (b) determining the post-surgical progression of colon rectal cancer in said subject based on the status of the at least one miR gene product.
- 30 10. The method according to claim 7 comprising the steps of: (a) analysing a sample, for instance a tumour sample, a circulating tumour cell or a tumour derived a vesicle such as a microvesicle or an exosome, from the subject with an assay that specifically detects at least one miR gene product of the microRNA signature; (b) determining whether or not the at least one miR gene product is differentially expressed; and (c) determining if the differential expression correlates with high risk colon rectal cancer.

11. The method according to any one of the claims 7 to 8 wherein the at least one miR gene product is detected by PCR, qRT-PCR, microarray analysis, in situ hybridization, in situ PCR, serial analysis of gene expression (SAGE) or deep sequencing.
12. The method according to any one of the claims 7 to 10, comprising the steps of (a) contacting the sample obtained from the subject with one or more reagent that specifically binds to at least one miR gene product of the microRNA signature; and (b) detecting in the sample an amount of the at least one miR gene product that binds to the reagent, relative to a control, and therefrom diagnosing high risk colon rectal cancer in the patient.
13. The method according to claim 12 comprising: providing a reagent that specifically binds to the at least one miR gene product to yield a detectable signal; processing the sample to produce a processed sample comprising miRNA; contacting the reagent with the processed sample to yield a detectable signal if the at least one miR gene product is present in the processed sample and if the reagent binds to the miR gene product; and quantifying the amount of miR gene product in the processed sample based on the detectable signal, wherein an alteration in the amount of miR gene product present in the processed sample, as compared to an amount of miR gene product present in a processed sample from a control, indicates that the subject has high risk colon rectal cancer.
14. The method according to any one of the claims 12 or 13 wherein the reagent is a nucleotide probe or primers that hybridize to the at least one miR gene product.
15. The method according to any one the claims 12 or 13 wherein the reagent comprises oligonucleotides specific for the at least one miR gene product placed on a microarray.
16. The method according to any one the claims 12 or 15 further comprising selecting a treatment and/or monitoring the levels of the at least one miR gene product in a subject diagnosed with high risk colon rectal cancer or undergoing treatment or being followed for progression.
17. A method for assessing the efficacy of a therapy for reducing high risk colon rectal cancer comprises: (a) detecting levels of the at least one miR gene product of a microRNA gene product signature of any one of claims 1 to 3 in a first sample from a subject obtained from the subject prior to providing at least a portion of the therapy to the patient; and (b) detecting levels of the at least one miR gene product in a second sample obtained from the patient following therapy, wherein a significant difference between the levels of the at least one miR gene product in the second sample relative

to the first sample or an abnormal state is an indication that the therapy is efficacious for reducing high risk colon rectal cancer.

18. A diagnostic kit for distinguish low risk disease and high risk disease characterized by biochemical failure, aggressive disease, recurrence and/or relapse in colon cancer patients, the diagnostic kit comprising probes or primers capable of detecting at least one, at least two, at least three, at least five, at least ten, at least twenty or all of the miR gene products of the microRNA gene product signature of any one of claims 1 to 3.
19. A diagnostic kit for the detection of colon cancer or to assist prognosis or determine the efficacy of a treatment regime for colon cancer comprising at least one oligonucleotide probe capable of binding to at least a portion of a circulating microRNA selected from the group consisting of hsa-miR-4532, hsa-miR-499a-5p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-135a-5p, hsa-miR-545-3p, hsa-miR-4802-5p, hsa-miR-4641, hsa-miR-3689f, hsa-miR-431-5p, hsa-miR-1266, hsa-miR-130b-3p, hsa-miR-16-1-3p and hsa-miR-155-5p.
20. A diagnostic kit for the detection of colon cancer or to assist prognosis or determine the efficacy of a treatment regime for colon cancer comprising at least one oligonucleotide probe capable of binding to at least a portion of a circulating microRNA selected from the group consisting of at least one miR gene product of hsa-miR-4532, hsa-miR-499a-5p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-135a-5p, hsa-miR-545-3p, hsa-miR-4802-5p, hsa-miR-4641, hsa-miR-3689f, hsa-miR-431-5p, hsa-miR-1266, hsa-miR-130b-3p, hsa-miR-16-1-3p and hsa-miR-155-5p, including their corresponding homologues and complementary sequences, and at least one gene product of hsa-miR-616-5p, hsa-miR-642a-5p, hsa-miR-499a-5p, hsa-miR-922, hsa-miR-654-3p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-199b-5p, hsa-miR-30a-5p, hsa-miR-23a-5p, hsa-miR-874, hsa-miR-148b-3p, hsa-miR-551b-5p, hsa-miR-181c-3p, hsa-miR-545-3p, hsa-miR-191-3p, hsa-miR-516a-3p, hsa-miR-548c-5p, hsa-miR-219-5p, hsa-miR-502-3p and hsa-miR-33a-3p.
21. A kit as claimed in any preceding claims 18 to 20 adapted for performance of an assay selected from a real-time PCR assay, a micro-array assay, histochemical assay or an immunological assay.
22. A kit as claimed in any preceding 18 to 20 for use in detecting colon cancer.
23. A method of identifying a therapeutic agent capable of preventing or treating cancers, including colon cancer, comprising testing the ability of the potential therapeutic agent

to enhance the expression of at least one circulating miRNA selected from the group consisting of hsa-miR-4532, hsa-miR-499a-5p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-135a-5p, hsa-miR-545-3p, hsa-miR-4802-5p, hsa-miR-4641, hsa-miR-3689f, hsa-miR-431-5p, hsa-miR-1266, hsa-miR-130b-3p, hsa-miR-16-1-3p and hsa-miR-155-5p.

24. The method of identifying a therapeutic agent capable of preventing or treating cancers, including colon cancer, comprising testing the ability of the potential therapeutic agent to enhance the expression of at least one circulating miRNA selected from the group consisting of at least one miR gene product of hsa-miR-4532, hsa-miR-499a-5p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-135a-5p, hsa-miR-545-3p, hsa-miR-4802-5p, hsa-miR-4641, hsa-miR-3689f, hsa-miR-431-5p, hsa-miR-1266, hsa-miR-130b-3p, hsa-miR-16-1-3p and hsa-miR-155-5p, including their corresponding homologues and complementary sequences, and at least one gene product of hsa-miR-616-5p, hsa-miR-642a-5p, hsa-miR-499a-5p, hsa-miR-922, hsa-miR-654-3p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-199b-5p, hsa-miR-30a-5p, hsa-miR-23a-5p, hsa-miR-874, hsa-miR-148b-3p, hsa-miR-551b-5p, hsa-miR-181c-3p, hsa-miR-545-3p, hsa-miR-191-3p, hsa-miR-516a-3p, hsa-miR-548c-5p, hsa-miR-219-5p, hsa-miR-502-3p and hsa-miR-33a-3p.

25. Use of a circulating miRNA selected the group consisting of hsa-miR-4532, hsa-miR-499a-5p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-135a-5p, hsa-miR-545-3p, hsa-miR-4802-5p, hsa-miR-4641, hsa-miR-3689f, hsa-miR-431-5p, hsa-miR-1266, hsa-miR-130b-3p, hsa-miR-16-1-3p and hsa-miR-155-5p to detect colon cancer, to stratify patients according to expected prognosis or to assess the efficacy of a medical treatment.

26. Use of a circulating miRNA selected the group consisting of at least one miR gene product of hsa-miR-4532, hsa-miR-499a-5p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-135a-5p, hsa-miR-545-3p, hsa-miR-4802-5p, hsa-miR-4641, hsa-miR-3689f, hsa-miR-431-5p, hsa-miR-1266, hsa-miR-130b-3p, hsa-miR-16-1-3p and hsa-miR-155-5p, including their corresponding homologues and complementary sequences, and at least one gene product of hsa-miR-616-5p, hsa-miR-642a-5p, hsa-miR-499a-5p, hsa-miR-922, hsa-miR-654-3p, hsa-miR-23b-5p, hsa-miR-30d-5p, hsa-miR-199b-5p, hsa-miR-30a-5p, hsa-miR-23a-5p, hsa-miR-874, hsa-miR-148b-3p, hsa-miR-551b-5p, hsa-miR-181c-3p, hsa-miR-545-3p, hsa-miR-191-3p, hsa-miR-516a-3p, hsa-miR-548c-5p, hsa-miR-219-5p, hsa-miR-502-3p and hsa-miR-33a-3p to detect colon

cancer, to stratify patients according to expected prognosis or to assess the efficacy of a medical treatment

27. Use as claimed in any one of the claims 24 to 26, wherein the detection is carried out in a blood sample or a sample derived from blood.

28. Any one of the previous claims wherein the colon rectal cancer is a stage III colon cancer.

29. A method for predicting a subject's response to a treatment for colorectal cancer (CRC), wherein the subject has been diagnosed with CRC, the method comprising:

obtaining a biological sample from the subject, wherein the biological sample is selected from the group consisting of a blood sample, a tumor, one or more a vesicle such as a microvesicle or an exosomes and any combination thereof;

preparing the biological sample for measurement of miRNA expression therein;

measuring expression of a miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p in the thus

prepared biological sample, wherein expression comprises MiRNA expression; and

comparing the expression of the miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p with a reference measurement;

wherein differential expression the miRNA of the group consisting of hsa-miR-155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p in the biological sample as compared to the reference measurement indicates that the subject will not respond to the treatment

30. A method for predicting a subject's response to a treatment for colorectal cancer (CRC), wherein the subject has been diagnosed with CRC, the method comprising:

obtaining a biological sample from the subject, wherein the biological sample is selected from the group consisting of a blood sample, a tumour, one or more a vesicle such as a microvesicle or an exosomes and any combination thereof;

preparing the biological sample for measurement of miRNA expression therein;

measuring expression of a miRNA of the group consisting of Hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p in the thus prepared biological sample, wherein expression comprises MiRNA expression; and

comparing the expression of the miRNA of the group consisting of Hsa-miR-874,
hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-
642a-5p and hsa-miR-616-5p with a reference measurement;

wherein differential expression the miRNA of the group consisting of Hsa-miR-874,
hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-
642a-5p and hsa-miR-616-5p indicates that the subject will respond to the treatment.

31. A method for predicting a subject's response to a treatment for colorectal cancer
(CRC), wherein the subject has been diagnosed with CRC, the method comprising:

obtaining a biological sample from the subject, wherein the biological sample is
selected from the group consisting of a blood sample, a tumor, one or more circulating
tumor cells (CTC), one or more a vesicle such as a microvesicle or an exosomes and
any combination thereof;

preparing the biological sample for measurement of MiRNA expression therein;

measuring expression of a miRNA of the group consisting of hsa-miR-155-5p, hsa-
miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p and of a
miRNA of the group consisting of Hsa-miR-874, hsa-miR-199b-5p, hsa-miR-23b-3p,
hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-642a-5p and hsa-miR-616-5p in the
thus prepared biological sample, wherein expression comprises MiRNA expression;
and comparing the expression of the miRNA of the group consisting of hsa-miR-155-
5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p with a
reference measurement;

comparing the expression of the a miRNA of the group consisting of Hsa-miR-874,
hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-miR-
642a-5p and hsa-miR-616-5p with a reference measurement

whereby the differential expression of a miRNA of the group consisting of hsa-miR-
155-5p, hsa-miR-16-1-3p, hsa-miR-33a-3p, hsa-miR-130b-3p and hsa-miR-1991-3p
correlates negative with relapse-free survival (RFS), and

whereby the differential expression of a miRNA of the group consisting of Hsa-miR-
874, hsa-miR-199b-5p, hsa-miR-23b-3p, hsa-miR-654-3p, hsa-miR-499a-5p, hsa-
miR-642a-5p and hsa-miR-616-5p correlates positive with relapse-free survival (RFS)

COLON CANCER

Figures

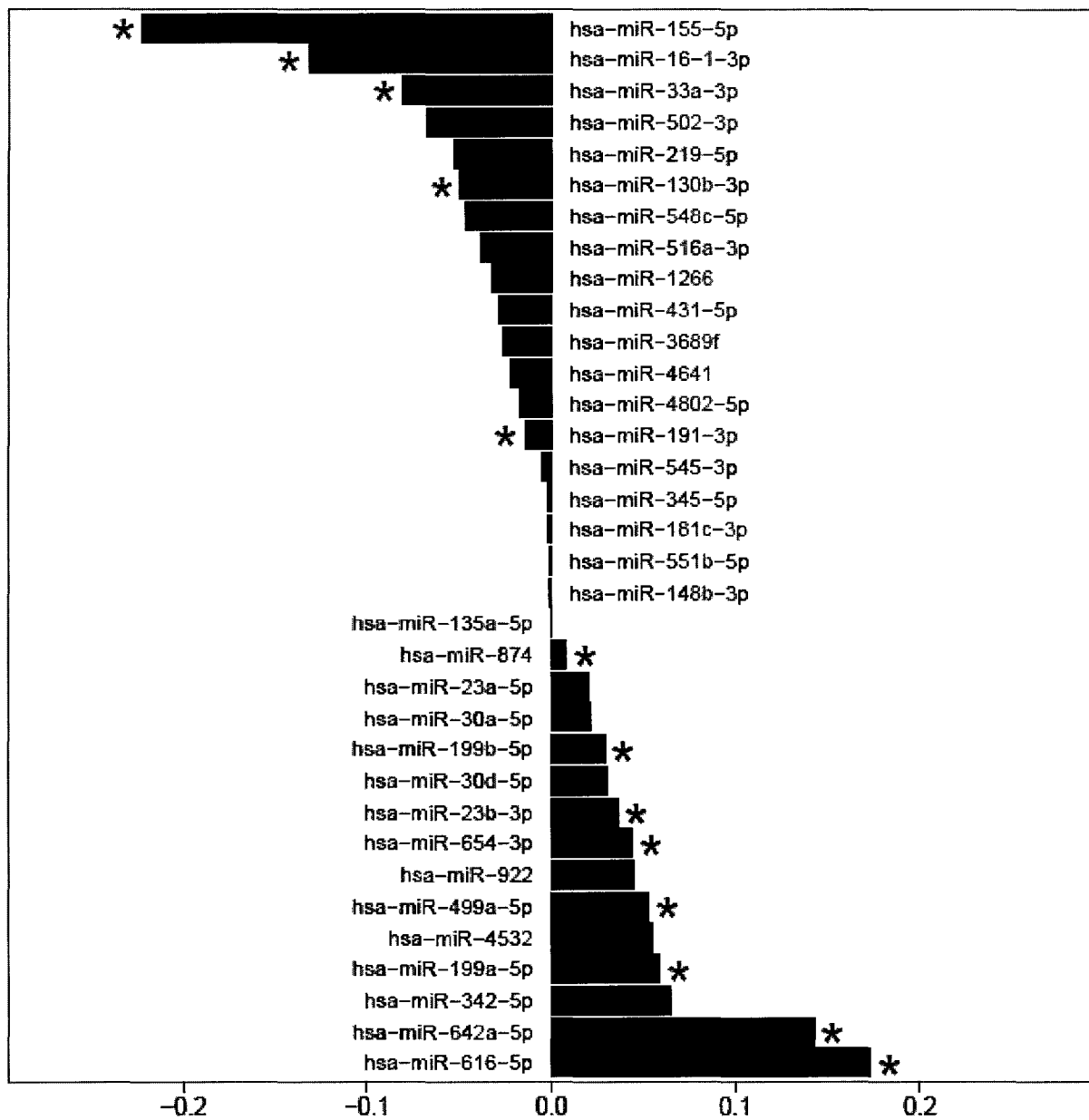


Fig. 1

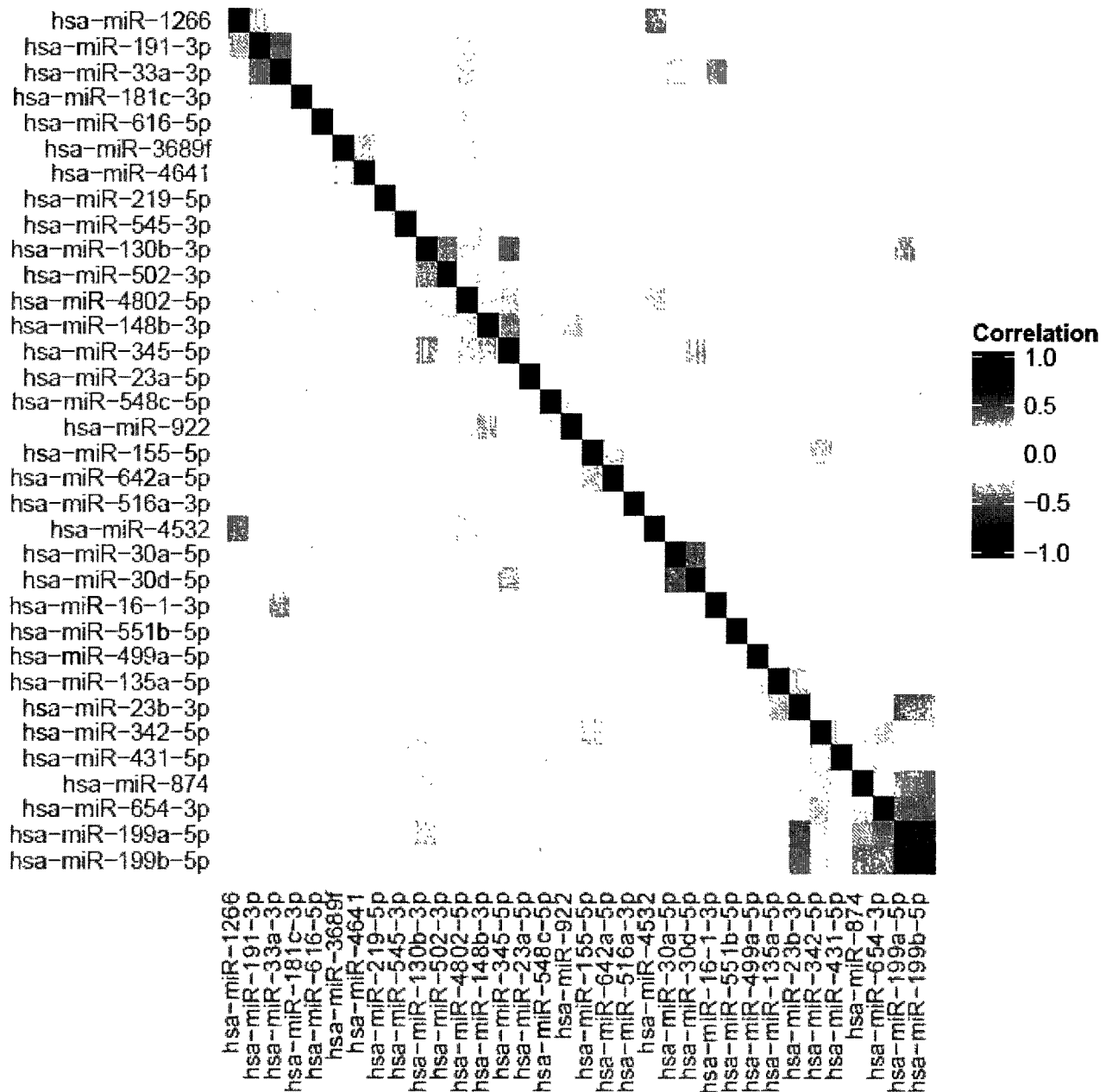


Fig 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/BE2016/000006A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

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)
C12Q

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, Sequence Search, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/107460 A2 (HERLEV HOSPITAL [DK]) 25 July 2013 (2013-07-25) claims 1,15; figures 3,4	1,2,4-31
X	EP 2 711 430 A1 (MAX PLANCK GESELLSCHAFT [DE]) 26 March 2014 (2014-03-26) paragraph [0057] ----- -/--	1,6-8, 10-15



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

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"&" document member of the same patent family

Date of the actual completion of the international search

2 June 2016

Date of mailing of the international search report

10/06/2016

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INTERNATIONAL SEARCH REPORT

International application No

PCT/BE2016/000006

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LV ZHONG-CHUAN ET AL: "Investigation of microRNA-155 as a serum diagnostic and prognostic biomarker for colorectal cancer", TUMOR BIOLOGY, KARGER, BASEL, CH, vol. 36, no. 3, 21 December 2014 (2014-12-21), pages 1619-1625, XP035476123, ISSN: 1010-4283, DOI: 10.1007/S13277-014-2760-9 [retrieved on 2014-12-21] the whole document -----	1,6-8, 10-15
X	WO 2014/048441 A1 (MIRRX THERAPEUTICS [DK]) 3 April 2014 (2014-04-03) discloses all miRs of claims 1-3 -----	1-3, 18-22
X	WO 2014/071205 A1 (DANA FARBER CANCER INST INC [US]) 8 May 2014 (2014-05-08) miR-545-3p,miR-642a-5p, miR-431 -5p,miR-4532, miR-874,miR-130b-3p, miR- 922, miR-499a-5p, miR-135a-5p, miR-148b-3p,miR-548c-5p, and miR-199a-5p,; claims 43, 46 -----	1,2, 18-22
E	WO 2016/034715 A2 (PRESTIZIA [FR]; INST REGIONAL DU CANCER DE MONTPELLIER ICM [FR]) 10 March 2016 (2016-03-10) hsa-miR-155-5p, hsa-miR-130b- 3p, hsa-miR- 30d-5p, hsa-miR-30a-5p; claims 1-3 -----	1,2,6-8, 10-22

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Information on patent family members

International application No

PCT/BE2016/000006

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