METHODS AND NUCLEIC ACIDS FOR DETERMINING THE PROGNOSIS OF A CANCER SUBJECT

FIELD OF THE INVENTION

[0001] The present invention relates to genomic DNA markers useful in determining the prognosis of a cancer subject, determining medical treatment for a cancer subject, determining if a tumor from a cancer subject indicates that the tumor is aggressive or has metastatic potential or indicates a reduced survival time for the subject, detecting an aggressive form of cancer in a subject, selecting a cancer subject for cancer treatment, or determining tumor load or cancer burden in a subject. Particular embodiments provide methods, nucleic acids, nucleic acid arrays and kits useful for determining the prognosis of a subject having cancer.

BACKGROUND

[0002] Methods for determining the prognosis, and thus methods and agents for determining treatment, of a cancer patient include determining the staging of the tumor based on various criteria. Often this determination includes invasive procedures to observe histological changes in tissue morphology and the level of invasion of the tumor into neighboring tissue and metastasis.

[0003] In particular, colorectal cancer is the second most frequent cancer in Europe and in the the US (412,900 and 150,000 individuals in 2006, respectively). In 75% of cases disease is removed by surgery. However, there is recurrence in 30-40% of stage II-III colorectal cancers, most within 3-5 years of initial diagnosis. Moreover, only 16-66% of patients are symptomatic at diagnosis of recurrence and of these tumors only 1.7 - 7% are resectable.Thus, 93 – 98.3 % of recurrent cases are identified past the time where resection is sufficient to remove all of the tumor or tumor cells. See Fakih, M.G. MD, CEA Monitoring in Colorectal Cancer, *What You Should Know*, Volume 20: Number 6: 2006.

[0004] Current practice guidelines for post-resection surveillance for Stage II and greater tumors include monitoring CEA (Carcinoembryonic antigen) every 3-6 months for 2 years then every 6 months for a total of 5 years, and/or colonoscopy after 1 year, optionally repeated every second year. For colorectal Stage I and II patients who are positive for CEA before surgery, only 3% to 32% of patients can be monitored by CEA-based monitoring, leaving 68 – 97% of Stage I & II patients who cannot be monitored at all with CEA. Furthermore, CEA sensitivity depends on the site of recurrence such that only a portion of the 3-32% of patients who can be monitored can benefit.

SUMMARY ~~OF THE INVENTION~~

[0009a] In a first aspect of the present disclosure, there is provided a method for

(i) determining the prognosis of a cancer subject after treatment removing the primary tumor,

(ii) determining the need for additional medical treatment for a cancer subject after treatment removing the primary tumor,

(iii) selecting a cancer subject for additional cancer treatment after treatment removing the primary tumor, or

(iv) determining, after treatment removing the primary tumor, if the cancer of a cancer subject is metastatic,

comprising the steps of:

a. measuring the level of methylated genomic DNA of a gene, or a fragment thereof, in a biological sample obtained from the subject prior to removing the primary tumor (pre-treatment sample);

b. measuring the level of methylated genomic DNA of the gene or a fragment thereof, in a biological sample obtained from the subject when the primary tumor has been removed (post-treatment sample),

whereby a presence of the methylated genomic DNA or fragment in the post-treatment sample after removal of the primary tumor indicates

(i) a bad prognosis,

(ii) the need for additional cancer treatment for the subject,

(iii) to select the cancer subject for additional treatment,

(iv) that the cancer is metastatic.

[0009b] In a second aspect of the present disclosure, there is provided a method for

(i) determining the prognosis of a cancer subject after treatment removing the primary tumor,

(ii) determining the need for additional medical treatment for a cancer subject after treatment removing the primary tumor,

(iii) selecting a cancer subject for additional cancer treatment after treatment removing the primary tumor, or

(iv) determining, after treatment removing the primary tumor, if the cancer of a cancer subject is metastatic,

comprising the step of:

measuring the level of methylated genomic DNA of the gene or a fragment thereof, in a biological sample obtained from the subject when the primary tumor has been removed (post-treatment sample),

whereby a presence of the methylated genomic DNA or fragment in the post-treatment sample after removal of the primary tumor indicates

(i) a bad prognosis,

(ii) the need for additional cancer treatment for the subject,

(iii) to select the cancer subject for additional treatment,

(iv) that the cancer is metastatic.

[0009c] In a third aspect of the present disclosure, there is provided the use of a methylated genomic SEPTIN9 nucleic acid or a fragment comprising at least 9, at least 16, at least 25, or at least 50 contiguous nucleotides of the nucleic acid and sequences complementary thereto in the determination of prognosis of a cancer subject when the primary tumor has been removed.

[0009d] In a fourth aspect of the present disclosure, there is provided the use of methylated genomic SEPTIN9 nucleic acid or a fragment comprising at least 9, at least 16, at least 25, or at least 50 contiguous nucleotides of the nucleic acid and sequences complementary thereto for determining the prognosis of a cancer subject, determining medical treatment for a cancer subject, determining if a tumor from a cancer subject indicates that the tumor is aggressive or has metastatic potential or indicates a reduced survival time for the subject, detecting an aggressive form of cancer in a subject, selecting a cancer subject for cancer treatment, or determining tumor load or cancer burden in a subject when the primary tumor has been removed.

[0009e] In a fifth aspect of the present disclosure, there is provided the use of a kit for determining the prognosis of a cancer subject, determining medical treatment for a cancer subject, for determining if a tumor from a cancer subject indicates that the tumor is aggressive or has metastatic potential or indicates a reduced survival time for the subject, for detecting an aggressive form of cancer in a subject, for selecting a cancer subject for cancer treatment, or for determining tumor load or cancer burden in a subject, when the primary tumor has been removed, the kit comprising:

a. a plurality of oligonucleotides or polynucleotides able to hybridise under stringent or moderately stringent conditions to methylated genomic DNA, wherein the methylated genomic DNA is SEPTIN9; and

b. means to detect the hybridisation.

[0009f] In a sixth aspect of the present disclosure, there is provided the use of a kit for determining the prognosis of a cancer subject, determining medical treatment for a cancer subject, for determining if a tumor from a cancer subject indicates that the tumor is aggressive or has metastatic potential or indicates a reduced survival time for the subject, for detecting an aggressive form of cancer in a subject, for selecting a cancer subject for cancer treatment, or for determining tumor load or cancer burden in a subject, when the primary tumor has been removed, the kit comprising:

a. a plurality of oligonucleotides or polynucleotides able to hybridise under stringent or moderately stringent conditions to methylated genomic DNA, wherein the methylated genomic DNA is RASSF2A; and

b. means to detect the hybridisation.

[0009g] In a seventh aspect of the present disclosure, there is provided the use of a kit for determining the prognosis of a cancer subject, determining medical treatment for a cancer subject, for determining if a tumor from a cancer subject indicates that the tumor is aggressive or has metastatic potential or indicates a reduced survival time for the subject, for detecting an aggressive form of cancer in a subject, for selecting a cancer subject for cancer treatment, or for determining tumor load or cancer burden in a subject, when the primary tumor has been removed, the kit comprising:

(a) a bisulfite reagent;

(b) at least one set of oligonucleotides containing two oligonucleotides whose sequences in each case are identical, are complementary, or hybridize under stringent or highly stringent conditions to an at least 9 or an at least 18 base long segment of a SEPTIN9 sequence.

[0009h] In an eighth aspect of the present disclosure, there is provided the use of a kit for determining the prognosis of a cancer subject, determining medical treatment for a cancer subject, for determining if a tumor from a cancer subject indicates that the tumor is aggressive or has metastatic potential or indicates a reduced survival time for the subject, for detecting an aggressive form of cancer in a subject, for selecting a cancer subject for cancer treatment, or for determining tumor load or cancer burden in a subject, when the primary tumor has been removed, the kit comprising:

(a) a bisulfite reagent;

(b) at least one set of oligonucleotides containing two oligonucleotides whose sequences in each case are identical, are complementary, or hybridize under stringent or highly stringent conditions to an at least 9 or an at least 18 base long segment of a RASSF2A sequence.

[0009i] In a ninth aspect of the present disclosure, there is provided a method for

(i) determining the prognosis of a cancer subject after treatment removing the primary tumor,

(ii) determining the need for additional medical treatment for a cancer subject after treatment removing the primary tumor,

(iii) selecting a cancer subject for additional cancer treatment after treatment removing the primary tumor, or

(iv) determining after treatment removing the primary tumor, if the cancer of a cancer subject is metastatic, comprising the steps of:

a. measuring the level of the Septin 9 gene expression and/or its promoter or regulatory elements or a fragment thereof, or of the RASSF2A gene and/or its promoter or regulatory elements or a fragment thereof, in a biological sample obtained from the subject prior to removing the primary tumor (pre-treatment sample);

b. measuring the level of the Septin 9 gene expression and/or its promoter or regulatory elements or a fragment thereof, or of the RASSF2A gene and/or its promoter or regulatory elements or a fragment thereof, in a biological sample obtained from the subject when the primary tumor has been removed (post-treatment sample);

whereby a presence of Septin 9 or RASSF2A gene expression in the post-treatment sample indicates

(i) a bad prognosis,

(ii) the need for additional cancer treatment for the subject,

(iii) to select the cancer subject for additional cancer treatment,

(iv) that the cancer metastatic.

[0009j] In a tenth aspect of the present disclosure, there is provided a method for

(i) determining the prognosis of a cancer subject after treatment removing the primary tumor,

(ii) determining the need for additional medical treatment for a cancer subject after treatment removing the primary tumor,

(iii) selecting a cancer subject for additional cancer treatment after treatment removing the primary tumor, or

(iv) determining after treatment removing the primary tumor, if the cancer of a cancer subject is metastatic, comprising the step of:

measuring the level of the Septin 9 gene expression and/or its promoter or regulatory elements or a fragment thereof, or of the RASSF2A gene and/or its promoter or regulatory elements or a fragment thereof, in a biological sample obtained from the subject when the primary tumor has been removed (post-treatment sample);

whereby a presence of Septin 9 or RASSF2A gene expression in the post-treatment sample indicates

(i) a bad prognosis,

(ii) the need for additional cancer treatment for the subject,

(iii) to select the cancer subject for additional cancer treatment,

(iv) that the cancer metastatic.

[0009k] In an eleventh aspect of the present disclosure, there is provided the use of a kit for carrying out the method as defined herein, the kit comprising:

a. a plurality of oligonucleotides or polynucleotides able to hybridise under stringent or moderately stringent conditions to the transcription products of the Septin 9 gene and/or its promoter or regulatory elements (including all transcript variants thereof), or of the RASSF2A gene and/or its promoter or regulatory elements (including all transcript variants thereof);and

b. means to detect the hybridisation.

[0009l] In a twelfth aspect of the present disclosure, there is provided the use of a kit for carrying out the method as defined herein, comprising:

a. a means for detecting polypeptides of Septin9 or RASSF2A, wherein the means can form complexes with the polypeptides; and

b. a means to detect the complexes of a).

[0009m] In a thirteenth aspect of the present disclosure, there is provided a method of detecting metastasis in a cancer subject or in tissue from the cancer subject, comprising detecting methylated genomic DNA from the subject when the primary tumor has been removed from the subject.

[0010] The ~~invention~~present disclosure provides a method for determining the prognosis of a cancer subject, comprising the steps of: measuring the pre-treatment level of methylated genomic DNA of a gene, or a fragment thereof, in a biological sample obtained from the subject; measuring the post-treatment level of methylated genomic DNA of the gene or a fragment thereof, in a biological sample obtained from the subject, whereby an increased or equivalent amount of the methylated genomic DNA or fragment in the post-treatment sample compared to the pre-treatment sample indicates additional cancer treatment for the subject. Within an embodiment, an increased amount of the methylated genomic DNA or fragment in the post-treatment sample compared to the pre-treatment sample indicates that the cancer is aggressive or has metastatic potential or reduced survival time for the subject. In a preferred embodiment the method of the ~~invention~~present disclosure provides a method for determining the prognosis of a cancer subject, comprising the steps of: a) measuring the pre-treatment level of methylated genomic DNA of a gene, or a fragment thereof, in a biological sample obtained from the subject; b) measuring the post-treatment level of methylated genomic DNA of the gene or a fragment thereof, in a biological sample obtained from the subject; and c) comparing the measured post-treatment level and the measured pre-treatment level of methylated DNA, whereby an increased or equivalent amount of the methylated genomic DNA or fragment in the post-treatment sample as compared to the pre-treatment sample indicates a bad prognosis and, thus, a need for additional cancer treatment for the subject. In a preferred embodiment of the method, the method comprises steps c) and d) as follows: c) comparing the measured post-treatment level and the measured pre-treatment level of methylated DNA and d) determining the prognosis of a cancer subject based on the result of the comparison of step c), whereby an increased or equivalent amount of the methylated genomic DNA or fragment in the post-treatment sample as compared to the pre-treatment sample indicates a bad prognosis and, thus, a need for additional cancer treatment for the subject.

[0011] Stable or even increased levels of the methylated genomic DNA, preferably, indicate that the chosen treatment failed to remove the cancer cells discharging the methylated DNA fragment or that their number increased despite of treatment, i.e. the cancer grew further. In contrast to this, decreased levels of the methylated genomic DNA, preferably, indicate that the number of cancer cells decreased, i.e. that the treatment was successful in reducing tumor load of the patient. In particular, a decrease to levels which are below the level of detection, indicates that all cancer cells may have been eradicated from the patient, i.e. a cure of the cancer. Typically, a cancer which responds poorly to treatment is considered aggressive.

[0012] If the applied cancer treatment is localized treatment, a decrease of the level of the methylated genomic DNA to a level below the limit of detection, preferably indicates a cure of the cancer. It will be understood by the person skilled in the art that – depending on the outcome of clinical studies – other threshold levels for defining a "cure" of a patient may be defined. The establishment of such threshold levels can be achieved by statistical methods conventional in the field of (medical) statistics.

[0013] However, if the level of the methylated genomic DNA measured after localized treatment is above the level of detection, this, preferably, indicates that localized treatment was insufficient to achieve a complete cure. This is typically the case if the cancer already spread beyond the area affected by the localized treatment. Therefore, even in the case of a decrease of the level of the methylated genomic DNA, the continued presence of detectable levels of the methylated genomic DNA indicates a poor prognosis because a cancer which spreads beyond its site of origin is, typically, much more difficult to treat.

[0014] The selection of further treatment of a cancer patient depends on his/her prognosis. If the prognosis is good, subsequent treatment does not need to be as aggressive as in cases with a bad prognosis. As the prognosis of the patient is an important parameter for the selection of further treatment of a cancer patient, the ~~invention~~present disclosure provides a method for determining medical treatment for a cancer subject, comprising the steps of: measuring the pre-treatment level of methylated genomic DNA of a gene, or a fragment thereof, in a biological sample obtained from the subject; measuring the post-treatment level of methylated genomic DNA of the gene or a fragment thereof, in a biological sample obtained from the subject, whereby an increased or equivalent amount of the methylated genomic DNA or fragment in the post-treatment sample compared to the pre-treatment sample indicates additional cancer treatment for the subject. In a preferred embodiment the ~~invention~~present disclosure also provides a method for determining which kind of medical treatment is suitable for a cancer subject, comprising the steps of: a) measuring the pre-treatment level of methylated genomic DNA of a gene, or a fragment thereof, in a biological sample obtained from the subject; b) measuring the post-treatment level of methylated genomic DNA of the gene or a fragment thereof, in a biological sample obtained from the subject; and c) comparing the measured post-treatment level and the measured pre-treatment level of methylated DNA, whereby an increased or equivalent amount of the methylated genomic DNA or fragment in the post-treatment sample compared to the pre-treatment sample indicates additional cancer treatment for the subject. In a preferred embodiment of the method, the method comprises steps c) and d) as follows: c) comparing the measured post-treatment level and the measured pre-treatment level of methylated DNA and d) determining based on the result of the comparison of step c) which kind of medical treatment is suitable for a cancer subject, whereby an increased or

equivalent amount of the methylated genomic DNA or fragment in the post-treatment sample compared to the pre-treatment sample indicates additional cancer treatment for the subject.

[0015] Preferably, a post-treatment level of the methylated genomic DNA which decreased below the level of detection indicates that no further medical treatment is required. In these cases, a monitoring of the patient for relapses may be sufficient. However, if the post-treatment level of the methylated genomic DNA does not decrease or even increases, additional medical treatment may be necessary. As an increasing level of the methylated genomic DNA indicates a failure of the the treatment, this situation, preferably, indicates the need to switch to a different kind of treatment.

[0016] It will be understood by the person skilled in the art that the choice of a suitable treatment of a cancer patient cannot be not exclusively based on the result of a single laboratory test. This decision is, preferably, based on medical judgement of the patient`s condition. Said judgement, preferably includes results of conventional diagnostic methods such as imaging methods as well as the general stat of health of the particular patient in addition to the results gained by applying the method of the present ~~invention~~disclosure.

[0017] The ~~invention~~present disclosure provides a method for determining if a tumor from a cancer subject indicates that the tumor is aggressive or has metastatic potential or indicates a reduced survival time for the subject comprising: measuring the pre-treatment level of methylated genomic DNA of a gene, or a fragment thereof, in a biological sample obtained from the subject; and measuring the post-treatment level of methylated genomic DNA of the gene or a fragment thereof, in a biological sample obtained from the subject, whereby an increased or equivalent amount of the methylated genomic DNA or fragment in the post-treatment sample compared to the pre-treatment sample indicates that the cancer is aggressive or has metastatic potential or indicates a reduced survival time for the subject. In a preferred embodiment the ~~invention~~present disclosure provides a method for determining if a tumor from a cancer subject indicates that the tumor is aggressive or has metastatic potential or indicates a reduced survival time for the subject comprising: a) measuring the pre-treatment level of methylated genomic DNA of a gene, or a fragment thereof, in a biological sample obtained from the subject; b) measuring the post-treatment level of methylated genomic DNA of the gene or a fragment thereof, in a biological sample obtained from the subject; and c) comparing the measured post-treatment level and the measured pre-treatment level of methylated DNA, whereby an increased or equivalent amount of the methylated genomic DNA or fragment in the post-treatment sample compared to the pre-treatment sample indicates that the tumor is aggressive or has metastatic potential or indicates a reduced survival time for the subject. In a preferred embodiment of the method, the method comprises steps c) and d) as follows: c) comparing the measured post-treatment level and the

measured pre-treatment level of methylated DNA and d) determining based on the result of the comparison of step c) if a tumor from a cancer subject indicates that the tumor is aggressive or has metastatic potential or indicates a reduced survival time for the subject, whereby an increased or equivalent amount of the methylated genomic DNA or fragment in the post-treatment sample compared to the pre-treatment sample indicates that the tumor is aggressive or has metastatic potential or indicates a reduced survival time for the subject.

[0018] Moreover, the ~~invention~~present disclosure provides a method for determining if a tumor from a cancer subject is aggressive and/or has metastatic potential comprising the steps of a) measuring the pre-treatment level of methylated genomic DNA of a gene, or a fragment thereof, in a biological sample obtained from the subject; b) measuring the post-treatment level of methylated genomic DNA of the gene or a fragment thereof, in a biological sample obtained from the subject; c) comparing the measured post-treatment level and the measured pre-treatment level of methylated DNA, whereby an increased or equivalent amount of the methylated genomic DNA or fragment in the post-treatment sample compared to the pre-treatment sample indicates that the tumor is aggressive and/or has metastatic potential. In a preferred embodiment of the method, the method comprises steps c) and d) as follows: c) comparing the measured post-treatment level and the measured pre-treatment level of methylated DNA and d) determining based on the result of the comparison of step c) if a tumor from a cancer subject is aggressive and/or has metastatic potential, whereby an increased or equivalent amount of the methylated genomic DNA or fragment in the post-treatment sample compared to the pre-treatment sample indicates that the tumor is aggressive and/or has metastatic potential.

[0019] The ~~invention~~present disclosure provides a method for detecting an aggressive form of cancer in a subject, comprising a) measuring the pre-treatment level of methylated genomic DNA of a gene, or a fragment thereof, in a biological sample obtained from the subject; b) measuring the post-treatment level of methylated genomic DNA of the gene or a fragment thereof, in a biological sample obtained from the subject, whereby an increased amount of the methylated genomic DNA or fragment in the post-treatment sample compared to the pre-treatment sample indicates that the cancer is an aggressive form. In a preferred embodiment the ~~invention~~present disclosure provides a method for detecting an aggressive form of cancer in a subject, comprising a) measuring the pre-treatment level of methylated genomic DNA of a gene, or a fragment thereof, in a biological sample obtained from the subject; b) measuring the post-treatment level of methylated genomic DNA of the gene or a fragment thereof, in a biological sample obtained from the subject; and c) comparing the measured post-treatment level and the measured pre-treatment level of methylated DNA, whereby an increased amount of the methylated genomic DNA or fragment in the post-treatment sample compared to the pre-treatment sample indicates that the cancer is an

aggressive form. In a preferred embodiment of the method, the method comprises steps c) and d) as follows: c) comparing the measured post-treatment level and the measured pre-treatment level of methylated DNA and d) detecting based on the result of the comparison of step c) an aggressive form of cancer in a subject, whereby an increased amount of the methylated genomic DNA or fragment in the post-treatment sample compared to the pre-treatment sample indicates that the cancer is an aggressive form.

[0020] The ~~invention~~present disclosure provides a method for selecting a cancer subject for cancer treatment comprising: measuring the pre-treatment level of methylated genomic DNA of a gene, or a fragment thereof, in a biological sample obtained from the subject; and measuring the post-treatment level of methylated genomic DNA of the gene or a fragment thereof, in a biological sample obtained from the subject, whereby an increase in the amount of methylated genomic DNA of the gene in the post-treatment sample compared to the pre-treatment sample indicates additional cancer treatment. In a preferred embodiment the ~~invention~~present disclosure provides a method for selecting a cancer subject for additional cancer treatment comprising: measuring the pre-treatment level of methylated genomic DNA of a gene, or a fragment thereof, in a biological sample obtained from the subject; and measuring the post-treatment level of methylated genomic DNA of the gene or a fragment thereof, in a biological sample obtained from the subject; comparing the measured post-treatment level and the measured pre-treatment level of methylated DNA, whereby an increase in the amount of methylated genomic DNA of the gene or the fragment thereof or an equivalent amount of said DNA or the fragment thereof in the post-treatment sample compared to the pre-treatment sample indicates the need for additional cancer treatment. In a preferred embodiment of the method, the method comprises steps c) and d) as follows: c) comparing the measured post-treatment level and the measured pre-treatment level of methylated DNA and d) selecting based on the result of the comparison of step c) a cancer subject for additional cancer treatment, whereby an increase in the amount of methylated genomic DNA of the gene or the fragment thereof or an equivalent amount of said DNA or the fragment thereof in the post-treatment sample compared to the pre-treatment sample indicates the need for additional cancer treatment.

[0021] Consequently, the present ~~invention~~disclosure provides a method for determining the success of a treatment against cancer in a subject comprising the steps of a) measuring the pre-treatment level of methylated genomic DNA of a gene, or a fragment thereof, in a biological sample obtained from the subject; and b) measuring the post-treatment level of methylated genomic DNA of the gene or a fragment thereof, in a biological sample obtained from the subject; c) comparing the measured post-treatment level and the measured pre-treatment level of methylated DNA, whereby (i) an decrease in the amount of methylated genomic DNA of the gene or the

fragment thereof in the post-treatment sample compared to the pre-treatment sample indicates that the treatment was successful and (ii) an increase in the amount of methylated genomic DNA of the gene or the fragment thereof or an equivalent amount of said DNA or the fragment thereof in the post-treatment sample compared to the pre-treatment sample indicates that the treatment was not successful. In a preferred embodiment of the method, the method comprises steps c) and d) as follows: c) comparing the measured post-treatment level and the measured pre-treatment level of methylated DNA and d) determining based on the result of the comparison of step c) the success of a treatment against cancer in a subject, whereby (i) an decrease in the amount of methylated genomic DNA of the gene or the fragment thereof in the post-treatment sample compared to the pre-treatment sample indicates that the treatment was successful and (ii) an increase in the amount of methylated genomic DNA of the gene or the fragment thereof or an equivalent amount of said DNA or the fragment thereof in the post-treatment sample compared to the pre-treatment sample indicates that the treatment was not successful.

[0022] Preferably, a treatment which was "successful" achieved at least one of the following effects: remission of the cancer, increase of the time to recurrence of the cancer, increase of the time to tumor progression, alleviation of the symptoms of the cancer, reduction of tumor mass and decrease of the number tumors. More preferably, a "successful treatment", characterized by a cure of the cancer, i.e. the complete eradication detectable and non-detectable tumor cells. A preferred indicator of the cure of the cancer is a recurrence free survival of the patient for at least 5 years or, more preferably, at least 10 years.

[0023] A treatment which was "not successful", preferably, failed to achieve any of the aims described above.

[0024] The ~~invention~~present disclosure provides a method for determining tumor load or cancer burden in a subject comprising: measuring the pre-treatment level of methylated genomic DNA of a gene, or a fragment thereof, in a biological sample obtained from the subject; and measuring the post-treatment level of methylated genomic DNA of the gene or a fragment thereof, in a biological sample obtained from the subject; whereby an increase in the amount of methylated genomic DNA of the gene in the post-treatment sample compared to the pre-treatment sample indicates that the subject has increased or equivalent tumor load or cancer burden or that the tumor load or cancer burden has not been diminished by the treatment. In a preferred embodiment the ~~invention~~present disclosure provides a method for determining the development of tumor load or cancer burden in a subject comprising: a) measuring the pre-treatment level of methylated genomic DNA of a gene, or a fragment thereof, in a biological sample obtained from the subject; b) measuring the post-treatment level of methylated genomic DNA of the gene or a fragment thereof, in a biological sample obtained from the subject; and c) comparing the measured post-treatment level

with the measured pre-treatment level of methylated DNA, whereby an increase in the amount of methylated genomic DNA of the gene or the fragment thereof or an equivalent amount of said DNA or the fragment thereof in the post-treatment sample compared to the pre-treatment sample indicates that the subject has increased or equivalent tumor load or cancer burden or that the tumor load or cancer burden has not been diminished by the treatment. In a preferred embodiment of the method, the method comprises steps c) and d) as follows: c) comparing the measured post-treatment level and the measured pre-treatment level of methylated DNA and d) determining based on the result of the comparison of step c) the development of tumor load or cancer burden in a subject, whereby an increase in the amount of methylated genomic DNA of the gene or the fragment thereof or an equivalent amount of said DNA or the fragment thereof in the post-treatment sample compared to the pre-treatment sample indicates that the subject has increased or equivalent tumor load or cancer burden or that the tumor load or cancer burden has not been diminished by the treatment.

[0025] The ~~invention~~present disclosure provides a method for determining tumor load or cancer burden in a subject comprising comparing the post-treatment level of methylated genomic DNA of a gene or a fragment thereof, in a biological sample obtained from the subject with the pre-treatment level of methylated genomic DNA of the gene or fragment, whereby an increase in the amount of methylated genomic DNA of the gene in the post-treatment sample compared to the pre-treatment sample indicates that the subject has increased or equivalent tumor load or cancer burden or that the tumor load or cancer burden has not been diminished by the treatment. In a preferred embodiment of the method, the method comprises steps c) and d) as follows: c) comparing the measured post-treatment level and the measured pre-treatment level of methylated DNA and d) determining based on the result of the comparison of step c) tumor load or cancer burden in a subject, whereby an increase in the amount of methylated genomic DNA of the gene in the post-treatment sample compared to the pre-treatment sample indicates that the subject has increased or equivalent tumor load or cancer burden or that the tumor load or cancer burden has not been diminished by the treatment.

[0026] The level of methylated DNA of the genes of the present ~~invention~~disclosure is generally useful as a marker for properties of a cancer such as aggressiveness or tumor load. A comparison of the levels of methylated DNA taken at different points in time, therefore, indicates independently of ongoing treatment how the properties of the cancer develop over time.

[0027] For this reason, the present ~~invention~~disclosure provides a method for monitoring a property of a cancer selected from the group consisting of tumor load, cancer burden, aggressiveness of a cancer and the prognosis of a cancer subject comprising the steps of a) measuring the level of methylated genomic DNA of a gene, or a fragment thereof, in a first biological sample obtained

from a subject suffering from cancer; b) measuring the level of methylated genomic DNA of the gene or a fragment thereof in a further biological sample obtained from the subject; and c) comparing the measured levels of methylated DNA in the further sample and the first sample, wherein an increased level of methylated DNA of the gene or the fragment thereof in the further sample indicates that the tumor load, tumor burden or the aggressiveness of the cancer increased or the prognosis of the patient worsened and (ii) a decreased level of methylated DNA of the gene or the fragment thereof in the further sample indicates that the tumor load, tumor burden or the aggressiveness of the cancer decreased or the prognosis of the patient improved.

[0028] In a preferred embodiment of the method, the method comprises steps c) and d) as follows: c) comparing the measured levels of methylated DNA in the further sample and the first sample and d) determining based on the result of the comparison of step c) whether the tumor load, tumor burden or the aggressiveness of the cancer increased or decreased or the prognosis of the patient worsened or improved.

[0029] The first and second sample can be taken any time provided that the second sample is taken after the first sample. Preferably, the second sample is taken at least 1 month, at least 2 months, at least 3 months, at least 6 months, at least 9 months orat least 12 months after the first sample.

[0030] The above-described method for monitoring a property of the cancer is especially suitable for monitoring a patient whose cancer has already been treated before for recurrence and/or progression of the cancer. Thus, in a particularly preferred embodiment of the present ~~invention~~disclosure, the patient is a cancer patient whose treatment apparently cured the cancer. The determination of methylation of the genes of the present ~~invention~~disclosure in at least 2 samples taken at different points in time after treatment can be used to detect a recurrence of the cancer. It is a general problem problem in the field of cancer therapy that a treatment may be apparently effective, i.e. it decreases the tumor burden of the patient below the level which is detectable with the available diagnostic methods, in particular imaging methods. Nevertheless, a few cancer cells may remain despite apparently successful treatment. These cells may proliferate and cause a relapse of the cancer even years after an apparently successful treatment. Therefore, a follow-up of treated patients for some period of time after treatment is good medical practice in order to detect a relapse as early as possible. As the method of the present ~~invention~~disclosure is both sensitive (Septin 9, in particular, may be used to detect early stages of colon carcinoma), easy to perform and non-invasive, it is particularly suited to monitor treated cancer patients during follow-up.

[0031] Within an ~~aspect~~embodiment of the methods of the ~~invention~~present disclosure, the gene is SEPTIN9 (SEQ ID NO:1) or RASSF2a (SEQ ID NO:16).

[0032] Within another ~~aspect~~embodiment of the methods of the ~~invention~~present disclosure, the gene is SEPTIN9 (SEQ ID NO:1).

[0033] Within another ~~aspect~~embodiment of the methods of the ~~invention~~present disclosure, the gene is RASSF2A (SEQ ID NO:16).

[0034] In a further preferred embodiment of the ~~invention~~present disclosure the above-described methods are based on the measurement of the level of methylated DNA of both SEPTIN9 and RASSF2A.

[0035] Within another ~~aspect~~embodiment of the method of the ~~invention~~present disclosure, the cancer is selected from the group consisting of: colon cancer; and colorectal cancer. Within an embodiment, the stage of the cancer is Stage I colorectal cancer. Within another embodiment, the stage of the cancer is Stage II colorectal cancer. Within another embodiment, the cancer is Stage III colorectal cancer. Within another embodiment, the cancer is Stage IV colorectal cancer.

[0036] Within another ~~aspect~~embodiment of the methods of the ~~invention~~present disclosure, the treatment is selected from the group consisting of: surgery or resection; immunotherapy; radiation; chemotherapy; therapy targeting solid tumors; therapy targeting soft-tissue tumors; and therapy targeting blood cells.

[0037] Within another ~~aspect~~embodiment of the methods of the ~~invention~~present disclosure, the treatment is localized to the region of cancer/tumor in the subject. Within another ~~aspect~~embodiment of the methods of the ~~invention~~present disclosure, the treatment is not localized to the region of cancer/tumor in the subject.

[0038] The term "localized treatment" preferably refers to surgical resection of the tumor and/or radiation therapy. The term "not localized treatment" is equivalent to systemic treatment and, preferably, refers to chemotherapy and/or immunotherapy.

[0039] Within another ~~aspect~~embodiment of the methods of the ~~invention~~present disclosure, the biological sample is selected from the group consisting of: tissue, blood, stool, urine, and lung lavage fluid, breast, prostate, colon, rectum, or a combination of these tissues. Within an embodiment, the sample is serum or plasma. The use of serum or plasma is preferred.

[0040] Within another ~~aspect~~embodiment of the methods of the ~~invention~~present disclosure, methylated genomic DNA or fragment thereof is measured quantitatively or measured quantitatively in part. Within another ~~aspect~~embodiment of the methods of the ~~invention~~present disclosure, methylated genomic DNA or fragment is measured qualitatively or measured qualitatively in part. Within another ~~aspect~~embodiment of the methods of the ~~invention~~present disclosure, methylated genomic DNA or fragment is measured quantitatively in part and qualitatively in part or semiquantitativley.

[0041] Within another ~~aspect~~embodiment of the methods of the ~~invention~~present disclosure, measuring the methylated genomic DNA or fragment comprises contacting genomic DNA from the biological sample with at least one reagent, or series of reagents that distinguishes between methylated and non-methylated CpG dinucleotides within at least one target region of the genomic DNA, wherein the target region comprises, or hybridizes under stringent conditions to a sequence of at least 9, at

least 16 or at least 25 contiguous nucleotides of SEQ ID NOs: 1, 2, 3 or 16 wherein said contiguous nucleotides comprise at least one CpG dinucleotide sequence. Within an embodiment, contacting the genomic DNA, or the fragment thereof in b), comprises use of a reagent selected from the group comprising of bisulfite, hydrogen sulfite, disulfite, and combinations thereof.

[0042] Within another ~~aspect~~embodiment of the methods of the ~~invention~~present disclosure comprise: a) extracting or otherwise isolating the genomic DNA or fragment thereof from the biological samples; b) treating the extracted or isolated genomic DNA or a fragment thereof with one or more reagents to convert cytosine bases that are unmethylated in the 5-position thereof to uracil or to another base that is detectably dissimilar to cytosine in terms of hybridization properties; c) contacting the treated genomic DNA or treated fragment, with an amplification enzyme and at least one primer comprising, a contiguous sequence of at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 19, at least 20, at least 25, or at least 50 nucleotides that is complementary to, or hybridizes under moderately stringent or stringent conditions to a the treated sequence or to a complement thereof, wherein the treated genomic DNA or the fragment thereof is either amplified to produce at least one amplificate, or is not amplified; and d) determining, based on a presence, absence or amount of, or on a property of said amplificate, the methylation state or level of at least one CpG dinucleotide of the gene, or an average, or a value reflecting an average methylation state or level of a plurality of CpG dinucleotides of the gene. The treated genomic DNA referred to above is preferably selected from the group consisting of SEQ ID NO: 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 18, 19 and 20.

[0043] Within another ~~aspect~~embodiment of the methods of the ~~invention~~present disclosure comprises a) extracting or otherwise isolating the genomic DNA or fragment thereof from the biological samples; b) digesting the extracted or isolated genomic DNA or a fragment thereof with one or more methylation sensitive restriction enzymes; c) contacting the DNA restriction enzyme digest of b), with an amplification enzyme and at least two primers suitable for the amplification of a sequence comprising at least one CpG dinucleotide of the gene; and d) determining, based on a presence, absence or class of an amplificate the methylation state or level of at least one CpG dinucleotide of the gene.

[0044] Further information on preferred methods for measuring the level of a methylated genomic DNA can be found further below in the application. In an especially preferred embodiment of the present ~~invention~~disclosure the method for measurement of methylation levels of genomic DNA is MethyLight™, HeavyMethl™ or methylation specific PCR.

[0045] Within another ~~aspect~~embodiment the ~~invention~~present disclosure provides a methylated genomic SEPTIN9 nucleic acid or a fragment comprising at at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 19, at least 20, at least 25, or at least 50 contiguous nucleotides of the nucleic acid and sequences complementary thereto for use in the determination of prognosis of a cancer subject. Another embodiment of the present ~~invention~~disclosure provides a methylated genomic RASSF2A nucleic acid or a fragment comprising at least 9, at least 16, at least 25, or at least 50 contiguous nucleotides of the nucleic acid and sequences complementary thereto for use in the determination of prognosis of a cancer subject.Within an embodiment the subject has is colorectal cancer.

[0046] Within another ~~aspect~~embodiment the ~~invention~~present disclosure provides the use of methylated genomic SEPTIN9 nucleic acid or a fragment comprising at least 9, at least 16, at least 25, or at least 50 contiguous nucleotides of the nucleic acid and sequences complementary thereto for determining the prognosis of a cancer subject. Within an embodiment, the subject has colorectal cancer.

[0047] Within another ~~aspect~~embodiment the ~~invention~~present disclosure provides a bisulfite treated genomic SEPTIN9 or RASSF2A DNA nucleic acid comprising at at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 19, at least 20, at least 25, or at least 50 contiguous nucleotides, or a complement thereto for use in determining the prognosis of a cancer subject. Preferably, the sequence of the bisulfite treated SEPTIN9 or RASSF2A DNA is definedby SEQ ID NO: 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 18, 19 or 20. Within an embodiment, the contiguous base sequence comprises at least one CpG, TpG or CpA dinucleotide sequence.

[0048] Within another ~~aspect~~embodiment the ~~invention~~present disclosure provides a kit for determining the prognosis of a cancer subject, determining medical treatment for a cancer subject, for determining if a tumor from a cancer subject indicates that the tumor is aggressive or has metastatic potential or indicates a reduced survival time for the subject, for detecting an aggressive form of cancer in a subject, for selecting a cancer subject for cancer treatment, or for determining tumor load or cancer burden in a subject comprising: a) a plurality of oligonucleotides or polynucleotides able to hybridise under stringent or moderately stringent conditions to the transcription products of the gene or methylated genomic DNA; and b) means to detect the hybridisation. Within an embodiment, the gene or methylated genomic DNA is SEPTIN9. Within an embodiment, the gene or methylated genomic DNA is RASSF2A.

[0049] Within another ~~aspect~~embodiment the ~~invention~~present disclosure provides a kit for determining the prognosis of a cancer subject, determining medical treatment for a cancer subject, for determining if a tumor from a cancer subject indicates that the tumor is aggressive or has metastatic potential or indicates a reduced survival time for the subject, for detecting an aggressive form of cancer in a

subject, for selecting a cancer subject for cancer treatment, or for determining tumor load or cancer burden in a subject comprising: (a) a bisulfite reagent; (b) at least one set of oligonucleotides containing two oligonucleotides whose sequences in each case are identical , are complementary, or hybridize under stringent or highly stringent conditions to a at at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 19, at least 20, at least 25, or at least 50 nucleotide long segment of a SEPTIN9 sequence or of a RASSF2A gene.

[0050] Within other ~~aspects~~embodiments the ~~invention~~present disclosure provides the use of the methods described herein, the nucleic acids as described herein and/or a kit as described herein for determining the prognosis of a cancer subject.

[0051] The present ~~invention~~disclosure provides a method for determining the prognosis of a subject having cancer, in a subject comprising determining the expression levels of at least one gene or genomic sequence wherein the genomic sequence is methylated in cancers and unmethylated in non-cancerous tissues. Methylation of the genomic DNA encoding a gene or, in particular, methylation of its promoter region decreases the expression of said gene. Consequently, methylation of the gene in question gives a similar diagnostic information as its underexpression.Thus, the level or amount of methylation/or expression of the gene in a biological sample isolated from said subject is indicative of the prognosis of said subject. Various ~~aspects~~embodiments of the present ~~invention~~disclosure provide genetic markers, whereby expression analysis of said marker enables the determination of the prognosis of a subject having cancer. In one embodiment said expression level is determined by detecting the presence, absence or level of mRNA transcribed from said gene. In a further embodiment said expression level is determined by detecting the presence, absence or level of a polypeptide encoded by said gene or sequence thereof.

[0052] The present ~~invention~~disclosure also provides a method for determining the prognosis of a subject having colorectal cancer (CRC) or colon cancer, in a subject comprising determining the DNA Methylation levels of Septin 9 (Septin9) or of RASSF2A in plasma isolated from said subject wherein after resection of the primary tumor the methylation status is indicative of the prognosis of said subject. In an embodiment the resection is curative.

[0053] The examples described herein showed that the Septin9 biomarker decreases in approximately 73% of the investigated CRC Stage II and only in 20 % of the Stage III patients after resection of the primary tumor. The presence of Septin9 in CRC patients after treatment with curative intention can be used an early prognostic indicator of disease recurrence. The fact that Septin9 is still detectable after resection of the primary tumor, indicates a high risk of the

presence of tumor cells (e.g. micro metastasis) that are still in the body of the patient and which can be sensitive detected by Septin9.

[0054] In further embodiments said expression is determined by detecting the presence, absence or amount of CpG methylation within said gene, and therefrom deducing the prognosis of said subject having cancer. Said method comprises the following steps: i) contacting genomic DNA isolated from a biological sample obtained from the subject with at least one reagent, or series of reagents that distinguishes between methylated and non-methylated CpG dinucleotides within at least one target region of the genomic DNA, wherein the nucleotide sequence of said target region comprises at least one CpG dinucleotide sequence of at least one gene or genomic sequence of this group of genes and ii) determining the prognosis of a subject having cancer. Preferably the target region comprises, or hybridizes under stringent conditions to a sequence of at least 16, at least 25 or at least 50 contiguous nucleotides.

[0055] Said use of the gene may be enabled by means of any analysis of the expression of the gene, by means of mRNA expression analysis or protein expression analysis. In an embodiment the determination of the prognosis of a subject having cancer, is enabled by means of analysis of the methylation status of at least one gene or genomic sequence that is methylated in cancer tissue but unmethylated in non-cancerous tissue, including isoforms, fragments, promoter or regulatory elements, and antisense versions thereof.

[0056] The ~~invention~~present disclosure provides a method for the analysis of biological samples for features associated with the progression of cancer, the method characterized in that the nucleic acid, or a fragment thereof is contacted with a reagent or series of reagents capable of distinguishing between methylated and non methylated CpG dinucleotides within the genomic sequence. In an embodiment, the gene is SEPTIN9 or RASSF2A.

[0057] Preferably, the sequence of SEPTIN9 is defined by SEQ ID NO: 1, 2 or 3. More preferably, the sequence of SEPTIN9 is defined by SEQ ID NO: 2 or 3.

The sequence of RASSF2A is, preferably, defined by SEQ ID NO: 16.

[0058] In a preferred embodiment of the present ~~invention~~disclosure the methylation status of the promotor region of SEPTIN9 and/or RASSF2A ist determined. In a more preferred embodiment, the methylation state of at least one cytosine comprised by the genomic sequence as defined by SEQ ID NO: 32 and/or 34 is determined. In an even more preferred embodiment of the ~~invention~~present disclosure, the methylation status of at least one cytosine selected from the group consisting of the cytosines in positions 21, 28, 30, 37 and 39 of SEQ ID NO: 32 and positions 25, 29, 46, 52, 58, 70, 74, 79 and 89 of SEQ ID NO: 34 is determined.In the most preferred embodiment, the methylation status of all aforementioned cytosine positions in SEQ ID NO: 32 and/or 34 is determined.

[0059] The present ~~invention~~disclosure provides a method for ascertaining epigenetic parameters of genomic DNA associated with the development of cancer.

[0060] The source of the test sample is a tissue, or body fluid, such as, for example, tissues and body fluids selected from the group consisting of tissue, blood, plasma, serum, urine, lung lavage fluid, stool, lung, breast, colon, rectum, intestine and combinations thereof.

[0061] Specifically, the present ~~invention~~disclosure provides a method for determining the prognosis of a subject having cancer suitable for use in a prognostic tool, comprising: obtaining a biological sample comprising genomic nucleic acid(s); contacting the nucleic acid(s), or a fragment thereof, with a reagent or a plurality of reagents sufficient for distinguishing between methylated and non methylated CpG dinucleotide sequences within a target sequence of the subject nucleic acid, wherein the target sequence comprises, or hybridises under stringent conditions to, a sequence comprising at least 16, at least 25 or at least 50 contiguous nucleotides of the gene said contiguous nucleotides comprising at least one CpG dinucleotide sequence; and determining, based at least in part on said distinguishing, the methylation state of at least one target CpG dinucleotide sequence, or an average, or a value reflecting an average methylation state of a plurality of target CpG dinucleotide sequences.

[0062] In distinguishing between methylated and non methylated CpG dinucleotide sequences within the target sequence comprises methylation state-dependent conversion or non-conversion of at least one such CpG dinucleotide sequence to the corresponding converted or non-converted dinucleotide sequence within a sequence selected from the group consisting of bisulfite converted sense and antisense strands of the genes and contiguous regions thereof corresponding to the target sequence.

[0063] Additional embodiments provide a method for the determination of the prognosis of a subject having cancer comprising: obtaining a biological sample having subject genomic DNA; extracting the genomic DNA; treating the genomic DNA, or a fragment thereof, with one or more reagents to convert 5-position unmethylated cytosine bases to uracil or to another base that is detectably dissimilar to cytosine in terms of hybridization properties; contacting the treated genomic DNA, or the treated fragment thereof, with an amplification enzyme and at least two primers comprising, in each case a contiguous sequence at least 9 nucleotides in length that is complementary to, or hybridizes under moderately stringent or stringent conditions to a sequence selected from the group consisting bisulfite converted sense and antisense strands, and complements thereof, wherein the treated DNA or the fragment thereof is either amplified to produce an amplificate, or is not amplified; and determining, based on a presence, absence or class of, or on a property of said amplificate, the methylation state or an average, or a value