

kit can further comprise sodium bisulfite and adapters for whole target genes amplification, and polynucleotides (e.g., detectably-labeled polynucleotides) to quantify the presence of the converted methylated and or the converted unmethylated sequence of at least one cytosine from a DNA region of epigenetic biomarkers in target DNA sequences as described herein.

5 Furthermore, the kit can further comprise methylation sensing restriction enzymes for whole target sequence or genes amplification.

[0022] In one embodiment, the kit further comprises one of more primers having sequences of SEQ ID NOs: 9 to 12 and 1 to 4 and/or one or more probes having sequences of SEQ ID Nos: 17, 18, 12 and 13 for assaying methylation status of epigenetic biomarkers in target DNA
10 sequences *GCM2* and *ITPRIPL1* or fragments thereof in combination with one or more target DNA sequences *C1orf114* or a fragment thereof, *ZNF177* or a fragment thereof, *C8orf47* or a fragment thereof, and ~~RKL0014~~RKL001 or any of combinations thereof.

[0023] In one embodiment, the kit can be used for detecting and/or characterizing the methylation profile of epigenetic biomarkers in target DNA sequences *GCM2* or a fragment
15 thereof and *ITPRIPL1* or a fragment thereof in combination with one or more target DNA sequences *C1orf114* or a fragment thereof, *ZNF177* or a fragment thereof, *C8orf47* or a fragment thereof, and ~~RKL0014~~RKL001 or any of combinations thereof. The kit further comprises one of more primers having sequences of SEQ ID NOs: 9 to 12 and 1 to 4 and/or one or more probes having sequences of SEQ ID Nos: 17, 18, 12 and 13 to detect or measure the methylation status
20 of the target DNA sequences.

[0024] In a further embodiment, the kit further comprises methylation sensing restriction enzymes for whole target sequence or genes amplification.

[0077] In some embodiments, the present disclosure provides a kit for detecting and/or characterizing the methylation profile of the target DNA sequences 19described herein. In some embodiments, the target DNA sequences comprises the combination selected from the group consisting of: *GCM2* and *ITPRIPL1*; *GCM2*, *ITPRIPL1* and *C1orf114*; *GCM2*, *ITPRIPL1*, *C1orf114* and *ZNF177*; *GCM2*, *ITPRIPL1*, *C1orf114*, *ZNF177* and ~~RKL1001~~RKL001; and *GCM2*, *ITPRIPL1*, *C1orf114* and *ZNF177*, ~~RKL1001~~RKL001 and *C8orf47*.

[0078] In some instances, the kit comprises a plurality of primers or probes to detect or measure the methylation status/levels of one or more target genes. Such kits comprise, in some instances, at least one polynucleotide that hybridizes to at least one of the methylation biomarker sequences described herein and at least one reagent for detection of gene methylation. Reagents for detection of methylation include, e.g., sodium bisulfate, polynucleotides designed to hybridize to sequence that is the product of a marker sequence if the marker sequence is not methylated (e.g., containing at least one C-U conversion), and/or a methylation-sensitive or methylation-dependent restriction enzyme. In some cases, the kits provide solid supports in the form of an assay apparatus that is adapted to use in the assay. In some instances, the kits further comprise detectable labels, optionally linked to a polynucleotide, e.g., a probe, in the kit. In some embodiments, the kit further comprises a process unit to obtain a weighted sum score as described herein.

[0079] Optionally, one or more detectably-labeled polypeptides capable of hybridizing to the amplified portion are also included in the kit. In some embodiments, the kits comprise sufficient primers to amplify the target DNA sequences described herein, and optionally include detectably-labeled polynucleotides capable of hybridizing to each amplified DNA region or

selected when β value from normal tissues is less than 0.15; $\Delta\beta$ value (the value of Tumor subtracts that of normal tissues) is higher than 0.3. The $\Delta\beta$ value of *GCM2*, *ITPRIPL1*, *C1orf114* and *ZNF177*, ~~RKL1001~~RKL001 and *C8orf47* is much higher than 0.3.

Breast Cancer	
	β value (T)
<i>GCM2</i>	0.70
<i>ITPRIPL1</i>	0.76
<i>C1orf114</i>	0.77
<i>ZNF177</i>	0.70
RKL001	0.52
<i>C8orf47</i>	0.49

β value (T), methylation level of tumor tissues;
 β value (T)>0.3 that labeled blue will be calculated as biomarkers for other cancers.

[0091] Figure 1 shows the difference of methylation status (β value) of target nucleic acid and genes between tumor tissues and adjacent normal tissues (n=97). Darker color indicates tissues with higher methylation status according to Illumina Methylation 450K array-based data.

Example 2 Detection of Difference of Methylation Status Between the Target Nucleic Acid and Genes of in Breast Cancer Tissue and Normal Tissue

[0092] Genomic DNA from matched pairs of primary tumors and adjacent breast tissues from the same patient from breast cancer patients were extracted and followed by bisulfite conversion. The methylation specific real-time PCR (qMSP) was used in detection of DNA methylation analyses in breast tumor tissues and adjacent normal tissues. The *ACTB* gene was used as a reference gene. Targets as considered hypermethylated when the methylation level of target genes relative to that of the *ACTB* gene was at least 2-fold higher in the breast tumor compared with the paired normal breast tissue sample. All the hypermethylation rate in breast

tumors compared to adjacent normal tissues of *GCM2*, *ITPRIPL1*, *C1orf114* and *ZNF177*, ~~RKL1001~~RKL001 and *C8orf47* is higher than 64%.

Breast Cancer	
Gene Name	Hypermethylation status No./Total patients(%)
<i>GCM2</i>	63/91 (69%)
<i>ITPRIPL1</i>	73/91 (80%)
<i>C1orf114</i>	71/91 (78%)
<i>ZNF177</i>	71/91 (78%)
RKL001	45/69 (65%)
<i>C8orf47</i>	54/83 (65%)

Example 3 Early Detection of Methylation Status of Epigenetic Biomarkers of Target Genes in Plasma Samples of Healthy Subjects and Breast Cancer Patients

[0093] The circulating cell-free DNA was extracted from plasma. Briefly, 3.5 mL of plasma was isolated immediately from 10 mL of peripheral blood. After circulating cell-free DNA (cfDNA) was extracted from plasma that obtained from 24 breast cancer patients and 25 healthy subjects, cfDNA was performed by bisulfite conversion. Probe-based methylation specific real-time PCR (qMSP) was used for cfDNA methylation analyses. The early prediction by *GCM2*, *ITPRIPL1*, *C1orf114* and *ZNF177* revealed 91.7% sensitivity and 96.0% specificity (AUC: 0.954).

	Sensitivity	Specificity	AUC
GCM2	54.2%	100%	0.771
ITPRIPL1	79.2%	96.2%	0.877
C1orf114	75.0%	76.9%	0.760
ZNF177	62.5%	82.6%	0.736
GCM2+ ITPRIPL1	87.5%	96%	0.927
GCM2+ ITPRIPL1+ C1orf114	95.8%	72.0%	0.944
GCM2+ ITPRIPL1+ C1orf114 (after weighted sum score analysis)	87.5%	96.7%	0.981

curves were calculated using the Kaplan–Meier method, and comparisons were performed using the log-rank and Wilcoxon test by SPSS software (SPSS Inc., Chicago, IL, USA). The Kaplan–Meier curve shown in Figure 5 indicates the methylation status of *ITPRIPL1* gene had poor prognosis in breast cancer patients. The overall survival curves *GCM2*, *ITPRIPL1*, *C1orf114*, *ZNF177*, *C8orf47* and/or ~~RKL1001~~RKL001 and/or any of their combinations were also calculated using the Kaplan–Meier method.

Claims

What is claimed is:

1. A method for detecting the methylation status in a human subject who is in a need of detection of a breast cancer, comprising (a) providing a biological sample, and (b) assaying methylation status of epigenetic biomarkers in target DNA sequences *GCM2* and *ITPRIPL1* or fragments thereof, wherein the presence of hypermethylation in the DNA sequences of the human subject relative to a methylation state of a control is indicative of a breast cancer;

wherein the control is a sample from a normal or healthy subject.

2. A method for detecting a predisposition to, or the incidence of, a breast cancer or predicting treatment response, prognosis or recurrence of a breast cancer in a human subject, comprising (a) providing a biological sample, and (b) assaying methylation status of epigenetic biomarkers in target DNA sequences *GCM2* and *ITPRIPL1* or fragments thereof, wherein the presence of hypermethylation in the DNA sequences of the human subject relative to a methylation state of a control is indicative of a predisposition to, or the incidence, poor treatment response, poor prognosis or recurrence of, the breast cancer;

wherein the control is a sample from a normal or healthy subject.

3. The method of Claim 1 or 2, wherein a *GCM2* methylation specific probe and a *ITPRIPL1* methylation specific probe are used to assay the methylation level of the target *GCM2* and *ITPRIPL1* DNA sequences or fragments thereof and a control DNA sequence in the sample;

wherein the control is a sample from a normal or healthy subject.

4. A method, comprising (a) providing a biological sample comprising *GCM2* and *ITPRIPL1* target DNA sequences or fragments thereof, and (b) assaying the methylation level of the target *GCM2* and *ITPRIPL1* DNA sequences or fragments thereof and a control DNA

sequence in the sample using a *GCM2* methylation specific probe and a *ITPRIPL1* methylation specific probe, (c) measuring the relative methylation status of the target *GCM2* and *ITPRIPL1* DNA sequences or fragments thereof compared to a control DNA sequence to detect the presence of hypermethylated *GCM2* and *ITPRIPL1*, (d) identifying the subject as in need of breast cancer treatment when the target DNA sequences *GCM2* and *ITPRIPL1* DNA sequences or fragments thereof of the human subject are hypermethylated relative to methylation of a control DNA sequence in said subject;

wherein the control is a sample from a normal or healthy subject.

5. A method, comprising (a) providing a biological sample comprising *GCM2* and *ITPRIPL1* target DNA sequences or fragments thereof, and (b) assaying the methylation level of the target *GCM2* and *ITPRIPL1* DNA sequences or fragments thereof and a control DNA sequence in the sample using a *GCM2* methylation specific probe and a *ITPRIPL1* methylation specific probe, (c) measuring the relative methylation status of the target *GCM2* and *ITPRIPL1* DNA sequences or fragments thereof compared to a control DNA sequence to detect the presence of hypermethylated *GCM2* and *ITPRIPL1*, (d) identifying the subject as having a predisposition to, or the incidence, poor treatment response, or having poor prognosis or recurrence of, the breast cancer when the target DNA sequences *GCM2* and *ITPRIPL1* of the human subject are hypermethylated relative to a methylation state of a control DNA sequence in said subject;

wherein the control is a sample from a normal or healthy subject.

6. The method of any of Claims 1 to 5, wherein the biological sample is a tissue, cell, blood, urine, serum or plasma.

7. The method of any of Claims 1 to 5, wherein the methylation in the DNA sequences *GCM2* and *ITPRIPL1* or fragments thereof is about 64% higher than that of the control.

8. The method of any of Claims 1 to 5, wherein the methylation in the DNA sequences *GCM2* and *ITPRIPL1* or fragments thereof is about 69% and about 80%, respectively, higher than that of the control.

9. The method of any of Claims 1 to 5, wherein the hypermethylation is indicated when the methylation of the target DNA sequences in a human subject is at least 0.5 times higher than that in a control.

10. The method of any of Claims 1 to 5, wherein the hypermethylation is indicated when the methylation of the target DNA sequences in a human subject is at least 2 times higher than that in a control.

11. The method of any of Claims 3 to 5, wherein the *GCM2* methylation specific probe has a sequence with homology of at least 85% to SEQ ID NO:15 and a *ITPRIPL1* methylation specific probe has a sequence with homology of at least 85% to SEQ ID NO:16.

12. The method of any of Claims 3 to 5, wherein the *GCM2* methylation specific probe has a sequence of SEQ ID NO:15 and a *ITPRIPL1* methylation specific probe has a sequence of SEQ ID NO:16.

13. The method of Claim 12, wherein the measure of the relative methylation status of the target *GCM2* and *ITPRIPL1* DNA sequences or fragments thereof compared to a control DNA sequence has higher than about 54% of sensitivity and about 100% specificity for *GCM2* and higher than about 79% of sensitivity and higher than about 96% of specificity for *ITPRIPL1*.

14. The method of any of Claims 1 to 5, which further comprises a step of assaying the methylation level of the following one or more target DNA sequences or any of their

combinations: *C1orf114* or a fragment thereof, *ZNF177* or a fragment thereof and *C8orf47* or a fragment thereof.

15. The method of any of Claim 14, wherein the methylation level of RKL001 is further assayed.

5 16. The method of any of Claim 14, wherein a *C1orf114* methylation specific probe, a *ZNF177* methylation specific probe, or a *C8orf47* methylation specific probe or any of their combinations are further used to assay the methylation level of the following one or more target DNA sequences or any of their combinations: *C1orf114* or a fragment thereof, *ZNF177* or a fragment thereof and *C8orf47* or a fragment thereof.

10 17. The method of any of Claim 16, wherein the methylation specific probes of target *C1orf114*, *ZNF177* and *C8orf47* or fragments thereof have a sequence with homology of at least 85% to SEQ ID NOs:17, 18 and 14, respectively.

15 18. The method of any of Claim 16, wherein the methylation specific probes of target *C1orf114* or a fragment thereof, *ZNF177* or a fragment thereof and *C8orf47* or a fragment thereof have a sequence of SEQ ID NOs:17, 18 and 14, respectively.

20 19. The method of any of Claim 16 or 17, wherein the measure of the relative methylation status of the target *C1orf114*, *ZNF177*, and/or *C8orf47* DNA sequences or fragments thereof compared to a control DNA sequence has higher than about 75% of sensitivity and about 76% specificity for *C1orf114*, and higher than about 62% of sensitivity and about 82% specificity for *ZNF177*, respectively.

20. The method of any of Claim 16, wherein the measure of the relative methylation status of the combination of target *GCM2* and *ITPRIPL1* or fragments thereof; target *GCM2*, *ITPRIPL1* and *C1orf114* or fragments thereof; and *GCM2*, *ITPRIPL1*, *C1orf114* and *ZNF177* or

fragments thereof has higher than about 87% of sensitivity and about 96% specificity, higher than about 95% of sensitivity and about 72% specificity, higher than about 87% of sensitivity and about 96% specificity, respectively, compared to a control DNA sequence.

21. The method of Claim 13, 19 or 20, wherein the measure ~~further~~further comprises a step of measuring the specificity and ~~sensitivity~~sensitivity by a weighted sum score analysis.

22. The method of Claim 21, wherein the measure of the relative methylation status of the combination of target *GCM2*, *ITPRIPL1* and *C1orf114* or fragments thereof or target *GCM2*, *ITPRIPL1*, *C1orf114* and *ZNF177* or fragments thereof has higher than about 87% of sensitivity and about 96% specificity and higher than about 91% of sensitivity and about 96% specificity, respectively.

23. The method of any of Claim 15, wherein a RKL001 methylation specific probe is further used to assay the methylation level of RKL001.

24. The method of any of Claim 23, wherein a RKL001 methylation specific probe has a sequence with homology of at least 85% to SEQ ID NO:13.

25. The method of any of Claim 23, wherein a RKL001 methylation specific probe has a sequence with homology of SEQ ID NO:13.

26. The method of any of Claims 1 to 5, wherein the methylation status is detected by polymerase chain reaction.

27. ~~A~~The method of any of claims 1 to 5, wherein assaying methylation status comprises using a probe having a sequence selected from the group consisting of: SEQ ID Nos: 14 to 18.

28. ~~A~~The method of any of claims 1 to 5, wherein assaying methylation status comprises using a primer having a sequence selected from the group consisting of: SEQ ID Nos: 1 to 13.

29. ~~A~~ The method of any of claims 1 to 5, wherein assaying methylation status comprises using a kit for detecting the methylation status in a human subject who is in a need of detection of a breast cancer or detecting a predisposition to, or the incidence of, a breast cancer or predicting treatment response, prognosis or recurrence of a breast cancer in a human subject,
5 ~~which comprises~~ comprising primers having sequences of SEQ ID NOs: 5 to 8 or probes having sequences of SEQ ID Nos: 15 and 16 ~~for assaying to detect or measure the~~ methylation status of epigenetic biomarkers in the target DNA sequences *GCM2* and *ITPRIPL1* or fragments thereof.

30. ~~The kit~~ method of Claim 29, which is used for detecting and/or characterizing the methylation profile of epigenetic biomarkers in target DNA sequences *GCM2* and *ITPRIPL1* or
10 fragments thereof in combination with one or more target DNA sequences *C1orf114* or a fragment thereof, *ZNF177* or a fragment thereof, *C8orf47* or a fragment thereof, higher than about 87% of sensitivity and about 96% specificity and RKL001 or any of combinations thereof,
further comprises one of more primers having sequences of SEQ ID NOs: 9 to 12 and 1 to 4 and/or one or more probes having sequences of SEQ ID Nos: 17, 18, 12 and 13 ~~for assaying~~
15 ~~methylation status of epigenetic biomarkers in target DNA sequences *GCM2* and *ITPRIPL1* or fragments thereof in combination with one or more target DNA sequences *C1orf114* or a fragment thereof, *ZNF177* or a fragment thereof, *C8orf47* or a fragment thereof, and RKL0014 or any of combinations thereof~~ to detect or measure the methylation status of the target DNA sequences.

20 31. ~~The kit~~ method of Claim 29 or 30, further comprising sodium bisulfite and adapters for whole target genes amplification, and polynucleotides (e.g., detectably-labeled polynucleotides) to quantify the presence of the converted methylated and or the converted

unmethylated sequence of at least one cytosine from a DNA region of epigenetic biomarkers in target DNA sequences as shown in any of Claims 1 to 23.

32. The ~~kit~~method of Claim 31, further comprising methylation sensing restriction enzymes for whole target sequence or genes amplification.

5 33. The method of Claim 4, wherein the method detects the methylation status in a human subject who has a need of detection of a breast cancer.