METHODS OF DNA METHYLATION DETECTION

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References Cited
U.S. PATENT DOCUMENTS
5,605,662 A * 2/1997 Heller et al. .................. 422/68.1
5,844,033 A * 12/1998 Lizardi ...................... 435/91.2

* cited by examiner

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ABSTRACT

The present invention provides for methods of DNA methylation detection. The present invention provides for methods of generating and detecting specific electronic signals that report the methylation status of targeted DNA molecules in biological samples.

Two methods are described, direct and indirect detection of methylated DNA molecules in a nano transistor based device. In the direct detection, methylated target DNA molecules are captured on the sensing surface resulting in changes in the electrical properties of a nano transistor. These changes generate detectable electronic signals. In the indirect detection, antibody-DNA conjugates are used to identify methylated DNA molecules. RNA signal molecules are generated through an in vitro transcription process. These RNA molecules are captured on the sensing surface thereby generating detectable electronic signals.

8 Claims, 5 Drawing Sheets
Figure 4
The present invention relates to methods for the detection of methylated DNA molecules. More specifically, the present invention relates to methods for the detection of DNA methylation in a nano electronic device.

BACKGROUND

Early detection is one of the most challenging issues in cancer diagnosis. Currently, diagnosis depends on a variety factors including illness symptoms, the detection of biomarkers in blood and/or body fluids, the microscopic analysis of biopsy samples, as well as imaging technology. Limited sensitivity in these methods and technologies can result in the delay of treatment. The National Cancer Institute has indicated there is an urgent need of new technologies for cancer early diagnosis.

Cancer is known as a disease driven by genetic abnormalities. Recently, however, research has suggested that epigenetic alterations of gene function may also be central to the pathogenesis of this disease. Epigenetic alterations include heritable changes in the gene expression that are not caused by direct alteration of the gene’s nucleotide sequence. Abnormal DNA methylation at gene transcription sites can result in epigenetic silencing of genes that protect against tumor formation or that repair DNA. Research into gene silencing has focused on the regions of high CpG content, known as CpG islands. CpG islands are often located near gene transcription sites. In normal cells, most promoter-associated CpG islands are unmethylated. But in cancer cells, promoter region CpG islands are most likely to be methylated. Thus, the detection of promoter methylation in tumor suppress genes is one of the most important assays in early cancer diagnosis. Conventional methods for DNA methylation detection use methylation specific and/or methylation sensitive restriction enzymes for restriction landmark analysis. Recently, several advanced methods have been developed for DNA methylation detection, including bisulfite sequencing, methylation-specific PCR, MethylLight, CpG island microarray. However, major drawback with these methods include complicated procedures, relatively low sensitivity, time consuming and false positive. Therefore, the development of new technologies to improve DNA methylation detection will have a significant impact in early cancer detection.

The p16\(^{NK}\) promoter was chosen as a target of the detection model, since its protein inhibits cell cycle progression. Methylation of CpG islands in p16 promoter and the first exon regions inactivates transcription of the p16 gene. This is significantly associated with lung cancer. Recently, field effect transistor (FET) based electronic charge detectors were introduced into DNA detection by several research groups. However, using charge detector to report DNA methylation events is a challenge, since both methylated and unmethylated DNA carry the same amount of electrical charge. In light of the foregoing there is a need for a DNA methylation detection system that has increased sensitivity yet is relatively easy to operate and without the need for bisulfite treatment or PCR amplification.

Accordingly, the present invention is directed to methods of detecting DNA methylation in biological samples that is based on nano-transistor based electronic detection, which is label free, no requirement of bisulfite treatment and PCR amplification.

Additional features and advantages of the invention will be set forth in the description which follows, and in part will be apparent from the description, or may be learned by practice of the invention. The objectives and other advantages of the invention will be realized and attained by method particularly pointed out in the written description and the claims hereof as well as the appended drawings.

To achieve these and other advantages and in accordance with the purpose of the invention, as embodied and broadly described, one object of the present invention is to provide a direct method of DNA methylation detection. In this direct method of DNA methylation detection is accomplished by exposing the DNA molecules from a biological sample to D-desthiobiotin labeled sequence specific probe, allowing hybridization thereby creating a target DNA; capturing the hybridized target DNA molecules with the nitro-streptavidin magnetic beads; removing unhybridized DNA and D-desthiobiotin molecules by washing; eluting the captured DNA molecules from the nitro-streptavidin magnetic beads using D-biotin; capturing the target DNA molecules with a monoclonal anti-methylcytosine antibody immobilized on a sensing surface of an electronic device; and sensing in the presence of methylated target DNA molecules by detecting the changed electrical properties of the sensing surface thereby generating a signal.

Another object of the present invention related to an indirect method DNA methylation detection. In this method, DNA methylation detection is accomplished by hybridizing DNA molecules from a biological sample with biotin-labeled sequence specific probes; capturing the DNA-biotin-labeled sequence probes with streptavidin magnetic beads; exposing the captured DNA-biotin-labeled sequence probes to a conjugate of monoclonal anti-5-methylcytosine antibody thereby creating a DNA template; generating RNA molecules from the DNA template by an in vitro transcription process; capturing the RNA molecules by peptide nucleic acids immobilized on a sensing surface of an electronic device through a hybridization process; generating an electronic signal changing the electronic properties of the sensing surface in the presence of captured RNA molecules.

In both methods the electronic signal is detected in an electronic device by a nano transistor, or other basic electronic device.

It is to be understood that both the following forgoing general description and the following detailed description are exemplary and explanatory and are intended to provide further explanation of the invention as claimed.

The accompanying figures are included to provide a further understanding the invention and are incorporated and constitute a part of this specification, illustrate several embodiments of the present invention and together with the description serve to explain the principals of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic diagram depicting the method of direct DNA methylation detection.

FIG. 2 is a schematic diagram depicting the method of indirect DNA methylation detection.
FIG. 3 is photomicroscopic image of nano transistor. FIG. 4 is a spectra of electronic detection of DNA methylation by using the direct detection method. FIG. 5 is a spectra of electronic detection of DNA methylation by using the indirect detection method.

DETAILED DESCRIPTION

Reference will now be made in detail to the present preferred embodiments of the invention, examples of which are illustrated in the accompanying figures.

Two methods described in this invention teach the generation of methylation specific signals that can be detected on a nano transistor based device. The detection sensitivity has been achieved at atom mole level.

An exemplary embodiment of the direct DNA methylation detection method is shown in FIG. 1. FIG. 1 is a diagram of the direct detection of DNA methylation method. Methylated 101 and non-methylated (not shown) target DNA molecules 102, are hybridized with a D-desthiobiotinlated specific probe 103, then these hybridized target DNA-D-desthiobiotinlated molecules are captured using nitro-streptavidin 104 magnetic beads 105. After washing away any unbound DNA and D-desthiobiotinlated specific probe molecules, D-biotin 106 is used to elute capture DNA molecules 102 from nitro-streptavidin 104. Those of ordinary skill in the art will readily understand that D-desthiobiotin and D-biotin can be other hapten or tags. Similarly, the nitro-streptavidin and streptavidin can be any biomolecules that recognize hapten or tags. Similarly, the nitro-streptavidin and streptavidin can be any biomolecules that recognize hapten or tags.

DNA molecules 102 are detected by changing electrical properties of the sensing surface 108. Those of ordinary skill in the art will readily understand that the conjugate of DNA molecules 102 with a monoclonal anti-methylcytosine antibody 107 immobilized on the sensing surface 108 of a nano-transistor 109. Non-methylated target DNA molecules (not shown) are washed away. Methylated 101 target DNA molecules 102 are detected as shown in the spectra.

Turning now to FIG. 2, which is a diagram of the indirect detection of DNA methylation method. In this embodiment, methylated 101 and non-methylated (not shown) target DNA molecules 102, are hybridized with a biotinylated specific probe 201, are captured by streptavidin 202 magnetic beads 203. Monoclonal anti-methylcytosine antibody 207 conjugated with a DNA template 203 binds to methylated cytosine 201. RNA signal molecules 204 are generated through in vitro transcription. RNA molecules 204 are captured by their complimentary PNA molecules 205 immobilized on the sensing surface 108 of nano-transistor 109, and detected by changing electrical properties of the sensing surface. It will be readily apparent to one of ordinary skill in the art that the conjugate can be antibody-beads with oligonucleotides which in turn are captured by PNA on the sensing surface. Similarly, the peptide nucleic acid can be other biomolecules capable of recognizing and capturing RNA or any oligonucleotide.

Looking now at FIG. 3, which is a photomicroscopic images of a nano transistor. Image 301 is an image of the nano transistor captured on Olympus B60 microscope under dark-field-reflected light using a 20x material objective. Image 302 is the image of low doped semiconductor nanowire and this image was taken at the Cornell Nanoscale Facility using a scanning electron micrograph at a Magnification of 252KX and 1.2 kV EHT.

Turning now to FIG. 4 which is a spectra of electronic detection of DNA methylation by using direct detection method. To generate these spectra, a 1 µl sample containing one (1) picomole of methylated 16 promoter DNA molecules was used as a target in the experiment. DNA sample was loaded onto the anti-methylcytosine antibody modified sensing surface, and incubate for 15 minutes. Unbound DNA molecules were washed away by using TE buffer. Bound DNA molecules were detected as shown in the spectra.

Turning now to FIG. 5 which is a spectra of electronic detection of DNA methylation by using the indirect detection method. To generate these spectra, a serial dilution of oligonucleotide A20 was made in TE buffer. Experiments were performed on the same nano transistor with PNA modified sensing surface. A 0.5 µl of sample containing 1x10^-12 to 1x10^-20 mole oligonucleotides was loaded onto the sensing surface in the tests. Hybridization was performed at room temperature for 15 minutes. Unbound oligonucleotides were washed away by using TE buffer. Hybrids were detected as shown in the spectra.

P16 promoter region target sequence

5' ACCAGAGGATGCGGGCCGCGCCGCGCCGCGCCGCGCCGCGGGGG

GGGAGAGGCGCGGGCGCGCGCGCGCGCGCGCGCGGAGGAGG

Top strand oligonucleotide

3' TGC GGA GAG GGG GAG AGC AGG CAG C 3'

Bottom strand oligonucleotide

5' TGC TCC CCC CGG CGG GCT CCA TGC TCC CCC CCC GCT GCC TCT CCC 3'

Overlap 15 bases, Tm=56.2

Probes

5' Amine-TGC CCC CGG CGG GCT CCA TGCC 3' (SEQ ID NO: 4)

D-Desthiobiotin labeling procedure was performed according to the product information sheet from Molecular Probes (Invitrogen).

5' Biotin-TGC TCC CCC CGG CGG GCT CCA TGCC 3' (SEQ ID NO: 5)

DNA Template for Antibody Conjugation

5' Thio-GAT AAT ACG ACT CAC TAT AGG GAA AAA AAA AAA AAA AAA AAA 3' (SEQ ID NO: 6)

T7 promoter sequence is under lined.
Nitro-streptavidin was prepared according to the published procedure as detailed by Laird P W and by Riodan, J. F., Sokolovsky, M. and Valee, B. L, hereby incorporated by reference in its entirety.

EXAMPLE 1

1. Target DNA Preparation and Methylation

To generate a full length of target DNA, annealing and extension of top and bottom strands oligonucleotides were performed. DNA polymerase Klenow fragment exo− was used for the strand extension. Methylation of target DNA was performed by using DNA methylation kit from New England Biolab. Purification of methylated DNA was performed through phenol extraction and ethanol precipitation procedures.

2. Antibody-DNA Conjugation

Antibodies were modified with bifunctional linker LC-SMCC (Pierce) and purified by using P-6 spin column. Sulfonated DNA template was reduced by using DTT and purified by using P-6 spin column. Modified antibody and reduced DNA template were then mixed together at 1:4 ratio. Conjugation reaction was carried out overnight. Conjugates were purified by using P-100 gravity flow column. The biological function of the conjugates was tested by antibody antigen reaction and in vitro transcription.

3. Synthesis and Characterization of PNA

PNAs were synthesized using Boc/Z or Fmoc/Bhoc protected monomers is described using manual and automated solid phase synthesis protocol as describe by Leif Christensen, Richard Fitzpatrick and Rolf H. Berg. Chemical synthesis of Boc/Z and Fmoc/Bhoc PNA monomers using solution phase chemistry is reported. PNA monomers, reagents and wash solution for PNA synthesis are commercially available through ASM Research Chemicals e.K. Germany. PNA products were released from the solid support and analyzed for purity by reverse phase HPLC and MALDI-TOF mass spectroscopy.

4. Sensing Surface Modification

The sensing surface was cleaned by plasma treatment and sonication. A mixture of MUA/MPA was used to functionize the surface with COOH groups. A mixture of EDAC/NHS was used as activated linker for covalently linking antibody or peptide nucleic acid on the sensing surface. Modified surface was blocked by PBS with 1 mM PEG, 1 mM glycine and 0.01% HSA.

5. Methylation DNA Detection

The principle of direct and indirect detection of methylated DNA was presented in FIGS. 1 and 2. The results of electronic detection were presented in FIGS. 4 and 5. Non-methylated DNA with identical sequence was used as negative control in both detection models.

SEQUENCE LISTING

accagagggt ggggcggacc gcgtgcgctc ggcggctgca gagaggggga gagcaggcag
The invention claimed is:

1. A method of detecting DNA methylation in a biological sample comprising:
   a) targeting DNA molecules hybridized with hapten-labeled sequence specific probes to produce DNA-hapten-labeled sequence probes;
   b) capturing the DNA-hapten-labeled sequence probes with a hapten recognizing biomolecule on solid material;
   c) exposing the captured DNA-hapten-labeled sequence probes to a conjugate of antibody and a DNA template, or a conjugate of antibody and a particle with oligonucleotides;
   d) generating RNA molecules from the DNA template by an in vitro transcription process, or releasing oligonucleotides from the particle;
   e) capturing RNA molecules or oligonucleotides by nucleic acid recognizing biomolecules immobilized on a sensing surface of an electronic device through a hybridization process;
   f) changing the electronic properties of the sensing surface by the presence of RNA molecules or oligonucleotides, thereby generating electronic signals; and
   g) detecting the generated electronic signals by an electronic signal detector, thereby detecting DNA methylation in the biological sample.
2. The method according to claim 1, wherein the hapten is D-biotin.
3. The method according to claim 1, wherein the hapten-recognizing biomolecule is nitro-streptavidin or streptavidin.
4. The method according to claim 1, wherein the solid material is magnetic beads.
5. The method according to claim 1, wherein the antibody is monoclonal anti-5-methylcytosine.
6. The method according to claim 1, wherein the particle is a nano-gold particle.
7. The method according to claim 1, wherein the nucleic acid recognizing biomolecule is peptide nucleic acid.
8. The method according to claim 1 wherein the electronic signal detector is a nano transistor, or other basic electronic device.

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