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(54) DETECTION REAGENT, DETECTION KIT AND DETECTION METHOD FOR ITGA4 GENE METHYLATION

NACHWEISREAGENZ, NACHWEISKIT UND NACHWEISVERFAHREN FÜR DIE ITGA4-GENMETHYLIERUNG

RÉACTIF DE DÉTECTION, KIT DE DÉTECTION ET PROCÉDÉ DE DÉTECTION DE LA MÉTHYLATION DU GÈNE ITGA4

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(73) Proprietor: **Creative Biosciences (Guangzhou) Co., Ltd.**
Guangzhou, Guangdong 510530 (CN)

(72) Inventors:
- **LIU, Xianglin**
  Guangzhou,
  Guangdong 510530 (CN)
- **ZOU, Hongzhi**
  Guangzhou
  Guangdong 510530 (CN)

(74) Representative: **Schulz Junghans**
Patentanwälte PartGmbB
Großbeerenstraße 71
10963 Berlin (DE)

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The complete document including Reference Table(s) and the Sequence Listing(s) can be downloaded from the EPO website

Description

Technical Field

[0001] The present invention relates to a detection reagent, a detection kit and a detection method for ITGA4 gene methylation.

Background

[0002] The fecal immunochemical test for DNA (FIT-DNA) was included in the screening methods for colorectal cancer in 2016 by the United States Preventive Services Task Force. One of the principles of this test is to detect methylation levels of specific genes in exfoliated cells in feces. The selection of DNA methylation markers with very high sensitivity and specificity to intestinal cancer is the basis of this test. The detection of ITGA4 gene methylation has high detection sensitivity and specificity to intestinal cancer in tissue and feces specimens.

[0003] The detection sensitivity of methylated ITGA4 to adenoid tumor in feces specimens is 69% (9/13) and the specificity is 79% (22/28). Other research shows that in detecting methylated ITGA4 in feces specimens, when the specificity is 100% (31/31), the detection rates for colorectal cancer and colorectal adenoid tumor are 36.7% (11/30) and 16% (4/25), respectively. These results suggest that there is still great room for improvement of ITGA4 detection sensitivity and specificity.

[0004] The key factor influencing the detection sensitivity and specificity of markers is the sequences of primers. Ausch et al. Clinical Chemistry 2009 disclose detection of methylated IGTA4 to predict whether colonic neoplasms are present in a patient.

Summary

[0005] To this end, the technical problem to be solved by the present invention is to provide a detection kit and a detection method for ITGA4 gene methylation. Both quantitative and qualitative kits provided by the present invention can achieve specificity and sensitivity detection for ITGA4 gene methylation.

[0006] The present invention provides:

a qualitative detection kit for ITGA4 gene methylation, which takes feces, tissue or cells as a sample to be detected and comprises an ITGA4 gene capturing reagent and an ITGA4 gene methylation qualitative detection reagent.

[0007] The ITGA4 gene capturing reagent comprises a magnetic bead probe complex comprising a sequence of the probe shown as SEQ ID NO: 1.

[0008] The ITGA4 gene methylation qualitative detection reagent comprises a detection primer, and a nucleotide sequence of the detection primer is shown as SEQ ID NO: 2-3.

[0009] The present invention further provides a quantitative detection kit for ITGA4 gene methylation, which takes feces, tissue or cells as a sample to be detected and comprises an ITGA4 gene capturing reagent and an ITGA4 gene methylation quantitative detection reagent.

[0010] The ITGA4 gene capturing reagent comprises a magnetic bead probe complex comprising a sequence of the probe shown as SEQ ID NO: 1.

[0011] In an example of the present invention, the ITGA4 gene methylation quantitative detection reagent comprises a detection primer and a detection probe, wherein a nucleotide sequence of the detection primer is shown as SEQ ID NO: 4-5; and a nucleotide sequence of the detection probe is shown as SEQ ID NO: 6.

[0012] The present invention further provides a detection kit for ITGA4 gene methylation, which takes feces, tissue or cells as a sample to be detected and comprises an ITGA4 gene capturing reagent, an ITGA4 gene methylation qualitative detection reagent and an ITGA4 gene methylation quantitative detection reagent.

[0013] The ITGA4 gene capturing reagent comprises a magnetic bead probe complex; in the magnetic bead probe complex, a nucleotide sequence of the probe is shown as SEQ ID NO: 1;

the ITGA4 gene methylation qualitative detection reagent comprises a detection primer; a nucleotide sequence of the detection primer is shown as SEQ ID NO: 2-3; and

the ITGA4 gene methylation quantitative detection reagent comprises a detection primer and a detection probe; a nucleotide sequence of the detection primer is shown as SEQ ID NO: 4-5, and a nucleotide sequence of the detection probe is shown as SEQ ID NO: 6.

[0014] ITGA4 genes are captured in feces specimens by using a capturing sequence of the nucleotide sequence shown as SEQ ID NO: 1, and the methylation level of ITGA4 is detected in 240 feces specimens (80 cases of intestinal cancer, 77 cases of adenoid tumor larger than or equal to 1 cm, and 83 cases of normal). Results show that when the

specificity is 95.2%, the sensitivities of methylated ITGA4 to intestinal cancer and adenoid tumor are 83.8% and 41.6%, respectively.

[0015] The methylation level of ITGA4 is detected in 105 pairs of paired intestinal cancer tissue, 109 cases of adenoid tumor tissue larger than or equal to 1 cm, and 41 cases of normal intestinal epithelial tissue by using the primer and the probe of the qMSP test. When the specificity is 97.6%, the detection rates for colorectal cancer and adenoid tumor are 96.2% and 71.6%, respectively.

[0016] The methylation level of ITGA4 genes is detected in six colorectal cancer cell lines including Widr, SW480, HCT15, HT29, Caco2 and DLD1 by using the primer of the MSP test. The result shows that ITGA4 is methylated in all the six colorectal cancer cells.

[0017] Preferably, the sample to be detected by the kit provided by the present invention is feces and/or tissue;

[0018] in some specific examples, the sample to be detected is feces.

[0019] The present invention further provides a qualitative detection method for ITGA4 gene methylation, comprising: taking a sample to be detected as a template, capturing ITGA4 genes by a capturing reagent, and then modifying with bisulfite, amplifying with a qualitative detection reagent, and determining whether the ITGA4 genes are methylated or not according to an amplification product;

wherein the sample to be detected is feces, tissue or cells;
the capturing reagent comprises a magnetic bead probe complex; in the magnetic bead probe complex, a nucleotide sequence of the probe is shown as SEQ ID NO: 1;
the qualitative detection reagent comprises a detection primer; a nucleotide sequence of the detection primer is shown as SEQ ID NO: 2-3.

[0020] Specifically, the qualitative detection method comprises the steps of taking the sample to be detected as the template, capturing the ITGA4 genes by an ITGA4 gene capturing sequence of the nucleotide sequence shown as SEQ ID NO: 1, then treating and modifying by bisulfite, then amplifying carried out with an ITGA4 gene methylation qualitative detection primer of the nucleotide sequence shown as SEQ ID NO: 2-3, and determining whether the ITGA4 genes are methylated or not according to the amplification product; and the sample to be detected is feces, tissue or cells.

[0021] Preferably, the sample to be detected is feces, colorectal cancer, colorectal adenoid tumor, normal intestinal epithelial tissue or cell lines. Preferably, the sample to be detected is feces.

[0022] In a magnetic bead capturing method, magnetic beads are taken as a solid phase adsorption carrier, and a specially designed reagent system and an extraction procedure ("Detection and Extraction of Free Methylated DNA in Urine by Applying Magnetic Bead Method", Journal of China Medical University, 2015, (10)) are used.

[0023] In the present invention, the sequence is shown as SEQ ID NO. 1. The ITGA4 genes in feces may be extracted and enriched by means of magnetic bead capturing.

[0024] The feces may optionally be treated by: uniformly mixing the feces in a buffer solution, centrifuging, taking a supernatant into another test tube, adding magnetic beads with specific complementary oligonucleotide capturing sequences to the supernatant, after incubating and hybridizing, adsorbing the magnetic beads on one side of a tube wall by using a magnet, and after repeatedly washing, eluting target gene DNA by using the buffer solution. The target genes may be captured by the method, and the enrichment process lasts about 2 hours.

[0025] The captured DNA is treated and modified with bisulfite for subsequent fluorescence quantitative PCR detection.

[0026] Amplification system: nuclease-free water 10.5 μL, 2× enzyme reaction solution 12.5 μL, forward primer and reverse primer (5 μM concentration) 0.5 μL each, and DNA to be detected 1 μL, totaling 25 μL.

[0027] Amplification procedure: 95 °C 5 min, (95 °C 30 s, 64 °C 30 s, and 72 °C 30 s) × 34 Cycles, 72 °C 5 min, and 37 °C 30 s.

[0028] According to the amplification result, if an amplification band occurs, the genes are methylated.

[0029] The present invention further provides a quantitative detection method for ITGA4 gene methylation, comprising:

taking a sample to be detected as a template, capturing ITGA4 genes by a capturing reagent, then modifying with bisulfite, performing quantitative detection with a quantitative detection reagent, and determining the methylation level of the ITGA4 genes according to a quantitative result;
wherein the sample to be detected is feces, tissue or cells;
the capturing reagent comprises a magnetic bead probe complex; in the magnetic bead probe complex, a nucleotide sequence of the probe is shown as SEQ ID NO: 1;
the quantitative detection reagent comprises a detection primer and a detection probe; a nucleotide sequence of the detection primer is shown as SEQ ID NO: 4-5, and a nucleotide sequence of the detection probe is shown as SEQ ID NO: 6.

[0030] Specifically, the quantitative detection method comprises the steps of taking the sample to be detected as the

template, capturing the ITGA4 genes by an ITGA4 gene capturing sequence of the nucleotide sequence shown as SEQ ID NO: 1, then treating and modifying by bisulfite, then quantitative detecting is carried out with an ITGA4 gene methylation quantitative detection primer of the nucleotide sequence shown as SEQ ID NO: 4-5 and an ITGA4 gene methylation quantitative detection probe of the nucleotide sequence shown as SEQ ID NO: 6, and the methylation level of the ITGA4 genes is determined according to the quantitative result; and the sample to be detected is feces, tissue or cells.

[0031] Preferably, the sample to be detected is feces, colorectal cancer, colorectal adenoid tumor, normal intestinal epithelial tissue or cell lines. Preferably, the sample to be detected is feces.

[0032] In a magnetic bead capturing method, magnetic beads are taken as a solid phase adsorption carrier, and a specially designed reagent system and an extraction procedure ("Detection and Extraction of Free Methylated DNA in Urine by Applying Magnetic Bead Method", Journal of China Medical University 2015, (10)) are used.

[0033] In the present invention, the sequence is shown as SEQ ID NO. 1. The ITGA4 genes in feces may be extracted and enriched by means of magnetic bead capturing.

[0034] The feces may optionally be treated by: uniformly mixing the feces in a buffer solution, centrifuging, taking a supernatant into another test tube, adding magnetic beads with specific complementary oligonucleotide capturing sequences to the supernatant, after incubating and hybridizing, adsorbing the magnetic beads on one side of a tube wall by using a magnet, and after repeatedly washing, eluting target gene DNA by using the buffer solution. The target genes may be captured by the method, and the enrichment process lasts about 2 hours.

[0035] The captured DNA is treated and modified with bisulfite for subsequent fluorescence quantitative PCR detection.

[0036] The amplification reaction system: nuclease-free water 8.2 μL, 5× enzyme reaction buffer solution 5 μL, $MgCl_2$ (25 mM) 5 μL, dNTPs(10 mM) 1 μL, reaction enzyme 0.5 μL, a forward primer (100 μM) 0.125 μL, a backward primer (100 μM) 0.125 μL, a probe (100 μM) 0.05 μL, and DNA to be detected 5 μL, totaling 25 μL.

[0037] The procedure: 95 °C 4 min, (95 °C 20 s, 56 °C 30 s, and 72 °C 30 s) × 45 Cycles, and 37 °C 30 s.

[0038] According to the quantitative result, the relative methylation level of the ITGA4 genes is obtained through calculation by taking an ACTB gene as a reference gene.

[0039] The primer and the probe provided by the present invention are matched, then detection can be carried out on a feces sample, and thus the detection is simple, convenient and rapid. In the present invention, the detection can also be carried out on tissue specimens. Moreover, in the process of detecting the samples, the detection specificity and sensitivity are also improved. The experiment proves that for the feces specimen, when the specificity is 95.2%, the sensitivities of the methylated ITGA4 to intestinal cancer and adenoid tumor are 83.8% and 41.6%, respectively; and for the intestinal cancer tissue, when the specificity is 97.6% (40/41), the detection rates for colorectal cancer and adenoid tumor are 96.2% (101/105) and 71.6% (78/109), respectively. The effects are superior to the detection effects obtained by adopting other primers or probes.

Brief Description of the Drawings

[0040]

Fig. 1 shows a dot plot of methylation levels of ITGA4 genes in cancer, adenoid tumor and a normal group, wherein **** indicates that P is less than 0.0001;

Fig. 2 shows an ROC graph, wherein AUC (cancer vs normal) is equal to 0.953 (95% CI: 0.920-0.985); and AUC (adenoid tumor vs normal) is equal to 0.735 (95% CI: 0.657-0.814);

Fig. 3 shows an amplification curve of a gradiently diluted S1-S6 and a standard curve constructed therefrom, wherein NTC represents a non-template control, WT represents wild-type DNA as a control, and each sample is provided with three complex holes;

Fig. 4 shows the standard curve with linearity R^2 being equal to 0.995 and amplification efficiency being equal to 97.96%; and

Fig. 5 shows amplification curves for different primers and probes.

Detailed Description

[0041] The present invention provides a detection reagent, a detection kit and a detection method for ITGA4 gene methylation, which can be achieved by a person skilled in the art by referring to the contents herein and appropriately improving process parameters. It is specifically noted that all similar substitutes and modifications apparent to a person skilled in the art are deemed to be included in the present invention. The methods and applications of the present invention have been described through preferred examples, and it will be apparent to a person skilled in the art that the technology of the present invention may be practiced and applied with modification or appropriate alteration and combination of the methods and applications herein.

[0042] The test materials, reagents and instruments used in the present invention are all common commercial products

and can be purchased in the market.

[0043] The present invention is further illustrated by the following examples:

Example 1 Qualitative Detection

[0044] Six colorectal cancer cell lines including Widr, SW480, HCT15, HT29, Caco2 and DLD1 were selected, and healthy cells CCD-18CO were additionally used as a control. The MSP test was used to detect whether ITGA4 genes were methylated in these cells; and each cell line was repeatedly detected 10 times.

[0045] The method comprises the following steps:

1. Cell pellets were collected and transferred to a new 15 mL centrifuge tube previously containing 3 mL of cell lysate.

   100 μL of capturing magnetic beads were added to the centrifuge tube, wherein the capturing magnetic beads contain capturing sequences (5'-TGCTTCTCCGGGTACGGGCCGCTGGG TGGGGTC-3) of the sequence shown as the capturing sequence SEQ ID NO: 1. The mixture was subjected to incubation in a water bath kettle at 92°C for 10 min and on a room temperature shaker at 100 rpm for 1 h, and then placed on a magnetic stand for 5 min after short centrifugation, with a supernatant removed.
   500 μL of a washing solution was added to the 15 mL centrifuge tube, oscillated and shaken to enable magnetic beads on a wall of the tube to be completely suspended, and transferred into a new 2 mL centrifuge tube after short centrifugation. The mixture was subjected to incubation in a room temperature dry bath incubator at 900 rpm for 1 min and then placed on the magnetic stand for 1 min, with a supernatant removed. The process was repeated 4 times.
   55 μL of an eluent was added, shortly centrifuged, incubated in the dry bath incubator at 92 °C and at 900 rpm for 10 min, shortly centrifuged, placed on the magnetic stand, and transferred into a new EP tube within 3 min.

2. Bisulfite transformation was carried out with EZ DNA methylation Kit (ZYMO RESEARCH).
3. Amplification: primers including:

   Upstream primer sequence: 5'-TCGGAGAAGTAGCGCGAGTATTC-3' (SEQ ID NO: 2)
   Downstream primer sequence: 5'-AAATCGACCCACCGCGAACG-3' (SEQ ID NO: 3)
   System: 25 μL (1× system: nuclease-free water 10.5 μL, GoTaq Hot Start Colorless Master Mix 12.5 μL, Forward primer and Reverse primer (concentration 5 uM) 0.5 μL each, and DNA to be detected 1 μL)
   Procedure: 95 °C 5 min, (95 °C 30 s, 64 °C 30 s, and 72 °C 30 s) × 34 Cycles, 72 °C 5 min, and 37 °C 30 s.

4. Amplification result observation.

[0046] According to the amplification result, agarose gel electrophoresis is carried out on a PCR product, and if bands occur, methylation occurs.

[0047] Results: the methylation of ITGA4 was detected in all the six intestinal cancer cells, with the accuracy of 10-repeated detection up to 100%, while the methylation was not detected in the healthy cells.

Example 2 Quantitative Detection

1. Feces as sample

[0048] 240 feces specimens were selected, including 80 cases of intestinal cancer, 77 cases of adenoid tumor larger than or equal to 1 cm, and 83 cases of normal, based on enteroscopy, and quantitative detection of the methylation level of ITGA4 genes was carried out.

[0049] Test process: ITGA4 genes were captured by using a capturing sequence (SEQ ID NO: 1) in the feces specimens; and the capturing conditions and process were the same as those in Example 1), and quantitative detection of the methylation level of the ITGA4 genes in the specimens was carried out through the qMSP test by using ACTB as a reference gene.

[0050] Quantitative detection primers and probes:

Upstream primer sequence: 5'-ACGCGAGTTTTGCGTAGTC-3' (SEQ ID NO: 4)
Downstream primer sequence: 5'-TCCGAATACGAACCGCTAA-3' (SEQ ID NO: 5)
Detection probe sequence: 5'-ACGGAGTTCGGTTTTGCGTTTTC-3'(SEQ ID NO: 6)
System: 25 μL (1× system: nuclease-free water 8.2 μL, 5× Colorless GoTaq Flexi Buffer 5μL, $MgCl_2$ (25mM) 5

μL, dNTPs (10 mM) 1 μL, GoTaq Hot Start polymerase 0.5 μL, Forward primer (100 uM) 0.125 μL, Reverse primer (100 μM) 0.125 μL, Probe (100 μM) 0.05 μL, and DNA 5 μL)
Procedure: 95 °C 4 min, (95 °C 20s, 56 °C 30 s, and 72 °C 30 s) × 45 Cycles, and 37 °C 30 s.

[0051] In 240 feces specimens (80 cases of intestinal cancer, 77 cases of adenoid tumor larger than or equal to 1 cm, and 83 cases of normal), the methylation level of ITGA4 was detected, and the result showed that when the specificity is 95.2%, the sensitivities of the methylated ITGA4 to intestinal cancer and adenoid tumor were 83.8% and 41.6%, respectively (Fig. 1-2).

2. Tissue as sample

[0052] Colorectal cancer, colorectal adenoid tumor and normal intestinal epithelial tissue specimens excised surgically or endoscopically were selected, and quantitative detection of the methylation level of ITGA4 genes was carried out, with the specimens including: 105 pairs of intestinal cancer and paracancerous paired tissue, 109 cases of adenoid tumor tissue larger than or equal to 1 cm, and 41 cases of normal intestinal epithelial tissue.

[0053] The DNA of each tissue was extracted and treated and modified with bisulfite, and then quantitative detection of the methylation level of ITGA4 genes in the specimens was carried out through the qMSP test.

[0054] An ROC curve (Fig. 2) was plotted according to the quantitative result, and the result showed: when the specificity was 97.6% (40/41), the detection rates for colorectal cancer and adenoid tumor were 96.2% (101/105) and 71.6% (78/109), respectively. The areas under the curve are respectively as follows: 0.958 (95% CI: 0.909-1) and 0.832 (95% CI: 0.761-0.902), (P of both is less than 0.001).

Example 3 Detection Limit

[0055] Bisulfite transformation was carried out by using CpGenomeUniversal Methylated DNA (purchased from Millipore) to obtain 20 ng/μL template DNA, and S1, S2, S3, S4, S5 and S6 were obtained by 5-fold gradient dilutions, with the concentrations of 20 ng/μL, 4 ng/μL, 0.8 ng/μL, 0.16 ng/μL, 0.032 ng/μL and 0.0064 ng/μL, respectively.

[0056] qPCR was carried out with a loading template amount of 5 μL (the method and the primer are same as those in Example 2). It can be seen that the actual DNA addition amounts of S1-S6 are 100 ng, 20 ng, 4 ng, 0.8 ng, 0.16 ng, and 0.032 ng, respectively. The qPCR result showed that the amount of DNA detectable by the gene primer probe ranged from 0.032 ng to 100 ng (Fig. 3).

Comparative Example 1

[0057] Bisulfite transformation was carried out by using CpGenomeUniversal Methylated DNA (purchased from Millipore); and ACTB was used as a reference gene, and then different primers and probes were used for quantitative detection to investigate the amplification effect of each set of probes and primers.

[0058] The primers and probes used were shown in Table 1:

Table 1 Primers and probes

Name	Type	Sequence	Serial number	Ct value
Set 1	Upstream primer	ACGCGAGTTTTGCGTAGTC	SEQ ID NO: 4	31.5
	Downstream primer	TCCGAATACGAACCGCTAA	SEQ ID NO: 5	
	Probe	ACGGAGTTCGGTTTTGCGTTTTC	SEQ ID NO: 6	
Set 2	Upstream primer	GTTTTCGTATTACGTTCGGG	SEQ ID NO: 7	31.7
	Downstream primer	TCGAACCGACCTAAAATACC	SEQ ID NO: 8	
	Probe	AATCGGGAGTGGGGTCGGGCGA	SEQ ID NO: 9	
Set 3	Upstream primer	TATCGAGAGCGTATGGTTTG	SEQ ID NO: 10	31.6
	Downstream primer	CCACGTTATAAAAACGACCG	SEQ ID NO: 11	
	Probe	AGGGTCGTCGTTCGGGAGACGGT	SEQ ID NO:12	
Set 4	Upstream primer	AGAGTTATTTCGCGTTTTGC	SEQ ID NO:13	31.7
	Downstream primer	ATCCCGAACGTAATACGAAA	SEQ ID NO:14	

(continued)

Name	Type	Sequence	Serial number	Ct value
	Probe	TGGGAGGTTCGGGTTAGGACGCGA	SEQ ID NO:15	
Set 5	Upstream primer	TGCGTTTTCGTATTACGTTC	SEQ ID NO:16	33.3
	Downstream primer	CCAACCGAAAACTTCGAATA	SEQ ID NO:17	
	Probe	GCGGTTCGTATTCGGAGAAGTAGCGCC	SEQ ID NO:18	
Set 6	Upstream primer	GCGGTTCGTATTCGGAGAAG	SEQ ID NO:19	35.8
	Downstream primer	TCTACCGCCAACCGAAAACT	SEQ ID NO:20	
	Probe	AGCGCGAGTATTC	SEQ ID NO:21	
Set 7	Upstream primer	TGCGGAGGCGTAGGGTC	SEQ ID NO:22	32.8
	Downstream primer	CAACCGAAATTCCCCAACG	SEQ ID NO:23	
	Probe	CCTACAACCGCGCGTAAACAAAAACG	SEQ ID NO:24	

[0059] The amplification results of each set of probes and primers were shown in Fig. 5, and the amplification result of Set 1 referred to the detection limit results of Example 3 (the detection template of S3 in the detection limit was the same as the detection template in this comparative example). As can be seen from the data in the table, in the specific amplification process aiming at the target gene, the amplification result Ct of the Set 1 was minimum, which showed the best amplification effect, and was superior to the probes of other sets.

Claims

1. A quantitative detection kit for determining ITGA4 gene methylation in at least one sample selected from a group consisting of feces, tissue or cells comprising:

   an ITGA4 gene capturing reagent comprising:
   a magnetic bead probe complex comprising:
   a first probe of nucleotide sequence as shown in SEQ ID NO: 1; and
   an ITGA4 gene methylation quantitative detection reagent comprising:

   a primer of nucleotide sequences as shown in SEQ ID NO: 4-5, and
   a second probe of nucleotide sequence as shown in SEQ ID NO: 6.

2. A detection kit for determining ITGA4 gene methylation in at least one sample selected from a group consisting of feces, tissue or cells, comprising:

   an ITGA4 gene capturing reagent comprising:
   a magnetic bead probe complex comprising:
   a first nucleotide sequence of a probe as shown in SEQ ID NO: 1;
   an ITGA4 gene methylation qualitative detection reagent comprising:
   a first primer of nucleotide sequence as shown in SEQ ID NO: 2-3; and
   an ITGA4 gene methylation quantitative detection reagent comprising:

   a second primer of nucleotide sequences as shown in SEQ ID NO: 4-5; and
   a second probe of nucleotide sequence as shown in SEQ ID NO: 6.

3. A method of detecting ITGA4 gene methylation in at least one sample selected from a group consisting of feces, tissue or cells, comprising:

   extracting DNA from said sample using an ITGA4 gene capturing reagent comprising a magnetic bead probe complex comprising a first probe of nucleotide sequence as shown in SEQ ID NO: 1;
   treating the DNA with bisulfite;

amplifying the DNA using a primer of nucleotide sequence as shown in SEQ ID NO: 4-5;
and
detecting the presence of said ITGA4 gene methylation in said DNA using a second probe of nucleotide sequence as shown in SEQ ID NO: 6.

Patentansprüche

1. Ein quantitatives Nachweis-Kit zum Bestimmen der ITGA4-Gen-Methylierung in zumindest einer Probe, ausgewählt aus einer Gruppe, bestehend aus Fäkalien, Gewebe oder Zellen, umfassend:

   ein ITGA4-Gen-Einfangreagenz, umfassend:
   einen Magnetkügelchen-Sondenkomplex, umfassend:
   eine erste Sonde aus einer Nukleotidsequenz, wie in SEQ ID NO: 1 gezeigt; und
   ein quantitatives ITGA4-Gen-Methylierungsnachweisreagenz, umfassend:

   einen Primer aus Nukleotidsequenzen, wie in SEQ ID NO: 4-5 gezeigt, und
   eine zweite Sonde aus einer Nukleotidsequenz, wie in SEQ ID NO: 6 gezeigt.

2. Ein Nachweis-Kit zum Bestimmen der ITGA4-Gen-Methylierung in zumindest einer Probe, ausgewählt aus einer Gruppe, bestehend aus Fäkalien, Gewebe oder Zellen, umfassend:

   ein ITGA4-Gen-Einfangreagenz, umfassend:
   einen Magnetkügelchen-Sondenkomplex, umfassend:
   eine erste Nukleotidsequenz einer Sonde, wie in SEQ ID NO: 1 gezeigt;
   ein qualitatives ITGA4-Gen-Methylierungsnachweisreagenz, umfassend:
   einen ersten Primer aus einer Nukleotidsequenz, wie in SEQ ID NO: 2-3 gezeigt; und
   ein quantitatives ITGA4-Gen-Methylierungsnachweisreagenz, umfassend:

   einen zweiten Primer aus Nukleotidsequenzen, wie in SEQ ID NO: 4-5 gezeigt; und
   eine zweite Sonde aus einer Nukleotidsequenz, wie in SEQ ID NO: 6 gezeigt.

3. Ein Verfahren zum Nachweisen von ITGA4-Gen-Methylierung in zumindest einer Probe, ausgewählt aus einer Gruppe, bestehend aus Fäkalien, Gewebe oder Zellen, umfassend:

   Extrahieren von DNA aus besagter Probe unter Verwendung eines ITGA4-Gen-Einfangreagenzes, umfassend einen Magnetkügelchen-Sondenkomplex, der eine erste Sonde aus einer Nukleotidsequenz, wie in SEQ ID NO: 1 gezeigt, umfasst;
   Behandeln der DNA mit Bisulfit;
   Amplifizieren der DNA unter Verwendung eines Primers aus einer Nukleotidsequenz, wie in SEQ ID NO: 4-5 gezeigt; und
   Nachweisen des Vorhandenseins der ITGA4-Gen-Methylierung in der DNA unter Verwendung einer zweiten Sonde aus einer Nukleotidsequenz, wie in SEQ ID NO: 6 gezeigt.

Revendications

1. Kit de détection quantitative pour déterminer une méthylation de gène ITGA4 dans au moins un échantillon sélectionné parmi un groupe constitué de selles, tissu ou cellules comprenant :

   un réactif de capture de gène ITGA4 comprenant :
   un complexe de sondes à billes magnétiques comprenant :
   une première sonde de séquence nucléotidique telle qu'illustrée dans SEQ ID N° : 1 ; et
   un réactif de détection quantitative de méthylation de gène ITGA4 comprenant :

   une amorce de séquences nucléotidiques telles qu'illustrées dans SEQ ID N° : 4-5, et
   une seconde sonde de séquence nucléotidique telle qu'illustrée dans SEQ ID N° : 6.

2. Kit de détection pour déterminer une méthylation de gène ITGA4 dans au moins un échantillon sélectionné parmi un groupe constitué de selles, tissu ou cellules, comprenant :

un réactif de capture de gène ITGA4 comprenant :
un complexe de sondes à billes magnétiques comprenant :
une première séquence nucléotidique d'une sonde telle qu'illustrée dans SEQ ID N° : 1;
un réactif de détection qualitative de méthylation de gène ITGA4 comprenant :
une première amorce de séquence nucléotidique telle qu'illustrée dans SEQ ID N° : 2-3 ; et
un réactif de détection quantitative de méthylation de gène ITGA4 comprenant :

une seconde amorce de séquences nucléotidiques telles qu'illustrées dans SEQ ID N° : 4-5 ; et
une seconde amorce de séquence nucléotidique telle qu'illustrée dans SEQ ID N° : 6.

3. Procédé de détection de méthylation de gène ITGA4 dans au moins un échantillon sélectionné parmi un groupe constitué de selles, tissu ou cellules, comprenant :

extraire de l'ADN dudit échantillon en utilisant un réactif de capture de gène ITGA4 comprenant un complexe de sondes à billes magnétiques comprenant une première sonde de séquence nucléotidique telle qu'illustrée dans SEQ ID N° : 1 ;
traiter l'ADN avec du bisulfite ;
amplifier l'ADN en utilisant une amorce de séquence nucléotidique telle qu'illustrée dans SEQ ID N° : 4-5 ; et
détecter la présence de ladite méthylation de gène ITGA4 dans ledit ADN en utilisant une seconde sonde de séquence nucléotidique telle qu'illustrée dans SEQ ID N° : 6.

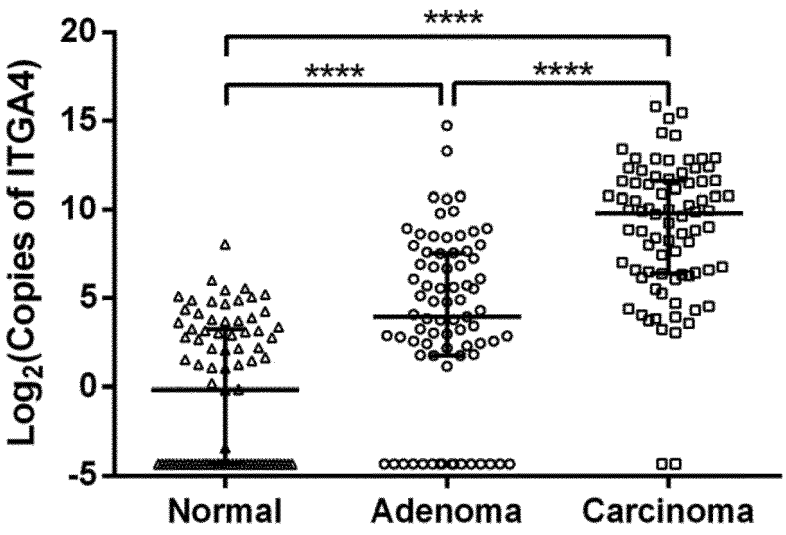
****
****
****
20
15
10
5
0
-5
Log2(Copies of ITGA4)
Normal
Adenoma
Carcinoma

Fig. 1

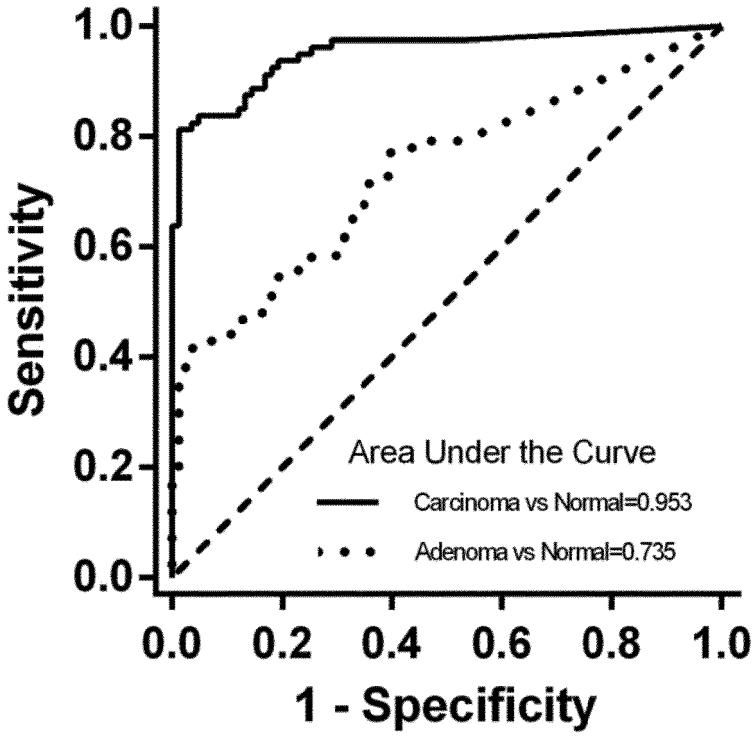
1.0
0.8
0.6
0.4
0.2
0.0
Sensitivity
Area Under the Curve
Carcinoma vs Normal=0.953
Adenoma vs Normal=0.735
0.0
0.2
0.4
0.6
0.8
1.0
1 - Specificity

Fig. 2

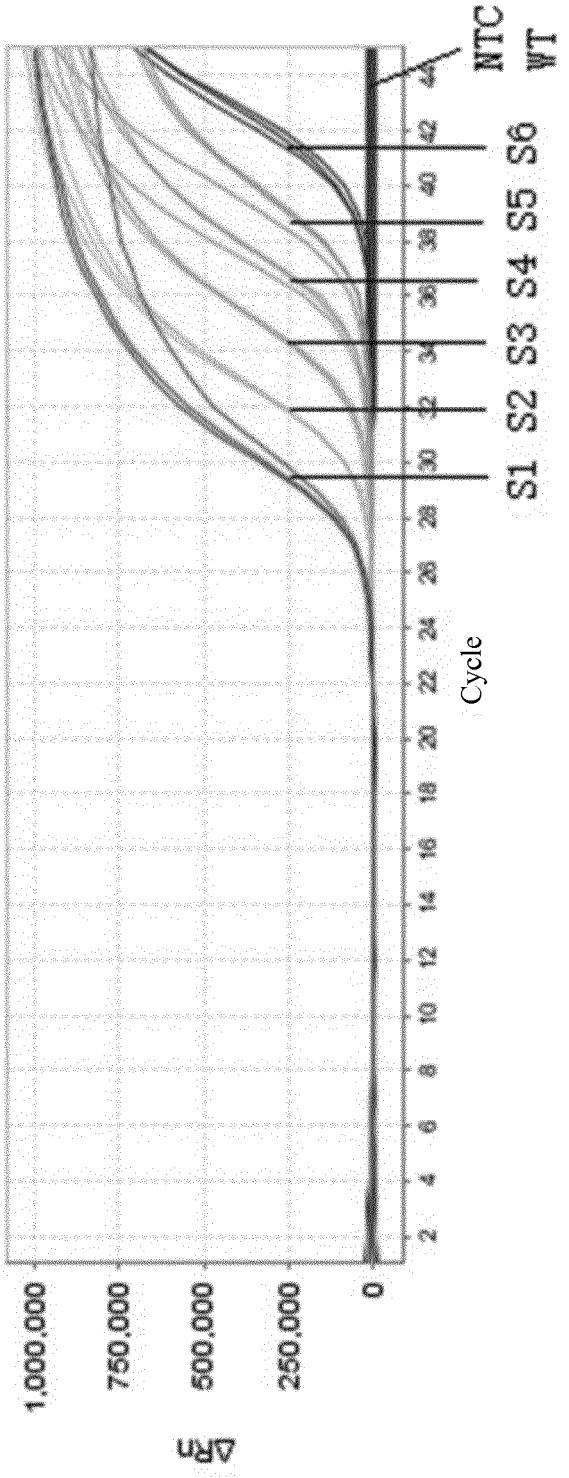
ΔRn
1,000,000
750,000
500,000
250,000
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44
Cycle
S1
S2
S3
S4
S5
S6
NTC
WT

Fig. 3

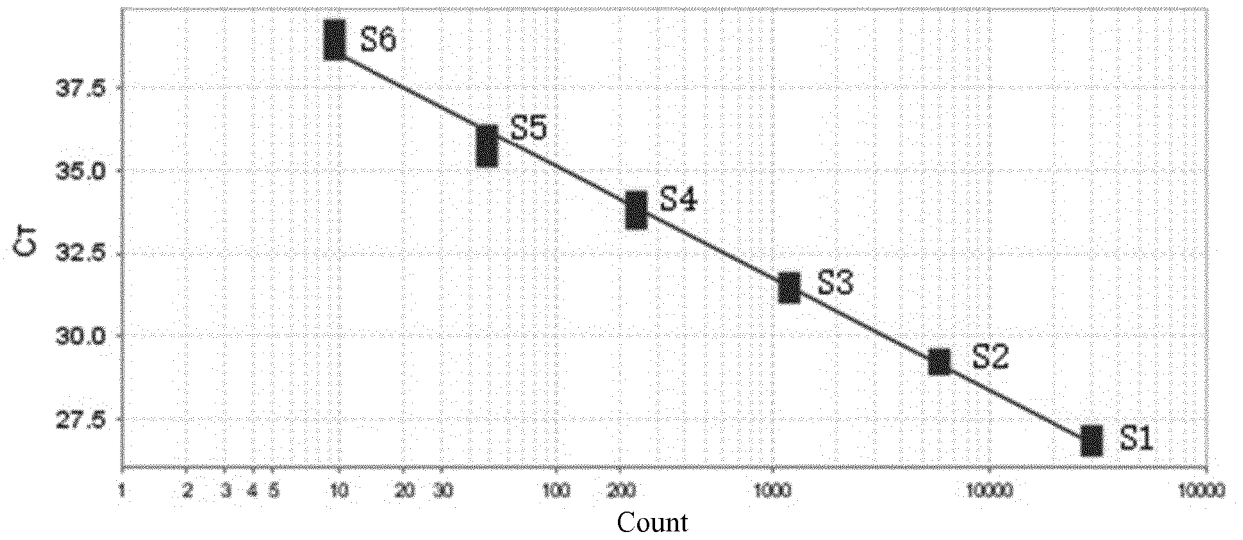
S6
S5
S4
S3
S2
S1
37.5
35.0
32.5
30.0
27.5
Ct
1
2
3
4
5
10
20
30
100
200
1000
10000
10000
Count

Fig. 4

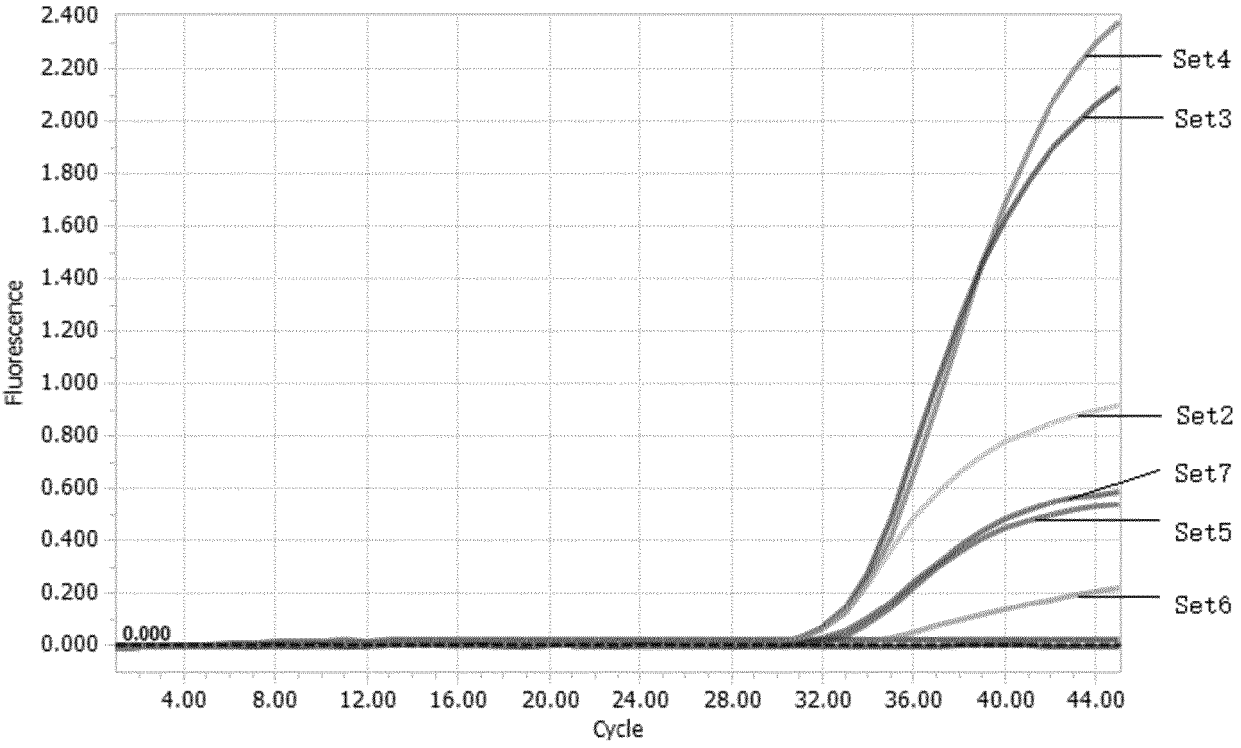
2.400
2.200
2.000
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Fluorescence
0.000
4.00
8.00
12.00
16.00
20.00
24.00
28.00
32.00
36.00
40.00
44.00
Cycle
Set4
Set3
Set2
Set7
Set5
Set6

Fig. 5

REFERENCES CITED IN THE DESCRIPTION

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Non-patent literature cited in the description

- **AUSCH et al.** *Clinical Chemistry,* 2009 **[0004]**
- Detection and Extraction of Free Methylated DNA in Urine by Applying Magnetic Bead Method. *Journal of China Medical University,* 2015, (10 **[0022] [0032]**