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methods for diagnosing colorectal cancer in plasma samples. Suitable panels in this context comprise, consist essentially of or consist of OSMR, NDRG4, GATA5 and ADAM23. This may be utilised in order to diagnose early stage

5 colorectal cancer, in particular stage 0 to II colorectal cancer. Additionally or alternatively, the at least one gene may be selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, such as from TFPI2, BNIP3, FOXE1, SYNE1 and SOX17, in particular TFPI2.

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In embodiments in which tissue samples are utilised, the methods may comprise, consist essentially of or consist of detecting an epigenetic change in a panel of genes comprising OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5,

15 wherein detection of the epigenetic change in at least one of the genes in the panel is indicative of a predisposition to, or the incidence of, colorectal cancer. The tissue sample may comprise, consist essentially of or consist of a colon and/or rectal and/or appendix sample.

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In embodiments where faecal samples are employed, the at least one gene may be selected from GATA4, OSMR, NDRG4, GATA5, SERP1, ADAM23, JPH3, SFRP2, APC and MGMT. In addition, or alternatively, the at least one gene may be

25 selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3, and JAM3, such as from TFPI2, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, in particular TFPI2. Two, three, four, five or six etc. gene panels selected from these genes are also envisaged in the present invention.

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CpG dinucleotides susceptible to methylation are typically concentrated in the promoter region, exons and introns of

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human genes. Promoter, exon and intron regions can be assessed for methylation. In one embodiment, the methylation status of the promoter region of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is determined. A "promoter" is a region extending typically between approximately 1 Kb, 500 bp or 150 to 300 bp upstream from the transcription start site. Frequently, the CpG island surrounding or positioned around the transcription start site of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is analysed to determine its methylation status. Alternatively, the methylation status of the exon and/or intron regions of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) may be determined as appropriate.

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In one embodiment of the methods of the invention, the methylation status of the promoter region of the NDRG4 gene is analysed. In another embodiment, the methylation status of the promoter region of the NDRG2 gene is analysed. Alternatively, the promoter region of NDRG2 and NDRG4 are analysed simultaneously.

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In one embodiment, the region of the NDRG4/NDRG2 subfamily gene comprising, consisting essentially of, or consisting of the nucleotide sequence of NDRG4 as set forth as SEQ ID NO: 524 (FIG. 3a) and/or the nucleotide sequence of NDRG2 as set
5 forth as SEQ ID NO: 525 (FIG. 3b) is analysed in order to determine its methylation status.

Various methylation assay procedures are known in the art, and can be used in conjunction with the present invention.
10 These assays rely onto two distinct approaches: bisulphite conversion based approaches and non-bisulphite based approaches. Non-bisulphite based methods for analysis of DNA methylation rely on the inability of methylation-sensitive enzymes to cleave methylation cytosines in their
15 restriction. The bisulphite conversion relies on treatment of DNA samples with sodium bisulphite which converts unmethylated cytosine to uracil, while methylated cytosines are maintained (Furuichi et al., 1970). This conversion results in a change in the sequence of the original DNA.
20 DNA methylation analysis has been performed successfully with a number of techniques including: sequencing, methylation-specific PCR (MS-PCR), melting curve methylation-specific PCR (McMS-PCR), MLPA with or without bisulfite treatment, QAMA (Zeschnigk et al, 2004), MSRE-PCR
25 (Melnikov et al, 2005), MethyLight (Eads et al., 2000), ConLight-MSP (Rand et al., 2002), bisulfite conversion-specific methylation-specific PCR (BS-MSP) (Sasaki et al., 2003), COBRA (which relies upon use of restriction enzymes to reveal methylation dependent sequence differences in PCR
30 products of sodium bisulfite - treated DNA), methylation-sensitive single-nucleotide primer extension conformation (MS-SNuPE), methylation-sensitive single-strand

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conformation analysis (MS-SSCA), Melting curve combined bisulfite restriction analysis (McCOBRA) (Akey et al., 2002), PyroMethA, HeavyMethyl (Cottrell et al. 2004), MALDI-TOF, MassARRAY, Quantitative analysis of methylated alleles
5 (QAMA), enzymatic regional methylation assay (ERMA), QBSUPT, MethylQuant, Quantitative PCR sequencing and oligonucleotide-based microarray systems, Pyrosequencing, Meth-DOP-PCR. A review of some useful techniques is provided in Nucleic acids research, 1998, Vol. 26, No. 10,
10 2255-2264, Nature Reviews, 2003, Vol.3, 253_266; Oral Oncology, 2006, Vol. 42, 5-13, which references are incorporated herein in their entirety. Any of these techniques may be utilised in accordance with the present invention, as appropriate.

15 Additional methods for the identification of methylated CpG dinucleotides utilize the ability of the methyl binding domain (MBD) of the MeCP2 protein to selectively bind to methylated DNA sequences (Cross et al, 1994; Shiraishi et
20 al, 1999). Alternatively, the MBD may be obtained from MBP, MBP2, MBP4 or poly-MBD (Jorgensen et al., 2006). In one method, restriction exonuclease digested genomic DNA is loaded onto expressed His-tagged methyl-CpG binding domain that is immobilized to a solid matrix and used for
25 preparative column chromatography to isolate highly methylated DNA sequences. Such methylated DNA enrichment-step may supplement the methods of the invention. Several other methods for detecting methylated CpG islands are well known in the art and include amongst others methylated-CpG
30 island recovery assay (MIRA). Any of these methods may be employed in the present invention where desired.

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In specific embodiments, the methylation status of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) (or portion thereof, especially the CpG islands, as discussed herein) is determined using methylation specific PCR (MSP), or an equivalent amplification technique. The MSP technique will be familiar to one of skill in the art. In the MSP approach, DNA may be amplified using primer pairs designed to distinguish methylated from unmethylated DNA by taking advantage of sequence differences as a result of sodium-bisulphite treatment (Herman et al., 1996; and WO 97/46705).

A specific example of the MSP technique is designated real-time quantitative MSP (QMSP), which permits reliable quantification of methylated DNA in real time. These methods are generally based on the continuous optical monitoring of an amplification procedure and utilise fluorescently labelled reagents whose incorporation in a product can be quantified and whose quantification is indicative of copy number of that sequence in the template. One such reagent is a fluorescent dye, called SYBR Green I that preferentially binds double-stranded DNA and whose fluorescence is greatly enhanced by binding of double-stranded DNA. Alternatively, labelled primers and/or labelled probes can be used. They represent a specific application of the well known and commercially available real-time amplification techniques such as hydrolytic probes (TAQMAN®), hairpin probes (MOLECULAR BEACONS®), hairpin primers (AMPLIFLUOR®), hairpin probes integrated into

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primers (SCORPION®), oligonucleotide blockers (such as the HeavyMethyl technique) and primers incorporating complementary sequences of DNazymes (DzyNA®), specific interaction between two modified nucleotides (Plexor™) etc
5 as described in more detail herein. Often, these real-time methods are used with the polymerase chain reaction (PCR). In Heavymethyl, described for example in WO02/072880 the priming is methylation specific, but non-extendable oligonucleotide blockers provide this specificity instead of
10 the primers themselves. The blockers bind to bisulfite-treated DNA in a methylation-specific manner, and their binding sites overlap the primer binding sites. When the blocker is bound, the primer cannot bind and therefore the amplicon is not generated. Heavymethyl can be used in
15 combination with real-time or end point detection in the methods of the invention.

Thus, in specific embodiments, the methylation status of the at least one gene selected from an NDRG2/NDRG4 subfamily
20 gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is determined by methylation specific PCR/amplification,
25 preferably real-time methylation specific PCR/amplification. In specific embodiments, the real time PCR/amplification involves use of hairpin primers (Amplifluor)/hairpin probes (Molecular Beacons)/hydrolytic probes (Taqman)/FRET probe pairs (Lightcycler)/primers incorporating a hairpin probe
30 (Scorpion)/primers incorporating complementary sequences of DNazymes that cleave a reporter substrate included in the reaction mixture (DzyNA®)/fluorescent dyes (SYBR Green

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etc.)/oligonucleotide blockers/the specific interaction between two modified nucleotides (Plexor). Primers and/or probes can be used to investigate the methylation status of the at least one gene.

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Real-Time PCR detects the accumulation of amplicon during the reaction. Real-time methods do not need to be utilised, however. Many applications do not require quantification and Real -Time PCR is used principally as a tool to obtain convenient results presentation and storage, and at the same time to avoid post-PCR handling. Analyses can be performed only to know if the target DNA is present in the sample or not. End point verification is carried out after the amplification reaction has finished. This knowledge can be used in a medical diagnostic laboratory to detect a predisposition to, or the incidence of, cancer in a patient. In the majority of such cases, the quantification of DNA template is not very important. Amplification products may simply be run on a suitable gel, such as an agarose gel, to determine if the expected sized products are present. This may involve use of ethidium bromide staining and visualisation of the DNA bands under a UV illuminator for example. Alternatively, fluorescence or energy transfer can be measured to determine the presence of the methylated DNA. The end-point PCR fluorescence detection technique can use the same approaches as widely used for Real Time PCR: TaqMan assay, Molecular Beacons, Scorpion, Amplifluor etc. For example, «Gene» detector allows the measurement of fluorescence directly in PCR tubes.

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In real-time embodiments, quantitation may be on an absolute basis, or may be relative to a constitutively methylated DNA

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standard, or may be relative to an unmethylated DNA standard. Methylation status may be determined by using the ratio between the signal of the marker under investigation and the signal of a reference gene where methylation status is known (such as β -actin for example), or by using the ratio between the methylated marker and the sum of the methylated and the non-methylated marker. Alternatively, absolute copy number of the methylated marker gene can be determined. Suitable reference genes for the present invention include beta-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal RNA genes such as 18S ribosomal RNA and RNA polymerase II gene (Radonic A. et al., Biochem Biophys Res Commun. 2004 Jan 23;313(4):856-62). In a particularly preferred embodiment, the reference gene is beta-actin.

In one embodiment, each clinical sample is measured in duplicate and for both C_t values (cycles at which the amplification curves crossed the threshold value, set automatically by the relevant software) copy numbers are calculated. The average of both copy numbers (for each gene) is used for the result classification. To quantify the final results for each sample two standard curves are used, one for either the reference gene (β -actin or the non-methylated marker for example) and one for the methylated version of the marker. The results of all clinical samples (when m-Gene was detectable) are expressed as 1000 times the ratio of "copies m-Gene"/"copies reference gene" or "copies m-Gene"/"copies u-Gene+m-Gene" and then classified accordingly (methylated, non-methylated or invalid) (u=unmethylated; m=methylated).

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In one embodiment, primers useful in MSP carried out on the promoter region of the NDRG4 gene are provided. These primers comprise, consist essentially of or consist of the following sequences:

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TABLE 2

SEQ ID NO.	NDRG4	Primer	Sense primer	SEQ ID NO.	Antisense primer	Annealing temp	Number of PCR cycles
5	Primer set 1	Flank	GGTTYGTTYGGGATTAGTTTT AGG	6	CRAACAACCAAAAACCCCT C	56	35
7	Primer set 1	U	GATTAGTTTTAGGTTTGGTATT GTTTTGT	8	AAAACCAAACTAAAAACAAT ACACCA	66	25
9	Primer set 1	M	TTTAGGTTTCGGTATCGTTTCG C	10	CGAACTAAAAACGATACGC CG	66	25
11	Primer set 2	Flank	ATYGGGGTGTTTTTAGGTTT	12	ATACCRAACCTAAAACTAAT CCC	56	35
13	Primer set 2	U	GGGTGTTTTTAGGTTTCGCG TCGC	14	CCTAAACTAATCCCAAACA AACCA	66	30
15	Primer set 2	M	TTTTTAGGTTTCGCGTCGC	16	AACTAATCCCGAACGAAC CG	66	30

Where "Flank" = Flanking primers

"U" = Unmethylated NDRG4 specific primers

10 "M" = Methylated NDRG4 specific primers

Primer set 1 is useful in particular applications for predicting the progression of adenomas. Primer set 2 may provide slightly more sensitive results although both primer
15 sets are clearly useful.

In a further embodiment, primers and probes useful in quantitative MSP carried out on the (promoter region of the) NDRG4 gene are provided. These primers and probes comprise,
20 consist essentially of or consist of the following sequences:

SEQ ID NO: 17 5' - GTATTTTAGTCGCGTAGAAGGC - 3' (forward primer)

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SEQ ID NO: 18 5' - AATTTAACGAATATAAACGCTCGAC - 3' (reverse primer) and

SEQ ID NO: 19 5'-FAM-CGACATGCCCCGAACGAACCGCGATCCCTGCATGTCTG-3'-DABCYL (molecular beacon probe)

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Further characteristics of these primers and probes are summarized in the experimental part.

In a further embodiment, primers useful in MSP carried out on the promoter region of the NDRG2 gene are provided. These primers comprise, consist essentially of or consist of the following sequences:

Flanking primers:

15 SEQ ID NO: 20 5'-YGTTTTTTATTTATAGYGGTTTTT-3' (flank up)
SEQ ID NO: 21 5'- TCCTAATACCTCTCCTCTCTTTACTAC -3' (flank down)

Unmethylated NDRG2 specific primers:

20 SEQ ID NO: 22 5'- TTTTATTTATAGTGGTTTTTGTATTTTTT -3' (sense)
SEQ ID NO: 23 5'- TCTCCTCTCTTTACTACATCCCAACA -3' (antisense)

Methylated NDRG2 specific primers:

SEQ ID NO: 24 5'- TTTATAGCGGTTTTTCGTATTTTTC -3' (sense)
25 SEQ ID NO: 25 5'- CCTCTCTTTACTACGTCCCGACG -3' (antisense).

In one embodiment, primers and/or probes useful in determining the methylation status of the at least one gene selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT (carried out on the promoter region of at least one gene selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT) are

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provided. These primers and/or probes comprise, consist essentially of or consist of the following sequences:

Table 3: Primer sequences and beacon (probe) sequences

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SEQ ID NO.			
26	B- Actin	forward primer	5' - TAGGGAGTATATAGGTTGGGGAAGTT - 3'
27		reverse primer	5' - AACACACAATAACAAACACAAATTCAC - 3'
28		beacon	5'-FAM-CGACTGCGTGTGGGGTGGTGATGGAGGAGGTTTAGGCAGTCG-3'-DABCYL
29	GATA4	forward primer	5' - AGGTTAGTTAGCGTTTATAGGGTC - 3'
30		reverse primer	5' - ACGACGACGAAACCTCTCG - 3'
31		beacon	5'-FAM-CGACATGCCTCGCGACTCGAATCCCCGACCCAGCATGTCG-3'-DABCYL
32	GATA5	forward primer	5' - AGTTCGTTTTTATAGGTTAGTTTTCGGC - 3'
33		reverse primer	5' - CCAATACAACATAACGAACGAACCG - 3'
34		beacon	5'-FAM-CGACATGCGTAGGAGGTAGAGGTTTCGGGATTCGTAGCATGTCG-3'-DABCYL
35	SFRP1	forward primer	5' -TGTAGTTTTTCGGAGTTAGTGTGCGCG- 3
36		reverse primer	5' -CCTACGATCGAAAACGACGCGAACG- 3'
37		beacon	5'-FAM-CGACATGCTCGGGAGTCGGGGCGTATTAGTTCGTAGCGGCATGTCG-3'-DABCYL
38	SFRP2	forward primer	5' - GGGTCGGAGTTTTTCGGAGTTGCGC - 3'
39		reverse primer	5' - CCGCTCTCTTCGCTAAATACGACTCG - 3'
40		beacon	5'-FAM-CGACATGCGGTGTTTCGTTTTTTCGCGTTTTAGTCGTGGGCATGTCG -3'-DABCYL
17	NDRG4	forward primer	5' - GTATTTTAGTCGCGTAGAAGGC - 3'
18		reverse primer	5' - AATTTAACGAATATAAACGCTCGAC - 3'
19		beacon	5'-FAM-CGACATGCCCGAACGAACCGCGATCCCTGCATGTCG-3'-DABCYL
41	APC	forward primer	5'-GAACCAAAACGCTCCCAT-3'
42		reverse primer	5'-TTATATGTCGGTTACGTGCGTTTATAT-3'
43		beacon	5' - FAM-CGTCTGCCCCGTCGAAAACCCGCCGATTAAACGCAGACG-3'-DABCYL
44	ADAM23	forward primer	5' - GAAGGACGAGAAGTAGGCG - 3'
45		reverse primer	5' - CTAACGAACATAACCTTACCGA - 3'
46		beacon	5'-FAM-CGACATGCCCCGACCCGACGCGCCCTGCATGTCG-3'-DABCYL
47	OSMR (3)	forward primer	5' - TTTGGTCGGGGTAGGAGTAGC - 3'
48		reverse primer	5' - CGAACTTTACGAACGAACGAAC - 3'
49		beacon	5'-FAM-CGACATGCCCGTACCCCGCGCGCAGCATGTCG-3'-DABCYL
47	OSMR (4)	forward primer	5' - TTTGGTCGGGGTAGGAGTAGC - 3'
50		reverse primer	5' - AAAAAGTTAAAAACCGAAAAGTCG - 3'
49		beacon	5'-FAM-CGACATGCCCGTACCCCGCGCGCAGCATGTCG-3'-DABCYL
51	JPH3	forward primer	5' - TTAGATTTTCGTAAACGGTGAAAAC - 3'
52		reverse primer	5' - TCTCCTCCGAAAAACGCTC - 3'
53		beacon	5'-FAM-CGTCTGCAACCGCCGACGACCGGACGACGAGACG-3'-DABCYL
54	MGMT	forward primer	5' - TTTCGACGTTTCGTAGGTTTTCGC - 3'
55		reverse primer	5' - GCACTCTTCCGAAAACGAAACG - 3'
56		beacon	5'-FAM-CGTCTCGCGTGCATGCTTTGCGATTGGTGAGTGTGGGGCGAGACG-3'-DABCYL

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In specific embodiments, the methods of the invention employ or rely upon or utilise primers and/or probes selected from the primers and probes comprising the nucleotide sequences set forth in Table 4 below to determine the methylation status of the at least one gene. The table presents specific primer and probe combinations for certain preferred genes whose methylation status may be determined according to the methods of the invention.

Table 4: Primer sequences and beacon (probe) sequences

SEQ ID NO			
26	β-Actin	forward primer	5' - TAGGGAGTATATAGGTTGGGGAAGTT - 3'
27		reverse primer	5' - AACACACAATAACAAACACAAATTCAC - 3'
28		beacon	5'-FAM-CGACTGCGTGTGGGGTGGTGATGGAGGAGTTTAGGCAGTCG-3'-DABCYL
29	GATA4	forward primer	5' - AGGTTAGTTAGCGTTTTAGGGTC - 3'
30		reverse primer	5' - ACGACGACGAAACCTCTCG - 3'
31		beacon	5'-FAM-CGACATGCCTCGCGACTCGAATCCCCGACCCAGCATGTCG-3'-DABCYL
32	GATA5	forward primer	5' - AGTTCGTTTTTAGGTTAGTTTTCGGC - 3'
33		reverse primer	5' - CCAATACAATAACGAACGAACCG - 3'
34		beacon	5'-FAM-CGACATGCGTAGGGAGGTAGAGGGTTCGGGATTCTAGCATGTCG-3'-DABCYL
35	SFRP1	forward primer	5' -TGTAGTTTTTCGGAGTTAGTGTGCGGC- 3'
36		reverse primer	5' -CCTACGATCGAAAACGACGCGAACG- 3'
37		beacon	5'-FAM-CGACATGCTCGGGAGTCGGGGCGTATTAGTTCGTAGCGGCATGTCG-3'-DABCYL
38	SFRP2	forward primer	5' - GGGTCGGAGTTTTTCGGAGTTGCGC - 3'
39		reverse primer	5' - CCGCTCTCTTCGCTAAATACGACTCG - 3'
40		beacon	5'-FAM-CGACATGCGGTGTTTCGTTTTTCGCGTTTTAGTCGTGGGCATGTCG-3'-DABCYL
17	NDRG4	forward primer	5' - GTATTTTAGTCGCGTAGAAGGC - 3'
18		reverse primer	5' - AATTTAACGAATATAAACGCTCGAC - 3'
19		beacon	5'-FAM-CGACATGCCCCGAACGAACCGCGATCCCTGCATGTCG-3'-DABCYL
41	APC	forward primer	5'-GAACCAAAACGCTCCCAT-3'
42		reverse primer	5'-TTATATGTCGGTTACGTGCGTTTATAT-3'
43		beacon	5' -FAM-CGTCTGCCCCGTCGAAAACCCGCCGATTACGCAGACG-3'-DABCYL
44	ADAM23	forward	5' - GAAGGACGAGAAGTAGGCG - 3'

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		primer	
45		reverse primer	5' - CTAACGAACTACAACCTTACCGA - 3'
46		beacon	5'-FAM-CGACATGCCCCGACCCGCGACGCCGCCCTGCATGTCG-3'-DABCYL
47	OSMR (3)	forward primer	5' - TTTGGTCGGGGTAGGAGTAGC - 3'
48		reverse primer	5' - CGAACTTTACGAACGAACGAAC - 3'
49		beacon	5'-FAM-CGACATGCCCGTACCCCGCGCGCAGCATGTCG-3'-DABCYL
47	OSMR (4)	forward primer	5' - TTTGGTCGGGGTAGGAGTAGC - 3'
50		reverse primer	5' - AAAAAGCTTAAAAACCGAAAACGTCG - 3'
49		beacon	5'-FAM-CGACATGCCCGTACCCCGCGCGCAGCATGTCG-3'-DABCYL
51	JPH3	forward primer	5' - TTAGATTTCTGTAACGGTGAAAAC - 3'
52		reverse primer	5' - TCTCCTCCGAAAAACGCTC - 3'
53		beacon	5'-FAM-CGTCTGCAACCGCCGACGACCGCGACGCGAGACG-3'-DABCYL
54	MGMT	forward primer	5' - TTTCGACGTTCTGAGGTTTTCGC - 3'
55		reverse primer	5' - GCACTCTTCCGAAAACGAAACG - 3'
56		beacon	5'-FAM-CGTCTCGCGTGCATATCGTTTGCATTGGTGAGTGTGTTGGGGCGAGACG-3'-DABCYL

In a further specific embodiment, the methods of the invention employ or rely upon or utilise primers and/or probes selected from the primers comprising the nucleotide sequences set forth in Table 5 below to determine the methylation status of NDRG4. The table presents specific primer and probe combinations for determining the methylation status of this gene and the primer pairs and corresponding probe may be selected according to the table.

Table 5 Primer pairs and probes for determining the methylation status of NDRG4, with predicted amplification product lengths shown.

Assay name	Amplification length	SEQ ID NO	Oligonucleotides & probes	5' to 3' Sequences (all the beacons are 5'-FAM and 3'-DABCYL)
NDRG4_1b	112	17	Forward primer	GTATTTTAGTCGCGTAGAAGGC
		18	Reverse primer	AATTTAACGAATATAACGCTCGAC
		57	Beacon	CGACATGCAGGGATCGCGGTTTCGTTCCGGGCATGTCG
NDRG4_138 30	105	58	Forward primer	GGTATTTTAGTCGCGTAGAAGGC

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		59	Reverse primer	GAATATAAACGCTCGACCCGC
		60	Beacon	CGACATGCGCGGTTTCGTTTCGGGATTAGTTTTAGGTTCCGCATGTCG
NDRG4_2(MvE)	88	9	Forward primer	TTTAGGTTCCGGTATCGTTTCGC
		10	Reverse primer	CGAACTAAAAACGATACGCCG
		61	Beacon	CGTACCCGCGTTTATATTCGTTAAATTTACGCGGGTACG
NDRG4_662_92	163	62	Forward primer	TAGTCGCGTAGAAGGCGGA
		63	Reverse primer	GACTACAAAAACGAAAACCGAAC
		64	Beacon	CGACATCGGGTACGTTTCGCGGCGATGTCTG
NDRG4_662_93	168	58	Forward primer	GGTATTTTAGTCGCGTAGAAGGC
		65	Reverse primer	CTACAAAAACGAAAACCGAAC
		66	Beacon	CGTTTCGCGGGTCGAGCGAAACG
NDRG4_662_94	152	62	Forward primer	TAGTCGCGTAGAAGGCGGA
		67	Reverse primer	CGAAAACCGAACTAAAAACGA
		68	Beacon	CGACATGCCGCGGTTTCGTTTCGGGATTAGTTTTAGGGCATGTCTG
NDRG4_662_95	90	69	Forward primer	TTTCGTTTCGTTTATCGGGT
		70	Reverse primer	CGAACCTAAACTAATCCCGAAC
		71	Beacon	CGACACGCGTAGAAGGCGGAAGTTACGCGCGTGTCTG
NDRG4_662_96	160	72	Forward primer	GGTTTCGTAGCGTATTTAGTATAGTTC
		73	Reverse primer	GTAACCTCCGCCTTCTACGC
		74	Beacon	CGACATGCGCGGATCGATCGGGGTGTTTTTAGGGCATGTCTG
NDRG4_662_97	143	75	Forward primer	GAGTTGTTTTTGTCGTTTCGTTT
		76	Reverse primer	AACACCTTCATCTCGACGC
		77	Beacon	CGACATGCGGTTTCGGTCGAGCGCGCATGTCTG
NDRG4_662_98	148	78	Forward primer	GTTGTGAGTTGTTTTGTCTGTTTC
		76	Reverse primer	AACACCTTCATCTCGACGC
		79	Beacon	CGACATGCCGTTGTTTCGACGTCGTTATTTAGAGTCGGCATGTCTG
NDRG4_662_99	144	80	Forward primer	TTTTAGTATTTTTATTTCCGGCGTTC
		81	Reverse primer	CTACTCCTACCGCTTCGCTC
		82	Beacon	CGACATCGCGCTCCTCTCCCCGATGTCTG
NDRG4_663_00	151	83	Forward primer	CGGTGTTTTAGTATTTTTATTTCCGG
		84	Reverse primer	AACTACTCCTACCGCTTCGCT
		85	Beacon	CGACATCGGTTTTTGGGTGGCGGCGATGTCTG
NDRG4_663_01	120	80	Forward primer	TTTTAGTATTTTTATTTCCGGCGTTC
		86	Reverse primer	CTCTCCTACCGCTCCGCTC
		87	Beacon	CGACATCGCTCCTCTCCCCGACTCGATGTCTG
NDRG4_663_02	125	83	Forward primer	CGGTGTTTTAGTATTTTTATTTCCGG
		86	Reverse primer	CTCTCCTACCGCTCCGCTC
		88	Beacon	CGACATGCCGAACGCGCTACCCCGCATGTCTG

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NDRG4_663 03	95	89 Forward primer	CGAGTCGTTTTAGTTTTCGGT
		90 Reverse primer	TACTCACAAATACCGCCCG
		91 Beacon	CGACATCGGAAAGTGGCGGTGCGTTGCGATGTCG
NDRG4_663 04	85	92 Forward primer	TTCGGTGAATTTTAGGAGGC
		93 Reverse primer	TCGAACGACGAACACGAAA
		94 Beacon	CGACATGCGCGGGGTGGGTGCGGCATGTCG

In a further specific embodiment, the methods of the invention employ or rely upon or utilise primers selected from the primers comprising the nucleotide sequences set forth in Table 6 and 7 below to determine the methylation status of GATA5. The table presents specific primer combinations for determining the methylation status of this gene and the primer pairs may be selected according to the table. Table 6 also sets forth specific probes which may be utilised to facilitate (quantitative) detection of the methylation status of GATA5 and Table 7 incorporates Amplifluor sequences which allow the primers to act as hairpin primers, thus facilitating quantitative detection (as discussed in detail herein).

Table 6 - Primer pairs and probes for determining the methylation status of GATA5, with predicted amplification product lengths shown.

Assay name	Amplicon length	SEQ ID NO	Oligonucleotides & probes	5' to 3' Sequences (all the beacons are 5'-FAM and 3'-DABCYL)
GATA5_126 56	94	95 Forward primer		TTCGGGTTGGAGTATTTATTAGC
		96 Reverse primer		CGAACTTCCAATCTTCGACC
		97 Beacon		CGACATGCGGCGGTGGCGGTGCGTCGGCATGTCTG
GATA5_126 59	102	98 Forward primer		GATTTTTCGGGGTTTACGAAG
		99 Reverse primer		GAAACTTAACGACAAAAACGCA
		100 Beacon		CGACATGCGTTTAGTTGTATTGGTTCGGGTTTCGCATGTCTG
GATA5_126 66	107	101 Forward primer		GGTTTGTATTTCGGATTCCGGTC
		102 Reverse		TCGATAACAACGTCTACACG

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		primer	
GATA5_126 69	111	103 Beacon	CGACATGCGAAGGTGGGTTTGCGGTTTGGGAGGTCGCATGTCG
		104 Forward primer	TAGGGTGCGGGTTTGTATTC
		105 Reverse primer	AACAACGTCCTACACGACC
		106 Beacon	CGACATGCGTATTTATCGAAGGTGGGTTTGCGGTTTGCATGTCG
GATA5_662 12	118	107 Forward primer	TAGTTGGTGTAGTAGAGGTCGGC
		108 Reverse primer	GACCTAAATCTCGCTTCCGT
		109 Beacon	CGACATGCCGAGGGAGATTGGAGTGAGTTTCGCATGTCG
GATA5_662 13	139	110 Forward primer	TATAGCGTGGTGTTGGTCGT
		111 Reverse primer	CTAAATCTCGCTTCCGTCC
		112 Beacon	CGACATGCGCGAGGGAGATTGGAGTGAGTTTCGCATGTCG
GATA5_662 15	80	113 Forward primer	GGTGTGAGGTTTTTAAGGTTTC
		114 Reverse primer	TCACTTTCTAACGAAAACGACT
		115 Beacon	CGACATGCGGGACGGGATGGGTTTTGCGGGCATGTCG
GATA5_662 16	124	116 Forward primer	GTAGTTTCGGAGTTGGGTGTC
		117 Reverse primer	AAAAACGACTCTTCCCGATT
		118 Beacon	CGACATGCGAGGGACGGGATGGGTTTTGCATGTCG
GATA5_662 17	118	116 Forward primer	GTAGTTTCGGAGTTGGGTGTC
		119 Reverse primer	GACTCTTCCCGATTACAACG
		120 Beacon	CGACATGCGAGGGACGGGATGGGTTTTGGCATGTCG
GATA5_662 18	71	121 Forward primer	TTTTGCGTTAAAGGGTCGG
		122 Reverse primer	CGAAACCTTAAAAACCTCGACA
		123 Beacon	CGACATGCCGGGGTTTTAAAGGTAGTTTCGGAGTTGGCATGTCG
GATA5_662 19	90	124 Forward primer	GATGTCGTTGCGTTCGTTT
		125 Reverse primer	CCGAAACCTTAAAAACCTCG
		126 Beacon	CGACATGCCGGCGGGGTTTTAAAGGTAGTTTCGGCATGTCG
GATA5_662 20	98	127 Forward primer	GTTTTGCGGATGTCGTTGC
		125 Reverse primer	CCGAAACCTTAAAAACCTCG
		126 Beacon	CGACATGCCGGCGGGGTTTTAAAGGTAGTTTCGGCATGTCG
GATA5_662 21	158	128 Forward primer	TAGGGGTTTTGCGGATGTC
		114 Reverse primer	TCACTTTCTAACGAAAACGACT
		126 Beacon	CGACATGCCGGCGGGGTTTTAAAGGTAGTTTCGGCATGTCG
GATA5_662 22	150	129 Forward primer	TCGAGATTGTGGAGTTTTCGT
		130 Reverse primer	TAAAAACCTCGTACTCCGCC
		131 Beacon	CGACATCGGTTTGGGAGGTCGTGTAGGACGATGTCG
GATA5_662 23	103	129 Forward primer	TCGAGATTGTGGAGTTTTCGT
		132 Reverse primer	GTAACCCAATCCTAAACTACCGA
		131 Beacon	CGACATCGGTTTGGGAGGTCGTGTAGGACGATGTCG
GATA5_662	112	133	
Forward			GGTTTGTATTCGGATTCGGT

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		primer	
24		134 Reverse primer	ACCCCTCGATAACAACGTCC
		135 Beacon	CGACATGCCGTATTTATCGAAGGTGGGTTTGCGGGCATGTCG
GATA5_662_25	76	136 Forward primer	GTTTCGAGATTGTGGAGTTTTC
		137 Reverse primer	GATAACAACGTCCTACACGACC
		138 Beacon	CGACATGCCGAAGGTGGGTTTGCGGTTTGGGGCATGTCG
GATA5_662_26	163	139 Forward primer	TTATTCGTTTCGTTTCGGG
		140 Reverse primer	AAACCCACCTTCGATAAATACG
		141 Beacon	CGACATCGTTTTTGGTAGGGAGGTTCCGATCGATGTCG
GATA5_662_27	164	142 Forward primer	CGGGGTGTTATTTAGGTTTATTC
		143 Reverse primer	AATACGAAACTCCACAATCTCG
		144 Beacon	CGACATGCGTTTTTGGTAGGGAGGTTCCGATCGCATGTCG
GATA5_662_28	76	145 Forward primer	CGTTTTTGGTAGGGAGGTTTC
		146 Reverse primer	ATCCGAATACAAACCCGCA
		147 Beacon	CGACATGCCGTGGGGGAGGATGAGGGGAGCGTTTCGGCATGTCG
GATA5_662_29	113	142 Forward primer	CGGGGTGTTATTTAGGTTTATTC
		148 Reverse primer	AAACCCGCACCCTACGAAA
		144 Beacon	CGACATGCGTTTTTGGTAGGGAGGTTCCGATCGCATGTCG
GATA5_662_30	161	149 Forward primer	ATTAGTGTAGTTAGACGGGCGG
		150 Reverse primer	GACTCAACCACCAACACGA
		151 Beacon	CGACATGCGTGGGTTTCGGGGAGTCGCATGTCG
GATA5_662_31	116	95 Forward primer	TTCGGGTGGAGTATTTATTAGC
		152 Reverse primer	AAACTACGAAACCTCAACGACC
		153 Beacon	CGACATGCGGTGGCGGTGGGTCCGATGTCG
GATA5_662_33	134	154 Forward primer	GTTACGGGAGTTTTGCGTTT
		155 Reverse primer	CGATTCCTCTCCCTCGAAT
		156 Beacon	CGACATGCCAGTTTATGTCGGGTAGGTGTCGCATGTCG
GATA5_662_34	105	157 Forward primer	AATCGTGTTTCGTTTCGTATTTTC
		158 Reverse primer	GATATACTCCGAACCCGCC
		159 Beacon	CGACATGCGCGGAGTAGTTTCGTAGGTTGCGGGCATGTCG
GATA5_662_35	121	160 Forward primer	GCGATTTAGGTTAGGGAATCGT
		158 Reverse primer	GATATACTCCGAACCCGCC
		161 Beacon	CGACATGCCGTGAGGGTTGTATGGAGGCGTCGGCATGTCG
GATA5_662_37	99	162 Forward primer	TTTCGGTGGGTTTTTAGTC
		163 Reverse primer	GATTCCCTAACCTAAATCGCCT
		164 Beacon	CGACATGCGCGTTAGAAATCGTGTGGGTAGGAGGCGCATGTCG
GATA5_662_38	72	165 Forward primer	ATTCGGTGGGTTTTTAGTC
		166 Reverse primer	CACACGCATTCTAACGCC

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GATA5_662 43	91	167	Beacon	CGACATGCCTCTTCCCGAATCCCGAAAAACCGCATGTCTG
		168	Forward primer	GGGTTTTATCGTCGCGTGT
		169	Reverse primer	CCGAAAACCTAACCTAAAAACGAA
		170	Beacon	CGACATGCCCGACCCCGCTCACCGGCATGTCTG
GATA5_662 44	100	171	Forward primer	GGGGTTTACGGGGTTTATC
		172	Reverse primer	CGAAAACCTAACCTAAAAACGAAC
		173	Beacon	CGACATGCGATAATCCCGACCCCGCTCACCGGCATGTCTG
GATA5_662 45	152	174	Forward primer	TTGTTTAGAAATCGAGGAAATCG
		175	Reverse primer	CGACGATAAAACCCCGTAA
		176	Beacon	CGACATGCGAGTTTCGGGTGCGGTTACGCATGTCTG
GATA5_662 47	163	177	Forward primer	TGTGGTTTCGTTTGTTAGAAATC
		175	Reverse primer	CGACGATAAAACCCCGTAA
		178	Beacon	CGACATGCGAGTTTCGGGTGCGGTTACGTAACGCATGTCTG
GATA5_662 50	151	177	Forward primer	TGTGGTTTCGTTTGTTAGAAATC
		179	Reverse primer	CCCGTAAACCCCTCGTTA
		180	Beacon	CGACATGCCCGCGGGGTTTTCGTTAGTGTATTCGGCATGTCTG
GATA5_662 51	85	181	Forward primer	CGTTTGTTTAGAAATCGAGGAAATC
		182	Reverse primer	CATAAAAACGACCGACTCGAA
		183	Beacon	CGACATGCGGGGTTTTTCGTTAGTGTATTCGTTTTAGCATGTCTG
GATA5_662 52	141	184	Forward primer	TTCGTATTTTCGTTATTTATTCGGTT
		185	Reverse primer	GAAACTATAAAACCCCGCA
		186	Beacon	CGACATGCCCGGGTTTTTCGATGGTAGCGTTTTGTACGGCATGTCTG
GATA5_662 54	131	187	Forward primer	CGAGTTTTTCGTTAGGTCGTTT
		188	Reverse primer	ACTCGACTCACACCCGAAC
		189	Beacon	CGACATGCGTACGTTTCGGGCGTCGGTTTTTCGGCATGTCTG
GATA5_662 55	119	190	Forward primer	CGCGAGTTTTTCGTTAGGTC
		191	Reverse primer	CGAACAAATAAAACAACATCGAA
		189	Beacon	CGACATGCGTACGTTTCGGGCGTCGGTTTTTCGGCATGTCTG
GATA5_662 56	95	192	Forward primer	TCGGGATTTTGAGGTTTC
		193	Reverse primer	CTACGAATACCGCTACGCC
		194	Beacon	CGACATGCGGGATTTCGTCGGTTTTTTGGCGTAGGGCATGTCTG

Table 7 - Additional assay designs: Primer and amplifluor sequences for determining the methylation status of GATA5, with predicted amplification product lengths shown.

Assay name	Amplification length	SEQ ID	Oligonucleotides	5' to 3' Sequences
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		NO	
GATA5_12671_S_AMP	90	195 Forward primer	AGCGATGCGTTCGAGCATCGCUTTTTTTCGATGTTGTTTTATTTGTTTC
		196 Reverse primer	ATAACTATCTACGCCCCAACCGA
GATA5_12671_A_S_AMP	90	197 Forward primer	TTTTTCGATGTTGTTTTATTTGTTTC
		198 Reverse primer	AGCGATGCGTTCGAGCATCGCUATAACTATCTACGCCCCAACCGA
GATA5_66214_S_AMP	70	199 Forward primer	AGCGATGCGTTCGAGCATCGCUTTTCGTGTAGTTTTATGTAGAGTTCG
		200 Reverse primer	GCTATAACGACGAAACTCGAA
GATA5_66214_A_S_AMP	70	201 Forward primer	TTCGTGTAGTTTTATGTAGAGTTCG
		202 Reverse primer	AGCGATGCGTTCGAGCATCGCUGCTATAACGACGAAACTCGAA
GATA5_66236_S_AMP	73	203 Forward primer	AGCGATGCGTTCGAGCATCGCUTTACGCGTTAGAAATGCGGTG
		204 Reverse primer	CACCGAAAATACGAACGAAA
GATA5_66236_A_S_AMP	73	205 Forward primer	TTAGGCGTTAGAAATGCGGTG
		206 Reverse primer	AGCGATGCGTTCGAGCATCGCUCACCGAAAATACGAACGAAA
GATA5_66239_S_AMP	101	207 Forward primer	AGCGATGCGTTCGAGCATCGCUGGTCTTAAGTTTGGGTTTATTC
		208 Reverse primer	AAAACCTACATAAAAACGCCGCTA
GATA5_66239_A_S_AMP	101	209 Forward primer	GGTCGTTAAGTTTGGGTTTATTC
		210 Reverse primer	AGCGATGCGTTCGAGCATCGCUAAAACCTACATAAAAACGCCGCTA
GATA5_66240_S_AMP	93	207 Forward primer	AGCGATGCGTTCGAGCATCGCUGGTCTTAAGTTTGGGTTTATTC
		211 Reverse primer	ATAAAAACGCCGCTACCGC
GATA5_66240_A_S_AMP	93	209 Forward primer	GGTCGTTAAGTTTGGGTTTATTC
		212 Reverse primer	AGCGATGCGTTCGAGCATCGCUATAAAAACGCCGCTACCGC
GATA5_66241_S_AMP	78	213 Forward primer	AGCGATGCGTTCGAGCATCGCUCGTTAAGTTTGGGTTTATTC
		214 Reverse primer	CTACCGCGAAACAACTCCG
GATA5_66241_A_S_AMP	78	215 Forward primer	CGTTAAGTTTGGGTTTATTCGGT
		216 Reverse primer	AGCGATGCGTTCGAGCATCGCUCTACCGCGAAACAACTCCG
GATA5_66248_S_AMP	86	217 Forward primer	AGCGATGCGTTCGAGCATCGCUGTTAGAAATCGAGGAAATCGC
		218 Reverse primer	GACTTCCATAAAAACGACCGA
GATA5_66248_A_S_AMP	86	219 Forward primer	GTTTAGAAATCGAGGAAATCGC
		220 Reverse primer	AGCGATGCGTTCGAGCATCGCUGACTTCCATAAAAACGACCGA
GATA5_66249_S_AMP	80	217 Forward primer	AGCGATGCGTTCGAGCATCGCUGTTAGAAATCGAGGAAATCGC
		182 Reverse primer	CATAAAAACGACCGACTCGAA
GATA5_66249_A_S_AMP	80	219 Forward primer	GTTTAGAAATCGAGGAAATCGC
		221 Reverse primer	AGCGATGCGTTCGAGCATCGCUCATAAAAACGACCGACTCGAA
GATA5_66257_S_AMP	78	222 Forward primer	AGCGATGCGTTCGAGCATCGCUTTTGCGTGGTCGTAAGGTC

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GATA5_66257_A S_AMP	78	223	Reverse primer	AAATAAACCCCGAACCGAA
		224	Forward primer	TTTGCGTGGTCGTAAGGTC
		225	Reverse primer	AGCGATGCGTTCGAGCATCGCUAAATAAACCCCGAACCGAA
GATA5_66246_S _AMP	70	226	Forward primer	AGCGATGCGTTCGAGCATCGCUCGGGGTTTCGTTAGTGTAT TTC
		227	Reverse primer	AAACCGACTTCCATAAAAACGA
GATA5_66246_A S_AMP	70	228	Forward primer	CGGGGTTTTCGTTAGTGTATTTTC
		229	Reverse primer	AGCGATGCGTTCGAGCATCGCUAAACCGACTTCCATAAAAAC GA

In a further specific embodiment, the methods of the invention employ or rely upon or utilise primers selected from the primers comprising the nucleotide sequences set forth in Tables 8 and 9 below to determine the methylation status of OSMR. The tables present specific primer combinations for determining the methylation status of this gene and the primer pairs may be selected according to the table. Table 8 also sets forth specific probes which may be utilised to facilitate (quantitative) detection of the methylation status of OSMR and Table 9 incorporates Amplifluor sequences which allow the primers to act as hairpin primers, thus facilitating quantitative detection (as discussed in detail herein).

Table 8 Primer pairs and probes (molecular beacons) for determining the methylation status of OSMR, with predicted amplification product lengths shown.

Assay name	Amplicon length	SEQ ID NO	Oligonucleotides & probes	5' to 3' Sequences (all the beacons are 5'-FAM and 3'-DABCYL)
OSMR_1	148	230	Forward primer	GTGTTAAGAGTGCGTAGTAAGACG
		231	Reverse primer	GAAACGAACGTACAAAAACGA
		232	Beacon	CGACATGCCGAAACTATAATCAACTACGAAACAAACGCGCAT GTCC
OSMR_2	142	233	Forward primer	TTAAGTAAACGTTGGGTAGAGGC
		234	Reverse primer	CTCGATAACTTTTCCGACGA

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OSMR_252 59	138	235 Beacon	CGACATGCCGAGGAGGGGAACGGGTTGTTGGCATGTCTG
		236 Forward primer	TGTTTCGTTTCGTTTCGTAAGTTC
		237 Reverse primer	TACAATTTCCCGTCTTACTACGC
		238 Beacon	CGACATGCCGCGGTCTGTTTTTTTCGGGATTGAAGGCATGTCTG
OSMR_252 60	139	47 Forward primer	TTTGGTCGGGTAGGAGTAGC
		239 Reverse primer	CACAACCCGAACTTTACGAAC
		240 Beacon	CGACATGCCGCGGGGTACGGAGTTTCGGTCGCATGTCTG
OSMR_5	130	241 Forward primer	ACGTTGGGTAGAGGCGGTATC
		242 Reverse primer	ATAACTTTTCCGACGAACGAAC
		243 Beacon	CGACATGCACCCATCCCGACTAAACGCCACGCATGTCTG
OSMR_663 07	120	244 Forward primer	GTATAGTACGGGGTTCGTTCTG
		245 Reverse primer	ACTCGTAAACCCCTTCGCC
		246 Beacon	CGACATGCCGTAGGGCGCGAGTAGAGCGCATGTCTG
OSMR_663 08	124	247 Forward primer	GGTAGAGGCGGTATCGAGG
		242 Reverse primer	ATAACTTTTCCGACGAACGAAC
		248 Beacon	CGACATGCCGGATGGGTTGCCAAGTTGTCTGCATGTCTG
OSMR_663 09	130	249 Forward primer	ACGTTGGGTAGAGGCGGTA
		242 Reverse primer	ATAACTTTTCCGACGAACGAAC
		250 Beacon	CGACACGCGTTTAGTCGGGATGGGTTGCGTGTCTG
OSMR_663 10	76	251 Forward primer	CGGTATCGAGGAGGGGAAC
		252 Reverse primer	AAATCCGACAACCTTCGCAA
		253 Beacon	CGACATGCGTTGTTGTATTTTCGGTCGCGTTTAGTCGCATGTCTG
OSMR_663 11	84	247 Forward primer	GGTAGAGGCGGTATCGAGG
		252 Reverse primer	AAATCCGACAACCTTCGCAA
		254 Beacon	CGACATGCCGGGTGTTGTATTTTCGGTCGCGGCATGTCTG
OSMR_663 12	120	255 Forward primer	TAGGTAGGTAGGTCTGGGGGC
		256 Reverse primer	CGAAAATACAACAACCCGTTT
		257 Beacon	CGACATGCGTTGGGTAGAGGCGGTATCGCATGTCTG
OSMR_Sid	142	258 Forward primer	TTCGTGCGTTTTTGGTCTG
		259 Reverse primer	CGAACTTTACGAACGAACG
		240 Beacon	CGACATGCCGCGGGTACGGAGTTTCGGTCGCATGTCTG

Table 9 - Additional assay designs: Primer and amplifluor sequences for determining the methylation status of OSMR, with predicted amplification product lengths shown.

Assay name	Amplification length	SEQ ID NO	Oligonucleotides	5' to 3' Sequences
OSMR_25258_S_AMP	135	260	Forward primer	AGCGATGCGTTTCGAGCATCGCUAGAGTGCCTAGTAAGACGGGA
		261	Reverse primer	ACGTACAAAAACGACCCGAAC
OSMR_25258_AS_AMP	135	262	Forward primer	AGAGTGCCTAGTAAGACGGGA
		263	Reverse primer	AGCGATGCGTTTCGAGCATCGCUACGTACAAAAACGACCCGAAC
OSMR_25264_S_AMP	65	264	Forward primer	AGCGATGCGTTTCGAGCATCGCUGCGTAGCGTTGTTTTGTTTC

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		265	Reverse primer	CGACTTACCTCTAATTCCGCC
OSMR_25264_AS_AMP	65	266	Forward primer	GCGTAGCGTTGTTTTGTTTC
		267	Reverse primer	AGCGATGCGTTTCGAGCATCGCUCGACTTACCTCTAATTCCGCC
OSMR_66305_S_AMP	142	260	Forward primer	AGCGATGCGTTTCGAGCATCGCUAGAGTGCCTAGTAAGACGGGA
		231	Reverse primer	GAAACGAACGTACAAAAACGA
OSMR_66305_AS_AMP	142	262	Forward primer	AGAGTGCCTAGTAAGACGGGA
		268	Reverse primer	AGCGATGCGTTTCGAGCATCGCUGAAACGAACGTACAAAAACGA
OSMR_66306_S_AMP	98	260	Forward primer	AGCGATGCGTTTCGAGCATCGCUAGAGTGCCTAGTAAGACGGGA
		269	Reverse primer	CTACGAAACAAACGCGAAA
OSMR_66306_AS_AMP	98	262	Forward primer	AGAGTGCCTAGTAAGACGGGA
		270	Reverse primer	AGCGATGCGTTTCGAGCATCGCUCTACGAAACAAACGCGAAA
OSMR_66313_S_AMP	71	271	Forward primer	AGCGATGCGTTTCGAGCATCGCUCGAGGATTTTTCGAGCGTC
		272	Reverse primer	ATACCGCCTCTACCCAACG
OSMR_66313_AS_AMP	71	273	Forward primer	CGAGGATTTTTCGAGCGTC
		274	Reverse primer	AGCGATGCGTTTCGAGCATCGCUATACCGCCTCTACCCAACG

In a further specific embodiment, the methods of the invention employ or rely upon or utilise primers selected from the primers comprising the nucleotide sequences set forth in Table 10 below to determine the methylation status of ADAM23. The table presents specific primer combinations for determining the methylation status of this gene and the primer pairs may be selected according to the table. Table 6 also sets forth specific probes which may be utilised to facilitate (quantitative) detection of the methylation status of ADAM23.

Table 10 - Primer pairs and probes (molecular beacons) for determining the methylation status of ADAM23, with predicted amplification product lengths shown.

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Assay name	Amplicon length	SEQ ID NO	oligonucleotide	5' to 3' Sequences (all the beacon sequences are 5'-FAM and 3'-DABCYL)
ADAM23_5	99	275	Forward primer	TAACGTAAAGGGTACGGGG
		276	Reverse primer	GTCCTTCTCCTACTACCTCCGCT
		277	Beacon	CGACATGCCCGGACTCGCCTAACCTCGCAAGCATGTCTG
ADAM23_66 258	98	278	Forward primer	GTAGTAGTTCGCGGTAGTCGTTT
		279	Reverse primer	AACGCTAACAAACACCGAA
		280	Beacon	CGACATGCGCGGGTTGTAGTTTTGTCGGCGGCATGTCTG
ADAM23_66 259	169	281	Forward primer	TTCGTAGTCGTTGAAGCGG
		282	Reverse primer	GCGAACTCGAACTAAACGA
		283	Beacon	CGACATCGGGAGTGGTTGCGAGGTTAGGCGATGTCTG
ADAM23_66 260	81	284	Forward primer	GCGTCGTTTTAGTATTTTTAGGTTT
		285	Reverse primer	GACTACTCCCTCCCCGAC
		286	Beacon	CGACATGCGTTTTTCGTAGTCGTTGAAGCGGTCGGCATGTCTG
ADAM23_66 261	104	287	Forward primer	GTTTTGCGTCGTTTCGTTT
		285	Reverse primer	GACTACTCCCTCCCCGAC
		288	Beacon	CGACATGCGGTTTCGGCGGTAGTTTTTCGTAGTCGGCATGTCTG
ADAM23_66 263	106	289	Forward primer	GGGTACGGGGTTATATTTATCGT
		290	Reverse primer	CTACCGCCTACTTCTCGTCC
		291	Beacon	CGACATCGGGACGAGGCGGCGCATGTCTG
ADAM23_66 264	90	289	Forward primer	GGGTACGGGGTTATATTTATCGT
		276	Reverse primer	GTCCTTCTCCTACTACCTCCGCT
		292	Beacon	CGACATGCCCCCGCGCCTAAAAAATACTACGGCATGTCTG
ADAM23_66 265	84	293	Forward primer	GGTACGGGGTTATATTTATCGTTG
		294	Reverse primer	TCTCCTACTACCTCCGCTCG
		295	Beacon	CGACATGCCTCGTCCCGACCCCGCGCATGTCTG
ADAM23_66 266	125	296	Forward primer	GTCGAGTCGGGGATAAGTTC
		297	Reverse primer	AAAACTACTACGCCCAACGA
		298	Beacon	CGACATGCGCGGGAAAGTTAACGTAAAGGGTACGCATGTCTG
ADAM23_66 267	97	296	Forward primer	GTCGAGTCGGGGATAAGTTC
		299	Reverse primer	AACCCCGTACCCTTTACGTT
		300	Beacon	CGACGCGCGTTTTTCGTTTTTTTTGTAGGGTTTCGCGTCTG
ADAM23_66 268	133	301	Forward primer	AAGGAAAGGTCGAGTCGGG
		297	Reverse primer	AAAACTACTACGCCCAACGA
		302	Beacon	CGACATGCGTAGGGTTTCGCGGGAAAGTTAACGGCATGTCTG
ADAM23_66 269	108	301	Forward primer	AAGGAAAGGTCGAGTCGGG
		303	Reverse primer	TATAACCCCGTACCCTTTACGTT
		304	Beacon	CGACATGCAGTTTCGGAGTATACGGATTCGCGCGCATGTCTG

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ADAM23_66 271	97	305	Forward primer	TTCGTCGGTTATACGGAGC
		306	Reverse primer	GACAAACTACAACCCGCCA
		307	Beacon	CGACATGCGGGAGTTATGAGTTATGAAGTCGTTGCGATGTCG
ADAM23_A	112	308	Forward primer	GAGGTTTTAAGTTGGCGGAGC
		309	Reverse primer	ACTCGAACTAAACGACGCCC
		277	Beacon	CGACATGCCCCGACTCGCCTAACCTCGCAAGCATGTCG

In a further specific embodiment, the methods of the invention employ or rely upon or utilise primers selected from the primers comprising the nucleotide sequences set forth in Table 11 below to determine the methylation status of JPH3. The table presents specific primer combinations for determining the methylation status of this gene and the primer pairs may be selected according to the table. Table 11 also sets forth specific probes which may be utilised to facilitate (quantitative) detection of the methylation status of JPH3.

Table 11 - Primer pairs and probes (molecular beacons) for determining the methylation status of JPH3, with predicted amplification product lengths shown.

Assay name	Amplicon length	SEQ ID NO	Oligonucleotides & probes	5' to 3' Sequences (all the beacons are 5'-FAM and 3'-DABCYL)
JPH3_1(MVE)	103	310	Forward primer	TTTAATATGGTGTAGTCGTTAGCGTC
		311	Reverse primer	CCCACCTACGACTACCGCG
		312	Beacon	CGACATGCACGAAACCCGCGAACGACGACGCATGTCG
JPH3_12608	90	313	Forward primer	GGGGTAGGTTTAATTTTGACGAC
		314	Reverse primer	TAAAACCGATACAAACGCCA
		315	Beacon	CGACATGCGGTTGGGAGGACGGTAAGCGGCATGTCG
JPH3_2	123	316	Forward primer	TGTAGTCGTTAGCGTCGTCGT
		317	Reverse primer	GAAAAACAACCTCAAACCCGAA
		318	Beacon	CGACATGCACCCGCGAACGACGACGACGCATGTCG
JPH3_3	88	319	Forward primer	GTAGGTTTAATTTTGACGACGGA
		320	Reverse primer	TAAAACCGATACAAACGCCA
		321	Beacon	CGACATGCCCCGTACGCCTTACCGTCCTCGCATGTCG
JPH3_4	134	322	Forward primer	GATATAGTAGAGTCGCGGTCGTC
		323	Reverse primer	CGATTAACTAAAATTCTCCGAAA
		324	Beacon	CGACATGCCCCGAAAAACGCTCGCGACCCAGCATGTCG

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JPH3_5	127	325	Forward primer	GGGGTAGTTTAGGTTCCGGTC
		326	Reverse primer	ATATAATACAACCGCCAACGCC
		327	Beacon	CGACATGCCCGCAACGCGACAACCGCAGCATGTCTG
JPH3_67326	122	328	Forward primer	GTAGTCGTTAGCGTCGTCGT
		317	Reverse primer	GAAAAACAACCTCAAACCCGAA
		329	Beacon	CGACATGCGCGGTAGTCGTAGGTGGGCATGTCTG
JPH3_67329	128	319	Forward primer	GTAGGTTTAATTTTGACGACGGA
		330	Reverse primer	GAAACCGTAACCTCCACGAAC
		331	Beacon	CGACATGCGAGGACGGTAAGGCGTACGGGCATGTCTG
JPH3_67330	92	319	Forward primer	GTAGGTTTAATTTTGACGACGGA
		332	Reverse primer	ACCCTTAAACCGATACAAACG
		331	Beacon	CGACATGCGAGGACGGTAAGGCGTACGGGCATGTCTG
JPH3_67331	90	313	Forward primer	GGGGTAGGTTTAATTTTGACGAC
		314	Reverse primer	TAAACCGATACAAACGCCA
		331	Beacon	CGACATGCGAGGACGGTAAGGCGTACGGGCATGTCTG
JPH3_67332	115	333	Forward primer	TACGGTTTAATCGGAGGACGTAG
		334	Reverse primer	AACGAAAAATAATACCGCGAA
		335	Beacon	CGACATGCGGGCGCGATCGGAAGTACGGCATGTCTG
JPH3_67333	109	333	Forward primer	TACGGTTTAATCGGAGGACGTAG
		336	Reverse primer	AATAAATACCGCGAACCAGAA
		335	Beacon	CGACATGCGGGCGCGATCGGAAGTACGGCATGTCTG
JPH3_67334	92	333	Forward primer	TACGGTTTAATCGGAGGACGTAG
		337	Reverse primer	GAACCGAACCAGAAACGAAA
		335	Beacon	CGACATGCGGGCGCGATCGGAAGTACGGCATGTCTG
JPH3_67335	96	51	Forward primer	TTAGATTTTCGTAACCGGTGAAAAC
		52	Reverse primer	TCTCCTCCGAAAAACGCTC
		338	Beacon	CGACATGCGCGGTCTGTCGGCGGTTTTGGCATGTCTG
JPH3_67336	108	339	Forward primer	TGTAATTCGGTTTTAGATTTCTG
		52	Reverse primer	TCTCCTCCGAAAAACGCTC
		338	Beacon	CGACATGCGCGGTCTGTCGGCGGTTTTGGCATGTCTG
JPH3_67337	91	340	Forward primer	GTTTCGTTTTCTGTTTTCTGTTT
		341	Reverse primer	CTAACCTACTAAACCGCGCC
		338	Beacon	CGACATGCGCGGTCTGTCGGCGGTTTTGGCATGTCTG
JPH3_67338	97	342	Forward primer	GTTTTCTGTTCTGTTTTCTGTTT
		341	Reverse primer	CTAACCTACTAAACCGCGCC
		338	Beacon	CGACATGCGCGGTCTGTCGGCGGTTTTGGCATGTCTG
JPH3_67339	120	343	Forward primer	AGTAGTAGTAGTAATGCGGCGGT
		344	Reverse primer	CGAACGAACGAAATACGAAC
		345	Beacon	CGACATGCGCGTTTCGGGTTCTGGTTCGGCATGTCTG
JPH3_67340	126	346	Forward primer	GGGTAGTTTAGGTTCCGGTC
		326	Reverse primer	ATATAATACAACCGCCAACGCC
		347	Beacon	CGACATGCGCGGGCGTTTCGAGGGCGCATGTCTG

In specific embodiments, the methods of the invention employ or rely upon or utilise primers and/or probes selected from the primers and probes comprising the nucleotide sequences

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set forth in Table 12 below to determine the methylation status of the at least one gene. The table presents specific primer and probe combinations for certain preferred genes whose methylation status may be determined according to the methods of the invention.

Table 12 - Primer sequences and beacon (probe) sequences

	SEQ ID No	
BNIP3	348 forward primer	5'-TACGCGTAGGTTTAAAGTCGC-3'
	349 reverse primer	5'-TCCCGAACTAAACGAAACCCCG-3'
	350 beacon	5'-FAM-CGACATGCCTACGACCGCGTCGCCATTAGCATGTCTG-3'-DABCYL
FOXE1	351 forward primer	5'-TTTGTTGTTTTTCGATTGTTTC-3'
	352 reverse primer	5'-TAACGCTATAAACTCCTACCGC-3'
	353 beacon	5'-FAM-CGTCTCGTCGGGGTTCGGGCGTATTTTTTAGGTAGGCGAGACG-3'-DABCYL
JAM3	354 forward primer	5'-GGGATTATAAGTCGCGTCGC-3'
	355 reverse primer	5'-CGAACGCAAAACCGAAATCG-3'
	356 beacon	5'-FAM-CGACACGATATGGCGTTGAGGCGGTTATCGTGTCTG-3'-DABCYL
JPH3	51 forward primer	5'-TTAGATTTCTGTAACGGTGAAC-3'
	52 reverse primer	5'-TCTCCTCCGAAAAACGCTC-3'
	53 beacon	5'-FAM-CGTCTGCAACCGCCGACGACCGCGACGACGAGACG-3'-DABCYL
PHACTR3	357 forward primer	TTATTTTGCGAGCGGTTTC
	358 reverse primer	GAATACTCTAATTCACGCGACT
	359 beacon	CGACATGCGGGTTCGGTCGGCGCGGGGCATGTCTG
TFPI2	360 forward primer	5'-GTTCTGTTGGGTAAGGCGTTC-3'
	361 reverse primer	5'-CATAAACGAACACCCGAACCG-3'
	362 beacon	5'-FAM-CGACATGCACCGCGCACCTCCTCCCGCCAAGCATGTCTG-3'-DABCYL
SOX17	363 forward primer	5'-GAGATGTTTCGAGGGTTGC-3'
	364 reverse primer	5'-CCGCAATATCACTAAACCGA-3'
	365 beacon	5'-FAM-CGACATGCGTTCTGTGTTTTGGTTTGTCTCGGTTTGGCATGTCTG-3'-DABCYL
SYNE1	366 forward primer	5'-GTTGGGTTTTCTAGTTTTGTAGATCGC-3'
	367 reverse primer	5'-CTACGCCCAAACTCGACG-3'
	368 beacon	5'-FAM-CGACATGCCCCGCCCTATCGCCGAAATCGCATGTCTG-3'-DABCYL

10 In a further specific embodiment, the methods of the invention employ or rely upon or utilise primers selected

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from the primers and beacons comprising the nucleotide sequences set forth in Table 13 below to determine the methylation status of BNIP3. The table presents specific primer combinations for determining the methylation status of this gene and the primer pairs may be selected according to the table.

Table 13 - Additional assay designs: Primer and probe sequences for determining the methylation status of BNIP3, with predicted amplification product lengths shown.

Assay name	Amplicon length	SEQ ID NO	Oligonucleotides & probes	5' to 3' Sequences (all the beacons are 5'-FAM and 3'-DABCYL)
BNIP3_1340 ₉	94	369	Forward primer	AGTGTTTAGAGAGTTCGTCGGTT
		370	Reverse primer	CGTAACGAATAAACTACGCGAT
		371	Beacon	CGACATGCCGAGAATTTCGGTTATCGTTCGTCGCGCATGTCG
BNIP3_6722 ₇	159	372	Forward primer	TTTAGGTGGAATTTAGTTCGC
		373	Reverse primer	CCCTCCTACGAACATACGAAA
		374	Beacon	CGACATGCCGTGCGGTTTCGATTTCGGGTTTAAGGCATGTCCG
BNIP3_6722 ₉	160	375	Forward primer	CGGTTTAATTGCGAGACGTAG
		376	Reverse primer	AACGTAAAAACCCGCGTA
		377	Beacon	CGACATGCCGTGCGGTTTCGATTTCGGGTCATGTCCG
BNIP3_6723 ₁	107	378	Forward primer	GTTTTCGGGTTTTTGTTCGT
		379	Reverse primer	GACTCTACTCGAACCTCCGCT
		380	Beacon	CGACATGCCGGGCGTTTCGTTTCGTAGGAAGAAGGCATGTCCG
BNIP3_6723 ₂	141	381	Forward primer	TGAGGACGTGTAGGGAAGC
		382	Reverse primer	AAACGAACAAAAACCCGAAA
		383	Beacon	CGACATGCCGAGCGGTGGTTCGGAGGCATGTCCG
BNIP3_6723 ₃	153	384	Forward primer	GCGTTAGAGGGTAATTGCG
		385	Reverse primer	CTATAAATTCCTCCGACCGAAC
		386	Beacon	CGACATGCCGCGTCGGGTTGCGGGCATGTCCG
BNIP3_6723 ₅	94	387	Forward primer	TTTGATTTTCGGGCGTTTC
		388	Reverse primer	GCAACTAAAACACATCCCGC
		389	Beacon	CGACATGCCGCGATATGGCGTTAGAGGGTAATTGCGCATGTCCG
BNIP3_6723 ₆	106	390	Forward primer	GGTTTTACGGAAGTCGGG
		391	Reverse primer	AATACAAACGCGATATAAACGAA
		392	Beacon	CGACATGCCGCGTTATTTTCGTTTCGTGGACGGGCATGTCCG
BNIP3_6723 ₉	151	393	Forward primer	GATTTTCGCGTATTGTTCCG
		394	Reverse primer	GATCCAACCTACGAAACGCA
		395	Beacon	CGACATGCCGTTTGGATTTCGGGTCGGATCGGCATGTCCG

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In a further specific embodiment, the methods of the invention employ or rely upon or utilise primers selected from the primers and beacons comprising the nucleotide sequences set forth in Table 14 below to determine the methylation status of FOXE1. The table presents specific primer combinations for determining the methylation status of this gene and the primer pairs may be selected according to the table.

- 10 Table 14 - Additional assay designs: Primer and probe sequences for determining the methylation status of FOXE1, with predicted amplification product lengths shown.

Assay name	Amplicon length	SEQ ID NO	Oligonucleotides & probes	5' to 3' Sequences (all the beacons are 5'-FAM and 3'-DABCYL)
FOXE1_13297	108	396	Forward primer	TTCGTTTCGAGAAGTATTACGC
		397	Reverse primer	GCGCTAAAACTCAACGTCC
		398	Beacon	CGACATGCGAGTCGTCGGTTAGCGGGTTATTTTCGGCATGTCTG
FOXE1_13307	133	399	Forward primer	TTCGTTTCGTTAGTTATGGC
		400	Reverse primer	GATCCCTAACTCTCCGC
		401	Beacon	CGACATGCCGGGTTTTGGATTTTCGCGGTTGTCGGCATGTCTG
FOXE1_13317	111	402	Forward primer	CGGAGAGTTTAGGGGATCGT
		403	Reverse primer	CTCTATCTACACCGCGCCA
		404	Beacon	CGACATGCGTTTAGGTTGGTACGCGTTGGAGGGCATGTCTG
FOXE1_67265	118	405	Forward primer	ATCGGTGTCGTTTACGTTTC
		406	Reverse primer	GTAAATCTCCAACCCTACGAAC
		407	Beacon	CGACATGCCGGGAGGGAGGAGTCGGGCATGTCTG
FOXE1_67266	125	408	Forward primer	TAGGAATCGGTGTCGTTTAC
		409	Reverse primer	CGTAAATCTCCAACCCTACGAAC
		410	Beacon	CGACATGCCGGGAGGGAGGAGTCGGTTTCGGGCATGTCTG
FOXE1_67267	108	411	Forward primer	TGAGGTTTTTCGAGTCGGTT
		412	Reverse primer	CCACAACGTCAAAACGAAA
		413	Beacon	CGACATGCCGGGTTTTAGTCGATCGGGGCATGTCTG
FOXE1_67268	100	414	Forward primer	ACGTTTCGCGTTATGATTGTC
		415	Reverse primer	CCGACCCCTACTACCGTCT
		416	Beacon	CGACATGCCGTAGTCGGAGGTGTTGGTTATCGGCATGTCTG
FOXE1_67270	124	417	Forward primer	GAGGTTATCGTCGTTGTTCTG
		397	Reverse primer	GCGCTAAAACTCAACGTCC
		418	Beacon	CGACATGCCGGGTTGAGTCGTCGGGCATGTCTG
FOXE1_67271	116	419	Forward primer	TTAGGGATTATTTTCGGATTITTC
		420	Reverse primer	TTCTCGAAACGAACAACGAC
		421	Beacon	CGACATGCCGTTTCGGTATTAGCGCGTAAGGGGCATGTCTG

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FOXEl_67274	92	422	Forward primer	CGGTAGAAGGGGAAGCGTT
		423	Reverse primer	CTCATCGCCATAACCATCG
		424	Beacon	CGACATGCGCGTGAGGCGGCGTTCGGCATGTCTG
FOXEl_67276	90	351	Forward primer	TTTGTTCTGTTTTTCGATTGTTT
		425	Reverse primer	CTATAAACTCCTACCGCGCC
		426	Beacon	CGACATGCCGGGGTTCGGGCGTATTTTTTAGGGCATGTCTG
FOXEl_67278	98	427	Forward primer	TGTGCGCGTAGAAGAGGTTTC
		428	Reverse primer	CGAAAACAAAACATAAACGACC
		429	Beacon	CGACATGCGGTTAGAGCGAGGGTAGTTAGTATTGGGCATGTCTG
FOXEl_67279	90	430	Forward primer	GTGCGCGTAGAAGAGGTTTC
		431	Reverse primer	AAAACATAAACGACCCCCG
		432	Beacon	CGACATGCGAGCGAGGGTAGTTAGTATTGGCGGCATGTCTG

In a further specific embodiment, the methods of the invention employ or rely upon or utilise primers selected from the primers and beacons comprising the nucleotide sequences set forth in Table 15 below to determine the methylation status of JAM3. The table presents specific primer combinations for determining the methylation status of this gene and the primer pairs may be selected according to the table.

Table 15 - Additional assay designs: Primer and probe sequences for determining the methylation status of JAM3, with predicted amplification product lengths shown.

Assay name	Amplicon length	SEQ ID NO	Oligonucleotides & probes	5' to 3' Sequences (all the beacons are 5'-FAM and 3'-DABCYL)
JAM3_12721	104	433	Forward primer	TGTGTCTGGTTTAGAGTATCGTTG
		434	Reverse primer	CAATTACCATAACGACCGCC
		435	Beacon	CGACATGCGTTATTATGGTGTCTGGTTCGGTTGGGCATGTCG
JAM3_67314	108	433	Forward primer	TGTGTCTGGTTTAGAGTATCGTTG
		436	Reverse primer	GCCCCAATTACCATAACGACC
		435	Beacon	CGACATGCGTTATTATGGTGTCTGGTTCGGTTGGGCATGTCG
JAM3_67315	113	437	Forward primer	ATTTATGTGTCTGGTTTAGAGTATCG
		436	Reverse primer	GCCCCAATTACCATAACGACC
		435	Beacon	CGACATGCGTTATTATGGTGTCTGGTTCGGTTGGGCATGTCG

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JAM3_67 317	90	438 Forward primer	TCGAGTTTTAGTTTTGGTTGC
		439 Reverse primer	AAATAACGATCCTAACTCCGAAA
		440 Beacon	CGACATGCCGGTTCGGGATTTCTGGGAGGCATGTCTG
JAM3_67 318	133	441 Forward primer	TTTAGTAAGTTTTAGCGTTTACGTC
		442 Reverse primer	GAATAAACTCCTCCCAAACGAA
		443 Beacon	CGACATGCGAGGGTCTGTGTTTATCGTTCTGGGCATGTCTG

In a further specific embodiment, the methods of the invention employ or rely upon or utilise primers selected from the primers and beacons comprising the nucleotide sequences set forth in Table 16 below to determine the methylation status of PHACTR3. The table presents specific primer combinations for determining the methylation status of this gene and the primer pairs may be selected according to the table.

Table 16 - Additional assay designs: Primer and probe sequences for determining the methylation status of PHACTR3, with predicted amplification product lengths shown.

Assay name	Amplification length	SEQ ID No	Oligonucleotides & probes	5' to 3' Sequences (all the beacons are 5'-FAM and 3'-DABCYL)
PHACTR3_672 95	111	444 Forward primer		ATTTAGGTAACGGGTGGGC
		445 Reverse primer		ACTCCCCGAATACAAACGAA
		446 Beacon		CGACATGCGGTTCTGAGGTAGGTGGCGTTGGCATGTCTG
PHACTR3_672 96	128	447 Forward primer		TTCGTAGAGTGATTTAGCGTTT
		448 Reverse primer		AACGCCACCTACCTCGAAC
		449 Beacon		CGACATGCGCGGACGTCGGGGAGAAATTTAGGGCATGTCTG
PHACTR3_672 97	92	450 Forward primer		TAATTTGTTTTCGCGTCGG
		451 Reverse primer		CTAAAATCACTCTACGAACGACC
		452 Beacon		CGACATGCGGACGGGACCGGTTGTTCTGGCATGTCTG
PHACTR3_672 98	118	453 Forward primer		CGTTTCGGATGTTTGTATTAC
		454 Reverse primer		ACTCTACGAACGACCCCGC
		455 Beacon		CGACATGCCGGAGGACGGGAGCGGGCATGTCTG
PHACTR3_672 99	136	456 Forward primer		TTCGTGCGGTGATTTTGGTC

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		454	Reverse primer	ACTCTACGAACGACCCCGC
		457	Beacon	CGACATGCCGTCGGTCGGGTTTATGGTCGCATGTCG
PHACTR3_673 02	128	458	Forward primer	ACGTTGTTACGAAATCGGG
		459	Reverse primer	AAACGCCTAACTCCAACGAAA
		460	Beacon	CGACATGCGGCGTACGTTTTTCGTTTTTTGTGCGGCGGCATGTCG
PHACTR3_673 03	118	458	Forward primer	ACGTTGTTACGAAATCGGG
		461	Reverse primer	CTCCAACGAAACCTAACGCA
		460	Beacon	CGACATGCGGCGTACGTTTTTCGTTTTTTGTGCGGCGGCATGTCG
PHACTR3_673 04	110	462	Forward primer	CGTTGTTACGAAATCGGGT
		463	Reverse primer	GAAACCTAACGCACCTAAACG
		460	Beacon	CGACATGCGGCGTACGTTTTTCGTTTTTTGTGCGGCGGCATGTCG
PHACTR3_673 05	103	462	Forward primer	CGTTGTTACGAAATCGGGT
		464	Reverse primer	AACGCACCTAAACGCGCTA
		460	Beacon	CGACATGCGGCGTACGTTTTTCGTTTTTTGTGCGGCGGCATGTCG
PHACTR3_673 06	93	465	Forward primer	GATACGAGGTAGTCGTTTTTCGTT
		358	Reverse primer	GAATACTCTAATTCACGCGACT
		466	Beacon	CGACATGCGCGGTTATGGGTTCCGGTCGGGCATGTCTG
PHACTR3_673 08	124	467	Forward primer	GACGTTGGGGTTATTTTGC
		358	Reverse primer	GAATACTCTAATTCACGCGACT
		468	Beacon	CGACATGCGCGATACGAGGTAGTCGTTTTTCGTTTTTCGGCATGTCTG
PHACTR3_673 09	92	469	Forward primer	CGTCGTTTTTCGTTTAGTTCGT
		470	Reverse primer	GCAAAATAACCCCAACGTCC
		471	Beacon	CGACATGCGCGGAGGAGGTGGTCGAGGCATGTCTG
PHACTR3_673 10	133	472	Forward primer	GATTGGGGATAGGAATCGC
		473	Reverse primer	AACGACGAACGAATCGAAA
		471	Beacon	CGACATGCGCGGAGGAGGTGGTCGAGGCATGTCTG
PHACTR3_673 11	113	472	Forward primer	GATTGGGGATAGGAATCGC
		474	Reverse primer	AACCCGAAACAAATAACGCT
		475	Beacon	CGACATGCGCGGTTTTTCGAATGTAGGCGGGCATGTCTG
PHACTR3_673 12	101	472	Forward primer	GATTGGGGATAGGAATCGC
		476	Reverse primer	ATAACGCTAAAAACAAACCCCG
		475	Beacon	CGACATGCGCGGTTTTTCGAATGTAGGCGGGCATGTCTG
PHACTR3_673 13	92	472	Forward primer	GATTGGGGATAGGAATCGC
		477	Reverse primer	AAAACAAAACCCCGCGAAA
		475	Beacon	CGACATGCGCGGTTTTTCGAATGTAGGCGGGCATGTCTG

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In a further specific embodiment, the methods of the invention employ or rely upon or utilise primers selected from the primers and beacons comprising the nucleotide sequences set forth in Table 17 below to determine the methylation status of TFPI2. The table presents specific primer combinations for determining the methylation status of this gene and the primer pairs may be selected according to the table.

Table 17 - Additional assay designs: Primer and probe sequences for determining the methylation status of TFPI2, with predicted amplification product lengths shown.

Assay name	Amplicon length	SEQ ID No	Oligonucleotides & probes	5' to 3' Sequences (all the beacons are 5'-FAM and 3'-DABCYL)
TFPI2_12620	117	478	Forward primer	CGGGGTGATAGTTTTCGTG
		479	Reverse primer	CGACTTTCTACTCCAAACGACC
		480	Beacon	CGACATGCGGGTCGGTCGGACGTTCCGGCATGTCTG
TFPI2_67243	98	481	Forward primer	TAGAAATTGTTGGCGTTGTTTTC
		482	Reverse primer	TACCGAACCCCTACTTCTCCGT
		483	Beacon	CGACATGCCGTATAGGAATTGGCGGTAGTTTTCGGTGGCATGTCG
TFPI2_67244	124	484	Forward primer	TAGTCGTCGGCGTAAGGAGC
		485	Reverse primer	AAACTACGAAAACAACGCCA
		486	Beacon	CGACATGCTGGGTGCGCGTAGGGTAGCATGTCTG
TFPI2_67245	120	487	Forward primer	GTGTTTCGTTTTATGCGGGG
		488	Reverse primer	TCTTACACAATTTACAACGCGAA
		489	Beacon	CGACATGCCGTTCCGGTCGATTTTCGTCGGGCATGTCTG
TFPI2_67246	115	490	Forward primer	TTTTTGTTTTAGGCGGTTTC
		491	Reverse primer	GACGAAATAACAATCCCCGT
		489	Beacon	CGACATGCCGTTCCGGTCGATTTTCGTCGGGCATGTCTG
TFPI2_67247	106	492	Forward primer	TTCGTTAGGAAAAGTAGTAGAATCG
		493	Reverse primer	GCCAAACGCTTTCTCGAAC
		494	Beacon	CGACATGCGGGTAAGGCGTTCGAGAAAAGCGGCATGTCTG
TFPI2_67248	117	478	Forward primer	CGGGGTGATAGTTTTCGTG
		479	Reverse primer	CGACTTTCTACTCCAAACGACC
		495	Beacon	CGACATGCGTCGGTCGGACGTTTCGTTCCGGCATGTCTG
TFPI2_67250	120	496	Forward primer	GTCGTTAGTTTTTGTACGGGG
		497	Reverse primer	GAAAATCCTAAATACGCGCAA
		498	Beacon	CGACATGCGGGAGGTTTTCGACGATGTTTGTGGGCATGTCTG

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In a further specific embodiment, the methods of the invention employ or rely upon or utilise primers selected from the primers and beacons comprising the nucleotide sequences set forth in Table 18 below to determine the methylation status of SOX17. The table presents specific primer combinations for determining the methylation status of this gene and the primer pairs may be selected according to the table.

- 10 Table 18 - Additional assay designs: Primer and probe sequences for determining the methylation status of SOX17, with predicted amplification product lengths shown.

Assay name	Amplicon length	SEQ ID NO	Oligonucleotides & probes	5' to 3' Sequences (all the beacons are 5'-FAM and 3'-DABCYL)
SOX17_66067	117	499	Forward primer	GGCGTTAGAGTTTAGTTTCGGT
		500	Reverse primer	TAATCCGAATCCCACGTCC
		501	Beacon	CGACATGCGGTGTAGTTTTGGGCGCGGGCATGTCTG
SOX17_66070	131	502	Forward primer	CGGTTTAGTGATATTGCGGG
		503	Reverse primer	ACGTAAACTCGAACCACGAC
		504	Beacon	CGACATGCGATGTGTTAATGGACGGCGAGGGCATGTCTG
SOX17_66071	110	505	Forward primer	TTAGTGATATTGCGGGCGT
		506	Reverse primer	CGACCTAAACGTAAACCTAACGA
		507	Beacon	CGACATGCGGAGCGGCGAGGGCGGCATGTCTG
SOX17_66073	92	508	Forward primer	TATTGAGATGTTTCGAGGGTTGC
		509	Reverse primer	CTAAATACGCTATAAACCAAACCG
		510	Beacon	CGACATGCCGGTTCGAAGTCGTCGTTCTGTCGTCATGTCTG
SOX17_66078	96	511	Forward primer	TCGAGTTAAGGGCGAGTTTC
		512	Reverse primer	TCTAAATCTACTACGCCAACCG
		513	Beacon	CGACATGCGGTGTGGGTTAAGGACGAGCGTAAGGCATGTCTG
SOX17_66079	91	511	Forward primer	TCGAGTTAAGGGCGAGTTTC
		514	Reverse primer	ATTCTACTACGCCAACCGCT
		515	Beacon	CGACATGCCGGCGGTGATGAACGTTTTATGGGCATGTCTG
SOX17_66080	117	516	Forward primer	CGAATAGCGGAGTATCGGTC
		517	Reverse primer	ACTACGCCAACCGCTTACG
		518	Beacon	CGACATCGCGGGTCGAGTTAAGGGCGATGTCTG
SOX17_66082	119	519	Forward primer	TTAGTATTTTGTTAATTCGGCGT
		520	Reverse primer	AACGAATCCCGTATCCGAC
		521	Beacon	CGACATGCGGATTTTGTTCGTTAGTCGTTTCGTTTCGCATGTCTG

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Each and all of these primers and probes form separate aspects of the invention. In particular, the invention relates to primer pairs selected from the primer pairs disclosed herein, including in the tables (which may
5 comprise additional sequence over above the basic sequence listed). Further characteristics of these primers are summarized in the detailed description (experimental part) below. It is noted that variants of these sequences may be utilised in the present invention. In particular,
10 additional sequence specific flanking sequences may be added, for example to improve binding specificity, as required. Variant sequences preferably have at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at
15 least 99% nucleotide sequence identity with the nucleotide sequences of the primers and/or probes set forth in any of tables 2, 3, 4, 5 or 6. The primers and probe (including hairpin) structures may incorporate synthetic nucleotide analogues as appropriate or may be RNA or PNA based for
20 example, or mixtures thereof. Similarly alternative fluorescent donor and acceptor moieties/FRET pairs may be utilised as appropriate. In addition to being labelled with the fluorescent donor and acceptor moieties, the primers may include modified oligonucleotides and other appending groups
25 and labels provided that the functionality as a primer in the methods of the invention is not compromised. Similarly alternative fluorescent donor and acceptor moieties/FRET pairs may be utilised as appropriate. Molecules that are commonly used in FRET include fluorescein, 5-
30 carboxyfluorescein (FAM), 2'7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), rhodamine, 6-carboxyrhodamine (R6G), N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), 6-

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carboxy-X-rhodamine (ROX), 4-(4'-dimethylaminophenylazo) benzoic acid (DABCYL), and 5-(2'-aminoethyl) aminonaphthalene-1-sulfonic acid (EDANS). Whether a fluorophore is a donor or an acceptor is defined by its
5 excitation and emission spectra, and the fluorophore with which it is paired. For example, FAM is most efficiently excited by light with a wavelength of 488 nm, and emits light with a spectrum of 500 to 650 nm, and an emission maximum of 525 nm. FAM is a suitable donor fluorophore for
10 use with JOE, TAMRA, and ROX (all of which have their excitation maximum at 514 nm).

Thus, in one embodiment, said donor moiety and said acceptor moiety are selected from 5-carboxyfluorescein (FAM), 2'7'-
15 dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), rhodamine, 6-carboxyrhodamine (R6G), N,N,N'-tetramethyl-6-carboxyrhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX), 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS), anthranilamide, coumarin, terbium chelate derivatives,
20 Malachite green, Reactive Red 4, DABCYL, tetramethyl rhodamine, pyrene butyrate, eosine nitrotyrosine, ethidium, and Texas Red. In a further embodiment, said donor moiety is selected from fluorescein, 5-carboxyfluorescein (FAM), rhodamine, 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid
25 (EDANS), anthranilamide, coumarin, terbium chelate derivatives, Malachite green, and Reactive Red 4, and said acceptor moiety is selected from DABCYL, rhodamine, tetramethyl rhodamine, pyrene butyrate, eosine nitrotyrosine, ethidium, and Texas Red.

30

In one particular embodiment, said donor moiety is fluorescein or a derivative thereof, and said acceptor

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moiety is DABCYL. In specific embodiments, the fluorescein derivative comprises, consists essentially of or consists of 6-carboxy fluorescein.

5 For all aspects and embodiments of the invention, the primers and in particular the stem loop/hairpin structures, and/or the probes (as appropriate upon the form of detection employed) may be labelled with donor and acceptor moieties during chemical synthesis of the primers or probes or the
10 label may be attached following synthesis using any suitable method. Many such methods are available and well characterised in the art.

It is noted that the specific exemplified probe types (such
15 as the hairpin probe type employed in tables 7 and 9) may be replaced as appropriate with a different probe (or primer) type as appropriate. Equivalents are discussed herein and may be utilised as appropriate.

20 In a further embodiment, bisulphite sequencing is utilised in order to determine the methylation status of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3
25 and JAM3 (in all permutations and combinations including panels as discussed herein). Primers may be designed for use in sequencing through the important CpG islands in the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1,
30 ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein). Thus,

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primers may be designed in both the sense and antisense orientation to direct sequencing across the promoter region of the relevant gene or genes.

5 In one embodiment, in which the NDRG4 and/or NDRG2 gene is sequenced, bisulphite sequencing may be carried out by using sequencing primers which comprise, consist essentially of or consist of the following sequences, and which may be used in isolation or in combination to sequence both strands:

10

NDRG4 primers

SEQ ID NO: 570 5'- gatyggggtgttttttaggttt -3' (forward)
wherein "Y" represents a pyrimidine nucleotide

15

SEQ ID NO: 6 5'- craacaacccaaaaccctc -3' (reverse)
Wherein "r" represents a purine nucleotide.

NDRG2 primers

20

SEQ ID NO: 522 5'- ttgtgttggtattttttttttattttt -3' (forward)
SEQ ID NO: 523 5'- cccccaactcaataataaaaac -3' (reverse)

25 These sequencing primers form a further aspect of the invention, with suitable variants being included within the scope of the invention (the discussion of which applies mutatis mutandis here).

30 Other nucleic acid amplification techniques, in addition to PCR (which includes real-time versions thereof and variants such as nested PCR), may also be utilised, as appropriate, to detect the methylation status of the at least one gene

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selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as
5 discussed herein). Such amplification techniques are well known in the art, and include methods such as NASBA (Compton, 1991) , 3SR (Fahy et al., 1991) and Transcription Mediated Amplification (TMA). Other suitable amplification methods include the ligase chain reaction (LCR) (Barringer
10 et al, 1990), selective amplification of target polynucleotide sequences (US Patent No. 6,410,276), consensus sequence primed polymerase chain reaction (US Patent No 4,437,975), arbitrarily primed polymerase chain reaction (WO 90/06995), invader technology, strand
15 displacement technology, and nick displacement amplification (WO 2004/067726). This list is not intended to be exhaustive; any nucleic acid amplification technique may be used provided the appropriate nucleic acid product is specifically amplified. Thus, these amplification
20 techniques may be tied in to MSP and/or bisulphite sequencing techniques for example.

Sequence variation that reflects the methylation status at CpG dinucleotides in the original genomic DNA offers two
25 approaches to primer design. Both primer types may be utilised in the methods of the invention either alone or in combination. Firstly, primers may be designed that themselves do not cover any potential sites of DNA methylation. Sequence variations at sites of differential
30 methylation are located between the two primers. Such primers are used in bisulphite genomic sequencing, COBRA and Ms-SnuPE for example. Secondly, primers may be designed

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that anneal specifically with either the methylated or unmethylated version of the converted sequence. If there is a sufficient region of complementarity, e.g., 12, 15, 18, or 20 nucleotides, to the target, then the primer may also
5 contain additional nucleotide residues that do not interfere with hybridization but may be useful for other manipulations. Examples of such other residues may be sites for restriction endonuclease cleavage, for ligand binding or for factor binding or linkers or repeats. The
10 oligonucleotide primers may or may not be such that they are specific for modified methylated residues.

One way to distinguish between modified and unmodified DNA is to hybridize oligonucleotide primers which specifically
15 bind to one form or the other of the DNA. After hybridization, an amplification reaction can be performed and amplification products assayed. The presence of an amplification product indicates that a sample hybridized to the primer. The specificity of the primer indicates whether
20 the DNA had been modified or not, which in turn indicates whether the DNA had been methylated or not.

Another way to distinguish between modified and unmodified DNA is to use oligonucleotide probes which may also be
25 specific for certain products. Such probes may be hybridized directly to modified DNA or to amplification products of modified DNA. Oligonucleotide probes can be labelled using any detection system known in the art. These include but are not limited to fluorescent moieties,
30 radioisotope labelled moieties, bioluminescent moieties, luminescent moieties, chemiluminescent moieties, enzymes, substrates, receptors, or ligands.

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In the MSP technique, amplification is achieved with the use of primers specific for the sequence of the gene whose methylation status is to be assessed. In order to provide
5 specificity for the nucleic acid molecules, primer binding sites corresponding to a suitable region of the sequence may be selected. The skilled reader will appreciate that the nucleic acid molecules may also include sequences other than primer binding sites which are required for detection of the
10 methylation status of the gene, for example RNA Polymerase binding sites or promoter sequences may be required for isothermal amplification technologies, such as NASBA, 3SR and TMA.

15 TMA (Gen-probe Inc.) is an RNA transcription amplification system using two enzymes to drive the reaction, namely RNA polymerase and reverse transcriptase. The TMA reaction is isothermal and can amplify either DNA or RNA to produce RNA amplified end products. TMA may be combined with Gen-probe's
20 Hybridization Protection Assay (HPA) detection technique to allow detection of products in a single tube. Such single tube detection is a preferred method for carrying out the invention.

25 Whilst the genes (in particular promoters) of the invention appear to be unmethylated in normal tissues, and thus the detection of methylation (or indeed a lack of methylation) in these genes is readily observable as being significant in terms of a cancer diagnosis and also in selecting suitable
30 treatment regimens and for determining the likelihood of successful treatment or resistance to treatment with certain anti-cancer agents etc, when determining methylation status,

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it may be beneficial to include suitable controls in order to ensure the method chosen to assess this parameter is working correctly and reliably. For example, suitable controls may include assessing the methylation status of a gene known to be methylated. This experiment acts as a positive control to ensure that false negative results are not obtained (i.e. a conclusion of a lack of methylation is made even though the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) may, in fact, be methylated). The gene may be one which is known to be methylated in the sample under investigation or it may have been artificially methylated, for example by using a suitable methyltransferase enzyme, such as SssI methyltransferase. In one specific embodiment, the NDRG4/NDRG2 subfamily gene, preferably the NDRG4 and/or NDRG2 gene, may be assessed in normal lymphocytes, following treatment with SssI methyltransferase, as a positive control.

Additionally or alternatively, suitable negative controls may be employed with the methods of the invention. Here, suitable controls may include assessing the methylation status of a gene known to be unmethylated or carrying out an amplification in the absence of DNA (for example by using a water only sample). The former experiment acts as a negative control to ensure that false positive results are not obtained (i.e. a conclusion of methylation is made even though the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5,

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SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein, such as at least one gene selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC in one specific embodiment) may, in fact, be unmethylated). The gene may be one which is known to be unmethylated in the sample under investigation or it may have been artificially demethylated, for example by using a suitable DNA methyltransferase inhibitor, such as those discussed in more detail below. In one specific embodiment, the NDRG4/NDRG2 subfamily gene, in particular the NDRG4 and/or NDRG2 gene, may be assessed in normal lymphocytes as a negative control, since it has been shown for the first time herein that the NDRG4 and/or NDRG2 gene is unmethylated in normal tissues.

The application of the methods of present invention to extremely small amounts of abnormally-methylated DNA, that are released into collected fluids, in particular stools, may require the generation and amplification of a DNA library before testing for methylation of any specific gene. Suitable methods on whole genome amplification and libraries generation for such amplification (e.g. Methylplex and Enzyplex technology, Rubicon Genomics) are described in US2003/0143599, WO2004/081225 and WO2004/081183 for example. In addition, WO2005/090507 describes library generation/amplification methods that require either bisulfite conversion or non-bisulfite based application. Bisulfite treatment may occur before or after library construction and may require the use of adaptors resistant to bisulfite conversion. Meth-DOP-PCR (Di Vinci et al, 2006), a modified degenerate oligonucleotide-primed PCR

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amplification (DOP-PCR) that is combined with MSP, provides another suitable method for specific detection of methylation in small amounts of DNA. Improved management of patient care may require these existing methods and
5 techniques to supplement the methods of the invention.

As discussed in the experimental section, epigenetic silencing resulting in methylation of the NDRG4/NDRG2 subfamily gene has been shown in a number of
10 gastrointestinal cancers such as colorectal cancer and/or gastric cancer, stomach and oesophageal cancers, in particular oesophageal carcinomas. Thus, in specific embodiments, the invention provides for a method of diagnosing a gastrointestinal cancer, such as colorectal
15 cancer and/or gastric cancer and/or oesophageal cancer or predisposition to a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer comprising detecting the methylation status of the NDRG4/NDRG2 subfamily gene, wherein methylation of the gene
20 is indicative for a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer, or predisposition to a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer. Preferably, the gene is NDRG2, or NDRG4,
25 or a combination of NDRG2 and NDRG4.

Whilst the epigenetic change, in particular methylation status, of any of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4,
30 OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed

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herein, such as at least one gene selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT) may be determined in order to diagnose a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer or a predisposition thereto. In specific embodiments, the at least one gene may be selected from GATA4, OSMR, NDRG4 and SFRP2, in particular where faecal samples are utilized. Detecting an epigenetic change, in particular methylation, in these genes results in a particularly sensitive and specific diagnostic method. In a further embodiment, where plasma or serum samples are utilised, the at least one gene may be selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC and particularly selected from OSMR, NDRG4, GATA5 and ADAM23, in particular where plasma or serum samples are utilised. Detecting an epigenetic change, in particular methylation, in these genes results in a particularly sensitive and specific diagnostic method.

Additionally or alternatively, the at least one gene may be selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, such as from TFPI2, BNIP3, FOXE1, SYNE1 and SOX17, in particular TFPI2.

In embodiments in which tissue samples are utilised, the methods may comprise, consist essentially of or consist of detecting an epigenetic change in a panel of genes comprising OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5, wherein detection of the epigenetic change in at least one of the genes in the panel is indicative of a predisposition to, or the incidence of, colorectal cancer. The tissue sample may comprise, consist essentially of or consist of a

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colon and/or rectal and/or appendix sample for example, as discussed herein above.

5 In embodiments where faecal samples are employed, the at least one gene may be selected from GATA4, OSMR, NDRG4, GATA5, SERP1, ADAM23, JPH3, SFRP2, APC and MGMT. in addition, or alternatively, the at least one gene may be selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3,
10 and JAM3, such as from TFPI2, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, in particular TFPI2.

Moreover, in order to improve the sensitivity of the methods of the invention the methods may comprise detecting an
15 epigenetic change in a panel of genes comprising at least two, three, four, five or six of the genes, wherein detection of an epigenetic change in at least one of the genes in the panel is indicative of a predisposition to, or the incidence of, cancer and in particular gastrointestinal
20 cancers as defined herein, such as colorectal cancer. The panel of genes may comprise/consist essentially of or consist of two, three, four, five or six genes.

Certain panels of genes have been found to result in
25 particularly sensitive methods for detecting a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer or a predisposition thereto - especially colorectal cancer. Accordingly, in one embodiment, the panel of genes comprises, consists
30 essentially of or consists of GATA4 and OSMR, GATA4 and NDRG4, GATA4 and SFRP2, OSMR and NDRG4, OSMR and SFRP2, NDRG4 and SFRP2, APC and SFRP2, APC and OSMR, APC and GATA4,

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APC and NDRG4, MGMT and OSMR, MGMT and GATA4, MGMT and NDRG4, MGMT and SFRP2, MGMT and APC, SFRP1 and MGMT, SFRP1 and OSMR, SFRP1 and GATA4, SFRP1 and NDRG4, SFRP1 and SFRP2, SFRP1 and APC, GATA5 and SFRP1, GATA5 and MGMT, GATA5 and OSMR, GATA5 and GATA4, GATA5 and NDRG4, GATA5 and SFRP2 or GATA5 and APC. Suitable panels incorporating other genes such as ADAM23 and/or JPH3 are also envisaged in the present invention. These embodiments are of particular applications to faecal test samples.

10

Further useful panels of genes comprise, consist essentially of or consists of SFRP1, SFRP2 and APC or SFRP2, OSMR and APC. Further panels of genes comprise, consist essentially of or consist of GATA4, OSMR and NDRG4, GATA4, OSMR and SFRP2, GATA4, NDRG4 and SFRP2 or OSMR, NDRG4 and SFRP2. One specific four gene panel consists of GATA4, OSMR, NDRG4 and SFRP2. One specific panel of at least six genes comprises, consists essentially of or consists of NDRG4, OSMR, SFRP1, ADAM23, GATA5 and MGMT. These panels may usefully be applied to faecal test samples in certain embodiments.

20

In a further specific embodiment, the panel of genes comprises, consists essentially of or consists of OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5. This embodiment applies in particular to tissue samples, which may be colon, rectal or appendix samples for example, as discussed herein.

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In certain embodiments, the panel of genes comprises, consists essentially of or consists of OSMR, NDRG4, GATA5 and ADAM23, where blood based samples and in particular plasma or serum samples are utilised.

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Thus, the invention provides a method of detecting a predisposition to, or the incidence of, early stage colorectal cancer and in particular stage 0 to II colorectal cancer in a blood sample, or derivative thereof such as a plasma or serum sample (preferably a plasma sample) comprising detecting an epigenetic change in at least one gene selected from OSMR, NDRG4, GATA5 and ADAM23, wherein detection of the epigenetic change is indicative of a predisposition to, or the incidence of, early stage colorectal cancer and in particular stage 0 to II colorectal cancer. This method may be applied to a panel consisting of these four genes.

It is noted that for each gene, it may be possible to detect an epigenetic change, in particular methylation of the gene, in a plurality of locations within the same gene. Thus, for example, a gene may incorporate more than one CpG island, or multiple sites within the same CpG island may be investigated as appropriate. As shown in the detailed description (experimental part) below, for example, OSMR can be assessed at two discrete locations, both providing useful diagnostically relevant results. The respective targets are designated herein as OSMR3 and OSMR4. In one embodiment, the panel of genes comprises, consists essentially of or consists of both OSMR3 and OSMR4. When OSMR is referred to herein, as for all other genes, reference is made to an investigation of an epigenetic change, in particular methylation which is relevant to colorectal cancer. Thus, the panels of genes in the present invention may incorporate assessment of multiple sites within the same gene as appropriate. Primers investigating multiple sites within the same genes are set forth in the tables above, see

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particularly tables 2 to 18 (and especially tables 5 to 11 and 13 to 18).

As discussed in greater detail herein, the detection of an
5 epigenetic change in each of the panel of genes may be carried out in a single reaction. Many suitable techniques allowing multiplexing are available and may be utilised in the present invention. Most depend upon use of suitable
10 fluorescent molecules having distinguishable emission spectra. The skilled person can readily select from the many fluorophores available to determine which can be used in a multiplexing context.

In one embodiment, a universal quencher is utilised together
15 with suitable fluorophore donors each having a distinguishable emission wavelength maximum. A particularly useful quencher is DABCYL. Together with a suitable quencher such as DABCYL the following fluorophores may each be utilised to allow multiplexing: Coumarin (emission
20 maximum of 475nm), EDANS (491nm), fluorescein (515nm), Lucifer yellow (523nm), BODIPY (525nm), Eosine (543nm), tetramethylrhodamine (575nm) and texas red (615nm) (Tyagi et al., Nature Biotechnology, Vol. 16, Jan 1998; 49-53).

25 It is noted that the methylation status of additional genes may also be determined in order to supplement the methods of the invention. No gene has been found to be epigenetically silenced in every similar tumour. For this reason, it may be advantageous to target multiple DNA alterations to attain
30 high rates of tumour detection. Thus, in one embodiment of the methods of the invention, the methylation status of the at least one gene selected from an NDRG2/NDRG4 subfamily

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gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is

5 analysed in combination with the methylation status of at least one other gene involved in the establishment of cancer. The at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1,

10 SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) may be combined with at least two other genes involved in the establishment of cancer. The at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4,

15 OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) may be combined with and at least three, four, five or six other genes involved in the establishment of cancer.

20 For colorectal cancer, the other genes involved in the establishment of cancer may be selected from the group consisting of SFRP1, SFRP2, GATA-4, GATA-5, CHFR, APC(2), MGMT, p16, Vimentin, p14, RASSF1a, RAB32, SEPTIN-9, RASSF2A, TMEFF2, NGFR or SMARCA3. However, any gene involved in the

25 establishment of colorectal cancer may be utilized in combination with the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all

30 permutations and combinations including panels as discussed herein) in the methods of present invention.

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Genes that become methylated early in the process of carcinogenesis are not only ideal for screening purposes, but also interesting targets for early cancer detection and for monitoring the progression or outcome of cancers. In a further aspect, the invention provides for a method of cancer prognosis (prognosis to cancer) comprising detecting epigenetic silencing of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein), wherein epigenetic silencing of the gene is indicative for cancer development. Preferably, epigenetic silencing is detected by determination of the methylation status and/or measurement of expression level of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein). In one embodiment, the subject is suffering from advanced adenomas or at risk for developing AJCC stage I, II, III or IV cancer. In another embodiment, the outcome is the survival of the subject after a surgical resection, e.g. a noncurative or curative surgical resection.

Early detection of epigenetic silencing of one of more genes may provide justification for more definitive follow up of patients who have molecular, but not yet all the pathological or clinical, features associated with the malignancy. Identification of cancer at its earliest stage while it is still localized and readily treatable may

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improve the clinical outcome in patients. Methods with a prognostic value should allow for the specific detection of tumours and not detect (benign) adenomas, and thus provide for a differential diagnosis between advanced adenoma versus
5 benign adenoma. As shown in the detailed description (experimental part), gene promoter hypermethylation of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17,
10 PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) was observed at a higher frequency in adenomas with concurrent colorectal cancer when compared to adenomas from patients that did not have colorectal cancer. This prognostic value is included
15 within the definition of diagnosis.

In a related aspect, the invention provides a method for determining the histopathological stage of cancer and in particular gastrointestinal cancer, such as colorectal
20 cancer in a sample comprising detecting an epigenetic change in at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and
25 combinations including panels as discussed herein), wherein detection of the epigenetic change is indicative of the histopathological stage of the cancer, such as colorectal cancer for example. All embodiments of the methods of the invention are hereby incorporated as appropriate and are not
30 repeated for reasons of conciseness. The epigenetic change is generally one causing gene silencing. Preferably, epigenetic silencing is detected by determination of the

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methylation status and/or measurement of expression levels of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) and the methylation status and/or expression level of the gene or genes is correlated to a histopathological stage of cancer. In this method, a sample is obtained from a subject suffering from, or suspected of suffering from any appropriate cancer in accordance with this invention, such as colorectal cancer for example. The methylation level of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein), the expression level of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein), or a combination thereof is determined and correlated to a histopathological stage of the cancer. The "stage" of a cancer is a descriptor (usually numbers I to IV) of how much the cancer has spread. The stage often takes into account the size of a tumour, how deep it has penetrated, whether it has invaded adjacent organs, if and how many lymph nodes it has metastasized to, and whether it has spread to distant organs. Staging of cancer is important because the stage at diagnosis is the biggest predictor of survival, and treatments are often changed

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based on the stage. As aforementioned, the description of suitable methods for determining epigenetic silencing of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) apply *mutatis mutandis* to these aspects of the invention and are not repeated here simply for reasons of conciseness.

10

In a specific embodiment, the invention provides a method for determining the histopathological stage of a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer or a predisposition thereto in a tissue or blood sample, or derivative thereof such as a plasma or serum sample. The most suitable genes and combinations of genes are described hereinabove for these specific test samples (at least one gene selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC for blood samples for example) and are not repeated for reasons of conciseness.

20

In a further specific embodiment, the invention provides a method for predicting or monitoring progression of an adenoma (to a carcinoma), in particular in the context of gastrointestinal cancers such as colorectal cancer, comprising determining the methylation status of an NDRG2/NDRG4 subfamily gene and in particular the NDRG4 gene in a suitable test sample, wherein an elevated or increased level of methylation indicates that the adenoma is more likely to progress to a carcinoma (than if the level of methylation is lower). This embodiment applies particularly

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to the region of the NDRG4 gene which is amplified using primer set 1 as set out in table 2 above. Thus, in one embodiment, these methods employ primer set 1 in order to determine the methylation status of the NDRG4 gene. As is
5 discussed below, primer pair 1 allows distinguishing of adenomas that progress to cancer from those that will not progress. This is highly important for cancer screening. The test sample may be any suitable sample, as discussed extensively above. However, the sample is generally a
.0 suitable tissue sample, in particular an adenoma sample.

In a related embodiment, detecting increased levels of methylation towards the transcription start site of the NDRG4 gene may also be useful for monitoring the progression
15 of cancer, and in particular gastrointestinal cancers such as colorectal cancer (CRC). As is shown herein, based upon the results obtained, it is predicted that spreading of methylation from more 5' regions of the promoter towards the transcription start site correlates with cancer progression
20 (for example from adenoma to carcinoma). Thus, the invention provides a method for predicting or monitoring progression of a gastrointestinal cancer, such as CRC, comprising determining the methylation status of an NDRG2/NDRG4 subfamily gene in a suitable test sample,
25 wherein an elevated or increased level of methylation towards the transcription start site indicates that the cancer is more progressed than if the level of methylation is lower. The transcription start site and promoter sequence are known from the published gene sequence
30 information. Primer set 1 and 2 as defined herein may be utilised as appropriate in these methods. Primer set 1 is used to determine the methylation status of the NDRG4 gene

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closer to the transcription start site than primer set 2. Thus, a comparison of such results may be useful in these methods.

5

As stated herein the methods of the invention for diagnostic, prognostic, or personalised medicinal care are preferentially used in connection with a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer. A number of techniques are currently available for detection of colorectal cancer. These include:

- Faecal occult blood tests (Guaiac and immunochemical)
- Colonoscopy and/or sigmoidoscopy
- 15 - X-ray after double-contrast barium enema or CT-colonography
- Faecal DNA test (PreGen-Plus®)

More accurate screening, surveillance of higher-risk patients and improved management of patient care may advantageously employ these existing methods and techniques to supplement the methods of the invention.

Faecal DNA testing is an emerging technology in screening for colorectal cancer. Pre-malignant adenomas and cancers shed DNA markers from their cells which are not degraded during the digestive process and remain stable in the stool. Capture, followed by amplification, for example using the Polymerase Chain Reaction, amplifies the DNA to detectable levels for assay. The faecal DNA integrity assay has been proposed as a useful tool for the detection of colorectal cancer. The presence of high-molecular-weight DNA fragments in stool is associated with colorectal cancer and may be

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related to disease-associated differences in the regulation of proliferation and apoptosis. Detecting colorectal cancer by testing stool for DNA may alternatively be based on identifying oncogene mutations characteristic of colorectal neoplasia that are detectable in exfoliated epithelial cells in the stool. While neoplastic bleeding is intermittent, epithelial shedding is continuous, potentially making stool-based DNA testing (also known as fecal DNA [f-DNA]) testing more sensitive than other methods. Commercially available stool-based DNA tests for colorectal cancer include PreGen-Plus™ (EXACT Sciences Corporation, Marlborough, MA 01752 USA) which is a single test that identifies the presence of 23 different microsatellite (MSI) mutations known to be associated with CRC, including mutations in BAT-26. Additionally, 21 other point mutations in other genes associated with CRC are included in this test: adenomatous polyposis coli (APC), K-ras, and protein and molecular size 53,000 daltons (p53). This test is also designed to detect long DNA fragments, which have been specifically associated with cells called non-apoptotic colonocytes, which are common in CRC.

Accordingly, molecular screening of faecal samples focused on oncogene mutations and/or DNA integrity may complement the methods of present invention. In specific embodiments, the methods of the invention are used in combination with detecting DNA integrity, or at least one DNA oncogene mutation, or a combination of both detecting DNA integrity and at least one DNA oncogene mutation in the sample in order to detect a predisposition to, or the incidence of, colorectal cancer. The methods may be carried out on a

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faecal sample. In one embodiment the method may also include the step of obtaining and/or processing the sample.

Testing can be performed diagnostically or in conjunction
5 with a therapeutic regimen. Epigenetic loss of function of
at least one gene selected from an NDRG2/NDRG4 subfamily
gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1,
ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1,
SOX17, PHACTR3 and JAM3 (in all permutations and
10 combinations including panels as discussed herein) can be
rescued by the use of DNA demethylating agents and/or DNA
methyltransferase inhibitors. Testing can be used to
determine what therapeutic or preventive regimen to employ
on a patient and be used to monitor efficacy of a
15 therapeutic regimen.

Accordingly, the invention also provides a method for
predicting the likelihood of successful treatment of a
cancer as defined herein and in particular gastrointestinal
20 cancer, such as colorectal cancer and/or gastric cancer
and/or oesophageal cancer with a DNA demethylating agent
and/or a DNA methyltransferase inhibitor and/or HDAC
inhibitor comprising detecting an epigenetic change in at
least one gene selected from an NDRG2/NDRG4 subfamily gene
25 (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23,
JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17,
PHACTR3 and JAM3 (in all permutations and combinations
including panels as discussed herein), wherein detection of
the epigenetic change is indicative that the likelihood of
30 successful treatment is higher than if the epigenetic
modification is not detected. Alternatively, the method
comprises measurement of expression levels of the at least

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one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein), wherein a reduced level of expression indicates the likelihood of successful treatment of cancer is higher than if the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is expressed at a higher level. For the avoidance of doubt it is stated that the description of suitable methods (sample types, cancer types, panels of genes etc.) for determining epigenetic silencing of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) apply *mutatis mutandis* to these aspects of the invention and are not repeated here simply for reasons of conciseness.

In an opposite scenario, the invention provides a method for predicting the likelihood of resistance to treatment of colorectal cancer with a DNA demethylating agent and/or DNA methyltransferase inhibitor and/or HDAC inhibitor comprising detecting an epigenetic change in at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed

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herein), wherein detection of the epigenetic change is indicative that the likelihood of resistance to treatment is lower than if the epigenetic modification is not detected.

- 5 Alternatively, the method comprises measurement of expression levels of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all
- 10 permutations and combinations including panels as discussed herein), wherein a higher level of expression indicates the likelihood of resistance to treatment of cancer is higher than if the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5,
- 15 SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is expressed at a reduced level.
- 20 Thus, the patient population may be selected for treatment on the basis of their methylation status with respect to the relevant at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1,
- 25 SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein - such as where the at least one gene is selected from GATA4, OSMR, NDRG4 and SFRP2 or selected from OSMR, NDRG4, GATA5 and ADAM23 where tissues or bodily fluid and in particular
- 30 faecal or blood based samples and in particular plasma samples are utilised), which leads to down regulation of gene expression of the corresponding gene. This leads to a

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much more focussed and personalised form of medicine and thus leads to improved success rates since patients will be treated with drugs which are most likely to be effective. The description of suitable methods for determining

5 epigenetic silencing of the at least one gene selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 apply *mutatis mutandis* to these aspects of the invention and are not repeated here simply for reasons of conciseness.

10

In certain aspects, epigenetic loss of function of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3

15 and JAM3 (in all permutations and combinations including panels as discussed herein) in adenoma can identify the need for treatment. Subjects having a disease such as colon neoplasia may be assayed for methylation of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in

20 particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein). Alternatively, the subject may be undergoing routine screening and may not necessarily be

25 suspected of having a disease such as colon neoplasia.

Detecting methylation of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all

30 permutations and combinations including panels as discussed herein) in an adenoma can be used to improve sensitivity and/or specificity for detecting a colon neoplasia, since

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such advanced adenoma may indicate that the probable course of the adenoma is development to a carcinoma. In such case, preventive treatment may be recommended and involve resection of the adenoma.

5

Accordingly, the invention provides a method for predicting suitable treatment of an adenoma obtained from a subject, comprising determining the methylation status of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) in an adenoma, wherein if the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is methylated, in particular hypermethylated, the need for treatment of the adenoma is identified. Preferably, the treatment comprises resection of the adenoma.

In an opposite scenario, the invention provides a method for predicting suitable treatment of an adenoma obtained from a subject, comprising determining the methylation status of at least one gene selected from at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) in an adenoma, wherein if the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular

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NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is unmethylated or methylated to a lesser degree, it is decided that there is no need of resection of the adenoma. The description of suitable methods for determining epigenetic silencing of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) apply *mutatis mutandis* to these aspects of the invention and are not repeated here simply for reasons of conciseness. The adenomas are typically of colonic origin in certain embodiments.

The invention further provides for a method of selecting a suitable treatment regimen for cancer or predisposition to cancer comprising determining epigenetic silencing of a NDRG4/2 family gene in a sample obtained from a subject, wherein if the gene is epigenetically silenced, in particular hypermethylated or reduced expressed, a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor is selected for treatment.

In an opposite scenario, the invention provides for a method of selecting a suitable treatment regimen for cancer or predisposition to cancer comprising determining the methylation status and/or expression level of a NDRG4/2 family gene in a sample obtained from a subject, wherein if the gene is unmethylated or higher expressed, treatment with a DNA demethylating agent and/or a DNA methyltransferase

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inhibitor and/or a HDAC inhibitor is contra-indicated.
Thus, alternative treatment should be explored.

In a related aspect, the invention also provides a method of
5 selecting a suitable treatment regimen for cancer, in
particular a gastrointestinal cancer such as colorectal
cancer (as defined herein), comprising detecting an
epigenetic change in at least one gene selected from an
NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4,
10 OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2,
BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all
permutations and combinations including panels as discussed
herein), wherein detection of the epigenetic change results
in selection of a DNA demethylating agent and/or a DNA
15 methyltransferase inhibitor and/or a HDAC inhibitor for
treatment and wherein if the epigenetic change is not
detected, a DNA demethylating agent and/or a DNA
methyltransferase inhibitor and/or a HDAC inhibitor is not
selected for treatment. In the event that the epigenetic
20 change is not detected (for example through gene expression
detection or any other suitable method), alternative
treatments should be explored. The description of suitable
methods for determining epigenetic silencing of the at least
one gene selected from an NDRG2/NDRG4 subfamily gene (in
25 particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3,
SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3
and JAM3 (in all permutations and combinations including
panels as discussed herein) apply *mutatis mutandis* to these
aspects of the invention and are not repeated here simply
30 for reasons of conciseness. In embodiments where blood and
in particular plasma or serum samples are utilised, the at
least one gene may be selected from OSMR, SFRP1, NDRG4,

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GATA5, ADAM23, JPH3, SFRP2 and APC. Suitable panels in this context comprise, consist essentially of or consist of OSMR, NDRG4, GATA5 and ADAM23. Additionally or alternatively, the at least one gene may be selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, such as from TFPI2, BNIP3, FOXE1, SYNE1 and SOX17, in particular TFPI2.

In embodiments where faecal samples are employed, the at least one gene may be selected from GATA4, OSMR, NDRG4, GATA5, SERP1, ADAM23, JPH3, SFRP2, APC and MGMT. In addition, or alternatively, the at least one gene may be selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3, and JAM3, such as from TFPI2, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, in particular TFPI2. Suitable panels, as defined herein, are also envisaged, such as a panel comprising, consisting essentially of or consisting of OSMR, NDRG4, GATA4 and SFRP2 for example.

In embodiments in which tissue samples are utilised, the methods may comprise, consist essentially of or consist of detecting an epigenetic change in a panel of genes comprising OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5. The tissue sample may comprise, consist essentially of or consist of a colon and/or rectal and/or appendix sample.

In another aspect, the invention provides for a method of treating cancer and in particular colorectal cancer in a subject comprising administration of a DNA demethylating agent and/or a HDAC inhibitor and/or a DNA methyltransferase inhibitor wherein the subject has been selected for treatment on the basis of a method of the invention. Accordingly, the description of suitable methods for

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determining epigenetic silencing of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3

5 (in all permutations and combinations including panels as discussed herein) apply *mutatis mutandis* to these aspects of the invention and are not repeated here simply for reasons of conciseness. Thus, in embodiments where blood and in particular plasma or serum samples are utilised, the at

10 least one gene may be selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC. Suitable panels in this context comprise, consist essentially of or consist of OSMR, NDRG4, GATA5 and ADAM23.

15 Additionally or alternatively, the at least one gene may be selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, such as from TFPI2, BNIP3, FOXE1, SYNE1 and SOX17, in particular TFPI2.

20 In embodiments where faecal samples are employed, the at least one gene may be selected from GATA4, OSMR, NDRG4, GATA5, SERP1, ADAM23, JPH3, SFRP2, APC and MGMT. In addition, or alternatively, the at least one gene may be selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3,

25 and JAM3, such as from TFPI2, FOXE1, SYNE1, SOZ17, PHACTR3 and JAM3, in particular TFPI2. Suitable panels, as defined herein, are also envisaged, such as a panel comprising, consisting essentially of or consisting of OSMR, NDRG4, GATA4 and SFRP2 for example.

30 In embodiments in which tissue samples are utilised, the methods may comprise, consist essentially of or consist of

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detecting an epigenetic change in a panel of genes comprising OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5. The tissue sample may comprise, consist essentially of or consist of a colon and/or rectal and/or appendix sample.

5

Thus, for the patient population where the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is methylated, which leads to decreased gene expression, this type of treatment is recommended. This method is referred to hereinafter as the "method of treatment" aspect of the invention.

15

In a related aspect, the invention also provides for the use of a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or HDAC inhibitor (in the manufacture of a medicament for use) in treating cancer, and in particular a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer in a subject, wherein the subject has been selected for treatment on the basis of the methods of the invention. Likewise, the invention provides a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or HDAC inhibitor for use in treating cancer, and in particular a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer in a subject, wherein the subject has been selected for treatment on the basis of the methods of the invention.

30

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For all of the relevant methods (pharmacogenetic methods, treatment regimen methods and methods of treatment) of the invention, the DNA demethylating agent may be any agent capable of up regulating transcription of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein - such as at least one gene selected from GATA4, OSMR, NDRG4, GATA5 and ADAM23). A preferred DNA demethylating agent comprises, consists essentially of or consists of a DNA methyltransferase inhibitor. The DNA methyltransferase inhibitor may be any suitable inhibitor of DNA methyltransferase which is suitable for treating cancer in the presence of methylation of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein - such as at least one gene selected from OSMR, NDRG4, GATA5 and ADAM23). As is shown in the experimental section below, methylation of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein such as at least one gene selected from OSMR, NDRG4, GATA5 and ADAM23) is linked to colorectal cancer and so preventing this methylation is predicted to help to treat a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer.

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The DNA methyltransferase inhibitor may, in one embodiment, be one which reduces expression of DNMT genes, such as suitable antisense molecules, or siRNA molecules which
5 mediate RNAi for example. The design of a suitable siRNA molecule is within the capability of the skilled person and suitable molecules can be made to order by commercial entities (see for example, www.ambion.com). In embodiments, the DNA methyltransferase gene is (human) DNMT1.

10

Alternatively, the agent may be a direct inhibitor of DNMTs. Examples include modified nucleotides such as phosphorothioate modified oligonucleotides (fig 6 of Villar-Garea, A. And Esteller, M. DNA demethylating agents
15 and chromatin-remodelling drugs: which, how and why? Current Drug Metabolism, 2003, 4, 11-31) and nucleosides and nucleotides such as cytidine analogues. Suitable examples of cytidine analogues include 5-azacytidine, 5-aza-2'-deoxycytidine, 5-fluoro-2'-deoxycytidine,
20 pseudoisocytidine, 5,6-dihydro-5-azacytidine, 1- β -D-arabinofuranosyl-5-azacytosine (known as fazabarine) (see figure 4 of Villar-Garea, A. And Esteller, M. DNA demethylating agents and chromatin-remodelling drugs: which, how and why? Current Drug Metabolism, 2003, 4, 11-
25 31).

30

In another embodiment, the DNA methyltransferase inhibitor comprises Decitabine. Full details of this drug can be found at www.supergen.com for example.

Additional DNMT inhibitors include S-Adenosyl-Methionine (SAM) related compounds like ethyl group donors such as L-

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- ethionine and non-alkylating agents such as S-adenosyl-homocysteine (SAH), sinefungin, (S)-6-methyl-6-deaminosine fungin, 6-deaminosinefungin, N4-adenosyl-N4-methyl-2,4-diaminobutanoic acid, 5'-methylthio-5'-deoxyadenosine
- 5 (MTA) and 5'-amino-5'-deoxyadenosine (Villar-Garea, A. And Esteller, M. DNA demethylating agents and chromatin-remodelling drugs: which, how and why? Current Drug Metabolism, 2003, 4, 11-31).
- 10 Further agents which may alter DNA methylation and which may, therefore, be useful in the present compositions include organohalogenated compounds such as chloroform etc, procianamide, intercalating agents such as mitomycin C, 4-aminobiphenyl etc, inorganic salts of arsenic and selenium
- 15 and antibiotics such as kanamycin, hygromycin and cefotaxim (Villar-Garea, A. And Esteller, M. DNA demethylating agents and chromatin-remodelling drugs: which, how and why? Current Drug Metabolism, 2003, 4, 11-31).
- 20 Useful DNMT inhibitors in the present invention comprise, consists essentially of or consists of 5-azacytidine and/or zebulaine.
- 25 As discussed above, one challenge faced by researchers investigating colorectal cancer is the diversity of DNA present in stool samples. The DNA of interest represents only a very small percentage of the total DNA isolated from stool. Therefore, along with the exploration of suitable DNA
- 30 markers, techniques for improved DNA isolation and enrichment of the human DNA component from faecal samples are required for more sensitive cancer detection.

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Most techniques for improved sensitivity of cancer detection from faecal samples focus on improvements in recovery of target human DNA from the total DNA. The inventors have
5 successfully improved the sensitivity of detection of colorectal cancer in faecal samples by increasing the amount of DNA used in the detection reactions. Increasing the amount of DNA in the detection reaction goes along with an increase in substances co-purified with the DNA. An
10 increase in the amount of impurities may be expected to result in PCR-inhibition, and therefore an increased level of input DNA in the detection reaction has not been previously explored for improving the sensitivity of cancer detection in faecal samples.

15 Accordingly, in a further aspect, the invention provides a method of processing a faecal sample to isolate and prepare DNA for use in detecting a predisposition to, or the incidence of, colorectal cancer in a faecal sample

20 comprising:

- (a) isolating DNA from the faecal sample
- (b) subjecting at least 2.5µg of the isolated DNA per amplification reaction required to treatment with a reagent which selectively modifies unmethylated cytosine residues in
25 the DNA contained in the sample to produce detectable modified residues but which does not modify methylated cytosine residues
- (c) amplifying the treated isolated DNA.

30 Thus, the inventors have found that by including at least 2.5µg of isolated DNA in the reagent treatment step for every downstream amplification that is required, improved

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detection methods using stool samples are achieved. The amount of DNA is expressed per amplification reaction required in particular to allow for multiple parallel reactions to be carried out on the same sample. For
5 example, test and control samples can then be run in parallel. Also, where detection of an epigenetic change, preferably methylation, in at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2,
10 BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is not carried out in the same reaction (by use of appropriate fluorophores for example) each of a panel of genes may be assessed in a separate reaction. Thus a single
15 starting sample may need to be split into a plurality of sub-samples, as required. This improves the accuracy of the results obtained by minimizing inter-sample variations. The amount of isolated DNA per amplification reaction is at least approximately 2.5µg, 3µg, 4µg, 5µg, 7.5µg, 10µg etc.
20 to improve sensitivity, and is most preferably approximately 2.5µg.

In one embodiment, the method further comprises, preferably prior to isolation of DNA from the sample, adding a
25 homogenization buffer to the faecal sample. Any suitable buffer may be utilized. Useful buffers are commercially available, for example from Amresco.

The reagent which selectively modifies unmethylated cytosine
30 residues in the DNA contained in the sample to produce detectable modified residues but which does not modify methylated cytosine residues the reagent preferably

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comprises, consists essentially of or consists of a
bisulphite reagent. Suitable reagents are discussed herein,
which discussion applies *mutatis mutandis*. In specific
embodiments, the bisulphite reagent comprises, consists
5 essentially of or consists of sodium bisulphite.

In a specific embodiment, between treatment of the isolated
DNA with the reagent and amplification of the treated
isolated DNA, the treated isolated DNA is concentrated. Any
10 suitable DNA concentration method may be utilised. For
example, a DNA-binding reagent may be utilised in order to
concentrate DNA from the sample. DNA-binding reagents may
be selected from DNA-binding buffers, DNA-binding filters,
DNA-binding columns etc. and may require use of a
15 centrifugation step. Suitable kits are commercially
available, such as the ZYMO Clean and Concentrator Kit
available from Zymo Research.

In order to achieve the necessary recovery of DNA from the
20 faecal sample, the faecal sample may be at least
approximately 4g in weight. The faecal sample may be
anywhere between approximately 2g and 10g in weight and is
most preferably around 4g in weight.

25 The methods of the invention may thus include steps such as:
- obtaining and processing a stool sample from the
subject under test, wherein preferably around (at least) 4g
stool is obtained
- adding homogenization buffer, preferably directly after
30 defecation (the subject may add this themselves). The
buffer may be added at any suitable ratio, such as at 1:7
for example

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- isolating DNA from the stool sample. As mentioned above, any suitable DNA isolation technique may be employed. This may involve (a double) low speed centrifugation.

Isolation may require RNase A and Proteinase K treatment

5 followed by DNA extraction, for example by phenol/chloroform extraction. Other DNA purification techniques can be used, as discussed herein

- subjecting the obtained DNA to bisulphite conversion, such as subjecting at least 18 to 32 µg DNA to bisulphite
10 conversion or subjecting at least 2.5 µg DNA to bisulphite conversion for each PCR reaction to be done (input expressed per PCR reaction for reason of multiplexing as discussed earlier)

- concentrating the amount of bisulphite treated DNA
15 obtained from the at least 18 to 32 µg untreated DNA

- amplifying the amount of bisulphite converted DNA. The amount to be amplified may be equivalent to 10 to 2.5 µg unconverted DNA (this equals the amounts for 4 marker panels down to use of a single marker).

20

The sensitivity of the methods for processing a faecal sample may be improved further by combining them with known methods for isolating DNA from a faecal sample. For

example, the human DNA component may be purified from a

25 stool sample using streptavidin-bound magnetic beads (Dong et al., 2001; Ahlquist et al., 2000). In a further

embodiment, an electrophoresis-driven separation of target DNA sequences, using oligonucleotide capture probes

immobilized in an acrylamide gel (Whitney et al., 2004) may

30 be utilised in order to purify human DNA from the stool

sample. In a still further embodiment, which may be used in the alternative or in combination with earlier embodiments,

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faecal samples may be frozen as quickly as possible after collection in order to preserve DNA integrity. As discussed above, DNA integrity may also be usefully tested in terms of diagnosing colorectal cancer. Additionally or
5 alternatively, stabilization buffer may be added to the faecal samples before transport of the samples (Olson et al., 2005). In a yet further complementary embodiment, Methyl-binding domain (MBD) protein may be utilised to enrich methylated human DNA from a faecal sample, in order
10 to specifically improve sensitivity for detecting methylated DNA markers in the sample (Zou et al., Clin Chem. 2007 Sep;53(9):1646-51).

In specific aspects, the methods of processing a faecal
15 sample according to the invention are combined with the other methods of the invention in order to provide improved diagnosis, histopathological analysis, pharmacogenomic analysis etc. of colorectal cancer. Accordingly, all embodiments of the methods of the invention apply *mutatis*
20 *mutandis*. Thus, the methods of the invention can be performed on the amplified treated DNA to provide particularly sensitive methods relating to colorectal cancer for example.

25

In a still further aspect, the invention provides a method of determining the methylation status of at least one gene in a blood sample, in particular a blood plasma or serum
30 sample, comprising:

(a) isolating DNA from a blood plasma or serum sample

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(b) subjecting the isolated DNA to treatment with a reagent which selectively modifies unmethylated cytosine residues in the DNA contained in the sample to produce detectable modified residues but which does not modify methylated cytosine residues

(c) amplifying the treated isolated DNA in order to determine the methylation status of at least one gene, characterised in that 0.07 to 0.72 ml blood plasma or serum sample equivalent of DNA is used per amplification reaction.

The methods thus utilise small volumes in the amplification reactions yet still maintain high sensitivity and specificity of detection. Thus, as discussed herein, a single blood sample may be advantageously utilised to determine the methylation status of a panel of genes in one embodiment. The volumes may be anywhere between around 0.07 and around 0.72 ml blood plasma or serum equivalent, and as discussed below preferably plasma equivalent, of DNA per amplification reaction. In specific embodiments, between around 0.07, 0.10, 0.15 and 0.50, 0.60, 0.70 ml, such as between 0.07 and 0.15, 0.16, 0.17, 0.18 or 0.19 ml blood plasma or serum equivalent of DNA is used per amplification reaction. In a specific embodiment, substantially the same selected volumes of blood plasma or serum sample, equivalent of DNA is used for each amplification reaction carried out. Thus, where multiple amplifications are carried out based upon a single blood sample taken from a subject, each amplification will utilise 0.07 to 0.72 ml blood plasma or serum sample, equivalent of DNA.

The blood plasma or serum sample may be derived from whole blood or any suitable plasma or serum containing

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parts/fractions thereof as appropriate. In specific embodiments, the blood plasma or serum sample comprises, consist essentially of or consists of plasma. The blood sample, from which the plasma or serum is derived may be collected using any suitable method. Many such methods are well known in the art. In one embodiment, the methods of the invention also incorporate the step of obtaining the blood sample and/or the plasma or serum sample from whole blood. Any appropriate blood sample may be utilised in the methods of the invention, provided it contains sufficient (free floating) DNA. In a specific embodiment, the volume of the blood sample, or derivative thereof that is utilised in the methods is around 5 to 15 ml, such as 10 ml.

Blood samples, or derivatives thereof and in particular plasma or serum samples, may be stored prior to use in the methods of the invention once obtained. They may be frozen, for example, at a suitable temperature. Suitable temperatures may be between around 0°C, -1°C, -2°C, -3°C, -4°C and -20°C, -30°C, -40°C, -50°C, -60°C, -70°C, -80°C, -90°C etc., such as around -80°C. They may also be stored at other temperatures, such as at 4°C or at room temperature depending upon their form. In one specific embodiment, plasma or serum is dried to allow storage at non-freezing temperatures. The drying may comprise lyophilization for example, although other dehydration techniques may be employed. Where plasma or serum is stored at temperatures greater (i.e. warmer) than freezing, and in particular greater than -80°C, antimicrobial agents such as antibiotics may be added to the sample to prevent spoiling.

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In one embodiment, stabilizers are added to the blood sample, or derivative thereof, in particular serum or plasma. This is particularly relevant where the sample is not frozen. In one specific embodiment, where the sample is serum, stabilizers such as stabilizers selected from EDTA and/or citrate and/or heparin are employed. In a further embodiment, where the sample is plasma, stabilizers such as stabilizers selected from citrate and/or heparin may be utilised.

10

It is preferred that the blood plasma or serum sample comprises, consists essentially of or consists of a plasma sample. Plasma may be derived from whole blood by any suitable means. In one embodiment, the plasma sample is obtained by centrifugation of whole blood. Centrifugation may be carried out at any suitable speed and for any suitable period of time and under any suitable conditions as may be determined by one skilled in the art. For example, centrifugation may be carried out at between around 1000 and 3000g. Centrifugation may be carried out for between around 1, 2, 3, 4, or 5 and 10, 11, 12, 13, 14 or 15 minutes for example. Centrifugation may be carried out at low temperatures, such as between around 0 and 5°C, for example 4°C, to maintain integrity of the sample. Multiple centrifugation steps may be employed in order to obtain the plasma sample. In a specific embodiment, two centrifugation steps are employed to obtain the plasma sample.

It has been shown that sensitivity of the methods of the invention may be improved by excluding samples with a plasma (or serum) volume less than around 1 to 3 ml and in particular around 2 ml (such as 1.5 to 2.5 ml) prior to

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isolating DNA. Thus, the methods may comprise determining the volume of plasma (or serum) obtained from a blood sample prior to DNA isolation. If the volume of the plasma (or serum) obtained from the blood sample is less than around 1
5 to 3 ml and in particular around 2 ml (such as 1.5 to 2.5 ml), the sample is excluded from further assessment.

As stated herein, the methods are useful for determining the methylation status of at least one gene. By "determining
10 the methylation status" is meant determining the presence or absence of 5-methylcytosine ("5-mCyt") at one or a plurality of (functionally relevant) CpG dinucleotides within the DNA sequence of the at least one gene. In particular, aberrant methylation, which may be referred to as hypermethylation,
15 of the at least one gene may be detected. Typically, the methylation status is determined in one or more CpG islands in the at least one gene. These CpG islands are often found in the promoter region of the gene(s). Thus, CpG dinucleotides are typically concentrated in the promoter
20 regions and exons of human genes and the methylation status of these CpG residues is of functional importance to whether the at least one gene is expressed. Since CpG dinucleotides susceptible to methylation are typically concentrated in the promoter region, exons and introns of human genes, promoter,
25 exon and intron regions may be assessed in order to determine the methylation status of the at least one gene. A "promoter" is a region extending typically between approximately 1 Kb, 500 bp or 150 to 300 bp upstream from the transcription start site. The CpG island may surround
30 or be positioned around the transcription start site of the at least one gene.

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The methods of the invention involve isolating/extracting/purifying DNA from the blood plasma or serum sample. Any suitable DNA isolation technique may be utilised, as discussed herein, which discussion applies here
5 mutatis mutandis. Likewise, suitable methods and kits for isolating DNA from blood samples which are commercially available are discussed and exemplified herein, which discussion applies here mutatis mutandis (see table 1). Thus, as can be derived from the table , DNA isolation may
10 be carried out using silica-membranes, isopropanol or magnetic bead based methods for example.

The methods of the invention may also, as appropriate, incorporate quantification of isolated/extracted/purified
15 DNA in the sample. Quantification of the DNA in the sample may be achieved using any suitable means. Quantitation of nucleic acids may, for example, be based upon use of a spectrophotometer, a fluorometer or a UV transilluminator. Examples of suitable techniques are described in standard
20 texts such as Molecular Cloning - A Laboratory Manual (Third Edition), Sambrook and Russell (see in particular Appendix 8 therein). In one embodiment, kits such as the Picogreen® dsDNA quantitation kit available from Molecular Probes, Invitrogen may be employed to quantify the DNA.

25

The methods of this aspect of the invention (and other aspects of the invention which involve certain types of methylation detection) rely upon a reagent which selectively modifies unmethylated cytosine residues in the DNA contained
30 in the sample to produce detectable modified residues but which does not modify methylated cytosine residues. Any suitable reagent may be utilised in the methods of the

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invention. Examples include bisulphite, hydrogen sulphite and disulphite reagents and suitable mixtures thereof. In an embodiment of the invention, the reagent comprises, consists essentially of or consists of a bisulphite reagent.

- 5 In particular, the reagent may comprise, consist essentially of or consist of sodium bisulphite.

In a specific embodiment, following treatment of the isolated DNA with the reagent, and preferably between
10 treatment of the isolated DNA with the reagent and amplification of the treated isolated DNA, the treated isolated DNA is concentrated. Any suitable DNA concentration method may be utilised. For example, a DNA-binding reagent may be utilised in order to concentrate DNA
15 from the sample. DNA-binding reagents may be selected from DNA-binding buffers, DNA-binding filters, DNA-binding columns etc. and may require use of a centrifugation step. Suitable kits are commercially available, such as the Zymo Clean and Concentrator Kit available from Zymo Research.

20 In one specific embodiment, the at least one gene whose methylation status is determined is selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC. As is discussed in detail herein, the methylation status of these
25 genes in blood plasma or serum samples is correlated with the incidence of cancer and in particular colorectal cancer. Details of these genes are provided herein which discussion applies to this aspect mutatis mutandis.

30 In a specific embodiment, the at least one gene is selected from OSMR, NDRG4, GATA5 and ADAM23 since these four genes have been shown to be particularly reliably linked to the

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incidence of colorectal cancer using blood derived samples, in particular plasma sample.

Also, these genes have been shown to be linked to early stage colorectal cancer. Accordingly, the invention provides a method of determining the methylation status of at least one gene selected from OSMR, NDRG4, GATA5 and ADAM23 in a blood sample, in particular a blood plasma or serum sample, comprising:

- 10 (a) isolating DNA from a blood plasma or serum sample
- (b) subjecting the isolated DNA to treatment with a reagent which selectively modifies unmethylated cytosine residues in the DNA contained in the sample to produce detectable modified residues but which does not modify methylated cytosine residues
- 15 (c) amplifying the treated isolated DNA in order to determine the methylation status of at least one gene, characterised in that 0.07 to 0.72 ml blood plasma or serum sample equivalent of DNA is used per amplification reaction.
- 20 This method may be utilised in order to diagnose early stage colorectal cancer, in particular stage 0 to II colorectal cancer. It may also be used to stage colorectal cancer - detection of methylated gene or genes indicates an early stage of cancer. Corresponding methods and kits are also
- 25 envisaged. These methods may additionally or alternatively be usefully applied applied to determine the methylation status of at least one gene selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, such as from TFPI2, BNIP3, FOXE1, STNE1 and SOX17, in particular TFPI2.

30

Moreover, in order to improve the sensitivity of the methods of the invention the methods may comprise determining the

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methylation status of a panel of genes comprising at least two, three, four, five or six (of the) genes. Thus, in one embodiment, the at least one gene forms part of a panel of genes comprising at least two, three, four, five or six
5 genes, wherein the methylation status of each of the genes is determined. The panel of genes may comprise, consist essentially of or consist of two, three, four, five or six genes. Suitable panels are discussed herein in respect of other aspects of the invention. That discussion and those
10 embodiments apply here mutatis mutandis.

In specific embodiments, the panel of genes comprises, consists essentially of or consists of OSMR, NDRG4, GATA5 and ADAM23. This panel may be useful in the diagnosis of
15 early stage colorectal cancer, such as stage 0 to II colorectal cancer.

It is noted that for each gene, it may be possible to determine the methylation status of the gene, in a plurality
20 of locations within the same gene (as discussed herein). Thus, for example, a gene may incorporate more than one CpG island, or multiple sites within the same CpG island may be investigated as appropriate.

25 As discussed in greater detail herein, the determination of the methylation status of each of the panel of genes may be carried out in a single reaction. Many suitable techniques allowing multiplexing are available and may be utilised in the present invention. Most depend upon use of suitable
30 fluorescent molecules having distinguishable emission spectra. The skilled person can readily select from the

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many fluorophores available to determine which can be used in a multiplexing context.

In one embodiment, a universal quencher is utilised together
5 with suitable fluorophore donors each having a distinguishable emission wavelength maximum. One suitable quencher is DABCYL. Together with a suitable quencher such as DABCYL the following fluorophores may each be utilised to allow multiplexing: Coumarin (emission maximum of 475nm),
10 EDANS (491nm), fluorescein (515nm), Lucifer yellow (523nm), BODIPY (525nm), Eosine (543nm), tetramethylrhodamine (575nm) and texas red (615nm) (Tyagi et al., Nature Biotechnology, Vol. 16, Jan 1998; 49-53).

15 As discussed above, the methylation status of additional genes may also be determined in order to supplement the methods of the invention. Other genes involved in the establishment of colorectal cancer may be selected from the group consisting of CHFR, MGMT, p16, Vimentin, p14, RASSF1a,
20 RAB32, SEPTIN-9, RASSF2A, ALX4 and SMARCA3.

The final step of the methods of the invention involve amplifying the treated isolated DNA in order to determine the methylation status of at least one gene. As discussed
25 above, this amplification utilises 0.07 to 0.72 ml blood plasma or serum sample, equivalent of DNA per amplification reaction. Any suitable amplification technique may be utilised. In a specific embodiment, the amplifying step comprises, consists essentially of or consists of the
30 polymerase chain reaction (PCR). It should be noted that whilst PCR is a preferred amplification method, to include variants on the basic technique such as nested PCR,

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equivalents may also be included within the scope of the invention. Examples include without limitation isothermal amplification techniques such as NASBA, 3SR, TMA and triamplification, all of which are well known in the art and commercially available. Other suitable amplification methods without limitation include the ligase chain reaction (LCR) (Barringer et al, 1990), MLPA, selective amplification of target polynucleotide sequences (US Patent No. 6,410,276), consensus sequence primed polymerase chain reaction (US Patent No 4,437,975), invader technology (Third Wave Technologies, Madison, WI), strand displacement technology, arbitrarily primed polymerase chain reaction (WO90/06995) and nick displacement amplification (WO2004/067726).

Various amplification based assays for determining the methylation status of at least one gene are known in the art, and can be used in conjunction with the present invention. These assays (including techniques such as methylation specific PCR) are described in greater detail herein, which description applies here mutatis mutandis and is not repeated simply for reasons of conciseness.

In specific embodiments, the methods of the invention employ or rely upon or utilise primers and/or probes selected from the primers and probes comprising the nucleotide sequences set forth in the relevant tables above (such as tables 2 to 18 and in particular tables 4 to 10) to determine the methylation status of the at least one gene. The tables present specific primer and probe combinations for certain preferred genes whose methylation status may be determined according to the methods of the invention.

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Sequence variation that reflects the methylation status at CpG dinucleotides in the original genomic DNA offers two approaches to primer design. Both primer types may be
5 utilised in the methods of the invention as discussed in detail herein, which discussion applies mutatis mutandis here. Suitable probes may also be employed, as described herein.

10 When determining methylation status, it may be beneficial to include suitable controls in order to ensure the method chosen to assess this parameter is working correctly and reliably. Suitable (positive and negative) controls are discussed in detail herein, which discussion applies mutatis
15 mutandis.

As can be derived from the discussion and examples herein, the methylation status of the at least one gene may be correlated with the incidence of a disease for specific
20 genes and specific diseases. Accordingly, the methods of the invention may be used in order to detect a predisposition to, or the incidence of, any disease for which gene methylation plays a role. In a specific embodiment, the disease comprises a cell proliferative
25 disorder, although in principle any disease may be diagnosed according to these methods provided that gene methylation can be determined in an appropriate blood plasma or serum sample. The cell proliferative disorder may comprise, consist essentially of or consist of cancer for example. In
30 particular, the cancer may comprise, consist essentially of or consist of a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and

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in particular colorectal cancer for example. Further specific gastrointestinal cancers are discussed above and each may be applicable to the present methods. As discussed herein, the methods may have particular application to early stage colorectal cancer, such as stage 0 to II colorectal cancer.

The invention also provides kits which may be used in order to carry out the methods of the invention. The kits may incorporate any of the various features, aspects and embodiments mentioned in connection with the various methods (and uses) of the invention above.

Thus, a kit is provided for:

- (a) predicting the likelihood of successful treatment of cancer (as defined herein) and in particular a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and/or the likelihood of resistance to treatment of cancer and in particular a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer with a DNA damaging agent and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor, and/or
- (b) selecting a suitable treatment regimen for cancer and in particular a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and/or
- (c) diagnosing cancer and in particular a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer or a predisposition thereto, and/or

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(d) determining the histopathological stage of cancer and in particular a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer or a predisposition thereto in a sample comprising carrier means containing therein a set of primers for use in detecting the methylation status of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein, in particular with respect to the methods of the invention). For example in one specific embodiment, the kit comprises carrier means containing therein a set of primers for use in detecting the methylation status of at least one gene selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC. Any of the NDRG2/NDRG4-family genes may be assessed using the kits of the invention. A more detailed discussion of family members is provided above.

Thus, the kit may include suitable primers for determining whether the NDRG2/NDRG4-family gene and preferably the NDRG4 and/or NDRG2 gene is methylated. These primers may comprise any of the primers discussed in detail in respect of the various methods of the invention which may be employed in order to determine the methylation status of the NDRG2/NDRG4-family gene and preferably the NDRG4 and/or NDRG2 gene. Thus, the primers in the kit may comprise, consist essentially of, or consist of primers for the purposes of amplifying methylated or unmethylated DNA (following bisulphite treatment). In one embodiment, the primers in the kit comprise, consist essentially of, or consist of primers which are capable of amplifying methylated and/or unmethylated DNA following bisulfite

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treatment which DNA comprises, consists essentially of, or consists of the nucleotide sequence set forth as SEQ ID NO: 524 and/or SEQ ID NO: 525.

5 The kit may alternatively or additionally employ bisulphite sequencing in order to determine the methylation status the NDRG2/NDRG4-family gene and in particular the NDRG4 and/or NDRG2 gene. Thus, the kit may comprise primers for use in sequencing through the important CpG islands in the
10 NDRG2/NDRG4-family gene, in particular the NDRG4 and/or NDRG2 gene. Thus, primers may be designed in both the sense and antisense orientation to direct sequencing across the promoter region of the gene. In one embodiment, the primers in the kit comprise, consist essentially of, or consist of
15 primers which are capable of sequencing of DNA following bisulfite treatment which DNA comprises, consists essentially of, or consists of the nucleotide sequence set forth as SEQ ID NO: 524 and/or SEQ ID NO: 525. Suitable primers are discussed herein in greater detail.

20

Similarly, the invention provides a kit for detecting a predisposition to, or the incidence of, a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and in particular colorectal
25 cancer in a sample comprising:

(a) means for detecting an epigenetic change in at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, and MGMT, and/or at least one gene selected from
30 TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein)

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(b) means for processing a faecal sample.

As discussed in more detail above, the at least one genemay
be selected from GATA4, OSMR, NDRG4 and SFRP2 since these
5 genes provide a particularly sensitive indication of
colorectal cancer. The at least one gene may be selected
from TFPI2, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, in
particular TFPI2.

10 The kit may comprise means for detecting an epigenetic
change in a panel of genes comprising at least two, three,
four, five or six of the genes, wherein detection of an
epigenetic change in at least one of the genes in the panel
is indicative of a predisposition to, or the incidence of,
15 colorectal cancer or is used in one of the other application
as discussed above. In one embodiment, the panel of genes
comprises two, three, four, five or six genes.

In specific embodiments, the panel of genes comprises,
20 consists essentially of or consists of GATA4 and OSMR, GATA4
and NDRG4, GATA4 and SFRP2, OSMR and NDRG4, OSMR and SFRP2
or NDRG4 and SFRP2. In a more specific embodiment, the
panel of genes comprises, consists essentially of or
consists of GATA4, OSMR and NDRG4, GATA4, OSMR and SFRP2,
25 GATA4, NDRG4 and SFRP2 or OSMR, NDRG4 and SFRP2. Further
panels comprise, consist essentially of or consist of GATA4,
OSMR, NDRG4 and SFRP2.

An alternative panel of genes comprises, consists
30 essentially of or consists of NDRG4, OSMR, SFRP1, ADAM23,
GATA5 and MGMT. The skilled person would appreciate that
other combinations and permutations may be formed as

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appropriate, as discussed in respect of the methods of the invention.

In one embodiment, the means for processing a faecal sample
5 comprise a sealable vessel for collection of a faecal
sample. Additionally or alternatively, the means for
processing a faecal sample in the kit comprises a
homogenization buffer. The means for processing a faecal
sample may further or alternatively comprise reagents for
10 extraction/isolation/concentration/purification of DNA.
Suitable reagents are known in the art and comprise, consist
essentially of or consist of alcohols such as ethanol and
isopropanol for precipitation of DNA. Salt-based
precipitation may require high concentrations of salts to
15 precipitate contaminants. The salt may comprise, consist
essentially of or consist of potassium acetate and/or
ammonium acetate for example. Organic solvents may also be
included in the kits to extract contaminants from cell
lysates. Thus, in one embodiment, the means for processing
20 the faecal sample comprise, consist essentially of or
consist of phenol, chloroform and isoamyl alcohol to extract
the DNA. Suitable combinations of reagents are envisaged as
appropriate.

25 As discussed herein, which discussion applies *mutatis*
mutandis, sensitivity of detection may be improved by
increasing the quantity of DNA in the sample. Accordingly,
in one embodiment the means for processing a faecal sample
comprises, consists essentially of or consists of primers
30 for directing amplification of DNA in the sample. Any
suitable primers which amplify the at least one gene
selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23,

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JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 may be utilised. The primers may not discriminate between methylated and unmethylated DNA (i.e. the primer binding sites lies outside of the CpG islands) thus providing a general increase in the amount of DNA prior to determining whether the methylated form of the gene or genes is present in the sample.

Similarly, the invention provides a kit for detecting a predisposition to, or the incidence of, a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and in particular colorectal cancer in a sample comprising:

(a) means for detecting an epigenetic change in at least one gene selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC and/or at least one gene selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3.

(b) means for processing a blood sample or derivative thereof.

As discussed in more detail above, the at least one gene may be selected from OSMR, NDRG4 GATA5 and ADAM23 since these genes provide a particularly sensitive indication of colorectal cancer in blood samples, or derivatives thereof and in particular plasma. The at least one gene may be selected from TFPI2, BNIP3, FOXE1, SYNE1, and SOX17, in particular TFPI2.

The kit may comprise means for detecting an epigenetic change in a panel of genes comprising at least two, three, four, five or six of the genes, wherein detection of an epigenetic change in at least one of the genes in the panel

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is indicative of a predisposition to, or the incidence of, colorectal cancer or is used in one of the other application as discussed above. In one embodiment, the panel of genes comprises two, three, four, five or six genes.

5

In one embodiment, the panel of genes comprises, consists essentially of or consists of OSMR, NDRG4, GATA5 and ADAM23. This kit may be used to diagnose early stage colorectal cancer, in particular stage 0 to II colorectal cancer.

10

In one embodiment, the means for processing a blood sample or derivative thereof comprises, consists essentially of or consists of a sealable vessel for collection of a blood sample. The means for processing a blood sample or derivative thereof may further or alternatively comprises consists essentially of or consists of a reagents for extraction/isolation/concentration/purification of DNA. Suitable reagents are known in the art and comprise, consist essentially of or consist of alcohols such as ethanol and isopropanol for precipitation of DNA. Salt-based precipitation may require high concentrations of salts to precipitate contaminants. The salt may comprise, consist essentially of or consist of potassium acetate and/or ammonium acetate for example. Organic solvents may also be included in the kits to extract contaminants from cell lysates. Thus, in one embodiment, the means for processing the blood sample or derivative thereof comprise, consist essentially of or consist of phenol, chloroform and isoamyl alcohol to extract the DNA. Suitable combinations of reagents are envisaged as appropriate. The means for processing a blood sample or derivative thereof may comprise, consist essentially of or consist of isopropanol,

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magnetic beads or a silica-based membrane for isolating DNA. The means for processing a blood sample or derivative thereof may comprise, consist essentially of or consist of a kit as shown in table 1.

5

The means for processing a blood sample or derivative thereof, in particular plasma or serum sample may comprise consist essentially of or consist of one or more stabilizers. In one embodiment, stabilizers are included in
10 the kit to be added to the blood sample, or derivative thereof. This is particularly relevant where the sample is not frozen. In one specific embodiment, where the sample is serum, stabilizers such as stabilizers selected from EDTA and/or citrate and/or heparin are included. In a further
15 embodiment, where the sample is plasma, stabilizers such as stabilizers selected from citrate and/or heparin may be included. Antimicrobial agents such as antibiotics may be also be included in the kits of the invention prevent spoiling (of serum and plasma samples).

20

Similarly, the invention provides a kit for detecting a predisposition to, or the incidence of, a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and in particular colorectal
25 cancer in a sample comprising:

- (a) means for detecting an epigenetic change in at least one gene selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT
- (b) means for processing a tissue sample, in particular a
30 colon, rectal or appendix sample.

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The kit may comprise means for detecting an epigenetic change in a panel of genes comprising at least two, three, four, five or six of the genes, wherein detection of an epigenetic change in at least one of the genes in the panel is indicative of a predisposition to, or the incidence of, a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and in particular colorectal cancer or is used in one of the other applications as discussed above. In one embodiment, the panel of genes comprises two, three, four, five or six genes.

In specific embodiments, the panel of genes comprises, consists essentially of or consists of OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5.

These kits may also be useful in predicting the likelihood of successful treatment of a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and in particular colorectal cancer and/or the likelihood of resistance to treatment of a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and in particular colorectal cancer with a DNA damaging agent and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor, and/or selecting a suitable treatment regimen for a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and in particular colorectal cancer and/or determining the histopathological stage of a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and in particular colorectal cancer in a sample, as discussed in

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respect of the methods of the invention (which discussion applies *mutatis mutandis*).

In a further embodiment, applicable to all relevant kits of the invention, the means for detecting an epigenetic change in the panel of genes enable the detection to be carried out in a single reaction. Multiplexing is made possible for example through use of appropriate fluorophores having separable emission spectra. TaqMan probes, Molecular Beacons, Scorpions, etc..., as discussed herein, allow multiple markers to be measured in the same sample (multiplex PCR), since fluorescent dyes with different emission spectra may be attached to the different probes. Accordingly, suitably labelled probes and primers are encapsulated by the kits of the invention.

In a particularly preferred embodiment, the epigenetic change which is detected using the kits of the invention is methylation. Many suitable reagents for methylation detection are known in the art, and are discussed herein (which discussion applies here *mutatis mutandis*). In particular, hypermethylation of the promoter region of the gene(s) may be detected using the kits of the invention. Thus, the means for detecting methylation may comprise methylation specific PCR primers. Suitable primers may be selected from the primers comprising, consisting essentially of or consisting of the nucleotide sequences presented in any one of tables 2 to 18 as appropriate depending upon the kit and gene or genes concerned.

30

The kit may also include means for carrying out the methylation specific PCR in real time or at end point. The

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means for carrying out the methylation specific PCR/amplification in real time or at end point may comprise hairpin primers (Amplifluor), hairpin probes (Molecular Beacons), hydrolytic probes (Taqman), FRET probe pairs (Lightcycler), primers incorporating a hairpin probe (Scorpion), fluorescent dyes (SYBR Green etc.), DzyNA primers or oligonucleotide blockers for example. Suitable probes may be selected from the probes comprising, consisting essentially of or consisting of the nucleotide sequences presented in tables 2 to 18 as appropriate for the respective genes. All appropriate combinations are envisaged by the invention. Primers and probes for detecting a suitable reference gene, such as beta-actin are displayed in some of these tables (3 and 4).

The end-point PCR fluorescence detection technique can use the same approaches as widely used for Real Time PCR - TaqMan assay, Molecular Beacons, Scorpion etc. Accordingly, the kits of the invention may, in certain embodiments, include means for carrying out end-point methylation specific PCR. The means for carrying out end-point methylation specific PCR/amplification may comprise primers and/or probes as explained for PCR/amplification in Real-time.

In the real-time and end-point detection embodiments, the probes for detection of amplification products may simply be used to monitor progress of the amplification reaction in real-time and/or they may also have a role in determining the methylation status of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT,

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TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein), themselves. Thus, the probes may be designed in much the same fashion as the primers to take advantage of sequence differences following treatment with a suitable reagent such as sodium bisulphite dependent upon the methylation status of the appropriate cytosine residues (found in CpG dinucleotides).

10 The probes may comprise any suitable probe type for real-time detection of amplification products as discussed above. Notably, however, with the AMPLIFLUOR and SCORPION embodiments, the probes are an integral part of the primers which are utilised. The probes are typically fluorescently
15 labelled, although other label types may be utilised as appropriate (such as mass labels or radioisotope labels). These probes are also suitable for end-point detection.

The kits of the invention may be kits for use in MSP and in
20 particular in a real-time or end point detection version of MSP.

The kits of the invention may incorporate reagents for quantification of DNA such as those found in the Picogreen®
25 dsDNA quantitation kit available from Molecular Probes, Invitrogen.

The kits of the invention may, additionally or alternatively comprise, consist essentially of or consist of a reagent
30 which selectively modifies unmethylated cytosine residues in the DNA contained in the sample to produce detectable modified residues but which does not modify methylated

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cytosine residues. The reagent preferably comprises, consists essentially of or consists of a bisulphite reagent. The bisulphite reagent most preferably comprises, consists essentially of or consists of sodium bisulphite. This
5 reagent is capable of converting unmethylated cytosine residues to uracil whereas methylated cytosines remain unconverted. This difference in residue may be utilised to distinguish between methylated and unmethylated nucleic acid in a downstream process, such as PCR using primers which
10 distinguish between cytosine and uracil (cytosine pairs with guanine, whereas uracil pairs with adenine). The reagent may be incorporated as the means for processing a faecal sample or means for processing a blood sample or derivative thereof depending upon the kit in question.

15
As discussed with respect to the methods of the invention, suitable controls may be utilised in order to act as quality control for the methods. Accordingly, in one embodiment, the kit of the invention further comprises, consists
20 essentially of or consists of one or more control nucleic acid molecules of which the methylation status is known. These (one or more) control nucleic acid molecules may include both nucleic acids which are known to be, or treated so as to be, methylated and/or nucleic acid molecules which
25 are known to be, or treated so as to be, unmethylated. One example of a suitable internal reference gene, which is generally unmethylated, but may be treated so as to be methylated, is β -actin.

30 Furthermore, the kit of the invention may further comprise, consist essentially of or consist of primers for the amplification of the control nucleic acid. These primers may be the same primers as those utilised to monitor

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methylation in the test sample in specific embodiments. Thus, the control nucleic acid may comprise at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein), for example taken from normal tissues in which it is known to be unmethylated. The control nucleic acid may additionally comprise at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) in methylated form, for example as methylated by a methyltransferase enzyme such as SssI methyltransferase for example.

Suitable probes and/or oligonucleotide blockers for use in determining the methylation status of the control nucleic acid molecules may also be incorporated into the kits of the invention. The probes may comprise any suitable probe type for real-time detection of amplification products. The discussion provided above applies *mutatis mutandis*.

The kits of the invention may additionally include suitable buffers and other reagents for carrying out the claimed methods of the invention. Thus, the discussion provided in respect of the methods of the invention as to the requirements for determination of the methylation status of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1,

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SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein), apply mutatis mutandis here.

5 In specific embodiments, the kit of the invention further comprises, consists essentially of, or consists of nucleic acid amplification buffers. Suitable reagents may be selected from $(\text{NH}_4)_2\text{SO}_4$, Tris (pH 8.8), MgCl_2 , β -mercaptoethanol and stock solutions of dNTPs. Reagents may
10 be supplied at any suitable concentration.

The kit may also additionally comprise, consist essentially of or consist of enzymes to catalyze nucleic acid amplification. Thus, the kit may also additionally
15 comprise, consist essentially of or consist of a suitable polymerase for nucleic acid amplification. Examples include those from both family A and family B type polymerases, such as Taq (such as the commercially available Jumpstart DNA Taq polymerase), Pfu, Vent etc.

20 The various components of the kit may be packaged separately in separate compartments or may, for example be stored together where appropriate.

25 The kit may also incorporate suitable instructions for use, which may be printed on a separate sheet or incorporated into the kit packaging for example.

In one specific aspect, the methods and kits of the
30 invention may be combined with the other methods and kits of the invention in order to provide improved diagnosis, histopathological analysis, pharmacogenomic analysis etc. of

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a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and in particular colorectal cancer. Accordingly, all embodiments of the methods and kits of the invention apply *mutatis mutandis* to
5 the respective aspects of the invention.

The invention will now be described with respect to the following non-limiting examples.

10 **DETAILED DESCRIPTION OF THE INVENTION**

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1.

- 15 a. NDRG4: Bisulfite sequencing of colorectal cancer tissue (T), normal colon mucosa (N), the methylated colorectal cancer cell line (HCT116) and the unmethylated cell line SW480. White and black squares represent methylated and unmethylated CpG
20 dinucleotides in NDRG4 respectively. Each row represents a single clone. Location of the CpG are relative to the transcription start site. The location of the MSP primers is positions 20 to 23 and 31 to 34 respectively.
- 25 b. NDRG2: Bisulfite sequencing of colon carcinoma cell lines (RKO and LS174T). White and black squares represent methylated and unmethylated CpG dinucleotides in NDRG2B respectively.

30 FIG. 2. Relative expression of NDRG4 after treatment with DAC and TSA compared to untreated cell lines. Cyclophilin was used as a reference gene for expression normalisation.

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FIG. 3. a. Methylated NDRG4 sequence (SEQ ID NO: 524) (NM_020465: -1000 to +1000 relative to TSS) Bisulfite sequence primers in mid-grey; Flank primers for the nested
5 MSP are underlined; Methylated MSP primers in light-grey; Unmethylated primers in dark-grey and light-grey

FIG. 3. b. Methylated NDRG2 sequence (SEQ ID NO: 525). Bisulfite sequence primers in mid-grey; Flank primers for
10 the nested MSP are underlined; Methylated MSP primers in light-grey; Unmethylated primers in dark-grey and light-grey

FIG. 4. Sentivity of different markers, with 100% specificity. X axis=: % positive in real time QMSP; Y
15 axis=: different markers. Case: n = 65 carcinoma's; Controls: n = 33 histologically normal resection ends
FIG. 5 shows a decision tree for determination of the methylation status of the gene of interest linked to colorectal cancer in clinical samples (real-time MSP).

20
FIG. 6 presents results of real-time MSP carried out on 9 different genes for 34 colon carcinoma tissue samples, 16 colon adenoma tissue samples and 63 breast (20), lung (21) and bladder (22) cancer samples. Sensitivity performance
25 for each gene is shown wherein the analytical cut-off was set to give 100% specificity (based on the non-cancerous controls).

FIG. 7. Presents results of real-time MSP carried out on 10
30 different genes for 34 colon carcinoma tissue samples, 16 colon adenoma tissue samples and 59 samples from patients with cancer other than CRC. Sensitivity performance for

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each gene is shown wherein the analytical cut-off was set to give 100% specificity (based on the non-cancerous controls), except for JPH3 where 95% specificity was obtained.

5 FIG. 8. Presents results of real-time MSP carried out on 8 different genes for plasma training set 1 and 5 different genes for plasma training set 2. Plasma training set 1 includes 34 samples with no suspicious findings, 25 samples from patients with cancers other than colon and 42 samples
10 from patients covering all stages of CRC, with 81% representing stages I-III of disease. Plasma training set 2 was tested on 64 samples with no suspicious findings, 49 adenomas, 25 samples from patients with cancer other than colon cancer and 78 samples from patients covering all
15 stages of CRC, with 76% representing stages I-III of disease.

FIG. 9. Is an overview of the NDRG4 study showing the patient groups which were investigated.

20 FIG. 10. Schematic representation of the promoter region of NDRG4. A dense CpG island from -556 to +869 relative to the transcription start site (TSS) (indicated by a curved arrow) is shown. Locations of CpG dinucleotides (represented by
25 |), ORF NDRG4 (as indicated with a grey rectangle) and the region of the hypermethylated fragment identified by Methylation Specific PCR (MSP), Quantative MSP (qMSP) and Bisulfite sequencing (BS) primers are indicated.

30 FIG. 11a. Results of methylation specific PCR (MSP) with primer pair 2 to detect DNA methylation in eight different CRC cell lines.

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FIG.11b Bisulfite sequencing of two CRC cell lines, namely HCT116 and SW480. Six different clones were sequenced. Each row represents an individual cloned allele that was
5 sequenced following sodium bisulfite DNA modification. Each box indicate a CpG dinucleotide (black box; methylated CpG site, white box; unmethylated CpG site)

FIG. 11c NDRG4 expression in colon cancer cell lines (RKO and HCT116) after treatment with the methylation inhibitor 5-aza-2'-doxycytidine (DAC).
10

FIG. 12a. Bisulfite sequencing of three cases of cancers (T) and their matched normal non malignant mucosa tissue
15 (N). Six different clones were sequenced.

FIG. 12b Levels of NDRG4 transcript expression measured by realtime PCR in colon cancer tissue (labelled for T) and matched normal colon tissue samples (labelled for N) for
20 three different persons. For each patient, levels of NDRG4 expression in the normal mucosa tissue were set to equal 1. The experiments were performed three times.

FIG. 12c Localization of NDRG4 expression.
25 Immunohistochemical staining of NDRG4 in normal mucosa and colon tumor shows no staining in cancer cells but clear staining in the nuclei of normal epithelial cells.

EXPERIMENTAL SECTION

30

1) NDRG EXPERIMENTS

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Cell culture

Colon cancer cell lines LS174T, HCT116, HT29, RKO, CaCo2, Colo205, SW48 and SW480 were used for MSP, bisulfite sequencing and real time (reexpression) RT-PCR (1 MM DAC and
5 300 nM TSA).

Study population

Formalin-fixed, paraffin-embedded colon mucosa tissue of colorectal cancer patients and controls over 50 years of age
10 was retrospectively collected from the archive of the dept. of Pathology of the University Hospital Maastricht. Approval was obtained by the Medical Ethical Committee (MEC) of the Maastricht University and the University Hospital Maastricht. If present, also normal and adenoma tissue was
15 collected from these cases. The control group consists of histologically normal biopsy material from patients undergoing endoscopy because of non-specific abdominal complaints, adenoma biopsies from patients which did not develop colorectal cancers within 5-10 years. Colorectal
20 cancers patients and controls were excluded if being diagnosed with additional cancers other than non-melanoma skin cancer.

Methylation-Specific PCR

25 DNA methylation in the CpG islands of the gene promoter was determined by bisulfite treatment of genomic DNA with sodium bisulfite followed by MSP. Briefly, bisulfite modification of genomic DNA was carried using the EZ DNA methylation kit (Zymo Research). MSP analysis on DNA retrieved from
30 formalin-fixed, paraffin embedded tissue was facilitated by first amplifying the DNA with flanking PCR primers which amplify bisulfite-modified DNA but do not make the

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- distinction between methylated or unmethylated DNA. This PCR product was used as a template for the MSP reaction. All PCRs were performed with controls for unmethylated DNA (DNA from normal lymphocytes), methylated DNA (normal lymphocyte DNA treated in vitro with SssI methyltransferase (New England Biolabs)), and a control without DNA. Ten µl of each MSP reaction were directly loaded onto 2% agarose visualized under UV illumination. Primer sequences and PCR conditions, are specified in Table 19.
- Alternatively, DNA methylation was determined by QMSP.

Table 19: NDRG4 and NDRG2b MSP primers

SEQ ID No	Gene	Primer name	Sequence 5'-3'	Size	Ann. Temp .	Cycles	Posn.*
5	NDRG4	Flank F	ggttygttygggattagttttagg	155 bp	56	35	-144
6	NDRG4	Flank R	craacaaccaaaccctc				+10
7	NDRG4	U sense	gattagttttaggtttggtattgtttgt	100 bp	66	25	-133
8	NDRG4	U antisense	aaaaccaaactaaaaacaatacacca				-34
9	NDRG4	M sense	tttaggttcggtatcgtttcgc	88 bp	66	25	-126
10	NDRG4	M antisense	cgaactaaaaacgatacgccg				-39
20	NDRG 2	Flank F	YGTTTTTTATTATAGYGGTTTTT				
21	NDRG 2	Flank R	TCCTAATACCTCTCCTCTTTACTAC				
22	NDRG 2	U sense	TTTTATTATAGTGGTTTTTGTATTTTT				
23	NDRG 2	U antisense	TCTCCTCTCTTACTACATCCCAACA				
24	NDRG 2	M sense	TTTATAGCGTTTTTCGTATTTTC				
25	NDRG 2	M antisense	CCTCTCTTACTACGTCCCGACG				

* Position relative to transcription start site

15

Bisulfite genomic sequencing

Genomic DNA was isolated using the Wizard Genomic DNA Purification kit (Promega, Leiden, the Netherlands).

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Bisulfite modification of genomic DNA was carried out using the EZ DNA methylation kit (Zymo Research). PCR products were subcloned using the TA cloning kit (Invitrogen, Breda, the Netherlands) and single colonies were selected and
 5 sequenced. Primer sequences and PCR conditions are specified in Table 20.

Table 20: NDRG4 and NDRG2b bisulfite sequencing primers

SEQ ID NO	Gene	Primer name	Sequence 5'-3	Size	Ann. Temp.	Cycles	Position
570	NDRG4	F	gatygggggtgttttttaggttt	262 bp	64	40	-251
6	NDRG4	R	craacaacccaaaacccctc				+10
522	NDRG2	F	TTGTGTTGGTTATTTTTTTTTTATTTTT				
523	NDRG2	R	CCCCCAAACCTCAATAATAAAAC				

10 Real-time RT-PCR

Total RNA isolation was isolated by use of the Rneasy Mini kit (Qiagen) cDNA synthesis using the Iscript cDNA synthesis kit (Bio-Rad). Quantitative real-time reverse transcription-PCR was done using SYBR Green PCR Master Mix (Applied
 15 Biosystems, Nieuwekerk a/d IJssel, the Netherlands). Primers and PCR conditions are specified in Table 21.

Table 21: NDRG4 Real time RT-PCR primers

SEQ ID NO	Gene	Primer name	Sequence 5'-3	Size	Ann. Temp.	Cycles
1	NDRG4	F	cctgaggagaagccgctg	101bp	60	40
2	NDRG4	R	atgtcatgttccttcagtcctgt			

20

Expression analysis of NDRG4

Expression of the NDRG4 gene was determined by real-time reverse-transcription PCR (RT-PCR). The NDRG4 gene was found
 25 to be well expressed in normal colon cell lines, whereas it

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was not expressed in the colon cancer cell lines. Since this on its own did not indicate that the silencing is epigenetic, the RKO and HCT116 cell lines were treated with the reagent DAC (5'dazacytidine) and TSA. Relative
5 expression of NDRG4 after treatment with DAC and TSA was compared to untreated cell lines. Cyclophilin was used as a reference gene for expression normalisation. FIG 2 shows that treatment resulted in a reactivation of NDRG4
10 expression, providing evidence for epigenetic silencing of the gene in colon cancer cells.

CpG island methylation status analysis of NDRG4 and NDRG2

Having observed that the silencing of NDRG4 expression was
15 reversed after treatment with DAC and TSA, the association between the transcriptional inactivation and the putative epigenetic aberration was further investigated. The NDRG CpG island methylation status was established by PCR
analysis of bisulfite-modified genomic DNA, which induces
20 chemical conversion of unmethylated, but not methylated, cytosine to uracil, using the procedures as specified. Table V shows that NDRG4 CpG island methylation analysed by MSP was observed in the cancer cell lines LS174T, HCT116, HT29, RKO, CaCO2 and SW48, whereas it was absent in the
25 unmethylated cell line SW480. Similarly, NDRG2 CpG island methylation analysed by MSP with different primer sets (a to d) was observed in most of the cancer cell lines. In all cancer cell lines LS174T, HCT116, HT29, RKO, CaCO2 and SW48, NDRG2 CpG island methylation was
30 observed with primer sets b of table 19.

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Table 22: Methylation status of colorectal cancer cell lines (analysed by MSP)

	LS174T	HCT116	HT29	RKO	CaC02	Colo205	SW48	SW480
NDRG4								U
NDRG2a	U	U	U	U	U	U	U	/
NDRG2b	U		U					/
NDRG2c	U		U					U?
NDRG2d(a)	U		U			U	U	M

5

Following the demonstration of the epigenetic loss of function of NDRG4 in cancer-cell lines, we assessed the prevalence of NDRG4 CpG island promoter hypermethylation in cancer patients. As expected, NDRG4 CpG island promoter hypermethylation was absent in normal mucosa from patients without cancer. As indicated in Table 23, NDRG4 CpG island promoter hypermethylation was observed with different frequency among each class of neoplasm. NDRG4 was methylated in 76% of the 88 investigated carcinoma tissues and in 57% of 57 adenomas with concurrent colorectal cancer. In adenomas from patients that did not have colorectal cancer (low-grade dysplastic non-progressed adenomas), NDRG4 methylation was significantly lower (14%), indicating the prognostic value of this NDRG4 methylation towards colorectal cancer development

20

Table 23: Prevalence of NDRG4 methylation in colorectal tissue

	Methylation (%)
Morphologically normal mucosa adjacent to tumor tissue	2.5 (n=82)
Adenomas from patients also presenting a colorectal carcinoma	57 (n=57)
Carcinoma tissue	76 (n=88)

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Normal mucosa from patients without cancer	0 (n=27)
Adenomas from patients that did not develop colorectal cancer (low-grade dysplastic non-progressed adenomas)	14 (n=51)

NDRG4 methylation compared to methylation of other markers

Samples from resected tumors and histologically normal resection were tested for hypermethylation of 13 genes. Representative results are shown in FIG 4. The highest

5 methylation was obtained for SFRP1, SFRP2, NDRG4, GATA4 and GATA5. All showed a sensitivity >40% for 100% specificity. We tested the ability of the NDRG4 methylation marker to improve the sensitivity of cancer detection with a number of methylation markers selected on their ability to detect

10 colorectal cancer. The other genes were selected from the group consisting of SFRP1, SFRP2, GATA-4, GATA-5, CHFR, APC(2), MGMT, p16, Vimentin, p14, RASSF1a and RAB32. In a first instance, the ability of NDRG4 to complement SFRP1 was analysed. 30% of colon carcinoma samples (n=18) for which

15 SFRP1 failed to be hypermethylated, showed hypermethylation for NDRG4 (n=6). Similarly, carcinoma samples which failed to be detected by way of SFRP2, GATA4, or GATA5 methylation analysis, showed hypermethylation for NDRG4. In fact, the combination of NDRG4 with any of the methylation markers

20 from FIG 4 improved diagnosis of cancer

NDRG-4 MSP on other cancer types (methylated cancers)

NDRG-4 methylation was assessed on other cancer types showing hypermethylation for certain genes. These cancer

25 types comprised melanoma, clear cell kidney cancer, ovarian carcinoma, prostate cancer, breast cancer and gastric cancer. The results were as follows:

Melanoma: 0 out of 8 samples were methylated for NDRG4

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Clear cell kidney cancer: only 1 out of 10 samples was methylated for NDRG4

Ovarium carcinoma: 0 out of 20 samples were methylated for NDRG4

5 Prostate cancer: 0 out of 10 samples were methylated for NDRG4

Breast cancer: 0/7 lobular cancers and 0/9 Ductal cancers were methylated for NDRG4

In contrast to these results, in all of the 6 gastric
10 cancers tested methylation for NDRG4 was observed. This seems to indicate that NDRG4 is a type-specific cancer methylation marker and is preferably used to detect colon cancer and/or gastric cancer.

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15

2) EXPERIMENTS ON FAECAL DNA

Example 1

MATERIALS AND METHODS IN RELATION TO FAECAL DNA

20

Sample collection and processing

- A standardized multicenter screening trial (The Netherlands) was initiated in 2006. In this trial, non symptomatic subjects aged 50 or above are screened with colonoscopy,
- 25 FOBT and real-time MSP using DNA from stool and blood. In addition, prospectively collected stool samples from multiple centers (Germany and The Netherlands) were used. In these trials, symptomatic patients, attending a Gastroenterology clinic and ultimately diagnosed with CRC,
- 30 provided a stool sample for use in real-time MSP. From the ongoing trials 147 stool samples were available for the present study. 3 main categories of stool samples were

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used: 67 samples with no suspicious findings, 58 adenomas and 22 samples from patients covering all stages of CRC, with 90% representing early stage disease.

5 After defecation in a special bucket, patients added 250 ml of stool homogenization buffer (Amresco, Solon, Ohio, USA) to the sample. Samples were shipped to the laboratory and further processed within 72 hours after defecation. Stool homogenization buffer was added to a ratio 1:7, and the
10 samples were homogenized and aliquoted in portions of 32 ml.

DNA extraction from stool

Single aliquots (32 ml containing the equivalent of 4 g of stool) were centrifuged for 5 minutes at 2540 rcf at 20°C.
15 The supernatant was retained and centrifuged a second time (10 minutes at 16500 rcf at 4°C). 22 ml of the supernatant obtained following the second centrifugation step was incubated with 5 µl Rnase A for 60 minutes at 37°C. Total DNA was then SodiumAcetate (pH 5.2) - isopropanol
20 precipitated and washed with 70% ethanol. The DNA was resuspended in 4 ml 1x TE (pH 7.4). 400 µl 10x buffer (240 mM EDTA (pH=8.0), 750 mM NaC), 400 µl 10% SDS, and 20 µl Proteinase K (20 mg/ml) was added and the samples were incubated at 48°C overnight at constant shaking (225 RPM).
25 After centrifugation (3000 RCF for 30 seconds at room temperature), 5 ml of Phenol: Chloroform:Isoamylalcohol (25:24:1, v/v; Invitrogen) was added and incubated for 10 minutes at room temperature shaking at 225 RPM and centrifuged for 5 minutes at 3000 RCF. The aqueous layer was
30 transferred to a new tube containing 5 ml of Phenol: Chloroform:Isoamylalcohol. Again, the samples were incubated for 10 minutes at room temperature shaking at 225 RPM and

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centrifuged for 5 minutes at 3000 RCF at room temperature. The aqueous layer was transferred to a new tube and DNA was precipitated by adding 500 µl 7.5 M Ammonium Acetate, 5 µl glycogen and 10 ml of cold 100% Ethanol (-20°C), further
5 incubated at -20°C for at least 1 hour and centrifuged at 15000 RCF for 30 minutes at 4°C. Pellets were washed with 3.5 ml freshly prepared 70% Ethanol and air dried. Pellets were finally resuspended in 2 ml of LoTE pH 8.0 and stored at -80°C, until further processing. Average yield of DNA was
10 462 µg (ranging from 46 - 2127 µg; SD 420)

DNA modification

An upscaled DNA modification step was applied to 32 µg of the obtained DNA. 16 Aliquots of 2 µg of DNA were subjected
15 to bisulfite modification in 96-wells format on a pipetting robot (Tecan), using the EZ-96DNA Methylation kit (Zymo Research), according to the manufacturer's protocol. Basically, aliquots of 45 µl were mixed with 5 µl of M-Dilution Buffer and incubated at 37°C for 15 minutes shaking
20 at 1100 rpm. Then 100 µl of the diluted CT Conversion Reagent was added and samples were incubated at 70°C for 3 hours, shaking at 1100 rpm in the dark. After conversion, the samples were desalted by incubation on ice for 10 minutes and addition of 400 µl of M-Binding buffer. The
25 samples were loaded on a Zymo-Spin I Column in a collection tube and after centrifugation washed with 200 µl of M-Wash Buffer. 200 µl of M-Desulphonation Buffer was put onto the column and incubated at room temperature for 15 minutes. After centrifugation of the columns, they were washed twice
30 with 200 µl of M-Wash Buffer. Finally, the DNA was washed from the column in 50 µl Tris-HCl 1mM pH8.0 and stored at -80°C, until further processing.

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DNA concentration

Bisulfite treated DNA is concentrated using the ZYMO Clean and Concentrator Kit (Zymo Research). To each aliquot of DNA
5 100 µl of DNA Binding Buffer was added. The equivalent of ~6 µg of DNA (quantified before bisulfite treatment) was transferred to a Zymo-Spin™ Column in a collection tube. (16 wells with bisulfite treated DNA per sample are divided over 5 Zymo-Spin™ columns.) The tubes were centrifuged at
10 ≥10,000 rpm for 30 seconds and washed twice with 200 µl of wash buffer. The DNA was eluted of the column by adding 6 µl of 1 mM Tris-HCl, pH=8.0, incubated for 1 minute and centrifugation at ≥10,000 rpm for 30 seconds. The eluates of columns with the same sample were pooled. The resulting
15 chemical treated DNA was used as template for real-time MSP.

DNA amplification

Real-time MSP was applied on a 7900HT fast real-time PCR system (Applied Biosystems). 2.4 µl of the modified DNA
20 (equivalent to 2,5 µg unconverted DNA) was added to a PCR mix (total volume 12 µl) containing buffer (16.6mM (NH₄)₂SO₄, 67 mM Tris (pH 8.8), 6.7 mM MgCl₂, 10 mM β-mercaptoethanol), dNTPs (5 mM), forward primer (6 ng), reverse primer (18 ng), molecular beacon (0.16 µM), BSA (0.1
25 µg), and Jumpstart DNA Taq polymerase (0.4 units; Sigma Aldrich). The primer sequences and molecular beacon sequences used for each of the genes are summarized in table 1. Cycle program used was as follows: 5 minutes 95°C, followed by 45 cycles of 30 seconds 95°C, 30 seconds 57°C
30 (51°C for APC), and 30 seconds 72°C, followed by 5 minutes 72°C. A standard curve (2x10⁶ - 20 copies) was included to

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determine copy numbers of unknown samples by interpolation of their Ct values to the standard curve.

RESULTS

5

Marker identification and validation in colon tissue samples.

Assay validity rate in tissue and stool: 230 FFPE and 147 stool samples were processed using real-time MSP. The real-time MSP assays produced valid results in 99% of the FFPE and stool samples.

Marker selection in colon tissue: Based on re-expression, 224 different gene assays representing 145 gene promoters were tested on the Base5 methylation profiling platform (data not shown, see reference 1 for details). The 37 most differentially methylated gene sequences assessing 29 gene promoters were validated on retrospectively collected tumors from 65 colorectal cancer patients (all stages) and 74 distant resection ends (histopathologically normal) using real-time MSP. Several markers reliably detected CRC in those tissue samples (data not shown). The results were confirmed on an independent test set containing 39 tissue controls (non-cancerous), 34 carcinomas and 16 adenomas. Several combinations of the tested markers reliably detected CRC with high specificity and sensitivity.

The ten best performing markers GATA5, GATA4, SFRP1, SFRP2, APC, MGMT, NDRG4, OSMR, JPH3 and ADAM23 were validated with primer sets and beacon probes as specified in Table 24. In addition to the colon test genes, the independent reference gene β -Actin (ACT) was also measured. The ratios between

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the colon test genes and ACT were calculated, and are the test result of the assay. The samples were classified as methylated, non-methylated, or invalid based on the decision tree shown in FIG. 5.

5

The individual performance of the ten markers is shown in Table 25. Dependent on the cutoff applied, different sensitivities were obtained for the individual markers. For 100% specificity of the marker, sensitivities (%) ranged from 56 to 66 for GATA5, 78 to 82 GATA4, 84 to 92 for SFRP1, 72 to 84 for SFRP2, 40 to 46 for APC, 44 for MGMT, 64 to 66 for NDRG4, 88 for OSMR, 82 for JPH3 and 50 for ADAM23.

Table 24: Primers sequences and beacon sequences

SEQ ID NO:			
26	β-Actin	forward primer	5' - TAGGGAGTATATAGGTTGGGGAAGTT - 3'
27		reverse primer	5' - AACACACAATAACAAACACAAATTCAC - 3'
28		beacon	5'-FAM-CGACTGCGTGTGGGGTGGTGATGGAGGAGGTTTAGGCAGTCG-3'-DABCYL
29	GATA4	forward primer	5' - AGGTTAGTTAGCGTTTATAGGGTC - 3'
30		reverse primer	5' - ACGACGACGAAACCTCTCG - 3'
31		beacon	5'-FAM-CGACATGCCTCGCGACTCGAATCCCCGACCCAGCATGTCG-3'-DABCYL
32	GATA5	forward primer	5' - AGTTCGTTTTTAGGTTAGTTTTCGGC - 3'
33		reverse primer	5' - CCAATACAACATAACGAACGAACCG - 3'
34		beacon	5'-FAM-CGACATGCGTAGGGAGGTAGAGGGTTCGGGATTCGTAGCATGTCG-3'-DABCYL
35	SFRP1	forward primer	5' -TGTAGTTTTTCGGAGTTAGTGTGCGCG- 3
36		reverse primer	5' -CCTACGATCGAAAACGACGCGAACG- 3'
37		beacon	5'-FAM-CGACATGCTCGGGAGTCGGGGCGTATTTAGTTTCGTAGCGGCATGTCG-3'-DABCYL
38	SFRP2	forward primer	5' - GGGTCGGAGTTTTTCGGAGTTGCGC - 3'
39		reverse primer	5' - CCGCTCTCTTCGCTAAATACGACTCG - 3'
40		beacon	5'-FAM-CGACATGCGGTGTTTCGTTTTTTCGCGTTTTAGTCGTGCGGCATGTCG -3'-DABCYL
17	NDRG4	forward primer	5' - GTATTTTAGTCGCGTAGAAGGC - 3'
18		reverse primer	5' - AATTTAACGAATATAACGCTCGAC - 3'
19		beacon	5'-FAM-CGACATGCCCGAACGAACCGGATCCCTGCATGTCG-3'-DABCYL
41	APC	forward primer	5'-GAACCAAACGCTCCCAT-3'

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42		reverse primer	5'-TTATATGTCGGTTACGTGCGTTTATAT-3'
43		beacon	5' -FAM-CGTCTGCCCCGTCGAAAACCCGCCGATTACGCAGACG-3'-DABCYL
44	ADAM23	forward primer	5' - GAAGGACGAGAAGTAGGCG - 3'
45		reverse primer	5' - CTAACGAACCTACAACCTTACCGA - 3'
46		beacon	5'-FAM-CGACATGCCCCGACCCGACGCCGCCCTGCATGTCG-3'-DABCYL
47	OSMR (3)	forward primer	5' - TTTGGTCGGGGTAGGAGTAGC - 3'
48		reverse primer	5' - CGAACTTTACGAACGAACGAAC - 3'
49		beacon	5'-FAM-CGACATGCCCCGTACCCCGCGCGCAGCATGTCG-3'-DABCYL
47	OSMR (4)	forward primer	5' - TTTGGTCGGGGTAGGAGTAGC - 3'
50		reverse primer	5' - AAAAACCTAAAAACCGAAAACTCG - 3'
49		beacon	5'-FAM-CGACATGCCCCGTACCCCGCGCGCAGCATGTCG-3'-DABCYL
51	JPH3	forward primer	5' - TTAGATTTCGTAAACGGTGAAAAC - 3'
52		reverse primer	5' - TCTCCTCCGAAAAACGCTC - 3'
53		beacon	5'-FAM-CGTCTGCAACCGCCGACGACCGCGACGACGACG-3'-DABCYL
54	MGMT	forward primer	5' - TTTGACGTTCTAGGTTTTCGC - 3'
55		reverse primer	5' - GCACTCTTCCGAAAACGAAACG - 3'
56		beacon	5'-FAM-CGTCTCGCGTGCATCGTTTTCGATTGGTGAGTGTGGGGCGAGACG-3'-DABCYL

Table 25: Individual performance of markers on adenoma and carcinoma colorectal tissue samples

Gene *	Cases (adenoma+carcinoma)	Controls	Cut off ratio**	Sensitivity (%)	Specificity (%)
GATA5	50	39	12 (5)	56 (66)	100
GATA4	50	39	17 (12)	78 (82)	100
SFRP1	50	39	47 (25)	84 (92)	100
SFRP2	50	39	28 (9)	72 (84)	100
APC	50	39	16 (5)	40 (46)	100
MGMT	50	39	18	44	100
NDRG4	50	39	7 (1)	64 (66)	100
OSMR (3)	50	39	47	88	100
JPH3	50	39	55 (75)	82 (82)	95 (100)
ADAM23	50	39	2	50	100

5 * (3) reflects the primer combinations used for assessing methylation of the OSMR gene

** In case two sets of cut off ratio were assessed, the second set and its corresponding sensitivity is indicated between ().

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Complementarity of markers

The different markers were tested on their complementarity. Several combinations of the tested markers reliably detected CRC with high specificity and sensitivity. Results are summarized in Table 26. For 100% specificity, sensitivities (%) ranged between 90 to 98 for combinations of two markers. A sensitivity of 100% was obtained for the 3-marker combinations SFRP1+SFRP2+APC and SFRP2+OSMR+APC.

10

Table 26: Complementarity of markers on adenoma and carcinoma colorectal tissue samples

Genes *	Sensitivity **	Specificity
NDRG4 + OSMR (4)	90%	100%
SFRP2 + APC	92%	100%
APC + OSMR (3)	92%	100%
MGMT + OSMR (3)	92%	100%
OSMR (3) + OSMR (4)	92%	100%
SFRP1 + APC	94%	100%
SFRP1 + GATA-4	94%	100%
SFRP1 + NDRG4	94%	100%
SFRP1 + OSMR (3)	94%	100%
SFRP1 + OSMR (4)	94%	100%
GATA-4 + OSMR (4)	94%	100%
NDRG4 + OSMR (3)	94%	100%
GATA-5 + SFRP1	96%	100%
GATA-5 + OSMR (3)	96%	100%
SFRP2 + OSMR (4)	96%	100%
GATA-4 + OSMR (3)	96%	100%
SFRP1 + SFRP2	98%	100%
SFRP2 + OSMR (3)	98%	100%
SFRP1 + SFRP2 + APC	100%	100%
SFRP2 + OSMR (3) + APC	100%	100%

* (3) and (4) reflect the primer combinations used for assessing methylation of the OSMR gene

** Sensitivity corresponding to the second cutoff set specified between () in Table 25.

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Performance of markers on adenoma and carcinoma tissue samples

5 Important for early cancer detection is the performance of the markers on early stage cancers. Therefore, the 50 cancer cases from the test set were further divided into 2 diagnosis groups: carcinomas and adenomas. Results are summarized in table 27 and 28. Sensitivity for carcinomas
 10 ranged from 35% to 88% for detection of colorectal cancer whereas sensitivity for adenomas ranged from 31% to 88% both with a corresponding specificity of 100%. These results indicate that the selected set of genes are highly specific for colorectal cancer and include some promising early stage
 15 detection markers.

Table 27: Performance of the markers on carcinoma samples

Gene *	Carcinoma	Controls	Cut off ratio	Sensitivity	Specificity
GATA5	34	39	12	53	100
GATA4	34	39	17	74	100
SFRP1	34	39	47	82	100
SFRP2	34	39	28	68	100
APC	34	39	16	35	100
MGMT	34	39	18	35	100
NDRG4	34	39	7	62	100
OSMR (3)	34	39	47	88	100
JPH3	34	39	55	82	100
ADAM23	34	39	2	59	100

* (3) reflects the primer combinations used for assessing
 20 methylation of the OSMR gene

Table 28: Performance of the markers on adenoma samples

Gene *	adenoma	Controls	Cut off ratio	Sensitivity	Specificity
GATA5	16	39	12	63	100
GATA4	16	39	17	88	100

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Gene *	adenoma	Controls	Cut off ratio	Sensitivity	Specificity
SFRP1	16	39	47	88	100
SFRP2	16	39	28	81	100
APC	16	39	16	50	100
MGMT	16	39	18	63	100
NDRG4	16	39	7	69	100
OSMR (3)	16	39	47	88	100
JPH3	16	39	55	81	100
ADAM23	16	39	2	31	100

* (3) reflects the primer combinations used for assessing methylation of the OSMR gene

Performance of markers in fecal samples

- 5 Nine of the best performing methylation markers in tissue (GATA4, GATA5, SFRP1, SFRP2, NDRG4, APC, ADAM23, OSMR3, and JPH3) were chosen to be evaluated in fecal samples. β -Actin copy numbers were also quantified as a control for sample quality and DNA yield.
- 10 Methylated copies of these genes were quantified in all available stool samples by real-time MSP on a 7900HT fast real-time PCR system (Applied Biosystems).
- The individual performance of the 9 genes (Actin, SFRP2, GATA5, GATA4, APC, SFRP1, NDRG4, OSMR3 and ADAM23) in fecal
- 15 samples from adenoma's and colorectal cancers is shown in Table 29. A specificity of 100% was obtained for most of the genes, except for SFRP2. The best performing genes in fecal samples from patients with CRC corresponded to GATA4 with 73% sensitivity, SFRP1 with 67% sensitivity, OSMR3 with
- 20 67% sensitivity, and NDRG4 with 60% sensitivity, all with a corresponding specificity of 100%.

Table 29: Performance of the markers in fecal samples

	Number	Act	SFRP2	GATA5	GATA4	APC	SFRP1	NDRG4	OSMR (3)	Adam23
cutoff (copies)	of sample s	200	1	1	4	1	1	0	10	1

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Sens adenoma	13	15%	38%	0%	15%	8%	8%	15%	8%	0%
Sens CRC	15	67%	67%	27%	73%	47%	67%	60%	67%	40%
Spec	19	95%	84%	100%	100%	100%	100%	100%	100%	100%

Performance of marker combination panels in fecal samples

Four candidate methylation markers were found to result in the best sensitivity and specificity in stool samples:

- 5 GATA4, SFRP2, NDRG4, OSMR. β -Actin copy numbers were also quantified as a control for sample quality and DNA yield. The performance of combination panels of these 4 methylation markers was investigated. Methylated copies of these genes were quantified in all available stool samples by real-time
- 10 MSP on a 7900HT fast real-time PCR system (Applied Biosystems). Table 30 shows the results and lists the cut-off (copies) applied. For instance for the most sensitive marker combination panel SFRP2+GATA4+NDRG4+OSMR, cutoff values of the individual markers were SFRP2= 2; GATA4= 4;
- 15 NDRG4= 0.1 and OSMR= 10. This combination panel had 95% specificity, 87% sensitivity for CRC, and 46% sensitivity for adenomas. The preferred 2-marker combination NDRG4+GATA4 had a 100% specificity, a sensitivity of 73% for CRC, and a 33% sensitivity for adenomas.

20

Table 30: Performance of marker combinations

	Cutoff (copies) and performance of combination panels *					
	SFRP2+ GATA4+ NDRG4	SFRP2+ NDRG4	SFRP2+ GATA4+ NDRG4+ OSMR (3)	SFRP2+ NDRG4+ OSMR (3)	NDRG4+ OSMR (3)	NDRG4+ GATA4
SFRP 2 cp	1	1	1	1	(-)	(-)
GATA4 cp	4	(-)	4	(-)	(-)	4
NDRG4 cp	0	0	0	0	0	0
OSMR (3)	(-)	(-)	10	10	10	(-)
Sens adenoma	46%	46%	46%	46%	23%	33%
Sens CRC	80%	73%	87%	80%	80%	73%
Specificity	95%	95%	95%	95%	95%	100%

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* Marker not used in the combination panel is indicated by (-)

- 5 The performance of the most sensitive marker combination panel SFRP2+GATA4+NDRG4+OSMR was evaluated for the different UICC stages. Results are summarized in Table 31.

Table 31: Performance of combination panel

- 10 SFRP2+GATA4+NDRG4+OSMR for different UICC stages

UICC stage	Neg	Pos	Total
?		1	1
I	1	4	5
II		4	4
III	1	3	4
IV		1	1
Total samples	2	13	15

Example 2

- Based on re-expression, 224 different gene assays representing 145 gene promoters were tested on the Base5 methylation profiling platform (data not shown, see reference 2 for details). The 37 most differentially methylated gene sequences assessing 29 gene promoters were validated on retrospectively collected tumors from 65 colorectal cancer patients (all stages) and 74 distant resection ends (histopathologically normal) using real-time MSP. Several markers reliably detected CRC in those tissue samples (data not shown). The results were confirmed on an independent test set containing 59 samples from patients with cancer other than CRC (20 breast, 21 lung and 22

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bladder cancer samples covering stages I-III), 39 non-cancerous controls, 34 carcinomas and 16 adenomas. After testing the non-CRC tissue samples, we had 59 results because 4 were invalid. The individual performance of the 9 best performing tissue markers is shown in Figure 2, when the analytical cut-off was set to give 100% specificity (based on the 39 non-cancerous controls). The most tissue specific markers include: NDRG4, OSMR, SFRP1, ADAM23, GATA5 and MGMT.

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implications for colorectal cancer screening.

10 **3) EXPERIMENTS ON PLASMA DNA**

MATERIALS AND METHODS IN RELATION TO PLASMA DNA

Sample collection and processing

Plasma samples were collected from multiple centers in
15 Germany, The Netherlands and Belgium.
10 ml of blood was obtained per individual using EDTA
Vacutainer™ tubes. Individuals with no suspicious findings,
adenomas or carcinomas based on colonoscopy were enrolled in
the present study. Within 4 hrs from the blood drawing, the
20 plasma fraction was separated from the cell fraction by
centrifugation at 1500 g for 15 min (4°C). The plasma was
transferred to new tubes and once again centrifuged (1500 g,
15 min, 4°C), after which the supernatant was transferred to
new tubes and stored at -80°C until further use. Samples
25 were shipped on dry ice.

Plasma samples from patients with stages I - IV of
colorectal cancers and different controls belonging to the
following groups were enrolled in this study. Tables 32 and
30 33 gives an overview of the collected samples sets.

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- **Colorectal cancer group:** patients with pathologically confirmed colorectal cancer with stage I to IV (according to the UICC stage grouping)
- **Adenomas**
- 5 - **Non-cancer controls:** patients without cancerous disease
- **Cancer controls:** patients with carcinomas other than colorectal cancer

Table 32: Plasma training set 1

10

Diagnosis group	Sample volume	Number of samples	Notes
Colorectal cancers	1.2 to 4.5 ml of plasma (corresponding to 0.07 to 0.27 plasma equivalent of DNA per PCR)	42	Stage I - IV Grade 1-3 (81% stage I-III)
Non-cancer controls		34	Symptomatic patients with non-acute conditions
Cancer controls	4 to 6 ml of plasma (corresponding to 0.24 to 0.36 plasma equivalent of DNA per PCR)	25	Predominantly ovarian and prostate cancers

Table 33: Plasma training set 2

Diagnosis group	Sample volume	Number of samples	Notes
Colorectal cancers	1.3 to 4.3 ml of plasma (corresponding to 0.16 to 0.52 plasma equivalent of DNA per PCR)	78	Stage I - IV Grade 1-3 (76% stage I-III)
Adenomas		49	
Non-cancer controls		64	Symptomatic patients with

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			non-acute conditions
Cancer controls	4 to 6 ml of plasma (corresponding to 0.48 to 0.72 plasma equivalent of DNA per PCR)	25	Predominantly ovarian and prostate cancers

DNA isolation from plasma samples

DNA isolation from plasma samples (1.2 to 6 ml) was performed using an upscaled phenol-chloroform DNA isolation method using the 15 ml of Heavy Phase lock Gel tubes (PLG tubes) (Eppendorf, cat# 0032 005.152) or alternatively the ChargeSwitch® gDNA 1 ml serum kit from Invitrogen (cat# CS11040).

10 *Phenol-Chloroform procedure*

Plasma samples were thawed and 1/10 volume of 10x buffer (240 mM EDTA (pH=8.0), 750 mM NaCl), 1/10 volume of 10% SDS and 5 µl of Proteinase K (20 mg/ml stock solution) per 1 ml of sample (e.g. 15 µl for 3 ml of sample) was added to each plasma sample. This mixture was incubated overnight at 48°C at constant shaking (200 RPM).

Subsequently the PLG tube was centrifuged at 2500 RCF for 3 min, sample mixture and approximately the same volume of phenol/chloroform (Invitrogen, cat# 15593049) were added to it. This solution was briefly vortexed, mixed for 10 min using a tube rocker at room temperature and centrifuged for 5 min at 2500 RCF. In case the retrieved sample volume was ≤ 4 ml, an equal volume of phenol/chloroform was added. The upper aqueous layer was phenol/chloroform-treated for a second time.

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DNA was precipitated from the the upper aqueous layer by adding 5 µl glycogen, 1/10 volume of 7.5 M Ammonium Acetate and 2-2.5 volumes of cold (-20°C) 100% ethanol. Tubes were gently inverted and incubated at -20°C for at least 1 h, followed by a centrifugation step at 17000 RCF for 30 min (4°C). Ethanol was carefully removed by pipetting. Pellets were washed with 2 ml freshly prepared 70% ethanol, vortexed gently and submitted to a centrifugation step at 17000 RCF for 15 min at 4°C. After careful removal of the remaining ethanol, pellets were air dried and resuspended in 45 µl of LoTE pH 8.0. The isolated DNA is stored at -80°C until further processing. This method allowed an average DNA recovery of $\approx$ 120 ng per ml of plasma.

15 *ChargeSwitch® gDNA 1 ml serum kit*

Plasma samples are thawed and DNA is isolated using the ChargeSwitch® gDNA 1 ml serum kit according to the manufacturer's instructions with the exception that the procedure is upscaled for larger sample volumes using the MagnaBot® large volume magnetic separation device from Promega (Cat# V3471). Results are presented in Table 41.

DNA modification

25 The complete content of DNA isolated in above procedure was subjected to sodium bisulfite treatment (BT) using the EZ-96 DNA Methylation kit from Zymo Research (Cat# D5003) performed on a pipetting robot (Tecan Freedom EVOII, Roma, Liha, Mca, Te-Vacs). Briefly, 45 µl of plasma DNA sample was mixed with 5 µl of M-Dilution Buffer (provided in kit) and incubated at 37°C for 15 min shaking at 1100 RPM. This mixture was further incubated with 100 µl of diluted CT

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conversion reagent (provided in kit) shaking at 70°C for 3 hours (protected from light). Subsequently the modified DNA was desalted and desulfonated according to manufacturer's instructions and eluted in either 40 µl or 20 µl of Tris-HCl 1mM pH8.0, depending on the applied concentration procedure. The eluted material was stored at - 80°C until further processing.

DNA amplification

Real-time MSP was performed on a 7900HT fast real-time PCR cyclor from Applied Biosystems.

2.4 µl of the modified DNA was added to a PCR mix (total volume 12 µl) containing home-made buffer solution (final concentrations are summarized: 16.6mM (NH₄)₂SO₄, 67 mM Tris (pH 8.8), 6.7 mM MgCl₂, 10 mM β-mercaptoethanol), dNTPs (5 mM; Amersham Biosciences cat# 27-2035-02), methylation specific forward primer (6 ng), methylation specific reverse primer (18 ng), molecular beacon (0.16 µM) and Jumpstart DNA Taq polymerase (0.4 units; Sigma Cat# D9307).

Cycling conditions are specified in Table 34.

A standard curve was included (9.6 x 10⁵ - 9.6 copies) to determine copy numbers of unknown samples by interpolation of their Ct values to the standard curve.

Table 34: Cycling profile

1	Activation	95°C	5 min
2	Denaturation	95°C	30 sec
3	Annealing and data	57°C (51°C for APC)	30 sec

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	collection		
4	extension	72°C	30 sec
5	cycling	Repeat step 2 to 4, 45 times	

RESULTS

- 5 Marker identification and validation in tissue and plasma samples.

Assay validity rate in tissue and plasma:

- 293 FFPE and 317 plasma samples were processed using real-time MSP (Table 35). The real-time MSP assays produced
 10 valid results in 98% of the FFPE samples and in 100% of the plasma samples.

- 15 **Table 35:** Summary of samples evaluated by real-time MSP

Sample Sets	Sample Types	Sample Numbers	Valid Tests [%]
Tissue Training Set	Cancer	65	65/65 [100]
	Controls	76	74/76 [97]
	Total	141	139/141 [99]
Tissue Test Set	CRC	34	34/34 [100]
	Controls	39	39/39 [100]
	Other Cancers	63	59/63 [94]
	Adenomas	16	16/16 [100]
	Total	152	148/152 [97]
Tissue Sets combined	CRC	99	99/99 [100]
	Controls	115	113/115 [98]
	Other Cancers	63	59/63 [94]
	Adenomas	16	16/16 [100]
	Total	293	287/293 [98]
Plasma Training set (1)	Cancer	42	42/42 [100]
	Controls	34	34/34 [100]
	Other cancers	25	25/25 [100]
	Total	101	101/101 [100]

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Plasma	Cancer	78	78/78 [100]
Training	Adenoma	49	49/49 [100]
set (2),	Controls	64	64/64 [100]
increased	Other cancers	25	25/25 [100]
plasma	Total	216	216/216 [100]
equivalent			
of DNA per			
real-time			
MSP assay			
Plasma Sets	Cancer	120	120/120 [100]
combined	Adenoma	49	49/49 [100]
	Controls	98	98/98 [100]
	Other cancers	50	50/50 [100]
	Total	317	317/317 [100]

Marker identification

Using re-expression profiles of colon cancerous cell lines,
 5 candidate genes were identified and the most promising
 markers (224 different gene assays representing 145 gene
 promoters) were tested on tissue using the Base5 methylation
 profiling platform (data not shown, see Straub, J. et al for
 details). Promoter sequences were linked with gene
 10 expression to identify epigenetically silenced genes. An
 established pharmacologic unmasking strategy (5-aza-2'-
 deoxycytidine (DAC) and trichostatin A (TSA)) for re-
 expression analysis of epigenetically targeted genes was
 combined with proprietary advanced bioinformatics tools to
 15 identify genes prone to promoter methylation.

Marker selection in colon tissue

Marker candidates identified by re-expression were screened
 using 37 real-time methylation specific PCR (real-time MSP)
 20 assays. These assays were used to assess the methylation
 status of 29 gene promoters in 293 formalin-fixed paraffin-
 embedded (FFPE) tissue samples collected from various
 clinics. Samples included 99 carcinomas of various stages,

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16 adenomas, 63 samples from patients with cancer other than CRC (20 breast [stages I-III], 22 bladder [stages I-III], 21 lung [stages I and II]), 39 samples from patients with no evidence of cancer and 76 distant resection ends (histopathologically normal) from CRC patients. These samples were divided into training and independent test sets, and used to select the gene methylation assays best able to discriminate between cancerous and non-cancerous samples. The training set included retrospectively collected tumors from 65 colorectal cancer patients (all stages) and 74 distant resection ends. Using the 10 best performing genes the results were confirmed on an independent test set containing 59 samples from patients with cancer other than CRC, 39 non-cancerous controls and 50 cancer cases (34 carcinomas and 16 adenomas). The individual performance of the 10 best performing tissue markers OSMR, SFRP1, GATA4, SFRP2, NDRG4, ADAM23, GATA5, MGMT, APC and JPH3 is shown in FIG.7, when the analytical cut-off was set to give 100% specificity, except for JPH3 where a specificity of 95% was obtained (based on the 39 non-cancerous controls). Corresponding primer and beacon sequences are summarized in Table 3 (above). In addition to the colon test genes, the independent reference gene β -Actin (ACT) was also measured. The ratios between the colon test genes and ACT were calculated, and are the test result of the assay. The samples were classified as methylated, non-methylated, or invalid based on the decision tree shown in Figure 5.

Complementarity of markers

The different markers were tested on their complementarity. Several marker combinations reliably detected CRC with high specificity and sensitivity. Results of the best 2 marker

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combinations are summarized in Table 36. For 100% specificity, sensitivities ranged between 94 to 100%.

Table 36: Performance of 2 combinations of the markers

5 reliably detecting CRC and adenomas when using real-time MSP (tissue test set: 34 carcinomas, 16 adenomas, 39 controls)

Panel 1 (OSMR, GATA4, ADAM23)			
Samples	# detected / tested	Sensitivity % [95% CI]	Specificity % (# detected / tested)
34 carcinomas 39 controls	33/34	97 [91 - 100]	100 (0/39)
16 adenomas 39 controls	16/16	100	100 (0/39)
50 neoplasms (34 carcinomas and 16 adenomas) 39 controls	49/50	98 [94 - 100]	100 (0/39)
Panel 2 (OSMR, GATA4, GATA5)			
Samples	# detected / tested	Sensitivity % [95% CI]	Specificity % (# detected / tested)
34 carcinomas 39 controls	32/34	94 [86 - 100]	100 (0/39)
16 adenomas 39 controls	16/16	100	100 (0/39)
50 neoplasms (34 carcinomas and 16 adenomas) 39 controls	48/50	96 [90 - 100]	100 (0/39)

10 Marker testing in plasma

Eight of the best performing markers in tissue were assessed (OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC) on 101 available plasma samples from multiple centers (plasma

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training set 1: Table 32). These plasma samples included 34 samples with no suspicious findings, 25 samples from patients with cancers other than colon cancer and 42 samples from patients covering all stages of CRC, with 81%
5 representing stages I-III of disease.

DNA was isolated following the upscaled phenol-chloroform procedure; subsequently the whole DNA sample was modified as described above. The plasma training set 1 was eluted in 40
10 μ l of BT elution volume of which 2.4 μ l was subjected to real-time MSP, the 2.4 μ l of eluted DNA corresponds to an equivalent of 0.07 to 0.36 ml of original plasma sample which went into the isolation procedure (= 0.07 to 0.36 plasma equivalent of DNA per PCR).

15

The individual performance (% sensitivity) of the 8 gene assays in plasma samples is shown in FIG.8, sensitivity values ranging from 14 to 33%. Corresponding specificity values are displayed in Table 37. Obtained specificity
20 values ranged from 97 to 100%.

Five of the best performing markers in training set 1 were further studied with an additional, independent sample set prospectively collected from multiple centers (plasma
25 training set 2: Table 33). Reducing the number of gene assays from 8 to 5 resulted in fewer assays per sample and a greater aliquot of plasma equivalent of DNA was added per PCR reaction. The modified DNA from sample set 2 was more concentrated by eluting in 20 μ l instead of 40 μ l of BT
30 elution volume. 2.4 μ l eluted DNA from sample set 2 was further processed through real-time MSP, this corresponds to

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0.16 to 0.72 ml plasma equivalent of DNA per PCR depending on the plasma volume prior to DNA isolation.

The plasma samples of training set 2 included 64 samples
5 with no suspicious findings, 49 adenomas, 25 samples from patients with cancers other than colon cancer and 78 samples from patients covering all stages of CRC, with 76% representing stages I-III of disease. The individual performance (% sensitivity) of the 5 gene assays is shown in
10 FIG. 8 with corresponding specificity values displayed in Table 37. Specificity values ranged from 96 to 99%, with sensitivity ranging from 23 to 47%.

Four candidate methylation markers were found to result in
15 the best sensitivity and specificity in plasma samples: OSMR, NDRG4, GATA5 and ADAM23; performance of this plasma panel is shown in Table 38. Performance characteristics (stages I-III CRC) of this panel of 4 methylation genes demonstrated 73% sensitivity and 92% specificity when
20 optimized for sensitivity, whereas 64% sensitivity and 98% specificity was obtained when optimizing for specificity. Sensitivity can be further improved (from 64% to 68%) when samples with a plasma volume less than 2 ml prior to DNA isolation are excluded from analysis. Results are presented
25 in Table 39.

Table 37: Individual gene assay performance displaying %
specificity for both plasma training sets and % sensitivity
30 for adenomas in plasma training set 2

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	OSMR	SFRP1	NDRG4	GATA5	ADAM23	JPH3	SFRP2	APC
% Specificity (all 59 controls), plasma set 1	100	98	100	97	98	97	97	97
% Specificity (all 89 controls), plasma set 2: increased plasma equivalent of DNA per real-time MSP assay	99	96	99	99	97	N/A	N/A	N/A
% Sensitivity adenomas plasma set 2: increased plasma equivalent of DNA per real-time MSP assay	2	2	0	6	2	N/A	N/A	N/A

Table 38: Performance of a plasma marker panel using real-time MSP (independent of recovered plasma volume prior to DNA isolation)

		Plasma panel (optimized for sensitivity) OSMR, NDRG4, GATA5 and ADAM23	
Sample sets	Sample groups	Sensitivity % (# detected / # total) [95% CI]	Specificity % (# detected / # total) [95% CI]
Plasma training set 1	Stages I-III CRC All Stages CRC All Controls	50 (17/34) 60 (25/42) [45 - 83]	97 (2/59) [93 - 100]
Plasma training set 2 (increased plasma equivalent of DNA per real-time MSP assay)	Stages I-III CRC All Stages CRC Adenomas All Controls	73 (43/59) 73 (57/78) [63 - 83] 12 (6/49)	92 (7/89) [86 - 98]
		Plasma panel (optimized for specificity) OSMR, NDRG4, GATA5 and ADAM23	
Sample sets	Sample groups	Sensitivity % (# detected / # total) [95% CI]	Specificity % (# detected / # total) [95% CI]
Plasma training set 1	Stages I-III CRC All Stages CRC All Controls	N/A	N/A
Plasma training set 2 (increased plasma equivalent of	Stages I-III CRC All Stages CRC Adenomas All Controls	64 (38/59) 64 (50/78) [53 - 75] 6 (3/49)	98 (2/89) [95 - 100]

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DNA per real-time MSP assay)			
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Table 39: Performance of a plasma marker panel using real-time MSP using at least 2 ml of plasma prior to DNA

5 isolation

		Plasma panel (optimized for sensitivity) OSMR, NDRG4, GATA5 and ADAM23	
Sample sets	Sample groups	Sensitivity % (# detected / # total) [95% CI]	Specificity % (# detected / # total) [95% CI]
Plasma training set 2 (increased plasma equivalent of DNA per real-time MSP assay)	Stages I-III CRC All Stages CRC Adenomas All Controls	73 (41/56) 74 (54/73) [64 - 84] 12 (6/49)	92 (7/89) [86 - 98]
		Plasma panel (optimized for specificity) OSMR, NDRG4, GATA5 and ADAM23	
Sample sets	Sample groups	Sensitivity % (# detected / # total) [95% CI]	Specificity % (# detected / # total) [95% CI]
Plasma training set 2 (increased plasma equivalent of DNA per real-time MSP assay)	Stages I-III CRC All Stages CRC Adenomas All Controls	68 (38/56) 67 (49/73) [56 - 76] 6 (3/49)	98 (2/89) [95 - 100]

Average DNA recovery yield from plasma samples

Plasma DNA (collected after double centrifugation step) from
 10 colorectal cancer patients was isolated according to the
 phenol/chloroform procedure and quantified using the
 PicoGreen dsDNA quantitation kit from Molecular Probes. The
 average plasma DNA recovery yield was 117 ng/ml of plasma,
 with a range of 41 to 384 ng/ml (data obtained from 25
 15 patients).

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Table 40: Average DNA recovery yield plasma samples

Sample	ng/ml plasma
1	41
2	66
3	264
4	163
5	54
6	121
7	87
8	107
9	53
10	121
11	88
12	201
13	53
14	47
15	384
16	87
17	115
18	107
19	70
20	72
21	122
22	146
23	71
24	195
25	94

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Phenol/Chloroform procedure versus ChargeSwitch® using plasma samples

5 This experiment was carried out to show the isolation of DNA from plasma by using the method of this invention. Plasma volumes ranging from 2.5 to 6 ml were processed according to the above discussed upscaled phenol/chloroform and ChargeSwitch® isolation procedure. Plasma derived from

10 ovarian, prostate and colon blood samples were investigated. The objective was to isolate DNA (according to both methods) and further process the samples in parallel through bisphite treatment and β -Actin real-time MSP to address the sample quality and DNA yield. The corresponding β -Actin

15 copies for both isolation procedures are summarized in Table 16.

Table 41: β -Actin copies phenol/chloroform versus ChargeSwitch® isolation procedure

20

Sample number	Sample origin	Plasma volume (ml)	B-Actin copies Phenol	B-Actin copies ChargeSwitch
1	ovarian cancer	6.0	4349	863
2	ovarian cancer	6.0	2710	466
3	ovarian cancer	6.0	3922	967
4	ovarian cancer	6.0	758	490

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5	ovarian cancer	6.0	4201	423
6	ovarian cancer	6.0	2644	139
7	ovarian cancer	6.0	1472	187
8	prostate cancer	2.6	145	7
9	colon cancer	2.5	317	52
10	pos control cell line	N/A	8702	1314

Updated results for plasma training set 2.

Corrected information was received from the clinics about plasma training set 2. For plasma training set 2: the cancer cases remained the same, a new category of "unknown" was created, the number of controls was 52 (instead of former 64) and the adenoma cases were 39 (instead of former 49). This allowed re-classification of sample types as provided in Table 42. Since the corrected information classified a number of unknown cancer cases (controls) as early stage cancers, additional conclusions on detection of early stage cancers could be drawn. As shown in table 43, the plasma panel allowed very sensitive detection (70%) of early stage samples. Improved detection could be obtained by excluding samples with a plasma volume less than 2 ml (Table 44)

Table 42: Summary of samples tested by real-time MSP and evaluability rate

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Sample sets	Sample types	Sample numbers	Valid tests [%]
Tissue training set	CRC	65	65/65 [100]
	Controls	76	74/76 [97]
	Total	141	139/141 [99]
Tissue test set	CRC	34	34/34 [100]
	Controls	39	39/39 [100]
	Other Cancers	63	59/63 [94]
	Adenomas	16	16/16 [100]
	Total	152	148/152 [97]
Tissue sets combined	CRC	99	99/99 [100]
	Controls	115	113/115 [98]
	Other Cancers	63	59/63 [94]
	Adenomas	16	16/16 [100]
	Total	293	287/293 [98]
Plasma training set (1)	CRC	42	42/42 [100]
	Controls	34	34/34 [100]
	Other cancers	25	25/25 [100]
	Total	101	101/101 [100]
Plasma training set (2)	CRC	78	78/78 [100]
	Adenoma	■	49/49 [100]
	Controls	■	64/64 [100]
	Other cancers	25	25/25 [100]
	■	■	22/22 [100]
	Total	216	216/216 [100]
Plasma sets combined	CRC	120	120/120 [100]
	Adenoma	39	49/49 [100]
	Controls	86	98/98 [100]
	Other cancers	50	50/50 [100]
	Unknown	22	22/22 [100]
	Total	317	317/317 [100]

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Sample groups (plasma training set 2)	Plasma panel			
	OSMR, GATA5, NDRG4 and ADAM23			
	optimized for sensitivity		optimized for specificity	
	Sensitivity % (# detected / # total) [95% CI]	Specificity % (# detected / # total) [95% CI]	Sensitivity % (# detected / # total) [95% CI]	Specificity % (# detected / # total) [95% CI]
Early stages CRC (0-II)	70% (23/33) [54-86]	92% (6/77) [86-98]	58% (19/33) [41-75]	99% (1/77) [96-100]
All stages CRC	73% (57/78) [63-83]		64% (50/78) [53-75]	
Adenomas	10% (4/39)		5% (2/39)	
Controls				

Table 43: Performance characteristics of a 4-gene marker panel using plasma set 2

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Sample groups (plasma training set 2)	Plasma panel			
	OSMR, GATA5, NDRG4 and ADAM23			
	optimized for sensitivity		optimized for specificity	
	Sensitivity % (# detected / # total) [95% CI]	Specificity % (# detected / # total) [95% CI]	Sensitivity % (# detected / # total) [95% CI]	Specificity % (# detected / # total) [95% CI]
Early stages CRC (0-II)	70% (23/33) [54-86]	92% (6/77) [86-98]	58% (19/33) [41-75]	99% (1/77) [96-100]
All stages CRC	74% (54/73) [64-84]		67% (49/73) [56-78]	
Adenomas	10% (4/39)		5% (2/39)	
Controls				

Table 44: Performance characteristics of a 4-gene marker panel using plasma set 2

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4) N-Myc downstream regulated gene 4 (NDRG4) promoter methylation is a sensitive and specific biomarker for colorectal cancer

5

Abstract

Background and aims: N-Myc downstream regulated gene 4 (NDRG4), a gene involved in cellular differentiation and neurite formation, is one of the four members of the NDRG family. Here we address the role of NDRG4 promoter methylation in CRC (CRC).

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- 5 Methods: NDRG4 promoter methylation was analyzed in CRC cell lines, well characterised series of normal colon mucosa, colorectal adenomas, carcinomas and other neoplasias using methylation specific PCR (MSP) and bisulfite sequencing. NDRG4 promoter methylation was also analyzed in fecal DNA of CRC patients and controls using quantitative MSP. Loss of heterozygosity (LOH) mapping of the NDRG4 locus and mutation analysis using direct sequencing of NDRG4 coding exons and
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their flanking intronic regions were performed. NDRG4 mRNA and protein expression was studied using RT-PCR and immunohistochemistry respectively.

5 Results: NDRG4 promoter methylation is observed in 7/8 CRC cell lines. The prevalence of NDRG4 promoter methylation in CRC tissue is 86% (71/83) compared to 4% (2/48) in normal colon mucosa. A second, independent series of CRCs confirmed the high prevalence (69%, 127/183) of NDRG4 methylation.

10 NDRG4 methylation was also observed in 81% (13/16) of oesophageal adenocarcinomas and 77% (17/22) of gastric cancers while no or little methylation was observed in skin (0/8), kidney (1/10), ovary (0/20), prostate (0/10), breast (0/16) and oesophageal squamous cell cancers (0/12). NDRG4

15 promoter methylation can be detected in fecal DNA of 76% (16/21) of CRC patients, while only 3% (2/67) of control patients tested positive yielding a sensitivity of 76% and a specificity of 97%. No mutations were found and 30,5% of tumors showed LOH on the NDRG4 locus. Expression of NDRG4 is

20 decreased at the RNA and protein level in CRC when compared to normal tissue.

Conclusions: NDRG4 is frequently methylated in CRC cell lines, colorectal adenomas and carcinomas and other

25 adenocarcinomas of the gastrointestinal tract. NDRG4 promoter methylation in fecal DNA can be used as a sensitive and specific biomarker for the detection of CRC.

Introduction

30 Previous microarray experiments to identify genes which are epigenetically regulated in tumor endothelial cells revealed 81 genes that are downregulated in tumor endothelial cells

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and reexpressed after 5-aza-2'-deoxycytidine (DAC) and trichostatin A (TSA) treatment. Silencing of these genes in tumor- endothelial cells was associated with promoter histone H3 deacetylation and loss of H3 lysine 4 methylation, however did not involve DNA methylation of promoter CpG islands. Interestingly, 21 of these 81 genes (26%) have been reported to be hypermethylated and silenced in various tumor types suggesting that many of the identified gene promoters have the potential to be regulated by promoter methylation in tumor cells (Hellebrekers, Melotte et al. 2007). Amongst the identified CpG island containing genes is N-myc downregulated gene-4 (NDRG4), also known as Smap-8 and Bdml. NDRG4 is part of the NDRG family which consists of four members, NDRG1, -2, -3 and -4 which have an amino acid sequence homology of 57-65% (Zhou, Kokame et al. 2001; Qu, Zhai et al. 2002). Phylogenetic analysis verified two subfamilies, one consisting of NDRG1 and -3 and the other consisting of NDRG-2 and -4 (Qu, Zhai et al. 2002). NDRG1 is the most extensively studied member of the NDRG family. Expression of NDRG1 is often downregulated in cancer cells (van Belzen, Dinjens et al. 1997; Kurdistani, Arizti et al. 1998; Guan, Ford et al. 2000; Bandyopadhyay, Pai et al. 2003; Bandyopadhyay, Pai et al. 2004; Shah, Kemeny et al. 2005) and upregulated by DAC treatment (Guan, Ford et al. 2000; Bandyopadhyay, Pai et al. 2004). In addition, NDRG2 has also been described as candidate tumor suppressor gene (Deng, Yao et al. 2003; Lusic, Watson et al. 2005) and reported to be methylated in meningiomas (Lusic, Watson et al. 2005) and different cancer cell lines (Liu, Wang et al. 2007). So far, the function of NDRG3 and NDRG4 in cancer has not been addressed. The NDRG4 gene is located on chromosome 16q21-q22.3, spans 26kb and contains 17 exons

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covering the entire sequence of three cDNA isoforms NDRG4-B, NDRG4-Bvar and NDRG4-H. NDRG4 mRNA is predominantly present in the cytoplasm. At present, expression of NDRG4 has only been described in brain and heart using Northern blot analysis. The molecular characterization of NDRG4 and the role of this protein in the nervous system has mainly been investigated in the rat (Nakada, Hongo et al. 2002; Ohki, Hongo et al. 2002; Maeda, Hongo et al. 2004; Hongo, Watanabe et al. 2006). NDRG4 protein may participate in processes that lead to cellular differentiation and neurite formation (Ohki, Hongo et al. 2002).

Here, we report NDRG4 to be expressed in normal colon mucosa and downregulated in colon cancer tissue. In addition, NDRG4 promoter methylation, loss of heterozygosity (LOH) and mutational inactivation were examined. We identified the NDRG4 promoter as being frequently methylated in CRC and other neoplasias of the gastrointestinal tract and investigated its potential as a biomarker in stool of CRC patients and controls.

Materials and Methods

Cell lines, study population and tissues

CRC cell lines HT29, SW480, Caco2, Colo205, RKO, LS174T, HCT116 and SW480 were cultured in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Hyclone). To investigate reexpression of NDRG4 following inhibition of DNA methyltransferases, HCT116 and RKO were treated with 1 μ M DAC (Sigma).

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NDRG4 promoter methylation was investigated in well-characterized series of colorectal carcinomas, adenomas and

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controls (FIG.9). The first series consists of formalin-fixed, paraffin-embedded CRCs (n=90) of patients over 50 years of age which were retrospectively collected from the archive of the dept. of Pathology of the University Hospital Maastricht. When present, also normal (n=79) and adenoma (n=60) tissue was collected from these patients. Histologically normal biopsy material from patients undergoing endoscopy for non-specific abdominal complaints (n=51), adenoma biopsies (n=22) from patients who did not develop CRC within 10 years, and resected colon mucosa of patients with various inflammatory bowel conditions (n=33) were selected as control tissue. This last group includes Crohn's disease (n=1), colitis ulcerosa (n=6), non-specific inflammation (n=9) and diverticulitis (n=18). A second independent series of CRCs (n=200) was randomly selected from the prospective Netherlands Cohort Study on diet and cancer (NLCS), which has been described in detail elsewhere (van den Brandt, Goldbohm et al. 1990; Brink, de Goeij et al. 2003). Series characteristics are shown in supplemental table 1 In addition, archival, formalin-fixed, paraffin-embedded skin- (n = 8), kidney- (n = 10), ovary- (n = 10), prostate- (n = 10), breast- (n = 15), stomach- (n = 22) and oesophagus (n= 28) cancer tissue was analyzed for NDRG4 promoter methylation. This study was approved by the Medical Ethical Committee (MEC) of the Maastricht University and the University Hospital Maastricht.

Table 45: Series characteristics

	Age*	Sex [†]	Location [‡]	
			Proximal	Distal
CRC+				
Normal tissue	71.0 ± 8.6	41/38	40/75 (53%)	35/75 (47%)
Adenoma tissue	71.7 ± 7.9	32/30	26/59 (44%)	33/59 (56%)
Carcinoma tissue	71.5 ± 8.3	44/46	49/88 (56%)	39/88 (44%)

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CRC-				
Normal tissue	65.2 ± 9.0	22/29	13/39 (33%)	26/39 (67%)
Adenoma tissue	63.1 ± 7.6	16/6	6/18 (33%)	12/18 (67%)
Inflamed tissue	65.3 ± 10.1	14/19	10/26 (39%)	16/26 (62%)
<i>P-value</i>	<0.001	NS		NS

Carcinoma tissue		CRC+		CRC-
		Adenoma tissue	Adenoma tissue	Adenoma tissue
Histological type		Histological type		
Adenocarcinoma	72/90 (80%)	Tubular	39/62 (63%)	16/22 (73%)
Mucinous carcinoma	18/90 (20%)	Tubulovillous	22/62 (36%)	6/22 (27%)
		Villous	1/62 (2%)	0/22 (0%)
Differentiation:		Dysplasia		
Poor	8/90 (9%)	Lowgrade	54/62 (87%)	22/22 (100%)
Moderate	70/90 (78%)	Highgrade	8/62 (13%)	0/22 (0%)
Well	12/90 (13%)			
TNM stage:				
I	13/90 (14%)			
II	29/90 (32%)			
III	36/90 (40%)			
IV	12/90 (13%)			

Table 4.5: Patient characteristics NDRG4b

*years ± SD, analyzed by One-way ANOVA

†Male/Female, analyzed by Pearson's χ^2 ‡analyzed by Pearson's χ^2 . Location could not be traced for all samples explaining different total sample numbers

CRC+: colorectal cancer patients

CRC-: patients without colorectal cancer

NS: not significant

TNM stage: 'Tumour Node Metastasis' Staging

DNA-isolation from tissues and cell lines

A 5 µm section of each tissue block was stained with haematoxylin and eosin and revised by a pathologist (AdB).

Five sections of 20 µm were deparaffinated prior to DNA-isolation. DNA was extracted from these tissue samples and from cell lines using the Puregene® DNA isolation kit (Gentra systems) according to the manufacturers instructions. In brief, cell lysis solution and proteinase K (20 mg/ml, Qiagen) were added to the tissue samples and incubated overnight at 55°C. Subsequently, DNA was extracted

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for 72 h at 37°C, protein was removed, and DNA was precipitated using 100% 2-propanol. Finally, DNA was rehydrated in hydration buffer.

5 Collection and preparation of fecal DNA

Colonoscopy negative control stool samples (n=67) were obtained from a population of healthy subjects over 50 years of age which are being screened within the framework of a workplace-based community CRC screening study at the University Hospital Maastricht. The Medical Ethical Committee (MEC) of the Maastricht University, the University Hospital Maastricht and the Dutch 'Wet op Bevolkingsonderzoek' (WBO) is approving this screening study. Stool samples from colonoscopy confirmed CRC patients (n=21) covering all CRC stages were collected at the Free University Medical Center in Amsterdam. For recovery of human DNA, whole stool samples were homogenized in a 7 excess volume of stool homogenization buffer (Exact sciences, Marlborough, MA, USA) and aliquoted in portions of 32 ml containing the equivalent of 4g of stool each. Single aliquots were centrifuged and the supernatants were incubated with 80 units per ml RNase A for 60 minutes at 37°C. Total DNA was then precipitated using sodium acetate isopropanol (PH 5.2), washed with 70% ethanol and resuspended in 4ml 1xTE (pH 7.4). 400 µl 10x buffer (240mM EDTA (pH 8.0), 750 mM NaC), 400 µl 10% SDS and 20 µl Proteinase K (20 mg/ml) was added, samples were incubated overnight at 48°C at constant shaking and centrifuged the next day. Additionally, 5 ml of phenol-chloroform-isoamylalcohol was added and samples were incubated for 10 minutes at RT before centrifugation. The phenol- chloroform-isoamylalcohol extraction was repeated, the aqueous layer

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was subsequently transferred in a new tube, DNA was precipitated, washed and pellets were resuspended in 2 ml of LoTE (pH 8.0).

5 Sodium bisulfite conversion, methylation-specific PCR and sodium bisulfite sequencing

Sodium bisulfite modification of 500 ng genomic DNA was performed using the EZ DNA methylation kit (ZYMO research Co., Orange, CA) according to the manufacturer's
10 instructions. NDRG4 MSP analysis on bisulfite treated DNA retrieved from cell lines and formalin-fixed, paraffin embedded tissue was facilitated by first amplifying the DNA with flanking PCR primers which amplify bisulfite-modified DNA but do not discriminate between methylated or
15 unmethylated DNA. This PCR product was used as a template for the MSP reaction (Herman, Graff et al. 1996; van Engeland, Weijnenberg et al. 2003). Flank primers, MSP primers and PCR conditions are listed in table 2 (see above). All PCRs were performed with controls for unmethylated DNA
20 (DNA from normal lymphocytes), methylated DNA (normal lymphocyte DNA treated in vitro with SssI methyltransferase (New England Biolabs), and a control without DNA. Ten µl of each MSP reaction were directly loaded onto 2% agarose gel and visualized under UV illumination. For sequencing of
25 sodium bisulfite-converted DNA, PCR products were amplified and cloned using the TOPO-TA cloning kit (Invitrogen, Breda, the Netherlands). Single colonies were picked and sequenced using an automated sequencer (Applied Biosystems, Foster City, CA). Primer sequences used are SEQ ID NO: 570 5'-
30 GATYGGGGTGTTTTTGTAGGTTT-3' (sense primer) and SEQ ID NO: 6 5'- CRAACAACCAAAAACCCCTC-3' (antisense primer).

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Quantitative MSP

Quantitative real-time MSP was performed using a 7900HT real-time PCR system (Applied Biosystems). 2.4 µl of the modified DNA (equivalent to 2,5 µg unconverted DNA) was added to a PCR mix (total volume 12 µl) containing buffer (16.6mM (NH₄)₂SO₄, 67 mM Tris (pH 8.8), 6.7 mM MgCl₂, 10 mM β-mercaptoethanol), dNTPs (5 mM), forward primer (6 ng), reverse primer (18 ng), molecular beacon (0.16 µM), BSA (0.1 µg), and Jumpstart DNA Taq polymerase (0.4 units; Sigma Aldrich). The PCR program was as follows: 5 minutes 95°C, followed by 45 cycles of 30 seconds 95°C, 30 seconds 57°C, and 30 seconds 72°C, followed by 5 minutes 72°C. Primer sequences used are SEQ ID NO: 17 5' - GTATTTTAGTCGCGTAGAAGGC - 3' (forward primer), SEQ ID NO: 18 5' - AATTTAACGAATATAAACGCTCGAC - 3' (reverse primer) and SEQ ID NO: 19 5'-FAM-CGACATGCCCCGAACGAACCGCGATCCCTGCATGTCG-3'-DABCYL (molecular beacon). A standard curve (2x10⁶ - 20 copies) was included to determine copy numbers of unknown samples by interpolation of their Ct values to the standard curve.

Loss of Heterozygosity Analysis

Allelic status was analyzed by PCR amplification with specific primer pairs flanking polymorphic microsatellite loci. The fluorescent dye-labeled microsatellite markers DS16S3089 (forward primer: SEQ ID NO: 526 AGCCCTGCCTGATGAA; reverse primer: SEQ ID NO: 527 TGTGTGGGTAGCACCAA) and DS16S3071 (forward primer: SEQ ID NO: 528 AGCTCTCTGATGGGCAGTG; reverse primer: SEQ ID NO: 529 TGGAAGATAGCCCCCAAAT) located on 16q21-22 were selected from genome public database. DS16S3089 is situated 1.9Mb downstream of NDRG4 and DS16S3071 1.8 Mb upstream of NDRG4.

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Matched tumor/normal DNA samples were amplified by PCR in a 15µl volume containing 0,25 mM dNTP, 0,3µM primers, 1,5mM MgCl₂ and 0,04 units Taq-polymerase (platinum, Invitrogen) using 50ng DNA as template. The reaction mixture was

5 subjected to 3 min of denaturing at 95°C and 30 cycles of 95°C for 1 min, 60°C annealing temperature for 1 min and 72°C for 1 min followed by a final extension step at 72°C for 10 min. PCR products were sequenced using an automated sequencer (Applied Biosystems, Foster City, CA) and analyzed

10 using Genemapper software version 4,0 (Applied Biosystems). Only genotypes demonstrating two different sizes, i.e. heterozygous MS alleles, were used for evaluating allelic status. The allelic ratio was calculated as $(N1/N2)/(T1/T2)$ for the ratio of area values of tumor (T) versus the normal

15 (N) alleles. LOH was defined as an allelic ratio more than 1.35 and less than 0.67.

Mutation analysis

The NDRG4 coding exons and their flanking intronic regions

20 were individually amplified using genomic DNA extracted from paraffine embedded colonic adenocarcinoma tissue. Mutation analysis was examined using the nested PCR approach. The outside PCR was performed with 125 ng genomic DNA, 50 pmol of each forward and reverse primer and 1 units of

25 TaqPolymerase mixture (Invitrogen). DNA amplification was done on a thermal cycler using Thermo-Fast 96-well plates (Corning) starting with an initial denaturation step at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30s, annealing with an specific temperature for each primer

30 for 30s and extension at 72°C for 30 sec. An additional final extension of 72°C for 5 min was added. Following the outside PCR an inside PCR was done using the same conditions

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as the outside PCR. PCR primer sets for each exon, including intron-exon boundary, are provided in detail in supplemental table 3. DNA was purified using the Millipore multiscreen 96 wells plate (Millipore). PCR products were amplified using the BigDye® Terminator v1.1 Cycle sequencing kit and amplified products were sequenced using an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA).

Table 46: *NDRG4* mutation analysis primer sequences and PCR conditions.

Exon No.	Primer	SEQ ID NO :	Sense primer	SEQ ID NO	Antisense primer	Annealing temperature
2	Outside	530	CCCAGCCCCGACTTGC	531	CTAAGACCTCAAAGGCGCG	56
	Inside	532	TGTCCTTCTCCGCCCGG	531	CTAAGACCTCAAAGGCGCG	62
3	Outside	533	CCCCTCTGTTTGCCTTCC	534	CTGGCCAGGTGGGGTG	56
	Inside	533	CCCCTCTGTTTGCCTTCC	535	GCCAGGTGGGGTGAGGG	62
4	Outside	536	CTGCGTCACCTCATTCCC	537	TCACCGCTCTGGCTGATG	56
	Inside	538	GAGGAGCCAAGAGCGGAGG	537	TCACCGCTCTGGCTGATG	62
5	Outside	539	CCCCTCTGCTCAGCCATAG	540	GCTGGAGACAGGCAGAGGG	56
	Inside	539	CCCCTCTGCTCAGCCATAG	541	GGAGACAGGCAGAGGGGG	56
6	Outside	542	GTAGGTACCCTGAGCCCCC	543	ACCCCTGGGCCCTAGC	56
	Inside	544	CCCTCTGCCTGTCTCCAGC	543	ACCCCTGGGCCCTAGC	62
7	Outside	545	GGAAATGGCACCCCTAGC	547	GGGGGCATGGGGAGAC	56
	Inside	548	GCACCCCTAGCCCTAGAGT	547	GGGGGCATGGGGAGAC	56
8	Outside	549	CCTTGAAGACTTTACAGAGTGTTC	550	GTATACCCACCCACCCC	56
	Inside	551	CTGCACCCATCCTGGCC	550	GTATACCCACCCACCCC	62
9	Outside	552	GGGGTGGGGTGGGTATAC	553	GCTGGGAGGGGCAAATC	56
	Inside	552	GGGGTGGGGTGGGTATAC	554	GGCAAATCCCAGATCACCC	62
10	Outside	555	GCCTCCATCCATCTCCCTG	556	GGCTGCTGATCCACCC	56
	Inside	557	CATGCCTCCATCCATCTCC	556	GGCTGCTGATCCACCC	62
11+12	Outside	558	CACCTCTGCCTCTGCCCC	559	CCCCAGTGAGCCACAGC	56
	Inside	560	CCTCTGCCCCCTCCCC	559	CCCCAGTGAGCCACAGC	62
13	Outside	561	TGCCTTGGCAATGGGG	562	CAGGGCTGGGGAAGAAAG	56
	Inside	563	CTTGGCAATGGGGTGG	562	CAGGGCTGGGGAAGAAAG	62
14+15	Outside	564	GGAGCTTGTCTGGAGTGAG	565	GTGGGGTGGAAATGTACTCAC	56
	Inside	566	TGGAGTGAGGGCCTGC	565	GTGGGGTGGAAATGTACTCAC	62
16	Outside	567	TGCCCGCCAGTCCTCAG	568	TAAAGGGAACATGAGCCGG	56
	Inside	569	CAGTCCTCAGGCCATCC	568	TAAAGGGAACATGAGCCGG	56

*Number of cycles in each case was 35

Quantitative reverse transcriptase PCR

Total RNA from cell lines, normal mucosa and tumor tissue was isolated using the Rneasy Mini kit (Qiagen) following the manufacturers instructions. Possible genomic DNA contaminations were removed by DNase treatment with the RNase-free DNase set (Qiagen). cDNA synthesis using the

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IscripT cDNA synthesis kit (Bio-Rad) was performed. Quantitative real-time (RT-PCR) was performed using SYBR Green PCR master mix (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands). Realtime RT-PCR mixes were
5 composed of 1x iQ SYBR Green Supermix (Bio-Rad), 400 nM of the forward (SEQ ID NO: 3 5'-GGCCTTCTGCATGTAGTGATCCG-3') and reverse (SEQ ID NO: 4 5'-GGTGATCTCCTGCATGTCCTCG-3') primer and cDNA corresponding to 30 ng total RNA per reaction. As
10 standard control, primers targeted against cyclophilin A were used. Reactions were run using the iCycler (Bio-Rad) for 40 cycles at a T_m of 60 °C. The comparative Ct method was used to calculate differences in mRNA expression. To do so, the Ct value of each sample was normalized to the reference gene ($[\Delta]Ct = Ct_{\text{sample}} - Ct_{\text{cyclo}}$). Next, the
15 fold difference in expression was calculated as $2^{-[\Delta][\Delta]Ct}$, with $[\Delta][\Delta]Ct = [\Delta]Ct_{\text{sample1}} - [\Delta]Ct_{\text{control}}$.

Immunohistochemistry

20 Immunohistochemistry was performed on formalin-fixed, paraffin embedded tissue sections (5 µm) of normal colon mucosa and CRC tissue. Sections were deparaffinized in xylene, rehydrated and incubated with 1% methanol for 30 minutes to inactivate the endogenous peroxidase. After
25 blocking, sections were stained with the NDRG4 monoclonal antibody (Abnova Corporation), 1:6000 diluted in Tris-buffered saline (TBS) with 0.1% Tween and 0.5% bovine serum albumin (BSA) and incubated for 60 minutes. Sections were incubated with the secondary antibody poly-HRP-GAM/R/R IgG
30 (Immunologic, Immunovision Technologies) and staining was visualized as a brown precipitate using DAB substrate chromogen (Dako) followed by haematoxylin counterstaining.

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Sections incubated without the primary antibody served as a negative control.

Data analysis

5 We used the Pearson's χ^2 or Fisher's Exact test and the One-way ANOVA, Kruskal-Wallis or Mann-Witney test where appropriate to compare non-parametric and categorical data respectively. Paired samples within the group of cases were analyzed using the McNemar test and the paired T-test to
10 compare non-parametric and categorical data respectively. Logistic regression analysis was used to compare categorical data adjusted for age and location of the tissue since significant differences in age and location of the different tissues were observed between CRC cases and controls. All
15 quoted p-values are two-sided, and a p-value 0.05 or lower was considered statistically significant. All statistical tests were corrected for multiple comparisons using the Bonferroni method. Data analysis was done using SPSS software (version 12.0.1).

20

Results

NDRG4 promoter methylation and expression in CRC cell lines

The structure of the NDRG4 gene shows a dense CpG island (GC
25 content > 60%, ratio of observed CpG / expected CpG > 0.6 and minimum length 200 bp (Gardiner-Garden and Frommer 1987)) located -556 to +869 relative to the transcription start site as shown in FIG.10. To assay this region for potential methylation we designed two different MSP primer
30 pairs (1 and 2) amplifying overlapping fragments in the CpG island. These primers were initially used to investigate eight CRC cell lines (LS174, HCT116, HT29, RKO, CACO2,

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COLO2, SW48 and SW480) for DNA methylation. All cell lines except SW480 were methylated as analyzed by MSP using both primer pairs as shown in FIG.11a. To further investigate the pattern of CpG island methylation we performed sodium bisulfite sequencing of HCT116 and SW480. The promoter region spanning 39 CpG sites was PCR-amplified using sodium bisulfite-modified genomic DNA as template and six clones of each cell lines were sequenced. Bisulfite sequencing confirmed MSP data in that HCT116 showed almost complete methylation at 39 sites as depicted in FIG.11b, whereas SW480 showed almost no methylated CpG sites. Endogenous NDRG4 mRNA levels in CRC cell lines HCT116 and RKO were significant increased after treatment with DAC (FIG.11c).

15 Methylation of NDRG4 in normal and CRC tissue

Methylation of NDRG4 was confirmed in three pairs of primary tumors and matched normal colonic mucosa by sodium bisulfite sequencing. The results depicted in FIG.12a show dense methylation of the three tumor samples while almost no methylation was observed in the normal colon mucosa. Interestingly, the density of methylation was higher in the upstream region of the NDRG4 CpG island when compared to more downstream region as shown in FIG.12a.

Subsequently, the methylation status of NDRG4 was investigated in colorectal carcinoma, adenoma and normal colorectal mucosa using two different primer pairs (1 and 2). The methylation frequencies using both primer pairs are depicted in table 47. A significant difference (table 47, $p=0.042$ 10^{-7}) was observed in methylation frequencies in normal mucosa of the control group (2/48 (4%)) compared to cancer tissue of CRC patients (71/83 (86%)) using primer pair 2. In addition, we compared NDRG4 promoter methylation

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in adjacent normal mucosa tissue of CRC patients (9/78 (12%)) and the normal mucosa of non-cancerous patients (2/48 (4%)) but did not find a significant difference among these two groups (table 47). Furthermore, to investigate NDRG4 methylation in premalignant lesions, we compared adenomas obtained from CRC patients that developed synchronously or metachronously to the tumour and adenomas obtained from patients that did not develop CRC after 10 years of follow-up. We observed a higher prevalence of NDRG4 methylation in adenomas from CRC patients although these differences did not reach statistical significance (table 47).

Table 47: Methylation frequencies (%) of normal, adenoma, carcinoma tissue from CRC patients and normal, adenoma tissue of non-cancerous patients. Methylation differences are analyzed by logistic regression adjusted for age (NDRG4p1, p2) and location (NDRG4 p1)

	Carcinoma tissue	Controls normal	P	Normal tissue		P	Adenoma tissue		P
				controls	CRC+		Controls	CRC+	
NDRG4 p1	71%	0%	0.02×10^{-2}	0%	3%	NS	13%	41%	NS
NDRG4 p2	86%	4%	0.042×10^{-7}	4%	12%	NS	55%	66%	NS

Abbreviations: CRC+, colorectal cancer patients; P, P-value; NS, not significant

To confirm the high prevalence of NDRG4 promoter methylation in CRC, we analyzed a second independent series of 183 CRC samples. Comparable to the results of the first study series we observed that 70% (127/183) of CRC patients presented NDRG4 methylation.

Further analysis of the clinicopathologic features of patients with primary CRC with regard to NDRG4 promoter methylation did not reveal any association with age at

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diagnosis, sex, location of the tumor or the TNM stage for both independent series using primer 2 (table 49).

To investigate NDRG4 promoter methylation during cancer progression we compared the frequency of methylation from normal mucosa to adenoma and carcinoma tissues in patients for which all the three tissues were available (table 48). Our results show that NDRG4 is significantly (table 48, $p < 0.02 \times 10^{-2}$) more frequently methylated in carcinomas (84%) compared to normal mucosa adjacent to the tumor (16%). In addition to the carcinomas, adenoma samples from CRC patients also exhibit significantly (table 48, $p < 0.03 \times 10^{-3}$) higher NDRG4 methylation frequencies (61%) compared to normal colon samples (14%). Finally, NDRG4 methylation was increased in carcinoma tissues (81%) compared to adenoma samples (63%) although this enhancement was not significant (primer pair 2, table 48).

Table 48: *NDRG4* Methylation frequencies (%) of carcinoma tissue, adenoma and normal tissue from colorectal cancer patients. Methylation differences were analyzed by Mc Nemar test.

CRC patients	Normal tissue	Adenoma tissue	<i>P</i>	Normal tissue	Carcinoma tissue	<i>P</i>	Adenoma tissue	Carcinoma tissue	<i>P</i>
NDRG4									
p1	0%	34%	0.003	0%	73%	0.01×10^{-4}	39%	76%	0.012
NDRG4									
p2	14%	61%	$< 0.03 \times 10^{-3}$	16%	84%	$< 0.02 \times 10^{-2}$	63%	81%	NS

Frequencies may vary because of missing data for some variables.
Abbreviations: CRC+, colorectal cancer patients; P, P-value; NS, not significant

The different series were analyzed using two different primer pairs 1 and 2 amplifying overlapping fragments in the CpG island, as depicted in FIG.10. Using primer pair 1 we observed overall the same results compared to primer pair 2

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however we found an increase of NDRG4 methylation for all the subgroups using primer pair 2 compared to primer pair 1. Interestingly, we found a significant difference (table 2, $p=0.012$) in promoter methylation in adenomas of CRC patients (55/77 (41%)) compared to the carcinomas (55/77 (71%)) which was not observed using primer pair 2. In addition, comparing the NDRG4 methylation status of adenomas obtained from CRC patients that developed synchronously or metachronously to the tumour (24/58 (41%)) and adenomas obtained from patients that did not develop CRC (4/31 (13%)) we observed a enormous increase of NDRG4 methylation in adenomas from CRC patients using primer pair 1 although these differences also did also not reach statistical significance. Further analysis of the clinicopathologic features of patients with primary CRC with regard to NDRG4 promoter methylation for both independent series did not reveal any association with age at diagnosis, sex or the TNM stage. However, we did find a significant correlation between promoter methylation and the location of the tumor using primer pair 1 (table 49, $p=0.034$).

20

Table 49: Prevalence (%) of promoter methylation of NDRG4 in relation to clinicopathological features of carcinoma tissue for two independent series. Methylation differences were analyzed by chi-square

Characteristics		%methylation	%methylation	% methylation
		NDRG4p1	NDRG4p2	NDRG4p2 Independent series
TNM stage*				
	I	15%	16%	23%
	II	33%	32%	33%
	III	40%	41%	30%
	IV	13%	11%	13%
	<i>P</i>	NS	NS	NS
Tumor Location‡				
	proximal	65%	56%	37%
	distal	35%	44%	63%
	<i>P</i>	0.034	NS	NS
Sex*				

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	Male	42%	48%	55%
	Female	58%	52%	44%
	<i>P</i>	NS	NS	NS
Age at diagnosis§				
	<= mean	40%	48%	48%
	> mean	60%	52%	52%
	<i>P</i>	NS	NS	NS

Abbreviations: P, P-value; NS, not significant

5 NDRG4 promoter methylation in other neoplasias

Next, we asked whether NDRG4 promoter methylation is present in other tumor tissues. Therefore 119 primary tumor specimens covering 7 different tumor types were analyzed using MSP primer pair 2. No or little methylation was found in skin (0/8, 0%), kidney (1/10, 10%), ovary (0/20, 0%), prostate (0/10, 0%) and breast (lobular (0/7, 0%) and ductal (0/9, 0%)) carcinomas. In contrast, NDRG4 promoter was frequently methylated in adenocarcinomas of the esophagus (13/16, 81%), while no methylation was found in esophageal squamous cancers (0/12, 0%). Both diffuse type (8/11, 73%) and intestinal type (9/11, 82%) carcinomas of the stomach were frequently methylated while the normal mucosa of the stomach did not show any methylation (0/5, 0%).

20 NDRG4 promoter methylation in fecal DNA

The high prevalence of NDRG4 promoter methylation in CRC and the absence of methylation in normal colon mucosa suggest that NDRG4 promoter methylation could be a sensitive and specific biomarker for non-invasive detection of CRC.

25 Therefore, we developed a quantitative MSP assay using molecular beacon technology and analyzed fecal DNA of 21 CRC patients and 67 healthy controls. NDRG4 promoter methylation could be detected in 16/21 CRC patients yielding a 76%

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sensitivity for the detection of CRC. Only 2/67 (3%) of healthy controls tested positive for NDRG4 methylation, which resulted in a clinical specificity for the assay of 97%. Stool samples were obtained from CRC patients covering all different TNM stages. The assay had a 75 % sensitivity among CRC patients with early stage colon cancer (stage I and II) and 80% of sensitivity among later stage patients (stage III and IV).

10 NDRG4 RNA and protein expression

To analyse whether methylation of the promoter CpG island of NDRG4 is associated with gene silencing we investigated mRNA expression of NDRG4 in CRC cell lines, three pairs of CRC tissues and matching normal colon mucosa. In all three CRCs, mRNA levels were significantly downregulated (97, 70% and 98% respectively) when compared to normal colon mucosa (FIG.12b).

To investigate the protein expression of NDRG4 in both normal colonic mucosa and colon cancers, we performed NDRG4 immunohistochemistry demonstrating the presence of NDRG4 protein expression in the cytoplasm of normal colon mucosa while protein expression is lost in half of CRCs (FIG.12c). Subsequently, we performed immunohistochemical analysis of NDRG4 expression on 19 CRC samples. Eleven of these patients had a methylated NDRG4 promoter. However, we could not find a significant association between NDRG4 promoter methylation and NDRG4 expression (data not shown). This observation suggests that other mechanisms might lead to NDRG4 inactivation.

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Loss of heterozygosity and mutation analysis of the NDRG4 gene in CRC

Macrodissected CRC tissue and corresponding normal tissues of 86 CRC patients were analyzed using the microsatellite markers DS16S3089 and DS16S3071. The two markers showed a heterozygosity of 77.4% and 35.4% respectively. Of these, 59 cases were informative; 18 tumors (30,5%) showed LOH with at least one marker on chromosome 16q.

Twelve primary CRC and CRC cell lines HCT116 and SW480 were analyzed for NDRG4 mutations. No inactivating mutations within the coding region of the NDRG4 gene were detected in 12 colorectal carcinomas. However, we found one novel nonsynonymous mutation in the SW480 cell line (40662A→AG Ile65Val). As part of the mutational analysis, 2 previously reported SNPs (NCBI SNP database) were detected. One SNP was observed in 1/12 CRC patients (43760G→GG Val224Val refSNP rs 17821543). The second SNP was observed in 9/12 CRC patients (48311A→AG Ser354Ser refSNP rs 42945).

20

Discussion

The progression of CRC from small benign colorectal adenomas to larger and more dysplastic lesions takes several decades and identifying early stages would improve management and treatment of this disease (Brenner and Rennert 2005). Colonoscopy is currently the best technique for detecting CRC or its precursor lesions from the age of 50 years onwards. Testing for the presence of fecal occult blood (FOBT) as preselection for colonoscopy is the only non-invasive screening method with proven effectiveness,

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reducing both the incidence and the risk of death from CRC when used programmatically.

However, both sensitivity and specificity of FOBT is low and therefore there is an urgent need for more sensitive and specific non-invasive screening tests. A promising option is analyzing (expression of) cancer-specific molecules such as DNA, RNA and protein in blood and tissue. First attempts to detect genetic alterations are promising (Dong, Traverso et al. 2001; Traverso, Shuber et al. 2002) although still need improvement. Markers of choice have been TP53, K-ras and APC mutations and in addition BAT-26 instability and long DNA (a marker for non-apoptotic shedding of epithelial colonocytes). Recently, CpG island hypermethylation can also be used as a (prognostic) marker for non-invasive detection of CRC in different biological samples (Esteller 2003; Chen, Han et al. 2005; Ebert, Model et al. 2006). Over the last years, several genes have been described to be methylated in CRC using different techniques.

Here we used MSP, quantitative MSP and bisulfite sequencing to analyse NDRG4 as a biomarker for the early detection of colorectal and other gastrointestinal cancers. (ARRAY) The NDRG4 promoter CpG island was demonstrated to be methylated in two independent large series of CRC cases. In the first series we included normal mucosa of non-cancerous patients since the normal mucosa from the CRC patients is situated within the same bowel segment as the tumor and can be contaminated with malignant cells or a field-effect could have change the molecular signature of this cell as described for MGMT (Issa, 2005). Nevertheless, by performing statistical analysis we could not find any significantly difference in methylation between these two groups. Chronic

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inflammation has previously been shown to accelerate DNA methylation in normal tissues (Issa, Ahuja et al. 2001). Therefore additional screens with inflamed colon mucosa are expected in a screening setting. In our study population, inclusion of inflamed mucosa to the normal mucosa of control patients slightly reduced the specificity of NDRG4 from 96% to 94%. Because we found a difference in the density of methylation in the promoter area of NDRG4 by bisulfite sequencing, we used two different primer sets to investigate the methylation status of NDRG4. Interestingly, using primer pair 2, we found 86% of methylation in carcinoma tissue while only 71% was observed by use of primer pair 1. This increased detection of methylation using primer pair 2 was observed for all the subgroups of this series as shown in table 47. Primer pair 2 is situated more to the 5' region of the gene. The frequencies of methylation were lower near the transcription start site. We hypothesize that NDRG4 hypermethylation initially occurs at the 5' end of the NDRG4 CpG island and spreads towards the transcription start site before ultimately shutting down NDRG4 mRNA expression, as has also been observed for RUNX3 (Turker 2002; Homma, Tamura et al. 2006). In addition, we found a significant difference ($p=0.012$) in methylation frequency using primer pair 1, between adenoma tissue and carcinoma tissue within the group of CRCs. Therefore, we speculated, that spreading of DNA methylation in the promoter area of NDRG4 towards the transcription start site occurs during cancer progression.

Remarkably, using primer pair 1, hypermethylation was more frequently present in progressed adenomas from the CRC patients (41%) when compared to the non progressing adenomas

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of the CRC- patients (13%). The capacity to distinguish adenomas that progress to cancer from those that will not progress is highly important for CRC screening (Hermesen, Postma et al. 2002). Whereas this difference can not be made
5 macroscopically, endoscopic screening strategies aiming to detect and remove all adenomas will be inherently unspecific. The majority of adenomas removed would not have progressed to cancer because only a small percentage of these benign precursor lesions will progress into a
10 carcinoma (Lengauer, Kinzler et al. 1998). These data might indicate that NDRG4 promoter methylation in adenoma tissue (in the region we investigated) is a possible risk factor for developing a colon tumor.

15 Recently, it has been reported that promoter methylation can be detected in biological fluids such as blood, urine or stool and may allow early diagnosis of various cancers, including CRC. Some studies have shown that methylation of one gene promoter can be used as a screening method for
20 fecal DNA methylation detection. For example, promoter methylation of SFR2, Vimentin and HIC1 can be detected in fecal DNA of CRC patients with a sensitivity of 77%, 43% and 42% respectively and a specificity of 77%, 90% and 95% respectively (Muller, Oberwalder et al. 2004; Chen, Han et
25 al. 2005; Lenhard, Bommer et al. 2005). NDRG4 methylation in fecal DNA as a single marker can differentiate cancer from controls with a sensitivity of 76% and a specificity of 97%.

In order to be a specific biomarker for CRC, analysis of
30 tissue specificity was performed; we found NDRG4 methylation in other tumors of the gastrointestinal tract, namely oesophagus and gastric cancers. This data indicate that

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methylation of NDRG4 may serve as a marker for other gastrointestinal tumors as well.

We next studied whether methylation of NDRG4 is associated
5 with downregulation of NDRG4 RNA and protein expression. So far, the expression of NDRG4 has only been documented in the brain and heart by use of Northern blotting. We observed expression of NDRG4 in normal colon tissue and downregulation in all three tumor tissues. Subsequently, we
10 performed immunohistochemical analysis of NDRG4 expression on 19 CRC samples from the CRC patients for which paraffin-embedded tissues were available. Eleven of these patients had a methylated NDRG4 promoter. However, we could not find a significant association between NDRG4 promoter methylation
15 and NDRG4 expression. Some tumors had a methylated NDRG4 promoter although still expressed NDRG4 protein. The methylation that we detected using MSP might reflect methylation of only a few cancer cells or methylation of only one of two NDRG4 alleles (and absence in the other).
20 Nevertheless, some tumors lack expression of NDRG4 protein while no promoter methylation was observed. This observation suggests that other mechanisms might lead to NDRG4 inactivation. No mutations were found, indicating that mutational inactivation of the NDRG4 gene might not play a
25 major role in CRC. Our results confirmed previous data on NDRG4 mutation studies (Sjoberg, Jones et al. 2006). However, LOH at 16q is seen in about 30% of the CRC cases. Frequent LOH of 16q had previously been described in a wide variety of solid tumor types as breast (Rakha, Green et al.
30 2006), liver (Sakai, Nagahara et al. 1992; Bando, Nagai et al. 2000), prostate (Elo, Harkonen et al. 1997), ovarian (Kawakami, Staub et al. 1999) and Wilms' tumors (Mason,

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Goodfellow et al. 2000) but until now it has not been described in CRC. Because NDRG4 is downregulated in most of the colon cancer cells compared to normal colonic epithelial cells we hypothesizes that NDRG4 has a tumor suppressor
5 function in cancer.

In conclusion, we are the first group who described a role for NDRG4 in cancer and our data indicate that NDRG4 is a potential novel marker for CRC with a very high sensitivity
10 and specificity of 76% and 100% respectively. Although the sensitivity and specificity of NDRG4 as a marker alone is already very high, the diagnostic accuracy of NDRG4 may be enhanced by the addition of other markers analyzed in patients with CRC as well. This may augment the ability to
15 identify patients with cancer in a multipanel methylation-based diagnostic test.

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5) Additional real-time MSP assays tested on plasmid material for NDRG4 and OSMR genes

5 Plasmid material corresponding to a promoter region of NDRG4 and OSMR gene was used to test additional assay designs. The plasmid for the standard curve was generated as follows: the promoter sequence as defined by the primers is PCR amplified and cloned (using suitable isolated and bisulphite modified
10 cell line DNA). The sequence is verified by sequencing and compared to the published promoter sequence. A serial dilution of either NDRG4 or OSMR plasmid material (2×10^6 to 2×10^1 copies/5 μ l) was loaded in duplicate. 5 μ l of plasmid dilution or buffer (non template control) was added to a 20
15 μ l PCR mix containing the specified primer and beacon detector sequences as previously described. Results were generated using the SDS 2.2 software from Applied Biosystems with automatic baseline and threshold settings. Data were exported as Ct values (cycle number at which the
20 amplification curves cross the threshold value, set automatically by the software).

NDRG4

Initial real-time results for 2 different NDRG4 assay
25 designs are presented in table 50 and 51. The primer and beacon combinations used for the respective assays NDRG4_1a and NDRG4_1b were previously described. Underscore 1a and 1b reflect the different primer and/or beacon combinations used for assessing the methylation status of the NDRG4 gene.
30 NDRG4_1a corresponds to the preferred NDRG4 assay design, also simply referred to as NDRG4 (see Table 4). Comparable results were obtained for both assay designs. Clinical

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sample data provided in this invention are generated using the preferred NDRG4 assay design (=NDRG4 = NDRG4_1a)

Table 50: Real time MSP results obtained for NDRG4_1a assay on plasmid material. Resulting standard curve ($y = -3.3321x + 39.862$; $R^2 = 0.9991$) corresponds to a PCR efficiency of 100%.

Assay	Task	Ct	Quantity	Log copies	Duplicate Ct	Average Ct	ΔCt
NDRG4_1a	Standard	18.82	200000	6.30	18.92	18.87	0.09
NDRG4_1a	Standard	22.09	200000	5.30	22.22	22.15	0.13
NDRG4_1a	Standard	25.42	20000	4.30	25.47	25.45	0.06
NDRG4_1a	Standard	28.86	2000	3.30	28.94	28.90	0.08
NDRG4_1a	Standard	32.58	200	2.30	32.48	32.53	0.10
NDRG4_1a	Standard	34.92	20	1.30	35.64	35.28	0.71
NDRG4_1a	NTC	Undetermined	0		Undetermined	Undeter	Undeter

Table 51: Real time MSP results obtained for NDRG4_1b assay on plasmid material. Resulting standard curve ($y = -3.4181x + 40.991$; $R^2 = 0.9991$) corresponds to a PCR efficiency of 99.2%.

Assay	Task	Ct	Quantity	Log copies	Duplicate Ct	Average Ct	ΔCt
NDRG4_1b	Standard	19.48	2000000	6.30	19.59	19.53	0.12
NDRG4_1b	Standard	22.93	200000	5.30	22.92	22.92	0.01
NDRG4_1b	Standard	26.26	20000	4.30	26.18	26.22	0.08
NDRG4_1b	Standard	29.65	2000	3.30	29.67	29.66	0.02
NDRG4_1b	Standard	32.82	200	2.30	32.83	32.82	0.01
NDRG4_1b	Standard	36.75	20	1.30	36.91	36.83	0.16
NDRG4_1b	NTC	Undetermined	0		Undetermined	Undet	Undet

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OSMR

5 Initial real-time results for 3 different OSMR assay designs are presented in below Tables 52 to 54. The primer and beacon combinations used for the respective assays OSMR_1, OSMR_3 [=OSMR (3)] and OSMR_4 [=OSMR (4)] were previously described. Underscore 1, 3 and 4 reflect the different
 10 primer and/or beacon combinations used for assessing the methylation status of the OSMR gene. Comparable results were obtained for all three assay designs.

Table 52: Real time MSP result obtained for OSMR_1 assays on
 15 plasmid material. Resulting standard curve ($y = -3.3326x + 41.136$; $R^2 = 0.9993$) corresponds to a PCR efficiency of 99.6%.

Assay	Task	Ct	Quantity	Log copies	Duplicate Ct	Average Ct	ΔCt
OSMR_1	Standard	20.04	2000000	6.30	20.14	20.09	0.09
OSMR_1	Standard	23.48	200000	5.30	23.41	23.44	0.07
OSMR_1	Standard	26.73	20000	4.30	26.85	26.79	0.12
OSMR_1	Standard	30.13	2000	3.30	30.26	30.19	0.13
OSMR_1	Standard	33.55	200	2.30	33.93	33.74	0.38
OSMR_1	Standard	36.54	20	1.30	36.58	36.56	0.04
OSMR_1	NTC	Undetermined	0		Undetermined	Undet	Undet

20

Table 52: Real time MSP result obtained for OSMR_3 assays on plasmid material. Resulting standard curve ($y = -3.3909x + 38.398$; $R^2 = 0.9999$) corresponds to a PCR efficiency of 97.2%.

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Assay	Task	Ct	Quantity	Log copies	Duplicate Ct	Average Ct	ΔCt
OSMR_3	Standard	16.93	2000000	6.30	17.16	17.04	0.23
OSMR_3	Standard	20.41	200000	5.30	20.29	20.35	0.12
OSMR_3	Standard	23.97	20000	4.30	23.83	23.90	0.14
OSMR_3	Standard	27.22	2000	3.30	27.16	27.19	0.06
OSMR_3	Standard	30.51	200	2.30	30.67	30.59	0.16
OSMR_3	Standard	34.18	20	1.30	33.77	33.98	0.41
OSMR_3	NTC	38.13	0		Undetermined	Undet	Undet

Table 54: Real time MSP result obtained for OSMR_4 assays on
 5 plasmid material. Resulting standard curve ($y = -3.2795x + 38.77$; $R^2 = 0.9997$) corresponds to a PCR efficiency of 100.8%

Assay	Task	Ct	Quantity	Log copies	Duplicate Ct	Average Ct	ΔCt
OSMR_4	Standard	18.24	2000000	6.30	17.90	18.07	0.33
OSMR_4	Standard	21.56	200000	5.30	21.05	21.31	0.51
OSMR_4	Standard	24.79	20000	4.30	24.64	24.72	0.15
OSMR_4	Standard	28.24	2000	3.30	27.91	28.08	0.33
OSMR_4	Standard	31.37	200	2.30	31.18	31.28	0.19
OSMR_4	Standard	34.63	20	1.30	34.12	34.37	0.50
OSMR_4	NTC	Undetermined	0		Undetermined	Undet	Undet

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6) Testing and validation of further CRC markers in bodily fluid test samples

New markers added: BNIP3, FOXE1, JAM3, PHACTR3, TPFI2, SOX17 and SYNE1 (and also JPH3 stool data). Suitable primers and probes for determining the methylation status of these genes are set forth in Tables 12 (and 13 to 18) above.

Methods and Results

10 Clinical samples

Samples were collected from centers in Germany and The Netherlands

Table 55: Samples for DNA extraction from plasma (blood origin)

type	Sample Numbers
Normal	10
Colorectal Cancer stage III	6
Colorectal Cancer stage IV	4

15

Table 56: Samples for DNA extraction from stool

type	Sample Numbers
Control (Normal)	7
Case (Colorectal Cancer)	1
Case (Colorectal Cancer)	6

20

Marker testing on clinical samples

Experiments were performed as previously described. Briefly DNA was extracted from stool and/or plasma followed by bisulfite treatment. Samples were tested by real-time MSP assays, using 384 well plates with a 12 µl final volume. The template volume is 2.4 µl with a mix volume of 9.6 µl.

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Results were generated using the SDS 2.2 software (Applied Biosystems), exported as Ct values (cycle number at which the amplification curves cross the threshold value, set automatically by the software). Copy numbers are
 5 extrapolated using a standard curve.

The individual performance of the 8 gene assays TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3, JAM3 and JPH3 in plasma and stool samples is shown in Table XV (except for JPH3:
 10 stool data only). Sensitivity values for plasma and stool are ranging from 30 to 70% and 0 to 57% respectively with a corresponding specificity of a 100%. When optimizing for sensitivity, 80% sensitivity for TFPI2 and 50% sensitivity for PHACTR3 is obtained in plasma samples with a
 15 corresponding specificity of 90%. It is observed that for some markers (TFPI2, BNIP3, FOXE1, SYNE1 and SOX17) sensitivity of colorectal cancer detection is higher when using plasma samples compared to stool samples.

20 Table 57: Individual gene performance: Sensitivity and specificity of TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3, JAM3 markers on stool and plasma samples. Sensitivity and specificity results for the JPH3 marker were only obtained for stool using this sample set, plasma data were enabled
 25 earlier with a different sample set.

	optimized for Specificity			optimized for Sensitivity		
	Specificity	Sensitivity	cutoff	Specificity	Sensitivity	cutoff
TFPI2 Stool	100	57	10			
TFPI2 Plasma	100	70	1	90	80	0
BNIP3 Stool	100	0	7			
BNIP3 Plasma	100	30	0			
FOXE1 Stool	100	57	0			
FOXE1 Plasma	100	60	0			

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SYNE1 Stool	100	57	2			
SYNE1 Plasma	100	60	0			
SOX17 Stool	100	57	30			
SOX17 Plasma	100	60	2			
PHACTR3 Stool	100	43	8			
PHACTR3 Plasma	100	40	2	90	50	0
JAM3 Stool	100	43	1			
JAM3 Plasma	100	30	1			
JPH3 Stool	100	14	20			
JPH3 Plasma	see previous colon results					

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various
5 modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims. Moreover, all embodiments
10 described herein are considered to be broadly applicable and combinable with any and all other consistent embodiments, as appropriate.

Various publications are cited herein, the disclosures of
15 which are incorporated by reference in their entireties.

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CLAIMS

1. A method of detecting a predisposition to, or the incidence of, cancer in a sample comprising detecting an
5 epigenetic change in at least one gene selected from an NDRG4/NDRG2 subfamily gene, GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, wherein detection of the epigenetic change is indicative of a predisposition to, or the
10 incidence of, cancer.
2. The method of claim 1 wherein the cancer is colorectal cancer and the at least one gene is selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and
15 MGMT.
3. The method of claim 1 or 2 wherein the test sample is a faecal test sample.
- 20 4. The method of claim 1 which comprises detecting epigenetic silencing of an NDRG4/NDRG2 subfamily gene, wherein epigenetic silencing of the gene is indicative for cancer or predisposition to cancer or advanced adenoma.
- 25 5. The method according to claim 1 wherein NDRG4/NDRG2 subfamily gene expression is determined, wherein reduced expression of the gene is indicative for colon cancer and/or gastric cancer, or predisposition to colon cancer and/or gastric cancer, or advanced adenoma.
- 0 6. The method according to claim 1 wherein NDRG4/NDRG2 subfamily gene methylation status is determined, wherein

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methylation of the gene is indicative for colon cancer and/or gastric cancer, or predisposition to colon cancer and/or gastric cancer, or advanced adenoma.

5 7. The method of claim 1 wherein the cancer is colorectal cancer, the sample is a blood sample, or derivative thereof and the at least one gene is selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC.

10 8. The method of claim 1 wherein the cancer is colorectal cancer, the sample is a tissue sample and the at least one gene is a panel of genes comprising OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5, wherein detection of the epigenetic change in at least one of the genes in the panel
15 is indicative of a predisposition to, or the incidence of, colorectal cancer.

9. The method of claim 1 wherein the cancer comprises a gastrointestinal cancer.

20

10. The method of claim 9 wherein the gastrointestinal cancer comprises colorectal cancer, gastric cancer, stomach cancer and/or oesophageal cancer.

25 11. The method of claim 10 wherein the gastrointestinal cancer is colorectal cancer.

12. The method of claim 10 wherein the oesophageal cancer is oesophageal adenocarcinoma.

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13. The method of claim 10 wherein the stomach cancer comprises a diffuse type and/or intestinal type carcinoma of the stomach.
- 5 14. The method of any preceding claim wherein the at least one gene is an NDRG4/NDRG2 subfamily gene.
15. The method of any preceding claim wherein the NDRG4/NDRG2 subfamily gene comprises or consists of the
10 NDRG4 gene.
16. The method of any of claims 1 to 13 wherein the at least one gene is selected from GATA4, OSMR, NDRG4 and SFRP2.
- 15 17. The method of any of claims 1 to 13 wherein the at least one gene is selected from OSMR, NDRG4, GATA5 and ADAM23.
- 20 18. The method of any preceding claim which comprises detecting an epigenetic change in a panel of genes comprising at least two, three, four, five or six of the genes, wherein detection of an epigenetic change in at least one of the genes in the panel is indicative of a
25 predisposition to, or the incidence of, cancer.
19. The method of claim 18 wherein the panel of genes comprises two, three, four, five or six genes.
- 30 20. The method of claim 18 or 19 wherein the panel of genes comprises GATA4 and OSMR, GATA4 and NDRG4, GATA4 and SFRP2, OSMR and NDRG4, OSMR and SFRP2, NDRG4 and SFRP2, APC and

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SFRP2, APC and OSMR, APC and GATA4, APC and NDRG4, MGMT and OSMR, MGMT and GATA4, MGMT and NDRG4, MGMT and SFRP2, MGMT and APC, SFRP1 and MGMT, SFRP1 and OSMR, SFRP1 and GATA4, SFRP1 and NDRG4, SFRP1 and SFRP2, SFRP1 and APC, GATA5 and
5 SFRP1, GATA5 and MGMT, GATA5 and OSMR, GATA5 and GATA4, GATA5 and NDRG4, GATA5 and SFRP2 or GATA5 and APC.

21. The method of any of claims 18 to 20 wherein the panel of genes comprises GATA4, OSMR and NDRG4, GATA4, OSMR and
10 SFRP2, GATA4, NDRG4 and SFRP2, SFRP1, SFRP2 and APC, SFRP2, OSMR and APC or OSMR, NDRG4 and SFRP2.

22. The method of any of claims 18 to 21 wherein the panel of genes consists of GATA4, OSMR, NDRG4 and SFRP2.

15 23. The method of any of claims 18 to 20 wherein the panel of genes comprises NDRG4, OSMR, SFRP1, ADAM23, GATA5, GATA4 and MGMT.

20 24. The method of claim 18 or 19 wherein the panel of genes is a three gene panel with the genes being selected from OSMR, NDRG4, GATAJ and ADAM23.

25 25. The method of claim 24 wherein the panel of genes consists of OSMR, NDRG4, GATA5 and ADAM23.

26. The method of claim 18 or 19 wherein the panel of genes comprises OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5.

30 27. The method of any of claims 18 to 20 wherein the detection of an epigenetic change in each of the panel of genes is carried out in a single reaction.

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28. The method of any preceding claim wherein the sample comprises a tissue sample and/or a bodily fluid sample.

5 29. The method of claim 28 wherein the bodily fluid sample comprises a faecal sample.

30. The method of claim 29 wherein the at least one gene is selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23,
10 JPH3, SFRP2, APC and MGMT and/or selected from TFPI2, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3.

31. The method of claim 28 wherein the tissue sample comprises a colon and/or rectal and/or appendix sample.

15

32. The method of claim 28 wherein the bodily fluid sample comprises a blood sample.

33. The method of claim 32 wherein the blood sample, or
20 derivative thereof comprises a plasma or a serum sample.

34. The method of claim 32 or 34 wherein the at least one gene is selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3, JPH3 and JAM3.

25

35. The method of claim 33 or 34 wherein the plasma sample is obtained by centrifugation of whole blood.

36. The method of claim 35 wherein multiple centrifugation
30 steps are employed to obtain the plasma sample.

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37. The method of claim 36 wherein two centrifugation steps are employed to obtain the plasma sample.

38. The method of claim 37 wherein plasma samples of volume
5 of less than 2 ml are excluded.

39. The method of any preceding claim wherein the cancer is colorectal cancer and wherein the colorectal cancer comprises early stage colorectal cancer.

10

40. The method of claim 39 wherein the early stage colorectal cancer comprises a stage 0-II colorectal cancer.

41. The method of any preceding claim wherein the
15 epigenetic change is methylation.

42. The method of claim 41 wherein hypermethylation of the promoter region of the gene(s) is detected.

20 43. The method of claim 41 or 42 wherein methylation is determined using methylation specific PCR/amplification.

44. The method of claim 43 wherein the methylation specific PCR/amplification is carried out in real time or at end
25 point.

45. The method of any of claims 41 to 44 wherein methylation is quantified against methylation of a reference gene.

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46. The method of any of claims 43 to 44 which utilises primers selected from primers comprising the nucleotide sequences set forth in tables 2 to 18.

5 47. The method of any of claims 43 to 46 which utilises probes selected from probes comprising the nucleotide sequences set forth in tables 2 to 18.

48. The method of any preceding claim which is used in
10 combination with detecting DNA integrity, or at least one DNA oncogene mutation, or a combination of both detecting DNA integrity and at least one DNA oncogene mutation in the sample in order to detect a predisposition to, or the incidence of, colorectal cancer.

15 49. The method of any preceding claim wherein the methylation status of the CpG island comprising the nucleotide sequence set forth as SEQ ID NO: 524 and/or SEQ ID NO: 525 is determined.

20 50. The method of any preceding claim wherein the epigenetic change is detected by determination of its effect at the level of gene expression.

25 51. The method of claim 50 wherein gene expression is determined at the protein level.

52. The method of claim 50 wherein gene expression is determined at the RNA level.

30 53. The method of claim 52 wherein real time or end-point detection is employed.

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54. A method for determining the histopathological stage of cancer and in particular a gastrointestinal cancer in a sample comprising detecting an epigenetic change in at least one gene selected from an NDRG4/NDRG2 subfamily gene, GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, wherein detection of the epigenetic change is indicative of the histopathological stage of the cancer.

55. The method of claim 54 wherein the cancer is colorectal cancer and the at least one gene is selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT.

56. The method of claim 54 or 55 wherein the test sample is a faecal sample.

57. The method of claim 51 which is for determining the histopathological stage of adenoma and/or colorectal cancer and comprises determining epigenetic silencing of the NDRG4/NDRG2 subfamily gene, wherein epigenetic silencing of the NDRG2/NDRG4-family gene is correlated to a histopathological stage of the cancer.

58. The method of claim 54 which is for determining the histopathological stage of colorectal cancer in a blood sample, or derivative thereof and comprises detecting an epigenetic change in at least one gene selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC and/or at least one gene selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, wherein detection of the epigenetic

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change is indicative of the histopathological stage of the colorectal cancer.

59. The method of claim 54 which is for determining the
5 histopathological stage of colorectal cancer in a tissue
sample and comprises detecting an epigenetic change in a
panel of genes comprising OSMR, GATA4 and ADAM23 or OSMR,
GATA4 and GATA5, wherein detection of the epigenetic change
in at least one of the genes in the panel is indicative of
10 the histopathological stage of the colorectal cancer.

60. A method for predicting or monitoring progression of an
adenoma to a carcinoma comprising determining the
methylation status of an NDRG2/NDRG4 subfamily gene in a
15 suitable test sample, wherein an elevated or increased level
of methylation indicates that the adenoma is more likely to
progress to a carcinoma than if the level of methylation is
lower.

20 61. The method of claim 60 wherein the methylation status
of the NDRG4 gene is determined.

62. The method of claim 61 wherein the methylation status
of the NDRG4 gene in the region between and including the
25 primer binding sites of the primers comprising the
nucleotide sequences set forth in table 2 is determined.

63. A method for predicting or monitoring progression of a
gastrointestinal cancer comprising determining the
30 methylation status of the NDRG4 gene in a suitable test
sample, wherein an elevated or increased level of
methylation towards the transcription start site indicates

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that the cancer is more progressed than if the level of methylation is lower.

64. The method of claim 63 wherein the gastrointestinal
5 cancer is colorectal cancer.

65. A method for predicting the likelihood of successful
treatment of cancer and in particular a gastrointestinal
cancer with a DNA demethylating agent and/or a DNA
10 methyltransferase inhibitor and/or HDAC inhibitor comprising
detecting an epigenetic change in at least one gene selected
from an NDRG4/NDRG2 subfamily gene, GATA4, OSMR, GATA5,
SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1,
SYNE1, SOX17, PHACTR3 and JAM3, wherein detection of the
15 epigenetic change is indicative that the likelihood of
successful treatment is higher than if the epigenetic
modification is not detected.

66. The method of claim 65 wherein the cancer is colorectal
20 cancer and the at least one gene is selected from GATA4,
OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and
MGMT.

67. The method of claim 65 or 66 wherein the test sample is
25 a faecal sample.

68. The method of claim 65 which comprises determining the
methylation status of a NDRG4/2 family gene in a sample
obtained from a subject, wherein if the NDRG4/2 family gene
30 is methylated, in particular hypermethylated, the likelihood
of successful treatment is higher than if the NDRG4/2 family
gene is unmethylated, or methylated to a lesser degree.

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69. The method of claim 65 which comprises measurement of expression levels of the NDRG4/2 family gene in a sample obtained from a subject, wherein a reduced level of
5 expression indicates the likelihood of successful treatment of cancer is higher than if the NDRG4/2 family gene is expressed at a higher level.

70. The method of claim 65 wherein the cancer is colorectal cancer, the at least one gene is selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC and/or selected from TFPI1, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 and the sample is a blood sample, or derivative thereof.

15 71. The method of claim 65 wherein the cancer is colorectal cancer and the method comprises detecting an epigenetic change in a panel of genes comprising OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5 in a tissue sample, wherein detection of the epigenetic change in at least one of the
20 genes in the panel is indicative that the likelihood of successful treatment is higher than if the epigenetic modification is not detected.

72. A method for predicting the likelihood of resistance to
25 treatment of cancer and in particular a gastrointestinal cancer with a DNA demethylating agent and/or DNA methyltransferase inhibitor and/or HDAC inhibitor comprising detecting an epigenetic change in at least one gene selected from an NDRG4/NDRG2 subfamily gene, GATA4, OSMR, GATA5,
30 SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, wherein detection of the epigenetic change is indicative that the likelihood of

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resistance to treatment is lower than if the epigenetic modification is not detected.

73. The method of claim 72 wherein the cancer is colorectal
5 cancer and the at least one gene is selected from GATA4,
OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and
MGMT.

74. The method of claim 72 or 73 wherein the test sample is
10 a faecal sample.

75. The method of claim 72 which comprises determining the
methylation status of a NDRG4/2 family gene in a sample
obtained from a subject, wherein if the NDRG4/2 family gene
15 is unmethylated, or methylated to a lesser degree, the
likelihood of resistance to treatment is higher than if the
NDRG4/2 family gene is methylated or hypermethylated.

76. A method of claim 72 which comprises measurement of
20 expression levels of the NDRG4/2 family gene in a sample
obtained from a subject, wherein a higher level of
expression indicates the likelihood of resistance to
treatment of cancer is higher than if the NDRG4/2 family
gene is expressed at a reduced level.

25

77. The method of claim 72 wherein the cancer is colorectal
cancer, the at least one gene is selected from OSMR, SFRP1,
NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC and/or selected
from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 and
30 the sample is a blood sample, or derivative thereof.

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78. The method of claim 72 wherein the cancer is colorectal cancer and the method comprises detecting an epigenetic change in a panel of genes comprising OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5 in a tissue sample, wherein
5 detection of the epigenetic change in at least one of the genes in the panel is indicative that the likelihood of successful treatment is higher than if the epigenetic modification is not detected.

10 79. A method of selecting a suitable treatment regimen for cancer in particular a gastrointestinal cancer comprising detecting an epigenetic change in at least one gene selected from an NDRG4/NDRG2 subfamily gene, GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1,
15 SYNE1, SOX17, PHACTR3 and JAM3, wherein detection of the epigenetic change results in selection of a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor for treatment and wherein if the epigenetic change is not detected, a DNA demethylating agent
20 and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor is not selected for treatment.

80. The method of claim 79 wherein the cancer is colorectal cancer and the at least one gene is selected from GATA4,
25 OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT.

81. The method of claim 79 or 80 wherein the sample is a faecal sample.

30

82. The method of claim 79 which comprises determining epigenetic silencing of a NDRG4/2 family gene in a sample

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obtained from a subject, wherein if the gene is epigenetically silenced, in particular hypermethylated or reduced expressed, a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor is
5 selected for treatment.

83. The method of claim 79 which is for selecting a suitable treatment regimen for cancer or predisposition to cancer or advanced adenoma and comprises determining the
10 methylation status and/or expression level of a NDRG4/2 family gene in a sample obtained from a subject, wherein if the gene is unmethylated or higher expressed, treatment with a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor is contra-indicated.

15 84. The method of claim 79 wherein the cancer is colorectal cancer, the at least one gene is selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC and/or is selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 and
20 the sample is a blood sample, or derivative thereof.

85. The method of claim 79 wherein the cancer is colorectal cancer and the method comprises detecting an epigenetic change in a panel of genes comprising OSMR, GATA4 and ADAM23
25 or OSMR, GATA4 and GATA5 in a tissue sample, wherein detection of the epigenetic change in at least one of the genes in the panel results in selection of a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor for treatment and wherein if the
30 epigenetic change in at least one of the genes in the panel is not detected, a DNA demethylating agent and/or a DNA

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methyltransferase inhibitor and/or a HDAC inhibitor is not selected for treatment.

86. The method of any one of claims 54 to 85 further
5 characterised by the features of the method as claimed in any of claims 1 to 53.

87. A method of treating cancer in a subject comprising
administration of a DNA demethylating agent and/or a HDAC
10 inhibitor and/or a DNA methyltransferase inhibitor wherein the subject has been selected for treatment on the basis of a method as claimed in any preceding claim.

88. The method of claim 87 wherein the subject has been
15 selected for treatment on the basis of measuring the methylation status of the NDRG2/NDRG4-family gene promoter, the expression level of the NDRG2/NDRG4-family gene, or a combination thereof.

20 89. The method of claim 87 or 88 wherein the cancer comprises a gastrointestinal cancer.

90. The method of claim 89 wherein the gastrointestinal
cancer comprises colorectal cancer, gastric cancer, stomach
25 cancer and/or oesophageal cancer, in particular colorectal cancer.

91. The method of claim 90 wherein the oesophageal cancer
is oesophageal adenocarcinoma.

30

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92. The method of claim 90 wherein the stomach cancer comprises a diffuse type and/or intestinal type carcinoma of the stomach.

5 93. A kit for;

(a) predicting the likelihood of successful treatment of cancer and/or the likelihood of resistance to treatment of cancer with a DNA damaging agent and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor,

10 (b) for selecting a suitable treatment regimen for cancer
(c) for diagnosing cancer or predisposition to cancer, comprising carrier means containing therein a set of primers for use in detecting the methylation status of at least one gene selected from a NDRG2/NDRG4-family gene, GATA4, OSMR,
15 GATA5, SERP1, ADAM23, JPH3, SFRP2, APC and MGMT, and/or at least one gene selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3.

94. The kit of claim 93 wherein the at least one gene is a
20 NDRG2/NDRG4 subfamily gene.

95. The kit of claim 94 wherein the NDRG2/NDRG4 subfamily gene is NDRG4.

96. The kit of any of claims 93 to 95 which further
25 comprises a reagent which selectively modifies unmethylated cytosine residues in the DNA contained in the sample to produce detectable modified residues but which does not modify methylated cytosine residues.

30 97. The kit of claim 96 wherein the reagent comprises a bisulphite reagent.

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98. The kit of claim 97 wherein the bisulphite reagent comprises sodium bisulphite.

99. A kit for detecting a predisposition to, or the incidence of a gastrointestinal cancer and in particular colorectal cancer in a sample comprising:

(a) means for detecting an epigenetic change in at least one gene selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT, and/or at least one gene selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3

(b) means for processing a faecal sample.

100. The kit of any of claims 93 to 99 wherein the at least one gene is selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT.

101. The kit of claim 93 wherein the at least one gene is selected from GATA4, OSMR, NDRG4 and SFRP2.

102. The kit of any of claims 93 to 101 which comprises means for detecting an epigenetic change in a panel of genes comprising at least two, three, four, five or six of the genes, wherein detection of an epigenetic change in at least one of the genes in the panel is indicative of a predisposition to, or the incidence of, colorectal cancer.

103. The kit of claim 102 wherein the panel of genes comprises two, three, four, five or six genes.

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104. The kit of claim 102 or 103 wherein the panel of genes comprises GATA4 and OSMR, GATA4 and NDRG4, GATA4 and SFRP2, OSMR and NDRG4, OSMR and SFRP2 or NDRG4 and SFRP2.

5 105. The kit of any of claims 102 to 104 wherein the panel of genes comprises GATA4, OSMR and NDRG4, GATA4, OSMR and SFRP2, GATA4, NDRG4 and SFRP2 or OSMR, NDRG4 and SFRP2.

106. The kit of any of claims 102 to 105 wherein the panel
10 of three or four genes selected from genes consists of GATA4, OSMR, NDRG4 and SFRP2.

107. The kit of claim 102 or 103 wherein the panel of genes comprises NDRG4, OSMR, SFRP1, ADAM23, GATA5 and MGMT.

15

108. The kit of any of claims 99 to 107 wherein the means for processing a faecal sample comprise a sealable vessel for collection of a faecal sample.

20 109. The kit of any of claims 99 to 108 wherein the means for processing a faecal sample comprises a homogenization buffer.

110. The kit of any of claims 99 to 109 wherein the means
25 for processing a faecal sample comprises reagents for extraction/isolation/concentration/purification of DNA.

111. The kit of any of claims 99 to 110 wherein the means for processing a faecal sample comprises primers for
30 directing amplification of DNA in the sample.

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112. A kit for detecting a predisposition to, or the incidence of, gastrointestinal cancer and in particular colorectal cancer in a sample comprising:

- (a) means for detecting an epigenetic change in at least
5 one gene selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC, and/or at least one gene selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3
(b) means for processing a blood sample, or derivative thereof.

10

113. The kit of claim 112 wherein the at least one gene is selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC.

- 15 114. The kit of claim 112 or 113 wherein the blood sample, or derivative thereof comprises a plasma or a serum sample.

115. The kit of any of claims 112 to 114 wherein the at least one gene is selected from OSMR, NDRG4, GATA5 and
20 ADAM23.

116. The kit of any of claims 112 to 115 which comprises means for detecting an epigenetic change in a panel of genes comprising at least two, three, four, five or six of the
25 genes, wherein detection of an epigenetic change in at least one of the genes in the panel is indicative of a predisposition to, or the incidence of, colorectal cancer.

117. The kit of claim 116 wherein the panel of genes
30 comprises two, three, four, five or six genes.

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118. The kit of claim 116 or 117 wherein the panel of genes consists of OSMR, NDRG4, GATA5 and ADAM23.

119. The kit of any of claims 112 to 118 wherein the
5 colorectal cancer comprises early stage colorectal cancer.

120. The kit of claim 119 wherein the early stage colorectal cancer comprises a stage 0-II colorectal cancer.

10 121. The kit of any of claims 112 to 120 wherein the means for processing a blood sample or derivative thereof comprise a sealable vessel for collection of a blood sample.

122. The kit of any of claims 112 to 121 wherein the means
15 for processing a blood sample, or derivative thereof comprises reagents for extraction/isolation/concentration/purification of DNA from a blood sample, or derivative thereof.

20 123. The kit of claim 122 wherein the reagents for extraction/isolation/concentration/purification of DNA from a blood sample, or derivative thereof are selected from the kits of table 1.

25 124. The kit of any of claims 112 to 123 wherein the means for processing a blood sample, or derivative thereof comprises a stabilizer.

125. A kit for detecting a predisposition to, or the
30 incidence of, colorectal cancer in a sample comprising:

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(a) means for detecting an epigenetic change in a panel of genes comprising OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5

(b) means for processing a colon, rectal or appendix tissue sample.

126. The kit of any of claims 93 to 125 wherein the means for detecting an epigenetic change in the panel of genes enable the detection to be carried out in a single reaction.

10

127. The kit of any of claims 93 to 126 wherein the epigenetic change is methylation.

128. The kit of claim 127 wherein hypermethylation of the promoter region of the gene(s) is detected.

15

129. The kit of claim 127 or 128 wherein the means for detecting methylation comprises methylation specific PCR primers.

20

130. The kit of claim 129 further comprising means for carrying out the methylation specific PCR in real time or at end point.

131. The kit of claim 129 or 130 wherein the methylation specific PCR primers are selected from primers comprising the nucleotide sequences set forth in tables 2 to 18.

25

132. The kit of claim 130 or 131 which comprises probes selected from probes comprising the nucleotide sequences set forth in tables 2 to 18.

30

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133. The kit of any of claims 99 to 132 wherein the means for processing a faecal sample or blood sample, or derivative thereof or a tissue sample comprises a reagent which selectively modifies unmethylated cytosine residues in the DNA contained in the sample to produce detectable modified residues but which does not modify methylated cytosine residues.

134. The kit of claim 133 wherein the reagent comprises a bisulphite reagent.

135. The kit of claim 134 wherein the bisulphite reagent comprises sodium bisulphite.

136. A method of determining the methylation status of at least one gene in a blood plasma or serum sample, comprising:

- (a) isolating DNA from a blood plasma or serum sample,
- (b) subjecting the isolated DNA to treatment with a reagent which selectively modifies unmethylated cytosine residues in the DNA contained in the sample to produce detectable modified residues but which does not modify methylated cytosine residues
- (c) amplifying the treated isolated DNA in order to determine the methylation status of at least one gene, characterised in that 0.07 to 0.72 ml blood plasma or serum sample equivalent of DNA is used per amplification reaction.

137. The method of claim 136 wherein the blood plasma or serum sample is a blood plasma sample.

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138. The method of claim 137 wherein the blood plasma sample is obtained by centrifugation of whole blood.

139. The method of claim 138 wherein multiple centrifugation
5 steps are employed to obtain the plasma sample.

140. The method of claim 138 wherein two centrifugation steps are employed to obtain the plasma sample.

10 141. The method of any of claims 136 to 140 wherein samples with a plasma volume less than 2 ml prior to isolating DNA are excluded.

142. The method of any of claims 136 to 141 wherein the
15 reagent comprises a bisulphite reagent.

143. The method of any of claims 136 to 142 wherein the volume of the blood sample, or derivative thereof is around
20 10 ml.

144. The method of any of claims 136 to 143 further comprising following step (b) concentrating the treated isolated DNA prior to amplification.

25 145. The method of any of claims 136 to 143 further comprising stabilization of the blood plasma or serum sample with a stabilizer.

146. The method of any of claims 136 to 145 wherein the at
30 least one gene is selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2, APC and, MGMT and/or is selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3.

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147. The method of claim 146 wherein the at least one gene is selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC.

5

148. The method of claim 147 wherein the at least one gene is selected from OSMR, NDRG4, GATA5 and ADAM23.

149. The method of any of claims 136 to 148 wherein the at least one gene forms part of a panel of genes comprising at least two, three, four, five or six genes, wherein the methylation status of each of the genes is determined.

150. The method of claim 149 wherein the panel of genes comprises two, three, four, five or six genes.

151. The method of claim 149 or 150 wherein the panel of genes consists of OSMR, NDRG4, GATA5 and ADAM23.

152. The method of any of claims 149 to 151 wherein the determination of the methylation status of each of the panel of genes is carried out in a single reaction.

153. The method of any preceding claim wherein the amplifying step comprises the polymerase chain reaction (PCR).

154. The method of claim 153 wherein the PCR is methylation specific PCR.

30

155. The method of claim 154 wherein the methylation specific PCR is carried out in real-time or at end point.

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156. The method of any of claims 136 to 155 which employs primers and/or probes selected from the primers and probes comprising the nucleotide sequences set forth in tables 2 to
5 18 to determine the methylation status of the at least one gene.

157. The method of any of claims 136 to 156 wherein the methylation status of the at least one gene is correlated
10 with the incidence of a disease and the method is used in order to detect a predisposition to, or the incidence of, the disease.

158. The method of claim 157 wherein the disease comprises a
15 cell proliferative disorder.

159. The method of claim 158 wherein the cell proliferative disorder comprises cancer.

20 160. The method of claim 159 wherein the cancer comprises a gastrointestinal cancer.

161. The method of claim 160 wherein the gastrointestinal cancer comprises a colorectal cancer.
25

162. The method of claim 161 wherein the colorectal cancer comprises early stage colorectal cancer.

163. The method of claim 162 wherein the early stage
30 colorectal cancer comprises a stage 0-II colorectal cancer.

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164. A method of processing a faecal sample to isolate and prepare DNA for use in detecting a predisposition to, or the incidence of, colorectal cancer in a faecal sample comprising:

- 5 (a) isolating DNA from the faecal sample
- (b) subjecting at least 2.5µg of the isolated DNA per amplification reaction required to treatment with a reagent which selectively modifies unmethylated cytosine residues in the DNA contained in the sample to produce detectable
- 10 modified residues but which does not modify methylated cytosine residues
- (c) amplifying the treated isolated DNA.

165. The method of claim 164 which comprises, prior to step

15 (a), adding a homogenization buffer to the faecal sample.

166. The method of claim 164 or 165 wherein the reagent comprises a bisulphite reagent.

20 167. The method of claim 166 wherein the bisulphite reagent comprises sodium bisulphite.

168. The method of any of claims 164 to 167 wherein following step (b) and prior to step (c) the treated

25 isolated DNA is concentrated.

169. The method of any of claims 164 to 168 wherein the faecal sample is at least 4g in weight.

30 170. The method of any of claims 164 to 169 further comprising carrying out the method of any of claims 1 to 58 on the amplified treated DNA.

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171. A primer pair selected from the primer pairs comprising the nucleotide sequences set forth in tables 2 to 18.

5 172. A probe selected from the probes comprising the nucleotide sequences set forth in tables 2 to 18.

173. A kit comprising a primer pair of claim 171 and corresponding probe of claim 172.

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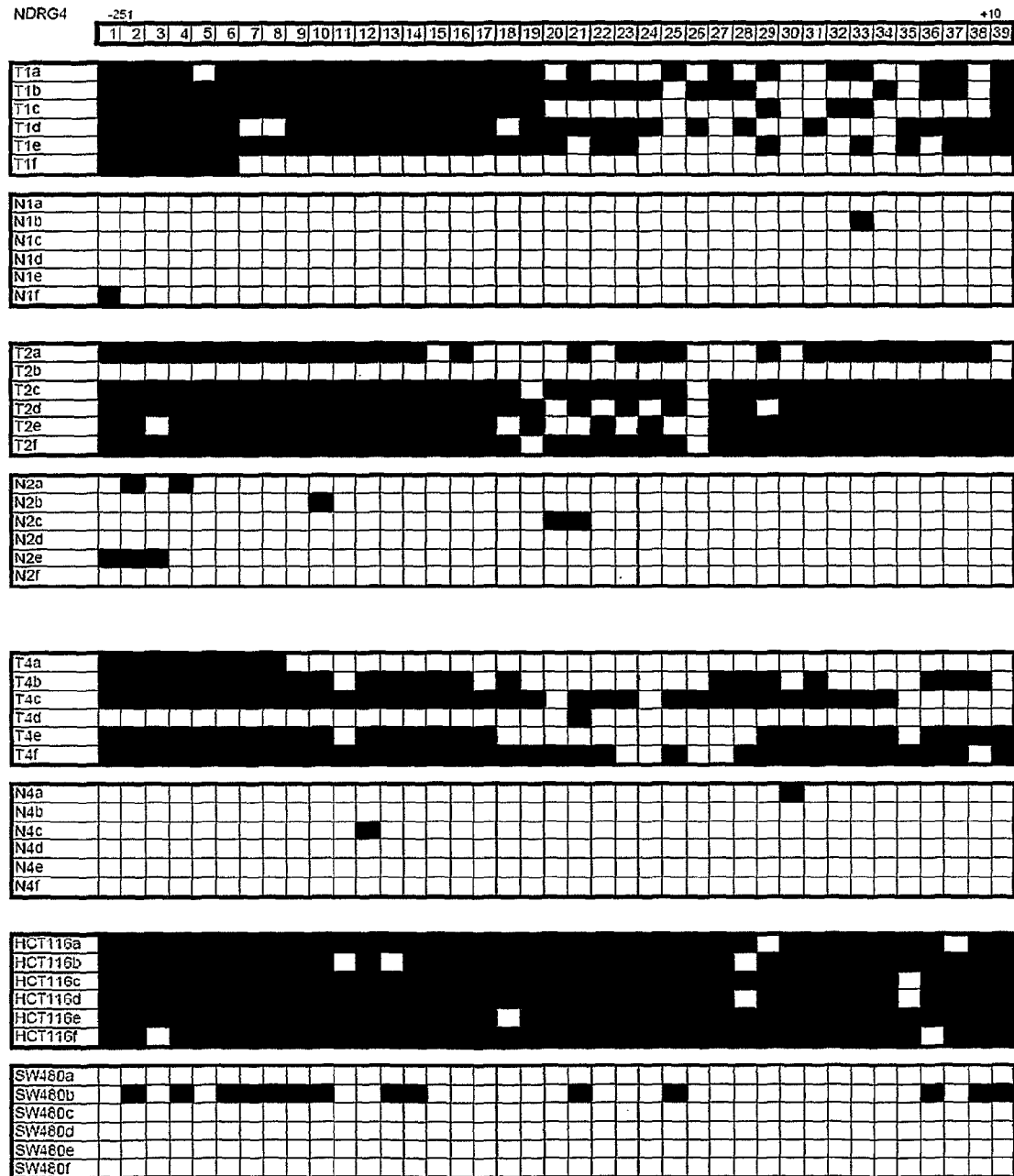


FIG. 1a

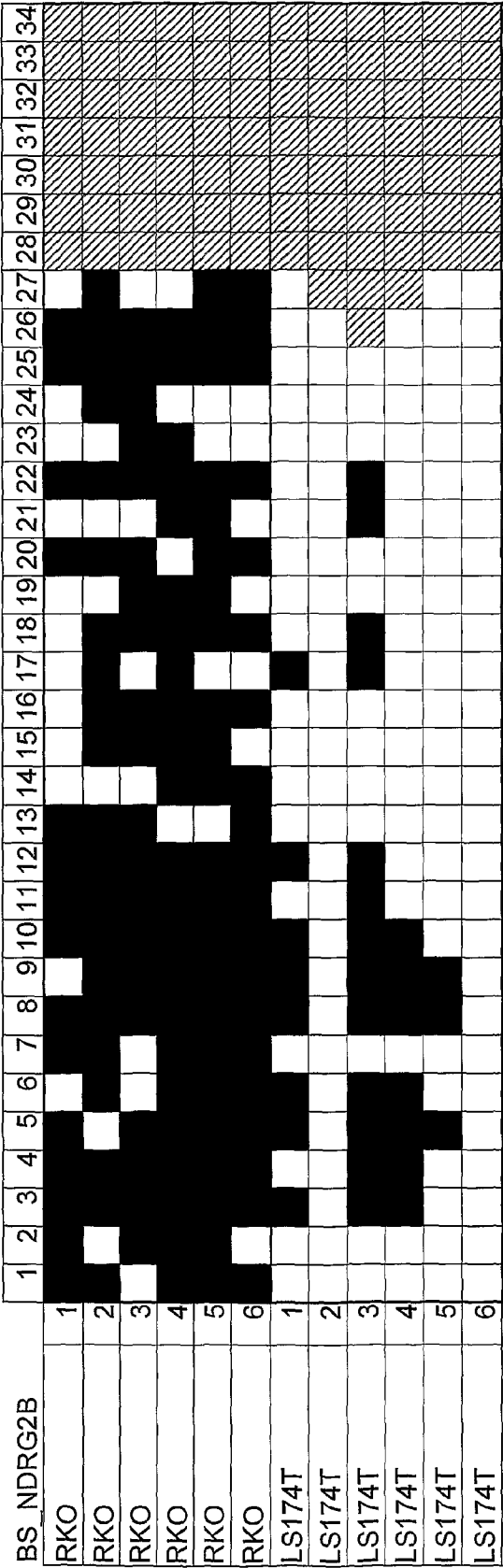


FIG. 1b

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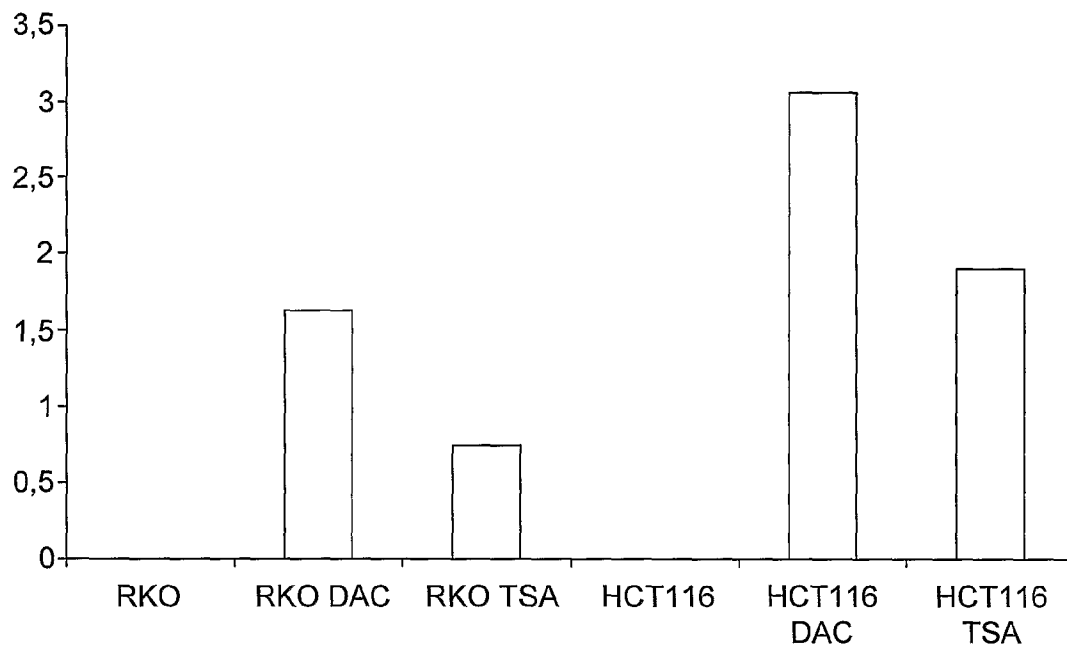


FIG. 2

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gtttaatagagttttgtatggaaaagattggaattttagggaggagtagagtttCGtttaa
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FIG. 3a

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[illegible]

FIG. 3b

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FIG. 3b
CONT'D

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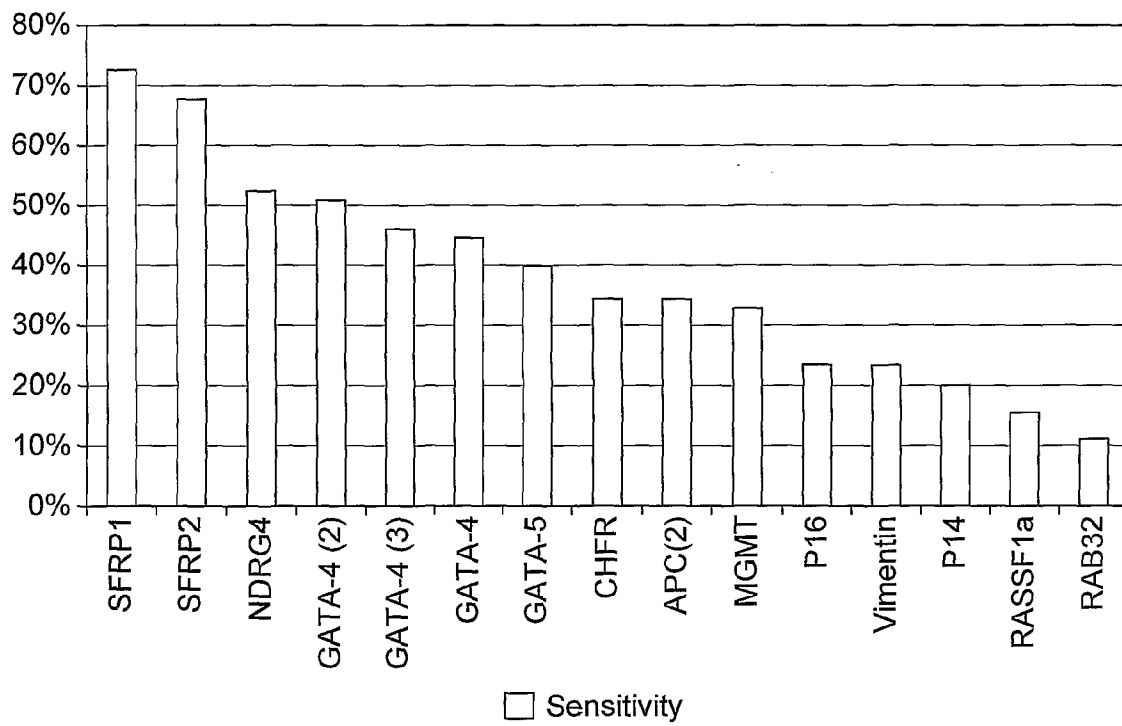


FIG. 4

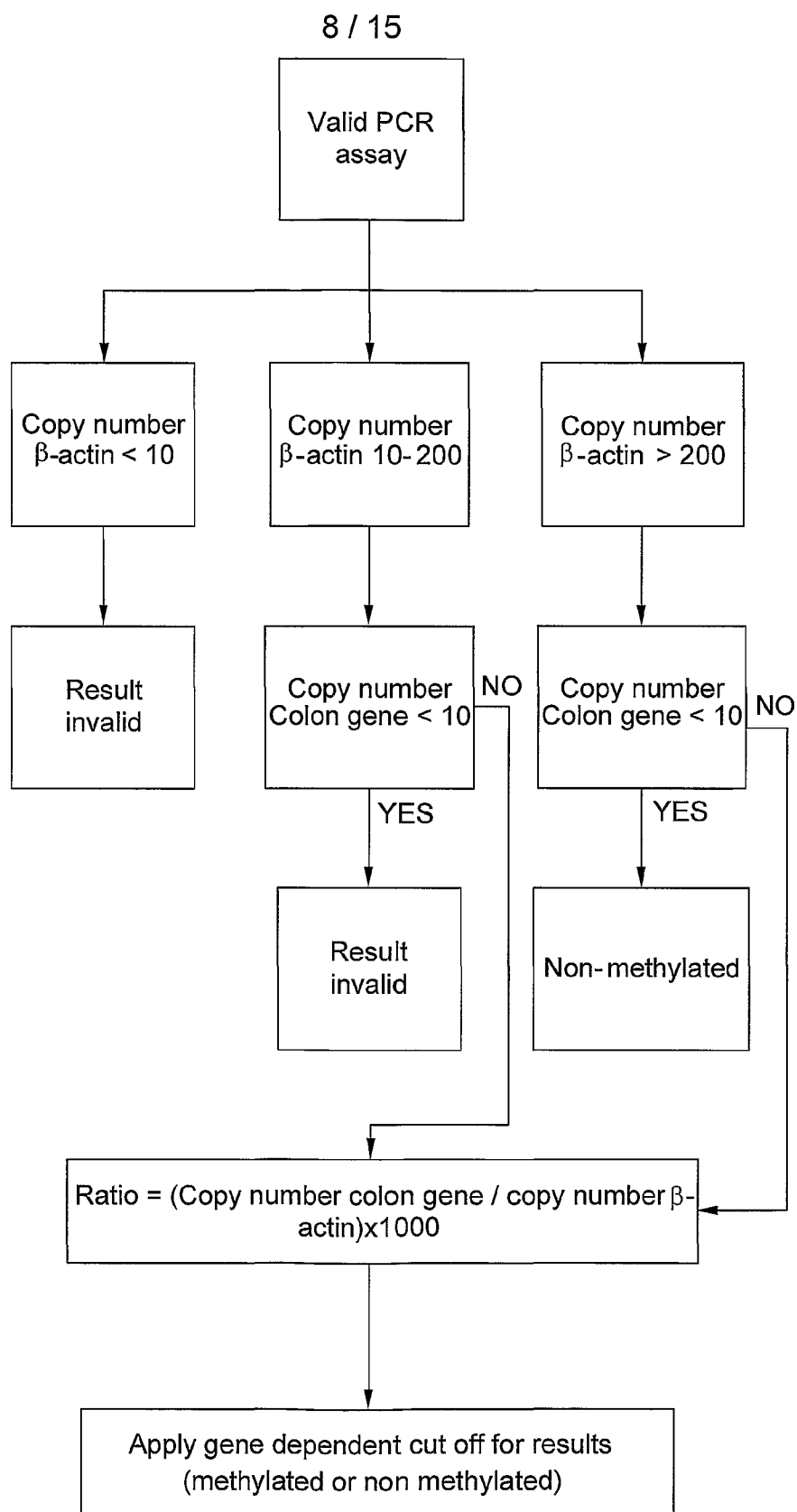


FIG. 5

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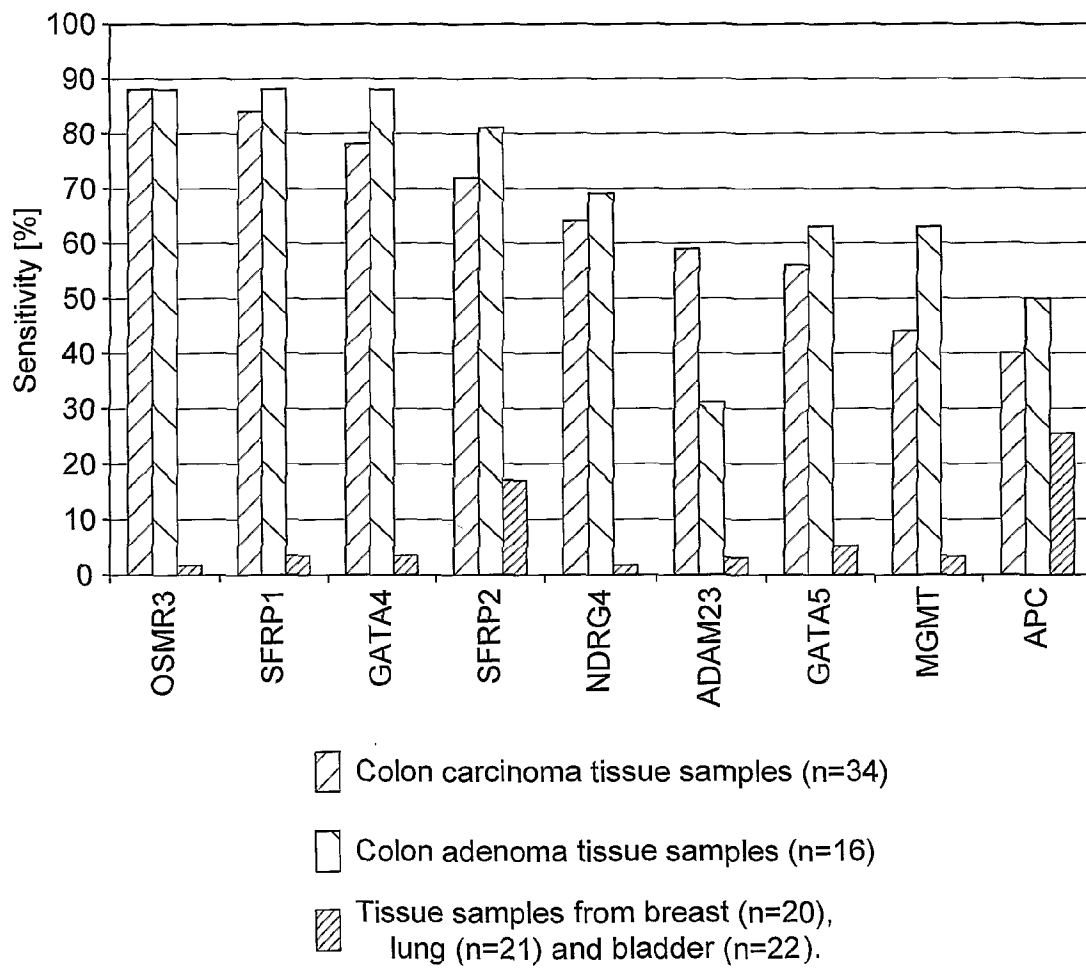


FIG. 6

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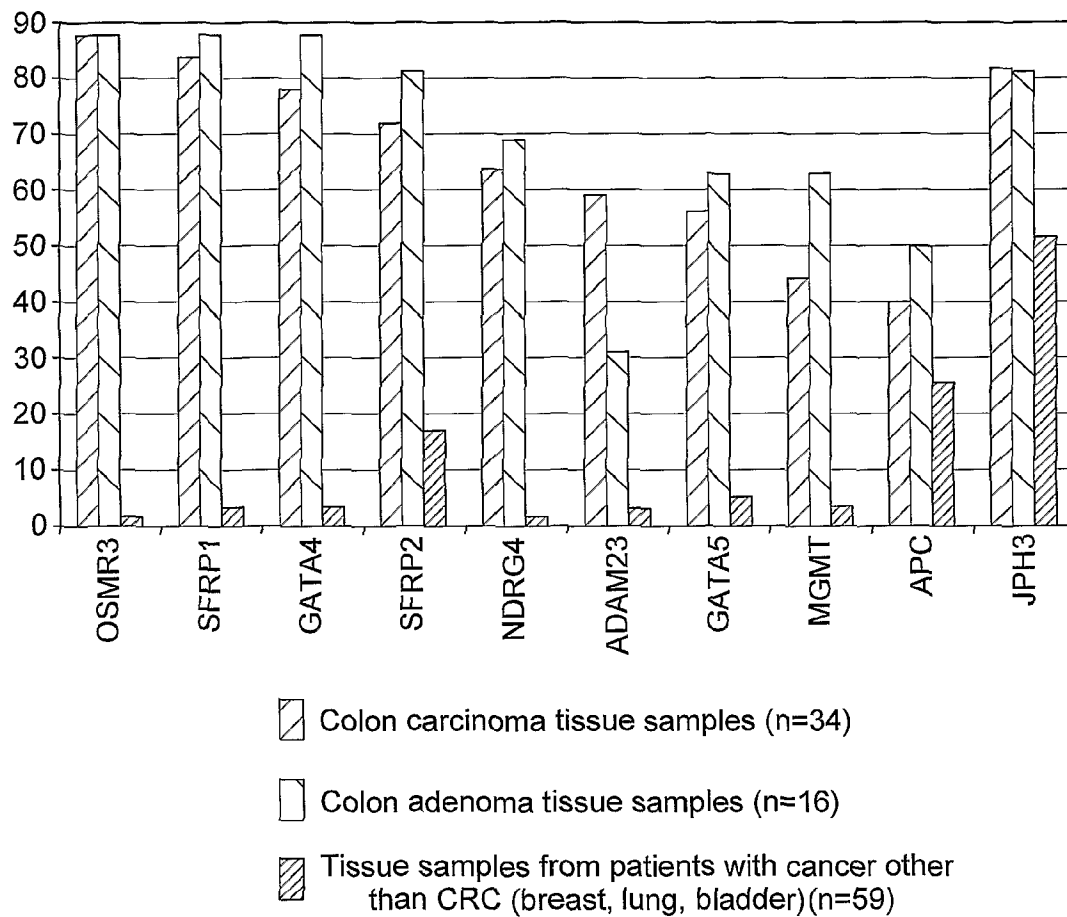


FIG. 7

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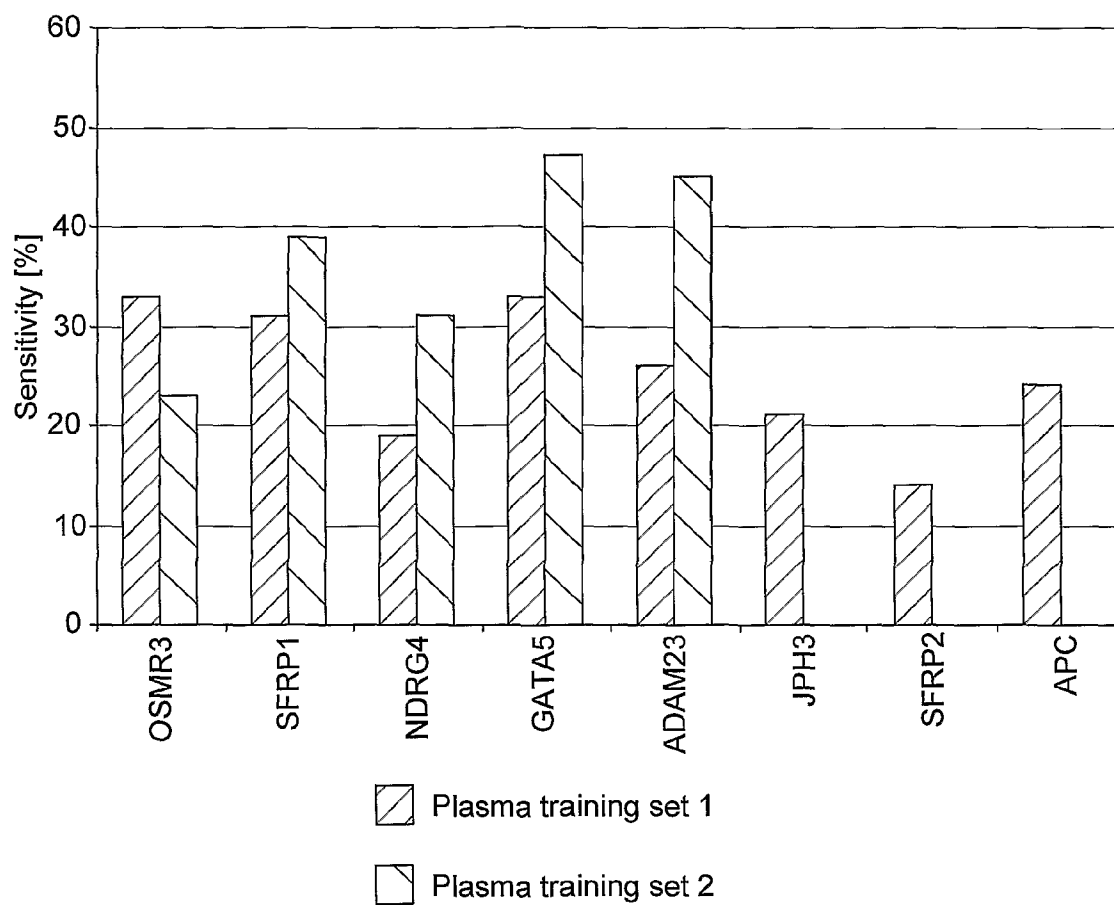


FIG. 8

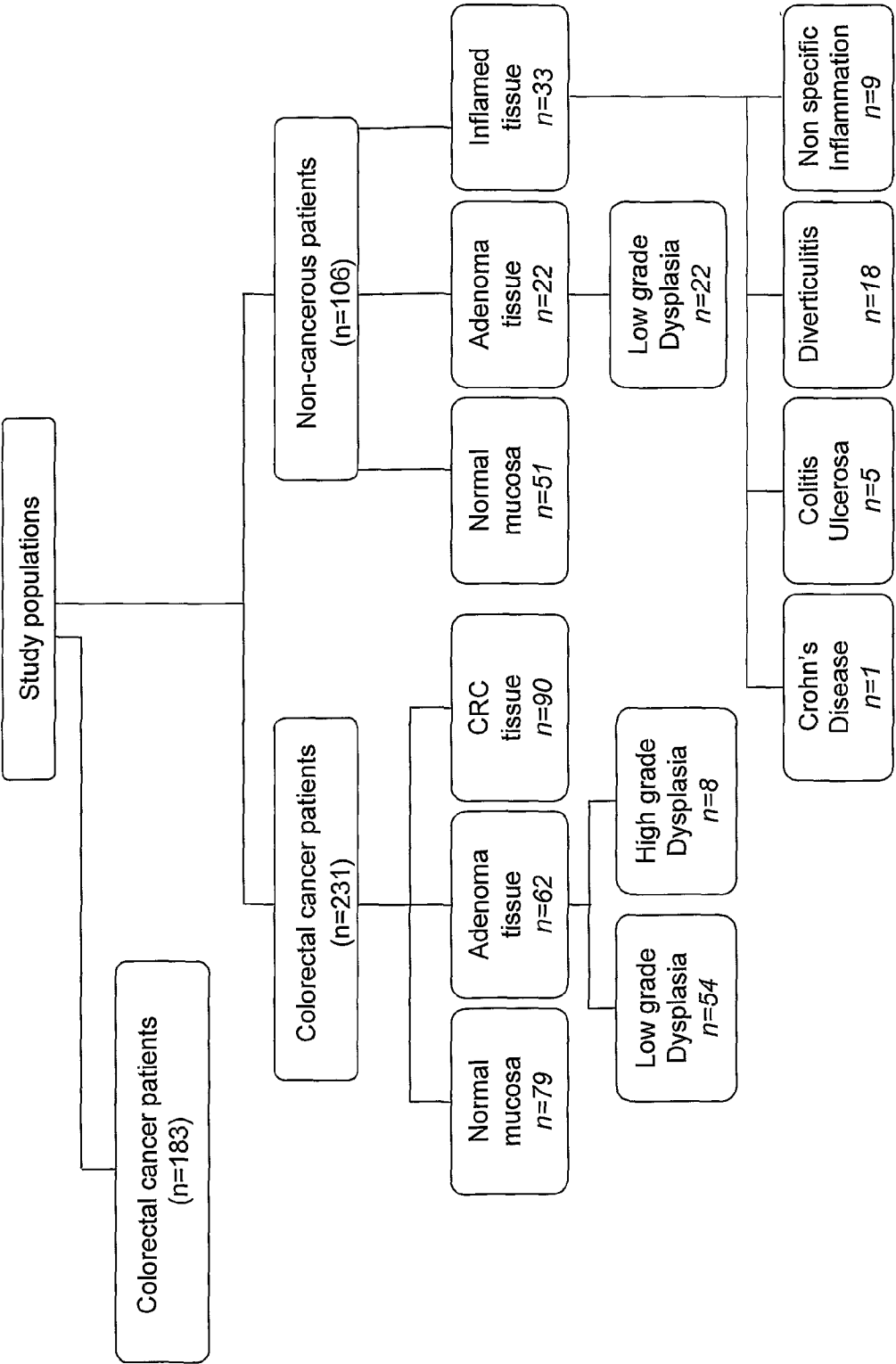


FIG. 9

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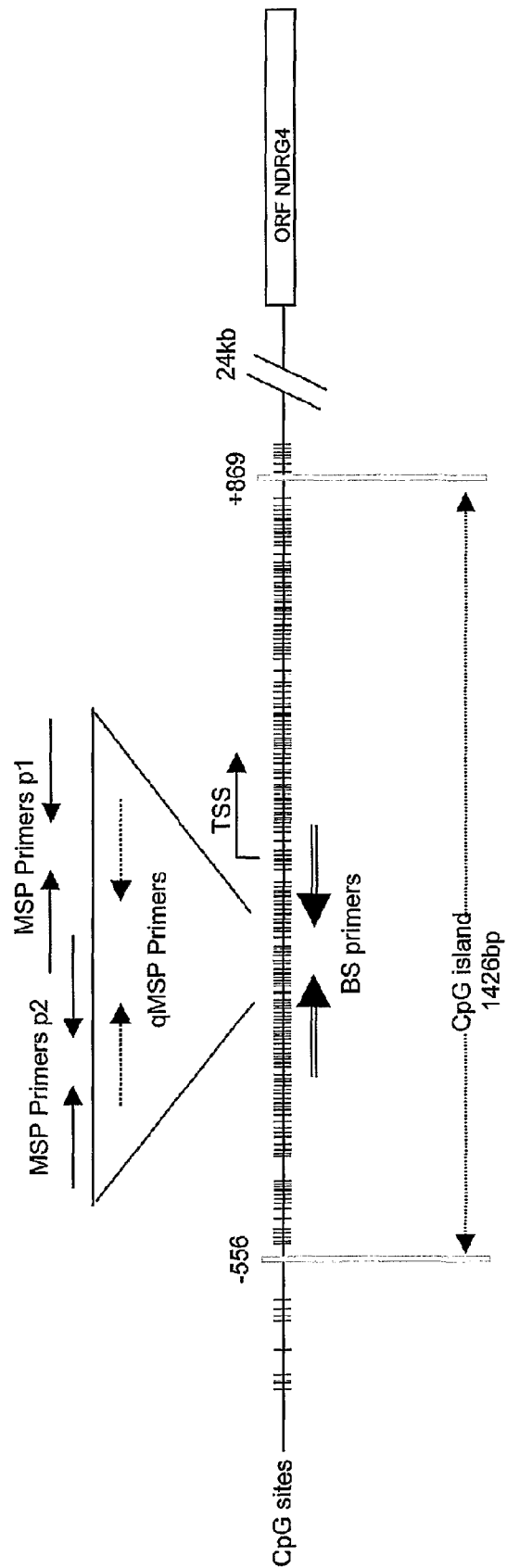


FIG. 10

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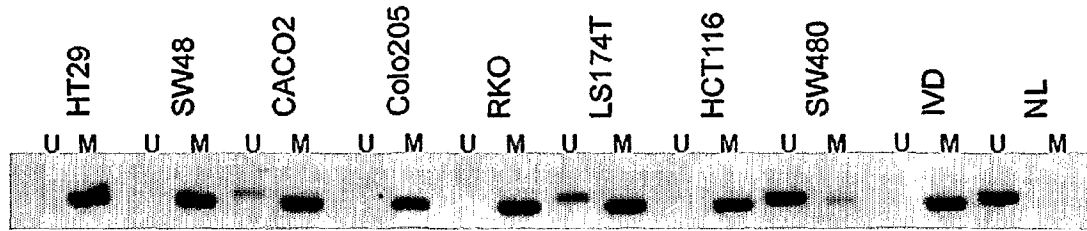


FIG. 11a

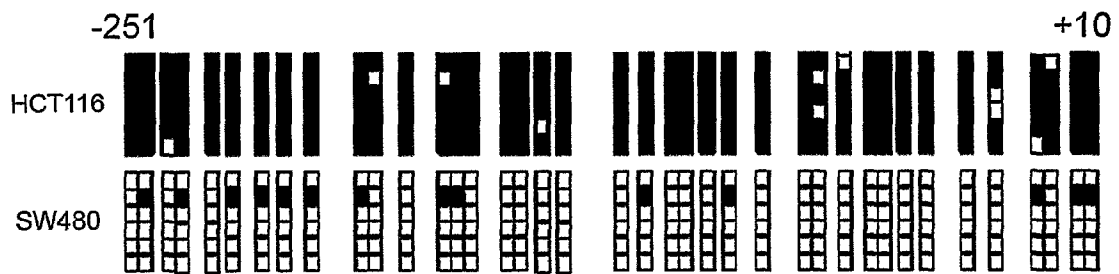


FIG. 11b

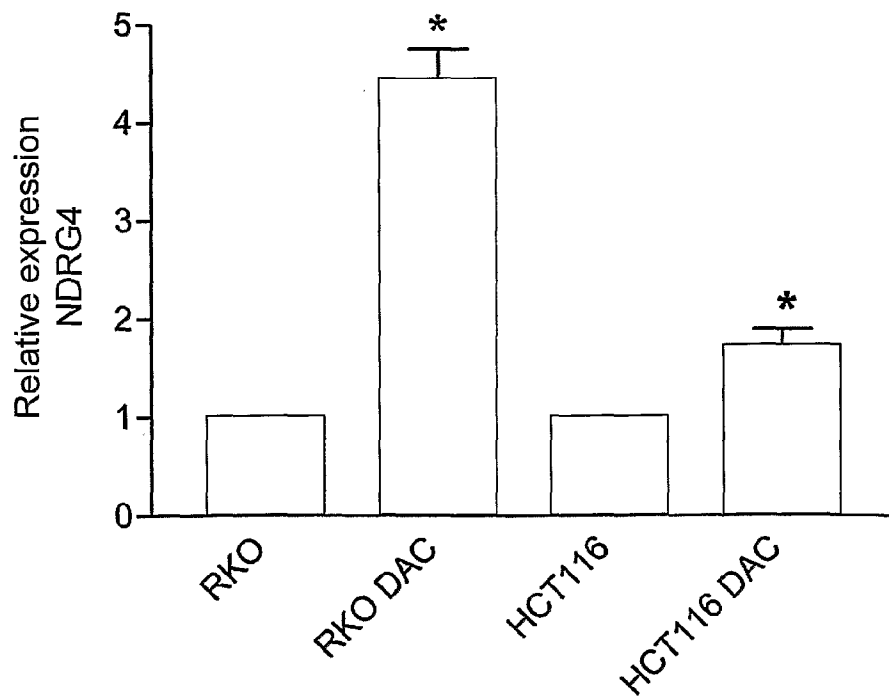


FIG. 11c

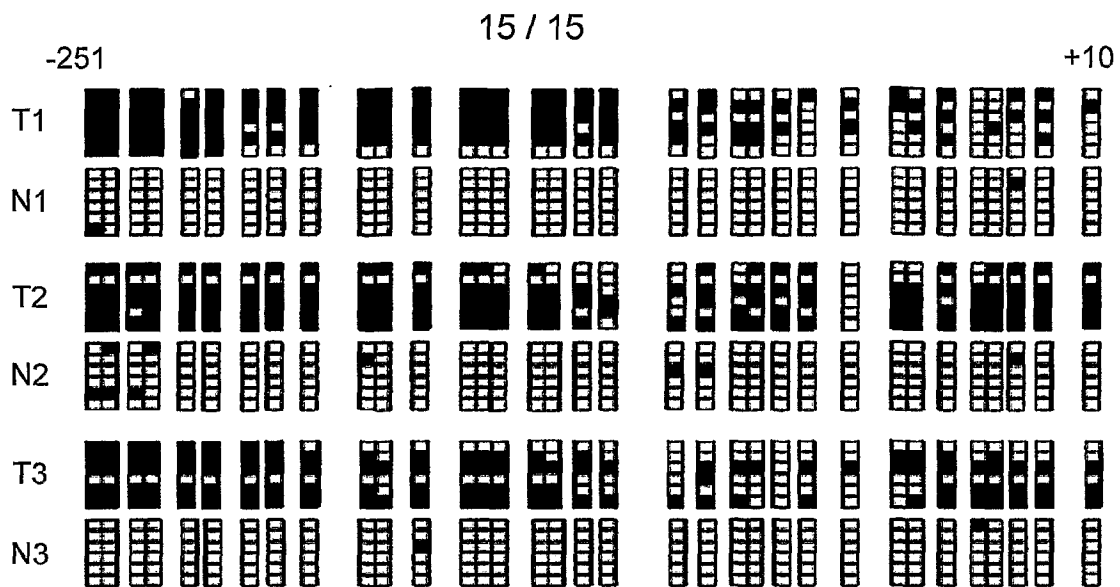


FIG. 12a

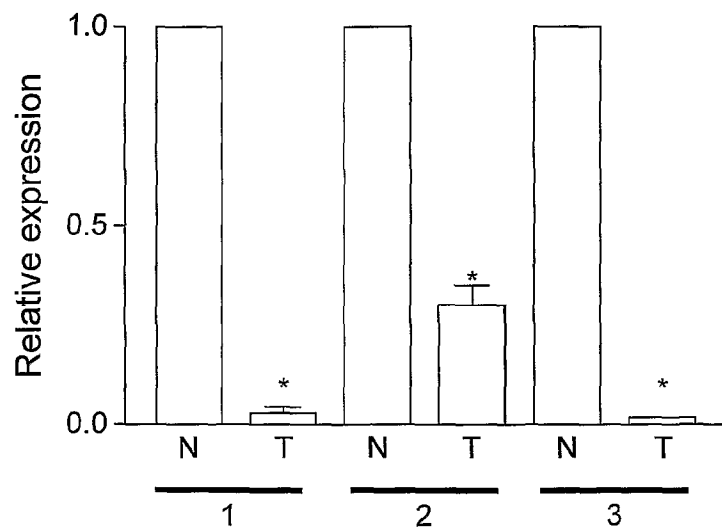


FIG. 12b

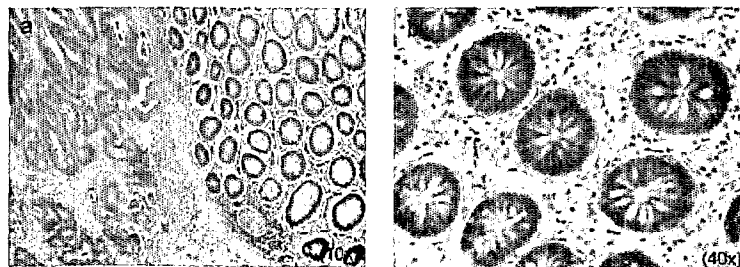


FIG. 12c

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2008/000056

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

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

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

EPO-Internal, BIOSIS, WPI Data, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/113770 A (EPIGENOMICS INC [US]; BALLHAUSE MATTHIAS [DE]; BERLIN KURT [DE]; DEVOS) 26 October 2006 (2006-10-26) abstract page 9, line 15 - line 35; figure 1 page 14, line 16 - page 15, line 11 page 19, line 13 - line 20 page 21, line 10 - page 24, line 15 page 67, line 25 - page 74, line 12 page 95, line 10 - page 101, line 14 claims 6,53-62,64 ----- -/--	99-134

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *Z* document member of the same patent family

Date of the actual completion of the international search

26 May 2008

Date of mailing of the international search report

20/06/2008

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Tilkorn, A

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2008/000056

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHOU R-H ET AL: "Characterization of the Human NDRG Gene Family: A Newly Identified Member, NDRG4, Is Specifically Expressed in Brain and Heart" GENOMICS, ACADEMIC PRESS, SAN DIEGO, US, vol. 73, no. 1, 1 April 2001 (2001-04-01), pages 86-97, XP004432256 ISSN: 0888-7543 abstract page 87, column 2, paragraph 2 - paragraph 5; figure 26 page 88, column 1, paragraph 1 page 94, column 2, paragraph 2 - page 95, column 1, paragraph 2 -----	171-173
A	QU XIANGHU ET AL: "Characterization and expression of three novel differentiation-related genes belong to the human NDRG gene family" MOLECULAR AND CELLULAR BIOCHEMISTRY, vol. 229, no. 1-2, January 2002 (2002-01), pages 35-44, XP002479836 ISSN: 0300-8177 abstract page 36, column 1, paragraph 3 - column 2, paragraph 1 page 38, column 1, paragraph 1 page 41, column 1, paragraph 2 - column 2, paragraph 2 page 43, column 2, paragraph 2 -----	1-7, 9-25, 27-33, 35-62, 65-70, 72-77, 79-84, 86-94, 96-124, 126-134, 153-163, 170-173
A	LUSIS ERIKS A ET AL: "Integrative genomic analysis identifies NDRG2 as a candidate tumor suppressor gene frequently inactivated in clinically aggressive meningioma." CANCER RESEARCH 15 AUG 2005, vol. 65, no. 16, 15 August 2005 (2005-08-15), pages 7121-7126, XP002479837 ISSN: 0008-5472 cited in the application abstract page 7122, column 2, paragraph 2 page 7124, column 2, paragraph 2 - page 7126, column 2, paragraph 1 page 7124, column 2, paragraph 2 - page 7126, column 2, paragraph 1 page 7122, column 2, paragraph 2 - page 7123, column 1, paragraph 1 ----- -/--	1-7, 9-25, 27-33, 35-62, 65-70, 72-77, 79-84, 86-94, 96-124, 126-134, 153-163, 170-173

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2008/000056

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	VERMA M ET AL: "Epigenetics in cancer: implications for early detection and prevention" LANCET ONCOLOGY, LANCET PUBLISHING GROUP, LONDON, GB, vol. 3, no. 12, 1 December 2002 (2002-12-01), pages 755-763, XP004811872 ISSN: 1470-2045 abstract page 757, column 2, paragraph 4 - page 759, column 1, paragraph 2 page 761, column 1, paragraph 3 - column 2, paragraph 3; figure 2; table 1 -----	1-7, 9-25, 27-33, 35-62, 65-70, 72-77, 79-84, 86-94, 96-124, 126-134, 153-163, 170-173

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2008/000056

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers allsearchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search reportcovers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB2008 /000056

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 61-64, 95 (completely),
1-7, 9-25, 27-33, 35-60, 65-70, 72-77, 79-84,
86-94, 96-124, 126-135, 153-163
, 170-173 (in part)

methods, kits, primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in NDRG4

2. claims:
1, 3-6, 9-14, 18, 19, 27-29, 31-33, 35-54, 56,
57, 60, 65, 67-69, 72, 75, 76, 79, 81
-83, 86-94, 96-98, 102, 103, 108-111, 153-163,
170-173

methods, kits primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in NDRG2

3. claims:
1-3, 8-13, 16, 18-23, 26-33, 35-48, 50-56, 59,
65-67, 70-74, 78-81, 85-87, 89-9
3, 96-106, 108-111, 125-130, 133-135,
153-163, 170-173 (in part)

methods, kits primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in GATA4

4. claims:
1-3, 7-13, 16-33, 35-48, 50-56, 58, 59, 65-67,
70-74, 77-81, 84-87, 89-93, 96-1
30, 133-135, 153-163, 170-173 (in part)

methods, kits primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in OSMR

5. claims:
1-3, 7-13, 17-20, 23, 25-33, 35-48, 50-56, 58,
59, 65-67, 70-74, 77-81, 84-87,
89-93, 96-100, 102, 103, 107-124,
126-130, 133-135, 153-163, 170-173

INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB2008 /000056

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

methods, kits, primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in GATA5

6. claims:

1-3,7,9-13,18-21,23,27-33,35-48,50-56,
58,65-67,70,72-74,77,79-81,84
,86,87,89-93,96-100,102,107-114,116,117,
119-124,126-135,153-163,170
-173 (in part)

methods, kits primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in SFRP1

7. claims:

1-3,7-13,17-19,23-33,35-48,50-59,65-67,
70-74,77-81,84-87,89-93,96-1
00,102,103,107-135,153-163,170-173 (in
part)

methods, kits primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in ADAM23

8. claims:

1-3,7,9-13,18,27-48,50-58,65-67,70,
72-74,77,79,81,84,86,87,89-93,96
-100,102,108-114,116,117,119-124,
126-135,153-163,170-173 (in part)

methods, kits primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in JPH3

9. claims:

1-3,7,9-13,16,18-22,27-33,35-48,50-58,
65-67,70,72-74,77,79-81,84,86
,87,89-93,96-106,108-114,116,117,
119-124,133-135,153-163,170-173
(in part)

methods, kits primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in SFRP2

INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB2008 /000056

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

10. claims:

1-3,7,9-13,18-21,27-33,34-48,50-58,
65-67,70,72-74,77,79-81,,84,86,8
7,89-93,96-100,102-103,108-114,116,
119-124,153-163,170-173 (in
part)

methods, kits primers and probes for detecting a
predisposition to or the incidence of cancer in a sample,
method of treatment and its likelihood of success on the
basis of an epigenetic change in APC

11. claims:

1-3,9-13,18-20,23,27-33,35-48,50-56,
65-67,72-74,79-81,86,87,89-93,96
-100,102,103,108-111,
126-135,153-163,170-173 (in part)

methods, kits primers and probes for detecting a
predisposition to or the incidence of cancer in a sample,
method of treatment and its likelihood of success on the
basis of an epigenetic change in MGMT

12. claims:

1,3,9-13,18,19,27-48,50-54,56,58,65,
67,72,74,77,79,81,84,86,89-93,9
6-99,102,103,108-112,114,116,117,
119-124,126-135,153-163,170-173
(in part)

methods, kits primers and probes for detecting a
predisposition to or the incidence of cancer in a sample,
method of treatment and its likelihood of success on the
basis of an epigenetic change in TFPI2

13. claims:

1,3,9-13,18,19,27-29,31-48,50-54,56,
58,65,67,70,72,74,77,79,81,84,8
6,87,89-93,96-98,102,103,108-112,114,
116,117,119-124,126-135,153-16
3,170-173 (in part)

methods, kits, primers and probes for detecting a
predisposition to or the incidence of cancer in a sample,
method of treatment and its likelihood of success on the
basis of an epigenetic change in BNIP3

14. claims:

INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB2008 /000056

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1,3,9-13,18,19,27-48,50-54,56,58,65,
67,70,72,74,77,79,81,84,86,87,8
9-93,96-98,102,103,108-112,114,116,
117,119-124,126-135,153-163,170-
173 (in part)

methods, kits, primers and probes for detecting a
predisposition to or the incidence of cancer in a sample,
method of treatment and its likelihood of success on the
basis of an epigenetic change in FOXE1

15. claims:

1,3,9-13,18,19,27-48,50-54,56,58,65,
67,70,72,74,77,79,81,84,86,87,8
9-93,96-98,102,103,108-112,114,116,
117,119-124,126-135,153-163,170-
173 (in part)

methods, kits, primers and probes for detecting a
predisposition to or the incidence of cancer in a sample,
method of treatment and its likelihood of success on the
basis of an epigenetic change in SYNE1

16. claims:

1,3,9-13,18,19,27-48,50-54,56,58,65,
67,70,72,74,77,79,81,84,86,87,8
9-93,96-98,102,103,108-112,114,116,
117,119-124,126-135,153-163,170-
173 (in part)

methods, kits, primers and probes for detecting a
predisposition to or the incidence of cancer in a sample,
method of treatment and its likelihood of success on the
basis of an epigenetic change in SOX17

17. claims:

1,3,9-13,18,19,27-48,50-54,56,58,65,
67,70,72,74,77,79,81,84,86,87,8
9-93,96-98,102,103,108-112,114,116,
117,119-124,126-135,153-163,170-
173 (in part)

methods, kits, primers and probes for detecting a
predisposition to or the incidence of cancer in a sample,
method of treatment and its likelihood of success on the
basis of an epigenetic change in PHACTR3

18. claims:

INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB2008 /000056

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1,3,9-13,18,19,27-48,50-54,56,58,65,
67,70,72,74,77,79,81,84,86,87,8
9-93,96-98,102,103,108-112,114,116,
117,119-124,126-135,153-163,170-
173 (in part)

methods, kits, primers and probes for detecting a
predisposition to or the incidence of cancer in a sample,
method of treatment and its likelihood of success on the
basis of an epigenetic change in JAM3

19. claims: 136-152 (completely), claims 153-157 (in part)

Method of determining the methylation status in a blood
sample

20. claims: claims 164-170

Method of processing a faecal sample to isolate and prepare
DNA

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2008/000056

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2006113770 A	26-10-2006	CA 2603815 A1 EP 1871912 A1	26-10-2006 02-01-2008



US011634781C1

(12) **EX PARTE REEXAMINATION CERTIFICATE** (12448th)
United States Patent
Louwagie

(10) **Number:** **US 11,634,781 C1**(45) **Certificate Issued:** **Dec. 4, 2023**(54) **FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD**(71) Applicant: **Exact Sciences Corporation**, Madison, WI (US)(72) Inventor: **Joost Louwagie**, Domach (CH)(73) Assignee: **EXACT SCIENCES CORPORATION**, Madison, WI (US)**Reexamination Request:**

No. 90/015,237, May 22, 2023

Reexamination Certificate for:Patent No.: **11,634,781**Issued: **Apr. 25, 2023**Appl. No.: **17/936,335**Filed: **Sep. 28, 2022****Related U.S. Application Data**

(63) Continuation of application No. 15/634,607, filed on Jun. 27, 2017, which is a continuation of application No. 15/010,436, filed on Jan. 29, 2016, now abandoned, which is a continuation of application No. 13/147,570, filed on Mar. 12, 2012, now abandoned.

(51) **Int. Cl.**
C12Q 1/6886 (2018.01)
G01N 33/574 (2006.01)(52) **U.S. Cl.**
CPC **C12Q 1/6886** (2013.01); **G01N 33/57446** (2013.01); **C12Q 2600/106** (2013.01); **C12Q 2600/154** (2013.01); **C12Q 2600/158** (2013.01); **G01N 2333/805** (2013.01); **G01N 2800/50** (2013.01); **G01N 2800/52** (2013.01)(58) **Field of Classification Search**
None
See application file for complete search history.(56) **References Cited**

To view the complete listing of prior art documents cited during the proceeding for Reexamination Control Number 90/015,237, please refer to the USPTO's Patent Electronic System.

Primary Examiner — Krisanne M Jastrzab(57) **ABSTRACT**

A method of processing a fecal sample from a human subject comprising removing a portion of a collected fecal sample and adding the removed portion of the sample to a buffer that prevents denaturation or degradation of blood proteins found in the sample, and detecting the presence of human blood in the removed portion of the fecal sample. The method further comprises stabilizing the remaining portion of the fecal sample.

US 11,634,781 C1

1

2

**EX PARTE
REEXAMINATION CERTIFICATE**

NO AMENDMENTS HAVE BEEN MADE TO
THE PATENT 5

AS A RESULT OF REEXAMINATION, IT HAS BEEN
DETERMINED THAT:

The patentability of claims 1-20 is confirmed. 10

* * * * *

INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i>				Complete if Known	
				Application Number	18/179,945
				Filing Date	03/07/2023
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	1634
				Examiner Name	WHISENANT, Ethan C.
Sheet	1	of	7	Attorney Docket Number	EXCTD-35239.307

U.S. PATENTS					
Exami ner Initials*	Cite No. ¹	Document Number Number-Kind Code ² (if known)	Issue or Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		5198365	1993-03-30	GROW et al.	
		5741650	1998-04-21	LAPIDUS et al.	
		5952178	1999-09-14	LAPIDUS et al.	
		7195878	2007-03-27	CLEATOR	
		9891223	2019-02-13	BEAULIEU et al.	
		11634781	2023-04-25	LOUWAGIE	

U.S. PUBLISHED PATENT APPLICATIONS					
Exami ner Initials*	Cite No. ³	Document Number Number-Kind Code ⁴ (if known)	Issue or Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		20060216714	2006-09-28	KANAOKA	
		20120164238	2012-06-28	JOOST	
		20160194723	2016-07-07	LOUWAGIE	

Note: Submission of copies of U.S. Patents and published U.S. Patent Applications is not required.

FOREIGN PATENT DOCUMENTS						
Exami ner Initials*	Cite No. ¹	Foreign Patent Document Country Code ⁵ Number ⁶ Kind Code ⁷ (if known)	Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	Translati on ⁸
		WO 1997/25925	1997-07-24	HULIOT PLASTICS INDUSTRIES et al.		
		WO 2005/113769	2005-12-01	EXACT SCIENCES CORPORATION		
		WO 2008/084219	2008-07-17	ONCOMETHYLOME SCIENCES SA		

Examiner Signature		Date Considered	
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EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i>				Complete if Known	
				Application Number	18/179,945
				Filing Date	03/07/2023
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	1634
				Examiner Name	WHISENANT, Ethan C.
Sheet	2	of	7	Attorney Docket Number	EXCTD-35239.307

FOREIGN PATENT DOCUMENTS						
		WO 2009/102788	2009-08-20	MAYO FONDATION FOR MEDICAL EDUCATION AND RESEARCH		

NONPATENT LITERATURE DOCUMENTS				
Examiner Initials*	Cite No. ¹	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and-or country where published.	Translatio n ⁶	
		AHLQUIST et al., A Stool Collection Device: The First Step in Occult Blood Testing, Annals of Internal Medicine, 108(4):609-612 (1988)		
		AHLQUIST et al., Colorectal Cancer Screening by Detection of Altered Human DNA in Stool: Feasibility of a Multitarget Assay Panel, Gastroenterology, 119(5):1219-1227 (2000)		
		AHLQUIST et al., Molecular Stool Screening for Colorectal Cancer. Using DNA Markers May Be Beneficial, But Large Scale Evaluation is Needed. BMJ, 29:321:254-5 (2000)		
		AHLQUIST et al., Stool DNA and Occult Blood Testing for Screen Detection of Colorectal Neoplasia. Ann Intern Med., 149(7):441-450, W81 (2008)		
		AHMED et al., Transcriptomic Molecular Markers for Screening Human Colon Cancer in Stool and Tissue, Cancer Genomics and Proteomics 4:1-20 (2007)		
		BOYNTON et al., DNA Integrity as a Potential Marker for Stool-based Detection of Colorectal Cancer, Clinical Chemistry, 49(7):1058-1065 (2003)		
		CHEN et al., Detection in Fecal DNA of Colon Cancer-Specific Methylation of the Nonexpressed Vimentin Gene, Journal of National Cancer Institute, 97(15):1124-1132 (2005)		
		Curriculum Vitae of Duncan H. Whitney, Ph.D., filed January 11, 2024, 10 pages		
		Declaration of Brendan T. Jones, dated January 11, 2024, 11 pages		
		Declaration of Duncan Whitney, Ph.D., dated January 10, 2024, 186 pages		

Examiner Signature		Date Considered	
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EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i>				Complete if Known	
				Application Number	18/179,945
				Filing Date	03/07/2023
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	1634
				Examiner Name	WHISENANT, Ethan C.
Sheet	3	of	7	Attorney Docket Number	EXCTD-35239.307

NONPATENT LITERATURE DOCUMENTS			
		DERKS et al., Promoter Methylation Precedes Chromosomal Alterations in Colorectal Cancer Development, Abstract, Cell Oncol, 28(5-6):247-257 (2006), retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4618222/ , on January 9, 2024, 2 pages	
		DERKS et al., Promoter Methylation Precedes Chromosomal Alterations in Colorectal Cancer Development, Cellular Oncology, 28(5-6):247-257 (2006)	
		EGUCHI et al., Mutations of the P53 Gene in Stool of Patients with Resectable Colorectal Cancer, Cancer, 77(8):1707-1710 (1996)	
		Exact Sciences and Mount Sinai School of Medicine, Stool DNA Testing For Colon Cancer, Press Release issued December 13, 2006, retrieved from https://www.sciencedaily.com/releases/2006/12/061213104115.htm# , on January 9, 2024, 3 pages	
		GUITTET et al., Comparison of a Guaiac Based and an Immunochemical Faecal Occult Blood Test In Screening For Colorectal Cancer In A General Average Risk Population, Gut, 56:210-214 (2007)	
		HIRATA et al., Usefulness of Fecal Lactoferrin and Hemoglobin in Diagnosis of Colorectal Diseases, World Journal of Gastroenterology, 14;13(10):1569-74 (2007)	
		HOEPFFNER et al., Comparative evaluation of a new bedside faecal occult blood test in a prospective multicentre study. Aliment Pharmacol Ther., 23(1):145-54 (2006)	
		IMPERIALE et al., Fecal DNA Versus Fecal Occult Blood for Colorectal-Cancer Screening in an Average-Risk Population, New England Journal of Medicine, 351(26):2704-2714 (2004)	
		ITZKOWITZ et al., Improved Fecal DNA Test for Colorectal Cancer Screening, Clinical Gastroenterology and Hepatology, 5(1):P111-117 (2007), retrieved from https://www.cghjournal.org/article/S1542-3565(06)01044-5/fulltext , on January 9, 2024, 26 pages.	
		ITZKOWITZ et al., Improved Fecal DNA Test for Colorectal Cancer Screening, Clinical Gastroenterology and Hepatology, 5:111-117 (2007)	
		ITZKOWITZ et al., A Simplified, Noninvasive Stool DNA Test for Colorectal Cancer Detection, American Journal of Gastroenterology, 103:2862-2870 (2008)	

Examiner Signature		Date Considered	
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EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i>				Complete if Known	
				Application Number	18/179,945
				Filing Date	03/07/2023
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	1634
				Examiner Name	WHISENANT, Ethan C.
Sheet	4	of	7	Attorney Docket Number	EXCTD-35239.307

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		JESSUP et al., Diagnosing Colorectal Carcinoma: Clinical and Molecular Approaches, A Cancer Journal for Clinicians, 47(2):70-92 (1997)	
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				Art Unit	1634
				Examiner Name	WHISENANT, Ethan C.
Sheet	5	of	7	Attorney Docket Number	EXCTD-35239.307

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		USPTO Order Granting Request for Ex Parte Reexamination of US Patent No. 11,634,781 dated June 29, 2023, 18 pages	
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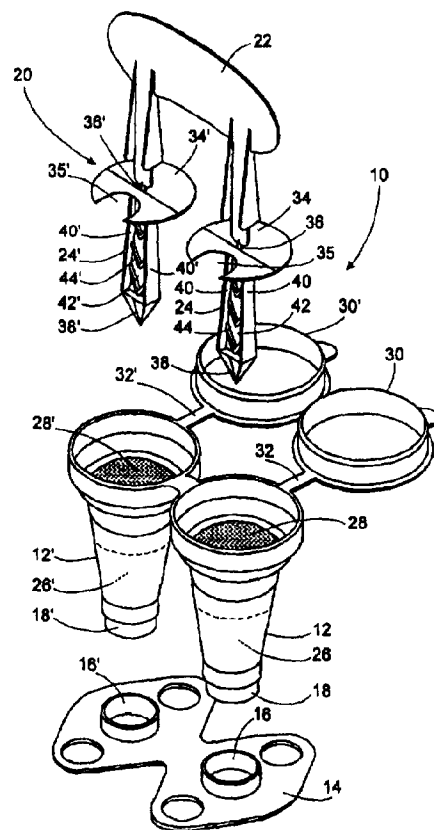
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(21) International Application Number: PCT/IL97/00017 (22) International Filing Date: 13 January 1997 (13.01.97) (30) Priority Data: 116824 19 January 1996 (19.01.96) IL (71) Applicants (for all designated States except US): HULIOT PLASTICS INDUSTRIES (IL/IL); Kibbutz Sdeh Nehemia, 12145 Mobile P.O. Upper Galilee (IL). QUALITY 9000 LTD. [IL/IL]; P.O. Box 593, 76103 Rehovot (IL). (72) Inventor; and (75) Inventor/Applicant (for US only): INBAR, Michael [IL/IL]; Neot Achva 10, 79800 D.N. Shikmim (IL). (74) Agent: REINHOLD COHN AND PARTNERS; P.O. Box 4060, 61040 Tel-Aviv (IL).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>

(54) Title: COLLECTION AND TRANSPORTATION OF A PASTY MATERIAL SAMPLE**(57) Abstract**

A system for collecting, storing and transporting of specimens of a pasty material, e.g. stool is provided. The system comprises at least one container holding a preservation medium for preserving a chemical entity or living matter to be assayed in a specimen, the container being sealed by a pierceable membrane having a closure arrangement for sealing the container after the membrane is pierced. The system further comprises a sampling device having a handle and at least one elongated specimen collecting member detachably attached thereto, the collecting member being adapted to be received within the container and having the pasty material retaining portion and a piercing tip for piercing the membrane at a lowermost end thereof remote from said handle, for piercing said film. Also provided is a stool collecting device for easy collecting of excreted stool.



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COLLECTION AND TRANSPORTATION OF A PASTY MATERIAL SAMPLE

FIELD OF THE INVENTION

The present invention concerns a system for the collection and transportation of a pasty material sample, for example of stool, which is to be distributed in a transport and/or suspension medium.

5

BACKGROUND OF THE INVENTION

Collection and transportation of a pasty material, such a stool, food samples, soil samples and the like for the purpose of laboratory manipulation such as detection of microorganism being bacteria or parasites, detection of cryptic blood, preparation of a biochemical profile, etc., is an essential component of modern medicine, health care and environmental management.

10

Often, when the pasty material is transported to the laboratory without a preservation medium, by the time it reaches a laboratory no viable micro-organisms can be detected, and the result of the laboratory tests are thus distorted.

15

In order to overcome this problem, various devices and systems for the collection and transportation of pasty material have been developed, in which the pasty material is distributed in a medium capable of maintaining viable microorganisms. Usually, such devices comprise a container holding the medium, and a sampler capable of picking up a small portion of the pasty material and placing it within said container.

20

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US 5,149,506 discloses a device for collection and transportation of a stool sample which is composed of a container with a lid having a spoon secured to the underside of the lid, the container being filled with a liquid capable of maintaining microorganisms in a viable form.

5 US 4,559,839 discloses a stool collection and concentration receiver comprising a container holding a medium and including within it a collecting-filtering device comprising a stick for picking up the stool sample and sieve through which the diluted stool is filtered.

10 US 4,678,559 discloses a sampling vessel for pasty sample material comprising a sample-taking cup fitted on the lid of a container and facing towards the container's bottom.

In the above three patents the sampling member, used to pick the sample of the pasty material, is held *a priori* within the container holding also the preservation medium. This feature has the following problems:

- 15 - in order to sample the pasty material the container holding both the medium and the sampling member has to be opened, thus increasing the chances of contamination of the sterile medium by ambient atmosphere, or by the hands of the user and increases the possibility of spillage due to mishandling;
- 20 - once the sampling member is taken out of the medium filled container it may drip the medium on to its surrounding, and where the medium contains harmful ingredients, e.g. formaldehyde or mercury which are present in some preservation media, this may cause some damage if it comes into contact with the user's skin or eyes;
- 25 - the wet sampling member, once coming into contact with the pasty material sample, adds to the sample also a certain amount of the transport or suspension medium itself. This may be problematic if another sample of the pasty material is to be transferred into another container holding a different medium, since the second sample may already be
- 30 contaminated by the first medium.

A stool sample is typically subjected to two different tests one being a bacteriological test and the other a parasitological test. The medium

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required to maintain viable bacteria and the medium required to maintain viable parasites are not only different but are mutually exclusive, i.e. one type of medium damages the other type of microorganism. Thus, in the case of a pasty material which is to be sampled for the purpose of two or more different laboratory assay, such as stool, it would be preferable to provide a system for collection and transportation of pasty material which comprises two or more separate containers each one holding a different transport or suspension medium.

Finally, the above-mentioned prior art devices for collection and transportation of stool, do not address the problem of obtaining the original stool sample in a convenient manner which enables easy access to the user and the prior art tends to leave the solution to the problem to the skills of each user.

The present invention intends to provide an answer to some of the above-noted problems.

GENERAL DESCRIPTION OF THE INVENTION

The present invention provides a system for collecting, storing and transporting of specimens of a pasty material, comprising:

at least one container holding a preservation medium for preserving a chemical entity or living matter to be assayed in the specimen, the container being sealed by a pierceable membrane and having a closure arrangement for sealing the container after the membrane is pierced; and

a sampling device having a handle and at least one elongated specimen collecting member detachably attached thereto, the collecting member being adapted to be received within the container and having a pasty material retaining portion and a piercing tip for piercing the membrane at a lowermost end thereof remote from said handle, for piercing said film.

The system of the present invention is suitable for collecting, storing and transporting of any pasty material, for example, stools, food samples, e.g. soft cheese, yogurt, various spreads; soil samples, e.g. mud samples; and many others. With appropriate preservation media, the

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samples may be stored for a lengthy period of time prior to assaying for the chemical entity or living matter. The composition of the medium depends on the type of entity which should be preserved and subsequently assayed: for example, if the entity is a chemical substance, the medium will have a composition which ensure that the substance is preserved in a manner to avoid its deterioration; if the assayed entity is microorganism or any other living matter, the medium will be designed so as to maintain such microorganisms or other living matter in a viable condition. A system for collecting, storing and transporting specimens to subsequent assays for living matter, e.g. microorganisms, yeasts or parasites thereon, is a preferred embodiment of the invention. In accordance with a particularly preferred embodiment, the pasty material is stool.

At times a variety of different entities have to be assayed in the pasty material. This is the case, for example, in stool, where it is generally desired to assay both bacteria and parasites, which as noted above, require different media. A system for assaying a plurality of entities, which require different media, comprises at least two containers, each holding a different preservation medium, each suitable for different entities. The containers in such a system may be permanently attached to one another; or alternatively, the attachment of the two containers to one another being in a detachable fashion to allow detachment when the containers with the sample reaches the laboratory.

The closure arrangement is typically a rigid lid member attached to the container through a flexible linker.

The system further comprises a sampling device which is an element distinct and separate from the container. The provision of the container and a separate sampling device ensures that until the pasty material sample is introduced into the container, the medium therein is kept totally sterile. As already noted above, in prior art devices, where a sampling device was stored within the container, it was necessary to open the container prior to sampling, with an inevitable loss of sterility.

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The sampling device comprises a handle which is detachably connected to the at least one collecting member. The detachable connection can be by a breakable portion, by a fastener capable of holding or releasing an attachment element in the collecting member, etc. The at least one
5 collecting member is adapted to be received within corresponding at least one container. (It will be appreciated, that the number of collecting members will typically correspond to the number of containers, and where the containers are held together, the spacing between the collecting members will be made to correspond to the distance between the container such that
10 all collecting members may be inserted simultaneously to the respective containers).

Each collecting member has a piercing tip for piercing the container's pierceable membrane and has a pasty material retaining portion. Once the membrane is pierced by the piercing tip, the collecting member is
15 pushed down until the material retaining portion is accommodated within the container and embedded in the medium. Then, the handle is detached from the collecting member. As will be appreciated, there are a number of different constructions which have the capability of retaining a specimen of the pasty material: for example, a rough surface having multiple depressions and/or protrusions; a helical structure; a ladder-like shaped structure having
20 straight, curved or V-shaped ribs; a structure comprising a planar stem with protrusions, which may have the form of straight, curved or V-shaped ribs, or which may comprise a plurality of bulges, etc.

In order to ensure that the pasty material will be retained on the
25 retaining portion and will not be smeared on the membrane, it is preferable that the pasty material retaining portion has the widest cross-section at the lowermost part, near the piercing tip. This will ensure that the hole in the membrane which will be made by the piercing tip, will be sufficiently large to ensure free passage of the remaining portions of the collecting member.
30 In accordance with an embodiment of the invention, the pasty material retaining portion is defined by two lateral side walls which are wider than the pasty material retaining portion. Thus during insertion, only these side