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(54) Title: METHOD AND NUCLEIC ACIDS FOR THE ANALYSIS OF COLORECTAL CELL PROLIFERATIVE DISORDERS

(57) Abstract: The invention provides methods and nucleic acids for detecting, differentiating or distinguishing between colon cell proliferative disorders as well as therapy thereof by analysis of the gene EYA4 and its promoter and regulatory sequences. The invention further provides novel nucleic acid sequences useful for the cell proliferative disorder specific analysis of said gene as well as methods, assays and kits thereof.



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Method and nucleic acids for the analysis of colorectal cell proliferative disorders**Field of the Invention**

Colorectal cancer is the fourth leading cause of cancer mortality in men and women. The 5-year survival rate is 61% over all stages with early detection being a prerequisite for curative therapy of the disease. Up to 95% of all colorectal cancers are adenocarcinomas of varying differentiation grades.

Sporadic colon cancer develops in a multistep process starting with the pathological transformation of normal colonic epithelium to an adenoma which consecutively progresses to invasive cancer. The progression rate of colonic adenomas is currently predicted based on their histological appearance, location, degree of spread and extent of bowel involvement.. For example, tubular-type benign adenomas rarely progress to malignant tumours, whereas villous benign adenomas, particularly if larger than 2 cm in diameter, have a significant malignant potential.

During progression from benign proliferative lesions to malignant neoplasms several genetic and epigenetic alterations are known to occur. Somatic mutation of the APC gene seems to be one of the earliest events in 75 to 80% of colorectal adenomas and carcinomas. Activation of K-RAS is thought to be a critical step in the progression towards a malignant phenotype. Consecutively, mutations in other oncogenes as well as alterations leading to inactivation of tumour suppressor genes accumulate.

Aberrant DNA methylation within CpG islands is among the earliest and most common alterations in human cancers leading to abrogation or overexpression of a broad spectrum of genes. In addition, abnormal methylation has been shown to occur in CpG rich regulatory elements in intronic and coding parts of genes for certain tumours. In contrast to the specific hypermethylation of tumour suppressor genes, an overall hypomethylation of DNA can be observed in tumour cells. This decrease in global methylation can be detected early, far before the development of frank tumour formation. Also, correlation between hypomethylation and increased gene expression was reported for many oncogenes. In colon cancer, aberrant DNA

methylation constitutes one of the most prominent alterations and inactivates many tumour suppressor genes such as p14ARF, p16INK4a, THBS1, MINT2, and MINT31 and DNA mismatch repair genes such as hMLH1.

In the molecular evolution of colorectal cancer, DNA methylation errors have been suggested to play two distinct roles. In normal colonic mucosal cells, methylation errors accumulate as a function of age or as time-dependent events predisposing these cells to neoplastic transformation. For example, hypermethylation of several loci could be shown to be already present in adenomas, particularly in the tubulovillous and villous subtype. At later stages, increased DNA methylation of CpG islands plays an important role in a subset of tumours affected by the so called CpG island methylator phenotype (CIMP). Most CIMP+ tumours, which constitute about 15% of all sporadic colorectal cancers, are characterised by microsatellite instability (MIN) due to hypermethylation of the hMLH1 promoter and other DNA mismatch repair genes. By contrast, CIMP- colon cancers evolve along a more classic genetic instability pathway (CIN), with a high rate of p53 mutations and chromosomal changes.

However, the molecular subtypes do not only show varying frequencies regarding molecular alterations. According to the presence of either micro satellite instability or chromosomal aberrations, colon cancer can be subclassified into two classes, which also exhibit significant clinical differences. Almost all MIN tumours originate in the proximal colon (ascending and transversum), whereas 70% of CIN tumours are located in the distal colon and rectum. This has been attributed to the varying prevalence of different carcinogens in different sections of the colon. Methylating carcinogens, which constitute the prevailing carcinogen in the proximal colon have been suggested to play a role in the pathogenesis of MIN cancers, whereas CIN tumours are thought to be more frequently caused by adduct-forming carcinogens, which occur more frequently in distal parts of the colon and rectum. Moreover, MIN tumours have a better prognosis than do tumours with a CIN phenotype and respond better to adjuvant chemotherapy.

The identification of markers for the differentiation of colon carcinoma as well as for early detection are main goals of current research.

EYA4 is the most recently identified member of the vertebrate Eya (eyes-absent) gene family, a group of four transcriptional activators that interact with other proteins in a conserved regulatory hierarchy to ensure normal embryologic development. The EYA4 gene is mapped to 6q22.3 and encodes a 640 amino acid protein. The structure of EYA4 conforms to the basic pattern established by EYA1–3, and includes a highly conserved 271 amino acid C-terminus called the eya-homologous region (eyaHR; alternatively referred to as the eya domain or eya homology domain 1) and a more divergent proline–serine–threonine (PST)-rich (34–41%) transactivation domain at the N-terminus (Borsani G, et al., EYA4, a novel vertebrate gene related to *Drosophila* eyes absent. *Hum Mol Genet* 1999 Jan;8(1):11-23). EYA proteins interact with members of the SIX and DACH protein families during early embryonic development. Mutations in the EYA4 gene are responsible for postlingual, progressive, autosomal dominant hearing loss at the DFNA10 locus (Wayne S, Robertson NG, DeClau F, Chen N, Verhoeven K, Prasad S, Tranebjarg L, Morton CC, Ryan AF, Van Camp G, Smith RJ: Mutations in the transcriptional activator EYA4 cause late-onset deafness at the DFNA10 locus. *Hum Mol Genet* 2001 Feb 1;10(3):195-200 with further references). A link between the Methylation of Cytosine positions in the EYA 4 gene and cancer has not yet been established.

5-methylcytosine is the most frequent covalent base modification in the DNA of eukaryotic cells. It plays a role, for example, in the regulation of the transcription, in genetic imprinting, and in tumorigenesis. Therefore, the identification of 5-methylcytosine as a component of genetic information is of considerable interest. However, 5-methylcytosine positions cannot be identified by sequencing since 5-methylcytosine has the same base pairing behaviour as cytosine. Moreover, the epigenetic information carried by 5-methylcytosine is completely lost during PCR amplification.

A relatively new and currently the most frequently used method for analysing DNA for 5-methylcytosine is based upon the specific reaction of bisulfite with cytosine which, upon subsequent alkaline hydrolysis, is converted to uracil which corresponds to thymidine in its base pairing behaviour. However, 5-methylcytosine remains unmodified under these conditions. Consequently, the original DNA is converted in such a manner that methylcytosine, which originally could not be distinguished from cytosine by its hybridisation behaviour, can now be detected as the only remaining cytosine using "normal" molecular biological techniques, for example, by amplification and hybridisation or sequencing. All of these techniques are based on base pairing which can now be fully exploited. In terms of

sensitivity, the prior art is defined by a method which encloses the DNA to be analysed in an agarose matrix, thus preventing the diffusion and renaturation of the DNA (bisulfite only reacts with single-stranded DNA), and which replaces all precipitation and purification steps with fast dialysis (Olek A, Oswald J, Walter J. A modified and improved method for bisulphite based cytosine methylation analysis. *Nucleic Acids Res.* 1996 Dec 15;24(24):5064-6). Using this method, it is possible to analyse individual cells, which illustrates the potential of the method. However, currently only individual regions of a length of up to approximately 3000 base pairs are analysed, a global analysis of cells for thousands of possible methylation events is not possible. However, this method cannot reliably analyse very small fragments from small sample quantities either. These are lost through the matrix in spite of the diffusion protection.

An overview of the further known methods of detecting 5-methylcytosine may be gathered from the following review article: Rein, T., DePamphilis, M. L., Zorbas, H., *Nucleic Acids Res.* 1998, 26, 2255.

To date, barring few exceptions (e.g., Zeschnigk M, Lich C, Buiting K, Doerfler W, Horsthemke B. A single-tube PCR test for the diagnosis of Angelman and Prader-Willi syndrome based on allelic methylation differences at the SNRPN locus. *Eur J Hum Genet.* 1997 Mar-Apr;5(2):94-8) the bisulfite technique is only used in research. Always, however, short, specific fragments of a known gene are amplified subsequent to a bisulfite treatment and either completely sequenced (Olek A, Walter J. The pre-implantation ontogeny of the H19 methylation imprint. *Nat Genet.* 1997 Nov;17(3):275-6) or individual cytosine positions are detected by a primer extension reaction (Gonzalzo ML, Jones PA. Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE). *Nucleic Acids Res.* 1997 Jun 15;25(12):2529-31, WO 95/00669) or by enzymatic digestion (Xiong Z, Laird PW. COBRA: a sensitive and quantitative DNA methylation assay. *Nucleic Acids Res.* 1997 Jun 15;25(12):2532-4). In addition, detection by hybridisation has also been described (Olek et al., WO 99/28498).

Further publications dealing with the use of the bisulfite technique for methylation detection in individual genes are: Grigg G, Clark S. Sequencing 5-methylcytosine residues in genomic DNA. *Bioessays.* 1994 Jun;16(6):431-6, 431; Zeschnigk M, Schmitz B, Dittrich B, Buiting K, Horsthemke B, Doerfler W. Imprinted segments in the human genome: different DNA

methylation patterns in the Prader-Willi/Angelman syndrome region as determined by the genomic sequencing method. Hum Mol Genet. 1997 Mar;6(3):387-95; Feil R, Charlton J, Bird AP, Walter J, Reik W. Methylation analysis on individual chromosomes: improved protocol for bisulphite genomic sequencing. Nucleic Acids Res. 1994 Feb 25;22(4):695-6; Martin V, Ribieras S, Song-Wang X, Rio MC, Dante R. Genomic sequencing indicates a correlation between DNA hypomethylation in the 5' region of the pS2 gene and its expression in human breast cancer cell lines. Gene. 1995 May 19;157(1-2):261-4; WO 97/46705 and WO 95/15373.

An overview of the Prior Art in oligomer array manufacturing can be gathered from a special edition of Nature Genetics (Nature Genetics Supplement, Volume 21, January 1999), published in January 1999, and from the literature cited therein.

Fluorescently labelled probes are often used for the scanning of immobilised DNA arrays. The simple attachment of Cy3 and Cy5 dyes to the 5'-OH of the specific probe are particularly suitable for fluorescence labels. The detection of the fluorescence of the hybridised probes may be carried out, for example via a confocal microscope. Cy3 and Cy5 dyes, besides many others, are commercially available.

Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-TOF) is a very efficient development for the analysis of biomolecules (Karas M, Hillenkamp F. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal Chem. 1988 Oct 15;60(20):2299-301). An analyte is embedded in a light-absorbing matrix. The matrix is evaporated by a short laser pulse thus transporting the analyte molecule into the vapour phase in an unfragmented manner. The analyte is ionised by collisions with matrix molecules. An applied voltage accelerates the ions into a field-free flight tube. Due to their different masses, the ions are accelerated at different rates. Smaller ions reach the detector sooner than bigger ones.

MALDI-TOF spectrometry is excellently suited to the analysis of peptides and proteins. The analysis of nucleic acids is somewhat more difficult (Gut I G, Beck S. DNA and Matrix Assisted Laser Desorption Ionization Mass Spectrometry. Current Innovations and Future Trends. 1995, 1; 147-57). The sensitivity to nucleic acids is approximately 100 times worse than to peptides and decreases disproportionally with increasing fragment size. For nucleic

acids having a multiply negatively charged backbone, the ionisation process via the matrix is considerably less efficient. In MALDI-TOF spectrometry, the selection of the matrix plays an eminently important role. For the desorption of peptides, several very efficient matrixes have been found which produce a very fine crystallisation. There are now several responsive matrixes for DNA, however, the difference in sensitivity has not been reduced. The difference in sensitivity can be reduced by chemically modifying the DNA in such a manner that it becomes more similar to a peptide. Phosphorothioate nucleic acids in which the usual phosphates of the backbone are substituted with thiophosphates can be converted into a charge-neutral DNA using simple alkylation chemistry (Gut IG, Beck S. A procedure for selective DNA alkylation and detection by mass spectrometry. *Nucleic Acids Res.* 1995 Apr 25;23(8):1367-73). The coupling of a charge tag to this modified DNA results in an increase in sensitivity to the same level as that found for peptides. A further advantage of charge tagging is the increased stability of the analysis against impurities which make the detection of unmodified substrates considerably more difficult.

Genomic DNA is obtained from DNA of cell, tissue or other test samples using standard methods. This standard methodology is found in references such as Sambrook, Fritsch and Maniatis eds., *Molecular Cloning: A Laboratory Manual*, 1989.

Description

The present invention discloses novel methods for the detection of cell proliferative disorders. Said invention discloses the use of the gene EYA4, as well as its promoter and regulatory elements as a marker for colon cell proliferative disorders. More specifically, the disclosed matter shows the applicability of said gene to the detection of colon cell proliferative disorders, distinguishing between different classes of colon cell proliferative disorders as well as the differentiation of colon cell proliferative disorders from cell proliferative disorders originating from other tissues.

In one aspect of the invention, the disclosed matters provides novel nucleic acid sequences useful for the analysis of methylation within said gene, other aspects provide novel uses of the gene and the gene product as well as methods, assays and kits directed to detecting, differentiating and distinguishing colon cell proliferative disorders, as well as therapeutic and diagnostic methods thereof.

In one embodiment the method discloses the use of the gene EYA4 as a marker for the differentiation, detection and distinguishing of colon cell proliferative disorders. Said use of the gene may be enabled by means of any analysis of the expression of the gene, by means of mRNA expression analysis or protein expression analysis. However, in the most preferred embodiment of the invention, the detection, differentiation and distinguishing of colon cell proliferative disorders is enabled by means of analysis of the methylation status of the gene EYA4 and its promoter or regulatory elements.

To detect the presence of mRNA encoding EYA4 in a detection system for colon cancer, a sample is obtained from a patient. The sample can be a tissue biopsy sample or a sample of blood, plasma, serum or the like. The sample may be treated to extract the nucleic acids contained therein. The resulting nucleic acid from the sample is subjected to gel electrophoresis or other separation techniques. Detection involves contacting the nucleic acids and in particular the mRNA of the sample with a DNA sequence serving as a probe to form hybrid duplexes. The stringency of hybridisation is determined by a number of factors during hybridisation and during the washing procedure, including temperature, ionic strength, length of time and concentration of formamide. These factors are outlined in, for example, Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2d ed., 1989). Detection of the resulting duplex is usually accomplished by the use of labelled probes. Alternatively, the probe may be unlabeled, but may be detectable by specific binding with a ligand which is labelled, either directly or indirectly. Suitable labels and methods for labelling probes and ligands are known in the art, and include, for example, radioactive labels which may be incorporated by known methods (e.g., nick translation or kinasing), biotin, fluorescent groups, chemiluminescent groups (e.g., dioxetanes, particularly triggered dioxetanes), enzymes, antibodies, and the like.

In order to increase the sensitivity of the detection in a sample of mRNA encoding EYA4, the technique of reverse transcription/polymerisation chain reaction can be used to amplify cDNA transcribed from mRNA encoding EYA4. The method of reverse transcription /PCR is well known in the art (for example, see Watson and Fleming, *supra*).

The reverse transcription /PCR method can be performed as follows. Total cellular RNA is isolated by, for example, the standard guanidium isothiocyanate method and the total RNA is reverse transcribed. The reverse transcription method involves synthesis of DNA on a

template of RNA using a reverse transcriptase enzyme and a 3' end primer. Typically, the primer contains an oligo(dT) sequence. The cDNA thus produced is then amplified using the PCR method and EYA4 specific primers. (Belyavsky et al, Nucl Acid Res 17:2919-2932, 1989; Krug and Berger, Methods in Enzymology, Academic Press, N.Y., Vol.152, pp. 316-325, 1987 which are incorporated by reference)

The present invention may also be described in certain embodiments as a kit for use in detecting a colon cancer disease state through testing of a biological sample. A representative kit may comprise one or more nucleic acid segments as described above that selectively hybridise to EYA4 mRNA and a container for each of the one or more nucleic acid segments. In certain embodiments the nucleic acid segments may be combined in a single tube. In further embodiments, the nucleic acid segments may also include a pair of primers for amplifying the target mRNA. Such kits may also include any buffers, solutions, solvents, enzymes, nucleotides, or other components for hybridisation, amplification or detection reactions. Preferred kit components include reagents for reverse transcription-PCR, in situ hybridisation, Northern analysis and/or RPA

The present invention further provides for methods to detect the presence of the polypeptide, EYA4, in a sample obtained from a patient. Any method known in the art for detecting proteins can be used. Such methods include, but are not limited to immunodiffusion, immunoelectrophoresis, immunochemical methods, binder-ligand assays, immunohistochemical techniques, agglutination and complement assays. (for example see Basic and Clinical Immunology, Sites and Terr, eds., Appleton & Lange, Norwalk, Conn. pp 217-262, 1991 which is incorporated by reference). Preferred are binder-ligand immunoassay methods including reacting antibodies with an epitope or epitopes of EYA4 and competitively displacing a labelled EYA4 protein or derivative thereof.

Certain embodiments of the present invention comprise the use of antibodies specific to the polypeptide encoded by the EYA4 gene. Such antibodies may be useful for diagnostic and prognostic applications in detecting the disease state, by comparing a patient's levels of colon disease marker expression to expression of the same markers in normal individuals. In certain embodiments production of monoclonal or polyclonal antibodies can be induced by the use of the EYA4 polypeptide as antigen. Such antibodies may in turn be used to detect expressed proteins as markers for human disease states. The levels of such proteins present in the

peripheral blood or prostate tissue sample of a patient may be quantified by conventional methods. Antibody-protein binding may be detected and quantified by a variety of means known in the art, such as labelling with fluorescent or radioactive ligands. The invention further comprises kits for performing the above-mentioned procedures, wherein such kits contain antibodies specific for the EYA4 polypeptides.

Numerous competitive and non-competitive protein binding immunoassays are well known in the art. Antibodies employed in such assays may be unlabeled, for example as used in agglutination tests, or labelled for use a wide variety of assay methods. Labels that can be used include radionuclides, enzymes, fluorescers, chemiluminescers, enzyme substrates or co-factors, enzyme inhibitors, particles, dyes and the like for use in radioimmunoassay (RIA), enzyme immunoassays, e.g., enzyme-linked immunosorbent assay (ELISA), fluorescent immunoassays and the like. Polyclonal or monoclonal antibodies to EYA4 or an epitope thereof can be made for use in immunoassays by any of a number of methods known in the art. One approach for preparing antibodies to a protein is the selection and preparation of an amino acid sequence of all or part of the protein, chemically synthesising the sequence and injecting it into an appropriate animal, usually a rabbit or a mouse (Milstein and Kohler *Nature* 256:495-497, 1975; Gutfre and Milstein, *Methods in Enzymology: Immunochemical Techniques* 73:1-46, Langone and Banatis eds., Academic Press, 1981 which are incorporated by reference). Methods for preparation of EYA4 or an epitope thereof include, but are not limited to chemical synthesis, recombinant DNA techniques or isolation from biological samples.

The invention provides significant improvements over the state of the art in that there are currently no markers used to detect colon cancer from body fluid samples. Current methods used to detect and diagnose colon cell proliferative disorders include colonoscopy, sigmoidoscopy, and fecal occult blood colon cancer. In comparison to these methods, the disclosed invention is much less invasive than colonoscopy, and as, if not more sensitive than sigmoidoscopy and FOBT. Compared to the previous descriptions of these markers in the literature, the described invention provides significant advantages in terms of sensitivity and specificity due to the advantageous combination of using highly sensitive assay techniques.

The objective of the invention can be achieved by analysis of the methylation state of the CpG dinucleotides within the genomic sequence according to SEQ ID NO: 1 and sequences

complementary thereto. SEQ ID NO: 1 discloses the gene EYA4 and its promoter and regulatory elements, wherein said fragment comprises CpG dinucleotides exhibiting a disease specific methylation pattern. The methylation pattern of the gene EYA4 and its promoter and regulatory elements have heretofore not been analysed with regard to cell proliferative disorders. Due to the degeneracy of the genetic code, the sequence as identified in SEQ ID NO: 1 should be interpreted so as to include all substantially similar and equivalent sequences upstream of the promoter region of a gene which encodes a polypeptide with the biological activity of that encoded by EYA4.

In a preferred embodiment of the method, the objective of the invention is achieved by analysis of a nucleic acid comprising a sequence of at least 18 bases in length according to one of SEQ ID NO: 2 to SEQ ID NO: 5 and sequences complementary thereto.

The sequences of SEQ ID NOS: 2 to 5 provide modified versions of the nucleic acid according to SEQ ID NO: 1, wherein the conversion of said sequence results in the synthesis of a nucleic acid having a sequence that is unique and distinct from SEQ ID NO: 1 as follows. (*see also* the following TABLE 1): SEQ ID NO: 1, sense DNA strand of EYA4 gene and its promoter and regulatory elements; SEQ ID NO: 2, converted SEQ ID NO: 1, wherein "C" "T," but "cp." remains "cp." (*i.e.*, corresponds to case where, for SEQ ID NO: 1, all "C" residues of cp. dinucleotide sequences are methylated and are thus not converted); SEQ ID NO: 3, complement of SEQ ID NO: 1, wherein "C" "T," but "cp." remains "cp." (*i.e.*, corresponds to case where, for the complement (*antisense* strand) of SEQ ID NO: 1, all "C" residues of cp. dinucleotide sequences are methylated and are thus not converted); SEQ ID NO: 4, converted SEQ ID NO: 1, wherein "C" "T" for all "C" residues, including those of "cp." dinucleotide sequences (*i.e.*, corresponds to case where, for SEQ ID NO: 1, all "C" residues of cp. dinucleotide sequences are unmethylated); SEQ ID NO: 5, complement of SEQ ID NO: 1, wherein "C" "T" for all "C" residues, including those of "CpG" dinucleotide sequences (*i.e.*, corresponds to case where, for the complement (*antisense* strand) of SEQ ID NO: 1, all "C" residues of CpG dinucleotide sequences are unmethylated).

TABLE 1. Description of SEQ ID NOS: 1 to 5

SEQ ID NO	Relationship to SEQ ID NO:1	Nature of cytosine base conversion
SEQ ID NO:1	Sense strand (EYA4 gene	None; untreated sequence

SEQ ID NO	Relationship to SEQ ID NO:1	Nature of cytosine base conversion
	including promoter and regulatory elements)	
SEQ ID NO:2	Converted sense strand	"C" to "T," but "CpG" remains "CpG" (all "C" residues of CpGs are methylated)
SEQ ID NO:3	Converted antisense strand	"C" to "T," but "CpG" remains "CpG" (all "C" residues of CpGs are methylated)
SEQ ID NO:4	Converted sense strand	"C" to "T" for all "C" residues (all "C" residues of CpGs are <u>un</u> methylated)
SEQ ID NO:5	Converted antisense strand	"C" to "T" for all "C" residues (all "C" residues of CpGs are <u>un</u> methylated)

Significantly, heretofore, the nucleic acid sequences and molecules according to SEQ ID NO: 1 to SEQ ID NO: 5 were not implicated in or connected with the ascertainment of colon cell proliferative disorders.

The described invention further disclose an oligonucleotide or oligomer for detecting the cytosine methylation state within pretreated DNA, according to SEQ ID NO: 2 to SEQ ID NO: 5. Said oligonucleotide or oligomer comprising a nucleic acid sequence having a length of at least nine (9) nucleotides which hybridises, under moderately stringent or stringent conditions (as defined herein above), to a pretreated nucleic acid sequence according to SEQ ID NO: 2 to SEQ ID NO: 5 and/or sequences complementary thereto.

Thus, the present invention includes nucleic acid molecules (*e.g.*, oligonucleotides and peptide nucleic acid (PNA) molecules (PNA-oligomers)) that hybridise under moderately stringent and/or stringent hybridisation conditions to all or a portion of the sequences of SEQ ID NOS: 2 to 5, or to the complements thereof. The hybridising portion of the hybridising nucleic acids is typically at least 9, 15, 20, 25, 30 or 35 nucleotides in length. However, longer molecules have inventive utility, and are thus within the scope of the present invention.

Preferably, the hybridising portion of the inventive hybridising nucleic acids is at least 95%, or at least 98%, or 100% identical to the sequence, or to a portion thereof of SEQ ID NOS: 2 to 5, or to the complements thereof.

Hybridising nucleic acids of the type described herein can be used, for example, as a primer (*e.g.*, a PCR primer), or a diagnostic and/or prognostic probe or primer. Preferably, hybridisation of the oligonucleotide probe to a nucleic acid sample is performed under stringent conditions and the probe is 100% identical to the target sequence. Nucleic acid duplex or hybrid stability is expressed as the melting temperature or T_m , which is the temperature at which a probe dissociates from a target DNA. This melting temperature is used to define the required stringency conditions.

For target sequences that are related and substantially identical to the corresponding sequence of SEQ ID NO: 1 (such as EYA41 allelic variants and SNPs), rather than identical, it is useful to first establish the lowest temperature at which only homologous hybridisation occurs with a particular concentration of salt (*e.g.*, SSC or SSPE). Then, assuming that 1% mismatching results in a 1°C decrease in the T_m , the temperature of the final wash in the hybridisation reaction is reduced accordingly (for example, if sequences having > 95% identity with the probe are sought, the final wash temperature is decreased by 5°C). In practice, the change in T_m can be between 0.5°C and 1.5°C per 1% mismatch.

Examples of inventive oligonucleotides of length X (in nucleotides), as indicated by polynucleotide positions with reference to, *e.g.*, SEQ ID NO: 1, include those corresponding to sets of consecutively overlapping oligonucleotides of length X, where the oligonucleotides within each consecutively overlapping set (corresponding to a given X value) are defined as the finite set of Z oligonucleotides from nucleotide positions:

n to (n + (X-1));

where n=1, 2, 3,...(Y-(X-1));

where Y equals the length (nucleotides or base pairs) of SEQ ID NO: 1 ;

where X equals the common length (in nucleotides) of each oligonucleotide in the set (*e.g.*, X=20 for a set of consecutively overlapping 20-mers); and

where the number (Z) of consecutively overlapping oligomers of length X for a given SEQ ID NO of length Y is equal to Y-(X-1). For example Z=2,785-19=2,766 for either sense or antisense sets of SEQ ID NO: 1, where X=20.

Preferably, the set is limited to those oligomers that comprise at least one CpG, TpG or CpA dinucleotide.

The present invention encompasses, for *each* of SEQ ID NOS: 2 to 5 (sense and antisense), multiple consecutively overlapping sets of oligonucleotides or modified oligonucleotides of length X, where, *e.g.*, X= 9, 10, 17, 20, 22, 23, 25, 27, 30 or 35 nucleotides.

The oligonucleotides or oligomers according to the present invention constitute effective tools useful to ascertain genetic and epigenetic parameters of the genomic sequence corresponding to SEQ ID NO: 1. Preferred sets of such oligonucleotides or modified oligonucleotides of length X are those consecutively overlapping sets of oligomers corresponding to SEQ ID NOS:1-5 (and to the complements thereof). Preferably, said oligomers comprise at least one CpG, TpG or CpA dinucleotide. Included in these preferred sets are the preferred oligomers corresponding to SEQ ID NO: 11 to SEQ ID NO: 15.

Particularly preferred oligonucleotides or oligomers according to the present invention are those in which the cytosine of the CpG dinucleotide (or of the corresponding converted TpG or CpA dinucleotide) sequences is within the middle third of the oligonucleotide; that is, where the oligonucleotide is, for example, 13 bases in length, the CpG, TpG or CpA dinucleotide is positioned within the fifth to ninth nucleotide from the 5'-end.

The oligonucleotides of the invention can also be modified by chemically linking the oligonucleotide to one or more moieties or conjugates to enhance the activity, stability or detection of the oligonucleotide. Such moieties or conjugates include chromophores, fluorophors, lipids such as cholesterol, cholic acid, thioether, aliphatic chains, phospholipids, polyamines, polyethylene glycol (PEG), palmityl moieties, and others as disclosed in, for example, United States Patent Numbers 5,514,758, 5,565,552, 5,567,810, 5,574,142, 5,585,481, 5,587,371, 5,597,696 and 5,958,773. The probes may also exist in the form of a PNA (peptide nucleic acid) which has particularly preferred pairing properties. Thus, the oligonucleotide may include other appended groups such as peptides, and may include hybridisation-triggered cleavage agents (Krol et al., *BioTechniques* 6:958-976, 1988) or intercalating agents (Zon, *Pharm. Res.* 5:539-549, 1988). To this end, the oligonucleotide may be conjugated to another molecule, *e.g.*, a chromophore, fluorophor, peptide, hybridisation-triggered cross-linking agent, transport agent, hybridisation-triggered cleavage agent, etc.

The oligonucleotide may also comprise at least one art-recognized modified sugar and/or base moiety, or may comprise a modified backbone or non-natural internucleoside linkage.

The oligomers according to the present invention are normally used in so called "sets" which contain at least one oligomer for analysis of each of the CpG dinucleotides of a genomic sequence comprising SEQ ID NO: 1 and sequences complementary thereto or to their corresponding CG, TG or CA dinucleotide within the pretreated nucleic acids according to SEQ ID NO: 2 to SEQ ID NO: 5 and sequences complementary thereto. Preferred is a set which contains at least one oligomer for each of the CpG dinucleotides within the gene EYA4 and its promoter and regulatory elements in both the pretreated and genomic versions of said gene, SEQ ID NO: 2 to 5 and SEQ ID NO: 1, respectively. However, it is anticipated that for economic or other factors it may be preferable to analyse a limited selection of the CpG dinucleotides within said sequences and the contents of the set of oligonucleotides should be altered accordingly. Therefore, the present invention moreover relates to a set of at least 3 n (oligonucleotides and/or PNA-oligomers) used for detecting the cytosine methylation state in pretreated genomic DNA (SEQ ID NO: 2 to SEQ ID NO: 5 and sequences complementary thereto) and genomic DNA (SEQ ID NO: 1 and sequences complementary thereto). These probes enable diagnosis and/or therapy of genetic and epigenetic parameters of cell proliferative disorders. The set of oligomers may also be used for detecting single nucleotide polymorphisms (SNPs) in pretreated genomic DNA (SEQ ID NO: 2 to SEQ ID NO: 5, and sequences complementary thereto) and genomic DNA (SEQ ID NO: 1, and sequences complementary thereto).

Moreover, the present invention makes available a set of at least two oligonucleotides which can be used as so-called "primer oligonucleotides" for amplifying DNA sequences of one of SEQ ID NO: 1 to SEQ ID NO: 5 and sequences complementary thereto, or segments thereof.

In the case of the sets of oligonucleotides according to the present invention, it is preferred that at least one and more preferably all members of the set of oligonucleotides is bound to a solid phase.

According to the present invention, it is preferred that an arrangement of different oligonucleotides and/or PNA-oligomers (a so-called "array") made available by the present invention is present in a manner that it is likewise bound to a solid phase. This array of

different oligonucleotide- and/or PNA-oligomer sequences can be characterised in that it is arranged on the solid phase in the form of a rectangular or hexagonal lattice. The solid phase surface is preferably composed of silicon, glass, polystyrene, aluminium, steel, iron, copper, nickel, silver, or gold. However, nitrocellulose as well as plastics such as nylon which can exist in the form of pellets or also as resin matrices may also be used.

Therefore, a further subject matter of the present invention is a method for manufacturing an array fixed to a carrier material for analysis in connection with cell proliferative disorders, in which method at least one oligomer according to the present invention is coupled to a solid phase. Methods for manufacturing such arrays are known, for example, from US Patent 5,744,305 by means of solid-phase chemistry and photolabile protecting groups.

A further subject matter of the present invention relates to a DNA chip for the analysis of cell proliferative disorders. DNA chips are known, for example, in US Patent 5,837,832.

The described invention further provides a composition of matter useful for detecting, differentiation and distinguishing between colon cell proliferative disorders. Said composition comprising at least one nucleic acid 18 base pairs in length of a segment of the nucleic acid sequence disclosed in SEQ ID NO: 2 to 5, and one or more substances taken from the group comprising :

1-5 mM Magnesium Chloride, 100-500 μ M dNTP, 0.5-5 units of taq polymerase, bovine serum albumen, an oligomer in particular an oligonucleotide or peptide nucleic acid (PNA)-oligomer, said oligomer comprising in each case at least one base sequence having a length of at least 9 nucleotides which is complementary to, or hybridises under moderately stringent or stringent conditions to a pretreated genomic DNA according to one of the SEQ ID NO: 2 to SEQ ID NO: 5 and sequences complementary thereto. It is preferred that said composition of matter comprises a buffer solution appropriate for the stabilisation of said nucleic acid in an aqueous solution and enabling polymerase based reactions within said solution.. Suitable buffers are known in the art and commercially available.

The present invention further provides a method for conducting an assay in order to ascertain genetic and/or epigenetic parameters of the gene EYA4 and its promoter and regulatory elements. Most preferably the assay according to the following method is used in order to detect methylation within the gene EYA4 wherein said methylated nucleic acids are present in

a solution further comprising an excess of background DNA, wherein the background DNA is present in between 100 to 1000 times the concentration of the DNA to be detected. Said method comprising contacting a nucleic acid sample obtained from said subject with at least one reagent or a series of reagents, wherein said reagent or series of reagents, distinguishes between methylated and non-methylated CpG dinucleotides within the target nucleic acid.

Preferably, said method comprises the following steps: In the first *step*, a sample of the tissue to be analysed is obtained. The source may be any suitable source, preferably, the source of the sample is selected from the group consisting of histological slides, biopsies, paraffin-embedded tissue, bodily fluids, plasma, serum, stool, urine, blood, and combinations thereof. Preferably, the source is biopsies, bodily fluids, , urine, or blood.

The DNA is then isolated from the sample. Extraction may be by means that are standard to one skilled in the art, including the use of detergent lysates, sonification and vortexing with glass beads. Once the nucleic acids have been extracted, the genomic double stranded DNA is used in the analysis.

In the second step of the method, the genomic DNA sample is treated in such a manner that cytosine bases which are unmethylated at the 5'-position are converted to uracil, thymine, or another base which is dissimilar to cytosine in terms of hybridisation behaviour. This will be understood as 'pretreatment' herein.

The above described treatment of genomic DNA is preferably carried out with bisulfite (hydrogen sulfite, disulfite) and subsequent alkaline hydrolysis which results in a conversion of non-methylated cytosine nucleobases to uracil or to another base which is dissimilar to cytosine in terms of base pairing behaviour. Enclosing the DNA to be analysed in an agarose matrix, thereby preventing the diffusion and renaturation of the DNA (bisulfite only reacts with single-stranded DNA), and replacing all precipitation and purification steps with fast dialysis (Olek A, et al., A modified and improved method for bisulfite based cytosine methylation analysis, *Nucleic Acids Res.* 24:5064-6, 1996). It is further preferred that the bisulfite treatment is carried out in the presence of a radical trap or DNA denaturing agent.

In the third step of the method, fragments of the pretreated DNA are amplified. Wherein the source of the DNA is free DNA from serum, or DNA extracted from paraffin it is particularly

preferred that the size of the amplificate fragment is between 100 and 200 base pairs in length, and wherein said DNA source is extracted from cellular sources (e.g. tissues, biopsies, cell lines) it is preferred that the amplificate is between 100 and 350 base pairs in length. It is particularly preferred that said amplificates comprise at least one 20 base pair sequence comprising at least three CpG dinucleotides. Said amplification is carried out using sets of primer oligonucleotides according to the present invention, and a preferably heat-stable polymerase. The amplification of several DNA segments can be carried out simultaneously in one and the same reaction vessel, in one embodiment of the method preferably six or more fragments are amplified simultaneously. Typically, the amplification is carried out using a polymerase chain reaction (PCR). The set of primer oligonucleotides includes at least two oligonucleotides whose sequences are each reverse complementary, identical, or hybridise under stringent or highly stringent conditions to an at least 18-base-pair long segment of the base sequences of SEQ ID NO: 2 to SEQ ID NO: 5 and sequences complementary thereto.

In an alternate embodiment of the method, the methylation status of preselected CpG positions within the nucleic acid sequences comprising SEQ ID NO: 2 to SEQ ID NO: 5 may be detected by use of methylation-specific primer oligonucleotides. This technique (MSP) has been described in United States Patent No. 6,265,171 to Herman. The use of methylation status specific primers for the amplification of bisulfite treated DNA allows the differentiation between methylated and unmethylated nucleic acids. MSP primers pairs contain at least one primer which hybridises to a bisulfite treated CpG dinucleotide. Therefore, the sequence of said primers comprises at least one CpG, TpG or CpA dinucleotide. MSP primers specific for non-methylated DNA contain a "T" at the 3' position of the C position in the CpG. Preferably, therefore, the base sequence of said primers is required to comprise a sequence having a length of at least 18 nucleotides which hybridises to a pretreated nucleic acid sequence according to SEQ ID NO: 2 to SEQ ID NO: 5 and sequences complementary thereto, wherein the base sequence of said oligomers comprises at least one CpG, TpG or CpA dinucleotide. In this embodiment of the method according to the invention it is particularly preferred that the MSP primers comprise between 2 and 4 CpG, TpG or CpA dinucleotides. It is further preferred that said dinucleotides are located within the 3' half of the primer e.g. wherein a primer is 18 bases in length the specified dinucleotides are located within the first 9 bases from the 3' end of the molecule. In addition to the CpG, TpG or CpA dinucleotides it is further preferred that said primers should further comprise several bisulfite converted bases (i.e. cytosine converted to thymine, or on the hybridising strand, guanine converted to

adenosine). In a further preferred embodiment said primers are designed so as to comprise no more than 2 cytosine or guanine bases.

In one embodiment of the method the primers may be selected from the group consisting of SEQ ID NO: 6 to SEQ ID NO: 10.

The fragments obtained by means of the amplification can carry a directly or indirectly detectable label. Preferred are labels in the form of fluorescence labels, radionuclides, or detachable molecule fragments having a typical mass which can be detected in a mass spectrometer. Where said labels are mass labels, it is preferred that the labelled amplicates have a single positive or negative net charge, allowing for better detectability in the mass spectrometer. The detection may be carried out and visualised by means of, e.g., matrix assisted laser desorption/ionisation mass spectrometry (MALDI) or using electron spray mass spectrometry (ESI).

Matrix Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-TOF) is a very efficient development for the analysis of biomolecules (Karas & Hillenkamp, *Anal Chem.*, 60:2299-301, 1988). An analyte is embedded in a light-absorbing matrix. The matrix is evaporated by a short laser pulse thus transporting the analyte molecule into the vapour phase in an unfragmented manner. The analyte is ionised by collisions with matrix molecules. An applied voltage accelerates the ions into a field-free flight tube. Due to their different masses, the ions are accelerated at different rates. Smaller ions reach the detector sooner than bigger ones. MALDI-TOF spectrometry is well suited to the analysis of peptides and proteins. The analysis of nucleic acids is somewhat more difficult (Gut & Beck, *Current Innovations and Future Trends*, 1:147-57, 1995). The sensitivity with respect to nucleic acid analysis is approximately 100-times less than for peptides, and decreases disproportionally with increasing fragment size. Moreover, for nucleic acids having a multiply negatively charged backbone, the ionisation process via the matrix is considerably less efficient. In MALDI-TOF spectrometry, the selection of the matrix plays an eminently important role. For the desorption of peptides, several very efficient matrixes have been found which produce a very fine crystallisation. There are now several responsive matrixes for DNA, however, the difference in sensitivity between peptides and nucleic acids has not been reduced. This difference in sensitivity can be reduced, however, by chemically modifying the DNA in such a manner that it becomes more similar to a peptide. For example, phosphorothioate nucleic acids, in which

the usual phosphates of the backbone are substituted with thiophosphates, can be converted into a charge-neutral DNA using simple alkylation chemistry (Gut & Beck, *Nucleic Acids Res.* 23: 1367-73, 1995). The coupling of a charge tag to this modified DNA results in an increase in MALDI-TOF sensitivity to the same level as that found for peptides. A further advantage of charge tagging is the increased stability of the analysis against impurities, which makes the detection of unmodified substrates considerably more difficult.

In a particularly preferred embodiment of the method the amplification of step three is carried out in the presence of at least one species of blocker oligonucleotides. The use of such blocker oligonucleotides has been described by Yu et al., *BioTechniques* 23:714-720, 1997. The use of blocking oligonucleotides enables the improved specificity of the amplification of a subpopulation of nucleic acids. Blocking probes hybridised to a nucleic acid suppress, or hinder the polymerase mediated amplification of said nucleic acid. In one embodiment of the method blocking oligonucleotides are designed so as to hybridise to background DNA. In a further embodiment of the method said oligonucleotides are designed so as to hinder or suppress the amplification of unmethylated nucleic acids as opposed to methylated nucleic acids or vice versa.

Blocking probe oligonucleotides are hybridised to the bisulfite treated nucleic acid concurrently with the PCR primers. PCR amplification of the nucleic acid is terminated at the 5' position of the blocking probe, such that amplification of a nucleic acid is suppressed where the complementary sequence to the blocking probe is present. The probes may be designed to hybridise to the bisulfite treated nucleic acid in a methylation status specific manner. For example, for detection of methylated nucleic acids within a population of unmethylated nucleic acids, suppression of the amplification of nucleic acids which are unmethylated at the position in question would be carried out by the use of blocking probes comprising a 'TpG' at the position in question, as opposed to a 'CpG.' In one embodiment of the method the sequence of said blocking oligonucleotides should be identical or complementary to molecule is complementary or identical to a sequence at least 18 base pairs in length selected from the group consisting of SEQ ID NOS: 2 to 5, preferably comprising one or more CpG, TpG or CpA dinucleotides. In one embodiment of the method the sequence of said oligonucleotides is selected from the group consisting SEQ ID NO: 15 and SEQ ID NO: 16 and sequences complementary thereto.

For PCR methods using blocker oligonucleotides, efficient disruption of polymerase-mediated amplification requires that blocker oligonucleotides not be elongated by the polymerase. Preferably, this is achieved through the use of blockers that are 3'-deoxyoligonucleotides, or oligonucleotides derivitised at the 3' position with other than a "free" hydroxyl group. For example, 3'-O-acetyl oligonucleotides are representative of a preferred class of blocker molecule.

Additionally, polymerase-mediated decomposition of the blocker oligonucleotides should be precluded. Preferably, such preclusion comprises either use of a polymerase lacking 5'-3' exonuclease activity, or use of modified blocker oligonucleotides having, for example, thioate bridges at the 5'-termini thereof that render the blocker molecule nuclease-resistant. Particular applications may not require such 5' modifications of the blocker. For example, if the blocker- and primer-binding sites overlap, thereby precluding binding of the primer (*e.g.*, with excess blocker), degradation of the blocker oligonucleotide will be substantially precluded. This is because the polymerase will not extend the primer toward, and through (in the 5'-3' direction) the blocker—a process that normally results in degradation of the hybridised blocker oligonucleotide.

A particularly preferred blocker/PCR embodiment, for purposes of the present invention and as implemented herein, comprises the use of peptide nucleic acid (PNA) oligomers as blocking oligonucleotides. Such PNA blocker oligomers are ideally suited, because they are neither decomposed nor extended by the polymerase.

In one embodiment of the method, the binding site of the blocking oligonucleotide is identical to, or overlaps with that of the primer and thereby hinders the hybridisation of the primer to its binding site. In a further preferred embodiment of the method, two or more such blocking oligonucleotides are used. In a particularly preferred embodiment, the hybridisation of one of the blocking oligonucleotides hinders the hybridisation of a forward primer, and the hybridisation of another of the probe (blocker) oligonucleotides hinders the hybridisation of a reverse primer that binds to the amplicate product of said forward primer.

In an alternative embodiment of the method, the blocking oligonucleotide hybridises to a location between the reverse and forward primer positions of the treated background DNA, thereby hindering the elongation of the primer oligonucleotides.

It is particularly preferred that the blocking oligonucleotides are present in at least 5 times the concentration of the primers.

In the fourth step of the method, the amplicates obtained during the third step of the method are analysed in order to ascertain the methylation status of the CpG dinucleotides prior to the treatment.

In embodiments where the amplicates were obtained by means of MSP amplification and/or blocking oligonucleotides, the presence or absence of an amplicate is in itself indicative of the methylation state of the CpG positions covered by the primers and or blocking oligonucleotide, according to the base sequences thereof.. All possible known molecular biological methods may be used for this detection, including, but not limited to gel electrophoresis, sequencing, liquid chromatography, hybridisations, real time PCR analysis or combinations thereof. This step of the method further acts as a qualitative control of the preceding steps.

In the fourth step of the method amplicates obtained by means of both standard and methylation specific PCR are further analysed in order to determine the CpG methylation status of the genomic DNA isolated in the first step of the method. This may be carried out by means of based-based methods such as, but not limited to, array technology and probe based technologies as well as by means of techniques such as sequencing and template directed extension.

In one embodiment of the method, the amplicates synthesised in step three are subsequently hybridised to an array or a set of oligonucleotides and/or PNA probes. In this context, the hybridisation takes place in the following manner: the set of probes used during the hybridisation is preferably composed of at least 2 oligonucleotides or PNA-oligomers; in the process, the amplicates serve as probes which hybridise to oligonucleotides previously bonded to a solid phase; the non-hybridised fragments are subsequently removed; said oligonucleotides contain at least one base sequence having a length of at least 9 nucleotides which is reverse complementary or identical to a segment of the base sequences specified in the SEQ ID NO: 2 to SEQ ID NO: 5; and the segment comprises at least one CpG , TpG or CpA dinucleotide.

In a preferred embodiment, said dinucleotide is present in the central third of the oligomer. For example, wherein the oligomer comprises one CpG dinucleotide, said dinucleotide is preferably the fifth to ninth nucleotide from the 5'-end of a 13-mer. One oligonucleotide exists for the analysis of each CpG dinucleotide within the sequence according to SEQ ID NO: 1, and the equivalent positions within SEQ ID NOS: 2 to 5. Said oligonucleotides may also be present in the form of peptide nucleic acids. The non-hybridised amplicates are then removed. The hybridised amplicates are detected. In this context, it is preferred that labels attached to the amplicates are identifiable at each position of the solid phase at which an oligonucleotide sequence is located.

In yet a further embodiment of the method, the genomic methylation status of the CpG positions may be ascertained by means of oligonucleotide probes that are hybridised to the bisulfite treated DNA concurrently with the PCR amplification primers (wherein said primers may either be methylation specific or standard).

A particularly preferred embodiment of this method is the use of fluorescence-based Real Time Quantitative PCR (Heid et al., *Genome Res.* 6:986-994, 1996; *also see* United States Patent No. 6,331,393). There are two preferred embodiments of utilising this method. One embodiment, known as the TaqMan™ assay employs a dual-labelled fluorescent oligonucleotide probe. The TaqMan™ PCR reaction employs the use of a non-extendible interrogating oligonucleotide, called a TaqMan™ probe, which is designed to hybridise to a GpC-rich sequence located between the forward and reverse amplification primers. The TaqMan™ probe further comprises a fluorescent "reporter moiety" and a "quencher moiety" covalently bound to linker moieties (*e.g.*, phosphoramidites) attached to the nucleotides of the TaqMan™ oligonucleotide. Hybridised probes are displaced and broken down by the polymerase of the amplification reaction thereby leading to an increase in fluorescence. For analysis of methylation within nucleic acids subsequent to bisulfite treatment, it is required that the probe be methylation specific, as described in United States Patent No. 6,331,393, (hereby incorporated by reference in its entirety) also known as the MethyLight assay. The second preferred embodiment of this technology is the use of dual-probe technology (Lightcycler®), each carrying donor or recipient fluorescent moieties, hybridisation of two probes in proximity to each other is indicated by an increase or fluorescent amplification primers. Both these techniques may be adapted in a manner suitable for use with bisulfite

treated DNA, and moreover for methylation analysis within CpG dinucleotides.

In a further preferred embodiment of the method, the fourth step of the method comprises the use of template-directed oligonucleotide extension, such as MS-SNuPE as described by Gonzalgo & Jones, *Nucleic Acids Res.* 25:2529-2531, 1997. In said embodiment it is preferred that the Ms-SNuPE primer is identical or complementary to a sequence at least nine but preferably no more than twenty five nucleotides in length of one or more of the sequences taken from the group of SEQ ID NO: 2 to SEQ ID NO: 5.

In yet a further embodiment of the method, the fourth step of the method comprises sequencing and subsequent sequence analysis of the amplificate generated in the third step of the method (Sanger F., et al., *Proc Natl Acad Sci USA* 74:5463-5467, 1977).

Additional embodiments of the invention provide a method for the analysis of the methylation status of genomic DNA according to the invention (SEQ ID NO: 1) without the need for pretreatment.

In the *first step* of such additional embodiments, the genomic DNA sample is isolated from tissue or cellular sources. Preferably, such sources include cell lines, histological slides, body fluids, or tissue embedded in paraffin. Extraction may be by means that are standard to one skilled in the art, including but not limited to the use of detergent lysates, sonification and vortexing with glass beads. Once the nucleic acids have been extracted, the genomic double-stranded DNA is used in the analysis.

In a preferred embodiment, the DNA may be cleaved prior to the treatment, and this may be by any means standard in the state of the art, in particular with methylation-sensitive restriction endonucleases.

In the *second step*, the DNA is then digested with one or more methylation sensitive restriction enzymes. The digestion is carried out such that hydrolysis of the DNA at the restriction site is informative of the methylation status of a specific CpG dinucleotide.

In the *third step*, which is optional but a preferred embodiment, the restriction fragments are amplified. This is preferably carried out using a polymerase chain reaction, and said

amplificates may carry suitable detectable labels as discussed above, namely fluorophore labels, radionuclides and mass labels.

In the *final step* the amplificates are detected. The detection may be by any means standard in the art, for example, but not limited to, gel electrophoresis analysis, hybridisation analysis, incorporation of detectable tags within the PCR products, DNA array analysis, MALDI or ESI analysis.

The present invention enables diagnosis and/or prognosis of events which are disadvantageous to patients or individuals in which important genetic and/or epigenetic parameters within the EYA4 gene and its promoter or regulatory elements may be used as markers. Said parameters obtained by means of the present invention may be compared to another set of genetic and/or epigenetic parameters, the differences serving as the basis for a diagnosis and/or prognosis of events which are disadvantageous to patients or individuals.

Specifically, the present invention provides for diagnostic and/or prognostic cancer assays based on measurement of differential methylation of EYA4 CpG dinucleotide sequences. Preferred gene sequences useful to measure such differential methylation are represented herein by SEQ ID NOS: 1 to 5. Typically, such assays involve obtaining a tissue sample from a test tissue, performing an assay to measure the methylation status of at least one of the inventive EYA4-specific CpG dinucleotide sequences derived from the tissue sample, relative to a control sample, and making a diagnosis or prognosis based thereon.

In particular preferred embodiments, inventive oligomers are used to assess EYA4 specific CpG dinucleotide methylation status, such as those based on SEQ ID NOS: 1 to 5, including the representative preferred oligomers corresponding to SEQ ID NOS: 11 to 15, or arrays thereof, as well as a kit based thereon are useful for the diagnosis and/or prognosis of cancer and/or other prostate cell proliferative disorders.

The present invention moreover relates to a diagnostic agent and/or therapeutic agent for the diagnosis and/or therapy colon cell proliferative disorders, the diagnostic agent and/or therapeutic agent being characterised in that at least one primer or probe based on SEQ ID NOS: 1 to 5 is used for manufacturing it, possibly together with suitable additives and ancillary agents. In one embodiment, the EYA4 polypeptide or a fragment or derivative

thereof may be administered to a subject to treat or prevent colon cancers.

In another embodiment, a vector capable of expressing EYA4, or a fragment or a derivative thereof, may also be administered to a subject to treat or prevent colon cancers.

In another embodiment, agonists which are specific for EYA4 may be used to stimulate or prolong the activity of EYA4 and may be administered to a subject to treat or prevent colon cancers.

In other embodiments, any of the therapeutic proteins or vectors described above may be administered in combination with other appropriate therapeutic agents. Selection of the appropriate agents for use in combination therapy may be made by one of ordinary skill in the art, according to conventional pharmaceutical principles. The combination of therapeutic agents may act synergistically to effect the treatment or prevention of colon cancer. Using this approach, one may be able to achieve therapeutic efficacy with lower dosages of each agent, thus reducing the potential for adverse side effects.

Expression vectors derived from retroviruses, adenovirus, herpes or vaccinia viruses, or from various bacterial plasmids may be used for delivery of nucleotide sequences to the targeted organ, tissue or cell population.

An additional embodiment of the invention relates to the administration of a pharmaceutical composition, in conjunction with a pharmaceutically acceptable carrier. Such pharmaceutical compositions may consist of EYA4 or agonists of EYA4. The compositions may be administered alone or in combination with at least one other agent, such as stabilising compound, which may be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The compositions may be administered to a patient alone, or in combination with other agents, drugs or hormones.

The pharmaceutical compositions utilised in this invention may be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual, or rectal means.

In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Further details on techniques for formulation and administration may be found in the latest edition of Remington's Pharmaceutical Sciences (Maack Publishing Co., Easton, Pa.).

Moreover, an additional aspect of the present invention is a kit comprising, for example: a bisulfite-containing reagent as well as at least one oligonucleotide whose sequences in each case correspond, are complementary, or hybridise under stringent or highly stringent conditions to a 18-base long segment of the sequences SEQ ID NOS: 1 to 5. Said kit may further comprise instructions for carrying out and evaluating the described method. In a further preferred embodiment, said kit may further comprise standard reagents for performing a CpG position-specific methylation analysis, wherein said analysis comprises one or more of the following techniques: MS-SNuPE, MSP, MethyLight, HeavyMethyl™, COBRA, and nucleic acid sequencing. However, a kit along the lines of the present invention can also contain only part of the aforementioned components.

Typical reagents (*e.g.*, as might be found in a typical COBRA-based kit) for COBRA analysis may include, but are not limited to: PCR primers for specific gene (or methylation-altered DNA sequence or CpG island); restriction enzyme and appropriate buffer; gene-hybridisation oligo; control hybridisation oligo; kinase labelling kit for oligo probe; and radioactive nucleotides. Additionally, bisulfite conversion reagents may include: DNA denaturation buffer; sulfonation buffer; DNA recovery reagents or kits (*e.g.*, precipitation, ultrafiltration, affinity column); desulfonation buffer; and DNA recovery components.

Typical reagents (*e.g.*, as might be found in a typical MethyLight®-based kit) for MethyLight® analysis may include, but are not limited to: PCR primers for specific gene (or methylation-altered DNA sequence or CpG island); TaqMan® probes; optimised PCR buffers and deoxynucleotides; and Taq polymerase.

Typical reagents (*e.g.*, as might be found in a typical Ms-SNuPE-based kit) for Ms-SNuPE analysis may include, but are not limited to: PCR primers for specific gene (or methylation-altered DNA sequence or CpG island); optimised PCR buffers and deoxynucleotides; gel

extraction kit; positive control primers; Ms-SNuPE primers for specific gene; reaction buffer (for the Ms-SNuPE reaction); and radioactive nucleotides. Additionally, bisulfite conversion reagents may include: DNA denaturation buffer; sulfonation buffer; DNA recovery reagents or kit (*e.g.*, precipitation, ultrafiltration, affinity column); desulfonation buffer; and DNA recovery components.

Typical reagents (*e.g.*, as might be found in a typical MSP-based kit) for MSP analysis may include, but are not limited to: methylated and unmethylated PCR primers for specific gene (or methylation-altered DNA sequence or CpG island), optimised PCR buffers and deoxynucleotides, and specific probes.

Definitions:

In the context of the present invention, the term “CpG island” refers to a contiguous region of genomic DNA that satisfies the criteria of (1) having a frequency of CpG dinucleotides corresponding to an “Observed/Expected Ratio” >0.6, and (2) having a “GC Content” >0.5. CpG islands are typically, but not always, between about 0.2 to about 1 kb in length.

In the context of the present invention, the term “methylation state” or “methylation status” refers to the presence or absence of 5-methylcytosine (“5-mCyt”) at one or a plurality of CpG dinucleotides within a DNA sequence. Methylation states at one or more particular palindromic CpG methylation sites (each having two CpG CpG dinucleotide sequences) within a DNA sequence include “unmethylated,” “fully-methylated” and “hemi-methylated.”

In the context of the present invention, the term “hemi-methylation” or “hemimethylation” refers to the methylation state of a palindromic CpG methylation site, where only a single cytosine in one of the two CpG dinucleotide sequences of the palindromic CpG methylation site is methylated (*e.g.*, 5'-CC^MGG-3' (top strand): 3'-GGCC-5' (bottom strand)).

In the context of the present invention, the term “hypermethylation” refers to the average methylation state corresponding to an *increased* presence of 5-mCyt at one or a plurality of CpG dinucleotides within a DNA sequence of a test DNA sample, relative to the amount of 5-mCyt found at corresponding CpG dinucleotides within a normal control DNA sample.

In the context of the present invention, the term “hypomethylation” refers to the average methylation state corresponding to a *decreased* presence of 5-mCyt at one or a plurality of CpG dinucleotides within a DNA sequence of a test DNA sample, relative to the amount of 5-mCyt found at corresponding CpG dinucleotides within a normal control DNA sample.

In the context of the present invention, the term “microarray” refers broadly to both “DNA microarrays,” and ‘DNA chip(s),’ as recognised in the art, encompasses all art-recognised solid supports, and encompasses all methods for affixing nucleic acid molecules thereto or synthesis of nucleic acids thereon.

“Genetic parameters” are mutations and polymorphisms of genes and sequences further required for their regulation. To be designated as mutations are, in particular, insertions, deletions, point mutations, inversions and polymorphisms and, particularly preferred, SNPs (single nucleotide polymorphisms).

“Epigenetic parameters” are, in particular, cytosine methylations. Further epigenetic parameters include, for example, the acetylation of histones which, however, cannot be directly analysed using the described method but which, in turn, correlate with the DNA methylation.

In the context of the present invention, the term “bisulfite reagent” refers to a reagent comprising bisulfite, disulfite, hydrogen sulfite or combinations thereof, useful as disclosed herein to distinguish between methylated and unmethylated CpG dinucleotide sequences.

In the context of the present invention, the term “Methylation assay” refers to any assay for determining the methylation state of one or more CpG dinucleotide sequences within a sequence of DNA.

In the context of the present invention, the term “MS.AP-PCR” (Methylation-Sensitive Arbitrarily-Primed Polymerase Chain Reaction) refers to the art-recognised technology that allows for a global scan of the genome using CG-rich primers to focus on the regions most likely to contain CpG dinucleotides, and described by Gonzalgo et al., *Cancer Research* 57:594-599, 1997.

In the context of the present invention, the term “MethylLight” refers to the art-recognised fluorescence-based real-time PCR technique described by Eads et al., *Cancer Res.* 59:2302-2306, 1999.

In the context of the present invention, the term “HeavyMethyl™” assay, in the embodiment thereof implemented herein, refers to a HeavyMethyl™ MethylLight assay, which is a variation of the MethylLight assay, wherein the MethylLight assay is combined with methylation specific *blocking* probes covering CpG positions between the amplification primers.

The term “Ms-SNuPE” (Methylation-sensitive Single Nucleotide Primer Extension) refers to the art-recognized assay described by Gonzalgo & Jones, *Nucleic Acids Res.* 25:2529-2531, 1997.

The term “MSP” (Methylation-specific PCR) refers to the art-recognised methylation assay described by Herman et al. *Proc. Natl. Acad. Sci. USA* 93:9821-9826, 1996, and by US Patent No. 5,786,146.

The term “COBRA” (Combined Bisulfite Restriction Analysis) refers to the art-recognized methylation assay described by Xiong & Laird, *Nucleic Acids Res.* 25:2532-2534, 1997.

The term “hybridisation” is to be understood as a bond of an oligonucleotide to a complementary sequence along the lines of the Watson-Crick base pairings in the sample DNA, forming a duplex structure.

“Stringent hybridisation conditions,” as defined herein, involve hybridising at 68°C in 5x SSC/5x Denhardt’s solution/1.0% SDS, and washing in 0.2x SSC/0.1% SDS at room temperature, or involve the art-recognised equivalent thereof (e.g., conditions in which a hybridisation is carried out at 60°C in 2.5 x SSC buffer, followed by several washing steps at 37°C in a low buffer concentration, and remains stable). Moderately stringent conditions, as defined herein, involve including washing in 3x SSC at 42°C, or the art-recognised equivalent thereof. The parameters of salt concentration and temperature can be varied to achieve the optimal level of identity between the probe and the target nucleic acid. Guidance regarding such conditions is available in the art, for example, by Sambrook et al., 1989, Molecular

Cloning, A Laboratory Manual, Cold Spring Harbor Press, N.Y.; and Ausubel et al. (eds.), 1995, Current Protocols in Molecular Biology, (John Wiley & Sons, N.Y.) at Unit 2.10.

“Background DNA” as used herein refers to any nucleic acids which originate from sources other than colon cells.

The invention will now be described in more detail based on the following examples, SEQ IDs, and Figures, without being limited thereto. In the sequence protocol and the Figures,

SEQ ID NO: 1 shows the sequence of the human gene EYA4,
SEQ ID NOS: 2 to 5 show chemically pretreated sequences of the gene EYA4,
SEQ ID NOS: 6 to 10 show the sequences of primers used in the examples, and
SEQ ID NOS: 11 to 15 show sequences of probes used in the examples.

Figure 1 shows the level of methylation determined by a MSP MethyLight assay and by a HeavyMethyl MethyLight assay according to examples 1 and 2. The Y-axis shows the degree of methylation within the region of the EYA4 gene investigated. Tumour samples are represented by white points, and normal colon tissue samples by white black points. A significantly higher degree of methylation was observed in tumour samples than in healthy tissue samples. The level of significance as measured using a t-test was $p=0.00000312$ (MSP-ML, Example 1) and $p=0.00000326$ (HM-ML, Example 2).

Figure 2 shows the Receiver Operating Characteristic curve (ROC curve) of the MSP-Methyl-Light-Assay for Adenocarcinomas according to Example 1. A ROC is a plot of the true positive rate against the false positive rate for the different possible cutpoints of a diagnostic test. It shows the tradeoff between sensitivity and specificity depending on the selected cutpoint (any increase in sensitivity will be accompanied by a decrease in specificity). The area under an ROC curve (AUC) is a measure for the accuracy of a diagnostic test (the larger the area the better, optimum is 1, a random test would have a ROC curve lying on the diagonal with an area of 0.5) The AUC for the MSP-Methyl-Light-Assay is: 0.94.

Figure 3 shows the Receiver Operating Characteristic curve (ROC curve) of the HM-Methyl-Light-Assay for Adenocarcinoma according to Example 2. The area under an ROC curve

(AUC) is a measure for the accuracy of a diagnostic test. The AUC for the HM-Methyl-Light-Assay is: 0.91.

Figure 4 shows the level of methylation determined by a HeavyMethyl MethyLight assay according to example 2, testing an additional set of colon samples (25 adenocarcinoma, 33 normals, and 13 adenomas). The Y-axis shows the degree of methylation within the region of the EYA4 gene investigated. Adenocarcinoma samples are represented by white squares, and normal colon tissue samples by black diamonds. A significantly higher degree of methylation was observed in tumour samples than in healthy tissue samples. The level of significance as measured using a t-test was 0.00424.

Figure 5 shows the Receiver Operating Characteristic curve (ROC curve) of the HM-Methyl-Light-Assay for Adenocarcinoma and Adenoma according to Example 2 (additional sets of samples). The area under an ROC curve (AUC) is a measure for the accuracy of a diagnostic test. The AUC for the HM-Methyl-Light-Assay is 0.81.

Figure 6 shows the Receiver Operating Characteristic curve (ROC curve) of the HM-Methyl-Light-Assay for Adenocarcinoma only according to Example 2 (additional sets of samples). The area under an ROC curve (AUC) is a measure for the accuracy of a diagnostic test. The AUC for the HM-Methyl-Light-Assay is: 0.844.

Figure 7 shows the Receiver Operating Characteristic curve (ROC curve) of the HM-Methyl-Light-Assay for Adenomas according to Example 2 (additional sets of samples). The area under an ROC curve (AUC) is a measure for the accuracy of a diagnostic test. The AUC for the HM-Methyl-Light-Assay is: 0.748.

Figure 8 shows the level of methylation in different tumour and healthy tissues determined by a HeavyMethyl MethyLight assay according to example 3. The Y-axis shows the degree of methylation within the region of the EYA4 gene investigated. Besides the colon cancer samples only one of the two breast cancer tissues were methylated.

Figure 9 shows the level of methylation in different breast cancer tissues determined by a HeavyMethyl MethyLight assay according to example 3. Only one tissue was methylated.

Figure 10 shows the level of methylation in serum samples determined by a HeavyMethyl MethyLight assay according to example 4. The Y-axis shows the degree of methylation within the region of the EYA4 gene investigated.

EXAMPLE 1

Analysis of methylation within colon cancer using an MSP- MethyLight Assay

DNA was extracted from 33 colon adenocarcinoma samples and 43 colon normal adjacent tissues using a Qiagen® extraction kit. The DNA from each sample was treated using a bisulfite solution (hydrogen sulfite, disulfite) according to the agarose-bead method (Olek et al 1996). The treatment is such that all non methylated cytosines within the sample are converted to thymidine. Conversely, 5-methylated cytosines within the sample remain unmodified.

The methylation status was determined with a MSP-MethyLight assay designed for the CpG island of interest and a control fragment from the *beta* actin gene (Eads et al., 2001). The CpG island assay covers CpG sites in both the primers and the taqman style probe, while the control gene does not. The control gene is used as a measure of total DNA concentration, and the CpG island assay (methylation assay) determines the methylation levels at that site.

Methods: The EYA4 gene CpG island assay was performed using the following primers and probes:

Forward Primer: CGGAGGGTACGGAGATTACG (SEQ ID NO:6);

Reverse Primer: CGACGACGCGCGAAA (SEQ ID NO:7); and

Probe: CGAAACCCTAAATATCCCGAATAACGCCG (SEQ ID NO:12).

The corresponding control assay was performed using the following primers and probes:

Primer: TGGTGATGGAGGAGGTTTAGTAAGT (SEQ ID NO:8);

Primer: AACCAATAAAACCTACTCCTCCCTTAA (SEQ ID NO:9); and

Probe: ACCACCACCCAACACACAATAACAAACACA (SEQ ID NO:13)

The reactions were run in triplicate on each DNA sample with the following assay conditions:

Reaction solution: (900 nM primers; 300 nM probe; 3.5 mM Magnesium Chloride; 1 unit of taq polymerase; 200 µM dNTPs; 7 dDNA, in a final reaction volume of 20 µl);

Cycling conditions: (95°C for 10 minutes; then 50 cycles of: 95°C for 15 seconds; 60°C for 1 minute). The data was analysed using a PMR calculation previously described in the literature (Eads et al 2001).

Results. The mean PMR for normal samples was 0.15, with a standard deviation of 0.18. The mean PMR for tumour samples was 17.98, with a standard deviation of 18.18. The overall difference in methylation levels between tumour and normal samples is significant in a t-test ($p=0.00000312$). The results are shown in Figure 1.

A Receiver Operating Characteristic curve (ROC curve) of the assay was also determined. A ROC is a plot of the true positive rate against the false positive rate for the different possible cutpoints of a diagnostic test. It shows the tradeoff between sensitivity and specificity depending on the selected cutpoint (any increase in sensitivity will be accompanied by a decrease in specificity). The area under an ROC curve (AUC) is a measure for the accuracy of a diagnostic test (the larger the area the better, optimum is 1, a random test would have a ROC curve lying on the diagonal with an area of 0.5; for reference: J.P. Egan. Signal Detection Theory and ROC Analysis, Academic Press, New York, 1975). The AUC for the MSP-Methyl-Light-Assay is: 0.94 (Figure 2).

EXAMPLE 2

Methylation within colon cancer was analyzed using a HeavyMethyl MethyLight[®] assay.

The same DNA samples were also used to analyse methylation of the CpG island with a HeavyMethyl MethyLight (or HM MethyLight) assay, also referred to as the HeavyMethyl assay. The methylation status was determined with a HM MethyLight assay designed for the CpG island of interest and the same control gene assay described above. The CpG island assay covers CpG sites in both the blockers and the taqman style probe, while the control gene does not.

Methods. The CpG island assay (methylation assay) was performed using the following primers and probes:

Forward Primer: GGTGATTGTTTATTGTTATGGTTTG (SEQ ID NO:10)

Reverse Primer: CCCCTCAACCTAAAACTACAAC (SEQ ID NO:11)

Forward Blocker: GTTATGGTTTGTGATTTTGTGTGGG (SEQ ID NO:15)

Reverse Blocker: AAACCTACAACCACTCAAATCAACCCA (SEQ ID NO:16)

Probe: AAAATTACGACGACGCCACCCGAAA (SEQ ID NO:14)

The reactions were each run in triplicate on each DNA sample with the following assay conditions:

Reaction solution: (400 nM primers; 400 nM probe; 10µM both blockers; 3.5 mM magnesium chloride; 1x ABI Taqman buffer; 1 unit of ABI TaqGold polymerase; 200 µM dNTPs; and 7 µl of DNA, in a final reaction volume of 20 µl);

Cycling conditions: (95°C for 10 minutes); (95°C for 15 seconds, 64°C for 1 minute (2 cycles)); (95°C for 15 seconds, 62°C for 1 minute (2 cycles)); (95°C for 15 seconds, 60°C for 1 minute (2 cycles)); and (95°C for 15 seconds, 58°C for 1 minute, 60°C for 40 seconds (41 cycles)).

Results. The mean PMR for normal samples was 1.12 with a standard deviation of 1.45. The mean PMR for tumour samples was 38.23 with a standard deviation of 33.22. The overall difference in methylation levels between tumour and normal samples is significant in a t-test ($p=0.000000326$). The results are shown in Figure 1. A ROC curve of the assay was also determined. The AUC for the MSP-Methyl-Light-Assay is 0.91 (Figure 3)

The assay was tested on an additional set of colon samples (25 adenocarcinoma, 33 normals, and 13 adenomas). The results showed a significant difference again (Figure 4). The ROC are shown in Figure 5-7.

EXAMPLE 3

The HeavyMethyl-MethyLight-assay was also tested against a panel of other tissues (Figure 8). Besides the colon cancer samples only one of the two breast cancer tissues were methylated. However, on a panel of 21 additional breast tumours (different stages), only one was methylated (Figure 9). So the marker is specific for colon tumour samples. All primers, probes, blockers and reaction conditions were identical to those used in the analysis of the colon cancer samples (Example 2).

EXAMPLE 4

Twelve of the colon tissues analysed by real-time PCR also had paired serum taken before surgery. We extracted DNA from 1 ml of that serum using a Qiagen UltraSens® DNA extraction kit, bisulfite treated the DNA sample, and ran the HeavyMethyl-MethyLight-assay on those samples. The control gene did not amplify for three of the cancer serum samples and three of the normal serum samples, so we can conclude that the sample preparation did not work in these cases. In the other cases, there was evidence of higher methylation in the cancer samples than the normal samples (Figure 10).

Patent Claims

1. A method of diagnosing a colon cell proliferative disorder in a subject, comprising the steps of:
 - (a) obtaining one or more test samples from colon tissue or serum or both of said subject; and
 - (b) detecting a decrease in the amount or expression of a polypeptide expressed from the EYA4 gene
2. The method of claim 1, wherein said colon cell proliferative disorders are taken from the group comprising adenocarcinomas, squamous cell cancers, carcinoid tumours, sarcomas, and lymphomas.
3. The method of claim 1 or 2, wherein said detection is by immunoassay, in particular by an ELISA.
4. The method of claim 3, wherein said immunoassay is a radioimmunoassay.
5. A method of diagnosing a colon cell proliferative disorder in a subject, comprising the steps of:
 - a) obtaining one or more test samples from colon tissue or serum or both of said subject; and
 - b) contacting said sample with an antibody immunoreactive with the EYA4 polypeptide to form an immunocomplex;
 - c) detecting said immunocomplex;
 - d) comparing the quantity of said immunocomplex to the quantity of immunocomplex formed under identical conditions with the same antibody and a control sample from one or more subjects known not to have colon cancer; wherein a decrease in quantity of said immunocomplex in the sample from said subject relative to said control sample is indicative of colon cancer.
6. The method of claim 5, wherein said immunocomplex is detected in a Western blot assay.

7. The method of claim 5, wherein said immunocomplex is detected in an ELISA.
8. The method of claim 1, wherein said detection is by expression analysis.
9. The method of claim 8, comprising detecting the presence or absence of mRNA encoding a EYA4 polypeptide in a sample from a patient, wherein a decreased concentration of said mRNA above the concentration for a healthy individual indicates the presence of colon cell proliferative disorder cells.
10. The method of claim 8, comprising the steps of:
 - (a) providing a polynucleotide probe which specifically hybridises or is identical to a polynucleotide consisting of SEQ ID NO: 1,
 - (b) incubating said sample with said polynucleotide probe under high stringency conditions to form a specific hybridisation complex between an mRNA and said probe;
 - (c) detecting said hybridisation complex.
11. The method according to claim 10 wherein the detecting step further comprises the steps of:
 - a) producing a cDNA from mRNA in the sample;
 - b) providing two oligonucleotides which specifically hybridise to regions flanking a segment of the cDNA;
 - c) performing a polymerase chain reaction on the cDNA of step a) using the oligonucleotides of step b) as primers to amplify the cDNA segment; and
 - d) detecting the amplified cDNA segment.
12. A method for repressing transformation in a colon cell, the method comprising contacting said cell with a EYA4 polypeptide in an amount effective to inhibit a transformed phenotype.
13. The method of claim 12, wherein said EYA4 polypeptide is introduced into said cell by the direct introduction of said EYA4 polypeptide.
14. The method of claim 12, wherein said EYA4 polypeptide is introduced into the cell through the introduction of a EYA4-encoding polynucleotide.

15. The method of claim 14, wherein said EYA4-encoding polynucleotide further comprises control sequences operatively linked to said EYA4 encoding polynucleotide.
16. The method of claim 14, wherein said EYA4-encoding polynucleotide is present in a vector.
17. Use of the vector according to claim 16 for therapy of colon cell proliferative disorders.
18. A method for preventing or treating a colon cell proliferative disorder in a subject which comprises administering to the subject a therapeutically effective amount of a compound that agonises EYA4.
19. Use of a polypeptide expressed from the EYA4 gene for detecting, differentiating or distinguishing between colon cell proliferative disorders.
20. Use of a polypeptide expressed from the EYA4 gene for therapy of colon cell proliferative disorders.
21. The method of claim 8, wherein said detection comprises methylation analysis of the gene EYA 4, its promoter and/or regulatory elements, in particular through the methylation analysis of a genomic DNA sequence according to SEQ ID NO: 1.
22. A nucleic acid comprising a sequence at least 18 bases in length of a segment of the chemically pretreated genomic DNA according to one of the sequences taken from the group comprising SEQ ID NO: 2 to SEQ ID NO: 5 and sequences complementary thereto.
23. An oligomer, in particular an oligonucleotide or peptide nucleic acid (PNA)-oligomer, said oligomer comprising in each case at least one base sequence having a length of at least 9 nucleotides which is complementary to, or hybridises under moderately stringent or stringent conditions to a pretreated genomic DNA according to one of the SEQ ID NO: 2 to SEQ ID NO: 5 and sequences complementary thereto.
24. The oligomer as recited in Claim 23; wherein the base sequence includes at least one

CpG, TpG or CpA dinucleotide.

25. The oligomer as recited in Claim 24; characterised in that the cytosine of the CpG dinucleotide is located approximately in the middle third of the oligomer.
26. A set of oligomers, comprising at least two oligomers according to any of claims 23 to 25.
27. A set of at least two oligonucleotides as recited in Claims 23 to 26, which can be used as primer oligonucleotides for the amplification of DNA sequences of one of SEQ ID NO: 2 to SEQ ID NO: 5 and sequences complementary thereto.
28. A set of oligonucleotides as recited in Claim 23, characterised in that at least one oligonucleotide is bound to a solid phase.
29. Use of a set of oligomer probes comprising at least ten of the oligomers according to any of claims 24 to 28 for detecting the cytosine methylation state and/or single nucleotide polymorphisms (SNPs) within one of the sequences according to SEQ ID NO: 1, and sequences complementary thereto.
30. A method for manufacturing an arrangement of different oligomers (array) fixed to a carrier material for analysing diseases associated with the methylation state of the CpG dinucleotides of one of SEQ ID NO: 1, and sequences complementary thereto wherein at least one oligomer according to any of the claims 23 through 26 and 28 is coupled to a solid phase.
31. An arrangement of different oligomers (array) obtainable according to claim 30.
32. An array of different oligonucleotide- and/or PNA-oligomer sequences as recited in Claim 31, characterised in that these are arranged on a plane solid phase in the form of a rectangular or hexagonal lattice.
33. The array as recited in any of the Claims 31 or 32, characterised in that the solid phase surface is composed of silicon, glass, polystyrene, aluminium, steel, iron, copper, nickel, silver, or gold.

34. A composition of matter comprising the following:

- A nucleic acid comprising a sequence at least 18 bases in length of a segment of the chemically pretreated genomic DNA according to one of the sequences taken from the group comprising SEQ ID NO: 1 to SEQ ID NO: 5 and sequences complementary thereto, and
- A buffer comprising at least one of the following substances: 1 to 5 mM Magnesium Chloride, 100-500 μ M dNTP, 0.5-5 units of taq polymerase, an oligomer, in particular an oligonucleotide or peptide nucleic acid (PNA)-oligomer, said oligomer comprising in each case at least one base sequence having a length of at least 9 nucleotides which is complementary to, or hybridises under moderately stringent or stringent conditions to a pretreated genomic DNA according to one of the SEQ ID NO: 2 to SEQ ID NO: 5 and sequences complementary thereto.

35. Use of the gene EYA 4, its promoter and/or regulatory elements for detecting, differentiating or distinguishing between colon cell proliferative disorders.

36. A method for detecting, differentiating or distinguishing between colon cell proliferative disorders according to claim 21, comprising:

- a) obtaining, from a subject, a biological sample having subject genomic DNA;
- b) treating the genomic DNA, or a fragment thereof, with one or more reagents to convert 5-position unmethylated cytosine bases to uracil or to another base that is detectably dissimilar to cytosine in terms of hybridisation properties;
- c) contacting the treated genomic DNA, or the treated fragment thereof, with an amplification enzyme and at least two primers comprising, in each case a contiguous sequence at least 18 nucleotides in length that is complementary to, or hybridises under moderately stringent or stringent conditions to a sequence selected from the group consisting of SEQ ID NOS: 2 to 5, and complements thereof, wherein the treated DNA or a fragment thereof is either amplified to produce one or more amplicates, or is not amplified; and
- d) determining, based on the presence or absence of, or on a property of said amplicate, the methylation state of at least one CpG dinucleotide sequence of SEQ ID NO: 1, or an average, or a value reflecting an average methylation state of a plurality of CpG dinucleotide sequences of SEQ ID NO: 1.

37. A method for detecting, differentiating or distinguishing between colon cell proliferative disorders according to claim 21, comprising the following steps of
- a) obtaining, from a subject, a biological sample having subject genomic DNA;
 - b) treating the genomic DNA, or a fragment thereof, with one or more reagents to convert 5-position unmethylated cytosine bases to uracil or to another base that is detectably dissimilar to cytosine in terms of hybridisation properties;
 - c) amplifying one or more fragments of the treated DNA such that only DNA originating from colon or colon cell proliferative disorder cells are amplified
 - d) detecting the amplicates or characteristics thereof and thereby deducing on the presence or absence of a colon cell proliferative disorder.
38. The method of one of claims 36 or 37, wherein in step a) the biological sample obtained from the subject is selected from the group consisting of histological slides, biopsies, paraffin-embedded tissue, bodily fluids, serum, plasma, stool, urine, blood, and combinations thereof.
39. The method of one of claims 36 to 38, wherein in step b) treating the genomic DNA, or the fragment thereof, comprises use of a solution selected from the group consisting of bisulfite, hydrogen sulfite, disulfite, and combinations thereof.
40. The method of one of claims 36 to 39, wherein treating in b) is subsequent to embedding the DNA in agarose.
41. The method of one of claims 36 to 40, where treating in b) comprises treating in the presence of at least one of a DNA denaturing reagent or a radical trap reagent.
42. The method of one of claims 36 to 41, wherein contacting or amplifying in step c) comprises use of at least one method selected from the group consisting of: use of a heat-resistant DNA polymerase as the amplification enzyme; use of a polymerase lacking 5'-3' exonuclease activity; use of a polymerase chain reaction (PCR); generation of a amplicate nucleic acid molecule carrying a detectable labels; and combinations thereof.

43. The method of claim 42, wherein the detectable amplificate label is selected from the label group consisting of: fluorescent labels; radionuclides or radiolabels; amplificate mass labels detectable in a mass spectrometer; detachable amplificate fragment mass labels detectable in a mass spectrometer; amplificate, and detachable amplificate fragment mass labels having a single-positive or single-negative net charge detectable in a mass spectrometer; and combinations thereof.
44. The method of claim 43, comprising in step d) use of mass spectrometry for detecting the amplificate, or detachable amplificate fragment mass labels.
45. The method of claim 44, wherein the mass spectrometry technique is selected from the group consisting of matrix assisted laser desorption/ionisation mass spectrometry (MALDI), electron spray mass spectrometry (ESI), and combinations thereof.
46. The method according to one of claims 36 to 45 wherein in step c) of the method 6 or more different fragments are amplified.
47. The method according to one of claims 36 to 46 wherein said amplificates are between 100 and 200 base pairs in length.
48. The method according to one of claims 36 to 47 wherein said amplificates are between 100 and 350 base pairs in length.
49. The method according to one of claims 36 to 48 wherein one or more of said primers comprise sequences taken from the group according to SEQ ID NO: 11 to SEQ ID NO: 15.
50. The method according to one of claims 36 to 48 wherein one or more of said primers comprise one or more CpG, TpG or CpA dinucleotides.
51. The method of claim 50 wherein said primers comprise between two to four CpG, TpG or CpA dinucleotides.
52. The method according to one of claims 50 or 51 wherein said one or more CpG, TpG or

CpA dinucleotides are located within the 3' half of the primer.

53. The method according to one of claims 50 to 52 wherein said primers comprise one or more bases which hybridise to positions that were converted in the treatment of step b).
54. The method of claim 53 wherein said bases are located within the 3' half of the primer.
55. The method according to one of claims 50 to 54 wherein said primers do not comprise more than 2 cytosine or guanine bases within the first 5 bases at the 3' end.
56. The method according to one of claims 36 to 55 wherein said amplicates obtained in step d) comprise at least one 20 base pair sequence that comprises 3 or more CpG, TpG or CpA dinucleotides.
57. The method according to one of claims 36 to 56, further comprising in step c) the use of at least one nucleic acid molecule or peptide nucleic acid molecule at least 18 base pairs in length comprising one or more CpG, TpG or CpA dinucleotides and wherein the sequence of said molecule is complementary or identical to a sequence selected from the group consisting of SEQ ID NOS: 2 to 5, and complements thereof, and wherein said nucleic acid molecule or peptide nucleic acid molecule suppresses amplification of the nucleic acid to which it is hybridised.
58. The method according to claim 57 wherein the sequence of said nucleic acid(s) or peptide nucleic acid(s) is selected from the group consisting SEQ ID NO: 6 to SEQ ID NO: 10, and sequences complementary thereto.
59. The method of claim 57, wherein amplification of DNA that was unmethylated prior to treatment of step b) is suppressed.
60. The method of one of claims 57 to 59, wherein said nucleic acid molecule or peptide nucleic acid molecule is in each case modified at the 5'-end thereof to preclude degradation by an enzyme having a 5'-3' exonuclease activity.
61. The method of one of claims 57 to 60, wherein said nucleic acid molecule or peptide

nucleic acid molecule in each case lack a 3' hydroxyl group.

62. The method of one of claims 57 to 61, wherein the amplification enzyme is a polymerase lacking 5'-3' exonuclease activity.
63. The method of one of claims 57 to 62, wherein the binding site of the oligonucleotide or PNA oligomer is identical to, or overlaps with that of the primer and thereby hinders hybridisation of the primer to its binding site.
64. The method of one of claims 57 to 63, wherein the binding sites of at least two of the oligonucleotides or PNA oligomers are identical to, or overlap with those of at least two of the primers, and thereby hinder hybridisation of the primers to their binding site.
65. The method of claim 64, wherein hybridisation of at least one of the oligonucleotides or peptide nucleic acid oligomers hinders hybridisation of a forward primer, and the hybridisation of at least one of the oligonucleotides or peptide nucleic acid oligomers hinders the hybridisation of a reverse primer that binds to the elongation product of said forward primer.
66. The method of one of claims 57 to 64, wherein said oligonucleotide or peptide nucleic acid oligomer hybridises between the binding sites of the forward and reverse primers.
67. The method of one of claims 36 or 37, wherein determining in step d), comprises hybridisation of at least one nucleic acid molecule or peptide nucleic acid (PNA) molecule in each case comprising a contiguous sequence at least 9 nucleotides in length comprising one or more CpG, TpG or CpA dinucleotides and wherein the sequence of said molecule that is complementary or identical to a sequence selected from the group consisting of SEQ ID NOS: 2 to 5, and complements thereof.
68. The method of claim 67, wherein at least one such hybridising nucleic acid molecule or peptide nucleic acid molecule is bound to a solid phase.
69. The method of claim 68, wherein a plurality of such hybridising nucleic acid molecules or peptide nucleic acid molecules are bound to a solid phase in the form of a nucleic acid or

peptide nucleic acid array selected from the array group consisting of linear, hexagonal, rectangular, and combinations thereof.

70. The method of one of claims 36 or 37, wherein determining in step d), comprises sequencing of the amplificate.
71. The method of one of claims 36 or 37, wherein determining in step d), comprises: hybridising at least one nucleic acid molecule comprising a contiguous sequence at least 9 nucleotides in length that is complementary to, or hybridises under moderately stringent or stringent conditions to a sequence selected from the group consisting of SEQ ID NOS: 2 to 5, and complements thereof; and extending at least one such hybridised nucleic acid molecule by at least one nucleotide base.
72. The method according to claim 71 wherein the sequence of said nucleic acid(s) or peptide nucleic acid(s) is selected from the group consisting SEQ ID NO: 11 to SEQ ID NO: 15, and sequences complementary thereto.
73. The method according to claim 67 wherein said oligonucleotides or PNA-oligomers are fluorescently labelled, and wherein detection thereof is by either an increase or a decrease in fluorescence or fluorescence polarisation.
74. The method according to claim 73 wherein the hybridisation of the oligonucleotides or PNA oligomers is detectable by fluorescence resonance energy transfer, and wherein the detection is by either an increase or a decrease in fluorescence.
75. The method of one of claims 36 or 37, wherein the background DNA concentration is at between 100 to 1000 fold excess of the concentration of the DNA to be investigated.
76. A method for detecting a colon cell proliferative disorder according to claim 21, comprising:
- a) obtaining, from a subject, a biological sample having subject genomic DNA;
 - b) extracting the genomic DNA;
 - c) contacting the genomic DNA, or a fragment thereof, comprising SEQ ID NO:1 or a sequence that hybridises under stringent conditions to SEQ ID NO:1, with one or more

methylation-sensitive restriction enzymes, wherein the genomic DNA is either digested thereby to produce digestion fragments, or is not digested thereby; and

d) determining, based on a presence or absence of, or on property of at least one such fragment, the methylation state of at least one CpG dinucleotide sequence of SEQ ID NO: 1, or an average, or a value reflecting an average methylation state of a plurality of CpG dinucleotide sequences of SEQ ID NO: 1, whereby at least one of detecting the prostate cell proliferative disorder, or distinguishing between a transitional and a peripheral zone of origin of the prostate cell proliferative disorder is, at least in part, afforded.

77. The method of claim 76, further comprising, prior to determining in step d), amplifying of the digested or undigested genomic DNA.
78. The method of claim 77, wherein amplifying comprises use of at least one method selected from the group consisting of: use of a heat resistant DNA polymerase as an amplification enzyme; generation of a amplificate nucleic acid carrying a detectable label; and combinations thereof.
79. The method of claim 78, wherein the detectable amplificate label is selected from the label group consisting of: fluorescent labels; radionuclides or radiolabels; amplificate mass labels detectable in a mass spectrometer; detachable amplificate fragment mass labels detectable in a mass spectrometer; amplificate, and detachable amplificate fragment mass labels having a single-positive or single-negative net charge detectable in a mass spectrometer; and combinations thereof.
80. The method of claim 79, comprising use of mass spectrometry for detecting amplificate, or detachable amplificate fragment mass labels.
81. The method of claim 80, wherein the mass spectrometry is selected from the group consisting of matrix assisted laser desorption/ionisation mass spectrometry (MALDI), electron spray mass spectrometry (ESI), and combinations thereof.
82. The method of claim 76, wherein the biological sample obtained from the subject is selected from the group consisting of histological slides, biopsies, paraffin-embedded

tissue, bodily fluids, urine, serum, plasma, stool, blood, and combinations thereof.

83. A kit useful for detecting, differentiating or distinguishing between colon cell proliferative disorders, comprising:
- a) a bisulfite reagent; and
 - b) at least one nucleic acid molecule or peptide nucleic acid molecule comprising, in each case a contiguous sequence at least 9 nucleotides in length that is complementary to, or hybridises under moderately stringent or stringent conditions to a sequence selected from the group consisting of SEQ ID NOS: 1 to 5, and complements thereof.
84. The kit of claim 83, further comprising standard reagents for performing a methylation assay selected from the group consisting of MS-SNuPE, MSP, MethyLight™, HeavyMethyl™, COBRA, nucleic acid sequencing, and combinations thereof.
85. The use of a nucleic acid according to Claim 22, of an oligonucleotide or PNA-oligomer according to one of the Claims 23 to 28, of a kit according to Claims 83 or 84, of an array according to one of the Claims 32 to 33, of a set of oligonucleotides according to one of claims 26 to 28, or a method according to claims 36 to 82, for the classification, differentiation and/or diagnosis of colon cell proliferative disorders or the predisposition to colon cell proliferative disorders.
86. The use of a nucleic acid according to Claim 22, of an oligonucleotide or PNA-oligomer according to one of the Claims 23 to 28, of a kit according to Claims 83 or 84, of an array according to one of the Claims 32 to 33, of a set of oligonucleotides according to one of claims 26 to 28, or a method according to claims 36 to 82, for the therapy of colon cell proliferative disorders.

FIGURE 2

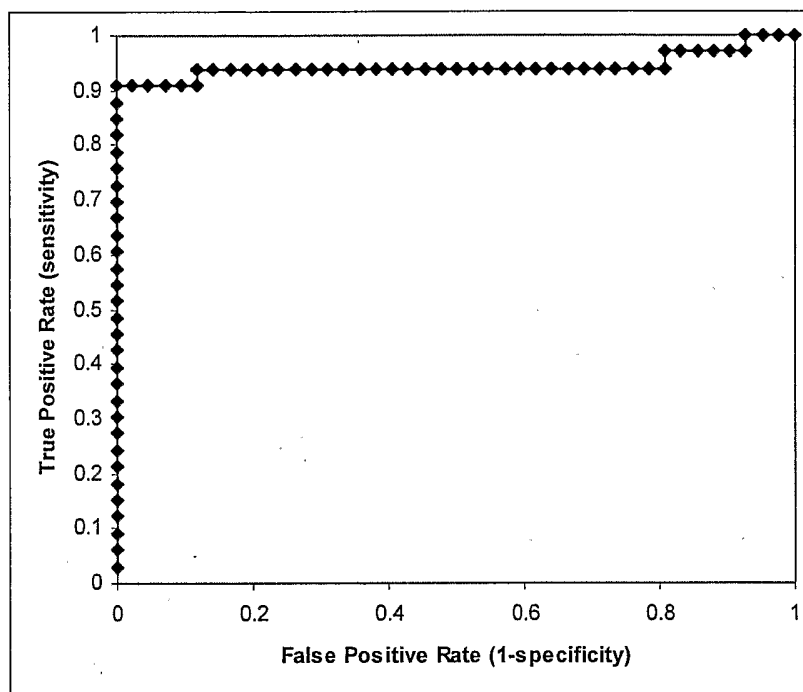


FIGURE 3

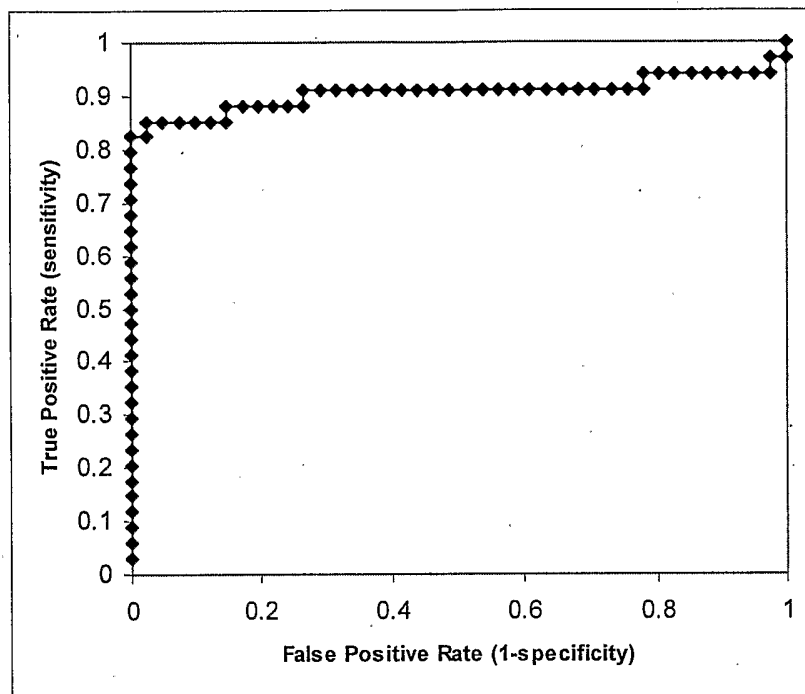


FIGURE 4

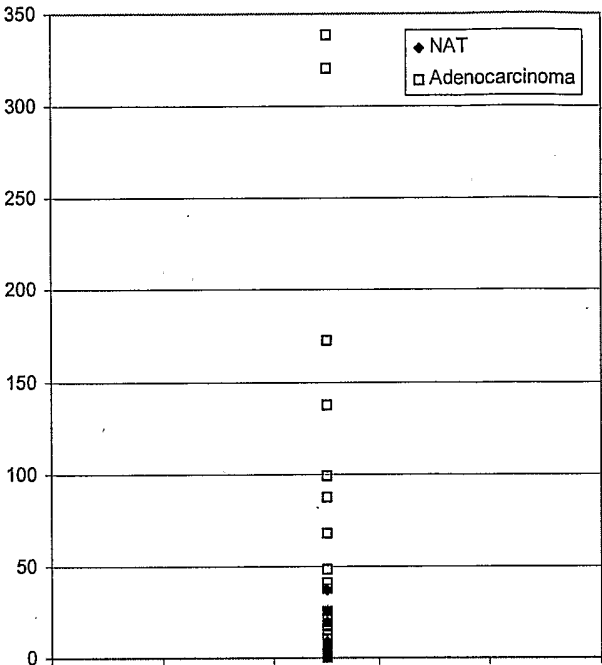


FIGURE 5

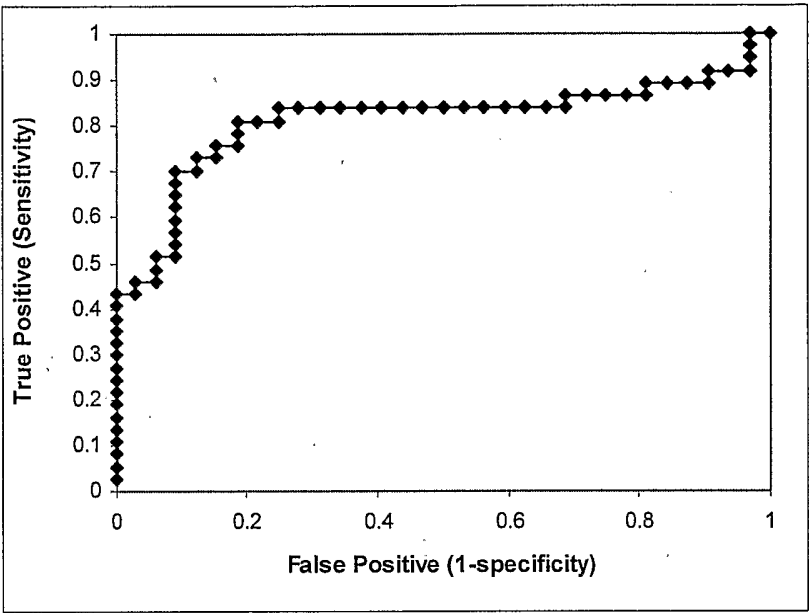
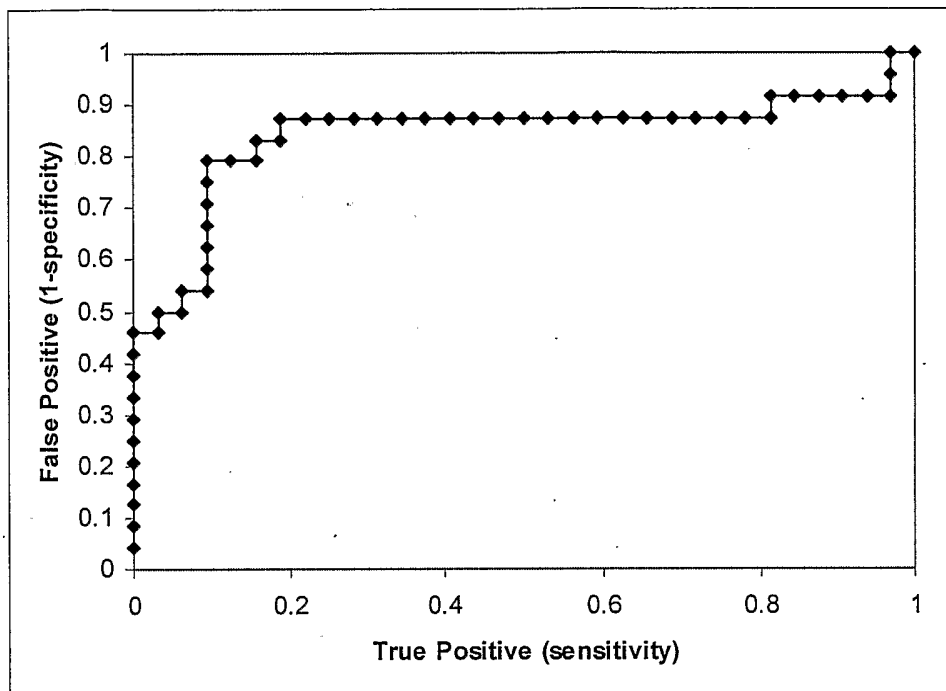


FIGURE 6



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FIGURE 7

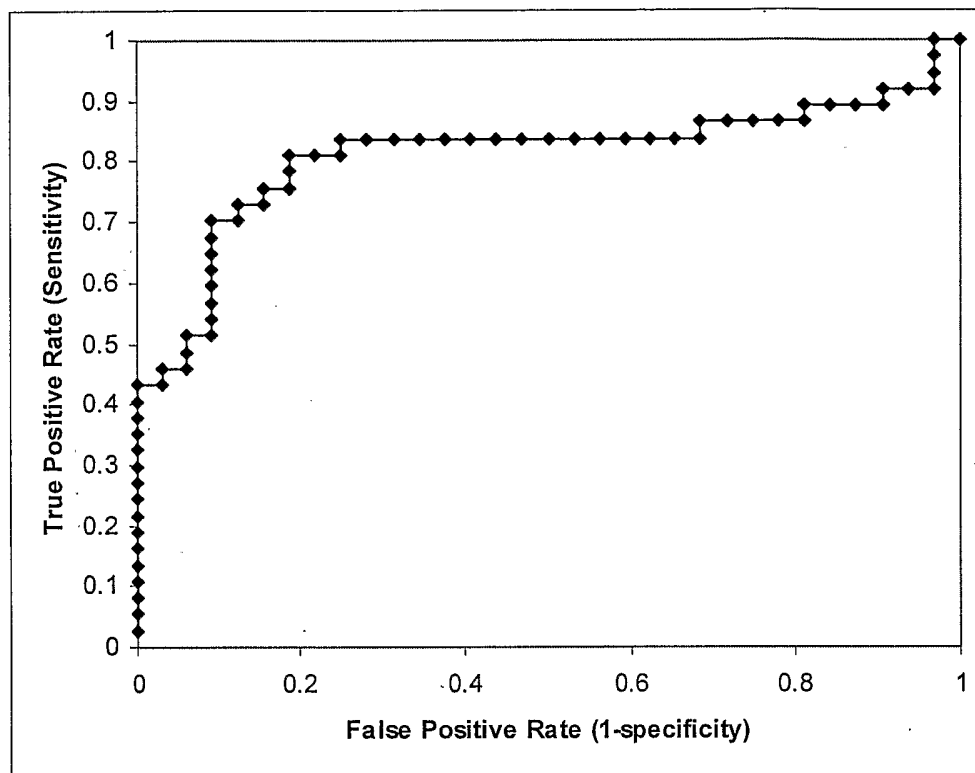


FIGURE 8

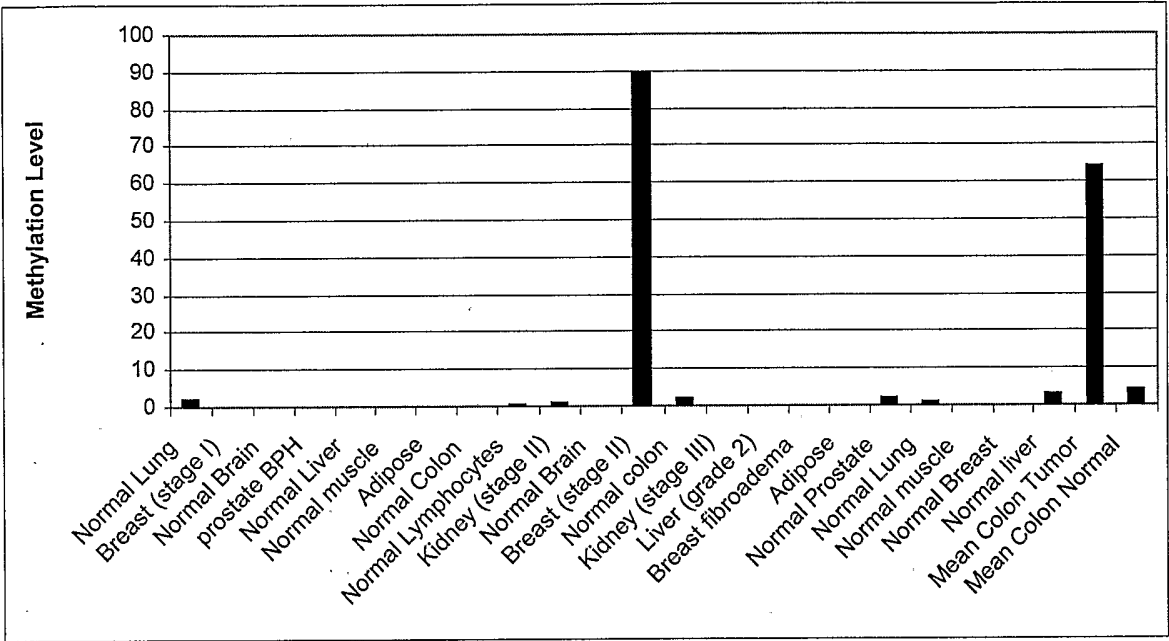
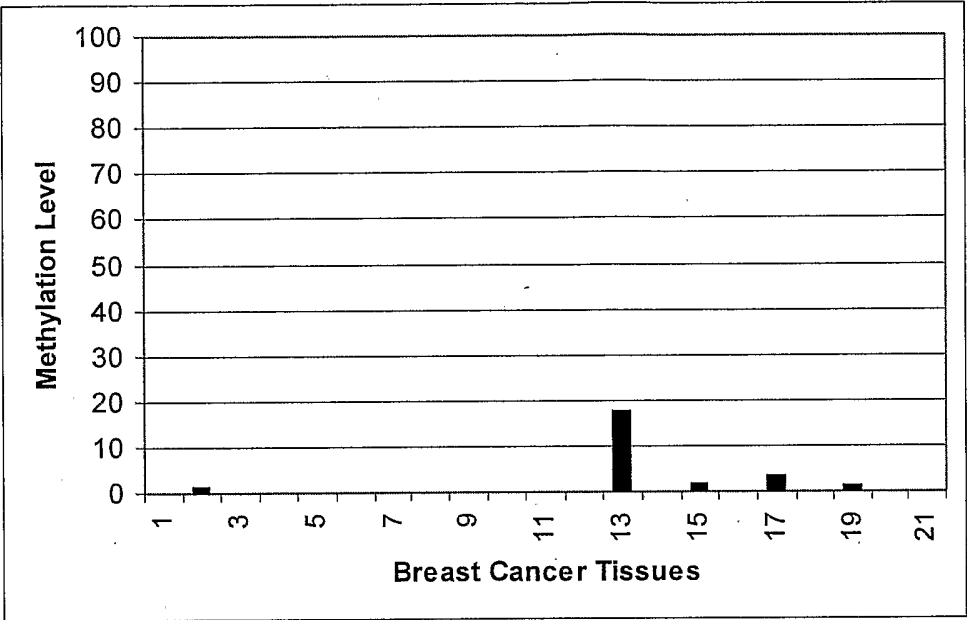


FIGURE 9



Sequence listing

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<213> Homo Sapiens

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