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(71) Applicant *(for all designated States except US)*: **EPIGENOMICS AG** [DE/DE]; Kastanienallee 24, 10435 Berlin (DE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **OLEK, Alexander** [DE/DE]; Schröderstrasse 13/2, 10115 Berlin (DE). **PIEPENBROCK, Christian** [DE/DE]; Schwartzkopffstrasse 7b, 10115 Berlin (DE). **BERLIN, Kurt** [DE/DE]; Marienkäferweg 4, 14532 Stahnsdorf (DE).

(74) Agents: **SCHOHE, Stefan** et al.; Boehmert & Boehmert, Franz-Joseph-Strasse 38, 80801 München (DE).

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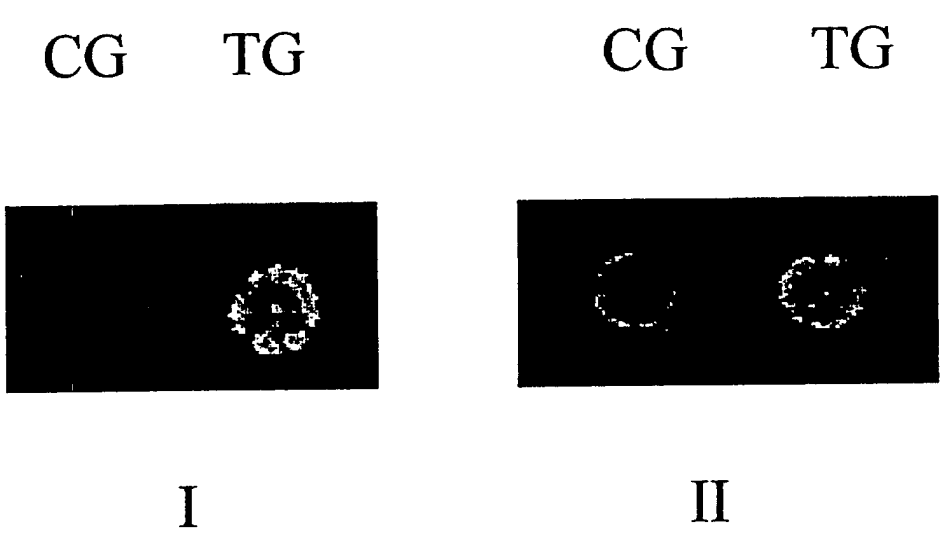
(54) Title: DIAGNOSIS OF DISEASES ASSOCIATED WITH THE CELL CYCLE



(57) Abstract: The present invention relates to the chemically modified genomic sequences of genes associated with cell cycle, to oligonucleotides and/or PNA-oligomers for detecting the cytosine methylation state of genes associated with cell cycle which are directed against the sequence, as well as to a method for ascertaining genetic and/or epigenetic parameters of genes associated with cell cycle.

WO 01/68911 A2

Diagnosis of Diseases Associated with the cell cycle

Field of the Invention

The levels of observation that have been well studied by the methodological developments of recent years in molecular biology, are the genes themselves, the translation of these genes into RNA, and the resulting proteins. The question of which gene is switched on at which point in the course of the development of an individual, and how the activation and inhibition of specific genes in specific cells and tissues are controlled is correlatable to the degree and character of the methylation of the genes or of the genome. In this respect, pathogenic conditions may manifest themselves in a changed methylation pattern of individual genes or of the genome.

The present invention relates to nucleic acids, oligonucleotides, PNA-oligomers and to a method for the diagnosis and/or therapy of diseases which have a connection with the genetic and/or epigenetic parameters of genes associated with cell cycle and, in particular, with the methylation status thereof.

Prior Art

The cell cycle is the series of events between each meitotic division, culminating in the division of a cell into two daughter cells. The cycle consists of 4 discrete phases, the G1, S, G2 and M phases. Nuclear and cytoplasmic division occur during the M (mitotic) phase and DNA replication takes place during the S phase. The period between the end of the M phase and the start of the S phase is termed G1, and the phase between the end of the S phase and the start of the M phase is termed G2.

The regulation of these phases and the progression from one phase to the next is a key factor in the regulation of ordered cell division. The unidirectional progression from one phase to the next is controlled at various biochemical 'checkpoints'. These 'checkpoints', or biochemical pathways are of considerable interest, as alterations within them may result in decreased fidelity of events associated with cell cycle, such as chromosome duplication and segregation.

In mammalian cells G1 represents the most important checkpoint. At this point, the cell commits itself to DNA replication and regulates proliferation. Other checkpoints are present during the S phase when the integrity of the replicated chromosomes is checked and G2 when commitment to meitotic division is made. If the cell does not divide at this point, it remains in the condition of having twice the normal complement of chromosomes.

The regulatory system consists of a series of enzymes that respond to external signals and checkpoints by phosphorylating or dephosphorylating the next member of the pathway. Phosphorylation is catalysed by cyclin dependant kinases. These holoenzymes consist of two protein subunits;a regulatory subunit (the cyclin), and an associated catalytic subunit (the cyclin-dependent kinase or CDK). They are subject to many levels of regulation, and are used both to control the activities of the regulatory circuit itself and to control the activities of the substrates that execute the decisions of the regulatory circuit. In general, different types of cyclins are designated by letters (e.g., cyclin A, cyclin B, etc.) and CDKs are distinguished by numbers (CDK1, CDK2, etc.;). The role of cell cycle regulators has been reviewed by Stephen Elledge, "J.Cell Cycle Checkpoints: Preventing an Identity Crisis" Science, 274; 1664-1672 (1996).

Misregulation of the the cell cycle can lead to proliferation, and cell cycle regulators have been implicated in a number of diseases, in particular tumor formation. Examples of diseases involving disruptions in cell cycle pathways include:

1. HIV infection;Zhou and Ratner "Phosphorylation of Human Immunodeficiency Virus Type 1 Vpr Regulates Cell Cycle Arrest" Journal of Virology, Vol. 74; 6520-6527, (2000).

2. Alzheimers; Bruckner et al. "Aberrancies in signal transduction and cell cycle related events in Alzheimer's disease." J Neural Transm Suppl;54:147-58 (1998)

3. Graft-versus-host disease; Boussiotis *et. al.* "Altered T-cell receptor + CD28-mediated signaling and blocked cell cycle progression in interleukin 10 and transforming growth factor-beta-treated alloreactive T cells that do not induce graft-versus-host disease." Blood. ;97(2):565-571 (2001)

4. Aging; Zhu *et. al.* "Neuronal CDK7 in hippocampus is related to aging and alzheimer disease" Neurobiol Aging. ;21(6):807-13 (2000)

5. Glomerular disease; Shankland *et. al.* "Differential expression of cyclin-dependent kinase inhibitors in human glomerular disease: role in podocyte proliferation and maturation." Kidney Int.;58(2):674-83 (2000)

6. Lewy body disease; Takahashi *et. al.* "Cyclin-dependent kinase 5 (Cdk5) associated with Lewy bodies in diffuse Lewy body disease." Brain Res. 17;862(1-2):253-6 (2000)

7. Arthirits; Kohsaka *et. al.* "Treatment of arthritis with cyclin-dependent kinase inhibitor p 16INK4a gene" Nihon Rinsho Meneki Gakkai Kaishi. ;22(6):397-9 (1999)

8. Neurodegenerative disorders; Patrick *et. al.* "Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration" Nature 9;402(6762):615-22 (1999)

9. Arterosclerosis; Ihling et. al. "Co-expression of p53 and MDM2 in human atherosclerosis: implications for the regulation of cellularity of atherosclerotic lesions." J Pathol;185(3):303-12 (1998)

10. Ataxia telangiectasia; Matsuoka *et. al.* "Linkage of ATM to Cell Cycle Regulation by the Chk2 Protein Kinase" Science, Vol. 282; 1893-1897 (1998).

11. Bladder cancer; Del Pizzo *et al.* "Loss of Cell Cycle Regulators p27Kip1 and Cyclin E in Transitional Cell Carcinoma of the Bladder Correlates with Tumor Grade and Patient Survival" American Journal of Pathology,Vol. 155;1129-1136 (1999).

12. Leukemia; Vrhovac *et al.* "Prognostic Significance of the Cell Cycle Inhibitor p27Kip1 in Chronic B-Cell Lymphocytic Leukemia" Blood Vol. 91; 4694-4700 (1998).

13. Colorectal cancer; Thomas G.V. "Down-Regulation of p27 Is Associated with Development of Colorectal Adenocarcinoma Metastases" American Journal of Pathology;153; 681-687 (1998).

14. Gastric cancer; Takani Y "Cyclin D2 Overexpression and Lack of p27 Correlate Positively and Cyclin E Inversely with a Poor Prognosis in Gastric Cancer Cases" American Journal of Pathology;156; 585-594 (2000).

15. Liver cancer; Albrecht J "Regulation of G1 cyclin-dependent kinases in the liver: role of nuclear localisation and p27 sequestration" Vol. 277;1207-G1216, (1999).

16. Lung cancer; Nabeyrat E "Retinoic acid-induced proliferation of lung alveolar epithelial cells is linked to p21CIP1 downregulation" Vol. 278;42-L50, (2000).

17. Prostate cancer; De Marzo A "Expression of the Cell Cycle Inhibitor p27Kip1 in, normal, Hyperplastic, and Neoplastic Cells" American Journal of Pathology;153;911-919 (1998).

The methylation of DNA is a necessary factor in the correct regulation of gene expression. For example, the p16 protein halts cell cycle progression at the G1/S boundary, and the loss of p16 function may lead to cancer progression by allowing unregulated cellular proliferation. Hypermethylation mediated inactivation of the p16 gene has been demonstrated in brain, breast, colon, head and neck, and non-small-cell lung cancer and in high grade non-Hodgkin's lymphoma. Other studies establishing a link between methylation and gene regualtion include, Jackson *et. al.* "Loss of genomic methylation causes p53 dependant apoptosis and epigenetic deregulation" Nature Genetics 27;31-39 (2001), which showed aberrant expression patterns of several key cell cycle genes.

The identification of the methylation dependant regulation of cell cycle genes opens up the possibility of developing alternative methods of cancer treatment and diagnosis. Treatment with DNA methylation inhibitors has been shown to restore gene expression of the key cell cycle gene p16, Bender *et. al.* "Inhibition of DNA methylation by 5-aza-2'-deoxycytidine suppresses the growth of human tumor cell lines." Cancer research 58; 95-101 (1998). This

resulted in heritable levels of gene expression leading to suppression of growth in tumor cell lines.

Methylation based therapies could have considerable advantages over current methods of treatment, such as chemotherapy, surgery and radiotherapy. They may even provide a means of treating tumors which are resistant to conventional methods of therapy, as demonstrated by Soengas *et al* "Inactivation of the apoptosis effector Apaf-1 in malignant melanoma" Nature 409; 207-211(2001). In addition to the development of methylation specific therapies, experiments with *Min* mice have shown that inhibition of DNA methylation can suppress tumor initiation, Laird *et. al.* 'Suppression of intestinal neoplasia by DNA hypomethylation' Cell 81; 197-205 (1995). Furthermore, DNA methylation analysis may provide novel means for cancer diagnosis.

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 behavior 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 analyzing 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 behavior. 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 hybridization behavior, can now be detected as the only remaining cytosine using "normal" molecular biological techniques, for example, by amplification and hybridization 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 analyzed 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 analyze 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 analyzed, a global analysis of cells for thousands of possible methylation events is not possible. However, this method cannot reliably analyze 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 (Gonzalgo 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 Application 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 hybridization 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, WO 95 15373 and WO 45560.

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 labeled probes are often used for the scanning of immobilized 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 hybridized 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 vapor phase in an unfragmented manner. The analyte is ionized 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 ionization 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 crystallization. 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 Fritsch and Maniatis eds., Molecular Cloning: A Laboratory Manual, 1989.

Object of the invention

In view of the above, it is the object of the present invention to provide the chemically modified DNA of genes associated with the cell cycle, as well as oligonucleotides and/or PNA-oligomers for detecting cytosine methylations, as well as a method which is particularly suitable for the diagnosis and/or therapy of genetic and epigenetic parameters of genes associated with the cell cycle. The present invention is based on the discovery that genetic and epigenetic parameters and, in particular, the cytosine methylation pattern of genes associated with cell cycle are particularly suitable for the diagnosis and/or therapy of diseases associated with the cell cycle.

This objective is achieved according to the present invention by providing a nucleic acid containing a sequence of at least 18 bases in length of the chemically pretreated DNA of genes associated with cell cycle according to one of Seq. ID No.1 through Seq. ID No.424 and sequences complementary thereto and/or of the chemically pretreated DNA of genes associated with cell cycle according to one of the sequences of the genes listed in table 1. In the

table, after the listed gene designations, the respective data bank numbers (accession numbers) are specified which define the appertaining gene sequences as unique. GenBank at the National Institute of Health was used as the underlying data bank at the Internet-address www.ncbi.nlm.nih.gov.

The chemically modified nucleic acid could heretofore not be connected with the ascertainment of genetic and epigenetic parameters.

The object of the present invention is further achieved by an oligonucleotide or oligomer for detecting the cytosine methylation state in chemically pretreated DNA, containing at least one base sequence having a length of at least 13 nucleotides which hybridizes to a chemically pretreated DNA of genes associated with the cell cycle according to Seq. ID No.1 through Seq. ID No.424 and sequences complementary thereto and/or of the chemically pretreated DNA of genes associated with cell cycle according to one of the sequences of the genes listed in table 1. The oligomer probes according to the present invention constitute important and effective tools which, for the first time, make it possible to ascertain the genetic and epigenetic parameters of genes associated with the cell cycle. The base sequence of the oligomers preferably contains at least one CpG dinucleotide. The probes may also exist in the form of a PNA (peptide nucleic acid) which has particularly preferred pairing properties. Particularly preferred are oligonucleotides according to the present invention in which the cytosine of the CpG dinucleotide is the 5^{th} - 9^{th} nucleotide from the 5'-end of the 13-mer; in the case of PNA-oligomers, it is preferred for the cytosine of the CpG dinucleotide to be the 4^{th} - 6^{th} nucleotide from the 5'-end of the 9-mer.

The oligomers according to the present invention are normally used in so called "sets" which contain at least one oligomer for each of the CpG dinucleotides of the sequences of Seq. ID No.1 through Seq. ID No.424 and sequences complementary thereto and/or of the chemically pretreated DNA of genes associated with cell cycle according to one of the sequences of the genes listed in table 1. Preferred is a set which contains at least one oligomer for each of the CpG dinucleotides from one of Seq. ID No.1 through Seq. ID No.424 and sequences complementary thereto and/or of the chemically pretreated DNA of genes associated with cell cycle according to one of the sequences of the genes listed in table 1.

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 through Seq. ID No.424 and sequences complementary thereto and/or of the chemically pretreated DNA of genes associated with cell cycle according to one of the sequences of the genes listed in table 1, or segments thereof.

In the case of the sets of oligonucleotides according to the present invention, it is preferred that at least one oligonucleotide is bound to a solid phase.

The present invention moreover relates to a set of at least 10 n (oligonucleotides and/or PNA-oligomers) used for detecting the cytosine methylation state in chemically pretreated genomic DNA (Seq. ID No.1 through Seq. ID No.424 and sequences complementary thereto and/or of the chemically pretreated DNA of genes associated with cell cycle according to one of the sequences of the genes listed in table 1). These probes enable diagnosis and/or therapy of genetic and epigenetic parameters of genes associated with the cell cycle. The set of oligomers may also be used for detecting single nucleotide polymorphisms (SNPs) in the chemically pretreated DNA of genes associated with the cell cycle according to one of Seq. ID No.1 through Seq. ID No.424 and sequences complementary thereto and/or of the chemically pretreated DNA of genes associated with cell cycle according to one of the sequences of the genes listed in table 1.

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 characterized 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, aluminum, 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 are possible as well.

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 diseases associated with the

cell cycle 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 diseases associated with the cell cycle which contains at least one nucleic acid according to the present invention. DNA chips are known, for example, from US Patent 5,837,832.

Moreover, a subject matter of the present invention is a kit which may be composed, for example, of a bisulfite-containing reagent, a set of primer oligonucleotides containing at least two oligonucleotides whose sequences in each case correspond or are complementary to an 18 base long segment of the base sequences specified in the appendix (Seq. ID No.1 through Seq. ID No.424 and sequences complementary thereto and/or of the chemically pretreated DNA of genes associated with cell cycle according to one of the sequences of the genes listed in table 1), oligonucleotides and/or PNA-oligomers as well as instructions for carrying out and evaluating the described method. However, a kit along the lines of the present invention can also contain only part of the aforementioned components.

The present invention also makes available a method for ascertaining genetic and/or epigenetic parameters of genes associated with the cycle cell by analyzing cytosine methylations and single nucleotide polymorphisms, including the following steps:

In the first step of the method, a genomic DNA sample is chemically 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 hybridization behavior. This will be understood as 'chemical pretreatment' hereinafter.

The genomic DNA to be analyzed is preferably obtained form usual sources of DNA such as cells or cell components, for example, cell lines, biopsies, blood, sputum, stool, urine, cerebral-spinal fluid, tissue embedded in paraffin such as tissue from eyes, intestine, kidney, brain, heart, prostate, lung, breast or liver, histologic object slides, or combinations thereof.

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 behavior.

Fragments of the chemically pretreated DNA are amplified, using sets of primer oligonucleotides according to the present invention, and a, preferably heat-stable polymerase. Because of statistical and practical considerations, preferably more than ten different fragments having a length of 100 - 2000 base pairs are amplified. The amplification of several DNA segments can be carried out simultaneously in one and the same reaction vessel. Usually, the amplification is carried out by means of a polymerase chain reaction (PCR).

In a preferred embodiment of the method, the set of primer oligonucleotides includes at least two olignonucleotides whose sequences are each reverse complementary or identical to an at least 18 base-pair long segment of the base sequences specified in the appendix (Seq. ID No.1 through Seq. ID No.424 and sequences complementary thereto and/or of the chemically pretreated DNA of genes associated with cell cycle according to one of the sequences of the genes listed in table 1). The primer oligonucleotides are preferably characterized in that they do not contain any CpG dinucleotides.

According to the present invention, it is preferred that at least one primer oligonucleotide is bonded to a solid phase during amplification. The different oligonucleotide and/or PNA-oligomer sequences can be arranged on a plane solid phase in the form of a rectangular or hexagonal lattice, the solid phase surface preferably being composed of silicon, glass, polystyrene, aluminum, steel, iron, copper, nickel, silver, or gold, it being possible for other materials such as nitrocellulose or plastics to be used as well.

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, it being preferred that the fragments that are produced have a single positive or negative net charge for better detectability in the mass spectrometer. The detection may be carried out and

visualized by means of matrix assisted laser desorption/ionization mass spectrometry (MALDI) or using electron spray mass spectrometry (ESI).

The amplificates obtained in the second step of the method are subsequently hybridized to an array or a set of oligonucleotides and/or PNA probes. In this context, the hybridization takes place in the manner described in the following. The set of probes used during the hybridization is preferably composed of at least 10 oligonucleotides or PNA-oligomers. In the process, the amplificates serve as probes which hybridize to oligonucleotides previously bonded to a solid phase. The non-hybridized fragments are subsequently removed. Said oligonucleotides contain at least one base sequence having a length of 13 nucleotides which is reverse complementary or identical to a segment of the base sequences specified in the appendix, the segment containing at least one CpG dinucleotide. The cytosine of the CpG dinucleotide is the 5^{th} to 9^{th} nucleotide from the 5'-end of the 13-mer. One oligonucleotide exists for each CpG dinucleotide. Said PNA-oligomers contain at least one base sequence having a length of 9 nucleotides which is reverse complementary or identical to a segment of the base sequences specified in the appendix, the segment containing at least one CpG dinucleotide. The cytosine of the CpG dinucleotide is the 4^{th} to 6^{th} nucleotide seen from the 5'-end of the 9-mer. One oligonucleotide exists for each CpG dinucleotide.

In the fourth step of the method, the non-hybridized amplificates are removed.

In the final step of the method, the hybridized amplificates are detected. In this context, it is preferred that labels attached to the amplificates are identifiable at each position of the solid phase at which an oligonucleotide sequence is located.

According to the present invention, it is preferred that the labels of the amplificates are fluorescence labels, radionuclides, or detachable molecule fragments having a typical mass which can be detected in a mass spectrometer. The mass spectrometer is preferred for the detection of the amplificates, fragments of the amplificates or of probes which are complementary to the amplificates, it being possible for the detection to be carried out and visualized by means of matrix assisted laser desorption/ionization mass spectrometry (MALDI) or using electron spray mass spectrometry (ESI).

The produced fragments may have a single positive or negative net charge for better detectability in the mass spectrometer. The aforementioned method is preferably used for ascertaining genetic and/or epigenetic parameters of genes associated with the cell cycle.

The oligomers according to the present invention or arrays thereof as well as a kit according to the present invention are intended to be used for the diagnosis and/or therapy of diseases associated with the cell cycle by analyzing methylation patterns of genes associated with the cell cycle. According to the present invention, the method is preferably used for the diagnosis and/or therapy of important genetic and/or epigenetic parameters within genes associated with cell cycle.

The method according to the present invention is used, for example, for the diagnosis and/or therapy of HIV infection, neurodegenerative disorders, graft-versus-host disease, aging, glomerular disease, Lewy body disease, arthirits, arterosclerosis, solid tumors and cancers.
The nucleic acids according to the present invention of Seq. ID No.1 through Seq. ID No.424 and sequences complementary thereto and/or of the chemically pretreated DNA of genes associated with cell cycle according to one of the sequences of the genes listed in table 1 can be used for the diagnosis and/or therapy of genetic and/or epigenetic parameters of genes associated with the cell cycle.

The present invention moreover relates to a method for manufacturing a diagnostic agent and/or therapeutic agent for the diagnosis and/or therapy of diseases associated with the cell cycle by analyzing methylation patterns of genes associated with the cell cycle, the diagnostic agent and/or therapeutic agent being characterized in that at least one nucleic acid according to the present invention is used for manufacturing it, possibly together with suitable additives and auxiliary agents.

A further subject matter of the present invention relates to a diagnostic agent and/or therapeutic agent for diseases associated with the cell cycle by analyzing methylation patterns of genes associated with the cell cycle, the diagnostic agent and/or therapeutic agent containing at least one nucleic acid according to the present invention, possibly together with suitable additives and auxiliary agents.

The present invention moreover relates to the diagnosis and/or prognosis of events which are disadvantageous to patients or individuals in which important genetic and/or epigenetic parameters within genes associated with the cell cycle 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.

In the context of the present invention the term "hybridization" is to be understood as a bond of an oligonucleotide to a completely complementary sequence along the lines of the Watson-Crick base pairings in the sample DNA, forming a duplex structure. To be understood by "stringent hybridization conditions" are those conditions in which a hybridization 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.

The term "functional variants" denotes all DNA sequences which are complementary to a DNA sequence, and which hybridize to the reference sequence under stringent conditions and have an activity similar to the corresponding polypeptide according to the present invention.

In the context of the present invention, "genetic parameters" are mutations and polymorphisms of genes associated with cell cycle 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).

In the context of the present invention, "epigenetic parameters" are, in particular, cytosine methylations and further chemical modifications of DNA bases of genes associated with the cell cycle and sequences further required for their regulation. Further epigenetic parameters include, for example, the acetylation of histones which, however, cannot be directly analyzed using the described method but which, in turn, correlates with the DNA methylation.

In the following, the present invention will be explained in greater detail on the basis of the sequences and examples and the accompanying figure without being limited thereto.

Figure 1

Figure 1 shows the hybridisation of fluorescent labelled amplificates to a surface bound oligonucleotide. Sample I being from healthy brain tissue and sample II being from pilocytic astrocytoma grade II (brain tumor) tissue. Flourescence at a spot shows hybridisation of the amplificate to the oligonucleotide. Hybridisation to a CG oligonucleotide denotes methylation at the cytosine position being analysed, hybridisation to a TG oligonucleotide denotes no methylation at the cytosine position being analysed. It can be seen that Sample I was unmethylated at position 156 of the amplificate whereas in comparison Sample II had a higher degree of methylation at the same position.

Sequence ID Nos. 1 to 424

Sequence ID Nos. 1 to 424 show sequences of the chemically pretreated genomic DNAs of different genes associated with cell cycle. In particular, sequences having odd sequence numbers (e.g., Seq. ID No. 1, 3, 5, ...) exhibit in each case sequences of the chemically pretreated genomic DNAs of different genes associated with cell cycle. Sequences having even sequence numbers (e.g., Seq. ID No. 2, 4, 6, ...) exhibit in each case the sequences of the chemically pretreated genomic DNAs of genes associated with cell cycle which are complementary to the preceeding sequences (e.g., the complementary sequence to Seq. ID No.1 is Seq. ID No.2, the complementary sequence to Seq. ID No.3 is Seq. ID No.4, etc.)

Seq. ID No. 425 to Seq. ID No. 428 show specific oligonucleotide sequences as used in Example 1.

The following example relates to a fragment of a gene associated with cell cycle, in this case, CDK4 in which a specific CG-position is analyzed for its methylation status.

Example 1:Methylation analysis in the gene CDK4 associated with the cell cycle.

The following example relates to a fragment of the gene cytosine dependant kinase 4 (CDK4) in which a specific CG-position is to be analyzed for methylation.

In the first step, a genomic sequence is treated using bisulfite (hydrogen sulfite, disulfite) in such a manner that all cytosines which are not methylated at the 5-position of the base are

modified in such a manner that a different base is substituted with regard to the base pairing behavior while the cytosines methylated at the 5-position remain unchanged.

If bisulfite solution is used for the reaction, then an addition takes place at the non-methylated cytosine bases. Moreover, a denaturating reagent or solvent as well as a radical interceptor is present. A subsequent alkaline hydrolysis then gives rise to the conversion of non-methylated cytosine nucleobases to uracil. Chemically converted DNA is then used for the detection of methylated cytosines. In the second method step, the treated DNA sample is diluted with water or an aqueous solution. Preferably, the DNA is subsequently desulfonated (10-30 min, 90-100 °C) at an alkaline pH value. In the third step of the method, the DNA sample is amplified in a polymerase chain reaction, preferably using a heat-resistant DNA polymerase. In the present case, cytosines of the gene CDK4 are analyzed. To this end, a defined fragment having a length of 474 bp is amplified with the specific primer oligonucleotides AAAAATAACACAATAACTCA (Seq. ID No. 425) and TTTTGGTAGTTGGTTATATG (Seq. ID No. 426). This amplificate serves as a sample which hybridizes to an oligonucleotide previously bonded to a solid phase, forming a duplex structure, for example GGGTTGGCGTGAGGTA (Seq. ID No. 427), the cytosine to be detected being located at position 179 of the amplificate. The detection of the hybridization product is based on Cy3 and Cy5 flourescently labeled primer oligonucleotides which have been used for the amplification. The hybridization reaction of the amplified DNA with the oligonucleotide takes place only if a methylated cytosine was present at this location in the bisulfite treated DNA. Thus, the methylation status of the specific cytosine to be analyzed may be inferred from the hybridization product.

In order to verify the methylation status of the position, a sample of the amplificate is further hybridized to another oligonucleotide previously bonded to a solid phase. Said oligonucleotide is identical to the oligonucleotide previously used to analyze the methylation status of the sample, with the exception of the position in question. At the position to be analysed said oligonucleotide comprises a thymine base as opposed to a cytosine base i.e. sequence GGGTTGGTGTGAGGTA (Seq. ID No. 428). Therefore, the hybridisation reaction only takes place if an unmethylated cytosine is present at the position to be analysed.

The analysis was carried out on two tissue samples, Sample 1 from healthy brain tissue, and Sample 2 from pilocytic astrocytoma (brain tumor) tissue. From the results (Figure 1) it can be seen that Sample 1 was unmethylated at position 156 of the amplificate whereas in comparison Sample 2 had a higher degree of methylation at the same position.

Example 2: Diagnosis of diseases associated with the cell cycle

In order to relate the methylation patterns to one of the diseases associated with the cell cycle, it is initially required to analyze the DNA methylation patterns of a group of diseased and of a group of healthy patients. These analyses are carried out, for example, analogously to example 1. The results obtained in this manner are stored in a database and the CpG dinucleotides which are methylated differently between the two groups are identified. This can be carried out by determining individual CpG methylation rates as can be done, for example, in a relatively imprecise manner, by sequencing or else, in a very precise manner, by a methylation-sensitive "primer extension reaction". It is also possible for the entire methylation status to be analyzed simultaneously, and for the patterns to be compared, for example, by clustering analyses which can be carried out, for example, by a computer.

Subsequently, it is possible to allocate the examined patients to a specific therapy group and to treat these patients selectively with an individualized therapy.

Example 2 can be carried out, for example, for the following diseases:
HIV infection, neurodegenerative disorders, graft-versus-host disease, aging, glomerular disease, Lewy body disease, arthirits, arterosclerosis, solid tumors and cancers.

Table 1

Listing of particularly preferred genes of the present invention associated with the cell cycle.

Gene	Database Entry No. (GenBank at www.ncbi.nlm.nih.gov)
CCNB1	(M25753)
CCNE1	(M73812); (M74093)
DENR	(O43583)
EPHA5	(L36644)
MCM4	(X74794)

NEK3	(Z29067)
PCTK3	(X66362)
PRKAR1B	(M65066)
PRKCG	(M13977); (Z15114)
PRKM3	(M84490); (Z11696)
PRKMK2	(L11285)
ZAP70	(L05148)
ACP1	(NM_004300)
AKT1	(NM_005163)
BMPR2	(NM_001204)
DDR1	(NM_013993)
CCND3	(NM_001760)
CHES1	(NM_005197)
CLK3	(NM_003992)
DYRK1	(NM_001396)
EFNA1	(NM_004428)
IFIT1	(NM_001548)
SCYB10	(NM_001565)
INPP5D	(NM_005541)
ISG20	(NM_002201)
LY6E	(NM_002346)
PCNA	(NM_002592)
PIK3CA	(NM_006218)
PPP1R3	(NM_002711)
PTPN7	(NM_002832)
PTPN9	(NM_002833)
RHOK	(NM_002929)
RYK	(NM_002958)
EPHB3	(NM_004443)
PPP3CA	(NM_000944)
PRKCA	(NM_002737)
PRKG1	(NM_006258)

MAPK10	(NM_002753)
MAPK6	(NM_002748)
PTPRC	(NM_002838)
PTPRD	(NM_002839)
PTPRG	(NM_002841)
RBL1	(NM_002895)
STK3	(NM_006281)
TGFBI	(NM_000358)

Claims

1. A nucleic acid comprising a sequence at least 18 bases in length of a segment of the chemically pretreated DNA of genes associated with the cell cycle according to one of the sequences taken from the group of Seq. ID No.1 to Seq. ID No.424 and sequences complementary thereto.

2. A nucleic acid comprising a sequence at least 18 bases in length of a segment of the chemically pretreated DNA of genes associated with the cell cycle according to one of the sequences according to the genes CCNB1 (M25753), CCNE1 (M73812) (M74093), DENR (O43583), EPHA5 (L36644), MCM4 (X74794), NEK3 (Z29067), PCTK3 (X66362), PRKAR1B (M65066), PRKCG (M13977) (Z15114), PRKM3 (M84490) (Z11696), PRKMK2 (L11285), ZAP70 (L05148), ACP1 (NM_004300), AKT1 (NM_005163), BMPR2 (NM_001204), DDR1 (NM_013993), CCND3 (NM_001760), CCNF (NM_001761), CHES1 (NM_005197), CLK3 (NM_003992), DYRK1 (NM_001396), EFNA1(NM_004428), IFIT1 (NM_001548), SCYB10 (NM_001565), INPP5D (NM_005541), ISG20 (NM_002201), LY6E (NM_002346), PCNA (NM_002592), PIK3CA (NM_006218), PPP1R3 (NM_002711), PTPN7 (NM_002832), PTPN9 (NM_002833), RHOK (NM_002929), RYK (NM_002958), EPHB3 (NM_004443), PPP3CA (NM_000944), PRKCA (NM_002737), PRKG1 (NM_006258), MAPK10 (NM_002753), MAPK6 (NM_002748), PTPRC (NM_002838), PTPRD (NM_002839), PTPRG (NM_002841), RBL1 (NM_002895), STK3 (NM_006281), TGFBI (NM_000358), and sequences complementary thereto.

3. 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 hybridizes to or is identical to a chemically pretreated DNA of genes associated with the cell cycle according to one of the Seq. ID Nos 1 through 424 according to claim 1 or to a chemically pretreated DNA of genes according to claim 2, and sequences complementary thereto

4. The oligomer as recited in Claim 3; wherein the base sequence includes at least one CpG dinucleotide.

5. The oligomer as recited in Claim 4; characterized in that the cytosine of the CpG dinucleotide is located approximately in the middle third of the oligomer.

6. A set of oligomers, comprising at least two oligomers according to any of claims 3 to 5.

7. A set of oligomers as recited in Claim 6, comprising oligomers for detecting the methylation state of all CpG dinucleotides from one of the sequences according to the Seq. ID Nos 1 through 424 according to claim 1 or to a chemically pretreated DNA of genes according to claim 2, and sequences complementary thereto.

8. A set of at least two oligonucleotides as recited in Claim 3 which can be used as primer oligonucleotides for the amplification of DNA sequences of one of Seq. ID 1 through Seq. ID 424 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to claim 2, and sequences complementary thereto, or segments thereof.

9. A set of oligonucleotides as recited in Claim 8, characterized in that at least one oligonucleotide is bound to a solid phase.

10. Use of a set of oligomer probes comprising at least ten of the oligomers according to any of Claims 6 through 9 for detecting the cytosine methylation state and/or single nucleotide polymorphisms (SNPs) in chemically pretreated genomic DNA according to claim 1 or a chemically pretreated DNA of genes according to claim 2.

11. A method for manufacturing an arrangement of different oligomers (array) fixed to a carrier material for analyzing diseases associated with the methylation state of the CpG dinucleotides of one of the Seq. ID 1 through Seq. ID 424 and sequences complementary thereto and/or a chemically pretreated DNA of genes according to claim 2,
wherein at least one oligomer according to any of Claims 3 through 5 is coupled to a solid phase.

12. An arrangement of different oligomers (array), obtainable according to Claims 11.

13. An array of different oligonucleotide- and/or PNA-oligomer sequences as recited in Claim 12, characterized in that these are arranged on a plane solid phase in the form of a rectangular or hexagonal lattice.

14. The array as recited in any of Claims 12 or 13,
characterized in that the solid phase surface is composed of silicon, glass, polystyrene, aluminum, steel, iron, copper, nickel, silver, or gold.

15. A DNA- and/or PNA-array for analyzing diseases associated with the methylation state of genes, comprising at least one nucleic acid according to one of the preceeding claims.

16. A method for ascertaining genetic and/or epigenetic parameters for the diagnosis and/or therapy of existing diseases or the predisposition to specific diseases by analyzing cytosine methylations, characterized in that the following steps are carried out:

a) in a genomic DNA sample, cytosine bases which are unmethylated at the 5-position are converted, by chemical treatment, to uracil or another base which is dissimilar to cytosine in terms of hybridization behavior;

b) fragments of the chemically pretreated genomic DNA are amplified using sets of primer oligonucleotides according to Claim 8 or 9 and a polymerase, the amplificates carrying a detectable label;

c) Amplificates are hybridized to a set of oligonucleotides and/or PNA probes according to the Claims 6 or 7, or else to an array according to one of the Claims 12 through 15;

d) the hybridized amplificates are subsequently detected.

17. The method as recited in Claim 16, characterized in that the chemical treatment is carried out by means of a solution of a bisulfite, hydrogen sulfite or disulfite.

18. The method as recited in one of the Claims 16 or 17, characterized in that more than ten different fragments having a length of 100 - 2000 base pairs are amplified.

19. The method as recited in one of the Claims 16 through 18, characterized in that the amplification of several DNA segments is carried out in one reaction vessel.

20. The method as recited in one of the Claims 16 through 19, characterized in that the polymerase is a heat-resistant DNA polymerase.

21. The method as recited in Claim 20, characterized in that the amplification is carried out by means of the polymerase chain reaction (PCR).

22. The method as recited in one of the Claims 16 through 21, characterized in that the labels of the amplificates are fluorescence labels.

23. The method as recited in one of the Claims 16 through 21, characterized in that the labels of the amplificates are radionuclides.

24. The method as recited in one of the Claims 16 through 21, characterized in that the labels of the amplificates are detachable molecule fragments having a typical mass which are detected in a mass spectrometer.

25. The method as recited in one of the Claims 16 through 21, characterized in that the amplificates or fragments of the amplificates are detected in the mass spectrometer.

26. The method as recited in one of the Claims 24 and/or 25, characterized in that the produced fragments have a single positive or negative net charge for better detectability in the mass spectrometer

27. The method as recited in one of the Claims 24 through 26, characterized in that detection is carried out and visualized by means of matrix assisted laser desorption/ionization mass spectrometry (MALDI) or using electron spray mass spectrometry (ESI).

28. The method as recited in one of the Claims 16 through 27, characterized in that the genomic DNA is obtained from cells or cellular components which contain DNA, sources of DNA comprising, for example, cell lines, biopsies, blood, sputum, stool, urine, cerebral-spinal fluid, tissue embedded in paraffin such as tissue from eyes, intestine, kidney, brain, heart, prostate, lung, breast or liver, histologic object slides, and all possible combinations thereof.

29. A kit comprising a bisulfite (= disulfite, hydrogen sulfite) reagent as well as oligonucleotides and/or PNA-oligomers according to any of Claims 3 through 5.

30. The use of a nucleic acid according to Claims 1 or 2, of an oligonucleotide or PNA-oligomer according to one of the Claims 3 through 5, of a kit according to Claim 29, of an array according to one of the Claims 12 through 15, of a set of oligonucleotides according to one of Claims 6 through 9 for the diagnosis of HIV infection, neurodegenerative disorders, graft-versus-host disease, aging, glomerular disease, Lewy body disease, arthirits, arteroscleroses, solid tumors and cancers.

31. The use of a nucleic acid according to Claim 1 or 2, of an oligonucleotide or PNA-oligomer according to one of Claims 3 through 5, of a kit according to Claim 29, of an array

according to one of the Claims 12 through 15, of a set of oligonucleotides according to one of Claims 6 through 9 for the therapy of HIV infection, neurodegenerative disorders, graft-versus-host disease, aging, glomerular disease, Lewy body disease, arthirits, arterosclerosis, solid tumors and cancers.

32. A kit, comprising a bisulfite (= disulfite, hydrogen sulfite) reagent as well as oligonucleotides and/or PNA-oligomers according to one of Claims 3 through 5.

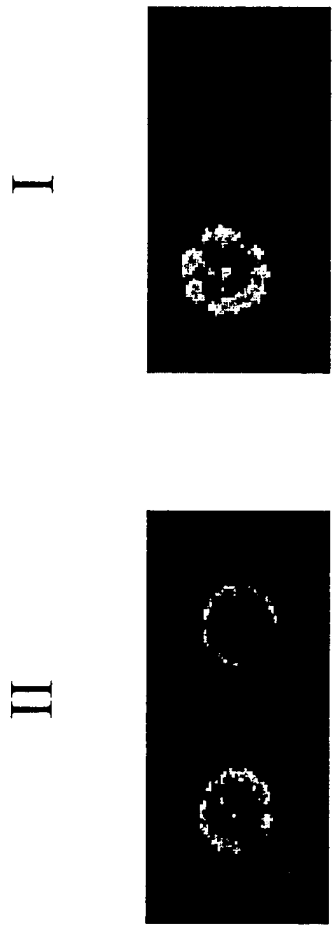


Fig. 1

专利名称(译)	诊断与细胞周期有关的疾病		
公开(公告)号	EP1283905A2	公开(公告)日	2003-02-19
申请号	EP2001921343	申请日	2001-03-15
[标]申请(专利权)人(译)	埃皮吉諾米克斯股份公司		
申请(专利权)人(译)	AG EPIGENOMICS		
当前申请(专利权)人(译)	AG EPIGENOMICS		
[标]发明人	OLEK ALEXANDER PIEPENBROCK CHRISTIAN BERLIN KURT		
发明人	OLEK, ALEXANDER PIEPENBROCK, CHRISTIAN BERLIN, KURT		
IPC分类号	G01N27/62 A61K31/7088 A61K48/00 A61P35/00 C07K14/47 C07K14/82 C12M1/00 C12N15/09 C12Q1/68 G01N21/78 G01N33/15 G01N33/50 G01N33/53 G01N33/566 G01N33/58 G01N37/00		
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优先权	10013847 2000-03-15 DE 10019058 2000-04-06 DE 10019173 2000-04-07 DE 10032529 2000-06-30 DE 10043826 2000-09-01 DE		
外部链接	Espacenet		

摘要(译)

本发明涉及与细胞周期相关的基因的化学修饰的基因组序列，涉及用于检测与细胞周期相关的基因的胞嘧啶甲基化状态的寡核苷酸和/或PNA寡聚体，其针对该序列，以及方法用于确定与细胞周期相关的基因的遗传和/或表观遗传参数。