



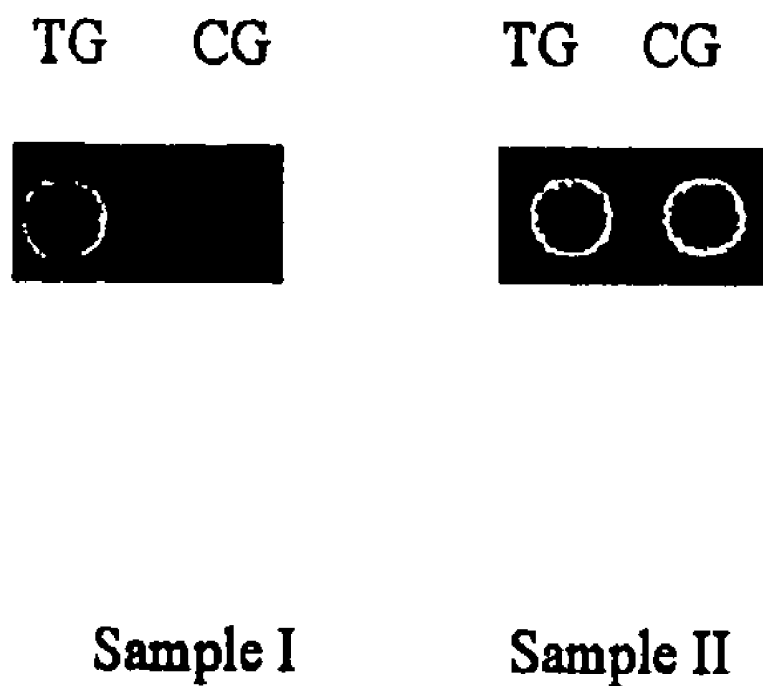
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536/23.1(21) Appl. No.: **10/240,708**(57) **ABSTRACT**(22) PCT Filed: **Apr. 6, 2001**(86) PCT No.: **PCT/EP01/03971**(30) **Foreign Application Priority Data**

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Chemically modified genomic sequences of genes associated with DNA replication, to oligonucleotides and/or PNA-oligomers for detecting the cytosine methylation state of genes associated with DNA replication which are directed against the sequence are disclosed. In addition, a method for ascertaining genetic and/or epigenetic parameters of genes associated with DNA replication is disclosed.

Figure 1



DIAGNOSIS OF DISEASES ASSOCIATED WITH DNA REPLICATION

FIELD OF THE INVENTION

[0001] 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.

[0002] 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 DNA replication and, in particular, with the methylation status thereof.

PRIOR ART

[0003] The replication of double stranded genomic DNA is a complex activity. It is carried out in three key stages, initiation, elongation and termination. Each stage involves specific protein and enzyme complexes. During initiation, the double helix is temporarily separated and stabilized into two single strands, each of which acts as a template for the replication of the DNA from the replication fork. Separation of the two strands is carried out by a helicase, and stabilisation of the strands is achieved using a single stranded binding protein. Replication of the DNA is then carried out by a polymerase after synthesis of a short 'primer' sequence. Replication is carried out in a semi-discontinuous fashion. The leading strand is continuously synthesised in the 5' to 3' direction. Whereas replication of the lagging strand, in the 3' to 5' direction is made by the synthesis of short fragments in the 5' to 3' direction. In the final stage, replication is terminated, and the lagging strand complementary DNA fragments are ligated into a continuous strand.

[0004] A further overview of the components of the DNA replication system is available from references such as Alberts et. al. 'Molecular Biology of the cell' Garland Publishing.

[0005] Disruptions to the ordered replication of DNA may impact on a wide variety of disease phenotypes. These range from chromosomal disorders to disorders at a molecular level. Malfunctions in the specific genes involved in DNA replication have been implicated in several disease phenotypes, including, but not limited to cancer:

[0006] Ataxia-telangiectasia; Meyn MS. 'Ataxia-telangiectasia, cancer and the pathobiology of the ATM gene'. Clin Genet. 1999 May; 55(5):289-304.

[0007] ATR-X; Wada T. 'Molecular genetic study of Japanese patients with X-linked alpha-thalassemia/mental retardation syndrome'. Am J Med Genet. 2000 Sep. 18; 94(3):242-8.

[0008] Bloom's syndrome; German J. 'Bloom's syndrome'. Dermatol Clin. 1995 January; 13(1):7-18.

[0009] Cancer; Sturgis et. al. 'XPD/ERCC2 polymorphisms and risk of head and neck cancer: a case-control analysis.' Carcinogenesis. 2000 December; 21(12):2219-23.

[0010] Neurological disorders; Hermon et. al. 'Expression of DNA excision-repair-cross-complementing proteins p80 and p89 in brain of patients with Down Syndrome and Alzheimer's disease.' Neurosci Lett. 1998 Jul. 17; 251(1):45-8.

[0011] The diversity of components involved in DNA replication provides an alternative target for therapies and diagnosis for diseases. In particular this may be relevant to diseases where current therapies may have unwanted side effects or fail to provide effective treatment. For cancer patients such methods constitute a considerable advantage over conventional methods such as chemotherapy, which with their massive side effects, sometimes result in unacceptable morbidity or lead up to the death of the patient. In practice, the unwanted side effects associated with cancer therapies frequently limit the treatment which could help a patient.

[0012] A global analysis of the status of DNA replication mechanisms would provide a basis for the development of appropriate and specific therapies for diseases associated with DNA replication. The current state of the art is such that the analysis may be carried out in a gene specific manner based on the results of gene expression, e.g. DNA micro array analysis of mRNA expression or proteomic analysis. The next step would then be to look at the causal factors involved at earlier stages in the regulatory mechanisms controlling DNA replication. DNA methylation provides such a novel level of information at which to analyse the genome.

[0013] 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 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.

[0014] 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.

[0015] 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 March-April; 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 November; 17(3):275-6) or individual cytosine positions are detected by a primer extension reaction (Gonzalzo M L, Jones P A. 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 P W. 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).

[0016] 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 June; 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 March; 6(3):387-95; Feil R, Charlton J, Bird A P, 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 M C, 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):2614; WO 97/46705, WO 95/15373 and WO 97/45560.

[0017] 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.

[0018] Fluorescently labelled probes are often used for the scanning of immobilised DNA arrays. The simple attach-

ment 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.

[0019] 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.

[0020] 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 disproportionately 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 I G, 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.

[0021] 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.

Description

[0022] The object of the present invention is to provide the chemically modified DNA of genes associated with DNA replication, 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 DNA replication. The present invention is

based on the discovery that genetic and epigenetic parameters and, in particular, the cytosine methylation pattern of genes associated with DNA replication are particularly suitable for the diagnosis and/or therapy of diseases associated with DNA replication.

[0023] This objective is achieved according to the present invention using a nucleic acid containing a sequence of at least 18 bases in length of the chemically pretreated DNA of genes associated with DNA replication according to one of Seq. ID No. 1 through Seq. ID No. 94 and sequences complementary thereto and/or a sequence of a chemically pretreated DNA of genes according to table 1 and sequences complementary thereto. 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 was used as the underlying data bank, which is located at the National Institute of Health, internet address www.ncbi.nlm.nih.gov.

[0024] The chemically modified nucleic acid could heretofore not be connected with the ascertainment of genetic and epigenetic parameters.

[0025] 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 DNA replication according to Seq. ID No. 1 through Seq. ID No. 94 and sequences complementary thereto and/or a sequence of a chemically pretreated DNA of genes according to table 1 and sequences complementary thereto. 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 DNA replication. 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 5th-9th 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 4th-6th nucleotide from the 5'-end of the 9-mer.

[0026] 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. 94 and sequences complementary thereto and/or a sequence of a chemically pretreated DNA of genes according to table 1 and sequences complementary thereto. 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. 94 and sequences complementary thereto and/or a sequence of a chemically pretreated DNA of genes according to table 1 and sequences complementary thereto.

[0027] 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. 94 and sequences complementary thereto and/or a sequence of a chemically pretreated DNA of genes according to table 1 and sequences complementary thereto, or segments thereof.

[0028] 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.

[0029] 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. 94 and sequences complementary thereto and/or a sequence of a chemically pretreated DNA of genes according to table 1 and sequences complementary thereto). These probes enable diagnosis and/or therapy of genetic and epigenetic parameters of genes associated with DNA replication. The set of oligomers may also be used for detecting single nucleotide polymorphisms (SNPs) in the chemically pretreated DNA of genes associated with DNA replication according to one of Seq. ID No. 1 through Seq. ID No. 94 and sequences complementary thereto and/or a sequence of a chemically pretreated DNA of genes according to table 1 and sequences complementary thereto.

[0030] 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, 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 are possible as well.

[0031] 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 DNA replication 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 U.S. Pat. No. 5,744,305 by means of solid-phase chemistry and photolabile protecting groups.

[0032] A further subject matter of the present invention relates to a DNA chip for the analysis of diseases associated with DNA replication which contains at least one nucleic acid according to the present invention. DNA chips are known, for example, for U.S. Pat. No. 5,837,832.

[0033] 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. 94 and sequences complementary thereto and/or a sequence of a chemically pretreated DNA of genes according to table 1 and sequences complementary thereto), 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.

[0034] The present invention also makes available a method for ascertaining genetic and/or epigenetic param-

eters of genes associated with the cycle cell by analyzing cytosine methylations and single nucleotide polymorphisms, including the following steps:

[0035] 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.

[0036] The genomic DNA to be analyzed is preferably obtained from 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.

[0037] 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.

[0038] 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).

[0039] In a preferred embodiment of the method, the set of primer oligonucleotides includes at least two oligonucleotides 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. 94 and sequences complementary thereto and/or a sequence of a chemically pretreated DNA of genes according to table 1 and sequences complementary thereto). The primer oligonucleotides are preferably characterized in that they do not contain any CpG dinucleotides.

[0040] 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, aluminium, steel, iron, copper, nickel, silver, or gold, it being possible for other materials such as nitrocellulose or plastics to be used as well.

[0041] 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 visualised by means of matrix assisted laser

desorption/ionization mass spectrometry (MALDI) or using electron spray mass spectrometry (ESI).

[0042] The amplicates 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 amplicates 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 5th to 9th 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 4th to 6th nucleotide seen from the 5'-end of the 9-mer. One oligonucleotide exists for each CpG dinucleotide.

[0043] In the fourth step of the method, the non-hybridized amplicates are removed.

[0044] In the final step of the method, the hybridized 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.

[0045] According to the present invention, it is preferred that the labels of the amplicates 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 amplicates, fragments of the amplicates or of probes which are complementary to the amplicates, 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).

[0046] 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 DNA replication.

[0047] 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 DNA replication by analyzing methylation patterns of genes associated with DNA replication. 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 DNA replication.

[0048] The method according to the present invention is used, for example, for the diagnosis and/or therapy of Ataxia

telangiectasia, ATR-X, Bloom's syndrome, neurological disorders, solid tumors and cancer.

[0049] The nucleic acids according to the present invention of Seq. ID No. 1 through Seq. ID No. 94 and sequences complementary thereto and/or a sequence of a chemically pretreated DNA of genes according to table 1 and sequences complementary thereto can be used for the diagnosis and/or therapy of genetic and/or epigenetic parameters of genes associated with DNA replication. 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 DNA replication by analyzing methylation patterns of genes associated with DNA replication, 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.

[0050] A further subject matter of the present invention relates to a diagnostic agent and/or therapeutic agent for diseases associated with DNA replication by analyzing methylation patterns of genes associated with DNA replication, 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.

[0051] 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 DNA replication 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.

[0052] 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×SSC buffer, followed by several washing steps at 37° C. in a low buffer concentration, and remains stable.

[0053] 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.

[0054] In the context of the present invention, "genetic parameters" are mutations and polymorphisms of genes associated with DNA replication 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).

[0055] 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 DNA replication 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.

[0056] In the following, the present invention will be explained in greater detail on the basis of the sequences and examples with reference to the accompanying FIGURE without being limited thereto.

[0057] FIG. 1

[0058] FIG. 1 shows the hybridisation of fluorescent labelled amplicates to a surface bound oligonucleotide. Sample I being from an oligodendroglyome grade II tumour sample and sample II being from astrocytoma grade II cerebrum tissue. Fluorescence at a spot shows hybridisation of the amplicate 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.

[0059] Seq ID Nos. 1 to 94

[0060] 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 DNA replication. 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 DNA replication which are complementary to the preceding 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.)

[0061] Seq ID Nos. 95 to 98

[0062] Seq ID Nos. 95 to 98 show the sequences of oligonucleotides used in Example 1.

[0063] The following example relates to a fragment of a gene associated with DNA replication, in this case, MLH1 in which a specific CG-position is analyzed for its methylation status.

EXAMPLE 1

Methylation Analysis in the Gene MLH1 Associated with DNA Replication

[0064] The following example relates to a fragment of the gene MLH1 in which a specific CG-position is to be analyzed for methylation.

[0065] 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.

[0066] If bisulfite solution is used for the reaction, then an addition takes place at the non-methylated cytosine bases. Moreover, a denaturing reagent or solvent as well as a radical interceptor must be present. A subsequent alkaline

hydrolysis then gives rise to the conversion of nonmethylated cytosine nucleobases to uracil. The chemically converted DNA (sequence ID 31) 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 MLH1 are analyzed. To this end, a defined fragment having a length of 866 bp is amplified with the specific primer oligonucleotides TTTAAGGTAAGAGAATAGGT (Sequence ID No. 95) and AAACAACCTTAAATACCAATC (Sequence ID No. 96). This amplificate serves as a sample which hybridizes to an oligonucleotide previously bonded to a solid phase, forming a duplex structure, for example GGTTTGTACGAGTAGTTT (Sequence ID No. 97), the cytosine to be detected being located at position 135 of the amplificate. The detection of the hybridization product is based on Cy3 and Cy5 fluorescently labeled primer oligonucleotides which have been used for the amplification. A 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 is inferred from the hybridization product.

[0067] 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. GGTTTGTATGAGTAGTTT (Sequence ID No. 98). Therefore, the hybridisation reaction only takes place if an unmethylated cytosine was present at the position to be analysed. The procedure was carried out on cell samples from 2 patients, sample I being from an oligodendroglyome grade II tumour sample and sample II being from a astrocytoma grade II cerebrum tumor sample.

[0068] From the results (FIG. 1) it can be seen that the sample I contained only unmethylated cells at position 135 of the amplificate whereas sample II contained a mixture of methylated and unmethylated cells at position 135 of the amplificate.

EXAMPLE 2

Diagnosis of Diseases Associated with DNA Replication

[0069] In order to relate the methylation patterns to one of the diseases associated with DNA replication, 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.

[0070] Subsequently, it is possible to allocate the examined patients to a specific therapy group and to treat these patients selectively with an individualized therapy.

[0071] Example 2 can be carried out, for example, for the following diseases:

[0072] *Ataxia telangiectasia*, ATR-X, Bloom's syndrome, neurological disorders, solid tumours and cancer

TABLE 1

Listing of particularly preferred genes of the present invention associated with the DNA replication	
Gene	Database Entry No. (Genbank, internet address www.ncbi.nlm.nih.gov)
CENPB	X05299
DNA2L	D42046
ATR	NM_001184
CHD1L	NM_004284
ERCC3	NM_000122
SNRPA1	NM_003090
RAD50	NM_005732
LIG2	

[0073]

SEQUENCE LISTING

The patent application contains a lengthy "Sequence Listing" section. A copy of the "Sequence Listing" is available in electronic form from the USPTO web site (<http://seqdata.uspto.gov/sequence.html?DocID=20050282157>). An electronic copy of the "Sequence Listing" will also be available from the USPTO upon request and payment of the fee set forth in 37 CFR 1.19(b)(3).

1-38. (canceled)

39. A method for ascertaining genetic and/or epigenetic parameters for the diagnosis and/or therapy of a disease or

the predisposition to the disease by analyzing cytosine methylations in genomic DNA, the method comprising the steps of:

- a) in a genomic DNA sample, converting, by chemical treatment, cytosine bases that are unmethylated at the 5-position to uracil or another base dissimilar to cytosine in terms of hybridization behavior so as to define chemically pretreated genomic DNA;
- b) amplifying fragments of the chemically pretreated genomic DNA using a set of primer oligonucleotides and a polymerase to produce amplicates, and
- c) analyzing sequences of the amplicates to determine a methylation status of genomic CpG sequences, wherein the genomic CpG sequences are located within at least one sequence associated with DNA replication.
- 40.** A method according to claim 39, wherein the at least one sequence associated with DNA replication is taken from the group of Seq. ID No. 1 to Seq. ID No. 98 and sequences complementary thereto.
- 41.** A method according to claim 39, wherein the at least one sequence associated with DNA replication is a gene associated with DNA replication selected from the group consisting of CENPB (X05299), DNA2L (D42046), ATR (NM_001184), CHD1L (NM_004284), ERCC3 (NM_000122), SNRPA1 (NM_003090), RAD50 (NM_005732), LIG2 and sequences complementary thereto.
- 42.** A method according to claim 39, wherein the amplification of the fragments of the chemically pretreated genomic DNA using a set of primer oligonucleotides and a polymerase is performed in a way that the amplicates carry a detectable label.
- 43.** A method according to claim 39, wherein step b) further comprises the step of hybridizing the amplicates to a set of oligomers, wherein the base sequence of the oligomers includes at least one CpG dinucleotide.
- 44.** A method according to claim 43, wherein the oligomers are oligonucleotides.
- 45.** A method according to claim 43, wherein the oligomers are PNA probes.
- 46.** A method according to claim 43, wherein the oligomers are in an array.
- 47.** A method according to claim 43, wherein the cytosine of the CpG dinucleotide is located approximately in a middle third of each oligomer.
- 48.** A method according to claim 43, wherein the set of oligomers comprises at least two different oligomers.
- 49.** A method according to claim 43, wherein the set of oligomers comprises oligomers for detecting the methylation state of one or more CpG dinucleotides of one or more sequences taken from the group of Seq. ID No. 1 to Seq. ID No. 98 and sequences complementary thereto.
- 50.** A method according to claim 43, wherein the set of oligomers comprises oligomers for detecting the methylation state of one or more CpG dinucleotides of a sequence selected from the group consisting of CENPB (X05299), DNA2L (D42046), ATR (NM_001184), CHD1L (NM_004284), ERCC3 (NM_000122), SNRPA1 (NM_003090), RAD50 (NM_005732), LIG2 and sequences complementary thereto.
- 51.** A method according to claim 39 wherein the chemical treatment is carried out using a bisulfite solution.
- 52.** A method according to claim 39, wherein the fragments comprise more than ten different fragments having a length of 100-2000 base pairs.
- 53.** A method according to claim 39, wherein the step of amplifying fragments of the chemically pretreated genomic DNA comprises amplification of more than one DNA segment in one reaction vessel.
- 54.** A method according to claim 39, wherein the set of primer oligonucleotides comprises oligonucleotides having 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 sequences associated with DNA replication taken from the group of Seq. ID No. 1 to Seq. ID No. 98 and sequences complementary thereto.
- 55.** A method according to claim 39, wherein the set of primer oligonucleotides comprises oligonucleotides having 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 DNA replication selected from the group within at least one gene associated with DNA replication selected from the group consisting of CENPB (X05299), DNA2L (D42046), ATR (NM_001184), CHD1L (NM_004284), ERCC3 (NM_000122), SNRPA1 (NM_003090), RAD50 (NM_005732), LIG2 and sequences complementary thereto.
- 56.** A method according to claim 39, wherein the polymerase is a heat-resistant DNA polymerase.
- 57.** A method according to claim 39, wherein the step of amplifying fragments of the chemically pretreated genomic DNA sequences using sets of primer oligonucleotides and a polymerase is carried out using a polymerase chain reaction (PCR).
- 58.** A method according to claim 42, wherein the detectable label is selected from the group consisting of fluorescent labels, radionuclides and detachable molecule fragments having a predetermined mass detectable in a mass spectrometer.
- 59.** A method according to claim 39, further comprising detecting amplicates or fragments of the amplicates in a mass spectrometer.
- 60.** A method according to claim 42, wherein the produced fragments comprise a single positive or negative net charge.
- 61.** A method according to claim 59, wherein the mass spectrometer uses matrix assisted laser desorption/ionization mass spectrometry (MALDI) or an electron spray mass spectrometry (ESI).
- 62.** A method according to claim 39, wherein the genomic DNA is obtained from cells or cellular components which contain DNA.
- 63.** A method according to claim 39, wherein the genomic DNA is obtained from a source selected from the group consisting of cell lines, biopsies, blood, sputum, stool, urine, cerebral-spinal fluid, tissue embedded in paraffin, intestine, kidney, brain, heart, prostate, lung, breast, liver, histologic object slides and combinations thereof.
- 64.** A method according to claim 39, further comprising the step of diagnosing a disease associated with DNA replication.
- 65.** A method according to claim 64, wherein the disease is selected from the group consisting of Ataxia telangiectasia, ATR-X, Bloom's syndrome, neurological disorders, solid tumours and cancer.
- 66.** A method according to claim 39, further comprising the step of treating a disease associated with DNA replication.

67. A method according to claim 66, wherein the disease is selected from the group consisting of Ataxia telangiectasia, ATR-X, Bloom's syndrome, neurological disorders, solid tumours and cancer.

68. An oligomer comprising 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 sequences associated with DNA replication taken from the group of Seq. ID No. 1 to Seq. ID. No. 98 and complementary sequences thereto or according to one of the genes associated with DNA replication selected from the group consisting of CENPB (X05299), DNA2L (D42046), ATR (NM_001184), CHD1L (NM_004284), ERCC3 (NM_000122), SNRPA1 (NM_003090), RAD50 (NM_005732), LIG2 and sequences complementary thereto.

69. An oligomer according to claim 68, wherein the oligomer is an oligonucleotide.

70. An oligomer according to claim 69, wherein the oligomer is a peptide nucleic acid (PNA)-oligomer.

71. An oligomer according to claim 68, wherein the base sequence includes at least one CpG dinucleotide.

72. An oligomer according to claim 68, wherein the cytosine of the CpG dinucleotide is located in about a middle third of the oligomer.

73. A set of oligomers comprising at least two oligomers, wherein each of the oligomers comprises 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 sequences associated with DNA replication taken from the group of Seq. ID No. 1 to Seq. ID. No. 98 and complementary sequences thereto or according to one of the genes associated with DNA replication selected from the group consisting of CENPB (X05299), DNA2L (D42046), ATR (NM_001184), CHD1L (NM_004284), ERCC3 (NM_000122), SNRPA1 (NM_003090), RAD50 (NM_005732), LIG2 and sequences complementary thereto.

74. A set of oligomers according to claim 73, wherein the at least two oligomers comprise oligomers for detecting the

methylation state of one or more CpG dinucleotides within at least one of the sequences according to Seq. ID Nos. 1 through 98 or according to at least one of the genes selected from the group consisting of CENPB (X05299), DNA2L (D42046), ATR (NM_001184), CHD1L (NM_004284), ERCC3 (NM_000122), SNRPA1 (NM_003090), RAD50 (NM_005732), LIG2 and sequences complementary thereto.

75. A set of oligomers according to claim 73, wherein at least one oligonucleotide is bound to a solid phase.

76. A method for manufacturing an arrangement of different oligomers (array) for analyzing diseases associated with the methylation state of genes associated with regulation, comprising:

coupling at least two oligomers according to claim 68 to a solid phase.

77. A method according to claim 76, wherein the solid phase has a surface selected from the group consisting of silicon, glass, polystyrene, aluminum, steel, iron, copper, nickel, silver and gold.

78. An array of oligomers comprising two or more oligomers of claim 68.

79. A kit for diagnosis or prognosis of diseases related to DNA replication, comprising (a) a container comprising at least one oligomer according to claim 68 and (b) a bisulfite reagent.

80. A kit according to claim 79, further comprising standard methylation assay reagents for performing a methylation assay selected from the group consisting of MS-SNuPE, COBRA and combinations thereof.

81. A nucleic acid comprising a sequence at least 18 bases in length of a segment of chemically pretreated DNA of genes associated with DNA replication according to

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摘要(译)

公开了与DNA复制相关的基因的化学修饰的基因组序列，涉及用于检测与DNA复制相关的基因的胞嘧啶甲基化状态的寡核苷酸和/或PNA-寡聚物，所述DNA复制针对所述序列。此外，公开了用于确定与DNA复制相关的基因的遗传和/或表现遗传参数的方法。

Figure 1

