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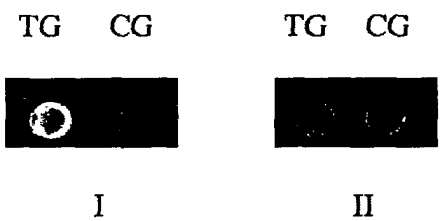
(54) Title: DIAGNOSIS OF DISEASES ASSOCIATED WITH CELL SIGNALLING



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

Diagnosis of Diseases Associated with cell signalling

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 signalling and, in particular, with the methylation status thereof.

Prior Art

Cells exist in social environment. The maintenance of a system of balances and checks on intercellular interactions has necessitated the development of a system of cell to cell signalling. During development cells are programmed to respond to specific sets of signals, that may act in various combinations. The majority of cell signalling takes the form of paracrine signalling, in which cells secrete and react to mediators which are rapidly utilised, thus localising the effects of the signal. Other forms of cell signalling include the endocrine system whereby hormones secreted from endocrine cells are carried in the bloodstream to distant parts of the body. A further form of cell signalling is synaptic signalling in which neurotansmitters secreted by nerve cells act on postsynaptic cells.

The signalling system consists of extracellular signalling molecules combined with intracellullar receptor molecules. In the case of small hydrophobic signalling molecules such as the steroid hormones, the molecules diffuse directly across the plasma membrane and activate intracellular

receptor molecules. However, most signalling molecules are hydrophillic and activate receptor, molecules situated on the target cell surface. There are three main classes of cell surface receptors, G-protein linked receptors, ion-channel linked receptors, and enzyme linked receptors. Although each functions in a unique manner, most act as signal transducers initiating the activation of a intracellular signals that alter the behaviour of the cell.

Failure of the cell signalling system has been implicated in a variety of disorders, and play a role in most diseases, for example, but not limited to cancer, immune disease, neurological disorders and developmental disorders.

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.

Aberrant DNA methylation within CpG islands is common in human malignancies leading to abrogation or overexpression of a broad spectrum of genes (Jones, P.A. Cancer Res 65:2463-2467, 1996). Abnormal methylation has also been shown to occur in CpG rich regulatory elements in intronic and coding parts of genes for certain tumours (Chan, M.F., et al., Curr Top Microbiol Immunol 249:75-86,2000). Using restriction landmark genomic scanning, Costello and coworkers were able to show that methylation patterns are tumour-type specific (Costello, J. F., et al., Nat Genet 24:132-138, 2000). Highly characteristic DNA methylation patterns could also be shown for breast cancer cell lines (Huang, T. H.-M., et al., Hum Mol Genet 8:459-470, 1999). Genome wide assessment of methylation status represents a molecular fingerprint of cancer tissues.

However, 5-methylcytosine positions cannot be identified by sequencing since 5-methylcytosine has the same base pairing behaviour as cytosine. Moreover, the epigenetic information carried by 5-methylcytosine is completely lost during PCR amplification.

A relatively new and currently the most frequently used method for analysing DNA for 5-methylcytosine is based upon the specific reaction of bisulfite with cytosine which, upon subsequent alkaline hydrolysis, is converted to uracil which corresponds to thymidine in its base pair-

ing 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 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 97/45560.

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.

Description

The object of the present invention is to provide the chemically modified DNA of genes associated with cell signalling, 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 cell signalling. 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 signalling are particularly suitable for the diagnosis and/or therapy of diseases associated with cell signalling.

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 cell signalling according to one of Seq. ID No.1 through Seq. ID No.516 and sequences complementary thereto and/or sequences 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 at internet address http://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 cell signalling according to Seq. ID No.1 through Seq. ID No.516 and sequences complementary thereto and/or sequences 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 cell signalling. 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.516 and sequences complementary thereto and/or sequences 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.516 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto.

Moreover, the present invention makes available a set of at least two oligonucleotides which can be used as so-called "primer oligonucleotides" for amplifying DNA sequences of one of Seq. ID No.1 through Seq. ID No.516 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto, or segments thereof.

In the case of the sets of oligonucleotides according to the present invention, it is preferred that at least one 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.516 and sequences complementary thereto and/or sequences 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 cell signalling. The set of oligomers may also be used for detecting single nucleotide polymorphisms (SNPs) in the chemically pretreated DNA of genes associated with cell signalling according to one of Seq. ID No.1 through Seq. ID No.516 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto.

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 cell signalling 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 cell signalling which contains at least one nucleic acid according to the present invention. DNA chips are known, for example, in 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.516 and sequences complementary thereto and/or sequences 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.

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 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.516 and sequences complementary thereto and/or sequences 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.

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 cell signalling.

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 cell signalling by analyzing methylation patterns of genes associated with cell signalling. 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 signalling.

The method according to the present invention is used, for example, for the diagnosis and/or therapy of solid tumours and cancer
The nucleic acids according to the present invention of Seq. ID No.1 through Seq. ID No.516 and sequences complementary thereto and/or sequences 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 cell signalling.

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 cell signalling by analyzing methylation patterns of genes associated with cell signalling, 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 cell signalling by analyzing methylation patterns of genes associated with cell signalling, 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 cell signalling 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.

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 signalling 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 cell signalling 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 with respect to 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 a astrocytoma tumor sample and sample II being from an oligo-

denroglioma grade II tumor sample. Flourescence at a spot shows hybridisation of the amplificate to the olignonucleotide. Hybridisation to a CG olignonucleotide denotes methylation at the cytosine position being analysed, hybridisation to a TG olignonucleotide denotes no methylation at the cytosine position being analysed. It can be seen that Sample II had a higher degree of methylation than Sample I.

Seq. ID No.1 through Seq. ID No.516

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 signalling. 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 signalling 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.517 through Seq. ID No.520

Seq. ID No.517 through Seq. ID No.520 show sequences of oligonucleotides used in Example 1.

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

Example 1:Methylation analysis of the gene Lmyc associated with cell signalling.

The following example relates to a fragment of the gene Lmyc 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 must be present. A subsequent alkaline hydrolysis then gives rise to the conversion of non-methylated

cytosine nucleobases to uracil. The 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 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 **Lmyc** are analyzed. To this end, a defined fragment having a length of 491 bp is amplified with the specific primer oligonucleotides AGGTTTGGGTTATTGAGTTT (Sequence ID 517) and CATTATTTCC TAACTACCTT (Sequence ID No. 518). This amplificate serves as a sample which hybridizes to an oligonucleotide previously bonded to a solid phase, forming a duplex structure, for example TTTTAGTTCG GAGTGGGT (Sequence ID No. 519), the cytosine to be detected being located at position 209 of the amplificate. The detection of the hybridization product is based on Cy3 and Cy5 fluorescently labelled 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.

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 olignonucleotide 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 TTTTAGTTTGGAGTGGGT (Sequence ID No. 520). Therefore, the hybridisation reaction only takes place if an unmethylated cytosine was present at the position to be analysed.

Example 2: Diagnosis of diseases associated with cell signalling

In order to relate the methylation patterns to one of the diseases associated with cell signalling, 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 cancer and solid tumours.

Table 1

List of preferred genes associated with cell signalling according to the invention

Gene	*Genbank Entry No. (http://www.ncbi.nlm.nih.gov)*
COT	D14497
EPHA5	L36644
FN1	M10905
PCTK3	X66362
NEK 3	Z29067
PRKAR1B	M65066
PRKCG	M13977 Z15114
PRKM3	M84490 Z11696
PRKMK2	L11285
ZAP70	L05148
ABR	NM_021962
IFIT1	NM_001548
PIK3CA	NM_006218
ACP1	NM_007099
ADRBK1	NM_001619
AKT1	NM_005163
AKT2	NM_001626
ARHA	NM_001664
BMPR2	NM_001204
CDH2	NM_001792
CLK3	NM_003992
CNK	NM_004073
CSNK1D	NM_001893
CTNND2	NM_001332
DGKG	NM_001346
EFNA1	NM_004428
EGR3	NM_004430
EPHB3	NM_004443
GRB2	NM_002086
INPP5D	NM_005541

Gene	*Genbank Entry No. (http://www.ncbi.nlm.nih.gov)*
ISG20	NM_002201
ITGA6	NM_000210
ITGAX	NM_000887
ITGB1	NM_002211
ITGB5	NM_002213
ITGB7	NM_000889
LY6E	NM_002346
MADH3	NM_005902
PCDH1	NM_002587
PPP1CC	NM_002710
PPP1R3	NM_002711
PPP2R1B	NM_002716
PPP3CA	NM_000944
PRKACG	NM_002732
PRKAG1	NM_002733
PRKAR1A	NM_002734)
PRKCZ	NM_002744
MAPK1	NM_002745
MAPK10	NM_002753
MAPK13	NM_002754
MAPK6	NM_002748
PTPN13	NM_006264)
PTPN7	NM_002832
PTPN9	NM_002833
PTPRD	NM_002839
PTPRN2	NM_002847
RGS7	NM_002924
RHOK	NM_002929
RSN	NM_002956
RYK	NM_002958
SFN	NM_006142
SRPK2	NM_003138
STK3	NM_006281
TGFBI	NM_000358
TYRO3	NM_006293
VASP	NM_003370
DVL3	NM_004423
SPTB	NM_000347
TIAM1	NM_003253
TTK	NM_003318
UBE1L	NM_003335
MAP3K12	NM_006301
ACTG1	NM_001614
CDC2L1	NM_001787
CDH4	NM_001794
MAP3K3	NM_002401

Gene	*Genbank Entry No. (http://www.ncbi.nlm.nih.gov)*
OCLN	NM_002538
PDPK1	NM_002613
PPP2R5D	NM_006245
PRKCA	NM_002737
PRKG1	NM_006258
PTPRG	NM_002841
SCYB10	NM_001565
PRKACB	NM_002731
CTNNB1	NM_001904
BMX	NM_001721
FLT4	NM_002020)
ITGAE	NM_002208
ITGB3	NM_000212

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 cell signalling according to one of the sequences taken from the group of Seq. ID No.1 to Seq. ID No.516 and sequences complementary thereto.

2. A nucleic acid comprising a sequence at least 18 base pairs in length of the chemically pretreated DNA of genes associated with cell signalling according to a sequence according to one of the genes COT (D14497), EPHA5 (L36644), FN1 (M10905), NEK3 (Z29067), PCTK3 (X66362), PRKAR1B (M65066), PRKCG (M13977) (Z15114), PRKM3 (M84490) (Z11696), PRKMK2 (L11285), ZAP70 (L05148), ABR (NM_021962), IFIT1 (NM_001548), PIK3CA (NM_006218), PIK3CA (NM_006218), ACP1 (NM_007099), ADRBK1 (NM_001619), AKT1 (NM_005163), AKT2 (NM_001626), ARHA (NM_001664), BMPR2 (NM_001204), CDH2 (NM_001792), CLK3 (NM_003992), CNK (NM_004073), CSNK1D (NM_001893), CTNND2 (NM_001332),DGKG (NM_001346), EFNA1 (NM_004428), EGR3 (NM_004430), EPHB3 (NM_004443), GRB2 (NM_002086), INPP5D (NM_005541), ISG20 (NM_002201), ITGA6 (NM_000210), ITGAX (NM_000887), ITGB1 (NM_002211), ITGB5 (NM_002213), ITGB7 (NM_000889), LY6E (NM_002346), MADH3 (NM_005902), PCDH1 (NM_002587), PPP1CC (NM_002710), PPP1R3 (NM_002711), PPP2R1B (NM_002716), PPP3CA (NM_000944), PRKACG (NM_002732), PRKAG1 (NM_002733), PRKAR1A (NM_002734),PRKCZ (NM_002744), MAPK1 (NM_002745), MAPK10 (NM_002753), MAPK13 (NM_002754), MAPK6 (NM_002748), PTPN13 (NM_006264), PTPN7 (NM_002832), PTPN9 (NM_002833), PTPRD (NM_002839), PTPRN2 (NM_002847), RGS7 (NM_002924), RHOK (NM_002929), RSN (NM_002956), RYK (NM_002958), SFN (NM_006142), SRPK2 (NM_003138), STK3 (NM_006281), TGFBI (NM_000358), TYRO3 (NM_006293), VASP (NM_003370), DVL3 (NM_004423), SPTB (NM_000347), TIAM1 (NM_003253), TTK (NM_003318), UBE1L (NM_003335), MAP3K12 (NM_006301), ACTG1 (NM_001614), CDC2L1 (NM_001787), CDH4 (NM_001794), MAP3K3 (NM_002401), OCLN (NM_002538), PDPK1 (NM_002613), PPP2R5D (NM_006245), PRKCA (NM_002737), PRKG1 (NM_006258), PTPRG (NM_002841), SCYB10 (NM_001565), PRKACB

(NM_002731), CTNNB1 (NM_001904), BMX (NM_001721), FLT4 (NM_002020), ITGAE (NM_002208), ITGB3 (NM_000212), 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 cell signalling according to one of the Seq ID Nos 1 to 516 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 3; 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 within one of the sequences according to Seq. ID Nos. 1 through 516 according to claim 1 or 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 516 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to claim 2, and sequences complementary thereto and 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 a 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 516 and sequences complementary thereto and/or chemically pretreated DNA of genes according to claim 2, wherein at least one oligomer according to any of the claims 3 through 5 is coupled to a solid phase.

12. An arrangement of different oligomers (array) obtainable according to claim 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 the Claims 12 or 13, characterized in that the solid phase surface is composed of silicon, glass, polystyrene, aluminium, 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:
- 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;
- 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;

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

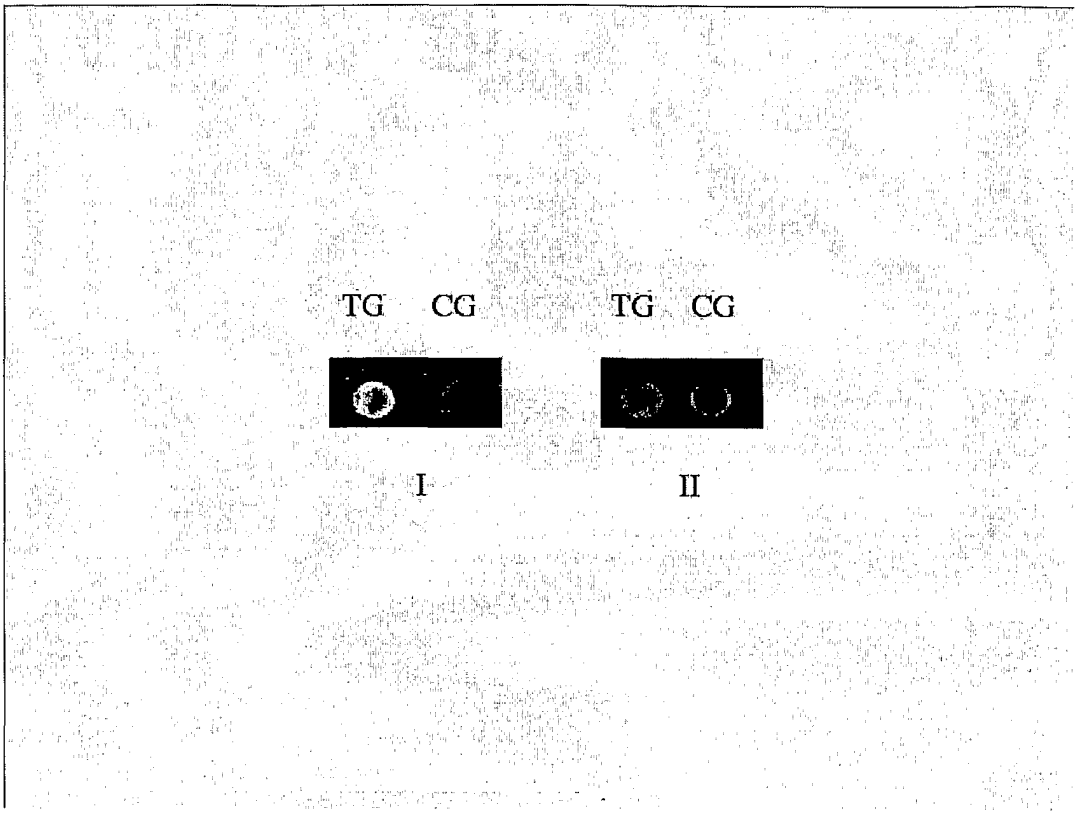
29. A kit comprising a bisulfite (= disulfite, hydrogen sulfite) reagent as well as oligonucleotides and/or PNA-oligomers according to one of the Claims 3 through 5.

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 diseases.

31. The use of a nucleic acid according to Claims 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 diseases.

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.

Figure 1



专利名称(译)	诊断与细胞信号传导相关的疾病		
公开(公告)号	EP1297182A2	公开(公告)日	2003-04-02
申请号	EP2001955325	申请日	2001-06-29
[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 C07K14/47 C07K14/82 C12M1/00 C12M1/34 C12N15/09 C12Q1/68 G01N33/53 G01N33/58 G01N37/00		
CPC分类号	C07K14/82 C07K14/4703 C12Q1/6883 C12Q1/6886 C12Q2523/125 C12Q2600/154 C12Q2600/156		
优先权	10032529 2000-06-30 DE 10043826 2000-09-01 DE		
外部链接	Espacenet		

摘要(译)

本发明涉及与细胞信号传导相关的基因的化学修饰的基因组序列，用于检测与细胞信号传导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，以及方法。用于确定与细胞信号传导相关的基因的遗传和/或表观遗传参数。