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(54) **Beta-catenin oligonucleotide michrochip and method for detecting beta-catenin mutations employing same**

Beta-Catenin Oligonukleotidemikrochip und Methode für das Ermitteln von beta-Catenin Mutationen unter Einsetzung dieses Mikrochips

Bêta-catenin microchip d' oligonucléotides et méthode pour détecter des bêta-catenin mutations utilisant ce microchip

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Description

Field of the Invention

[0001] The present invention relates to a β-catenin oligonucleotide microchip for detecting mutations in the mutational hot spot regions of β-catenin gene, a manufacturing process thereof and a method for detecting β-catenin mutations employing same.

Background of the Invention

[0002] β-Catenin, which functions as a downstream transcriptional activator in the Wnt signaling pathway, is a submembrane component of the cadherin-mediated cell-cell adhesion system (Abraham, S. C. et al., *Am. J. Pathol.* 158: 1005-1010, 2001; Abraham, S. C. et al., *Am. J. Pathol.* 158:1073-1078, 2001). APC (adenomatus polyposis coli) tumor suppressor protein, along with GSK-3β (glycogen synthase kinase-3β), promotes the phosphorylation of the serine/thereonine residues in exon 3 of the β-catenin gene (Abraham, S. C. et al., *Am. J. Pathol.* 158:1073-1078, 2001). Mutation of the *APC* gene or the β-catenin gene was found to result in the accumulation of β-catenin protein and the loss of β-catenin regulatory activity (Abraham, S. C. et al., *Am. J. Pathol.* 158:1073-1078, 2001). The majority of β-catenin mutations have been reported at specific GSK-3β phosphorylation sites, i.e., Ser-33, Ser-37, Thr-41, Ser-45, and other residues (Asp-32 and Gly-34) in many human cancers, including endometrial, gastric, ovarian, hepatoblastomas, and colorectal cancers (Saegusa, M. and Okayasu, I. *J. Pathol.* 194:59-67, 2001). In colorectal cancers, various frequencies of the β-catenin mutations have been reported, ranging from 0 to 16% (Nilbert, M. and Rambech, E. *Cancer Genet. Cytogenet.* 128:43-45, 2001; Mirabelli-Primdahl, L. et al., *Cancer Res.* 59:3346-51, 1999). Most β-catenin mutations are restricted at some codons in exon 3, and substitution mutations causing amino acid changes predominate in the β-catenin gene (Devereux, T. R. et al., *Mol. Carcinog.* 31:68-73, 2001; Udatsu, Y et al., *Pediatr. Surg. Int.* 17:508-512, 2001; Koch, A. et al., *Cancer Res.* 59:269-273, 1999; de La Coste, A. et al., *Proc. Natl. Acad. Sci. USA* 95:8847-8851, 1998; Morin, Bioessays, 21, 1021-1030).

[0003] Although it seems easy to detect β-catenin gene mutations using conventional methods, such as PCR-SSCP (single strand conformation polymorphism) and direct sequencing, technical problems associated with the low sensitivity of such β-catenin mutation detection methods have been reported (Abraham, S. C. et al., *Am. J. Pathol.* 158:1005-1010, 2001). Thus, there has been a need to develop a more reliable and faster mutation detection technique for β-catenin gene which can be used for various cancer studies, e.g., elucidation of the Wnt signaling related mechanism.

[0004] Studies have suggested that the high frequency MSI (microsatellite instability-H, MSI-H) colorectal cancer is not linked to APC mutations (Mirabelli-Primdahl, L. et al., *Cancer Res.* 59:3346-51, 1999), and that β-catenin gene mutations are mainly induced in MSI-H colorectal carcinomas **(Mirabelli-Primdahl, L. et al.,** *Cancer Res.* 59:3346-51, 1999; Shitoh, K. et al., *Genes Chromosomes Cancer* 30:32-37, 2001).

[0005] Traverso et al. used MSI in the stool as a marker for the diagnosis of proximal colon cancers in stools (Traverso, G. et al., *Lancet.* 359:403-404, 2002), and several other markers, such as *APC, p53,* long DNA and *K-ras,* have been also used for colorectal cancer diagnosis using fecal DNA (Ahlquist, D. A. et al., *Gastroenterology* 119:1219-1227, 2000; Dong S. M. et al., *J. Natl. Cancer. Inst.* 93:858-865, 2001).

[0006] The fact that β-catenin mutations are prone to occur in proximal colon cancers suggests β-catenin mutations might be used to diagnose proximal colon cancer. Accordingly, the present inventors have developed a β-catenin oligonucleotide microchip manufactured by fixing oligonucleotides on the surface of a solid matrix using an automatic microarrayer, the oligonucleotides being designed to detect various mutations at mutational hot spot regions of β-catenin gene. The β-catenin oligonucleotide microchip of the present invention can be used in studies to detect β-catenin mutations and to unravel the signal transduction mechanism and tumorigenesis related to β-catenin gene.

Summary of the Invention

[0007] Accordingly, an object of the present invention is to provide a β-catenin oligonucleotide microchip which can be used as a fast and reliable genetic diagnostic device for studying the signal transduction mechanism and tumorigenesis related to β-catenin gene.

[0008] In accordance with one aspect of the present invention, there is provided a β-catenin oligonucleotide microchip for detecting β-catenin mutations comprising a plurality of oligonucleotides fixed on the surface of a solid matrix, wherein the oligonucleotides are designed to detect mutations in the mutational hot spots of β-catenin gene and a manufacturing process thereof.

[0009] In accordance with still another aspect of the present invention, there is provided a method for detecting β-catenin mutations employing same.

Brief Description of the Drawings

[0010] The above and other objects and features of the present invention will become apparent from the following description of the invention, when taken in conjunction with the accompanying drawings which respectively show;

Fig. 1: the result of detecting β-catenin mutation in colon cancer tissue using the inventive β-catenin oligonucleotide microchip;

Fig. 2: the direct sequencing result of colon cancer tissue which has a β-catenin mutation confirmed by the inventive β-catenin oligonucleotide microchip;

Fig. 3: the PCR-SSCP analysis result of colon cancer tissue which has a β-catenin mutation confirmed by the inventive β-catenin oligonucleotide microchip.

Detailed Description of the Invention

[0011] The present invention provides a β-catenin oligonucleotide microchip (hereinafter, referred to as "β-catenin oligo chip") for detecting β-catenin mutations, which comprises oligonucleotides fixed on the surface of a solid matrix using an automatic microarrayer, wherein the oligonucleotides are capable of detecting various mutations at mutational hot spot regions of β-catenin gene.

[0012] First, the oligonucleotides are designed to detect all possible missense mutations and in-frame deletions at 11 codons (codons 29, 31, 32, 33, 34, 35, 37, 38, 41, 45 and 48) in exon 3, mutational hot spots of β-catenin gene.

[0013] β-Catenin mutations have been identified in a variety of human malignancies, most of being missense mutations resctricted at hot-spot areas in exon 3. β-Catenin mutations are known to be associated with colorectal cancers with MSI. More than 70% of β-catenin mutations have been reported in colorectal cancers, and about 90% of those at the 11 codons in the hot spot region (codons 29, 31, 32, 33, 34, 35, 37, 38, 41, 45 and 48).

[0014] The present invention provides oligonucleotides which can be used to detect all possible mutations at the above mentioned hot spots of β-catenin gene, which occur at a frequency of more than 90% of all cases examined. Therefore, the β-catenin oligo chip of the present invention makes it possible to detect mutation at a confidence level of over 90%. In addition, since the oligonucleotides used in the inventive β-catenin oligo chip are designed to detect all possible missense mutations at the 11 codons, it is capable of detecting any missense mutation at these codons which have not yet been discovered. Further, the inventive β-catenin oligo chip also includes the oligonucleotides for detecting in-frame deletion (3-bp deletion) at each of the hot spot codons. Namely, as the inventive oligonucleotides are specifically designed to detect mutations at the hot spots of β-catenin gene taking the gene characteristics into consideration, the inventive β-catenin oligo chip provides improved accuracy and efficiency in detecting β-catenin gene mutation.

[0015] According to one aspect of the present invention, the inventive β-catenin oligo chip has 121 types of oligonucleotides spotted and fixed on the surface of a solid matrix, the oligonucleotides being capable of detecting various missense mutations and in-frame deletions at the 11 hot spot codons of β-catenin gene. Each oligonucleotide is spotted 4 times horizontally for increased accuracy of measured signals. Nine oligonucleotides (M) are designed to cover all possible substitutions at each hot spot codon, and one oligonucleotide (W) for the wild type. Thus, a total of 10 oligonucleotides are designed to detect missense mutations for codons 23, 29, 31, 32, 33, 34, 35, 38, 41 and 48. Further, 11 oligonucleotides (D) are designed to detect in-frame deletions (3-bp deletion) for each hot spot codon. In total, the 121 oligonucleotides cover all substitutions and in-frame deletions in the above 11 codons of exon 3.

[0016] Specifically, used for codon 29 are 9 types of substituted oligonucleotides obtained by replacing TCT (serine) with ACT (threonine), GCT (alanine), CCT (proline), TAT (tyrosine), TGT (cytosine), TTT (phenylalanine), TCA (serine), TCG (serine) and TCC (serine), respectively, and one deletion oligonucleotide obtained by deleting 3 bp of TCT. Used for codon 31 are 9 types of substituted oligonucleotides obtained by replacing CTG (leucine) with ATG (methionine), TTG (leucine), GTG (valine), CAG (glutamine), CGG (arginine), CCG (proline), CTA (leucine), CTC (leucine) and CTT (leucine), respectively; and one deletion oligonucleotide obtained by deleting 3 bp of CTG. Used for codon 32 are 9 types of substituted oligonucleotides obtained by replacing GAC (aspartic acid) with CAC (histidine), TAC (tyrosine), AAC (asparagines), GCC (alanine), GTC (valine), GGC (glycine), GAG (glutamic acid), GAT (aspartic acid) and GAA (glutamic acid), respectively; and one deletion oligonucleotide obtained by deleting 3 bp of GAC. Used for codon 33 are 9 types of substituted oligonucleotides obtained by replacing TCT (serine) with ACT (threonine), GCT (alanine), CCT (proline), TGT (cysteine), TAT (tyrosine), TTT (phenylalanine), TCA (serine), TCG (serine) and TCC (serine), respectively; and one deletion oligonucleotide obtained by deleting 3 bp of TCT. Used for codon 34 are 9 types of substituted oligonucleotides obtained by replacing GGA (glycine) with TGA (stop codon), AGA (arginine), CGA (arginine), GTA (valine), GCA (alanine), GAA (glutamic acid), GGT (glycine), GGG (glycine) and GGC (glycine), respectively; and one deletion oligonucleotide obtained by deleting 3 bp of GGA. Used for codon 35 are 9 types of substituted oligonucleotides obtained by replacing ATC (isoleucine) with GTC (valine), CTC (leucine), TTC (phenylalanine), ACC (threonine), AGC (serine), AAC (asparagine), ATG (methionine), ATA (isoleucine) and ATT (isoleucine), respectively; and one deletion oligonu-

cleotide obtained by deleting 3 bp of ATC. Used for codon 37 are 9 types of substituted oligonucleotides obtained by replacing TCT (serine) with ACT (threonine), CCT (proline), GCT (alanine), TAT (tyrosine), TGT (cysteine), TTT (phenylalanine), TCA (serine), TCG (serine) and TCC (serine), respectively; and one deletion oligonucleotide obtained by deleting 3 bp of ACT. Used for codon 38 are 9 types of oligonucleotides obtained by replacing GGT (glycine) with AGT (serine), CGT (arginine), TGT (cysteine), GAT (aspartic acid), GCT (alanine), GTT (valine), GGA (glycine), GGG (glycine) and GGC (glycine), respectively; and one deletion oligonucleotide obtained by deleting 3 bp of GGT. Used for codon 41 are 9 types of substituted oligonucleotides obtained by replacing ACC (threonine) with TCC (serine), GCC (alanine), CCC (proline), AGC (serine), ATC (isoleucine), AAC (asparagine), ACA (threonine), ACT (threonine) and ACG (theronine), respectively; and one deletion oligonucleotide obtained by deleting 3 bp of ACC. Used for codon 45 are 9 types of substituted oligonucleotides obtained by replacing TCT (serine) with ACT (threonine), GCT (alanine), CCT (proline), TGT (cysteine), TAT (tyrosine), TTT (phenylalanine), TCA (serine), TCG (serine) and TCC (serine), respectively; and one deletion oligonucleotide obtained by deleting 3 bp of ACT. Used for codon 48 are 9 types of substituted oligonucleotides obtained by replacing GGT (glycine) with AGT (serine), TGT (cysteine), CGT (arginine), GAT (aspartic acid), GCT (alanine), GTT (valine), GGA (glycine), GGC (glycine) and GGG (glycine), respectively; and one deletion oligonucleotide obtained by deleting 3 bp of GGT.

[0017] One wild type of oligonucleotide (W) is designed for each codon to be directly compared with mutation types and to cover both homozygous and heterozygous mutations. For example, 12 oligonucleotides are spotted for codon 29, one is to detect a normal base sequence and the rest, the mutated base sequences. As a whole, 110 mutant oligonucleotides are designed for the 99 missense mutation types and 11 in-frame deletion types at the 11 hot spot codons, and 11 oligonucleotides, for the wild types and positive controls.

[0018] The β-catenin oligo chip of the present invention **is** manufactured by fixing 121 oligonucleotides on the surface of a solid matrix using an automatic microarrayer by a process comprising the steps of:

1) mixing each of the oligonucleotides in a micro spotting solution and distributing to a well plate;
2) spotting the oligonucleotide on the surface of a solid matrix using a microarrayer;
3) fixing the oligonucleotides on the solid matrix surface and washing;
4) denaturing the fixed oligonucleotides by soaking the solid matrix in 95°C water, and then, treating the solid matrix with a sodium borohydride solution; and
5) washing and drying the solid matrix.

[0019] Each of the oligonucleotides used in step (1) preferably has a functional group that can be used to form a stable bond with the solid matrix surface. For example, each oligonucleotide may be linked with a 12 carbon spacer having a 5' amino modification, e.g., H_2N-$(CH_2)_{12}$-oligonucleotide. This amine group undergoes Schiff's base reaction with an aldehyde group on the solid matrix to form a firm bond therebetween. The 12 carbon spacer serves to enhance the hybridization rate by facilitating the contact between the oligonucleotide and a fluorescent dye-labeled target DNA.

[0020] The micro spotting solution used in step (1) may contain suitable salts and polymers to facilitate the application of the oligonucleotides on the solid matrix.

[0021] The solid matrix used in step (2) may be made of glass; modified silicone; plastic cassette; or polymer such as polycarbonate or a gel thereof. The surface of a solid matrix may be coated with a chemical compound that can serve to bind the oligonucleotide to the matrix substrate. Preferable chemicals that can be used for such coating have functional groups such as aldehyde or amine groups. In one preferred embodiment, the present invention uses a slide glass coated with an aldehyde.

[0022] According to one embodiment of steps (1) and (2), a total of 484 oligonucleotides are arranged in a specified manner on a solid matrix using an automatic pin microarrayer. Each oligonucleotide spot is preferably of circular shape with a diameter ranging from 100 to 500 μm. A preferable example of the solid matrix is a 3.7 cm $\times$ 7.6 cm slide glass, which can accommodate approximately 100 to 10,000 spots per chip. Preferably, a total of 484 oligonucleotide spots, each of 130 μm diameter, may be arranged in multiple columns and rows at intervals of 200 to 800 μm.

[0023] In step (3), the oligonucleotides are fixed on the solid matrix surface by way of forming covalent bonds between the amine groups of the oligonucleotide and the aldehyde groups of the solid matrix via Schiff's base reaction. Free unreacted oligonucleotides are removed by washing the solid matrix with SDS, SSC, SSPE, etc.

[0024] In step (4), the fixed oligonucleotides are denatured, and unreacted aldehyde groups remaining on the solid matrix are reduced and inactivated by sodium borohydride treatment.

[0025] The β-catenin oligo chip of the present invention manufactured by the above process may be advantageously used to detect gene mutation, and this inventive method is much simpler and more economical than any of the conventional gene mutation detection methods: It takes several days to months on the average when the presence of gene mutation is examined using such conventional methods as SSCP (single strand conformation polymorphism), PTT (protein truncation test), RFLP (restriction fragment length polymorphism), cloning, direct sequencing, etc. However, analysis of a DNA sample for β-catenin gene mutation takes less than 10 to 11 hours when the inventive β-catenin oligo chip is

employed. In addition, the β-catenin oligo chip of the present invention can be manufactured much more simply at a much less production cost than conventional chips. Once the required oligonucleotides are synthesized, it is possible to mass-produce the inventive slides. The amounts of reagents required when the inventive β-catenin oligo chip is used are far less than those required in any of the conventional methods.

[0026] The β-catenin oligo chip of the present invention is easy to manufacture using a pin microarrayer, while the existing Affymetrix oligo chip must be prepared using a complicated and expense photolithography technique.

[0027] Further, it is possible with the β-catenin oligo chip of the present invention to purify and modify the oligonucleotides, in contrast to the case of Affymetrix oligo chip which is prepared by directly synthesizing oligonucleotides on the surface of a solid matrix, wherein it is not possible to purify or modify the oligonucleotides. Accordingly, the inventive β-catenin oligo chip is capable of providing greater experimental accuracy than was possible before.

[0028] The present invention provides a method for detecting the β-catenin mutation employing the β-catenin oligo chip, which comprises the steps of:

1) preparing a fluorescent dye-labeled DNA sample from the blood of a subject patient;
2) reacting the labeled DNA sample with oligonucleotide spots on the β-catenin oligo chip;
3) washing the reacted oligo chip to remove unbound sample DNA;
4) detecting the mode of hybridization of specific oligonucleotide spots using a fluorescence reader; and
5) examining the presence of gene mutation.

[0029] In step (1), a DNA sample is prepared by incorporating a fluorescent dye into a blood DNA sample obtained from a subject patient. In the hybridization of fluorescent dye-labeled DNA with certain oligonucleotide spot on the oligo chip, it can be analyzed with a fluorescence reader using an appropriate software. Preferable fluorescent dyes include, but are not limited to, Cy5, Cy3, Texas Red, Fluorescein and Lissamine.

[0030] In step (2), the florescent dye-labeled DNA sample prepared in step (1) is mixed with a hybridization solution and transferred to each of the oligonucleotide. The hybridization reaction is performed in a 45~60°C incubator saturated with water vapor for 3~9 hours. Then, the oligo chip is washed to remove unbound sample DNA and dried (step 3), and the resulting fluorescence is analyzed with a fluorescence reader using an appropriate software (step 4). In step (5), setting a maximum value at 99% reliable range as a threshold value, any signal showing a fluorescence level higher than the threshold is regarded positive for the presence of mutation.

[0031] The β-catenin oligo chip of the present invention can be effectively used to diagnose such cancer as colorectal carcinomas, endometrial cancer, stomach cancer, ovary cancer, hepatoblastoma cancer, etc. Since β-catenin gene function as a downstream transcriptional activator in the Wnt signaling pathway, the inventive β-catenin oligo chip can be used as an effective diagnostic tool for the study of signal transduction mechanism and tumorigenesis related to β-catenin gene.

[0032] The present invention investigated 74 colorectal carcinomas and 31 colorectal cancer cell lines for the presence of β-catenin mutations (see Fig. 1). All 5 β-catenin mutations were identified in proximal colon cancers (N=34), but β-catenin mutations were absent in 40 distal colorectal cancers. Four out of the 5 β-catenin mutations were point mutations at codons 32, 41 and 45, and the remaining one was in-frame deletion (3 bp deletion) at codon 45. In 31 colorectal cancer cell lines, 4 β-catenin mutations were identified. Three of these 4 mutations occurred at codon 45, and the remaining one occurred at codon 41.

[0033] In total 9 mutations were identified in the 74 colorectal carcinomas and 31 colorectal cancer cell lines. Six of the 9 mutations were found at codon 45 and 2 were at codon 41. Of the 6 mutations at codon 45, 4 were the identical missense mutations (TCT→TTT, Ser→Phe; in samples 395, 400, SNU-1047 and LSI17T) and 2 were the same in-frame deletion in samples 396 and HCT116. Codons 41 and 45 are known as GSK-3β phosphorylation sites and mutations at these sites might cause nuclear β-catenin accumulation (Saegusa, M. and Okayasu, I. *J. Pathol.* 194:59-67, 2001).

[0034] The remaining β-catenin mutation occurred at codon 32 in colon tissue 207. It has been proposed that codon 32 is important for β-catenin ubiquitination and proteasome-dependent degradation (Tong, J. H. et al., *Cancer Lett.* 163: 125-130, 1999). Mutations at codon 32 might influence serine 33 accessibility by GSK-3β kinase, thus preventing its phosphorylation (Koch, A. et al., *Cancer Res.* 59:269-273, 1999). It has been reported that specific codon 45 mutation (Ser45Phe) was frequent in colorectal carcinomas, and that codon 41 mutations, which predominate in hepatoblastomas, are rare in colorectal carcinomas (Koch, A. et al., *Cancer Res.* 59:269-273, 1999).

[0035] In the present invention, three of the 5 β-catenin mutations from colorectal cancers and three of the 4 β-catenin mutations from cell lines were identified at codon 45, and two of the 6 mutations at codon 45 were in-frame deletions. The in-frame deletion at codon 45 was previously reported in a colorectal cancer cell line and in colorectal carcinomas, but not in other types of cancer (Ilyas, M. et al., *Proc. Natl. Acad. Sci. USA* 94:10330-10334, 1997; Muller, O. et al., *Genes Chromosomes Cancer* 22:37-41, 1998). The in-frame deletion at codon 45 may result in the loss of highly conserved serine residues in a region of the protein that serves as a target for the enzyme GSK-3β (Ilyas, M. et al., *Proc. Natl. Acad. Sci. USA* 94:10330-10334, 1997). These results indicate that codon 45 mutations, including the in-frame

deletion, are common in colorectal carcinomas but are not common in other types of cancer.

[0036] Mutational analysis of the β-catenin gene was performed using the oligonucleotide microarray. As the result of mutational analysis using the inventive β-catenin oligo chip, the 9 β-catenin mutation positive samples in a total of 60 samples were detected. The present inventors compared the 9 β-catenin mutations detected by the β-catenin oligo chip with several techniques, e.g., PCR-SSCP, DHPLC, direct sequencing, cloning-sequencing (see Table 2). Automatic direct sequencing, which has been widely used for mutational analysis was not capable of clearly detecting 2 of the 9 β-catenin mutations (see Fig, 2), and PCR-SSCP also missed one β-catenin mutation (see Fig. 3). These results might have been caused by excessive wild-type DNA in cancer tissues or by the low sensitivity of these two methods.

[0037] In the MSI study using the BAT-26 marker, the present invention confirms that MSI is intimately associated with proximal colon cancer, which agrees with previous reports ($p<0.01$) (Mirabelli-Primdahl, L. et al., *Cancer Res.* 59: 3346-51, 1999; Traverso, G. et al., *Lancet.* 359:403-404, 2002). MSI was shown in 10 of 34 proximal colon carcinomas (29%), but in only 2 of 40 distal colorectal carcinomas (5%). In terms of the correlation between MSI and β-catenin mutations, β-catenin mutations were detected in 5 of the 12 (42%) colorectal carcinomas with MSI, but none of the 62 (0%) MSS colorectal carcinomas were found to harbor β-catenin mutations. All 5 β-catenin mutations detected in colon carcinomas with MSI were found in proximal colon cancers. These results confirm that MSI is involved in β-catenin mutations and demonstrate that β-catenin mutations are directly associated with proximal colon cancer.

[0038] It has been previously suggested that β-catenin mutations account for approximately are half of colorectal cancers with intact APC (Sparks, A. B. et al., *Cancer Res.* 59998:1130-1134, 1998). In the present invention, only one colorectal cancer cell line (SNU-1047) of the 9 samples with β-catenin mutations had *APC* mutations in the MCR. Recently, several groups have tried to diagnose colorectal cancers by using molecular markers such as *APC, p53,* long DNA, *K-ras,* etc (Traverso, G. et al., *Lancet.* 359:403-404, 2002; Ahlquist, D. A. et al., *Gastroenterology* 119:1219-1227, 2000; Dong, S. M. et al., *J. Natl. Cancer Inst*. 93:858-865, 2001). Three of 5 markers including MSI have been used for colorectal cancer diagnosis using fecal DNA (Traverso, G. et al., *Lancet*. 359:403-404, 2002). In addition, MSI has been used for the diagnosis of proximal colon cancers, which is difficult to detect because, among colorectal cancers, they are located furthest from the anus. β-Catenin may be a diagnostic marker for proximal colon cancer if β-catenin mutations correlate with the tumor's location in the proximal colon. The results of the present invention show MSI in 29% and β-catenin mutations in 15% of proximal colon cancers, respectively. Although all samples with β-catenin mutations exhibited MSI, β-catenin, alone or with MSI, may be used for the diagnosis of proximal colon cancer. Practically, such a system should not only be highly automated but also be usable as a high throughout diagnostic tool, especially if the substrate used in fecal DNA.

[0039] The following Examples and Test Examples are given for the purpose of illustration only, and are not intended to limit the scope of the invention.

Example 1: Manufacture of β-catenin oligo chip

[0040] Nine oligonucleotides were designed to cover all possible substitutions at 11 mutational hot spot codons of β-catenin gene (codons 23, 29, 31, 32, 34, 35, 37, 38, 41, 45 and 48), and one oligonucleotide for the wild-type. Further, one oligonucleotide was designed to detect in-frame deletions (3-bp deletion) at each of those codons. A total of 121 oligonucleotides was designed to cover all substitutions and in-frame deletions at the above 11 codons of exon 3.

[0041] The oligonucleotides described in SEQ ID Nos. 1, 12, 23, 34, 45, 56, 67, 78, 89, 100 and 111 are wild types. Oligonucleotides having missense mutation at one of the hot spot codons are: the oligonucleotides described in SEQ ID Nos. 2 to 10, at codon 29; the oligonucleotides described in SEQ ID Nos. 13 to 21, at codon 31; the oligonucleotides described in SEQ ID Nos. 24 to 32, at codon 32; the oligonucleotides described in SEQ ID Nos. 35 to 43, at codon 33; the oligonucleotides described in SEQ ID Nos. 46 to 54, at codon 34; the oligonucleotides described in SEQ ID Nos. 57 to 65, at codon 35; the oligonucleotides described in SEQ ID Nos. 68 to 76, at codon 37; the oligonucleotides described in SEQ ID Nos. 79 to 87, at codon 38; the oligonucleotides described in SEQ ID Nos. 90 and 98, at codon 41; the oligonucleotides described in SEQ ID Nos. 101 and 109, at codon 45; and the oligonucleotides described in SEQ ID Nos. 112 and 120, at codon 48.

[0042] Further, oligonucleotides having in-frame deletion at one of the hot spot codons are: the oligonucleotide described in SEQ ID No. 11, at codon 29; the oligonucleotide described in SEQ ID No. 22, at codon 31; the oligonucleotide described in SEQ ID No. 33, at codon 32; the oligonucleotide described in SEQ ID No. 44, at codon 33; the oligonucleotide described in SEQ ID No. 55, at codon 34; the oligonucleotide described in SEQ ID No. 66, at codon 35; the oligonucleotide described in SEQ ID No. 77, at codon 37; the oligonucleotide described in SEQ ID No. 88, at codon 38; the oligonucleotide described in SEQ ID No. 99, at codon 41; the oligonucleotide described in SEQ ID No. 110, at codon 45; and the oligonucleotide described in SEQ ID No. 121, at codon 48.

[0043] All 121 oligonucleotides, each having a 12-carbon spacer to 5'-terminal modified with an amine residue which can undergo Schiff's base reaction with aldehyde groups, were obtained from MWG-Biotech (Ebersberg, Germany) and purified by HPLC.

<Table 1a>

SEQ ID No.	Oligonucleotide	Exon	Codon	Sequence
1	29W	3	29	5'-CAGCAACAGTCTTACCTGGAC-3'
2	29M1			5'- GCAGCAACAG**ACT**TACCTGGA-3'
3	29M2			5'- GCAGCAACAG**GCT**TACCTGGA-3'
4	29M3			5'- GCAGCAACAG**CCT**TACCTGGA-3'
5	29M4			5'-CAGCAACAG**TAT**TACCTGGAC-3'
6	29M5			5'-CAGCAACAG**TGT**TACCTGGAC-3'
7	29M6			5'-CAGCAACAG**TTT**TACCTGGAC-3'
8	29M7			5'-AGCAACAG**TCA**TACCTGGACT-3'
9	29M8			5'-AGCAACAG**TCG**TACCTGGACT-3'
10	29M9			5'-AGCAACAG**TCC**TACCTGGACT-3'
11	29D			5'-GGCAGCAACAGTACCTGGACT-3'
12	31W		31	5'-CAGTCTTACCTGGACTCTGGA-3'
13	31M1			5'-ACAGTCTTAC**ATG**GACTCTGG-3'
14	31M2			5'-ACAGTCTTAC**TTG**GACTCTGG-3'
15	31M3			5'-ACAGTCTTAC**GTG**GACTCTGG-3'
16	31M4			5'-CAGTCTTAC**CAG**GACTCTGGA-3'
17	31M5			5'-CAGTCTTAC**CGG**GACTCTGGA-3'
18	31M6			5'-CAGTCTTAC**CCG**GACTCTGGA-3'
19	31M7			5'-AGTCTTAC**CTA**GACTCTGGAA-3'
20	31M8			5'-AGTCTTAC**CTC**GACTCTGGAA-3'
21	31M9			5'-AGTCTTAC**CTT**GACTCTGGAA-3'
22	31D			5'-AACAGTCTTACGACTCTGGAA-3'

<Table 1b>

SEQ ID No.	Oligonucleotide	Exon	Codon	Sequence
23	32W	3	32	5'-TCTTACCTGGACTCTGGAATC-3'
24	32M1			5'-GTCTTACCTG**CAC**TCTGGAAT-3'
25	32M2			5'-GTCTTACCTGB**TAC**TCTGGAAT-3'
26	32M3			5'-GTCTTACCTG**AAC**TCTGGAAT-3'
27	32M4			5'-TCTTACCTG**GCC**TCTGGAATC-3'
28	32M5			5'-TCTTACCTG**GTC**TCTGGAATC-3'
29	32M6			5'-TCTTACCTG**GGC**TCTGGAATC-3'
30	32M7			5'-CTTACCTG**GAG**TCTGGAATCC-3'
31	32M8			5'-CTTACCTG**GAT**TCTGGAATCC-3'
32	32M9			5'-CTTACCTG**GAA**TCTGGAATCC-3'
33	32D			5'-AGTCTTACCTGTCTGGAATCC-3'
34	33W		33	5'-TACCTGGACTCTGGAATCCAT-3'

(continued)

SEQ ID No.	Oligonucleotide	Exon	Codon	Sequence
35	33M1			5'-TTACCTGGAC**ACT**GGAATCCA-3'
36	33M2			5'-TTACCTGGAC**GCT**GGAATCCA-3'
37	33M3			5'-TTACCTGGAC**CCT**GGAATCCA-3'
38	33M4			5'-TACCTGGAC**TGT**GGAATCCAT-3'
39	33M5			5'-TACCTGGAC**TAT**GGAATCCAT-3'
40	33M6			5'-TACCTGGAC**TTT**GGAATCCAT-3'
41	33M7			5'-ACCTGGAC**TCA**GGAATCCATT-3'
42	33M8			5'-ACCTGGAC**TCG**GGAATCCATT-3'
43	33M9			5'-ACCTGGAC**TCC**GGAATCCATT-3'
44	33D			5'-TTACCTGGACGGAATCCATTC-3'

<Table 1c>

SEQ ID No.	Oligonucleotide	Exon	Codon	Sequence
45	34W	3	34	5'-CTGGACTCTGGAATCCATTCT-3'
46	34M1			5'-CCTGGACTCT**TGA**ATCCATTC-3'
47	34M2			5'-CCTGGACTCT**AGA**ATCCATTC-3'
48	34M3			5'-CCTGGACTCT**CGA**ATCCATTC-3'
49	34M4			5'-CTGGACTCT**GTA**ATCCATTCT-3'
50	34M5			5'-CTGGACTCT**GCA**ATCCATTCT-3'
51	34M6			5'-CTGGACTCT**GAA**ATCCATTCT-3'
52	34M7			5'-TGGACTCT**GGT**ATCCATTCTG-3'
53	34M8			5'-TGGACTCT**GGG**ATCCATTCTG-3'
54	34M9			5'-TGGACTCT**GGC**ATCCATTCTG-3'
55	34D			5'-CCTGGACTCTATCCATTCTGG-3'
56	35W		35	5'-GACTCTGGAATCCATTCTGGT-3'
57	35M1			5'-GGACTCTGGA**GTC**CATTCTGG-3'
58	35M2			5'-GGACTCTGGA**CTC**CATTCTGG-3'
59	35M3			5'-GGACTCTGGA**TTC**CATTCTGG-3'
60	35M4			5'-GACTCTGGA**ACC**CATTCTGGT-3'
61	35M5			5'-GACTCTGGA**AGC**CATTCTGGT-3'
62	35M6			5'-GACTCTGGA**AAC**CATTCTGGT-3'
63	35M7			5'-ACTCTGGA**ATG**CATTCTGGTG-3'
64	35M8			5'-ACTCTGGA**ATA**CATTCTGGTG-3'
65	35M9			5'-ACTCTGGA**ATT**CATTCTGGTG-3'
66	35D			5'-GGACTCTGGACATTCTGGTGC-3'

<Table 1d>

SEQ ID No.	Oligonucleotide	Exon	Codon	Sequence
67	37W	3	37	5'-GGAATCCATTCTGGTGCCACT-3'
68	37M1			5'-TGGAATCCAT**ACT**GGTGCCAC-3'
69	37M2			5'-TGGAATCCAT**CCT**GGTGCCAC-3'
70	37M3			5'-TGGAATCCAT**GCT**GGTGCCAC-3'
71	37M4			5'-GGAATCCAT**TAT**GGTGCCACT-3'
72	37M5			5'-GGAATCCAT**TGT**GGTGCCACT-3'
73	37M6			5'-GGAATCCAT**TTT**GGTGCCACT-3'
74	37M7			5'-GAATCCAT**TCA**GGTGCCACTA-3'
75	37M8			5'-GAATCCAT**TCG**GGTGCCACTA-3'
76	37M9			5'-GAATCCAT**TCC**GGTGCCACTA-3'
77	37D			5'-TGGAATCCATGGTGCCACTAC-3'
78	38W		38	5'-ATCCATTCTGGTGCCACTACC-3'
79	38M1			5'-AATCCATTCT**AGT**GCCACTAC-3'
80	38M2			5'-AATCCATTCT**CGT**GCCACTAC-3'
81	38M3			5'-AATCCATTCT**TGT**GCCACTAC-3'
82	38M4			5'-ATCCATTCT**GAT**GCCACTACC-3'
83	38M5			5'-ATCCATTCT**GCT**GCCACTACC-3'
84	38M6			5'-ATCCATTCT**GTT**GCCACTACC-3'
85	38M7			5'-TCCATTCT**GGA**GCCACTACCA-3'
86	38M8			5'-TCCATTCT**GGG**GCCACTACCA-3'
87	38M9			5'-TCCATTCT**GGC**GCCACTACCA-3'
88	38D			5'-AATCCATTCTGCCACTACCAC-3'

<Table 1e>

SEQ ID No.	Oligonucleotide	Exon	Codon	Sequence
89	41 W	3	41	5'-GGTGCCACTACCACAGCTCCT-3'
90	41M1			5'-TGGTGCCACT**TCC**ACAGCTCC-3'
91	41M2			5'-TGGTGCCACT**GCC**ACAGCTCC-3'
92	41M3			5'-TGGTGCCACT**CCC**ACAGCTCC-3'
93	41M4			5'-GGTGCCACT**AGC**ACAGCTCCT-3'
94	41M5			5'-GGTGCCACT**ATC**ACAGCTCCT-3'
95	41M6			5'-GGTGCCACT**AAC**ACAGCTCCT-3'
96	41M7			5'-GTGCCACT**ACA**ACAGCTCCTT-3
97	41M8			5'-GTGCCACT**ACT**ACAGCTCCTT-3'
98	41M9			5'-GTGCCACT**ACG**ACAGCTCCTT-3'
99	41D			5'-TGGTGCCACTACAGCTCCTTC-3'
100	45W		45	5'-ACAGCTCCTTCTCTGAGTGGT-3'
101	45M1			5'-CACAGCTCCT**ACT**CTGAGTGG-3'

(continued)

SEQ ID No.	Oligonucleotide	Exon	Codon	Sequence
102	45M2			5'-CACAGCTCCT**GCT**CTGAGTGG-3'
103	45M3			5'-CACAGCTCCT**CCT**CTGAGTGG-3'
104	45M4			5'-ACAGCTCCT**TGT**CTGAGTGGT-3'
105	45M5			5'-ACAGCTCCT**TAT**CTGAGTGGT-3'
106	45M6			5'-ACAGCTCCT**TTT**CTGAGTGGT-3'
107	45M7			5'-CAGCTCCT**TCA**CTGAGTGGTA-3'
108	45M8			5'-CAGCTCCT**TCG**CTGAGTGGTA-3'
109	45M9			5'-CAGCTCCT**TCC**CTGAGTGGTA-3'
110	45D			5'-CCACAGCTCCTCTGAGTGGTA-3'

<Table 1f>

SEQ ID No.	Oligonucleotide	Exon	Codon	Sequence
111	48W	3	48	5'-TCTCTGAGTGGTAAAGGCAAT-3'
112	48M1			5'-TTCTCTGAGT**AGT**AAAGGCAA-3'
113	48M2			5'-TTCTCTGAGT**TGT**AAAGGCAA-3'
114	48M3			5'-TTCTCTGAGT**CGT**AAAGGCAA-3'
115	48M4			5'-TCTCTGAGT**GAT**AAAGGCAAT-3'
116	48M5			5'-TCTCTGAGT**GCT**AAAGGCAAT-3'
117	48M6			5'-TCTCTGAGT**GTT**AAAGGCAAT-3'
118	48M7			5'-CTCTGAGT**GGA**AAAGGCAATC-3'
119	48M8			5'-CTCTGAGT**GGC**AAAGGCAATC-3'
120	48M9			5'-CTCTGAGT**GGG**AAAGGCAATC-3'
121	48D			5'-TTCTCTGAGTAAAGGCAATCC-3'

[0044] Each oligonucleotide was mixed with a micro spotting solution (TeleChem International Inc, Sunnyvale, CA) at a mix ratio of 1:1, and 40 $\mu\ell$ of each oligonucleotide was transferred to a 96 well plate. Twenty pmol/$\mu\ell$ of oligonucleotides were spotted for codons 37, 41 and 45, and 40 pmol/$\mu\ell$ for the remaining eight codons. After the charged 96 well plate was placed in a pin microarrayer (Microsys 5100 Cartesian, Cartesian Technologies Inc, Irvine, CA), each oligonucleotide was printed on an aldehyde-coated glass slide (26$\times$ 76$\times$ 1 mm, CEL Associates Inc, Houston, TX). Spots, each of 130 μm diameter in size, were arranged in multiple columns and rows at intervals of 300 μm. The glass slide spotted with the oligonucleotides was washed twice with 0.2% SDS, and then, once with distilled water. The glass slide was soaked in hot water (95°C) to denature the oligonucleotides, and then, in sodium borohydride solution for 5 minutes to inactivate unreacted aldehyde groups. Then, the glass slide was washed twice with 0.2% SDS, and then, once with distilled water, centrifuged, and dried.

Example 2: Examination of β-catenin mutation using β-catenin oligo chip

(Step 1) Preparation of DNA sample

[0045] Specimens of 74 colorectal carcinomas were collected from Seoul National University Hospital and 31 colorectal cancer cell lines were obtained from the Korean Cell Line Bank (KCLB). Of the 74 colorectal cancers, 34 were from the proximal colon (cecum to splenic flexure) and 40 were from the distal colorectum (splenic flexure to rectum). Of 31 colorectal cancer cell lines, 7 originated from the proximal colon and 6 from the distal colorectum. The origin of the remaining 18 colorectal cancer cell lines was unknown. The gastric cancer cell lines SNU-638 and SNU-719 were used

as positive controls for β-catenin mutations (Woo, D. K. et al., *Int. J. Cancer* 95:108-113, 2001). SNU-638 has β-catenin mutation at codon 41 (ACC→GCC, Thr→ Ala) and SNU-791 mutation at codon 34 (GGA→GTA, Gly→Val).

[0046] Genomic DNA was extracted from frozen specimens using TRI reagent (Molecular Research Center, Cincinnati, OH, USA) or the automatic magnetic bead-based system (KingFisher, ThermoLabsystems, Finland), following the manufacture's instructions. To generate a fluorescent dye-labeled DNA sample, PCR amplification was performed using the extracted DNA as a template and two pairs of primers described in SEQ ID Nos. 122 to 125 (MWG-Biotech, Ebersberg, Germany). As shown in table 2, PCR primers of SEQ ID Nos. 122 and 123 for exon 3 were used as described in Mirabelli-Primdahl, L. et al. (*Cancer Res.* 59:3346-51, 1999), and PCR primers of SEQ ID Nos. 124 and 125 for interstitial large deletion of β-catenin gene were used as described in Udatsu Y. et al. (*Pediatr. Surg. Int.* 17:508-512, 2001).

<Table 2>

SEQ ID No.	Primer	Amplified region	Amplified size	Sequence
122	Exon-3F	Exon 3	218 bp	5'-GATTTGATGGAGTTGGACATGG-3'
123	Exon-3R			5'-TGTTCTTGAGTGAAGGACTGAG-3'
124	Long-3F	Part of exon 2~ part of exon 3	1115 bp	5'-AAAATCCAGCGTGGACAATGG-3'
125	Long-3R			5'-TGTGGCAAGTTCTGCATCATC-3'

[0047] Each PCR reaction solution (25 μℓ) contained 100 ng of genomic DNA, 10 pmol of each primer, 40 μM of dCTP, 20 μM of fluorescent dye Cy5-dCTP (MEN) or Cy3-dCTP (Amersham-biotech Ltd., Buckinghamshire, UK). Reactions were initiated by denaturation for 5 min at 94 °C in a programmable thermal cycler (Perkin Elmer Cetus 9600; Roche Molecular Systems, Inc., NJ). PCR conditions consisted of 35 cycles of 30 sec at 94°C, 30 sec at 56°C, and 1 min at 72°C , with a final elongation of 7 min at 72 °C. After the PCR amplification, Cy5- or Cy3-labeled PCR product was purified using a purification kit (Qiagen Inc, Valencia, CA) and digested with 0.25 U of DNase I (Takara, Shiga, Japan) at 25°C for 10 min. Remaining enzyme was inactivated at 85°C for 10 min, removed by repeating the above purification procedure, and the Cy5- or Cy3-labeled DNA sample was recovered.

(Step 2) Hybridization reaction and analysis

[0048] The Cy5- or Cy3-labeled DNA samples prepared in step (1) were mixed and resuspended in 5× hybridization solution (TeleChem International Inc, Sunnyvale, CA) to a volume of 2~4 μℓ. Two μℓ of the mixed DNA sample prepared in Example 1 was dropped on the glass slide and the glass slide was covered with a cover glass. The hybridization reaction was performed by incubating the glass slide in a saturated vapor tube at 56°C for 3 hours. The hybridized glass slide was rinsed at room temperature in a buffer of 0.2% SDS + 2× SSC for 15~30 min, and then, in distilled water for 5 min, followed by centrifuging and drying. The glass slide was scanned using a ScanArray Lite (Parkard Instrument Co, Meriden, CT) and analyzed using Imagine (Biodiscovery, version 4.2) and Quantitative Microarray analysis software (QuantArray, version 2.0).

[0049] Eleven wild type signals were compared to each other and adjusted to be equal by signal normalization. The remaining 110 signals at each codon were also adjusted in the same way as the wild type signals. After signal normalization, all signals were re-analyzed as previously described (Kim, I. J. et al., *Clin. Cancer Res.* 8:457-463, 2002). The mean (BA) and the standard deviation (BSD) of the background signals were calculated, and the cutoff level was established to be BA+2.58BSD. (BA+2.58BSD) indicated the upper limit of the 99% confidence interval, and signals over this value were identified as meaningful signals. All data analysis was carried out using a SigmaPlot (SPSS Inc., San Rafael, CA), and means and standard deviations were calculated using Microsoft Excel program. The results of mutational analyses of colorectal carcinomas and colorectal cancer cell lines are shown in Table 3.

<Table 3>

Sample		β-catenin mutation			MSI	APC
Name	Type	Location	Codon	Mutation		mutation
207	Tumor	Ascending[c]	32	GAC→ AAC	+[f]	-[g]
395	Tumor	Ascending	45	TCT→ TTT	+	-
396	Tumor	Ascending	45	In-frame deletion	+	-

(continued)

Sample		β-catenin mutation			MSI	APC
Name	Type	Location	Codon	Mutation		mutation
400	Tumor	Ascending	45	TCT→ TTT	+	-
435	Tumor	Ascending	41	ACC→ GCC	+	-
SNU-407[a]	Cell line	Transverse[d]	41	ACC→ GCC	+	-
SNU-1047[a]	Cell line	Transverse	45	TCT→TTT	+	4107delC
LS174T[b]	Cell line	Colon[e]	45	TCT→ TTT	+	-
HCT116[b]	Cell line	Colon	45	In-frame deletion	+	-

[a] Oh, J. H. et al., *Int. J. Cancer* 81:902-910, 1999
[b] Ilyas, M. et al., *Proc. Natl. Acad. Sci. USA* 94:10330-10334, 1997
[c] Ascending colon
[d] Transverse colon
[c] Detailed information on the origin of these cell lines could not be found. It was confirmed that these cell lines originated from human colon adenocarcinomas.
[f] MSI in BAT-26
[g] No APC mutation was found in MCR

[0050] As shown in Table 3, mutations of the β-catenin gene were identified in 5 (tissue samples 207, 395, 396, 400 and 435) of 74 colorectal carcinomas (5/14, 7%). These five β-catenin mutations were identified in 34 proximal colon cancers (5/34, 15%) and none were found in 40 distal colorectal cancers (0/40, 0%). Of 34 proximal colon cancers, five β-catenin mutations were found in 25 right-sided colon cancers, and no mutation, in the 9 traverse colon cancers. These results suggest that β-catenin mutations are associated with the tumors in the proximal colon (p=0.017).

[0051] In 31 colorectal cancer cell lines, 4 β-catenin mutations were found in cell lines SNU-407, SNU-1047, LS174T and HCT116. Of these 4 β-catenin mutaions, two (SNU-407 and SNU-1047) were found in cell lines originating from the proximal colon (traverse colon). The origins of the other 2 cell lines (LS174T and HCT116) harboring β-catenin mutations were not determined.

[0052] A total of 9 mutations were found among 74 colorectal cancer tissues and 31 colorectal cancer cell lines. Eight mutations out of these 9 mutations were identified at GSK-3β phosphrylation sites. All point mutations were amino acid substitutions and occurred at codons 32, 41 and 45. Six mutations were concentrated at codon 45. Four of these 6 point mutations at codon 45 were the identical missense mutations (TCT→ TTT, Ser→ Phe; in samples 395, 400, SNU-1047 and LS 174T) and the remaining 2 mutations, the same in-frame deletions as in samples 396 and HCT116. No interstitial large deletion of the β-catenin gene was detected.

[0053] In the case of tissue 400, an additional signal in combination with wild type signals was observed, which indicated a missense mutation at codon 45 (TCT→TTT, Ser→Phe) (Fig. 1). Eight of the 9 samples with β-catenin mutations showed both wild type signals at each codon and an aberrant signal, which indicated the presence of heterozygous mutation. Meanwhile, LS 174T showed only an abnormal signal in the absence of a wild type signal at codon 45, which means that LS 174T has homozygous β-catenin mutation.

[0054] All 9 samples with the β-catenin mutations were investigated for APC mutations in the MCR (codons 1263-1513). Only one cell line (SNU-1047) harbored APC truncation mutation at codon 1369 (4107delC). The cell line LS 174T, which had been reported not to carry β-catenin mutation was found to harbor β-catenin mutation (codon 45, TCT→ TTT, Ser→ Phe) in the present invention (Ilyas, M. et al., *Proc. Natl. Acad. Sci. USA* 94:10330-10334, 1997).

Example 3: Confirmation of β-catenin mutations detected by β-catenin oligo chip

[0055] In order to confirm β-catenin mutations detected by the inventive β-catenin oligo chip, the nine β-catenin mutation samples were subjected to PCR-SSCP, DHPLC, PTT, cloning sequencing and direct sequencing as follows.

[0056] PCR-SSCP and DHPLC analyses were performed as previously described (Kim, I. J. et al., *Int. J. Cancer* 86: 529-532, 2000; Wagner, T. et al., *Genomics* 62:369-376, 1999). DHPLC analysis was done using WAVE (Transgenomic, Omaha, NE) and running conditions were optimized using WAVEMAKER software. A protein truncation test (PTT) was performed for mutation detection of the mutation cluster region (MCR, codon 1263-1513) of the *APC* gene, as previously described (Won, Y. J. et al., *J. Hum. Genet.* 44:103-108, 1999). During the cloning, fresh PCR products were ligated into PCR-TOPO vectors, and subcloned using the TA cloning system (Invitrogen, Carlsbad, CA). Bi-directional sequenc-

ing was performed using a Taq dideoxy terminator cycle sequencing kit and an ABI 3100 DNA sequencer (Applied Biosystems, Forster City, CA).

<Table 4>

Sample	SSCP	DHPLC	Direct sequencing	Cloning sequencing	β-catenin oligo chip
207	+[a]	+	ND	+	+
395	+	+	+	+	+
396	+	+	+	+	+
400	ND[b]	+	ND	+	+
435	+	+	+	+	+
SNU-407	+	+	+	+	+
SNU-1047	+	+	+	+	+
LS174T	+	+	+	+	+
HCT116	+	+	+	+	+

[a]Detected
[b] Not detected

[0057] Among the conventional techniques, the automatic direct sequencing method, which has been widely used for mutational analysis, did not clearly detect 2 of the 9 β-catenin mutations (Fig. 2). PCR-SSCP also missed one β-catenin mutation. These results might have been caused by excessive wild type DNA in cancer tissues or by the low sensitivity of these two methods.

Example 4: Relationship between β-catenin mutations and MSI

[0058] It has been reported that MSI status can be meaningfully correlated with proximal colon cancers, and MSI may be used as a diagnostic marker for the diagnosis of proximal colon cancer (Traverso, G. et al., *Lancet.* 359:403-403, 2002). To determine the MSI status, genomic DNAs extracted from 74 colorectal carcinomas were subjected to PCR using BAT-26 marker (Shitoh, K. et al., *Genes Chromosomes Cancer* 30:32-37, 2001; Samowits, W. S. et al., *Am. J. Pathol.* 158:1517-1524, 2001).

[0059] Each PCR reaction solution (25 $\mu\ell$) contained 100 ng of genomic DNA extracted from normal and cancer tissues using Picoll-Paque and Trizol reagents, 10 pmol of each BAT26-F and BAT26-R primers of SEQ ID Nos. 126 and 127 0.25 $\mu\ell$, 2.5 mM of dNTP 0.5 $\mu\ell$, 10x PCR buffer solution 2.5 $\mu\ell$, [α-^{32}P]dCTP 0.25 $\mu\ell$, and Taq DNA polymerase (5 unit/$\mu\ell$). Reactions were initiated by denaturation for 5 min at 94°C in a programmable thermal cycler (Perkin Elmer Cetus 9600; Roche Molecular Systems, Inc., NJ). PCR conditions consisted of 35 cycles of 30 sec at 94°C , 30 sec at 52°C, and 1 min at 72 °C, with a final elongation of 7 min at 72°C. The reaction mixture was heated at 95°C for 5 min and cooled down in a ice bath. Thirty-five $\mu\ell$ of the cooled reaction mixture was subjected to 40% polyacrylamide gel (29:1) electrophoresis, and the gel was dried and exposed to X-ray.

[0060] To determine the correlations between the β-catenin mutations, MSI and tumor location, statistical analyses were performed using the χ^2 or Fisher's exact test, setting a=0.05 as the significance level using the STATISTICA software (StatSoft Inc., Tulsa, OK).

[0061] As a result, 12 of 74 colorectal cancer tissues (16%) showed MSI in the BAT-26 marker. 10 out of 34 proximal colon cancers (29%) were found to carry MSI and only 2 of 40 distal colorectal cancers (5%) were found to harbor MSI. MSI was statistically correlated with the proximal location ($p<0.01$). All 5 β-catenin mutations were found in 12 colorectal cancers with MSI (5/12, 42%) and none were found in 62 colorectal cancers with MSS (microsatellite stability). β-catenin mutations were more common in colorectal carcinomas with MSI than in those with MSS ($p<0.001$).

[0062] While the embodiments of the subject invention have been described and illustrated, it is obvious that various changes and modifications can be made therein without departing from the scope of the appended claims.

[0063] The features in the foregoing description, in the claims and/or in the accompanying drawings may, both separately and in any combination thereof, be material for realizing the invention in diverse forms thereof.

SEQUENCE LISTINGS

[0064]

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Claims

1. A β-catenin oligonucleotide microchip for detecting β-catenin mutations comprising a set of oligonucleotides fixed on the surface of a solid matrix, wherein said oligonucleotides have the nucleotide sequences of SEQ ID NOs: 1 to 121.

2. A manufacturing process for the β-catenin oligonucleotide microchip of claim 1, comprising

   1) mixing each of the oligonucleotides having the nucleotide sequences of SEQ ID NOs: 1 to 121 in a micro spotting solution and distributing to a well plate;
   2) spotting the oligonucleotides on the surface of a solid matrix using a microarrayer;
   3) fixing the oligonucleotides on the solid matrix surface and washing;
   4) denaturing the fixed oligonucleotides by soaking the solid matrix in 95°C water, and then, treating the solid matrix with a sodium borohydride solution; and
   5) washing and drying the solid matrix.

3. The manufacturing process of claim 2, wherein each of the oligonucleotides used in step (1) has a 12 carbon spacer

with 5' amino modification.

4. The manufacturing process of claim 2, wherein the solid matrix of step (2) is a glass, modified silicone, plastic cassette or polymer plate.

5. The manufacturing process of claim 4, wherein the solid matrix is coated with an aldehyde or amine.

6. The manufacturing process of claim 2, each oligonucleotide spot of step (2) is of circular shape with a diameter ranging from 100 to 500 μm.

7. The manufacturing process of claim 6, the oligonucleotide spots of step (2) are arranged in multiple column and rows of intervals of 200 to 800 μm.

8. A method for detecting the β-catenin mutation using the β-catenin oligonucleotide microchip of claim 1, comprising

1) preparing a fluorescent dye-labelled DNA sample from the blood of a subject patient;
2) reacting the labelled DNA sample with oligonucleotide spots on the β-catenin oligo chip;
3) washing the reacted oligo chip to remove unbound sample DNA;
4) detecting the mode of hybridization of specific oligonucleotide spots using a fluorescence reader; and
5) examining the presence of gene mutation.

9. The method of claim 8, wherein the fluorescent dye of step (1) is selected from the group consisting of Cy5, Cy3, Texas Red, Fluorescein and Lissamine.

10. The method of claim 8, wherein the reaction of step (2) is performed in a 45 ~ 60°C incubator saturated with water vapour for 3 ~ 9 hours.

Patentansprüche

1. β-Catenin Oligonukleotid-Mikrochip zum Nachweis von β-Cateninmutationen, umfassend ein Set von Oligonukleotiden, die auf der Oberfläche einer festen Matrix fixiert sind, wobei die Oligonukleotide die Nukleotidsequenzen der SEQ ID NOs: 1 bis 121 aufweisen.

2. Herstellungsverfahren für den β-Catenin Oligonukleotid-Mikrochip nach Anspruch 1, umfassend

1) Mischen jedes der Oligonukleotide, die die Nukleotidsequenzen der SEQ ID NOs: 1 bis 121 aufweisen, in einer Mikrospotting-Lösung und Verteilen auf eine Lochplatte;
2) Spotten der Oligonucleotide auf der Oberfläche einer festen Matrix unter der Verwendung eines Microarray-Gerätes;
3) Fixieren der Oligonukleotide auf der festen Matrixoberfläche und Waschen;
4) Denaturieren der fixierten Oligonukleotide durch Eintauchen der festen Matrix in 95°C Wasser, und dann, Behandeln der festen Matrix mit einer Lösung von Natriumborhydrid; und
5) Waschen und Trocknen der festen Matrix.

3. Herstellungsverfahren nach Anspruch 2, wobei jedes der in Schritt (1) verwendeten Oligonukleotide einen 12 Kohlenstoffspacer mit 5' Aminomodifikation aufweist.

4. Herstellungsverfahren nach Anspruch 2, wobei die feste Matrix von Schritt (2) eine Glas-, modifiziertes Silikon, Plastikkassette oder Polymerplatte ist.

5. Herstellungsverfahren nach Anspruch 4, wobei die feste Matrix mit einem Aldehyd oder Amin beschichtet ist.

6. Herstellungsverfahren nach Anspruch 2, wobei jeder Oligonukleotidspot von Schritt (2) eine kreisförmige Form mit einem Durchmesser im Bereich von 100 bis 500 μm aufweist.

7. Herstellungsverfahren nach Anspruch 6, wobei die Oligonukleotidspots von Schritt (2) in multiplen Spalten und Reihen von Intervallen von 200 bis 800 μm angeordnet sind.

8. Verfahren zum Nachweis der β-Catenin-Mutation unter der Verwendung des β-Catenin Oligonukleotid-Mikrochips nach Anspruch 1, umfassend

1) Präparieren einer Fluoreszenzfarbstoff-markierten DNA-Probe aus dem Blut eines bestimmten Patienten;
2) Reagieren der markierten DNA-Probe mit Oligonukleotidspots auf dem β-Catenin Oligochip;
3) Waschen des reagierten Oligochips, um nicht gebundene Proben- DNA zu entfernen;
4) Nachweisen der Art von Hybridisierung auf spezifischen Oligonukleotidspots unter der Verwendung eines Fluoreszenz-Lesegeräts; und
5) Untersuchen der Anwesenheit an Genmutation.

9. Verfahren nach Anspruch 8, wobei der Fluoreszenzfarbstoff von Schritt (1) ausgewählt ist aus der Gruppe bestehend aus Cy5, Cy3, Texasrot, Fluoreszein und Lissamin.

10. Verfahren nach Anspruch 8, wobei die Reaktion von Schritt (2) in einem mit Wasserdampf gesättigten 45 - 60°C Inkubator für 3 ~ 9 Stunden durchgeführt wird.

Revendications

1. Micropuce à oligonucléotide β-caténine destinée détecter les mutations de la β-caténine comprenant un ensemble d'oligonucléotides fixés sur la surface d'une matrice solide, dans lequel lesdits oligonucléotides ont les séquences de nucléotides des n° SEQ ID : 1 à 121.

2. Processus de fabrication de la micropuce à oligonucléotide β-caténine selon la revendication 1, comprenant les étapes consistant à :

1) mélanger chacun des oligonucléotides ayant les séquences de nucléotides des N° SEQ ID : 1 à 121 dans une solution de micro-répartition et distribuer sur microplaque ;
2) répartir les oligonucléotides sur la surface d'une matrice solide en utilisant une micropipette automatique ;
3) fixer les oligonucléotides sur la surface de la matrice solide et laver ;
4) dénaturer les oligonucléotides fixés en trempant la matrice solide dans de l'eau à 95°C, et ensuite, en traitant la matrice solide avec une solution de borohydrure de sodium ; et
5) laver et sécher la matrice solide.

3. Processus de fabrication selon la revendication 2, dans lequel chacun des oligonucléotides utilisés dans l'étape (1) a un espaceur à 12 carbones avec une modification amino en 5'.

4. Processus de fabrication selon la revendication 2, dans lequel la matrice solide de l'étape (2) est un verre, une silicone modifiée, une cassette en plastique ou une plaque en polymère.

5. Processus de fabrication selon la revendication 4, dans lequel la matrice solide est enduite avec un aldéhyde ou une amine.

6. Processus de fabrication selon la revendication 2, dans lequel chaque dépôt d'oligonucléotide de l'étape (2) est de forme circulaire avec un diamètre dans la gamme de 100 à 500 μm.

7. Processus de fabrication selon la revendication 6, dans lequel les dépôts d'oligonucléotides de l'étape (2) sont disposés en de multiples colonnes et rangées à intervalles de 200 à 800 μm.

8. Procédé pour détecter la mutation de la β-caténine en utilisant la micropuce à oligonucléotide β-caténine selon la revendication 1, comprenant les étapes consistant à :

1) préparer un échantillon d'ADN marqué avec un colorant fluorescent à partir du sang d'un patient sujet ;
2) faire réagir l'échantillon d'ADN marqué avec les dépôts d'oligonucléotides sur la puce à oligonucléotide β-caténine ;
3) laver la puce à oligonucléotide ayant réagi pour retirer l'ADN de l'échantillon non lié ;
4) détecter le mode d'hybridation de dépôts d'oligonucléotides spécifiques en utilisant un détecteur de fluorescence ; et

5) examiner la présence d'une mutation génétique.

9. Procédé selon la revendication 8, dans lequel le colorant fluorescent de l'étape (1) est choisi dans le groupe comprenant la Cy5, la Cy3, le Texas Red, la fluorescéine et la lissamine.

10. Procédé selon la revendication 8, dans lequel la réaction de l'étape (2) est effectuée dans un incubateur à 45-60°C saturé avec de la vapeur d'eau pendant 3 à 9 heures.

Fig. 1

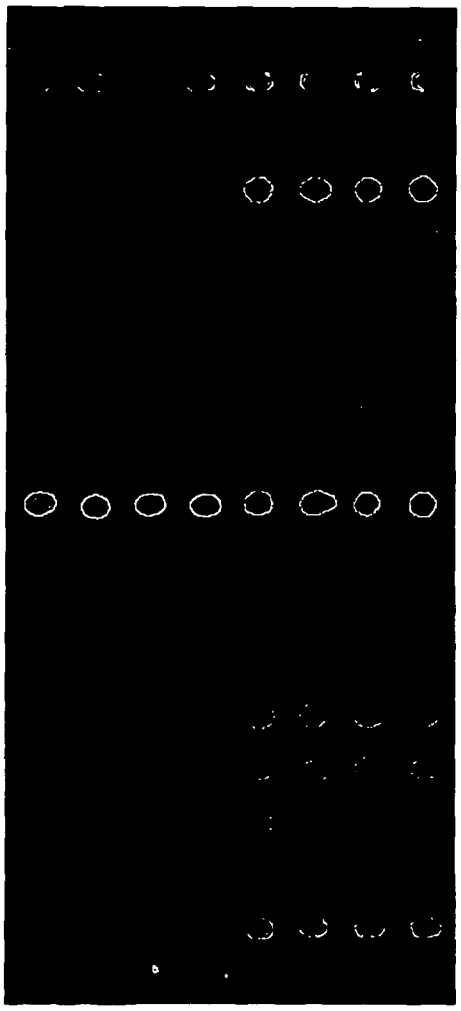

Fig. 2

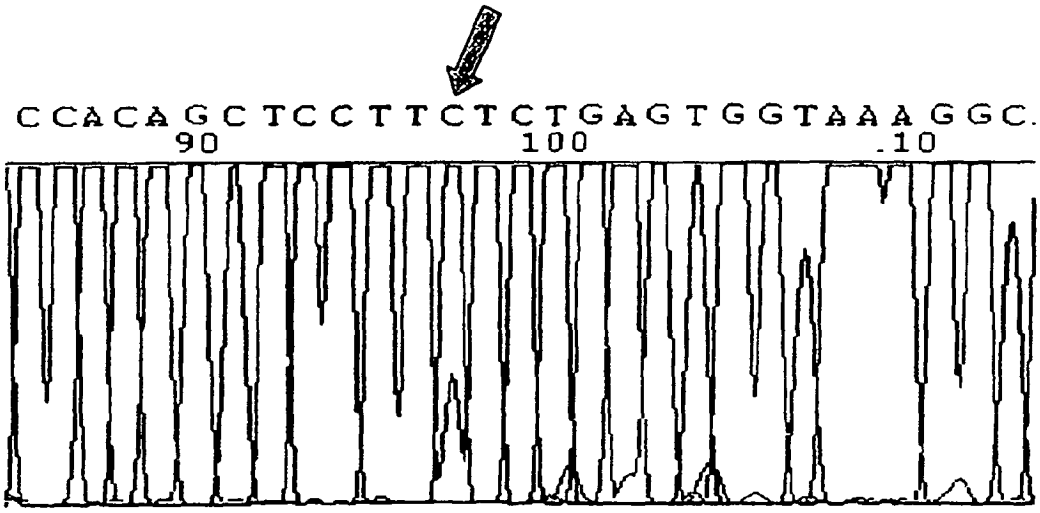

Fig. 3

专利名称(译)	β-连环蛋白寡核苷酸michrochip和用于检测β-连环蛋白突变的方法		
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摘要(译)

本发明涉及用于检测β-连环蛋白基因突变热点区突变的β-连环蛋白寡核苷酸微芯片及其制备方法和用其检测β-连环蛋白突变的方法，其中特异性寡核苷酸选择性地设计为检测β-catenin基因突变热点的各种错义突变和框内缺失。本发明的β-连环蛋白寡核苷酸芯片可用于检测β-连环蛋白突变的研究，并揭示与β-连环蛋白基因相关的信号转导机制和肿瘤发生。

Fig. 1