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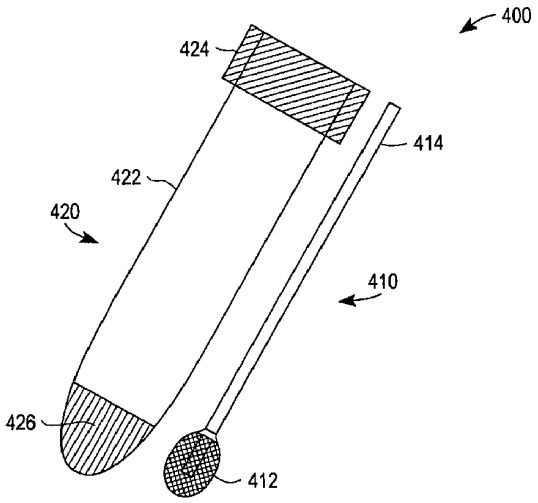
(54) Title: DRUG SCREENING AND MOLECULAR DIAGNOSTIC TEST FOR EARLY DETECTION OF COLORECTAL CANCER: REAGENTS, METHODS AND KITS THEREOF



(57) Abstract: A novel approach to the early detection of colorectal cancer ("CRC"), using a molecular diagnostic test to evaluate grossly normal-appearing colonic tissue for the early detection of colorectal cancer is disclosed. Such grossly normal-appearing colonic mucosal cells may be collected from non-invasive or minimally invasive procedures. The use of novel biomarker panels for drug screening also is disclosed. Such biomaker panels may be used wholly or in part as surrogate endpoints for monitoring effectiveness of a prospective drug in the intervention of pathologies, such as cancers, for example CRC, lung, prostate, and breast, and neurodegenerative diseases, for example Alzheimer's and ALS.

WO 2006/039405 A2

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DRUG SCREENING AND MOLECULAR DIAGNOSTIC TEST FOR EARLY DETECTION OF COLORECTAL CANCER: REAGENTS, METHODS, AND KITS THEREOF

Claim of Priority

U.S. Provisional Patent Application No. 60/614,746 entitled MOLECULAR DIAGNOSTIC TEST FOR EARLY DETECTION OF COLORECTAL CANCER: REAGENTS, METHODS, AND KITS THEREOF, by Nancy M. Lee, *et al.*, filed September 30, 2004 (Attorney Docket No. NLEE-01001US0);

U.S. Provisional Patent Application No. 60/651,344 entitled METHODS OF USE OF A BIOMARKER PANEL FOR DRUG SCREENING, by Nancy M. Lee, *et al.*, filed February 8, 2005 (Attorney Docket No. NLEE-01002US0); and

U.S. Patent Application No. 11/___,___ entitled DRUG SCREENING AND MOLECULAR DIAGNOSTIC TEST FOR EARLY DETECTION OF COLORECTAL CANCER: REAGENTS, METHODS, AND KITS THEREOF, by Nancy M. Lee, filed September 29, 2005 (Attorney Docket No. NLEE-01001US1).

Cross-Reference to Related Applications

This application is related to PCT/US2004/022594, entitled "Biomarker Panel for Colorectal Cancer," by Nancy M. Lee et al., filed July 14, 2004 (Attorney Docket No. NLEE-01000WO0), which claims priority to U.S. Provisional Application No. 60/488,660, entitled "Molecular Biomarker Panel for Determination of Colorectal Cancer," by Nancy M. Lee et al., filed July 18, 2003 (Attorney Docket No. CPMC-01000US0), and also to U.S. Patent Application No. 10/690,880, entitled "Biomarker Panel for Colorectal Cancer," by Nancy M. Lee et al., filed October 22, 2003 (Attorney Docket No. CPMC-01000US1), each of which is incorporated herein in full, by reference.

Nucleotide and/or amino acid sequence listings are included in this application in computer-readable form and in hard-copy. The information included in computer-readable form is incorporated herein in full by reference. The information in computer-readable form is also included on diskette, and such information submitted on diskette is incorporated herein in full by reference. Compact diskette No. 1 contains the following file: NLEE1001WO0.ST25.txt (created 9/30/2005, 96K). The total number of diskettes submitted is one.

Background

The field of art of this disclosure concerns reagents, methods, and kits for the early detection of colorectal cancer ("CRC"), and methods for drug screening effective in the treatment of pathologies, such as cancers, for example, CRC, lung, prostate, and breast, and neurodegenerative diseases, for example Alzheimer's and ALS. These reagents, methods, and kits are based on a panel of biomarkers that are useful for risk assessment, early detection, establishing prognosis, evaluation of intervention, recurrence of CRC and other such pathologies, and drug discovery for therapeutic intervention.

In the field of medicine, clinical procedures providing for the risk assessment and early detection of CRC have been long sought. Currently, CRC is the second leading cause of cancer-related deaths in the Western world. One picture that has clearly emerged through decades of research into CRC is that early detection is critical to enhanced survival rates.

Thus, one long-sought approach for the early detection of CRC has been the search for biomarkers that are effective in the early detection of CRC, and therefore that are effective for the treatment of CRC. For more than four decades, since the discovery of carcinogenic embryonic antigen ("CEA"), the search for biomarkers effective for early detection of CRC has continued. It is further advantageous for sampling methods used in conjunction with an early diagnostic test for CRC to be minimally invasive or non-invasive. Non-invasive and minimally invasive sampling methods increase patient compliance, and generally reduce cost. Additionally, bioinformatic methods for analysis of complex, multivariate data typical of bioanalysis, yielding a reliable diagnostic evaluation based on such data sets, are also desirable.

Therapeutic intervention for numerous types of cancers, such as CRC, lung, prostate, and breast, includes surgery, chemotherapy, and radiation treatment, and combinations thereof. For CRC, a current area of continued research and development, in addition to search for non-invasive methods for early detection, is in the area of drug development.

One picture that has clearly emerged through decades of research into CRC is that early detection, coupled with effective therapeutic intervention is critical to enhanced survival rates. To date, the most commonly used drug in the treatment of CRC is 5-fluoruracil ("5FU"), which frequently is administered intravenously, in combination with the folic acid vitamin, leucovorin. A strategy referred to as primary chemotherapy is used when metastasis has occurred, and the cancer has spread to different parts of the body. For CRC, the current strategy for primary chemotherapy is the administration of an oral form of

5FU, capecitabine, in combination with Camptosar, a topoisomerase I inhibitor, or Eloxatin, an organometallic, platinum-containing drug that inhibits DNA synthesis.

Currently, strategies for new drug development for CRC include two areas of research: angiogenesis inhibitors, and signal transduction inhibitors.

Novel biopharmaceutical drugs include both protein- and ribozyme-based therapeutics. Humanized antibody-based therapeutics include examples such as Erbitux and Avastin. Erbitux, a signal transduction inhibitor, is aimed at inhibiting epidermal growth factor receptors ("EGFR") on the surface of cancerous cells. Avastin, an angiogenesis inhibitor, is aimed at inhibiting vascular endothelial growth factor ("VEGF"), which is known to promote the growth of blood vessels. Additionally, Angiozyme, an example of a ribozyme-based therapeutic, is an angiogenesis inhibitor directed against the expression of the VEGF-R1 receptor. New traditional small molecule-based drugs include examples such as Iressa, based on a quinazoline template, and acting as a signal transduction inhibitor, and SU11248, based on an indolinone template, which acts as an anti-angiogenesis inhibitor.

Still, a number of potential drawbacks and uncertainties remain for these nascent drug therapies for CRC. In addition to typical contraindications such as nausea, vomiting, headache, and diarrhea, other more serious side effects, such as gastrointestinal perforation, elevated or lowered blood pressure, extreme fatigue, and internal bleeding have been observed for many of the promising candidates. Additionally, though many of the drug therapies based on angiogenesis inhibition or signal transduction inhibition appear promising, they are in the very early stages of clinical trials.

Accordingly, a need exists in the art for biomarkers that are effective in the early detection of CRC, coupled with sampling methods that are minimally or non-invasive, and bioinformatic methods, which together produce a robust diagnostic test for the early detection of CRC. A need also exists in the art for drug development, which can provide effective treatment prior to the development of cancer for individuals diagnosed with pathologies, such as cancers, for example CRC, lung, prostate, and breast, and neurodegenerative diseases, for example Alzheimer's and ALS, while minimizing serious side effects.

Brief Description of Figures

Fig. 1 is a table listing an embodiment of sequence listings for a panel of biomarkers of the disclosed invention.

Fig. 2 is a distribution plot of control subjects versus test subjects evaluated using an aspect of the panel of biomarkers of **Fig. 1**, and an aspect of a bioinformatic evaluation of the disclosed invention.

Fig. 3 shows the distribution of the log (base2) expression values for genes, PPAR-*γ*, IL-8, SAA 1 and COX-2 and their cut-off points.

Figs. 4A and 4B show that expression of different genes is altered at different sites of MNCM from individuals with a family history of colon cancer.

Fig. 5 displays a flow diagram of an aspect of the bioinformatic process used for evaluating data.

Fig. 6 is an embodiment of a swab sampling and transport system for the minimally invasive sampling of colonic mucosal cells.

Fig. 7 is a flow chart depicting one aspect of the drug screening disclosure.

Fig. 8 is a flow chart depicting another aspect of the drug screening disclosure.

Detailed Description

To date, a greater understanding of the biology of CRC has been gained through the research on adenomatous polyposis coli ("APC"), p53, and Ki-ras genes, as well as the corresponding proteins, and related pathways involved regulation thereof. However, there is a distinct difference between research on a specific gene, its expression, protein product, and regulation, and understanding what genes are critical to include in a panel used for the analysis of CRC that is useful in the management of patient care for the disease. Panels that have been suggested for CRC are comprised of specific point mutations of the APC, p53, and Ki-ras, as well as BAT-26, which is a gene that is a microsatellite instability marker.

For CRC, biomarkers for risk assessment and early detection of CRC long have been sought. The difference between risk assessment and early detection is the degree of certainty regarding acquiring CRC. Biomarkers that are used for risk assessment confer less than 100% certainty of CRC within a time interval, whereas biomarkers used for early detection confer an almost 100% certainty of the onset of the disease within a specified time interval. Risk factors may be used as surrogate end points for individuals not diagnosed with cancer, providing that there is an established relationship between the surrogate end point and a definitive outcome. An example of an established surrogate end point for CRC is the example of adenomatous polyps. What has been established is that the occurrence of adenomatous polyps is a necessary, but not sufficient condition for an individual later to develop CRC. This is demonstrated by the fact that 90% percent of all preinvasive

cancerous lesions are adenomatous polyps or precursors, but not all individuals with adenomatous polyps go on later to develop CRC.

Adenomatous polyps have been established as surrogate end points for CRC, and adenomatous polyps are macroscopically identifiable by colonoscopy or sigmoidoscopy. During such invasive procedures, biopsy samples can be taken from polyps or lesions for histological evaluation of the tissue. The molecular diagnostic approach disclosed herein may be used on grossly normal-appearing colonic mucosal cells that are not from a macroscopically identifiable polyp or lesion. However, as further disclosed herein, an invasive procedure need not be used to obtain a patient sample for histological evaluation. A non-invasive or minimally-invasive procedure can be employed to obtain, for example, a blood sample, stool sample, or swab of grossly normal-appearing rectal cells, upon which a molecular diagnostic test can be performed to evaluate the presence or absence of CRC. No previously-described approach for early detection of CRC has disclosed the non-invasive or minimally invasive collection of grossly normal-appearing colonic mucosal cells (biopsy or swab of rectal cells), blood samples, and/or stool samples, followed by a molecular and/or protein expression diagnostic test, which can detect changes in the tissue before any untoward histological changes indicating CRC are manifest.

Fig. 1 is a table that gives an overview of the sequence listings included with this disclosure. The table of **Fig. 1** lists a panel of biomarkers useful in practicing the disclosed invention. One embodiment of a biomarker panel is the 16 identified coding sequences given by SEQ. ID NOs 1-16, while another embodiment of a biomarker panel is the 16 identified proteins given by SEQ. ID NOs 17-32. These two embodiments represent molecular marker panels that provide the selectivity and sensitivity necessary for the early detection of CRC. It is to be understood that fragments and variants of the biomarkers described in the sequence listings are also useful biomarkers in embodiments of panels used for the early detection of CRC. What is meant by fragment is any incomplete or isolated portion of a polynucleotide or polypeptide in the sequence listing. Further, it is recognized that almost daily, new discoveries are announced for gene variants, particularly for those genes under intense study, such as genes implicated in diseases like cancer. Therefore, the sequence listings given are exemplary of what now is reported for a gene, but it is recognized that for the purpose of an analytical methodology, variants of the gene and their fragments also are included.

In **Fig. 1,** the entries 1-16 in the table are one aspect of a panel of biomarkers, which are polynucleotide coding sequences, and include the name and abbreviation of the gene. Entries 17-32 in **Fig. 1** are another embodiment of a panel of biomarkers, which are protein,

or polypeptide, amino acid sequences that correspond to the coding sequences for entries 1-16. A biomarker, as defined by the National Institutes of Health ("NIH") is a molecular indicator of a specific biological property; a biochemical feature or facet that can be used to measure the progress of disease or the effects of treatment. A panel of biomarkers is a selection of biomarkers, which taken together can be used to measure the progress of disease or the effects of treatment. Biomarkers may be from a variety of classes of molecules. As previously mentioned, there remains a need for biomarkers for CRC having the selectivity and sensitivity required to be effective for early detection of CRC. Therefore, one embodiment of what is disclosed herein is the selection of an effective set of biomarkers that is differentiating in providing the basis for early detection of CRC.

In one aspect of this disclosure, for the early detection of CRC, expression levels of polynucleotides indicated as SEQ. ID NOs 1-16 are determined from cells in samples taken from patients by non-invasive or minimally invasive methods. The contemplated methods include blood sampling, stool sampling, and rectal cell swabbing or biopsy. Such analysis of polynucleotide expression levels frequently is referred to in the art as gene expression profiling. For gene expression profiling, levels of mRNA in a sample are measured as a leading indicator of a biological state -- in this case, as an indicator of CRC. One of the most common methods for analyzing gene expression profiling is to create multiple copies from mRNA in a biological sample (said sample taken from a patient as disclosed above, by non- or minimally-invasive methods) using a process known as reverse transcription. In the process of reverse transcription, the mRNA from the sample is isolated from cells in the biological sample, by methods well-known in the art. The mRNA then is used to create copies of the corresponding DNA sequence from which the mRNA was originally transcribed. In the reverse transcription amplification process, copies of DNA are created without the regulatory regions in the gene (*i.e.*, introns). These multiple copies made from mRNA are therefore referred to as "cDNA," which stands for complementary, or copy DNA. Entries 33-64 are the sets of primers that can be used in the reverse transcription process for each biomarker gene listed in entries 1-16. All nucleotide and amino acid biomarker sequences identified in SEQ. ID NOs 1-64 are found in a printout attached and included as subject matter of this application, and are found on a diskette also included as part of this application and incorporated herein by reference.

Since the reverse transcription procedure amplifies copies of cDNA proportional to the original level of mRNA in a sample, it has become a standard method that allows the identification and quantification of even low levels of mRNA present in a biological sample.

Genes either may be up-regulated or down-regulated in any particular biological state, and hence mRNA levels shift accordingly.

In one aspect of this disclosure, a method for gene expression profiling comprises the quantitative measurement of cDNA levels for at least two of the biomarkers of the panel of biomarkers selected from SEQ. ID NOs. 1-16, in a biological sample taken from a patient by a non- or minimally-invasive procedure, such as blood sampling, stool sampling, rectal cell swabbing, and/or rectal cell biopsy. The tissue taken need not be apparently diseased; in fact, the disclosed invention is contemplated to be useful in evaluating even grossly normal-appearing cells for detection of CRC. Such a method for gene expression profiling requires the use of primers, enzymes, and other reagents for the preparation, detection, and quantifying of cDNAs. The method of creating cDNA from mRNA in a sample is referred to as the reverse transcriptase polymerase chain reaction ("RT-PCR"). The primers listed in SEQ. ID NOs 33-64 are particularly suited for use in gene expression profiling using RT-PCR based on the disclosed biomarkers in the biomarker panel. A series of primers were designed using Primer Express Software (Applied Biosystems, Foster City, CA). Specific candidates were chosen, and then tested to verify that only cDNA was amplified, and not contaminated by genomic DNA. The primers listed in SEQ. ID NOs 33-64 were specifically designed, selected, and tested accordingly.

The primers listed in SEQ. ID NOs 33-64 are important in the step subsequent to creating cDNA from isolated cellular RNA, for quantitatively amplifying copies in the real time PCR of gene expression products of interest. Optimal primer sequence, and optimal primer length are key considerations in the design of primers. The optimal primer sequence may impact the specificity and sensitivity of the binding of the primer with the template. A primer length between 18-30 bases is considered an optimal range. Theoretically, 18 bases is the minimal length representing a unique sequence, which would hybridize at only one position in most eukaryotic genomes. The primers listed in SEQ. ID NOs 33-64 range in primer length between 21-27 bases, and were designed and validated to amplify cDNA for the panel of nucleotides selected from SEQ. ID NOs 1-16. The specificity of the primers was demonstrated by a single product on 10% polyacrylamide gel electrophoresis ("PAGE"), and a single dissociation curve of the PCR product.

Once the primer pairs have been designed, and validated for specificity, they may be synthesized in large quantities, and stored for convenient future use. Since the PCR reaction is sensitive to buffer concentration and buffer constituents, primers should be maintained in a suitable diluent that will not interfere in the amplification reaction. One example of a suitable diluent is 10 mM Tris buffer, with or without 1mM EDTA, depending on

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摘要(译)

本发明公开了一种早期检测结肠直肠癌（“CRC”）的新方法，其使用分子诊断试验来评估大体上正常出现的结肠组织以用于早期检测结肠直肠癌。可以从非侵入性或微创手术收集这种非常正常出现的结肠粘膜细胞。还公开了用于药物筛选的新型生物标志物组的用途。这些生物制剂组可以全部或部分用作替代终点，用于监测前瞻性药物在病理干预中的有效性，例如癌症，例如CRC，肺，前列腺和乳腺，以及神经退行性疾病，例如阿尔茨海默氏病和ALS。 。