(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: NESTED METHYLATION-SPECIFIC POLYMERASE CHAIN REACTION CANCER DETECTION METHOD

(57) Abstract: A molecular marker-based method for monitoring and detecting cancer in humans. Aberrant methylation of gene promoters is a marker for cancer risk in humans. A two-stage, or "nested" polymerase chain reaction method is disclosed for detecting methylated DNA sequences at sufficiently high levels of sensitivity to permit cancer screening in biological fluid samples, such as sputum, obtained non-invasively. The method is for detecting the aberrant methylation of the p16 gene, O 6 -methylguanine-DNA methyltransferase gene, Death-associated protein kinase gene, RAS-associated family 1 gene, or other gene promoters. The method offers a potentially powerful approach to population-based screening for the detection of lung and other cancers.

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NESTED METHYLATION-SPECIFIC POLYMERASE CHAIN REACTION CANCER DETECTION METHOD

CROSS-REFERENCE TO RELATED APPLICATIONS

[1] This application is a continuation-in-part application of U.S. Patent Application Serial No. 60/228,057, entitled "Nested Methylation Specific Polymerase Chain Reaction", to Belinsky, et al., filed on August 25, 2001, and the specification thereof is incorporated herein by reference.

GOVERNMENT RIGHTS

[2] The Government has certain rights to this invention pursuant to National Institutes of Health Grants 50 CA8184 and RO1 CA70190, and pursuant to Cooperative Agreement DE-FC04-96AL76406 and Contract DE-FG02-90ER60939 with the Office of Biological and Environment Research, U.S. Department of Energy.

BACKGROUND OF THE INVENTION

Field of the Invention:

[3] The present invention relates to cancer detection, specifically a methylation specific polymerase chain reaction method for detecting the presence of gene-specific promoter methylation in tumor cells, and more specifically a nested, two-stage polymerase chain reaction method for amplifying the gene that may be altered in a particular cancer, thereby permitting cancer detection and monitoring by detecting gene inactivation in biological fluids such as sputum and blood. The invention may also find application in the field of pharmacogenomics.

Background Art:

[4] Cancer is one of the leading causes of death in the U.S. The high mortality from this disease stems from the inability in many cases to detect the cancer at a stage where it can be removed surgically and has not metastasized to other sites in the body. Current radio- and chemotherapy often result in disease remission, but actual cures (long-

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term survival) are rare for advanced stage cancer. However, 5-year survival for cancers that are surgically removed with no evidence of distant disease is 60% to 80%. The development of biomarkers usable for early detection, to follow therapy, and to predict outcome of the disease, could greatly aid in managing cancer and increasing long-term

5   survival.

     [5] Chemoprevention studies in current and former cancer-free subjects have largely been focused on the effect of defined interventions on cytologic and genetic changes in bronchial epithelium obtained through biopsy. This approach is predicated on

10   the fact that virtually the entire lower respiratory tract is exposed to inhaled carcinogens within cigarette smoke. The resulting "field cancerization" involves the generation of multiple, independently initiated sites throughout the lungs of persons with a long history of smoking. Cytological and genetic changes have been detected throughout the bronchial tree. Many of the genetic changes detected in the bronchial epithelium may have limited

15   utility for assessing lung cancer risk and response to intervention therapy. For example, assays for loss of heterozygosity (LOH) in cytologically normal tissue have limited sensitivity, and quite often subjects will not be informative for the microsatellite marker used to assess LOH at a specific chromosome locus. Moreover, some LOH changes appear less frequently in former smokers compared to current smokers, suggesting they

20   may be associated more with exposure. In addition, the conduct of large population-based prevention trials will necessitate the use of biological fluids such as sputum that can be obtained non-invasively for monitoring drug response. Neither polymerase chain reaction- nor fluorescent in situ hybridization (FISH)-based assays have the sensitivity to detect LOH in sputum. At present, there is no extensive data on methylation in the bronchial

25   epithelium in either persons with prevalent lung cancer or cancer-free smokers. Still further, the effect of smoking cessation on these markers has not been evaluated.

     [6] The development of cancer involves inactivation of many different types of genes in a cell. It is this inactivation that is largely responsible for a normal cell becoming

30   a tumor cell. One type of gene inactivation that occurs frequently in cancer is called CpG island methylation of gene promoters. This process involves a change in the portion of the

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gene, the promoter, which is responsible for maintaining its activity. CpG island methylation is responsible for inactivating many different cellular genes.

[7] Because current and even former cigarette smokers have increased bronchial secretions that contain exfoliated cells from the bronchial tree, the analysis of sputum from these

individuals has been an area of research for marker development. The late Dr. Geno Saccomanno demonstrated that premalignant cytologic changes can be detected several years prior to a clinical diagnosis of lung cancer in high-risk subjects. Unfortunately, these studies were difficult to replicate, most likely because of the skills required for identifying subtle nuclear changes in cells that often comprise < 5% of the sputum slide. Subsequent studies suggested that molecular assays could be used to enhance the predictive value of sputum samples. Mutations within the K-ras gene have been detected in sputum specimens collected prior to tumor resection; identical microsatellite alterations have been detected in primary tumors and corresponding sputum samples. However, the known methods to detect both alterations lack sensitivity, and the overall prevalence of these changes in non-small cell lung cancer (NSCLC) is less than 25%.

[8] Recently, Drs. James Herman and Stephen Baylin developed a technique, called methylation specific polymerase chain reaction (MSP), to detect the presence of gene-specific promoter methylation in tumor cells. This technique is described in U.S. Patent Nos. 5,786,146 to Baylin et al., and 6,017,704 to Herman, et al., the teachings of which are incorporated herein by reference. With the MSP approach, it is possible to detect one copy of a specific methylated gene in the background of 1,000 unmethylated gene copies. This approach is useful for detection of specific methylated genes in primary tumors, but has extremely limited utility for detecting gene inactivation in biological fluids such as sputum, plasma, urine, and fecal stool. Evaluating biological fluids is the most noninvasive and economical approach for population-based screening. Because these fluids contain DNA or cells that are mainly normal cells, increased sensitivity of the MSP procedure is essential for detecting the presence of the tumor cells.

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[9] Against the foregoing background, the present invention was developed.

SUMMARY OF THE INVENTION (DISCLOSURE OF THE INVENTION)

[10] Broadly described, the invention is a nested, two-stage polymerase chain

5   reaction (PCR) method for the monitoring and/or early detection of cancer. The method

first expands the number of specific gene copies by using PCR to amplify the gene that

may be altered in a particular cancer. This amplification includes the portion of the gene

where the promoter methylation resides. At this point, an aliquot of the specific gene

amplification product generated through the first PCR is used in a second PCR. The

10   second PCR is methylation-specific, and may utilize the methylation-specific technology

of U.S. Patent No. 6,017,704 to detect the presence or absence of inactivation for that

specific gene. Using this inventive nested, two-stage MSP method, the sensitivity for

detecting methylation is increased by 50-fold to offer practical utility. The inventive

method has been successfully applied to the detection of cancer through monitoring of

15   sputum.

[11] Two of the genes that have been evaluated are the p16 gene and the

O6-methylguanine-DNA methyltransferase (MGMT) gene. Both are inactivated through

CpG island methylation of their promoters. A "CpG island" is a stretch of DNA that can

20   range in size from about 200 to about 1000 base pairs, with an average percent of G + C

over 50%, and an observed/expected CpG ratio above 0.6. DNAs isolated from biological

fluids such as sputum or plasma are subjected to bisulfite modification, and PCR is

performed to amplify a 280 base pair and 289 base pair fragment of the p16 and MGMT

genes, respectively, including a portion of their CpG rich promoter region. "CpG rich"

25   means that the density of cytosine-guanosine runs is denser than normally expected. This

first step of the method is central to the improved sensitivity for the inventive procedure.

Specific PCR primers (small sequences of DNA bases that are complementary to a

specific gene, like p16) are designed to amplify through PCR the gene fragments.

Following this amplification, an aliquot of the PCR products that are generated, containing

30   the p16 or MGMT gene fragments, is subjected to a second PCR. Primers specific to

methylated DNA regions (only recognized methylated gene fragments -- this is the MSP

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step) are employed to determine whether the gene promoter (p16 or MGMT) is methylated. If a gene promoter is methylated, inactivation is assumed to occur.

BRIEF DESCRIPTION OF THE DRAWINGS:

5   [12] The accompanying drawings, which are incorporated into and form a part of the specification, illustrate several embodiments of the present invention and, together with the description, serve to explain the principles of the invention. The drawings are only for the purpose of illustrating a preferred embodiment of the invention and are not to be construed as limiting the invention. In the figures:

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[13] Figure 1 is a table setting forth the methylation of p16 and MGMT genes in tumor/sputum pairs at time of diagnosis. In the table, Tob is the abbreviation for tobacco, SCC is the abbreviation of squamous cell carcinoma, M is the abbreviation of methylated, U is the abbreviation of unmethylated, and Mod. Dyspl. is the abbreviation of moderate 15   dysplasia.

[14] Figure 2 is a table setting forth the methylation of the p16 and MGMT genes in sputum preceding clinical diagnosis of squamous cell carcinoma. In the table, Tob is the abbreviation for tobacco, SCC is the abbreviation of squamous cell carcinoma, M is 20   the abbreviation of methylated, U is the abbreviation of unmethylated, CIS is the abbreviation of carcinoma in situ, Dyspl. is the abbreviation of dysplasia, and Suspicious means suspicious for malignancy.

[15] Figure 3 is a diagram showing that P16 and MGMT methylation in sputum 25   are biomarkers for squamous cell carcinoma, depicting results of the stage-2 PCR using methylation-specific primers for the p16 and MGMT gene. Sputum (samples collected months prior to cancer) and primary SCC (time zero) are shown for five cases. The presence of 145 bp and 83 bp PCR products indicate that the sputum or tumor was positive for p16 and MGMT methylation, respectively. The CaLu6 and SKuLu1 cell lines are the 30   positive controls for p16 and MGMT methylation, respectively, and normal lung served as a negative control. Sputum cytology varied by case and is indicated for each specimen.

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[16] Figure 4 is a graph showing the prevalence for pl6 and MGMT methylation in sputum from cancer-free subjects. The frequency for pl6 and MGMT methylation in cancer-free subjects in sputum is expressed as a percentage. Above the bar graphs is indicated the total number of positive samples per total sample population.

[17] Figure 5 is a table setting forth the genes, methylation specific polymerase chain reaction primers, and annealing temperatures (°C) employed to run the first stage reaction according to one preferred embodiment of the invention.

[18] Figure 6 is a table setting forth the genes, methylation specific polymerase chain reaction primers, and annealing temperatures (°C) employed to run the second stage reaction according to one preferred embodiment of the invention.

[19] Figure 7 is a graphical depiction of a CaLu6 cell line containing a methylated pl6 gene, illustrating the dramatically increased sensitivity of the methylation specific polymerase chain reaction method of the invention for detecting pl6 gene methylation. Depicted in Lanes 1-5 are the results of the stage-2 PCR with methylation-specific primers. The PCR product indicative of methylation is readily seen in DNA diluted up to 50,000 fold with normal DNA (lanes 1-5).

[20] Figure 8 is a table summarizing selected demographic variables, by study group, of cases and controls involved in an exemplar test demonstrative of the invention.

[21] Figure 9 depicts methylation of the p16 gene in bronchial epithelial cells and corresponding sputum. Representative scenarios for detection of methylation in bronchial epithelial cells and sputum from five different controls involved in an exemplar test demonstrative of the invention are shown

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[22] Figure 10 is a graph showing the number of bronchial epithelial sites positive (by frequency of methylation) per subject involved in an exemplar test demonstrative of the invention.

5   [23] Figure 11 is a graph showing relative frequency of methylation for p16 gene, DAP-Kinase gene, MGMT gene, and RASSF1A gene in exemplar tests demonstrative of the invention.

[24] Figure 12 is a graph comparing sputum samples by frequency of methylation, 10   and number of subjects, in exemplar tests demonstrative of the invention.

DESCRIPTION OF THE PREFERRED EMBODIMENTS
(BEST MODES FOR CARRYING OUT THE INVENTION)

15   [25] Lung cancer is the leading cause of cancer-related death in the U.S. and is projected to reach epidemic levels in the world during the 21st century. Mortality from this disease could be reduced greatly through the development of molecular markers that identify individuals at the earliest stages of lung cancer where curative resection is feasible. Candidate biomarkers should have high sensitivity and specificity, and appear 20   sufficiently early in the course of disease for medical intervention to improve prognosis. Finally, the markers must be present in a biological fluid that can be obtained noninvasively, making its collection feasible for population-based screening.

[26] The present invention provides a molecular-based marker method for the 25   early detection of human cancer, especially lung cancer. The inventive method involves the detection of gene promoter regions that are aberrantly hypermethylated in human tumors. This change is associated with an epigenitically mediated gene silencing that constitutes an alternative to coding region mutations for loss of tumor suppressor gene function in cancer. The applicants have determined that aberrant promoter methylation of 30   the P16 INK4a (p16) tumor suppressor gene, which plays a key role in cell cycle regulation, is an early and very frequent event in squamous cell carcinoma (SCC) of the

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lung. Another gene frequently inactivated by aberrant promoter methylation in human non-small-cell lung cancer is O 6 -methylguanine-DNA methyltransferase (MGMT). MGMT is a DNA repair enzyme that protects cells from the carcinogenic effects of alkylating agents by removing adducts from the O 6 position of guanine. Thus, the p16

5   and MGMT genes are strong candidate biomarkers for early detection of lung cancer.

[27] That aberrant methylation of the p16 promoter region can be detected in DNA from exfoliated cells in sputum of patients with this disease supports the inventive methodology for the monitoring and early diagnosis of cancer. According to the

10   invention, aberrant methylation of the p16 gene promoter was detected in sputum from three of seven patients with lung cancer, and five of 26 cancer-free individuals at high-risk. The present invention involves a modification, to achieve even higher detection efficiency, of the methylation-specific polymerase chain reaction (PCR) assay used initially. The invention has utility for monitoring purposes irrespective of the physological

15   state of the cell (e.g. pre-malignancy) and also irrespective of the precise location of the methylated gene. Thus, the inventive method is used to extend not only analysis of the p16 promoter, but to study the methylation status of a similar region of MGMT or other specific and particular genes that may be altered by diseases such as cancer. Aberrant methylation of one of these two promoter regions was detected in sputum of 100% of

20   patients with proven SCC, not only at the time of diagnosis, but also in all sputum samples taken from patients five to 35 months prior to clinical tumor detection. Furthermore, these sputum markers were detected in a subset of cancer-free individuals with very high risk for developing lung tumors. Detection of aberrant promoter region methylation is central to the inventive approach for using DNA-based markers for the early detection of lung and

25   other common human cancers.

[28] Applicants have identified several biomarkers with great potential for the early detection of a common, non-inherited, cancer. Using two markers, which are frequent in non-small cell lung tumors, a perfect record for detecting a DNA methylation

30   abnormality in sputum up to three years prior to clinical diagnosis of SCC was obtained in a group of patients. With these two biomarkers, 100% of SCCs also were detected

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through analysis of sputum collected at the time of clinical diagnosis. This conclusion is supported by applicants' finding of p16 and MGMT methylation in cancer-free, high-risk subjects at prevalences that approximate lifetime risk for lung cancer. Thus, aberrant gene methylation is a useful molecular marker approach to population-based screening for

5   detecting lung cancer and monitoring the efficiency of chemopreventative agents. Furthermore, in these individuals, sputum samples were positive in both patients in this cohort who developed SCC to date.

[29] The inventive biomarker approach also aids the sensitivity and accuracy of

10 other diagnostic approaches for lung cancer that are currently receiving much attention. For example, the spiral computerized tomography (spiral CT) imaging procedure can detect very small peripheral lung cancers where routine chest X-rays are negative. However, in terms of the actual presence of lung cancer, many false positive results are the rule, and actual cancer detection rates using spiral CT are currently 0.3% to 0.48% . This

15 scanning diagnostic approach might be enhanced by combining it with the determination of hypermethylation biomarkers in the sputum according the present invention.

[30] Patients with Stage-I or -II disease have a 5-year cure rate of 60% to 80%, whereas Stage III patients have a median survival of 13 months. Lung cancer rates could

20 be reduced by
the development of a cost-effective screening approach according to the invention. Key to any screening approach is the identification of markers of lung cancer that can be detected through sensitive and specific diagnostic assays. It is clear that mutation of the K-ras and p53 genes and microsatellite instability constitute alterations important for lung

25 carcinogenesis. However, current methods for examining the concordance of detecting these changes in bronchoalveolar lavage fluid from patients with early stage lung cancer show limited sensitivity.

[31] The inventive method improves upon all previous efforts by incorporating a

30 nested, two-stage PCR approach, which is highly sensitive (one methylated allele in > 50,000 unmethylated alleles). Due to the inherent difficulties (e.g., DNA degradation)

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of assaying for methylation in DNA recovered from fixed tissues, previous efforts and studies have likely underestimated the actual frequency of methylation. In the development of the invention, the applicants observed a strong correlation between loss of expression of both p16 and MGMT and methylation.

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[32] The present invention also accounts for detecting at least one methylation marker in both sputum and corresponding tumor from every patient at the time of tumor diagnosis or within 3 years of tumor diagnosis. This contrasts with the applicants' previous study, where using only the one-stage PCR approach, p16 methylation was
10   detected in sputum from 43% of lung cancer cases. (Belinsky, S.A., et al., "Aberrant methylation of p16 is an early event in lung cancer and a potential biomarker for early diagnosis." Proc. Nat'l Acad. Sciences (USA), 95:11891-11896 (1998).) There were a few instances in which there were discordant findings for one of the two markers. In each case, the other marker allowed for a positive sputum change to correlate with presence of a
15   cancer, or the development of one, within three years or less.

[33] Possible explanations exist for the instances in which a positive marker was found in the sputum but not in the corresponding tumor. First, the presence of promoter hypermethylation for a given gene can be heterogeneous within a tumor, and sampling
20   would then determine whether it is detected or not. Second, the cancer may arise within a widespread process of bronchial epithelial changes, or so-called "field cancerization" in which cells have a very high risk for malignant transformation. The applicants have detected p16 methylation in non-malignant bronchial epithelial cells from different lung lobes of the same patient with a defined lung cancer, and such high-risk cell populations
25   could contribute exfoliated cells that contain a hypermethylation marker to the sputum. Referring to Fig. 2, and specifically Cases 10 and 11 thereof, the finding that MGMT methylation was not present in two sputum samples collected 3 years prior to clinical diagnosis may be related to the timing for inactivation of this gene in those tumors. Similarly, with reference to Fig. 1 (Case 6), MGMT methylation was not seen in the
30   sputum sample collected 15 months prior to cancer, but was present at 9 months.

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[34] Inactivation of genes by methylation is a major mechanism in cancer. Hypermethylation- associated inactivation of pl6 is an early and frequent event in non-small-cell lung cancers (NSCLCs) (SCC: 60% to 80%; adenocarcinoma: 30 to 45%) and other cancers. In experimental systems, such loss appears to act as a "gate keeper" in permitting cells to pass through early steps of cellular immortalization. The invention therefore can find application in the area of chemoprevention, where markers are needed to identify high-risk subjects and to evaluate efficacy of preventive agents.

EXAMPLE ONE:

[35] Sputum samples and matched SCCs were obtained from 21 people previously enrolled in a Lung Cancer Surveillance Study conducted through St Mary's Hospital, Grand Junction, Colorado. The SCCs were obtained through biopsy or surgical resection. Sputum was collected by standardized procedures at Johns Hopkins Medical Institutions. Sputum was collected from 32 patients being evaluated for possible lung cancer through referral from their primary care physician, and from 91 cancer-free, former uranium miners from Grants, New Mexico, participating in a cancer surveillance study. The study was approved by the respective Institutional Review Boards; all participants gave written informed consent.

[36] Sputum was considered unsatisfactory for evaluation if alveolar lung macrophages were absent or if a marked inflammatory component was present that diluted the concentration of pulmonary epithelial cells.

[37] SCC tumors were obtained as frozen or formalin-fixed specimens. Frozen tumors were not microdissected. Sequential sections (5μm) were prepared from tumors or biopsies, deparafinized, and stained with toluidine blue to facilitate dissection. A 25-gauge needle attached to a tuberculin syringe was used to remove the lesions under a dissecting microscope. Because some SCCs are extensively contaminated with normal tissue or very small in size (the situation for the biopsy), it was essential to include normal-appearing cells to ensure that after bisulfite modification and column clean-up of the DNA template, enough sample remained to conduct the MSP assay as described

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below. Thus, because the goal of the process was to determine whether p16 methylation was present microdissection was used to enrich the samples examined.

[38] DNA was isolated from frozen tumors, microdissected tumors, and sputum
5   by digestion with Pronase in sodium dodecyl sulfate (1%), followed by standard phenol-chloroform extraction and ethanol precipitation.

[39] The methylation status of the p16 and MGMT genes was then determined by the inventive modified MSP method. The nested, two-stage PCR approach was utilized,
10 which improved the sensitivity (one methylated allele in > 50,000 unmethylated alleles) to detect methylated alleles by over 50-fold over known MSP methods. DNAs were subjected to bisulfite modification, and PCR was performed to amplify a 280 base pair and 289 base pair fragment of the p16 and MGMT genes, respectively, including a portion of their CpG rich promoter region. The primers recognize the bisulfite-modified template,
15 but do not discriminate between methylated and unmethylated alleles.

[34] The stage-1 PCR products were diluted 50-fold, and 5 µl of the product was subjected to a stage-2 PCR in which primers specific to methylated or unmethylated template were used. Primer sequences used in the "stage 1" amplification of the p16 and
20 MGMT genes are as follows:

p16-Forward, 5'-GAAGAAAGAGGAGGGGTTGG-3 ',

p16- Reverse, 5'-CTACAAACCCTCTAC CCACC-3 ',

25   MGMT-Forward, 5'-GGATATGTTGGGATAGTT-3 ', and

MGMT-Reverse, 5' -CCAAAAACCCCAAACCC-3 '.

[41] TaqTM Gold polymerase (Perkin Elmer) in a 50 µl volume was used in all
30 PCRs. The PCR amplification protocol for stage 1 was as follows: 95 degrees C for 10 minutes, then denature at 95 degrees C for 30 seconds, anneal at 60 degrees C (for p16) or

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52 degrees C (for MGMT) for 30 seconds, extension at 72 degrees C for 30 seconds for 40 cycles, followed by a 10 minute final extension.

[42] Primers suitable to selectively amplify unmethylated or methylated alleles of the p16 and MGMT genes in the second, or "stage 2," PCR have been described previously in the art. (Esteller, M., et al., "Inactivation of the DNA repair gene O6-methylguaine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia." Cancer Res., 59:793-797 (1999); Herman, J.G., et al., "MSP: a novel PCR assay for methylation status of CpG islands." Proc. Nat'l Acad. Sciences (USA), 93:9821-9826 (1996).) Annealing temperatures for the second PCR were increased to 70 degrees C (for p16) and 62 degrees C (for MGMT), and all cycling times reduced to 15 seconds for a total of 40 cycles. Normal human tissue collected from autopsy of never smokers, and cell lines positive for p16 (Calu6) and MGMT (SkuLu1) methylation, served as negative and positive controls. All assays were conducted in at least duplicate. (Sensitivity for detecting methylated alleles was determined by mixing DNA isolated from either Calu6 or SkuLu1 cells with DNA isolated from lung tissue of a never smoker to achieve dilutions up to 1 in 100,000. The mixed DNA sample was then subjected to bisulfite modification and subsequent analysis by the two-stage MSP approach.)

[43] Sputum samples that rendered positive methylation products were also analyzed by a second method using restriction enzyme digestion of the resulting PCR product. Second-stage PCRs were performed in duplicate for each sample. Then one of each sample pair was incubated with the restriction enzyme FNU 4HI that cuts (G/CGGCG) at two sites within the amplified region of the methylated p16 gene promoter. (That is, the restriction enzyme is employed after the second stage PCR.) The restriction enzyme only cuts template that is methylated at the two CpG sites, since the unmethylated cytosines would be modified by the bisulfite treatment to uracils. Since FNU 4HI cuts at two different sites within the promoter, applicants confirmed that four CpGs were methylated, which verified the methylation status of the sample. Thus, by using a selected restriction enzyme, one is able to quantify, to a certain extent, the number of different methylated gene sites.

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[44] An identical approach was used for the MGMT gene using the Taq 1 and BstU1 restriction enzymes to assay three different CpG sites. All sputum samples scored as positive for
5   methylation were confirmed by both procedures.

[45] Statistical comparisons were then done by Fisher's Exact test. Aberrant methylation of p16 and/or MGMT is detected in the sputum of all tested lung cancer patients at the time of diagnosis. Sputum samples and matched SCCs were obtained from
10   21 people previously enrolled in a Lung Cancer Surveillance Study conducted through St Mary's Hospital, Grand Junction, Colorado. All subjects had a history of smoking, and approximately 50% were exposed to radon through uranium mining at the Colorado Plateau. (At sufficiently high concentrations, radon (222Ra) and the associated α-particle-emitting decay products polonium-214 and -218, cause an excess of lung
15   cancers in smokers who mined uranium.) Sputum was collected from 10 of the above 21 individuals at the time of diagnosis of SCC; as seen in Fig. 1, only four samples were diagnostic of cancer by cytologic criteria. In marked contrast to cytology findings, one or both gene promoters tested were abnormally methylated in all of these sputum samples. Continuing reference to Fig. 1 shows that abnormal p16 gene methylation was present in
20   sputum from all eight patients whose tumors were also positive for this marker, but not in sputum from the two individuals whose tumors were negative for this change. Four of the six patients with abnormal methylation of MGMT in their tumors also had this change detected in their sputum, including the two patients whose tumors lacked the p16 change. As indicated by Cases 4 and 5 in Fig. 1, methylation of p16 was present in sputum of the
25   two individuals whose sputum was negative for MGMT. Aberrant MGMT methylation was not detected in sputum or tumor from three cases. In Case 8 of Fig. 1, MGMT methylation was detected in the sputum, but not in the tumor.

[46] The foregoing disclosure indicates that the detection of p16 and MGMT
30   promoter methylation precedes clinical cancer. Reference is made to Fig. 2. For the other 11 of 21 original individuals with SCC, sputum samples were obtained well before

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the diagnosis of SCC at times ranging from 5 to 35 months. In only one patient (Fig. 2, Case 3) was the sputum sample thought to have unequivocal signs of cancer by cytologic criteria.

5   [47] However, abnormal methylation of the p16 promoter region was detected in DNA from sputum of all 11 subjects with the longest time to tumor diagnosis being 35 months. With continued reference to Fig. 2, where multiple sputum samples were available either as replicate specimens (Case 2), or as temporal samples (e.g. Case 6), methylation of p16 was always present. A 90% concordance was also observed between

10   p16 methylation in the primary SCC and paired sputum samples, as indicated by Figs. 2 and 3. Fig. 3 shows the results of the stage-2 PCR using methylation-specific primers for the p16 and MGMT gene. Sputum (samples collected months prior to cancer) and primary SCC (time zero) are shown for five cases. The presence of a 145 bp and 83 bp PCR products indicate that the sputum or tumor was positive for p16 and MGMT methylation,

15   respectively. The CaLu6 and SKuLu1 cell lines are the positive controls for p16 and MGMT methylation, respectively, and normal lung served as a negative control. Sputum cytology varied by case and is indicated in Fig. 3 for each specimen. Sputum from seven of 11 cases (Figs.1 and 3) also showed methylation of the MGMT gene, as indicated in Figs. 1 and 3. A 78% concordance was noted between MGMT methylation in the primary

20   SCC and the paired sputum sample. For the two discordant samples, MGMT methylation was detected in the tumor but not in the single sputum sample obtained 34 or 35 months prior to cancer (Fig. 2).

          [48] The demonstration of this Example 1 also considered the methylation of p16

25   and MGMT in cancer-free, high-risk subjects. The excellent concordance between detecting p16 and/or MGMT methylation in sputum samples prior to and at the time of SCC diagnosis warranted defining whether these two markers are present in any sputum samples from cancer-free subjects who are at high risk for lung cancer development. This information is valuable for the ultimate development of risk estimates for lung cancer.

30   Sputum samples were collected from 123 cancer-free subjects. People were divided into three groups based on exposure: 1) tobacco, 2) tobacco plus radon, and 3) radon alone.

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Approximately 50% of these people were considered heavy smokers with more than 30 pack-year history. Radon exposure ranged from 3 to 577 working level months; 75% of the people had more than 100 working level months. These former uranium miners worked in Grants, New Mexico, where exposures were much lower than in Colorado.

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[49] Fig. 4 shows the prevalences for p16 and MGMT methylation in sputum from cancer-free subjects. The frequency for p16 and MGMT methylation in cancer-free subjects in sputum is expressed as a percentage. Above the bar graphs of Fig. 4 are indicated the total number of positive samples per total sample population. As seen in

10   Fig. 4, the frequency for detecting aberrant methylation of the p16 or MGMT genes was similar across all groups, occurring in 12- 19% and 16 -36%, respectively. Smoking status was available for a subset (n =35) of the uranium miners and the methylation markers were present in sputum from both current and former smokers, consistent with the continued risk for lung cancer in former smokers. Bisulfite sequencing of sputum samples

15   (n = 5 for each gene) positive for p16 or MGMT revealed methylation at all CpG sites between the PCR primers (not shown).

[50] Furthermore, the examination of DNA isolated from lungs of autopsy cases, bronchial epithelial cells obtained by bronchoscopy, and lymphocytes (a component of the

20   sputum) from never smokers and all were proven negative for the methylation markers (not shown).

[51] Both p16 and MGMT methylation was detected in only four of 123 cancer-free subjects (3%), as opposed to 10 of 21 (48%) of the patients with SCC. The

25   much lower incidence (p < 0.001) of detecting both sputum markers in cancer-free subjects than in the 21 individuals studied having proven lung cancer reinforces two determinations. First, hypermethylation changes in sputum DNA do not simply reflect exposure to risk factors for lung cancer, but rather track with either a very high-risk status or actual presence of cancer. Second, the average incidence of -25% for detection of

30   either sputum marker in the cancer-free individuals is approximately equal to the known risk of lung cancer development for the populations studied. This indicates that these

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hypermethylation markers may potentially identify those patients at high risk who are most likely to actually get the disease.

[52]   To date, three lung cancers have been reported in the follow-up on this cancer-free population, which now ranges from two to six years after sputum collection: two in former uranium miners who smoked, and one in a smoker. Eight deaths have occurred not related to lung cancer. Two lung cancers were diagnosed as SCC, one and three years following sputum collection. The MGMT gene was methylated in sputums from both of these subjects. The other tumor was an adenocarcinoma. The incidence of p16 and MGMT hypermethylation is lower in this tumor type as opposed to SCC, and neither marker was present in the sputum sample collected two years prior to diagnosis.

EXAMPLE TWO:

[53]   Cases (n=52) and controls (n=89) were recruited from veterans who use the New Mexico Veterans Health Care System (NMVHCS) for their primary care. Current or former cigarette smokers (>100 cigarettes smoked during their lifetime) were eligible for the test. In addition, subjects could not have had prior diagnosis of cancer of the aerodigestive tract, have undergone chemotherapy or radiotherapy to the chest within the last year, or be unable to tolerate all procedures required to obtain tissue samples, as further described below.

[54]   Cases were recruited from patients referred to the Multispecialty Chest Clinic at NMVHCS, where > 90% of the NMVHCS lung cancer cases are evaluated for diagnosis and treatment recommendations. Patients are referred to this clinic because of signs or symptoms suggestive of lung cancer. All patients referred for diagnostic evaluation for lung cancer were recruited for participation in the test. Only those patients with histologic confirmation of lung cancer and who gave consent were enrolled into the study population as cases. All stages and histologic types of lung cancer were included. Controls were enrolled from the population of lung cancer-free individuals who received their primary care at the NMVHCS. Patients were approached randomly during primary-care clinic visits and asked to participate in the study. All subjects were asked to volunteer for a

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bronchoscopy; however, participation was not dependent on agreeing to this procedure. In addition, patients undergoing bronchoscopy for reasons other than a diagnosis of lung cancer (e.g., interstitial lung disease) were included as controls.

5   [55] The distribution of tumor histology among the 52 cases was 44% SCC, 31% adenocarcionoma, 2% large cell carcinoma, 8% small cell carcinoma, and 15% NSCLC (subtype not specified). Selected demographic variables by case-control status are summarized in the table of Fig. 8. The only significant differences seen between groups were for smoking history where duration and pack years were greater in cases, while years

10   since quit for former smokers were greater in the control group.

           [56] All participants enrolled for this study were approached to undergo bronchoscopy. A high proportion (> 85%) of cases underwent bronchoscopy since it is performed routinely as a diagnostic or staging procedure in lung cancer patients, and 45%

15   of controls consented to undergo this procedure. Bronchial epithelial cells (BECs) were obtained during bronchoscopy as previously described. BECs were harvested from up to four different sites at anatomically normal appearing bifurcations in the lungs, usually within each upper and lower lobe, using a standard cytology brush. These sites were sampled because: (1) they are high-deposition areas for particles; (2) they are frequently

20   associated with histological changes in smokers; and (3) they represent common sites of tumors. Sites selected for brushing were first washed with saline to remove non-adherent cells. Sites were not brushed if a tumor was visualized within 3 cm of the site. After brushing, the brush was placed in serum-free medium and kept on ice until processed. Up to three brushings were performed at each site. In addition, BECs from never smokers

25   were obtained from two different sources: cells derived at autopsy by Clonetics, Inc. (San Diego, California USA) from four never smokers and cells from three never smokers recruited through the NMVHS.

           [57] Tumor tissue was available either by resection or biopsy from 18 persons

30   diagnosed with lung cancer. Tumor tissue was fixed in formalin and embedded in paraffin for subsequent analysis for methylation of the p16 gene.

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[58] Cells were flash-frozen in liquid nitrogen for DNA isolation. Cells from one brush from each bronchial collection site were prepared for cytologic analysis by smearing the cells across a microscope slide. The cells were then fixed with 96% ethanol and

5   stained according to the Papanicolaou procedure to facilitate morphologic evaluation by a cytopathologist.

[59] On enrollment, patients were asked to provide an uninduced sputum specimen. If unsuccessful, participants underwent sputum induction. A variation of the

10   ultrasonic nebulization technique described by Saccomanno was used for sputum induction. Subjects used water or saline to gently brush tongue, buccal surfaces, teeth, and gingiva to remove superficial epithelial cells and bacteria, followed by gargling and rinsing with tap water. Patients then inhaled a nebulized 3% saline solution from an ultrasonic nebulizer for 20-30 minutes. Sputum was collected in a sterile specimen cup,

15   and an equal volume of Saccomanno solution was added immediately. Sputum samples were processed for methylation analysis by extensive mixing by vortex, washed once with Saccomanno solution, and stored at room temperature until analyzed. In addition, at least two slides from sputum samples were prepared and underwent Papanicoleau staining for morphologic examination by a certified cytopathologist.

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[60] DNA was isolated from BECs, tumors, and sputum by digestion with Pronase in 1% sodium dodecyl sulfate, followed by standard phenol-chloroform extraction and ethanol precipitation.

25   [61] The methylation status of the p16, MGMT, DAP-kinase, and RASSF1A genes was determined by the inventive method of MSP. Nested, two-stage PCR method was conducted, which improved the sensitivity to detect methylated alleles by > 50-fold over the original "one-stage" method (one methylated allele in > 50,000 unmethylated alleles). DNAs were subjected to bisulfite modification, and PCR was performed to

30   amplify 280 base pair, 289 base pair, 209 base pair, and 260 base pair fragment of the p16, MGMT, DAP-kinase, and RASSF1A genes, respectively, including a portion of their CpG

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rich promoter region. The primers recognize the bisulfite-modified template, but do not discriminate between methylated and unmethylated alleles. The stage-1 PCR products were diluted 50-fold, and 5μl was subjected to a stage-2 PCR in which primers specific to methylated or unmethylated template were used. Primer sequences and conditions used in

5   stage-1 and -2 amplification of the p16 and MGMT promoters have been described. Primer sequences used in the stage-1 amplification of the DAP-kinase and RASSF1A genes are as follows:

         Dap-kinase-Forward, 5 '-GGTTGTTTCGGAGTGTGAGGAG-3',

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         DAP-Kinase- reverse, 5 '-GCTATCGA-AAACCGACCATAAAC-3',

         RASSF1A-Forward, 5 '-GGAGGGAAGGAAGGGTAAGG-3',

15   RASSF1A-Reverse, 5 '-CAACTCAATAAACTCAAACTCCC-3'.

         [62] TaqTM Gold polymerase (Perkin Elmer) in a 50-μl volume was used in all PCRs. The PCR amplification protocol for stage 1 was as follows: 95° C 10 minutes, then denature at 95° C 30 seconds, anneal at 58° C (for DAP-kinase) or 60° C (for RASSF1A)

20   30 seconds, extension at 72° C 30 seconds for 40 cycles, followed by a 5-minute final extension. Primers used to selectively amplify unmethylated or methylated alleles of the DAP-kinase gene are as follows:

         Forward unmethylated, 5'-GGAGGATAGTTGGATTGAGTTAATGTT-3',

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         Reverse unmethylated, 5'-CAAATCCCT-CCCAAACACCAA-3',

         Forward methylated, 5'-ATAGTCGG-ATCGAGTTAACGTC-3', and

30   Reverse methylated, 5'-AAAACTAACCGAAA-CGACGACG-3'.

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[63] Primers used to selectively amplify unmethylated or methylated alleles of the RASSF1A gene are forward unmethylated, 5'-GGTTTTGTGAGAGTGTGTTTAG-3', reverse unmethylated, 5'-ACACTAACAAACACAA-ACCAAAC-3', forward methylated, 5'-GGGGGTTTTGCGAGAGCGC-3', and reverse methylated,

5   5'-CCCGATTAAACCCGTACTTCG-3'. Annealing temperatures were increased to 70° C and 62° C (for DAP-kinase) and 68° C and 62° C (for RASSF1A) to amplify methylated and unmethylated sequences, respectively, and all cycling times were reduced to 15 seconds for a total of 40 cycles.

10   [64] It will be immediately understood that other primers besides those specified may be employed without departing from the scope of the invention. The primer sequences can be changed; that is, both the inner primers and the outer primers may be shifted either direction a number of bases, yet within the nested approach of the invention. Modifying the location of one or more primers is a contemplated aspect of the invention

15   within the understanding of a person of ordinary skill in the art.

[65] Product sizes for each gene promoter were as follows: 153 base pairs and 106 base pairs for methylated and unmethylated DAP-kinase; and 204 base pairs and 170 base pairs for methylated and unmethylated FASSF1A. Normal human tissue collected from

20   autopsy of never smokers and cell lines positive for p16 (Calu6), MGMT (SkLU1), DAP-kinase (H2009), and RASSF1A (A549) methylation served as negative and positive controls. These cell lines were purchased from the ATCC (Mansius, Virginia USA) and cultured using the suggested protocols.

25   [66] Samples that gave positive methylation products were also analyzed by methylation-sensitive restriction enzyme digestion of the resulting PCR product. Second-stage PCRs were performed in duplicate for each sample. Then for p16, one of each sample pair was incubated with the restriction enzyme FNU 4HI that cuts (G/CGGCG) at two sites within the amplified region of the methylated p16 gene promoter. Thus, this

30   restriction enzyme will only cut template that is methylated at the two CpG sites, since the unmethylated cytosines would be modified by the bisulfite treatment to uracils. Since

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FNU 4HI cuts at two different sites within the portion of the p16 promoter being examined, applicants confirmed that four CpGs were methylated, which verified the methylation status of the sample. An identical approach was used for the DAP-kinase and RASSF1A genes using the BstUI restriction enzymes were used to assay three different

5   CpG sites within the MGMT gene. All samples scored as positive for methylation were confirmed by restriction analysis.

[67] Data were summarized using percents for discrete variables and medians with ranges for continuous variables. Differences between groups were assessed using Fisher's

10 exact test for discrete variables and the Wilcoxon rank sum test for continuous variables. Results from pairs of markers were examined using Fisher's exact test to assess the association between markers and MacNemar's test to assess differences in proportions positive for the markers. Logistic regression models with case-control status as the outcome were used to control for multiple predictor variables simultaneously. All

15 analyses were conducted in SAS software (SAS Institute In., Cary, North Carolina USA).

[68] Cytologic changes were characterized in BECs obtained from up to four sites in 51 cases (cytology not available for one case) and 41 controls. A total of 166 and 150 sites were evaluated in cases and controls, respectively. Metaplasia (reactive) and mild

20 dysplasia (atypia) were the predominant cytological abnormalities observed, and their distribution did not differ among groups. One case had severe dysplasia within a bronchial epithelial site. Cytologic changes were seen in at least one site in approximately 54% of both cases and controls. The number of cases with at least two or three sites positive for cytology exceeded those observed in controls: 33% versus 20% ($p = 0.24$) and

25 25% versus 3% ($p = 0.007$), respectively. These cytologic changes were observed in $< 10\%$ of cells recovered from the diagnostic brush. There was no apparent association between the presence of cytology and tumor histology or smoking status (current versus former); however, cases with cytologic changes in their bronchial epithelium smoked more than those without cytologic changes (median pack years, 78 versus 59; $p = 0.10$).

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[69] Fig. 9 depicts methylation of the p16 gene in BECs and corresponding sputum. Representative scenarios for detection of methylation in BECs and sputum from five different controls are shown. The sample number corresponds to an individual and the fraction (e.g. 1.1, 1.2) of BECs collected from different lung lobes from that

5   individual. For example, in the first control, only the second bronchial epithelial site was positive for p16 methylation. CaLu6 and H2009 are cell lines known to contain a methylated and unmethylated p16 gene, respectively. Fig. 10 illustrates aberrant promoter hypermethylation in nonmalignant BECs from lung cancer cases and controls. Summary data are presented as the frequency of methylation (percent of persons positive) at one to

10   three bronchial epithelial sites for the p16 and DAP-kinase genes.

[70] BEC cultures were successfully established from 94% of the collected sites allowing the methylation state of the p16 MGMT, DAP-kinase, and RASSF1A genes to be examined in 160 and 137 sites from cases and controls, respectively. Aberrant promoter

15   methylation of the p16 gene was seen in at least one site from 44% of both cases and controls, and 14% and 15% of cases and controls, respectively, had two sites positive, as seen in Figs. 9 and 10. Further, two cases and one control had three sites positive, but no subjects had methylation of p16 in all four BEC sites (Fig. 2). The prevalence for methylation of the DAP-kinase gene in bronchial epithelium was significantly less than

20   observed for p16 irrespective of case-control status (p -- 0.001). Only five cases (10%) and four controls (10%) had a BEC site positive for methylation of the DAP-kinase gene; as indicated in Fig. 10, methylation was never seen in more than one site in a subject. Methylation of the RASSF1A gene was not detected in any sites. Interestingly, methylation of both p16 and DAP-kinase was seen in two cases and one control. Only in

25   the control subject was the methylation found at the same site. Methylation of p16, DAP-kinase, or RASSF1A was not detected in any of the 17 sites from seven never-smokers (not shown), a finding consistent with previous studies that have reported virtually no genetic alterations in bronchial epithelium from this population).

30   [71] In marked contrast to the findings for these three genes in BECs, methylation of the MGMT gene was observed in > 75% of BEC sites from cases and controls. In

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addition, MGMT methylation was seen in 14 of 17 sites from the never-smokers (not shown). This finding has also been observed in normal human fibroblasts, which displayed extensive methylation of the MGMT CpG island as they became confluent. The process was partially reversed when fibroblasts were placed back into logarithmic growth.

5   Thus, in the tissue culture setting, it is apparent that confluence-induced growth constraint must cause transient alterations in epigenetic stability within the MGMT promoter, leading to aberrant methylation. The fact that the applicants' BECs were grown to 80% confluence, coupled with the use of the inventive two-stage MSP approach, facilitated the detection of this in vitro methylation. MGMT nevertheless may be used as a biomarker in

10   sputum, since the high prevalence for the changes seen in vitro occur in a context quite distinct from that of the airway epithelium. Furthermore, this determination is not recapitulated in either primary NSCLCs or exfoliated cells within sputum where the prevalence for MGMT methylation is 25% and 15%, respectively.

15   [72] For the cases, there was no association between tumor histology and methylation of the p16 of DAP-kinase genes in the bronchial epithelium. This finding is consistent with the fact that these genes are methylated in both SCC and adenocarcinome. Smoking status had no association with the detection of methylation of either the p16 or DAP-kinase gene in the bronchial epithelium. Thus, promoter methylation was seen as

20   frequently in BECs from current smokers as from former smokers. In addition, none of the smoking variables categorized (duration, pack years, time quit) was associated with the detection of promoter methylation in the bronchial epithelium.

[73] A correlation was observed between p16 status in BECs and lung tumors.

25   Tumor tissue was available from 18 of the cases diagnosed with lung cancer. To determine where p16 methylation in the BECs within these cases predicts methylation at this locus in tumors, DNA was isolated from microdissected tumors for MSP analysis of p16. Methylation of the p16 gene was detected in four of eight SCCs, four of eight adenocarcinomas, and one of two NSCLCs (not shown). Seventeen of the 18 tumors

30   showed an absolute concordance (p < 0.001), being either both methylated in the tumor and at least one BEC site (nine pairs) or unmethylated in the tumor and BECs (eight pairs).

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In the one discordant tumor-BEC pair, the bronchial epithelium was methylated, and the tumor was unmethylated.

[74] The studies of Example One above for p16 and MGMT methylation in a limited number of cancer-free smokers suggest that the prevalence for these changes approximate lifetime risk for lung cancer. Thus, during the recruitment of subjects for this case-control study, the protocol was amended to include the collection of sputum from controls in order to gather additional information on the prevalence of the p16, MGMT, DAP-kinase, and RASSF1A genes in this population of veterans. Sputum was obtained from 66 controls; 18 underwent bronchoscopy.

[75] Fig. 11 shows aberrant promoter hypermethylation in sputum from cancer-free controls. Summary data are presented as the frequency for methylation of the p16, MGMT, DAP-kinase, and RASSF1A genes in sputum collected from 66 cancer-free controls. The total number of positive samples per total sample population is depicted above the bar graphs in the figure. Reference also is made to Fig. 12, showing the relationship between p16 methylation in BECs and sputum. The overall frequency for methylation of p16 in BECs and matched sputum samples from 18 cancer-free controls is depicted on the left horizontal axis of the figure. The relationship between methylation in the BECs and sputum is displayed by four possible situations: (1) positive sputum, positive BEC; (2) negative sputum, negative BEC; (3) negative sputum, positive BEC; and (4) positive sputum, negative BEC. The number of subjects who fit into each category is indicated above the bar graph.

[76] Abnormal sputum cytology was seen in 25% of controls. Metaplasia was the most common change, being present in 19% of sputum samples, while mild dysplasia was observed in 6% of sputum samples. Methylation of the p16 gene was detected in sputum from 23 of 66 controls (35%; Fig. 11). DAP-kinase methylation was also seen in sputum from 16 controls, and eight of these subjects were positive for methylation of p16, as seen in Fig. 11. MGMT methylation was seen in nine controls, four of these subjects were also positive for either p16 (one subject) or DAP-kinase (three subjects). In addition, three

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subjects positive for MGMT methylation also had methylation of both p16 and DAP-kinase in their sputum (not shown). Methylation of RASSF1A was only detected in two sputum samples; however, one person positive for this gene contained p16, DAP-kinase, and MGMT methylation in the sputum sample. The other person's sputum was positive

5   for DAP-kinase and MGMT methylation. Thus, three (5%) and one (2%) of the control subjects were positive for three or four of the methylation markers, respectively, in their sputum. As seen in Example One above, sputum cytology was not associated with positivity for aberrant promoter methylation. Smoking status had no association with the detection of gene promoter methylation in sputum. In addition, none of the smoking

10   variables (duration, pack years, time quit) was associated with whether methylation was detected in the sputum.

      [77] The concordance between finding p16 methylation in sputum and bronchial epithelium from the 18 controls was examined. Reference is made to Fig. 12. In this

15   subset, p16 methylation was seen in BECs and sputum of 10 and seven controls, respectively. Of the seven subjects whose sputum was positive for this market, five displayed the methylation change in their bronchial epithelium. Continuing reference to Fig. 12 shows that there were five controls whose bronchial epithelium was positive and sputum negative for p16 methylation.

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      [78] The results of the test indicate that aberrant promoter hypermethylation of the p16 gene and to a lesser extent, DAP-kinase, occurs frequently in the bronchial epithelium of lung cancer cases and cancer-free controls who smoked. These methylation changes persist after smoking cessation. The changes are detected within exfoliated cells,

25   supporting their use as markers in assessing lung cancer risk reduction in response to cancer chemopreventative agents. Prevalence and tissue multiplicity for the five genetic and epigenetic markers examined in this Example did not differentiate cases from controls. This determination has been corroborated by investigators examining other genetic markers, and is likely due to the overwhelming damage to the bronchial epithelium

30   through years of exposure to tobacco carcinogens. Furthermore, the lack of any

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association between these markers and smoking does or duration may be due to the fact that 92% of cases and 73% of controls had >30 pack-years of smoking.

[79] The fact that promoter methylation was seen at similar prevalences in cases and controls irrespective of smoking status (current versus former) does not challenge the suggestion that these methylation changes confer increased risk for lung cancer. Approximately half of the lung cancers are diagnosed in former smokers. While cessation of smoking is associated with a decrease in lung cancer risk, the cumulative risk for lung cancer by age 75 for a person who quits smoking at age 50 is still 5 times greater than a never smoker. Furthermore, the strong association seen between p16 methylation in the bronchial epithelium and corresponding primary tumor reinforces the concept that inactivation of genes such as p16, while not transforming by themselves, are most likely permissive for the acquisition of additional genetic and epigenetic changes that ultimately lead to malignant cancer. Supporting this concept are the findings from Example One above, where methylation of both p16 and MGMT was seen in sputum from 43% of persons with confirmed SCC, but only in 3% to 6% of cancer-free controls. Therefore, the time to acquire the additional genetic and epigenetic changes that promote tumor progression is likely a critical determinant for lung cancer risk.

[80] Inactivation of the p16 gene by promoter hypermethylation was seen more frequently in bronchial epithelium than DAP-kinase, while RASSF1A methylation was never detected. This determination may reflect the timing and role of these genes in the development of lung cancer. Applicants have determined that p16 methylation is detected at the earliest cytologic stages of SCC and adenocarcinoma. Inactivation of p16 has been proposed as an early step to immortalization by allowing cells to escape the immortality checkpoint "MO". The importance of p16 in the development of NSCLC is evident by the fact that it is inactivated by promoter methylation in 60% to 70% of SCCs and up to 48% of adenocarcinomas.

[81] The fact that promoter methylation was not seen in bronchial epithelium, and that only two sputum samples were positive for RASSF1A methylation, suggest that

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inactivation of this gene is a later event in malignant transformation. This conclusion is supported by the determination that both control subjects with RASSF1A methylation in their sputum also had methylation of at least two other genes (p16, MGMT, and/or DAP-kinase). These determinations indicate the potential utility of multiple markers for the

5   ultimate development of risk profiles.

[82] The p16 and DAP-kinase genes are inactivated by promoter hypermethylation in nonmalignant bronchial epithelium of current and former smokers, which is also detected in exfoliated cells within sputum. Sputum samples contain cells from the large

10   bronchi and cells to a lesser extent from the smaller airways that include the small bronchi, bronchioles, and alveoli. A positive sputum sample reflected methylation in the bronchial epithelium 70% of the time.

[83] The foregoing disclosure indicates that bronchial epithelial cells that harbor

15   p16 or DAP-kinase genes inactivated through promoter hypermethylation persist after cessation of smoking. The impact that silencing of these and other critical regulatory genes has on absolute risk for lung cancer will be better addressed through the analysis of exfoliated cells within a biological fluid such as sputum that represents a composite of the genetic damage throughout the aerodigestive tract. Thus, the presence of promoter

20   methylation in sputum from cancer-free smokers signals a higher risk for lung cancer.

[84] The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples.

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[85] Figs. 5 and 6 summarize in tabular form the PCR primers and annealing temperatures, in degrees centigrade to perform, respectively, the first- and second-stage PCR method of the invention for various specified genes, including H-Cadherin, retinoic acid receptor beta, and fragile histidine triad. It is seen therefore, that the inventive

30   method finds applicability on other gene types besides p16, MGMT, DAP-kinase, and RASSF1A.

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[86] Fig. 7 illustrates the improved sensitivity for detection of p16 methylation according to the invention. The CaLu6 cell line contains a methylated p16 gene. DNA isolated from this cell line was serially diluted with DNA isolated from normal human

5   lung. The DNA was then modified by bisulfite, and primers flanking the CaLu6 promoter region were used for stage-1 PCR. Depicted in Lanes 1-5 of the figure are the results of the stage-2 PCR with methylation-specific primers. The PCR product indicative of methylation is readily seen in DNA diluted up to 50,000 fold with normal DNA (lanes 1-5).

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[87] The invention offers a highly sensitive, robust, and reproducible test. Equally important, the invention contributes to cancer diagnosis and monitoring of all forms of cancer. The nested approach reduces the problem encountered in amplification of DNA targets (specific gene fragments) that are of suboptimal quality (partially degraded). The

15   quality of DNA recovered from sputum and/or plasma is often partially degraded, meaning that the size of the DNA is less than what is found in healthy tissue. The impact of this on PCR is a reduction in the actual amount of specific DNA target that can be amplified and, ultimately, the sensitivity to detect the altered gene. Because the invention first expands the number of copies of the gene being evaluated, the amount of material used in the

20   second PCR can be adjusted based on the quantity of product generated through the first PCR. In addition, because the annealing temperatures (the temperature by which the specific primers attach to the complementary target DNA) are raised in the second PCR, this greatly reduces the chance for getting a false positive result, a critical issue in the development of an accurate diagnostic test.

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[88] Further, while this disclosure has provided examples where a single gene (e.g. p16) has been the subject of the examination for a given polymerase chain reaction, it should be noted that the inventive method may be practiced where a plurality of two or more genes are examined for methylation in the course of a single procedure. For

30   example, subject to limitations requiring significant differences in molecular weights, a single nested PCR procedure according to the invention may be practiced to look

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simultaneously for more than one marker, such as methylation of both p16 and MGMT. Risk profiles, for example, may be developed by looking at combinations of markers assayed with the invention. Accordingly, the inventive method may entail the concurrent assaying in a single reaction of multiple markers in biological fluids to determine risk, and

5   perform monitoring, prevention, and prognosis. Further, different markers may be the subject of separate procedures to offer additive detection monitoring value, and increased efficiency and reliability of the overall evaluation.

[89] The method of the invention may be practiced through the use of a laboratory

10   kit. Kits for practicing the invention may be adapted to provide the materials needed to perform analysis specific to a particular type of body fluid, and/or customized to pertain to specific promotor genes of interest to the technician.

[90] The development of methylation biomarkers using the invention allows the

15   detection of many types of early cancers that would include but not be limited to lung, liver, head and neck, leukemia, colorectal, prostate, and bladder. Once the resulting tumor is identified and appropriate intervention is taken, the biomarkers can also be used to monitor disease reoccurrence and response to adjuvant therapy such as chemotherapeutics. An additional application of the technology relates specifically to lung cancer where often

20   the tumor is not accessible for large-scale tissue sampling. Using biological fluids such as serum, plasma, or sputum, it is possible to determine non-invasively the genes inactivated by promoter hypermethylation. This information can be used to customize therapy.

[91] Finally, many cancers take years to develop and susceptible persons go

25   through a period of premalignancy where cells or DNA may be shed that harbor methylation biomarkers. The invention is useful for identifying individuals who subsequently could be enrolled in prevention intervention studies using dietary supplements that are designed to impede or reverse this premalignant state. The methylation biomarkers may also be used to monitor the efficacy of the interventions, by

30   determining whether the previously detected methylation biomarker persists or disappears during the course of the intervention. The efficacy of any of the interventions known in

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the art, including but not limited to immunological modulation, gene therapy, antisense treatment, alkylating treatments, and the like, may be monitored.

[92] Although the invention has been described in detail with particular reference
5   to these preferred embodiments, other embodiments can achieve the same results. Variations and modifications of the present invention will be obvious to those skilled in the art. The entire disclosures of all references, patents, and publications cited above are hereby incorporated by reference.

32

CLAIMS

What is claimed is:

5   1.   A method of monitoring for cancer in cells, comprising detecting the

presence of gene-specific promoter methylation in the cells, comprising the steps of:

expanding the number of copies of at least one specific gene by using a

polymerase chain reaction to amplify a portion of the gene where the promoter

methylation resides, thereby generating an amplification product; and

10   using an aliquot of the amplification product generated by the first

polymerase chain reaction in a second, methylation-specific, polymerase chain reaction to

detect the presence of inactivation of the at least one specific gene.

2.   The method of claim 1 wherein the step of expanding the number of copies

15   of at least one specific gene comprises expanding the number of copies of more than one

specific gene.

3.   The method of claim 2 wherein the step of expanding the number of copies

of more than one gene comprises expanding the number of copies of at least two genes

20   selected from the group consisting of p16, MGMT, RASSF1A gene, H-Cadherin, retinoic

acid receptor beta gene, and fragile histidine triad gene.

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4.   The method of claim 1 wherein the step of expanding the number of copies of a specific gene comprises amplifying a single gene.

5.   The method of claim 4 wherein the step of expanding the number of copies of a single gene comprises amplifying the p16 gene.

6.   The method of claim 5 wherein the step of amplifying the p16 gene comprises amplifying the 280 base pair.

7.   The method of claim 4 wherein the step of expanding the number of copies of a single gene comprises amplifying the MGMT gene.

8.   The method of claim 7 wherein the step of amplifying the MGMT gene comprises amplifying the 289 base pair.

9.   The method of claim 4 wherein the step of expanding the number of copies of a single gene comprises amplifying the DAP-kinase gene.

10.   The method of claim 9 wherein the step of amplifying the DAP-kinase gene comprises amplifying the 209 base pair.

11.   The method of claim 4 wherein the step of expanding the number of copies of a single gene comprises amplifying the RASSF1A gene.

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12.   The method of claim 11 wherein the step of amplifying the RASSF1A gene comprises amplifying the 260 base pair.

5   13.   A method of monitoring for a cancer in a human by detecting the presence of promoter methylation in the genes of the human's cells, comprising the steps of:

            expanding the number of copies of the gene that is altered by the cancer, by using a polymerase chain reaction to amplify a portion of the gene where the promoter methylation resides, thereby generating an amplification product; and

10   using, in a second methylation-specific polymerase chain reaction, an aliquot of the amplification product generated by the first polymerase chain reaction to detect the presence of inactivation of the gene.

14.   The method of claim 13 further comprising the step of obtaining a sample

15   of biological fluid containing the gene that is altered by the cancer.

15.   The method of claim 14 wherein the step of obtaining a sample comprises obtaining sputum.

20   16.   The method of claim 14 wherein the step of obtaining a sample comprises obtaining a biological fluid selected from the group consisting of plasma, ejaculate, cerebrospinal fluid, serum, mammary duct fluid, urine, and fecal stool.

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17.   The method of claim 15 wherein the step of expanding the number of copies of the gene comprises amplifying the p16 gene.

18.   The method of claim 17 wherein the step of amplifying the p16 gene
5   comprises amplifying the 280 base pair.

19.   The method of claim 15 wherein the step of expanding the number of copies of the gene comprises amplifying the MGMT gene.

10   20.   The method of claim 19 wherein the step of expanding the number of copies of a specific gene comprises amplifying the 289 base pair.

21.   The method of claim 15 wherein the step of expanding the number of copies of a specific gene comprises amplifying the DAP-kinase gene.

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22.   The method of claim 21 wherein the step of expanding the number of copies of a specific gene comprises amplifying the 209 base pair.

23.   The method of claim 15 wherein the step of expanding the number of
20   copies of a specific gene comprises amplifying the RASSF1A gene.

24.   The method of claim 23 wherein the step of expanding the number of copies of a specific gene comprises amplifying the 260 base pair.

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25.   A method of monitoring for cancer in a human, comprising detecting gene

inactivation in a biological fluid by ascertaining the presence of gene-specific promoter

methylation in the cells of the biological fluid, and further comprising the steps of:

5   obtaining a sample the biological fluid containing a specific gene, wherein

the step of obtaining a sample comprises the step of selecting a member from the group

consisting of plasma, urine, fecal stool, and sputum;

               expanding the number of copies of the gene by using a polymerase chain

reaction to amplify a portion of the gene where the promoter methylation resides, thereby

10   generating an amplification product; and

               using an aliquot of the amplification product generated by the first

polymerase chain reaction in a second, methylation-specific, polymerase chain reaction to

detect the presence of inactivation of the specific gene in the biological fluid.

15   26.   The method of claim 25 wherein the step of expanding the number of

copies of the gene comprises amplifying the p16 gene.

27.   The method of claim 26 wherein the step of amplifying the p16 gene

comprises amplifying the 280 base pair.

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28.   The method of claim 25 wherein the step of expanding the number of

copies of the gene comprises amplifying the MGMT gene.

37

29.   The method of claim 28 wherein the step of expanding the number of copies of a specific gene comprises amplifying the 289 base pair.

30.   The method of claim 25 wherein the step of expanding the number of
5   copies of the gene comprises amplifying the H-Cadherin gene.

31.   The method of claim 25 wherein the step of expanding the number of copies of the gene comprises amplifying the retinoic acid receptor beta gene.

10   32.   The method of claim 25 wherein the step of expanding the number of copies of the gene comprises amplifying the fragile histidine triad gene.

33.   A nested, two-stage polymerase chain reaction method for amplifying the gene that may be altered in a particular cancer, thereby permitting cancer detection and
15   monitoring by detecting gene inactivation in a biological fluid, comprising the steps of:

        expanding the number of copies of the gene that is altered by the cancer, by using a polymerase chain reaction to amplify a portion of the gene where the promoter methylation resides, thereby generating an amplification product containing fragments of a gene selected from the group consisting of the p16 gene, the MGMT gene, the DAP-
20   kinase gene and the RASSF1A; and

        using, in a second methylation-specific polymerase chain reaction, an aliquot of the amplification product generated by the first polymerase chain reaction to detect the presence of inactivation of the gene that is altered by the particular cancer.

38

34.   The method of claim 33 further comprising the step of obtaining a sample of the biological fluid containing the gene that is altered by the cancer.

5   35.   The method of claim 34 wherein the step of obtaining a sample comprises obtaining sputum.

36.   A method of monitoring for cancer in a human, comprising detecting gene inactivation in a biological fluid by ascertaining the presence of gene-specific promoter

10   methylation in the cells of the biological fluid, and further comprising the steps of:

subjecting DNA in the biological fluid to bisulfite modification;

expanding the number of copies of a specific gene in the DNA by using primers which recognize the bisulfite-modified DNA template, but which not discriminate between methylated and unmethylated alleles, in a polymerase chain reaction to amplify a

15   CpG-rich portion of the gene where the promoter methylation resides, thereby generating an amplification product containing fragments of the gene;

using an aliquot of the amplification product generated by the first polymerase chain reaction in a second, methylation-specific, polymerase chain reaction employing primers specific to a methylated DNA template to detect the presence of

20   inactivation of the specific gene.

39

37.   The method of claim 36 wherein the biological fluid is selected from the group consisting of sputum, plasma, ejaculate, mammary duct fluid, cerebrospinal fluid, urine, and fecal stool.

5   38.   The method of claim 37 wherein the step of expanding the number of copies of the specific gene further comprises amplifying the p16 gene.

39.   The method of claim 38 wherein the step of amplifying the p16 gene comprises amplifying the 280 base pair.

10

40.   The method of claim 37 wherein the step of expanding the number of copies of the gene further comprises amplifying the MGMT gene.

41.   The method of claim 40 wherein the step of expanding the number of

15   copies of a specific gene comprises amplifying the 289 base pair.

Methylation of the p16 and MGMT Genes in Tumor/Sputum Pairs at Time of Diagnosis

Case	Exposure	Sample	Cytology	Methylation p16	Methylation MGMT
1	Tob/Radon	SCC / Sputum	NA / Mild Atypia	M / M	U / U
2	Tob/Radon	SCC / Sputum	NA / SCC	U / U	M / M
3	Tob/Radon	SCC / Sputum	NA / SCC	U / U	M / M
4	Tob	SCC / Sputum	NA / SCC	M / M	M / U
5	Tob	SCC / Sputum	NA / SCC	M / M	M / U
6	Tob/Radon	SCC / Sputum	NA / Mod. Dyspl.	M / M	U / U
7	Tob	SCC / Sputum	NA / Marked Dyspl.	M / M	M / M
8	Tob	SCC / Sputum	NA / Mod. Dyspl.	M / M	U / M
9	Tob	SCC / Sputum	NA / Mod. Dyspl.	M / M	U / U
10	Tob/Radon	SCC / Sputum	NA / Marked Dyspl.	M / M	M / M

Methylation state of the p16 and MGMT genes in tumors and sputums obtained at the time of diagnosis.
Abbreviations: Tob, tobacco; SCC, squamous cell carcinoma; NA, not applicable; M, methylated; U,
unmethylated; Mod Dyspl. moderate dysplasia.

FIGURE 1

Methylation of the p16 and MGMT Genes in Sputum Precedes Clinical Diagnosis of SCC

Case	Exposure	Sample	Months Prior To Cancer	Cytology	Methylation	
					p16	MGMT
1	Tob/Radon	SCC Sputum	0 5	NA Mod. Dysp.	U M	M M
2	Tob/Radon	SCC Sputum Sputum Sputum	0 5 5 5	NA Suspicious Suspicious Marked Dysp.	M M M M	M M M M
3	Tob	SCC Sputum	0 6	NA SCC	M M	M M
4	Tob/Radon	SCC Sputum	0 10	NA Mod. Dysp.	M M	U U
5	Tob/Radon	SCC Sputum Sputum	0 8 13	NA Suspicious Suspicious	M M M	M M M
6	Tob/Radon	SCC Sputum Sputum Sputum	0 2 9 15	NA SCC Mod. Dysp. Mild Atypia	M M M M	M M M U
7	Tob/Radon	SCC Sputum Sputum	0 5 15	NA Mod. Dysp. Mod. Dysp.	M M M	M M M
8	Tob/Radon	SCC Sputum Sputum	0 8 20	NA Suspicious Suspicious	M M M	M M M
9	Tob/Radon	SCC Sputum Sputum	0 0 20	NA SCC CIS	M M M	U U U
10	Tob	SCC Sputum	0 34	NA Severe Dysp.	M M	M U
11	Tob	SCC Sputum	0 35	NA Severe Dysp.	M M	M U

Methylation state of the p16 and MGMT genes in tumors and sputums obtained from 11 different cases. Abbreviations: Tob, tobacco; SCC, squamous cell carcinoma; NA, NA, not applicable:not applicable; M, methylated; U, unmethylated; CIS, carcinoma in situ; Dysp, dysplasia; Suspicious, suspicious for malignancy.
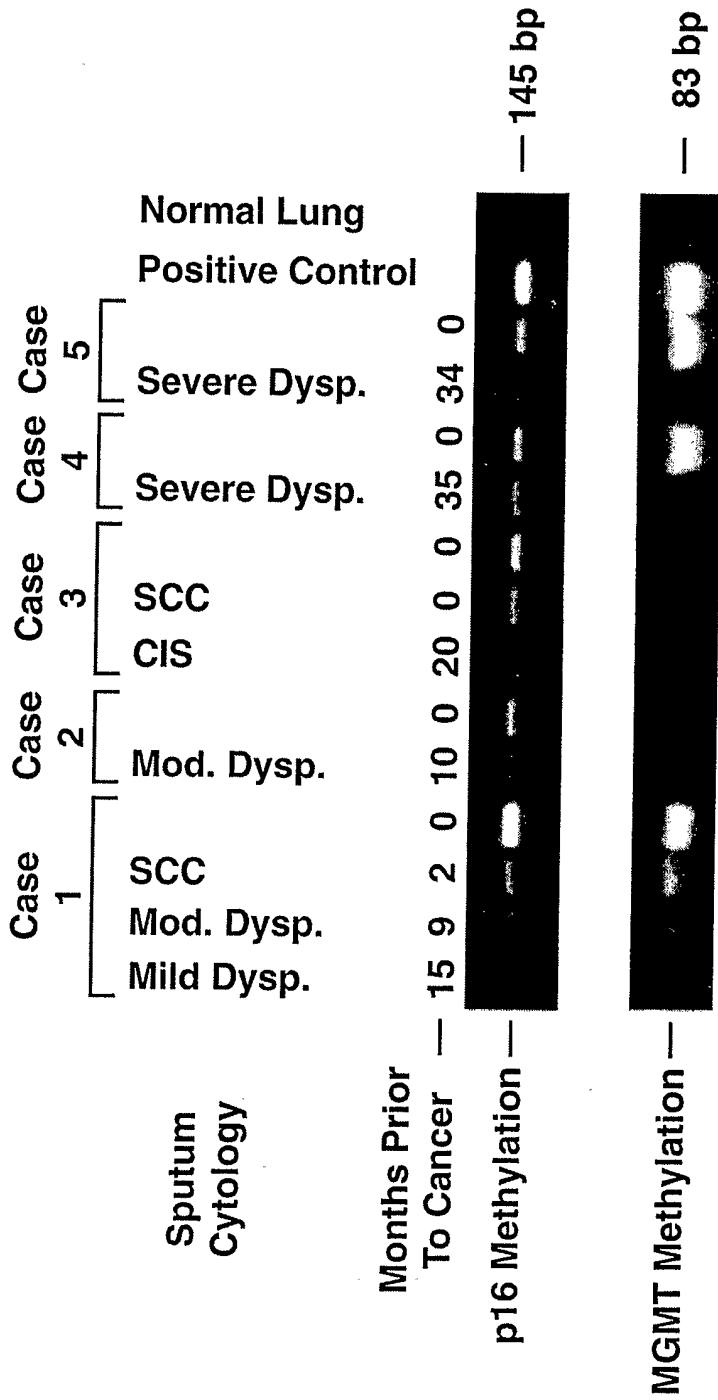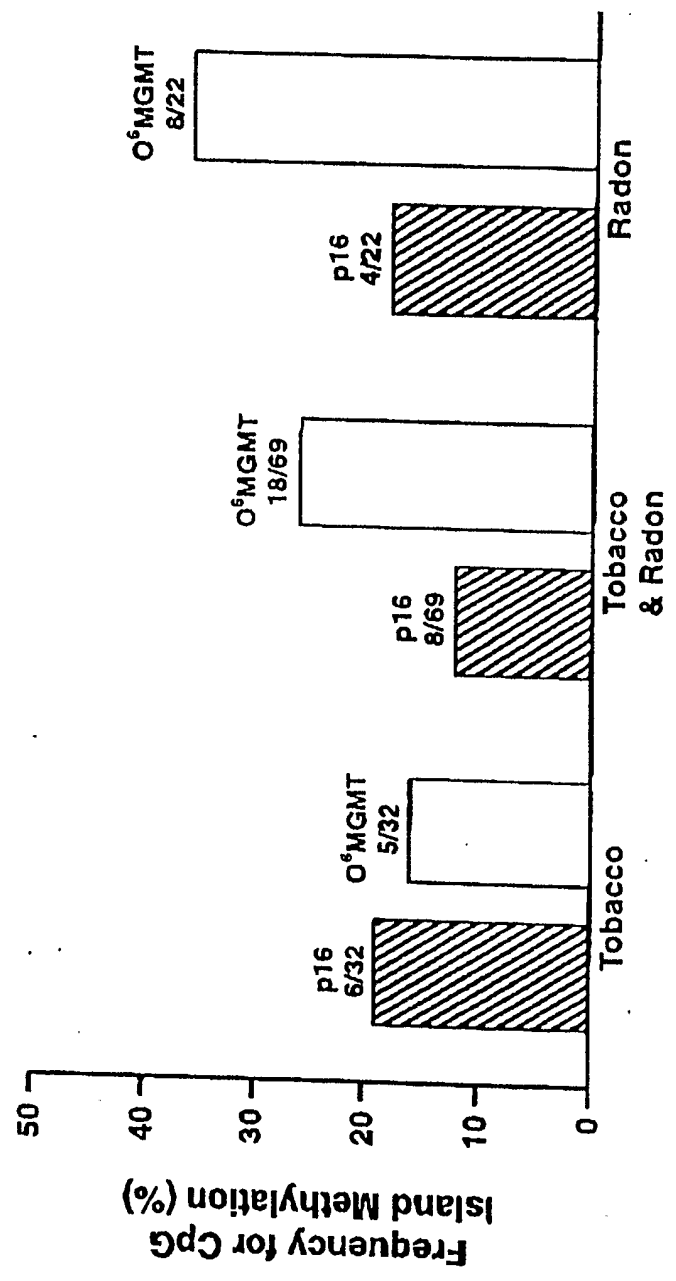
FIGURE 2

Figure 3

FIGURE 4

Gene	Primers (5' – 3')	Annealing Tm
P16 cyclin dependent kinase inhibitor 4a	GAGGAAGAAAGAGGAGGGGTTG ACAAACCCTCTACCCACCTAAATC	60 60
O6-methylguanine DNA methyltransferase	GTTTYGGATATGTTGGGATAGTT AACACTTAAAAACRCACCTAAAACTC	60 60
Death-associated protein kinase	GGTTGTTTYGGAGTGTGAGGAGG ACGCTATCGAAAAACCGACCATAAAC	60 60
RAS –accociated family 1 gene	GGAGGGAAGGAAGGGTAAGG CAACTCAATAAACTCAAACTCCC	60 60
H-Cadherin	TTTTTAYGGAAAATATGTTTAGTGTAGT TAAACTCRAAATAACCTCCCTACC	60 60
Retinoic acid receptor beta	TTTATGYGAGTTGTTTGAGGATTGG AATCCAAATAATCATTTACCATTTTCC	60 60
Fragile histidine triad	GGTTATTTAGTGGGTATATTTTTAGG RAATCCCCACCTAAAACCCTC	60 60

Y = C + T, R = A + G

FIGURE 5

STAGE 2 PCR

Gene	Primers (5' - 3')	Annealing Tm
P16 cyclin dependent kinase inhibitor 4a	GAGGGTGGGGCGGATCGC GACCCCGAACCGCGACCG	70 70
O6-methylguanine DNA methyltransferase	ACGTTTTGCGTTTCGACGTTC ACCCCCACCCGACGACG	68 68
Death-associated protein kinase	ATAGTCGGATCGAGTTAACGTC AAAACTAACCGAAACGACGACG	70 70
RAS –associated family 1 gene	GGGGGGTTTGCGAGAGCGC CCCGATTAAACCCGTACTTCG	68 68
H-Cadherin	GAATGAAAACGTCGTCGGGC ATCTATCTTCGCCGCCGCG	68 68
Retinoic acid receptor beta	GTCGAGAACGCGAGCGATTC CGACCAATCCAACCGAAACG	68 68
Fragile histidine triad	GGCGGGCGTTTCGGTTTCGC GCCCCGTAAAACGAGCGCCG	68 68

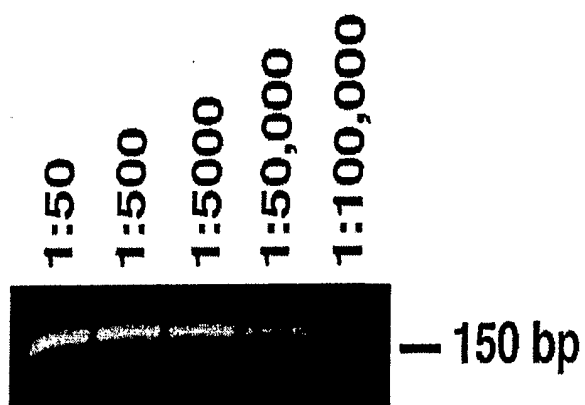FIGURE 6

Dilution of CaLu-6
with Normal DNA

1:50   1:500   1:5000   1:50,000   1:100,000

— 150 bp

FIGURE 7

Summary of selected demographic variables by study group

Variable	Cases (n = 52)	Controls (n = 89)
Age (years)	68 (44, 79)[a]	67 (45, 81)[a]
Gender (% male)	96	99
Ethnicity (%)		
Caucasian	67	69
Hispanic	21	27
African American	9	1
Native American	2	2
Smoking Status (% Current)	28	32
Smoking, Duration (yrs)	50 (18, 66)[a]	36 (3, 64)[a,b]
Smoking, Pack Years	66 (18, 177)[a]	48 (1, 183)[a,b]
Smoking, Time Quit (yrs)	3 (1, 34)[a]	20 (1, 42)[a,b]

Statistical comparisons are between cases and controls.
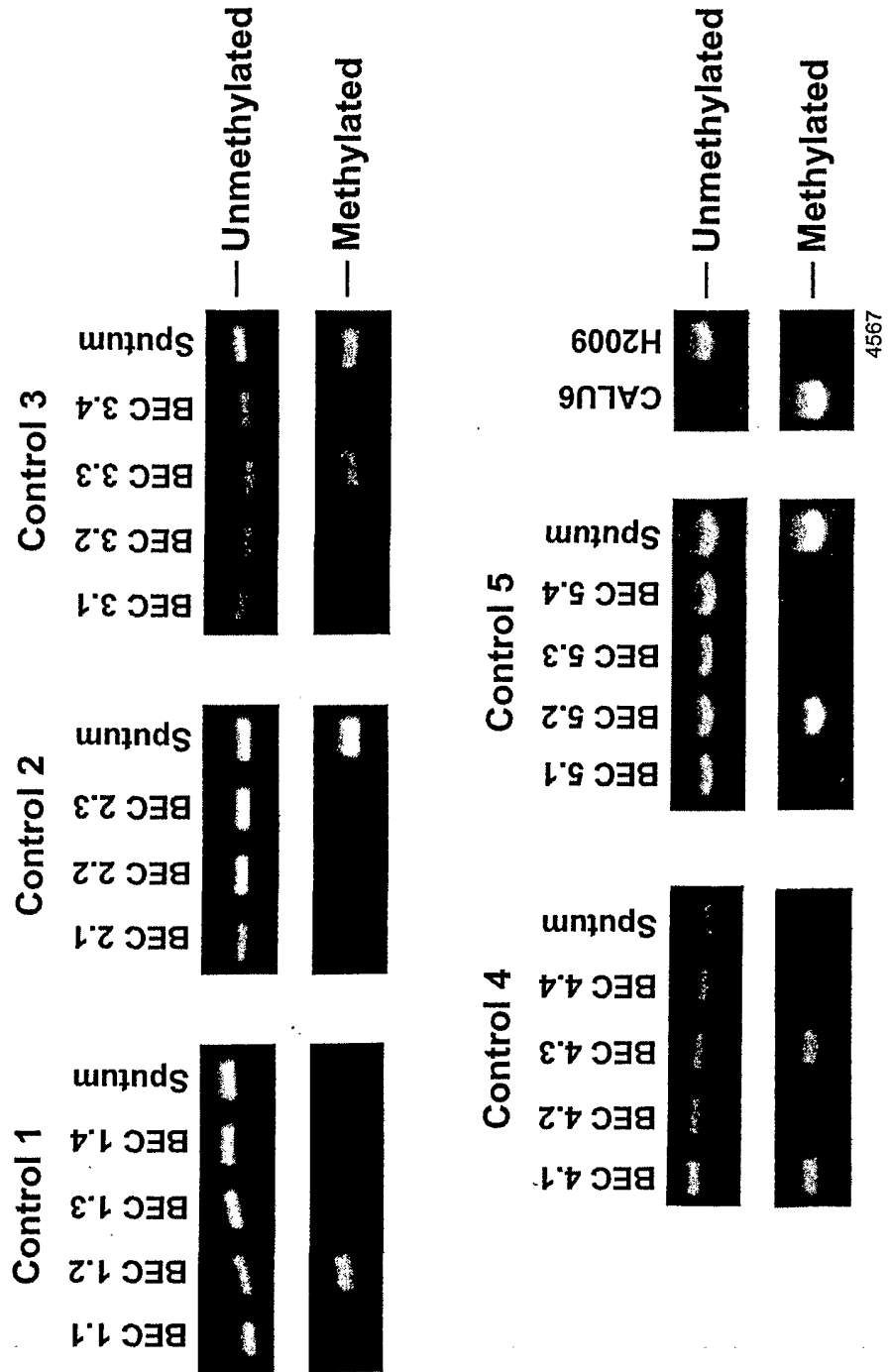
[a]Median (minimum, maximum).
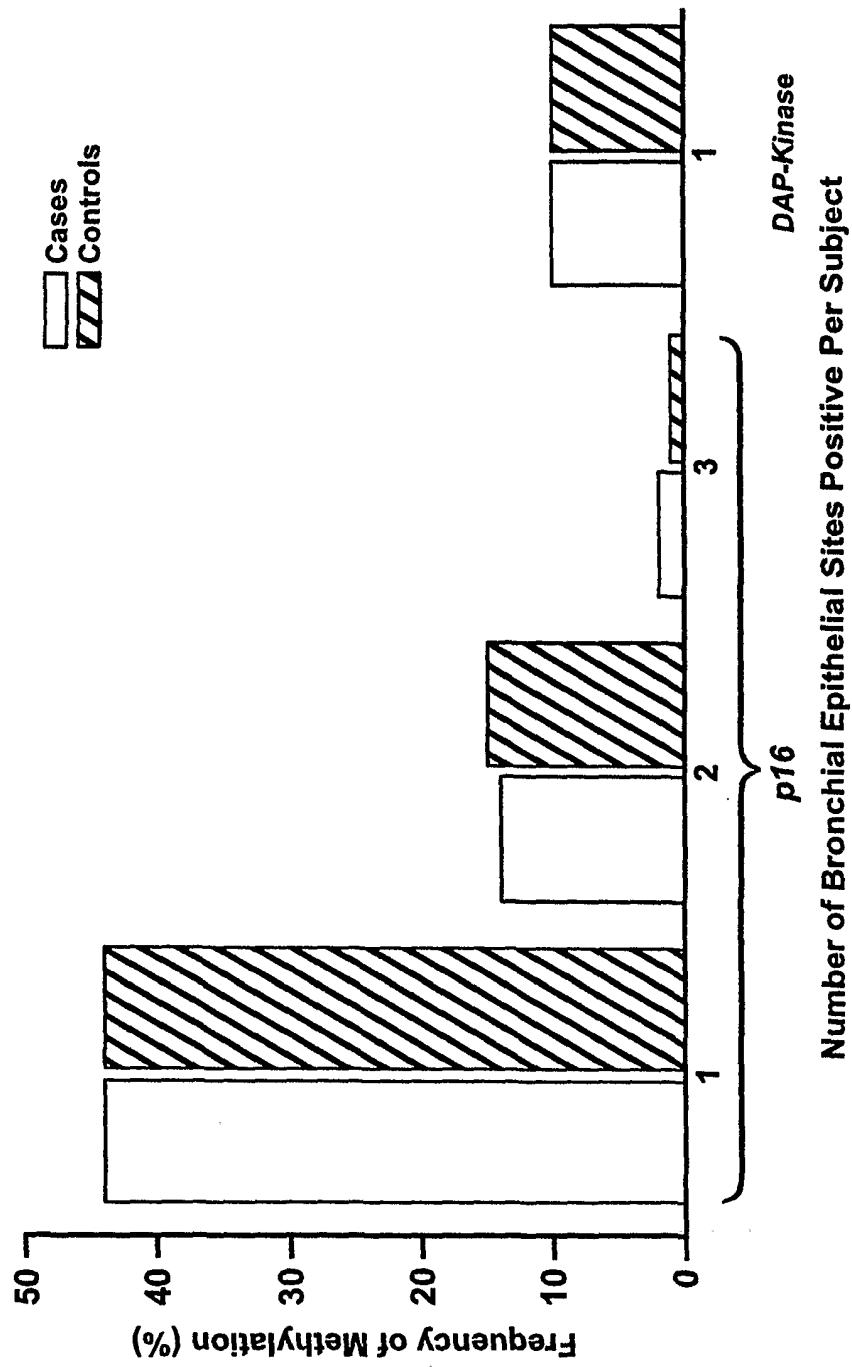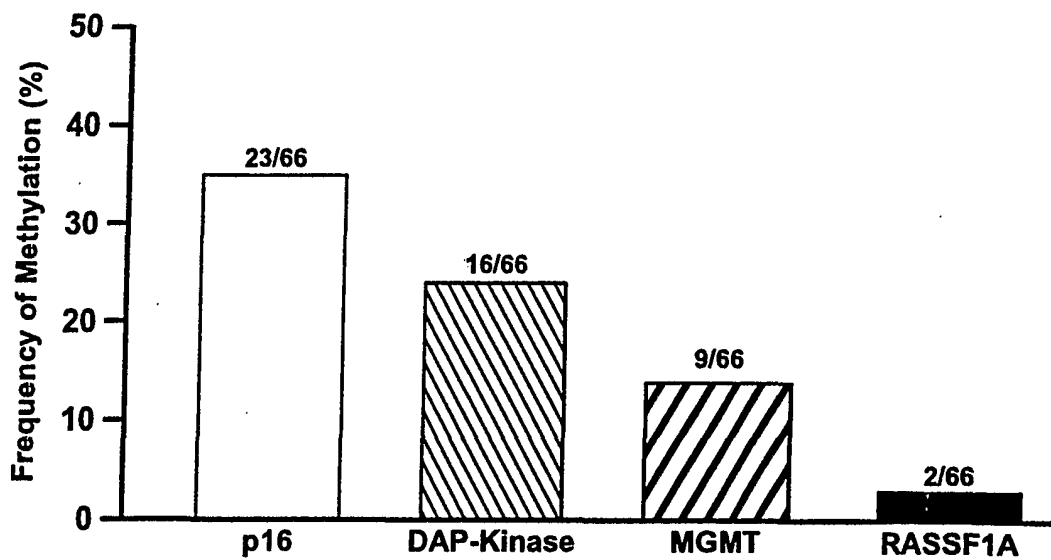
[b]$p < 0.005$

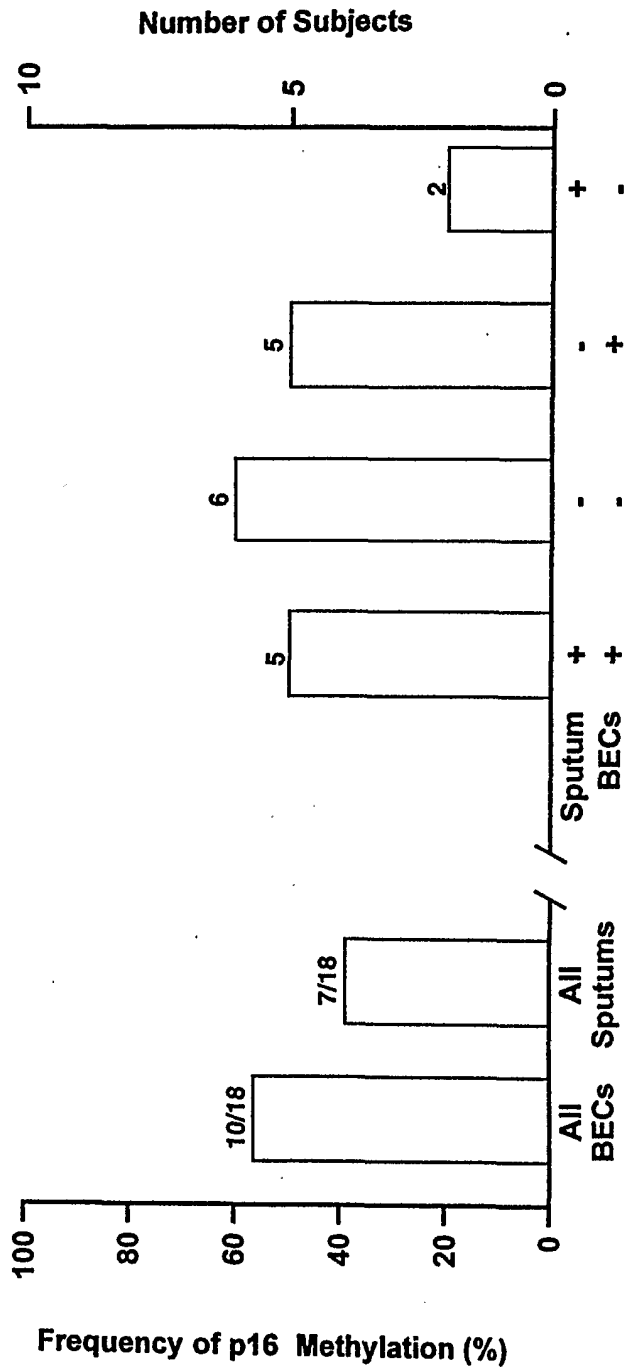FIGURE 8

Figure 9

FIGURE 10

FIGURE 11

FIGURE 12

INTERNATIONAL SEARCH REPORT

Int█████onal application No.

PCT/USoi/2645ª

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7)   :C12Q 1/68; C12P 19/34; CO7H 21/04
US CL   :435/6, 91.2; 536/24.33, 24.31

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/6, 91.2; 536/24.33, 24.31

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,786,146 A (HERMAN et al.), 28 July 1998, the Abstract.	1-41
Y	HERMAN et al. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc. Natl. Acad. Sci. USA.September 1996, Vol. 93, pages 9821-9826, the entire document.	1-41
Y	BELINSKY et al. Aberrant methylation of p16ink4a Is an early event in lung cancer and a potential biomarker for early diagnosis. Proc. Natl. Acad. Sci. USA,September 1998, Vol. 95, pages 11896-11896, the Abstract.	1-41

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 NOVEMBER 2001	**2 5 JAN 2002**

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer JOYCE TUNG
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	ZHENG et al. Correlations of partial and extensive methylation at the p14 (ARF) locus with reduced mRNA expression in colorectal cancer cell lines and clinicopathological features in primary tumors. Carcinogenesis, 2000, Vol. 21,no.11, pages 2057-2064, the entire document.	1-41

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

West 2.0, STN searched, uspat, Jpo, Epo, Derwent, medline, caplus, biosis
search terms: cancer, methylation, polymerase chain reaction, two step, two stage, p16, MGMT, RASSF1A