Towards Clinical Application of Methylated DNA Sequences as Cancer Biomarkers: A Joint NCI's EDRN and NIST Workshop on Standards, Methods, Assays, Reagents and Tools

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Abstract

The workshop report, entitled Towards Clinical Application of Methylated DNA Sequences as Cancer Biomarkers: A Joint National Cancer Institute's Early Detection Research Network and National Institute of Standards and Technology Workshop, presents a summary of the main issues, current challenges, outcomes, and recommendations toward application of methylated DNA sequences as cancer biomarkers. [Cancer Res 2007;67(10):4545–9]

Introduction

Cancer cells frequently exhibit genome-wide hypomethylation, as well as more localized hypermethylation typically in the promoter region and extending into the first exon of a gene. Hypermethylation of an allele(s) of a gene is associated with loss of expression and serves as a mechanism for the inactivation of tumor suppressor genes (TSG) and other cancer genes (1, 2).

Numerous studies have shown the promise of DNA methylation of cancer genes as potential biomarkers for clinical application in the risk, diagnosis, and prognosis of cancer.

Furthermore, demonstration of the detection of an identical pattern of DNA methylation in cancer cells and in circulating DNA in body fluids, such as serum, urine, sputum, and nipple aspirates, of the same patient opened the possibility for the development of noninvasive or minimally invasive diagnostic tests (3–7).

In spite of the promise of such biomarkers, there are several barriers that impede fast progress toward clinical application. For example, investigators frequently experience difficulties in comparing the performance of hypermethylated genes as cancer biomarkers among different laboratories due to a variety of reasons, including utilization of different genes as well as different DNA sequences (in the promoter region of the same gene); utilization of different technologies by different laboratories with a range of detection sensitivities and varying emphasis on quantitation; and utilization of different sample processing methodologies and different reference materials as controls for the degree of hypermethylation by the same technology (Appendix).

To identify and overcome barriers in the application of methylated genes as cancer biomarkers and to promote validation studies of these biomarkers, the National Cancer Institute's (NCI) Early Detection Research Network (EDRN) joined forces in August 2005 with the National Institute of Standards and Technology (NIST) to conduct a workshop on Standards and Metrology for Cancer Detection and Diagnostics focusing upon DNA methylation. The objectives of the workshop were:

- to evaluate methods and standards for robust, sensitive, and preferentially, quantitative measurements of DNA methylation in clinical specimens;
- to evaluate demands stemming from different types of specimens (e.g., tissue versus biological fluids);
- to identify and evaluate variables (e.g., amount of DNA template) influencing the robustness of the particular assay;
- to evaluate the need, and develop recommendations, for Standard Reference Materials for the discovery and validation of methylated DNA biomarkers (including cross-validation between laboratories and platforms);
- and to evaluate the need and make recommendations regarding the necessity to establish a common collection of data standards that can be used to transmit cancer-related clinical research data among investigators, clinicians, and regulators.

Choice and Handling of Clinical Specimens

The standardization of the methylation assays will be strongly influenced by the type, collection, and processing of the clinical specimen to be examined. Most methylation assays, even of low sensitivity, will be adequate to assess methylation in tumor tissue with the caveat that microdissection of tumor cell–rich areas and knowledge of the ratio of tumor cells to normal cells in the tumor biopsy may be necessary depending upon the question to be asked and technology to be used. The examination of methylation in normal appearing tissue adjacent to tumor may require more sensitive assays if the objective is to assess field cancerization or tumor spread (8). Similarly, analysis of needle biopsies and, in particular, body fluids such as blood or urine from cancer patients will require assays of greater sensitivity dependent on the origin and nature of the disease. Essentially, the ratio of tumor to normal cells, which can vary considerably from one organ system to another and from one individual to another, determines the appropriate technology to be used.

Note: The complete list of speakers is available as supplemental data at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Circulating tumor cells and/or free DNA from dead tumor cells determines the choice of, and processing protocol of, the body fluid specimen. For example, for the detection of genitourinary cancer, urine is readily accessible, and large amounts (10–100 mL) can typically be obtained (6, 9). An important question is whether tumor cell DNA content is higher in a first void of the morning specimen versus a routine void. That urine is better than serum/plasma for detection of genitourinary cancer has not been well examined, but is intuitive. However, large urine volume effectively precludes direct extraction of DNA by phenol/chloroform without prior centrifugation, which could reduce the amount of free tumor DNA extracted. Whereas for high-grade bladder cancer, it is clear that tumor cells are present in voided urine, it is unclear whether urine from prostate, renal, and low-grade bladder cancer patients will contain a greater proportion of free tumor DNA or intact tumor cells. Similar issues regarding optimal collection and processing procedures exist for sputum, ductal lavage, stool, and other clinical specimens.

Alternatively, or in the absence of an accessible body fluid for the cancer of interest, gene methylation can be tested for in DNA isolated from plasma or serum. Because plasma/serum generally contains few genome copy numbers of tumor DNA, stochastic sampling issues can arise. Approximately 50 to 100 ng of DNA/mL can be recovered as measured by quantitative real-time PCR, spectrophotometer, nanodrop or ethidium plate with standards analysis (10, 11). Some losses of DNA are likely during sodium bisulfite treatment. That plasma is preferable to serum has been suggested; however, in practice, the choice depends on what is available in retrospective collections. It will be important to determine the optimal blood collection tube and processing protocol for analysis of methylation in circulating tumor cell DNA. For prospective studies, it should be possible to obtain sufficient amounts of plasma/serum from a single blood draw to allow the distribution of 2 to 4 mL to each of several laboratories to facilitate comparison testing.

Bisulfite Modification of DNA

Sodium bisulfite treatment of DNA converts unmethylated cytosine to uracil, whereas methylated cytosines are protected. The combination of bisulfite treatment of genomic DNA with PCR amplification and sequence analysis revolutionized the analysis of methylated DNA (12). Because any unmodified wild-type DNA will be similar to the methylated DNA sequence, i.e., cytosines remain after modification, a less than optimal PCR stringency can yield false positives. According to the panel discussants, Drs. Stephen Baylin, Paul Cairns, Adi Gazdar, Steve Belinsky, Katja Bierau, Jean-Pierre Issa, Rene Myers, Mathias Ehrich, Jacob Kagan, Dave Hoon, and Pearlly Yan, incomplete modification of DNA is one of the most common sources of false-positive results in methylation analysis. Appropriate primer design, care with modification, analysis of DNA after modification with wild-type primers, and awareness of bisulfite block due to secondary structure can address this potential problem.

Assessment of Promoter Methylation

Various approaches to assess promoter methylation of candidate genes were discussed: direct sequencing of PCR products of individual alleles after cloning (12); methylation-specific PCR (MSP; ref. 13); CpG island microarray (14); and a variety of platforms based on different technologies, such as restriction analysis coupled with the Agilent 2100 Bioanalyzer platform (15), universal bead array (16), a combination of base-specific cleavage and mass spectrometry (17), and real-time pyrosequencing (18). The sensitivity and the affordability of these different assays will dictate their application to clinical specimens. In addition, for future clinical application, the chosen technology should have a high-throughput capability of testing multiple methylated sequences in multiple samples.

Quantitative Real Time MSP

Much of the discussion centered on quantitative real-time MSP (qMSP) or "MethylLight" technology (19, 20). The discussants noted that the provision of a quantitative value is not the only, or even main, advantage of qMSP for those groups previously using conventional gel-based MSP. The advantages of qMSP include that there is evidence for increased sensitivity of 1 in 10,000, equivalent to 20 pg of target methylated DNA or approximately three genome copies (six alleles) compared with 1 in 1,000 for conventional MSP. An important question to be asked is whether cancer patient body fluids that are negative for methylation by conventional gel-based MSP are positive by qMSP. Although not all qMSP technologies use the TaqMan probe as a fluorescence source, for reasons of cost or convenience, an application of a third oligonucleotide will likely provide increased specificity. It is also clear that post-PCR time and labor, i.e., running a gel, is eliminated by this approach. The use of a universal control gene (*β-actin*) sequence and standards facilitates run-to-run and operator reproducibility and can facilitate validation of the same set of specimens among different laboratories. Lastly, when working with genes, which may have low-level methylation in normal or benign cells, a quantitative result can allow the investigation of cutoff points in empirical methylation levels between normal, benign, and cancer DNAs (21). A quantitative methylation value in itself may also be of prognostic significance (22–24) and provide means for monitoring minimal residual disease and early recurrence following resection and treatment. Two issues with qMSP mentioned were that currently, not all primer sets yield a sensitivity of 1 in 10,000, suggesting that careful optimization is necessary, and second, that more template, i.e., input DNA, may be required for successful amplification.

Nested or two-step MSP is semiquantitative but has a very high theoretical sensitivity and can also be applied to the TaqMan approach. The major limitation of a nested strategy is the concern for bias or contamination, which may affect the fidelity and, therefore, the specificity. Proper experimental design and the use of optimal controls can minimize some of these issues (5, 13).

Other Methylation Technologies

Participants felt that bisulfite sequencing is best suited for marker discovery, i.e., choosing the most favorable area of a CpG island to design MSP primers. It is semiquantitative (12), whereas pyrosequencing has the advantage of being very quantitative (to 1% or so above a 5% background; ref. 18). Pyrosequencing works better with small PCR products (50–100 bp) that may contain only a limited number of the relevant CpG sequences; however, one has to consider that CpG islands are typically several hundred base pairs in size. At present, pyrosequencing would be useful in the analysis of tumor tissue sections (and perhaps needle biopsies)

or adjacent normal tissue, where a "yes or no" and/or the quantitation of methylation is required, and in the analysis of partially degraded DNA. However, the 5% minimum sensitivity level may limit its utility for detection of scant tumor cells/DNA in body fluids or occult cancer cells in margins, nodes, etc. Pyrosequencing is a direct analysis in that sequence readout is provided compared with the presence or absence of a PCR amplification product for MSP (13).

Sequenom recently introduced a high-throughput DNA methylation analysis by matrix-assisted laser desorption/ionization–time of flight mass spectrometry. The technology is based on sequence-specific cleavage of bisulfite-treated DNA combined with mass spectrometric analysis. This procedure can be applied to the discovery and semiquantitative analysis of DNA methylation. The procedure is very rapid and potentially low cost (after purchase of relatively expensive instrumentation; ref. 17). It should be noted that there are many other technologies for methylation analysis (25) that were not discussed because of the discussants' particular interests as well as time constraints.

Specificity Issues

The main topic of discussion here was whether, and if so, which, genes methylated in tumor cells have been found to be methylated in normal or non-neoplastic cells. It is important to understand that there are different types or classes of methylated genes in both normal and cancer cells. Classic TSGs should rarely, if ever, be methylated in normal cells. Other types of cancer genes, e.g., *myoD1* or *GDF15*, show low levels of methylation in normal cells and denser methylation in tumor cells (26, 27). Other genes show tissue-specific methylation patterns between different types of normal cells (28). Where a classic TSG shows methylation of functional significance (it is conceivable that methylation of part of an island with minimal functional effect can be tolerated in normal cells) in an apparently normal cell, however, it can be argued that such a cell is not a normal cell, even if it is non-neoplastic. If a gene does show low-level methylation accumulation in normal or non-neoplastic cells from aging individuals (29, 30), it should be possible to investigate if a quantitative cutoff point for the amount of methylation can be established by real-time MSP that would indicate the presence of cancer (31). It is important to note that only a few studies have been carefully designed to address this question (8).

Tumor-specific inactivation of a TSG via promoter hypermethylation may precede clinically relevant disease, which raises the question of optimal timing of detection. Overdetection can potentially be addressed by understanding the timing of the alteration screened for in tumorigenesis. For diagnostic or screening purposes, it might be useful to choose a TSG in which hypermethylation is highly associated with progression to a clinically relevant disease.

Finally, if we consider the need for differential diagnosis in a body fluid, although relatively few methylated genes show absolute tissue specificity, e.g., *VHL, BRCA1* or *p15*, a greater number of genes show more frequent methylation in some cell types than others, e.g., *GSTP1* is methylated in 90% of prostate cancer, 30% of breast cancer, and 25% of hepatic cancer. Given that breast cancer is predominantly a disease of females and that hepatic cancer is relatively rare in the Western world, inclusion of *BRCA1* and *GSTP1* in a detection panel would provide some specificity for breast cancer. As more methylated genes are identified, algorithms could be developed to score the specificity of a particular gene hypermethylation panel for the detection of breast cancer, compared with other cancer types, in serum, for example (1, 32).

Discussion Outcomes and Recommendations

There were several recommendations made concerning on how to establish a standard assay for methylation of a specific gene. First, it is critical to design methylation-specific primers in areas within the CpG island that are associated with dense methylation and loss of transcription. This region often encompasses the transcriptional start site and may extend into exon 1 of the gene. MSP interrogates only the limited number of CpG sites in the promoter that are in the sequences covered by the primers or probe. Second, primer design should incorporate sufficient CpG within the 3′-region of the sequence to allow for maximal discrimination between methylated and unmethylated alleles and, in addition, contain cytosines alone to provide specificity to modified DNA. Modification is harsh on DNA, and smaller products of 100 to 150 bp will amplify better. Annealing temperatures for MSP should be at, or even exceed, the melting temperature for the primers. Generally, the best controls are *in vitro* methylated normal cell DNA, tumor cell lines known to be positive or negative for methylation of the particular gene, or plasmid clones of a methylated allele. Finally, the amount of DNA template used for interrogation of methylation of a specific gene depends greatly on the clinical specimen. In general, with a highly specific assay, using 75 to 150 ng of DNA will ensure adequate template to evaluate methylation. Using <75 ng of DNA can result in diminished sensitivity. This can occur if the quality of the DNA is poor (e.g., formalin-fixed tissue, circulating free DNA in plasma), the specimen is very heterogenous with respect to cell types (e.g., sputum), or when the goal is to detect field effects or age-related changes in a background of normal tissue. In instances where only low amounts of specimen DNA are available, experimental design should include more stringent controls.

One outcome from this workshop was the necessity to have non-gel–based detection for methylation, especially if these markers will be used for diagnostic purposes by a clinical laboratory. This can be accomplished through use of TaqMan, Molecular Beacon, or Sybr Green real-time MSP technology or pyrosequencing technologies. Critical issues for all of these techniques include the design of the fluorescent probe and the appropriate control for validating amplification of the target gene, i.e., an always unmethylated reference gene such as *β-actin* rather than the unmethylated sequence of the gene of interest as in conventional MSP. Another major issue with this technology is how the quantitative data received will be used in the clinical setting. Threshold values are set by the instrumentation; to call samples positive or negative for methylation and a quantitative value can be obtained as a ratio of signal intensity from the methylated gene to the reference gene. However, very few studies have been conducted to evaluate on different days or in different laboratories the variation in the quantitation. It was noted that methylation of the *MGMT* gene was scored similarly between two different Oncomethylome Science laboratories using similar models of real-time PCR machines. Investigators can publish the thresholds they used for determining that a specimen is positive for methylation and provide the values used to divide the

samples into quartiles. Finally, it is unclear how reporting levels of methylation would influence clinical practice.

In regard to comparison of approaches and technologies for the analysis of methylation status of a gene, it was proposed, as a first step, to standardize "yes or no" scoring for methylation by different methodologies through the distribution of dilutions of a tumor cell line with mutually agreed upon methylated genes and unmethylated genes for analysis and the appropriate corresponding sequences and primers. The second step would be to similarly analyze tumor tissue DNA and, third, body fluid DNA.

It was clear from this workshop that one standard cannot be developed for addressing all the applications for methylation in the basic and translational research fields as well as the clinical testing. The best technology depends on the question being asked. However, the development of standard assays will require standard specimens for clinical comparison. The most straightforward set of specimens are tumor cell lines, which can be regenerated and provide an unlimited source of DNA. Tumor tissue and adjacent tissue from a cancer that is common and always resected, such as colon cancer, could be a second valuable standard for assay validation. It also is clear that there is a pressing need for perhaps unexciting, but important, studies to determine the optimal parameters for choice, storage, and preparation of clinical specimen for DNA isolation, bisulfite modification, and technology controls. In summary, we hope that the discussion as reported above will provide impetus to existing investigators and aid investigators new to DNA methylation analysis.

Appendix. Toward the Application of Methylation Biomarkers

Characteristics of an ultimate disease biomarker
- distinguishes healthy from diseased individuals with a high degree of accuracy
- present during early stages of disease
- measurable in a readily accessible body fluid/s (e.g., serum/plasma, urine, sputum, saliva, ductal lavage)
- leads to development of a diagnostic test that ultimately impacts mortality rates

Methylated DNA sequences as clinical biomarkers
- reproducible, preferentially quantitative measurement is important in all clinical applications
- absolute methylation measurement (% methylation at individual sites) is more amenable to precise quantitation
- individual gene methylation measurement will likely be clinically useful in cancer detection, diagnosis and prognosis and classification and possibly in risk assessment
- the performance of a biomarker is highly dependent on the choice of methylation detection method

Factors that impact sensitivity
- choice of clinical specimen, e.g., urine or serum
- specimen stability/degradation
- processing of specimen, e.g., urine pellet (sediment) or supernatant
- choice of target gene/s and primers
- choice of technology for analysis
- amount of input DNA available

Factors that impact specificity
- choice of target gene/s and primers
- efficacy of bisulfite modification
- choice of PCR parameters, e.g., annealing temperature
- negative and no template controls used

Choice of technology recommendations
- bisulfite sequencing is optimal for the analysis of CpG island methylation of new genes
- pyrosequencing is optimal for quantitation of individual CpG sites
- quantitative MSP is optimal for sensitive detection of methylated alleles

Standardization issues and recommendations
- different genes used in detection assays: establish optimal gene panel by interlaboratory testing
- different area of promoter of same gene: establish optimal gene panel by interlaboratory testing
- Different technology used for analysis of methylation status: establish by interlaboratory testing of aliquots from universal standard
- Different reference or controls used with same technology: establish by interlaboratory testing of aliquots from universal standard

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