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Commentary

Quantitative DNA Methylation Analysis

The Promise of High-Throughput Epigenomic Diagnostic Testing in Human Neoplastic Disease

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The term "epigenetics" was first introduced by Conrad Waddington in 1942 to describe "the interactions of genes with their environment, which bring the phenotype into being."[1] Later that same decade came the identification and characterization of DNA methylation,[2] the first epigenetic mark to be discovered. The major target for DNA methylation in the mammalian genome is cytosine, where enzymatic attachment of a methyl group to the 5 position of the pyrimidine ring produces 5-methylcytosine,[3] which has been referred to as the fifth base of genomic DNA. Although 5-methylcytosine is indistinguishable from cytosine within the structure of the DNA molecule where it base-pairs with guanine, the presence of the methyl group has considerable biological implications for DNA function.[3] Alterations in DNA methylation affecting target sequences within the transcriptional control regions of genes produce changes in gene expression, with hypomethylation leading to increased expression and hypermethylation leading to decreased expression. In contemporary terms, epigenetics refers to modifications of the genome that are heritable during cell division but do not involve a change in the DNA sequence.[4] Thus, epigenetics describes heritable changes in gene expression that are not simply attributable to nucleotide sequence variation.[5] It is now recognized that epigenetic regulation of gene expression reflects contributions from both DNA methylation and complex modifications of histone proteins and chromatin structure.[6] Nonetheless, DNA methylation plays a central role in nongenomic inheritance and in the preservation of epigenetic states and remains the most accessible epigenomic feature because of its inherent stability.[4] Thus, DNA methylation represents a target of fundamental importance in the characterization of the epigenome, in defining the role of epigenetics in disease pathogenesis, and in the development of useful molecular tools for diagnostic testing and prediction of prognosis (clinical responses and patient outcomes) in neoplastic and non-neoplastic diseases.[7–9] In this issue of *The Journal of Molecular Diagnostics*, Ogino et al[10] investigate one such molecular tool, sodium bisulfite conversion of DNA followed by MethyLight real-time polymerase chain reaction (PCR), and describe the factors that influence the variability of quantitative analysis. However, to fully understand these results, it is important to comprehend the importance of DNA methylation in cancer and the significance of such information to cancer diagnosis and prognosis.

Cancer Epigenetics

Aberrant DNA methylation represents a hallmark of cancer, and methylation-dependent epigenetic mechanisms have been implicated in the molecular pathogenesis of many forms of human neoplasms.[4,11] Alterations in normal DNA methylation profiles were first characterized in human cancer nearly 25 years ago.[12,13] Since that time, a number of cancer-related epigenetic alterations have been described, including two apparently contradictory DNA methylation phenomena: 1) a profound loss of 5-methylcytosine content across the entire genome, and 2) discrete regions of dense hypermethylation.[14] However, the functional implications of these two disparate phenomena complement each other in the cancer phenotype. DNA hypomethylation is associated with activation and inappropriate expression of proto-oncogenes

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(and other positive mediators of cell proliferation) and contributes to chromosomal instability.[15] In contrast, DNA hypermethylation is associated with silencing of specific genes that control cellular proliferation, including some tumor suppressor genes.[16,17]

The majority of 5-methylcytosine occurs in the context of the CpG dinucleotide, which is underrepresented in the mammalian genome but is enriched in specific regions of the genome termed CpG islands.[18] Computational analysis of the DNA sequence of the human genome predicts about 29,000 discreet CpG islands and suggests that approximately 60% of structural genes are associated with a CpG island.[19] CpG islands are often contained within the promoters and early exons of genes, suggesting a role for these structural features in the regulation of these genes.[3,20] Hypermethylation of CpG islands occurs in a nonrandom fashion in cancer cells, and the DNA methylation patterns observed appear to be tumor specific,[21] suggesting that gene-specific methylation events represent potentially useful markers for molecular diagnostic testing in cancer.

Evidence in the literature suggests that epigenetic events target genes that are required for tumorigenesis in a tissue or cell type (perhaps resulting in a recapitulation of developmental gene expression patterns), leading to altered gene expression signatures that are unique to specific types of neoplasms. In most cases, genes that are subject to methylation-dependent silencing function normally in the control of cell growth (tumor suppressor genes and other negative mediators of cell proliferation), and their loss contributes to the uncontrolled cell division that characterizes neoplastic cells. The *Rb1* gene in sporadic retinoblastoma was the first example of a tumor suppressor gene in human cancer that was silenced through DNA methylation.[22] Subsequently, a number of methylation-sensitive tumor suppressor genes have been identified, including *BRCA1*, *p16/CDKN2A*, *APC*, and others.[16] However, loss of gene expression through promoter hypermethylation is not limited to tumor suppressor genes or other genes that directly control cell proliferation. For example, it is well-established that hypermethylation of *MLH1* in colorectal cancer cosegregates with DNA mismatch repair deficiency and genomic instability.[23] In this manner, epigenetic alterations in evolving neoplastic cell populations can contribute to genetic events (and genetic instability) that ultimately result in critical gene mutations that drive carcinogenesis. Likewise, DNA methylation can lead to loss of expression of genes that control other biological aspects of tumor cells, like the ability to invade and metastasize.

The tremendous potential for exploiting epigenetic alterations and particularly aberrant DNA methylation events in the development of useful molecular tests for human cancer has emerged from a few published studies. Methylation can be assessed in DNA samples from tissues and/or from minimally invasive biological samples (such as sputum, plasma, urine, stool, saliva, or others).[24] It has now been shown that methylated DNA sequences can 1) facilitate the identification and diagnosis of some occult cancers, 2) predict biological aggressiveness of certain tumors, and 3) predict clinical responsiveness to treatment for some neoplasms. *GSTP1* is methylated in >90% of prostate cancers, *APC* is methylated in >90% of esophageal cancers, and both markers provide excellent test specificity in plasma DNA analysis.[25] Methylation of *DAPK* is associated with early recurrence of bladder cancer, and methylation of *RASSF1A* and *APC* are associated with poor prognosis in breast cancer.[24] Hypermethylation of *MGMT* predicts clinical responsiveness of gliomas to certain alkylating agents and good survival among glioma patients that receive multidrug treatment regimens.[24] These examples from the literature illustrate the potential for using DNA methylation analysis in the molecular evaluation of cancer.

Analysis of Epimutations in Human Cancer

The analysis of DNA methylation at the level of the individual nucleotide was revolutionized by the introduction of sodium bisulfite conversion of genomic DNA.[26] Sodium bisulfite efficiently converts cytosine to uracil, whereas 5-methylcytosine is resistant to this chemical reaction. Thus, coupling this reaction with PCR amplification and DNA sequencing results in a methodology that allows for the positive display of methylated cytosines in the sequence of genomic DNA.[26] A significant number of techniques have been developed to interrogate specific DNA sequences for CpG methylation (using either PCR-based approaches or methylation-sensitive restriction enzymes), most of which incorporate sodium bisulfite conversion to facilitate the identification of 5-methylcytosine.[25] Although sodium bisulfite conversion of DNA represents a powerful method for identification of 5-methylcytosine, it is also a tremendously harsh treatment that can result in significant levels of DNA degradation (approaching 85 to 95% after 4 hours at 55°C).[27] Thus, it is essential that the reproducibility of the sodium bisulfite conversion reaction be considered as new methods for DNA methylation are developed. At present, sodium bisulfite conversion of DNA followed by PCR and DNA sequencing remains the gold standard for methylation analysis.[5] Although this approach provides a method for detailed analysis of DNA methylation within a given gene sequence, it is labor intensive, time consuming, relatively expensive, and not readily adaptable to high-throughput analysis of DNA samples. Recognition of these limitations led to the development of real-time PCR assays, such as MethyLight.[28] This real-time PCR technique represents an extremely flexible platform for high-sensitivity quantitative DNA methylation analysis of small DNA samples. The major advantage of this technique is the sensitive real-time detection of PCR products, eliminating the need for gel electrophoresis or other downstream analysis of PCR products and enabling analysis of minute quantities of DNA.

With methods for DNA methylation analysis being broadly applied to human cancer, numerous reports have appeared in recent years describing alterations in gene-specific DNA methylation patterns related to the disease phenotype. Initial characterization of the

methylation profile of a gene promoter typically requires application of brute-force methodologies like sodium bisulfite conversion followed by PCR and DNA sequencing.[26] However, once the methylation profile of a given gene promoter has been characterized (in normal and disease tissue), it is possible to develop specific assays that facilitate direct comparison of a given sequence between tissue/cell samples. It follows that DNA methylation analyses are then applied to clinical samples to investigate whether relationships between methylation patterns of specific genes and the behaviors of tumors hold when patient samples are analyzed. This type of retrospective study is extremely important for establishing the role of gene-specific methylation in disease pathogenesis and/or to examine potential relationships with clinicopathological characteristics of tumors. In addition, these studies typically require analysis of DNA samples from paraffin-embedded tissue samples, which is necessarily of lower yield and poorer quality, making analysis of DNA methylation more challenging. Thus, there is a need for robust methods for DNA methylation analysis that can be applied to the analysis of minute quantities of DNA that are prepared from paraffin-embedded tumor samples.

Critical Parameters for Quantitative DNA Methylation Analysis

In this issue of *The Journal of Molecular Diagnostics*, Ogino et al[10] describe an in-depth investigation of critical parameters that influence the success of quantitative DNA methylation analysis after sodium bisulfite conversion of DNA samples from archived paraffin-embedded tumor specimens. This investigation makes several significant contributions to the field of quantitative DNA methylation analysis. First, Ogino et al have performed a thorough investigation of factors that influence variability in the analysis of DNA methylation using sodium bisulfite conversion and MethyLight real-time PCR. Specifically, Ogino et al examined the day-to-day and run-to-run reproducibility of the sodium bisulfite conversion reaction and the MethyLight real-time PCR assay. Second, Ogino et al performed sodium bisulfite conversion and MethyLight assays on a large sample of paraffin-embedded colorectal cancer specimens for *CDKN2A*, *MLH1*, and *MGMT*, with parallel immunohistochemical staining of their protein products. This aspect of the study facilitated a comparative analysis of the results of these assays and a rigorous assessment of the predictive value of gene promoter methylation (as determined by sodium bisulfite-MethyLight) for loss of protein expression of select genes.

To measure the precision and reproducibility of sodium bisulfite conversion and quantitative MethyLight assay, Ogino et al[10] performed sodium bisulfite conversion on multiple replicate aliquots ($n = 7$) from four DNA samples prepared from individual cases of colorectal carcinoma, using paraffin-embedded tumor samples as the source of DNA. Two sources of technical variation were explored: 1) reaction-to-reaction variation in the sodium bisulfite conversion and 2) run-to-run variation in the results of the MethyLight real-time PCR. The variability of the sodium bisulfite conversion was investigated by performing the chemical reaction on multiple replicates from the same source of DNA. The variation in real-time PCR results was investigated by performing multiple MethyLight assays on converted DNA samples on multiple days ($n = 5$ independent PCR runs). Given the nature of the quantitative DNA methylation assay described by Ogino et al, the individual contributions of the sodium bisulfite conversion and the MethyLight PCR to the overall variation in results could not be completely separated. Nonetheless, the experimental design used by Ogino et al provided for a robust analysis of the general methodology. When multiple sodium bisulfite conversions and/or multiple MethyLight runs were performed on replicate samples from a single DNA source, excellent reproducibility was observed. Ogino et al show that the threshold cycle (Ct) values obtained were remarkably consistent using the described methodology, based on 1) mean Ct values (and corresponding SD) for multiple sodium bisulfite conversion reactions within a single MethyLight run and 2) mean Ct values (and corresponding SD) for a single sodium bisulfite conversion over multiple MethyLight runs.[10] In fact, when SD measures of variability were calculated for each of the genes analyzed (*CDKN2A*, *MLH1*, *MGMT*, *ACTB*, and *COL2A1*) in each of the four test cases, excellent reproducibility was observed in all cases.

To produce a measure of gene methylation, Ogino et al[10] calculated the "percentage of methylated reference" (PMR) for each MethyLight result using Ct values for the gene of interest (*CDKN2A*, *MLH1*, or *MGMT*) and a control gene (*ACTB* or *COL2A1*), for assays using sodium bisulfite-converted DNA samples representing both test case samples (individual colorectal carcinomas) and control human DNA that had been treated with M-Sss I (presumably fully methylated).[10] Values for PMR for individual genes showed some variation between replicates of sodium bisulfite-converted DNA and among multiple MethyLight runs. For example, the PMR values for *MGMT* across five MethyLight runs from a single sodium bisulfite-converted DNA sample varied from 22.7 to 43.9 in one case. Some of the variation observed in calculated PMR values was attributed to the reference gene used, with larger coefficients of variance observed in calculations using *ACTB* as the control gene.[10] Overall, the coefficients of variance indicated excellent reproducibility in the determination of PMR for the test genes using the MethyLight assays, especially when *COL2A1* was used as a reference gene. Using PMR as a measure of gene methylation, Ogino et al[10] observed that tumor DNAs tend to produce gene-specific PMR values that are either low or high but that are rarely intermediate. Thus, this assay may be able to tolerate some variability without loss of predictive power for an individual tumor based on the use of a threshold PMR value. Ogino et al show that although the PMR values for *MGMT* vary from 22.7 to 61.0 across multiple sodium bisulfite conversions and multiple MethyLight runs, 100% of 35 independent assays produced PMR values >20. Likewise, Ogino et al de-

scribe several cases in which PMR values suggested a lack of gene methylation. Among these several cases, >99% of the individual MethyLight results ($n = 175$) produced a PMR value of <1, and >90% produced PMR values of 0, consistent with a lack of gene methylation.[10] These observations indicate that the results of individual MethyLight assays are highly informative for individual tumor samples, producing PMR values that reflect the average PMR result and that can be easily interpreted as consistent with either gene methylation (high PMR) or the lack of gene methylation (low PMR).

DNA methylation-dependent silencing of gene expression in cancer results in loss of protein expression and consequently protein function. Ogino et al[10] performed an investigation to determine the value of PMR (as a measure of gene methylation) for *CDKN2A*, *MLH1*, and *MGMT* in the prediction of loss of protein expression (as measured by immunohistochemistry) in a group of 274 paraffin-embedded colorectal carcinoma specimens. The reliability of the sodium bisulfite conversion and MethyLight real-time PCR assay for large-scale testing of paraffin-embedded samples proved to be excellent because DNA samples from 272 of 274 tumors (>99%) were successfully amplified using this methodology. Ogino et al found that the majority of tumors produced values for PMR that were <1 (no methylation) or >10 (methylated). Intermediate PMR values (>1 but <10) were obtained for 3.8, 3.5, and 4.1% of tumors for *CDKN2A*, *MLH1*, and *MGMT*, respectively.[10] Given the spread of the PMR data, Ogino et al used a cutoff value for PMR of 4, where PMR <4 was defined as unmethylated and PMR >4 was defined as methylated. Subsequently, the colorectal tumors were immunostained for the protein products of *CDKN2A*, *MLH1*, and *MGMT*, and the results were correlated with the values of PMR obtained for the same tumors. This rigorous evaluation of the MethyLight assay is essential for establishing the predictive value of the quantitative DNA methylation analysis as a surrogate for the more time-consuming and labor-intensive immunohistochemical approach to determine loss of protein expression/function. PMR of *MLH1* proved to be highly predictive for loss of MLH1 protein among these tumors: 98% correct assignments overall, 99% positive predictive value (PMR suggests unmethylated, protein is present), and 96% negative predictive value (PMR suggests methylated, protein is absent).[10] PMR for *CDKN2A* had an excellent positive predictive value (96% correct assignments) but was less useful as a negative predictor (62% correct assignments). Likewise, PMR for *MGMT* provided a very good positive predictive value (86% correct assignments) but was not as good as a negative predictor (66% correct assignments). These results suggest that regulation of *MLH1* is tightly linked to the methylation status of the gene and that the specific MethyLight assay used by Ogino et al is optimized for this gene. Thus, PMR can be used effectively and with few errors to predict the presence or absence of MLH1 protein in these tumors. In contrast, PMR was not as powerful in the prediction of *CDKN2A* and *MGMT* expression among these tumors using the assay described. When these genes were unmethylated (low PMR), the protein products were expressed in most cases. However, when high PMR values were obtained for the *CDKN2A* and *MGMT* genes (indicative of promoter methylation), the corresponding protein products were present in a significant percentage of cases (38 and 34%, respectfully). Given that these genes have been extensively characterized and are well-known to be methylation sensitive, it is unlikely that mechanisms exist to drive their expression when critical regulatory regions of the gene promoters are methylated. Therefore, these results appear to suggest that the MethyLight assays used for *CDKN2A* and *MGMT* are not optimized for negative predictive value, resulting in significant numbers of errors when PMR is used to predict protein expression status. Thus, alteration of the MethyLight assay (by modification of the design of the PCR primers and/or real-time probes) may significantly improve the ability to predict the loss of protein expression based on PMR for these genes. Nonetheless, the assays for *CDKN2A* and *MGMT* as described may still prove useful in tumor prognostication, especially in cases where the continued expression of the protein products of these genes correlates with response to therapy or favorable patient outcome.

The Promise of Epigenomic Medicine

Cancer epigenomics remains an emerging field of experimental and clinical investigation 25 years after the discovery of abnormal DNA methylation in cancer.[12,13] Our current understanding of epigenetic regulation of gene expression recognizes the significant contributions of DNA methylation, histone protein modification, and chromatin remodeling,[6] although the mechanisms that regulate some of the most fundamental aspects of these processes remain unknown. Nonetheless, investigators have begun to harness epigenetic marks for the purpose of molecular testing in cancer.[7] DNA methylation is a stable epigenetic mark that can be readily examined using methodologies developed over the last decade, and analysis of DNA methylation forms the basis for many new molecular diagnostics. Eventually, specific DNA methylation assays might be used in early cancer diagnosis, identification of occult cancer, diagnosis of specific forms of cancer, prediction of tumor behavior (tumor staging), prediction of response to therapy (specific types of drugs), and/or prediction of long-term patient outcome. However, before these potential applications of epigenomics can be realized in clinical oncology, additional experimental investigations will be required to examine potential relationships between DNA methylation (or other epigenetic) events and the clinicopathological characteristics of cancer. The study by Ogino et al[10] illustrates several important considerations for development of quantitative DNA methylation analyses and their application to the molecular evaluation of cancer. First, it is necessary to investigate potential sources of experimental variation (such as the sodium bisulfite conversion reaction) and to establish the day-to-day and run-to-run reproducibility of the assay system to be used.

Second, the readout from DNA methylation analyses must be informative for individual tumor DNA samples using small numbers of replicates. Third, it is essential to determine the value of DNA methylation analyses for prediction of protein expression, because this will provide an indication of the usefulness of the assay as a surrogate for immunohistochemical detection of protein expression in tissue samples. Use of the investigative model provided by Ogino et al[10] to evaluate new quantitative DNA methylation assays will facilitate the effective application of these high-throughput methodologies to the analysis of human neoplasms, leading to an expansion of our knowledge of the relationships between DNA methylation-dependent regulation of gene expression and the molecular pathogenesis of cancer.

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