Early Insights into Cancer Epigenetics: Gene Promoter Hypermethylation Emerges as a Potential Biomarker for Cancer Detection

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DNA methylation is one of the most intensely studied epigenetic modifications in mammals. In normal cells, it plays an essential role in core biologic processes by assuring the proper regulation of gene expression and stable gene silencing. In cancer cells, genome-wide DNA methylation patterns are altered and often represent an early and fundamental step in neoplastic transformation. The landmark study from Esteller and colleagues, published in *Cancer Research* in 2001, was the first to reveal high frequency promoter methylation across multiple cancer types. They highlighted that widespread alterations in DNA methylation may be a key characteristic of oncogenesis and proposed aberrant DNA methylation of gene promoters could provide markers for sensitive detection of nearly all cancer types. The authors used a candidate gene approach to show promoter hypermethylation occurred across 12 cancer-associated genes in DNA from over 600 primary tumor samples, representing 15 major tumor types. The profile of promoter hypermethylation differed in every tumor type, suggesting that alterations in DNA methylation are pervasive, but the genes affected may be tumor-specific and impact multiple signaling pathways. Over the past 20 years since this publication, the cancer epigenetics field has exploded to generate thousands of normal and cancer methylome maps and developed sophisticated informatic tools for genome-wide methylome analyses. These methylomes are providing roadmaps for the study of cancer biology and discovery of DNA methylation biomarkers for early detection and monitoring of cancer.

See related article by Esteller and colleagues, Cancer Res 2001;61:3225–29.

DNA methylation is an epigenetic modification that occurs during normal mammalian development and involves the addition of a methyl (CH_3) group onto the C_5 position of cytosine to form 5-methylcytosine typically next to guanine residues, termed CpG methylation. Regions known as CpG islands, which are refractory to DNA methylation, are often associated with active or bivalent gene promoters and play a central role in normal gene regulation. Notably, tumor suppressor genes commonly harbor unmethylated CpG island promoters. Interestingly back in 1971, Alfred Knudson proposed that retinoblastoma tumors are caused by two mutational events in the retinoblastoma tumor suppressor gene (*Rb*), one in the germline and one in somatic cells, commonly termed The Knudson hypothesis, or two-hit hypothesis. In 1989, Greger and colleagues (1) showed the first evidence of hypermethylation of the *Rb* CpG island promoter in retinoblastoma tumors, using methylation-sensitive restriction enzymes, which was the only tool available to detect DNA methylation.

With development of bisulphite sequencing in the early 1990s, methylation analyses expanded beyond single restriction enzyme sites to enable the analysis of contiguous CpGs in the genome. In 1997, Stirzaker and colleagues (2) reported the first detailed methylation sequence analysis of *Rb* promoter CpG to reveal that methylation is not confined to a specific CpG site, as detected by the restriction enzyme studies, but extends to essentially all CpG dinucleotides spanning the CpG island. The CpG methylation pattern from each retinoblastoma tumor DNA sample was found to be different, ranging from densely to sparsely methylated profiles, suggesting that DNA methylation dynamics in cancer cells was different from normal cells. In 1999, Toyota and colleagues (3) described that methylation of multiple genes, termed CpG island methylator phenotype, occurred in a significant subset of colorectal cancers. In the same year, Melki and colleagues (4) also found abnormal methylation of multiple CpG island promoters associated with 8 tumor-related genes (*calcitonin*, *estrogen receptor*, *E-cadherin*, *p16(INK4a)*, *p15(INK4b), Rb, GSTP1, and HIC1*) in acute myeloid leukemia. These early studies demonstrated there was a general deregulation of CpG island methylation in the cancer cells and that hypermethylation is not limited to single genes, but a number of genes are methylated concurrently. With increasing evidence for tumor suppressor gene hypermethylation in cancer, the two-hit hypothesis now includes both mutational and epigenetic silencing as a cause of phenotypic change.

In their 2001 landmark paper, Esteller and colleagues (5) expanded methylation analyses from single tumor types by demonstrating that tumors arising in different tissues displayed different profiles of methylation for CpG islands associated with 12 candidate genes [*p16(INK4a), p15(INK4b), p14(ARF), p73, APC, BRCA1, hMLH1, GSTP1, MGMT, CDH1, TIMP3*, and *DAPK*], using methylation-specific PCR (MSP). MSP is based on bisulphite treatment of DNA followed by PCR amplification using primers specific for methylated versus unmethylated DNA, allowing for rapid and cost-effective analysis of a large number of clinical samples. The authors highlighted the potential for using DNA methylation markers for cancer detection, as unlike point mutations or other DNA alterations, aberrant DNA methylation can occur commonly and in different cancers, and as such, DNA methylation provides a positive signal easily detected within a

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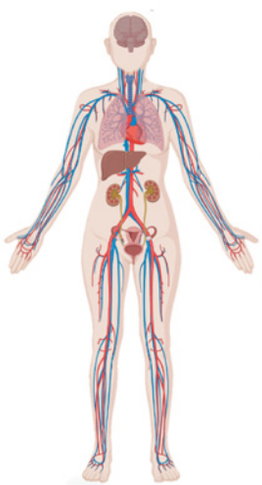
background of normal DNA with the potential for cancer detection in readily available sites, such as serum and sputum. These predictions are being borne out today in the development of numerous commercially available DNA methylation–based tests ranging from single genes to large panels of CpG sites, in both tissues and bodily fluids.

Prior to this publication, nearly all studies had been based on targeted candidate genes, as approaches for scanning genome site-specific differences in DNA methylation were in their infancy. Across the ensuing decade a wide variety of genome-wide array-based approaches were developed for detection of differential methylation and applied to detection of specific differences in methylation between tumor and normal tissues. A major milestone in this development was the Illumina Infinium 450K array (6) and the subsequent EPIC array. The 450K array provided reliable, quantitative measurement of methylation levels at approximately 450,000 CpG sites across the human genome and became the mainstay of extensive characterization of tumor methylomes. Using this technology, The Cancer Genome Atlas consortium has now assessed genome-wide DNA methylation in over 20,000 primary cancer and matched normal samples representing 33 different cancer types. This extensive resource has been widely used for the identification and/or validation of DNA methylation biomarkers for specific cancers. In a pan-cancer analysis of DNA methylation profiles, Hoadley and colleagues (7) undertook hierarchical clustering of 1,035 CpG sites that were unmethylated in normal tissues and leukocytes but hypermethylated in at least one cancer type. While the CpG sites did not differ between normal tissues, tumor methylation profiles distinguished different tumor types and clustered largely according to cell-of-origin. More recently, with the advent of whole genome bisulphite sequencing, alterations to all CpG sites in the cancer methylome, regardless of location, can now be assessed alongside changes to the cancer genome to determine the role of mutations in epigenetic readers, writers, and erasers in remodeling the cancer epigenome.

Over the past decade, based on candidate gene or genome-wide array-based approaches, there has been a slow but steady development of commercially available tests that target single or small sets of methylated genes for different cancer types (summarized in **Fig. 1**). These include tissue-based tests used in follow-up to histologic evaluation or for prediction of response to chemotherapy. Two tests for detection of cancer-derived methylated target DNA in blood plasma (Epi proColon) or stool samples (Cologuard) have received regulatory approval for colorectal cancer screening in asymptomatic subjects. Other methylation-based assays are used for monitoring minimal residual disease or cancer recurrence and for primary cancer detection in at-risk patient subgroups (8).

The steadily decreasing costs of DNA sequencing have also led to a rapid expansion in the application of targeted multiplexed amplicon sequencing to detect individual tumor-derived mutations in circulating cell-free DNA. More recently, the large-scale application of whole genome bisulphite sequencing to circulating cell-free DNA, combined with machine learning, has been used to develop a blood-based DNA methylation test designed to detect multiple cancer types (9); the test, named Galleri, targets over 100,000 markers, enabling high sensitivity for detection of cancer-derived DNA in plasma. In a recent case control study of over 4,000 subjects across multiple cancer types using this test, Klein and colleagues (9) reported very high specificity (99.5%) in

Cancer type	Target gene/s	Test indication	Commercial test
Prostate biopsy	*GSTP1, APC, RASSF1*	Detection of occult disease	ConfirMDX, MDxHealth
Cervical cancer	*ASTN1, DLX1, ITGA4, RXFP3, SOX17, ZNF671*	Triage of unclear patients	GynTect, Oncgnostics
Cervical cancer	*FAM19A4, hsa-mir124-2*	Triage of unclear patients	QIAsure, Qiagen
Breast cancer	*PITX2*	Chemotherapy response	Therascreen PITX2 RGQ, Qiagen
Glioblastoma	*MGMT*	Response to alkylating agents	Xiamen SpaceGen
Glioblastoma	*MGMT*	Response to alkylating agents	PredictMDx, LabCorp



Body fluid	Cancer type	Target gene/s	Test indication	Commercial test
Urine	Bladder cancer	*TWIST1, OTX1, ONECUT2*	Cancer detection	AssureMDx, MDxHealth #
	Bladder cancer	15 marker panel	Monitoring recurrence	Bladder EpiCheck, Nucleix
Stool	Colorectal cancer	*NDRG4, BMP3*	Screening, cancer detection	Cologuard, Exact Sciences #
Blood plasma	Colorectal cancer	*SEPT9*	Screening, cancer detection	Epi proColon, Epigenomics
	Colorectal cancer	*BCAT1, IKZF1*	Monitoring recurrence, detection of minimal residual disease	COLVERA, Clinical Genomics
	Liver	*SEPT9*	Detection of HCC in cirrhotic patients	HCCBlood test, Epigenomics
	Lung	*SHOX2, PTGER4*	Cancer detection in increased risk patients	Epi proLung, Epigenomics
	Colorectal cancer	Undisclosed markers	Monitoring disease recurrence, minimal residual disease	REVEAL, Guardant Health #
	Multiple cancers	>100,000 site panel (undisclosed)	Primary cancer detection	Galleri, GRAIL

Figure 1.
Summary of commercial diagnostic tests using DNA methylation markers. The panel to the left shows a selection of currently available commercial cancer tests that incorporate DNA methylation assays to analyze cancer tissue–derived DNA. The right panel shows tests that assay cancer DNA methylation found in urine, stool, or as circulating cell-free DNA in blood plasma. The target genes for each methylation tests are listed as well as the test indication. Tests indicated by a # also include immuno and/or mutation assays. Further details of all the diagnostic tests summarized can be found at the company websites and in ref. 8.

identifying the cell of origin of cancers. Sensitivity varied with stage and for different cancers, being low for poorly vascularized cancers, such as prostate cancer, but was high (>70%) for stage II and above for a number of cancers, including colorectal, liver, lung, head and neck, and ovarian. The Galleri test is now commercially available for individuals with specific predispositions and is undergoing a large clinical trial through the British National Health System.

In the years since the discovery that CpG island hypermethylation occurs in most cancer types, genome-wide sequencing-based technologies have advanced to explore cancer cell heterogeneity at single cell level and map genetic and epigenetic changes with tumor evolution. We anticipate that with developing spatial 'omic' technologies, future studies will explore epigenomic changes beyond the cancer epithelial cell and determine if these changes also impact different cell types in the tumor microenvironment. Beyond the impact of DNA methylation changes in cancer diagnosis, the important role of epigenetics in cancer has expanded to encompass chromatin proteins and modifications. Mutations in a range of DNA methylation and chromatin modifier genes have been characterized across multiple cancer types. There is now growing evidence that epigenetic drugs targeting these modifiers will provide new and exciting additions to current therapy approaches for cancer treatments (10).

Authors' Disclosures

P.L. Molloy is an inventor on pending patents PCT/AU2011001176, PCT/AU2011000999, and PCT/AU2013000481 licensed to Clinical Genomics Pty Ltd. No disclosures were reported by the other author.

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