DNA Methylation in Peripheral Blood: Providing Novel Biomarkers of Exposure and Immunity to Examine Cancer Risk

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ABSTRACT

DNA methylation is an epigenetic phenomenon that can alter and control gene expression. Because methylation plays a key role in cell differentiation, methylation markers have been identified that are unique to a given cell type; these markers are stable and can be measured in tissue or whole blood. The article by Katzke and colleagues, published in this issue, uses methylation markers to estimate proportions of immune cell subtypes in peripheral blood samples that were collected prior to diagnosis, thus allowing them to directly examine associations with pancreatic cancer risk. Given that immune-cell counts cannot be measured from archived blood, and that retrospective case–control studies rely on blood that is collected after cancer diagnosis, few studies have been able to examine the role of the systemic immune response in cancer risk. Measurement of DNA methylation in peripheral blood, primarily through development of whole-genome approaches, has also opened new doors to examining cancer etiology.

See related article by Katzke et al., p. 2179

Aberrant DNA methylation in cancer cells is widespread and has long been known to play a key role in carcinogenesis (1); for example, changes in DNA methylation can drive the process of dedifferentiation of normal cells through the induction of alterations in the expression of oncogenes and suppression of tumor suppressor genes. Similarly, the role of the immune response in tumor promotion and progression is well established and has led to the development of effective cancer immunotherapies. In recent years, differentially methylated regions (DMR), akin to those identified in the studies of normal cell dedifferentiation into cancer cells, have been identified that reflect lineage in differentiated immune cell subtypes. Methylation in these regions is stable and can be measured in blood that has been previously frozen (as buffy coats or whole blood). Thus, these normal, lineage-defining DMRs (unique for different immune-cell subtypes) can be used to estimate immune cell proportions many years after being in storage [complete blood count (CBC) cannot be obtained from archived blood samples]. Hence, study of DNA methylation from blood leukocytes provides new opportunities to understand the role of the immune response in cancer risk.

Different approaches have been used to measure methylation levels in genes known or suspected to be associated with carcinogenesis. Bisulfite genomic sequencing is considered the 'gold standard' method to measure DNA methylation status. Unfortunately, bisulfite sequencing is not high-throughput and is very expensive. Other methods used to measure DNA methylation at targeted genomic regions include qPCR and pyrosequencing. Using these methodologies, hundreds of studies have examined methylation hotspots believed to play a role in gene expression of critical cancer genes, in a manner similar to examining mutations or genetic variants in genes or gene pathways known or suspected to play a role in cancer development.

Over the past 20 years, technology has been developed that moves toward a more whole-genome assessment of DNA methylation, rather than targeting localized regions. Only a few whole-genome methylation studies have been conducted using next-generation sequencing because of they are expensive and technically demanding. Most whole-genome methylation studies performed in the past 10 years have used BeadChip microarray technology. Initially, methylation arrays included CpG sites in known genes (e.g., 27 k targeted methylation arrays), but these arrays were rapidly developed to include hundreds of thousands of CpG sites across the whole human genome. The most recent high-dimensional DNA methylation array (developed by Illumina in 2015) includes more than 850,000 CpG sites. These high-resolution, high-throughput arrays have allowed researchers to make significant advances in understanding cancer pathways by agnostically interrogating a large number of CpG sites for methylation in tumor samples.

One of the most important consideration when measuring whole-blood DNA methylation levels (regardless of the laboratory method used), is immune cell composition. Peripheral blood is composed of many different immune cell subtypes derived from either myeloid or lymphoid progenitors, and the relative proportions of immune cell subsets can differ dramatically across individuals. Unique DMRs have be identified for numerous immune subsets and can be used to adjust for the varying distribution of immune cells in peripheral blood (a method called "deconvolution"). In a recent study (2), DMRs were identified for 12 leukocyte subtypes [neutrophils, eosinophils, basophils, monocytes, B cells, $CD4^+$ and $CD8^+$ naïve and memory cells, natural killer, and T regulatory cells (Treg cells)]. The methodology used to identify DMRs from high-dimensional arrays involves numerous steps, including assessing the methylation of groups of immune cells isolated using flow cytometry; validation is accomplished using reconstruction experiments and further flow cytometry confirmation (3, 4). The identified DMRs can be used to adjust for immune

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subtypes when considering associations between blood DNA methylation markers and disease outcomes.

DMRs that identify immune cell in peripheral blood can also be used to directly interrogate the relationship between immune cell proportions, or ratios of cell proportions such as the neutrophil-to-lymphocyte ratio (NLR), and cancer risk. DNA methylation measured in archived blood can thus be used to examine the role of the immune response in cancer risk using cohort studies. Using this method, we recently conducted a study examining the role of immune response on pancreatic cancer risk using the Illumina 850K DNA methylation array (using an older reference library that did not include Treg cells; ref. 5). In contrast, in the study published in this issue, Katzke and colleagues (6) used qRT-PCR to quantify methylation status in regions previously linked to immune-cell lineage (e.g., FOXP3 gene for Treg cells) to identify neutrophils, $CD3^+$, $CD4^+$, $CD8^+$, and Treg cells. Results from these two studies were similar in that no overall associations were noted for the different immune subsets examined in each study and the risk of pancreatic cancer. In the study by Katzke and colleagues (6), positive associations were observed for higher Treg proportions and lower $CD8^+$ proportions when the analysis was restricted to blood samples collected close to cancer diagnosis, suggesting, as pointed out by the authors, that the changes in methylation may well have been a consequence of the cancer development, rather than a cause. In a prior study conducted in the same cohort and using the same approach, a relatively higher proportion of Treg cells and lower $CD8^+$ T cells were associated with elevated risks of lung, colorectal, and breast cancer (7). Of course, without a cell blood count done at the time of the blood draw it also is not possible to know the absolute direction of these changes. Future work to understand the role of the immune response in cancer risk might require additional DMRs to identify rarer immune subsets, allowing for assessment of their role in cancer risk and/or the immune response to cancer.

There is a growing number of studies that are using methylation levels in blood to examine changes that occur with environmental exposures, including important cancer risk factors such as smoking history (8–10), arsenic (11, 12), and air pollution (13–15). Regardless of causal impact on disease, methylation at CpGs associated with exposure can provide valuable objective data reflecting the biological response to long-term exposures that can also be examined in relation to cancer risk. Identifying DNA methylation markers that reflect lifetime exposures, which are generally difficult to capture with questionnaires, could reduce measurement error and provide more accurate estimations for risk. Studies have shown that DNA methylation markers associated with smoking more accurately predict lung cancer risk than self-reported smoking history (9, 16). Efforts to understand whether these changes are causally associated with cancer are ongoing (17). In addition, numerous studies have identified unique methylation alterations in peripheral blood of individuals with medical conditions that are known cancer risk factors, including obesity (18), diabetes (19), and hypertension (20, 21). Methylation markers of known risk factors could, in turn, be used to evaluate how they impact the systemic immune response.

One strength of the study by Katzke and colleagues (6) is the prospective cohort study design. Cohort studies with archived blood samples collected prior to disease diagnosis are superior to retrospective studies as they are less prone to reflect changes that occur as a result of the disease. While cohort studies measuring DNA methylation markers in blood have opened promising new research avenues that may help us understand the role of the immune response, these studies may still be prone to biases, including measurement error (e.g., batch-to-batch variation observed in array data), confounding (e.g., immune cell composition, reverse causation), and chance (e.g., multiple comparisons, overall or by subanalyses). As such, including replication datasets and confirming findings in other populations are critical steps to identifying robust associations. Another important consideration includes conducting time-lag analyses that provide insight into the causal role of methylation changes. The immune response is very dynamic and individualized; hence, consideration of both temporal changes in exposures, diseases and outcomes will be necessary.

New technology and high-dimensional analyses have offered new opportunities for cancer epidemiologists. With these tools, significant strides will be made to cement causal risk factors, identify new risk factors, unravel important biological pathways, and combine methylation with genomic data to identify high-risk individuals. With appropriate study designs, careful analysis of high-dimensional data, and replication datasets, this field will certainly reveal many new insights into cancer risk.

Authors' Disclosures

K.T. Kelsey reports personal fees from Cellintec outside the submitted work; in addition, K.T. Kelsey has a patent for PCT/US2012/039699 and the title is "Methods Using DNA Methylation for Identifying a Cell or a Mixture of Cells for Prognosis and Diagnosis of Diseases, and for Cell Remediation Therapies." United States, serial No. 14/089,398 filed November 25, 2013; European Patent, serial No. 12789375.8 filed October 7, 2013; Canada, serial No, 2,869,295 filed May 25, 2012 issued. No disclosures were reported by the other authors.

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