Immunomethylomics: A Novel Cancer Risk Prediction Tool

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Abstract

There is emerging evidence that the immune biology associated with lung and other solid tumors, as well as patient immune genetic traits, contributes to individual survival. At this time, dramatic advances in immunologic approaches to the study and management of human cancers are taking place, including lung and head and neck squamous cell carcinoma. However, major obstacles for therapies are the profound immune alterations in blood and in the tumor microenvironment that arise in tandem with the cancer. Although there is a significant current effort underway across the cancer research community to probe the tumor environment to uncover the dynamics of the immune response, little similar work is being done to understand the dynamics of immune alterations in peripheral blood, despite evidence showing the prognostic relevance of the neutrophil/lymphocyte ratio for these cancers. A prominent feature of cancer-associated inflammation is the generation of myeloid-derived suppressor cells, which arise centrally in bone marrow myelopoiesis and peripherally in response to tumor factors. Two classes of myeloid-derived suppressor cells are recognized: granulocytic and monocytic. To date, such immune factors have not been integrated into molecular classification or prognostication. Here, we advocate for a more complete characterization of patient immune profiles, using DNA from archival peripheral blood after application of methylation profiling (immunomethylomics). At the heart of this technology are cell libraries of differentially methylated regions that provide the "fingerprints" of immune cell subtypes. Going forward, opportunities exist to explore aberrant immune profiles in the context of cancer-associated inflammation, potentially adding significantly to prognostic and mechanistic information for solid tumors.

Keywords: immunology; cancer; methylation; epidemiology

(Received in original form June 19, 2017; accepted in final form July 26, 2017)

Supported by National Institutes of Health grants 1R01CA207360 (J.K.W.) and CA207110 (K.T.K.).

Author Contributions: K.T.K. and J.K.W.: Substantial contributions to the conception or design of the work; drafting the work and revising it critically for important intellectual content; and final approval of the version submitted for publication. K.T.K.: Accountability for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Ann Am Thorac Soc Vol 15, Supplement 2, pp S76–S80, Apr 2018

DOI: 10.1513/AnnalsATS.201706-477MG
Internet address: www.atsjournals.org

It has long been known that DNA methylation patterns are completely erased and reprogramed in preimplantation embryos, enabling the developmental differentiation processes associated with the genesis of somatic lineages (1). In general, genomic methylation patterns in somatic differentiated cells are stable and heritable (1) and include such generally irreversible states as those associated with genomic imprinting and X-inactivation. Our early data (2) are consistent with those of numerous other labs showing that patterns of methylation in promoters, enhancers, and gene bodies strongly correlate with expression (3) and define essentially invariant, stable differentially methylated regions (DMRs) that can be used to predict lineage (4, 5). These DNA methylation changes act to form barriers to ensure that cell-type specification, within the context of the normal development of an organism, is a one-way street. We note that there is some evidence of somatic cell plasticity, but this has been observed under unusual circumstances (e.g., in cancers or in inducing pluripotent cells) (6–8). The Epigenome Roadmap Consortia recently confirmed this, noting that DMRs define lineage (in the developmental context) and that the environment has little (if any) influence on this process (9). Of course, the "environment" does influence cellular lineage, but it does so with some specificity, in the sense that it modifies the major differentiation pathways so as to hone the genome to best respond to the anticipated challenges for the individual. Finally, DNA methylation has been explored extensively in hematopoietic lineage differentiation. In the case of T-cell differentiation, stable and heritable changes in DNA methylation, impervious to minimally responsive environmental perturbations, define the major lineage (10), although it

must be noted that the environment can alter the relative size and nature of the differing cell lineages and the pool of cells in each lineage. Regulatory T cells, as one example of a lineage, have been exhaustively shown to have stable and invariant methylation marks that define their phenotype (11, 12). As a consequence, methylation marks with lineage specificity have a potentially profound utility as biomarkers, signaling programmed cellular responses to regulatory stimuli that are distinct from environmental influences upon methylation. Thus, it is now clear that DMRs are specific, genetically determined, developmentally programmed, invariant marks of lineage. Hence, we have applied these methylation biomarkers to probe the immune response status of patients with nonhematopoietic cancer, seeking to enhance our understanding of the normal immune response and devise predictors of disease outcome.

DNA Methylation Data Can Be Used to Define the Immune Profile in Peripheral Blood

Epigenetic modifications, specifically DNA methylation, dictate programmed lineage differentiation within the immune system (13–16). Remodeling the epigenome during development leads to progressively restricted immune subtypes, and DNA methylation provides a chemically stable mark for these cell fate decisions that are immutable and unchanged by lifestyle or exposures (17). This fact, in conjunction with our empirical observations of isolated leukocyte DNA methylation array data, led our group to hypothesize that all lineage-specific immune cells in the peripheral blood could be distinguished by a signature or "fingerprint" of DMRs. In 2012, with our group, Dr. Houseman developed the first statistical algorithm for estimating leukocytes solely by reference to DNA methylation data (18). We have since performed several more extensive validation experiments and continue to evolve ever more sophisticated and accurate bioinformatic methods for immunomethylomics; that is, immune cell typing using methylation (19–22). Using pure cell type reference DNA methylation data (cell type libraries), we deconvolute separate target DNA methylation data sets into constituent cell-type proportions. At this time, there are DMR libraries based on the Illumina 450K methylation platform (Illumina) for normal leukocyte subtypes, including CD4, CD8 T-cells, B cells, natural killer cells, dendritic cells, monocytes, neutrophils, basophils, eosinophils (23), activated natural killer cells (24), and cord blood (25).

Host Immunity Has a Significant Effect on Cancer Survival

Although the classification of tumors has improved our understanding of lung cancer prognosis, immune factors are notably absent in existing prognostic models of lung cancer (26). This omission is significant both because immune evasion is a recognized hallmark of cancer (27) and because of the abundant evidence that patients with lung and aerodigestive cancer suffer systemic immune defects (28–46), a portion of which are now known to respond to immunotherapy (47, 48). Blood lymphocyte counts (particularly CD4 T-cells) and T-cell function are altered in patients with cancer, with T-regulatory cells having been of significant interest to date in the literature (30, 38, 40, 42, 45, 46). Several studies have reported that the increased frequency of T-regulatory $CD4^+$ lymphocytes in the peripheral circulation correlates with prognosis (49–51). In addition to the inhibitory signaling alterations mediated by T-regulatory cells that are associated with cancers, it is also clear that natural killer cells play an important role in aerodigestive cancers (32, 38, 52). In addition to mediating direct cytotoxicity, they participate in the regulation of the antitumorous adaptive immune response, as they produce cytokines such as interferon-γ, tumor necrosis factor-α, interleukin-10, several chemokines, and growth factors. Thus, natural killer cells exert an influence on macrophages, neutrophils, and dendritic cells during the immune response (53). We demonstrated depressed natural killer cell numbers in patients with head and neck squamous cell carcinoma, consistent with their importance in the immune response to head and neck squamous cell carcinoma (54). In addition, the solid tumor microenvironment is highly immunosuppressive (30, 33, 37, 40, 44, 52, 53) through secretion of soluble factors, most notably transforming growth factor-β, interleukin-4, interleukin-10, interleukin-13, and other mechanisms. At the same time, polymorphonuclear granulocytes have been shown to play an important role in the immune and inflammatory responses in head and neck squamous cell carcinoma and lung cancers (28, 29, 41–43, 52, 53).

Neutrophil–Lymphocyte Ratio as a Prognostic Biomarker

Shifts in the distribution of blood leukocytes are important predictors of cancer patient survival. The neutrophil–lymphocyte ratio (NLR) in whole blood has received a great deal of attention as a marker of cancer inflammation (55). The NLR can be derived using the common five-part white blood cell differential (neutrophil, basophil, eosinophil, monocytes, and lymphocytes) from automated cell analyzers. Because the NLR reflects the relative balance of the myeloid and lymphocytic lineages in peripheral blood, it is sensitive to the altered myelopoiesis arising in chronic inflammation and cancer. Extensive studies show that the NLR is a remarkably consistent prognostic factor for survival in malignant and cardiovascular disease (56–61). An NLR <3 is widely considered a favorable predictor for solid tumors as well as related disease mortalities, and an NLR >5 has often been used as the threshold that predicts poor outcome (62). A recent meta-analysis of solid tumor prognosis including 100 studies and 40,559 subjects showed that a higher NLR was significantly associated with reduced overall survival, reduced cancer-specific survival, and reduced progression-free and disease-free survival (55).

There are now numerous studies that all show shorter survival times in patients with lung cancer with an elevated NLR, and a recent meta-analysis confirms the data are consistent (63). Although the thresholds for defining an elevated NLR were somewhat different in these studies, an NLR >5 was associated with poor prognosis independent of known risk factors (e.g., age, stage). We have devised an algorithm using DNA methylation to estimate (64) NLR from 27K and 450K methylation data, and our approach is easily adaptable to the new 850K array platform. In published studies, we found that this DNA methylation-derived NLR at values >5 was independently associated with significantly shorter survival time in studies of multiple solid tumors (65).

Because the conventional NLR is based on simple normal cell morphology, the presumed pathologic cell types within the blood cannot be phenotypically (or otherwise) distinguished in blood smears or automated differential counters. Similarly, the current epigenetic methylation-derived NLR measure is based on the methylomes of normal mature leukocyte populations. We have also created an algorithm that estimates the common clinical NLR parameter using only DMR information from normal leukocyte libraries. Because cancer inflammation leads to aberrant myeloid populations in the blood and associated shifts toward higher values of the NLR, immune biomarkers specific to these pathologic cell types (driving immunosuppression) will provide the greatest power to evaluate the role of cancer inflammation in lung cancer survival. Importantly, new preliminary data are poised to answer the obvious question about the predictive power of the NLR. Using either complete blood cell counts or blood methylation data, emerging data suggest the NLR can also prospectively predict solid tumor risk (65). Confirmation of these results awaits further studies.

Mechanistic Considerations

As a result of the overwhelming concordance of this body of literature, there is now an urgent need to investigate the molecular drivers of this phenotype. Researchers long ago observed that tumors affect the host's hematopoietic progenitor cells, resulting in expansion of myeloid lineage populations and a decrease in circulating lymphoid cells (66). Chronic inflammation, infection, and aging lead to the same reciprocal dynamic between myeloid and lymphoid lineages (67, 68). Today, cancer-associated shifts in myelopoiesis are actively studied, fueled by the realization that inflammation-induced myeloid cells suppress host immune cells (69–71). These myeloid-derived suppressor cells (MDSCs) (72) suppress antigen-specific $CD8^+$ T-cell activity via production of reactive oxygen species and nitric oxide (73, 74), and increase L-arginine metabolism via arginase secretion (75), leading to arginine depletion. These effects lead to downregulation of crucial T-cell receptor components (76), as well as natural killer cell–suppression and cytokine secretion (77). MDSCs also downregulate the NKG2D gene (an activation receptor on natural killer cells), rendering them ineffective in attacking malignant cells (78). Importantly, MDSCs contribute to maternofetal tolerance, modulating immune response via a presence in cord blood (79). Although much research has focused on the effects cancer cells have on bone marrow MDSC precursors, compelling evidence exists that circulating MDSCs may arise from cancer-associated normal blood monocytes and bone marrow precursors, likely via tumor-derived soluble factors such as lactate dehydrogenase and prostaglandin E2 (80, 81). As the myeloid cascade is induced by the presence of a cancer, there exists an opportunity to capture these cells in the peripheral blood; this is a particularly attractive opportunity to use immunomethylomic methods (see Figure 1). The role of MDSCs in lung cancer has been studied, with recent work highlighting the potentially crucial role they might play in personalized medicine (82–85).

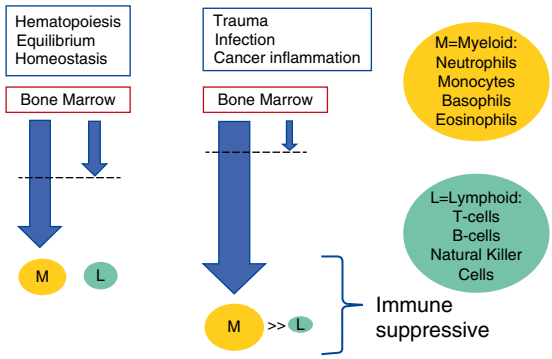

Figure 1. Immune suppression detectable in peripheral blood. Myeloid-derived neutrophil suppressors diminish lymphocytes in many inflammatory conditions, including cancer. The dashed lines indicate cells leaving the bone marrow.

Challenges and Opportunities

Immunomethylomic approaches have demonstrated a completely novel approach to the interrogation of the peripheral blood immune profile. This can be accomplished using archived DNA from blood and does not require flow cytometry. As a consequence, new opportunities have arisen for application of epidemiologic techniques to study of the immune response. At this time, there are few quantitative data enumerating the immune subtype response to environmental insult; these new tools offer promise for in-depth studies of the effects of the environment on the immune response. There are opportunities for assessing the immune profile prospectively, as well as the immune correlates of treatment. Application of this tool will require building additional immune subtype libraries and devising rich quantitative approaches to detection of more rare cell subtypes. Using the tools derived from the stable, developmental methylome, a rich new array of biomarkers and mechanistically based epidemiologic assessments of the immune response is likely to be discovered moving ahead. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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