Editorial Commentary

CLOCK'ing differences in DNA methylation signatures to understand the molecular etiology of lung cancer

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Lung adenocarcinoma (LUAD) is the most commonly diagnosed form of non-small cell lung cancer, and is associated with high frequency of tumor recurrence, severe malignancy and vast heterogeneity (1). Moreover, this histological diversity is compounded by the LUAD tumor microenvironment's (TME) extensive cellular diversity and accompanying immune infiltration patterns. These properties have been linked to unpredictable tumor recurrence, poor patient response to therapy, and mortality (2). Integrative application of existing tools and methods geared towards understanding epigenetic contributions to LUAD heterogeneity is imperative to answering these questions (3).

In the recent issue of *Clinical Cancer Research*, Guidry *et al.* repurposed established DNA methylation patterns associated with cellular age to define signatures that can act as an index for distinct subtypes of LUAD (4). In establishing the relationship between epigenetic signatures and LUAD subtypes, they were able to ascribe phenotypic and molecular characteristics to each subclassification, including enrichment for oncogenic driver mutations and immune composition of the TME (5-9). Further to this, Guidry drew correlations between immune microenvironment composition, ethnic background and patient smoking history.

Previous studies have utilized genome-wide methylation alterations within LUAD to define relevant subtypes. These investigations yielded three [3] major DNA methylation classifications in LUAD; CpG-island methylation phenotype (CIMP)-low, CIMP-intermediate, and CIMP-high (10). DNA methylation patterns have also been associated with TME molecular characteristics and immune cell composition within LUAD tumors (11). The study by Guidry *et al.* improves on these associations by refining genome-wide methylation patterning to the 353 CpG sites within the Horvath DNA methylation CLOCK (6). The authors were then able to use methylation levels at these sites to subdivide LUAD into six [6] distinct subgroups, each associated with distinct pathologic and molecular features of LUAD. Subgroups with higher DNAm age were associated with better patient survival and specific oncogenic driver mutations.

Aberrant oncogenic driver gene expression plays an integral role in tumorigenesis and progression in LUAD. To understand the relationship between DNAm-defined LUAD subclusters and oncogenic drivers, Guidy *et al.* conducted mutational analysis on tumor samples and focused on established LUAD oncogenic driver mutations that included: *KRAS*, *TP53*, *EGFR*, *STK11*, *ATM* and

KEAP1. Their results indicate that specific oncogenic driver mutations were associated with disparate immune cell composition and thus not only influences tumor immune response, but also TME heterogeneity. This was consistent with previous work that linked oncogenic drivers with TME immune cell composition (12) and innovatively connected these observations with DNA methylation-defined age. Having performed pathway analysis and quantifiably contrasting oncogenic expression to delineate which pathways underlie LUAD tumorigenesis, Guidry *et al.* underscored which included MAPK, RAS, PI3K-AKT and cellular senescence. They observed that not only were these genes the most differentially methylated, but that they were also key players in tumor progression, invasiveness and metastasis. These findings suggest a connection between differentially methylated pathways in LUAD to observed varying degrees of malignancy.

To examine how the DNAm-defined subclusters influenced cellular composition of the TME, the bioinformatic tool MethylCIBERSORT (13) was used to determine immune cell composition in each DNAm-defined subcluster. Varying DNAm age elicited distinct immune responses consisting of unique assortments of immune cell populations. The resulting tumor subtypes were further categorized as being either immune "hot" or "cold". Global DNA methylation loss and CpG hypermethylation has previously been associated with the "cold" immune refractory state (14). The Guidry *et al.*, study extends these findings and furthers our understanding of how tumor immune response capitulates more precise and targeted therapeutics and advances the immunotherapeutic clinical model.

The study also comparatively assessed immune cell composition based on specific oncogenic driver mutations and uncovered differences in immune infiltration in tumors based on *TP53* or *KRAS* mutational status. Intriguingly, oncogenic driver mutations elicited contrasting DNAm-age and unique immune infiltration patterns across LUAD mutational subtypes. Further characterization of driver mutations and correlative methylation characteristics may advance our understanding relative to LUAD histologic subtypes that are confounded by race, smoking and sex.

Differential oncogenic mutation prevalence within and across ethnic groups has been well established. For example, *KRAS* G12C most prevalently occurs in White populations while *EGFR* exon 21 L858R point mutation is seen predominantly in Asians when compared to other racial groups (15,16). Guidry *et al.*'s deconvolution of LUAD heterogeneity by integrating well established genetic and molecular analysis platforms with DNA methylation analysis may address demonstrative health disparities that exist in cancer care. Currently, there is no consensus around the prevalence of oncogenic driver mutations in Blacks as driver mutations are not well defined in this demographic when compared to their White and Asian counterparts (15,17).

Guidry's approach prompts continued characterization of oncogenic drivers, further stratification of diverse patient populations, enhanced biomarker identification and improved patient selection for specific therapies. Additionally, this platform may advise about differential efficacy and adverse effects of therapeutics across different racial groups. Employing DNA methylation analysis may enhance our understanding of molecular pathogenesis in underrepresented minority populations.

It is known that TME composition may influence sensitivity to immunotherapy and that the unique TME cellular composition may modify the interplay observed between a tumor and subsequent immune response (18). This discovery has ushered in the use of novel immunotherapeutics; including checkpoint inhibitors with adjuvant therapy, that have transformed the clinical intervention arsenal. Among the most notable of these discoveries is the humanized antibody pembrolizumab which has yielded improved NSCLC overall survival to 5 years (19). Enhanced activation of tumor-specific T cells are instrumental in the engineering of effective immunotherapy. Immune checkpoints are a rapidly emerging area of focus in cancer. PD-L1, TIM-3, LAG-3 and CTLA 4 are among the most notable and exploited immune checkpoints. Deconvoluting T cell phenotypic heterogeneity, uncovering additional relevant immune checkpoints and further characterization of antigen presenting cells (APCs) and major histocompatibility complex (MHC) proteins within TME, may yield improved biomarker and immunotherapeutic discovery and development. Understanding the interchange between DNA methylation of checkpoint gene promoter regions and oncogenic aberrations and DNAm-age holds significant implications for refinement of diagnostic criteria used in precision medicine while raising the possibility that combination small molecule inhibitor and immunotherapy may be optimized to increase positive responses in diverse patient populations.

Finally, Guidry *et al.*'s classification system revealed a DNAm signature associated with variation in the aggressiveness of LUAD presentation and suggests that smoking history, ethnicity and age confound poorer

outcomes. DNA methylation has a well-established role in gene regulation and has been exploited clinically as biomarkers for cancer screening (20). Multiple consortia are actively pursuing the identification of DNA methylation signatures in LUAD (21-23), with special emphasis on their use as a companion diagnostic for CT-scanning. This innovative diagnostic combination may serve as a way to manage the exceedingly high false positive rates or as a companion surveillance method for patient response to therapy (24). However, these efforts have had inconclusive results, which have often failed to recapitulate in diverse patient populations. The Guidry *et al.*, study may improve on the current state of DNAm as a biomarker of disease by both reducing the complexity of DNAm signatures from genome-wide to the DNAm-age associated CpGs and classifying LUAD subtypes.

Taken together, the vast, heterogenous landscape of LUAD has been a major factor impeding progress in treatment and patient outcomes. While lung cancer survival has increased ~10% over the past 10 years, it is still the deadliest form of cancer (25). The associated poor survival is further exacerbated as a result of specific oncogenic mutations and inadequate stratification modalities. Another caveat is variable patient response to therapeutics. While significant progress has been made in cancer care, many patients simply do not benefit from current therapeutic modalities. This is largely in part due to immunosuppressive TMEs, inadequate discovery and development of targeted small molecule inhibitors of oncogenic driver mutations, insufficient early detection biomarkers, and a poor understanding of the societal and ancestral genetic contributions to cancer disparities. Untangling LUAD heterogeneity therefore represents the future of personalized medicine. Employing a multifaceted approach that utilizes DNAm-age to stratify clinically relevant LUAD subtypes may reshape the framework of clinical intervention and management. Guidry *et al.*'s multiplexed approach featuring DNAm-age will enhance discovery and development of more diverse and specific biomarkers, thereby providing a specific epigenetic signature that can serve as an index for diagnosis, metastasis and tumor recurrence. This integrative approach will advance the precision medicine model for LUAD and provide a viable tool to address health inequities.

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