Editorial Commentary

Clinical applications of DNA methylation profiling in lung cancer

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Lung cancer is one of the most common and deadly types of cancer, particularly in industrialized countries. Based on the histological subtype, lung cancer can be divided into 2 major groups: small cell lung cancer (SCLC) with 15% of cases and non-small cell lung cancer (NSCLC) with the remaining 85% (1). Lung adenocarcinoma (LUAD) is the most frequent histological subtype of NSCLC, which continues to be the leading cause of cancer-related deaths worldwide (1-3). Patients with NSCLC can be treated with surgery, chemotherapy, radiotherapy, targeted therapy, or a combination of all. Surgical resection still represents the standard treatment for patients during the early stages of LUAD. Even though it increases patient survival, almost half of them will die due to disease recurrence (4,5). In addition to surgical resections, LUAD patients usually receive adjuvant chemotherapy that can help to improve this poor patient outcome (6,7). However, despite the research advances made in first-line and complementary treatments, such as molecule-targeted therapies [epidermal growth factor receptor (EGFR), phosphatidylinositol-3 kinase (PI3K)/Akt kinase (AKT)/mammalian target of rapamycin (mTOR) and neurotrophic tropomyosin receptor kinase (NTRK)/c-ros oncogene 1 (ROS1) inhibitors], immunotherapy (anti-PD-1), and non-invasive resection, LUAD patients still remain among cancer with the worst prognoses (8-10). One of the main reasons for these challenging clinical outcomes is our limited ability to characterize LUAD patients in terms of prognosis and potential individualized therapeutic plans. For the majority of NSCLC tumors without identifiable targeted therapy, a combination of chemotherapy regimens is the mainstay with a median overall survival of less than 2 years (11). Due to the molecular heterogeneity of LUAD patients, risk assessments using traditional factors such as tumor size, stage, or lymph node status have difficulties in accurately predicting patient prognosis and determining which patients would benefit more from specific therapies (12,13). Therefore, it is essential to find new methods to identify high-risk LUAD patients and classify them for precise therapeutic strategies. To address this issue, research groups have recently pursued the identification of useful and powerful predictive biomarkers for the molecular characterization of tumors. Most of these studies are based on genetic studies, while the information we have from epigenetic studies is more limited and difficult to interpret. DNA methylation (DNAm) is one of the most studied epigenetic mechanisms for the regulation of gene expression. Abnormal DNAm signatures have an important biological significance in cancer, as they can

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modify important processes involved in carcinogenesis. This suggests that their study may have clinical utility (14). As the matter of fact, several prognostic models in LUAD have been developed in the last years using DNAm data (15-17). Despite all these proposed models, developing more accurate predicting tools is still required to better stratify patients into more specific risk subgroups and molecular subtypes for clinical evaluation and explore more precise targeted therapies. In this regard, in a recent study by Guidry and colleagues (18), the genome-wide DNAm profile from 88 resected LUAD samples was evaluated to provide additional insight into the tumor microenvironment (TME) heterogeneity and to allow the stratification of patients into clinically relevant molecular LUAD subgroups. The TME is the environment around a tumor, including the surrounding blood vessels, immune cells, fibroblasts, signaling molecules, and the extracellular matrix. TME heterogeneity may be partly responsible for the differences in the response to the treatment of patients. It has been widely established that the presence of specific immune cells within the TME greatly affects cancer development and progression in general, and LUAD in particular (18,19). Some of the immune cell types typically included in the evaluation of TME are (I) the cytotoxic cells such as $CD8^+$ T-lymphocytes ($CD8^+$ T) and natural killers (NK), (II) effectors $CD4^+$ T-lymphocytes ($CD4^+$ T), $CD19^+$ B-lymphocytes (B-cells), (III) and regulatory T-lymphocytes (Treg), which are a subpopulation of lymphocytes with an immune suppressive activity that tunes down the immune system during pro-inflammatory responses. Given the relevance of TME in LUAD progression, Guidry and colleagues used methyl-CIBERSORT (Cell type Identification By Estimating Relative Subsets Of known RNA Transcripts), a bioinformatic tool able to deconvolute *in silico* TME cell composition (19) and classify those LUAD tumors into cold or hot subgroups based on immune cell infiltration profiles. Immune hot or immune cold refers to the immunogenicity associated with the cell types infiltrated within the tumor, finding $CD8^+$ T and NK cells at the highest portion of the scale, in opposition to the low immunogenicity associated with cells such as fibroblasts or Treg cells. Interestingly, Guidry and colleagues observed that TME immune composition was affected by driver mutations in key cancer genes (like *KRAS* or *TP53*), smoking history, and ethnicity. Additionally, a direct correlation was found between the loss of *TP53* and tumor-infiltrated Treg cells, which supports previous studies in the field (20). They also observed that hot tumors exhibited a higher $CD8^+$ T to Treg ratio compared to cold tumors. While this ratio seems to be associated with clinical outcomes in other cancers, Guidry *et al.* found no differences in terms of overall survival between the two immunological LUAD phenotypes. For their study, Guidry *et al.* also measured the DNAm age, an epigenetic clock that measures the cumulative effect of a system of epigenetic maintenance such that it is close to zero for induced embryonic and induced pluripotent stem cells and increases with the number of cell divisions (21). They also determined that DNAm age has an implication in LUAD TME composition. Specifically, they observed that higher levels of tumor DNAm age are associated with an increase in the population of infiltrated $CD4^+$ T and NK cells and a reduction in the levels of Treg and B cells. Paradoxically, whereas DNAm age strongly correlated with chronological age, they found that overall patient survival directly correlates with DNAm age while it does it inversely for chronological age.

Finally, they performed an unsupervised clustering analysis based on DNAm profiles and identified 6 different molecular subgroups among LUAD tumors. Interestingly, these subgroups have immunological infiltration profiles and, most importantly, unique clinical characteristics. For instance, the subgroup with the best clinical outcome (subgroup number 1), was characterized by the highest DNAm age and no tumor recurrence or reported death whatsoever. This phenotype seemed to be associated with the repression of the PI3K-AKT signaling pathway through hypermethylation, which is in line with previous studies *in silico* (22). Nevertheless, it would be very interesting to confirm whether this repression significantly reduces protein levels and whether that could be somehow related to the increase in DNAm age observed in this subgroup. Additionally, although DNAm age directly correlated with overall survival, the subgroup with the lowest DNAm age (subgroup 6) had overall survival levels comparable to those observed for subgroup 1 (highest DNAm age). Remarkably, all tumors included in subgroup 6, turned out to have a mutation on *TP53* and the highest infiltration of Treg cells. Again, it would be worthwhile to further evaluate the potential association between mutations in TP53, DNA age, and overall survival. On the other side of the clinical spectrum, they identified a subgroup with the lowest survival rates (subgroup 4), which were hot tumors with a high $CD8^+$/Treg ratio and high tumor recurrence. Besides the mentioned subgroups, they identified another 3 clusters with unique molecular features, immunological composition, and overall survival and recurrence. All in all, although this study would have benefited from additional drug responsiveness data, it does provide relevant new

insight into the understanding of TME heterogeneity and shows a great power of prediction for LUAD patient classification. For instance, this unsupervised methylation-based analysis was able to successfully cluster tumors from patients with both smoking habits and mutations in *KRAS* within those with the best clinical outcomes.

In conclusion, if applied to larger cohorts and supported by prospective studies, this work would improve our capacity for the identification of reliable biomarkers to classify patients in need of specific immunotherapy, more or less aggressive therapy, or even closer monitoring. Moreover, further efforts in the study of LUAD-associated DNAm profiles, combined with specific de-methylating tools, such as the recently developed CRISPR-DiR strategy (23), could open new therapeutic approaches for improving the current survival rates.

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