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RNA Sequencing: A Potentiator of Discovery-based Research

In 1989, a team of Toronto-based researchers first published the discovery that mutations of the *CFTR* (cystic fibrosis transmembrane conductance regulator) gene led to the development of CF (1–3). Over the intervening 30 years since that discovery, incredible advances have been made in CF treatment. For many patients, a disease that once ensured death in childhood or early adulthood is now managed well into adulthood as a chronic illness. The most exciting novel therapies for CF are undoubtedly the targeted modulator therapies. For those with amenable mutations, CFTR modulators improve both physiologic and patient-centered endpoints (4).

Ivacaftor was the first available CFTR potentiator approved by the U.S. Food and Drug Administration for treatment of CF with the missense mutation G551D (5). *In vitro* data showed that ivacaftor restored the gating function of G551D CFTR (6), rescuing transmembrane chloride transport. Clinical trials of ivacaftor demonstrated rapid and durable improvements in lung function, quality of life as measured by the Cystic Fibrosis Questionnaire Revised (CFQ-R) score, weight, and frequency of pulmonary exacerbations (7). Further studies demonstrated effectiveness in treating a number of mutations similar to G551D (8), and in 2017 the Food and Drug Administration expanded the clinical indications for ivacaftor to an additional 23 class III ("gating") mutations (9). More recently, ivacaftor in combination with other modulators was shown to be efficacious for the treatment of patients with Phe508 del homozygous *CFTR*, the most common genotype in patients with CF (10, 11). In the face of an increasing number of CFTR modulators and the option to use varying combinations of drugs for treating different *CFTR* genotypes, novel approaches to predicting response to therapy are needed.

Next-generation sequencing technologies such as RNA sequencing (RNA-seq) and machine learning (ML) have the potential to revolutionize translational research. Insights from next-generation sequencing are currently guiding the generation of novel hypotheses regarding the pathobiological underpinnings of disease and facilitating biomarker discovery. Likewise, ML models integrating biomarker and clinical data are being trained to predict clinical outcomes and drug response. These technologies are already driving innovations in CF research, ranging from the identification of a novel cell type (the ionocyte) that likely contributes to the pathogenesis of lung disease in CF (12, 13) to the discovery of biomarkers of CF pulmonary exacerbations based on RNA-seq of neutrophils (14).

In this issue of the *Journal*, Sun and colleagues (pp. 643–652) report on studies investigating the transcriptomic landscape of CF and how it changes after the initiation of ivacaftor therapy (15). They performed RNA-seq analysis of peripheral blood mononuclear cells (PBMCs) from 56 patients with CF carrying at least one copy of the G551D mutation, using paired blood samples before and 1 month after initiation of ivacaftor therapy. The authors estimated differential gene expression (DGE) changes associated with the initiation of ivacaftor and identified 102 genes that were significantly different. They applied pathway analysis to these genes and found enrichment for cellular processes that regulate innate immunity and inflammation. They used a consensus clustering algorithm to classify patients as "good" or "moderate" responders to ivacaftor based on clinical variables including forced expiratory volume in 1 second (FEV_1)% predicted and CFQ-R score. Compared with moderate responders, good responders were significantly older (17.3 vs. 28.0 yr), had significantly lower FEV_1% predicted (69.4% vs. 98.3%), and had significantly lower CFQ-R scores (56.4 vs. 81.7). The authors performed another DGE analysis comparing pre-ivacaftor PBMCs between these good and moderate responders, and identified 65 differentially expressed genes, which were enriched for a previously published set of CFTR modifier genes. Finally, they built a prospective predictive model for clinical response to ivacaftor using an ML-based random-forest algorithm trained on combined clinical data and expression levels of *PPARG*, one of the genes with the highest log-fold difference between good and moderate responders.

Two of the most notable strengths of the report by Sun and colleagues are the large size of the study population and the focus on a relatively homogeneous population of patients with at least one copy of the same mutation. Genetic homogeneity in a disease locus in human RNA-seq studies is rare and helps to bolster the expression signal of ivacaftor treatment. Furthermore, the pathway analysis serves as a nice companion to the established clinical improvements seen in previous clinical trials, in that it highlights the salutary effects of improved mucociliary clearance along with decreased inflammation and infectious burden. Crucially, Sun and colleagues are committed to making the RNA-seq data described in this report publicly available. This is essential for maximizing the value of their work because it enables investigators to increase power or perform validation studies in future transcriptomic analyses. Finally, their use of RNA-seq for biomarker discovery and application of ML-based methods that incorporate both clinical and genomic variables are promising approaches that may aid in the generation of predictive models for CF and other disease processes.

This report also has important limitations. Although the authors noted low expression of *CFTR* by PBMCs, it is not possible to determine whether the transcriptomic changes observed in this study are a direct effect of ivacaftor on these cells or instead reflect systemic effects of improved CFTR function in stakeholder tissues such as the lungs. The baseline differences in age, lung function, and symptoms between patients classified as good or moderate responders suggest that the patients labeled as good responders were sicker than the moderate responders, and those labeled as

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moderate responders were close enough to ideal health that they simply may not have had as much room to improve with ivacaftor treatment. Finally, the use of ML-based methods on a single training dataset without confirmation in an independent validation dataset is vulnerable to overfitting, a form of modeling error common to ML methods where the predictive power of the algorithm performs well on the generative dataset but breaks down when applied more broadly. The authors explain that they compared model test errors among candidate biomarker genes from their DGE analysis and selected *PPARG* alone over a set of candidate genes because it resulted in the lowest test error for their model. This approach could actually worsen overfitting because the gene with the lowest test error in a single training dataset may turn out to have a higher test error than other genes when the model is applied more broadly.

Treatment with ivacaftor as part of standard of care for patients with CF and at least one G551D mutation will probably not change with this report, but the approach presented here can be adapted to future prospective studies of CF modulators, including combination therapies, for patients with different CF genotypes. Thoughtful experimental designs including large, prospectively defined, independent training and validation cohorts can yield successful predictive models of response to therapy that can enter clinical practice for CF and other diseases. ■

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