Perspective

Multiplex Proteomic Analysis of Cells in Fine Needle Aspiration Samples:
How Soon Can We Expect to Individualize Therapy by Comparing Pre- and Posttreatment Biopsies?

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Individualized therapy of the future will be guided by panels of biomarkers that include proteins, nucleic acids, and metabolites. Among these analyte classes, proteins can provide direct functional information central to the disease. The added bonus is that the protein, or phosphoprotein, biomarker is the drug target itself and/or the direct readout of the signaling activity being inhibited. An example is the selection of kinase inhibitor therapy based on phosphorylated receptor tyrosine kinases, such as human epidermal growth factor receptor 2, protein kinase B, epidermal growth factor receptor, MET receptor tyrosine kinase, or cytoplasmic signaling proteins, such as mammalian target of rapamycin. Yet it has not been easy to quantitatively measure a panel of protein/phosphoprotein biomarkers in tissue biopsy samples with high precision, sensitivity, and accuracy, which are essential attributes if the data are used to select patient therapies. Addressing this challenge, Ullal et al. *(1)* described a new technology that uses DNA barcode labeling of antibodies for multiplex proteomic/phosphoprotein analysis of cells in a tumor fine needle aspiration (FNA).[2] FNAs are challenging clinical specimens that contain relatively few cells compared to a tissue biopsy. Why is this method important, how does it compare to existing multiplexed clinical proteomic technologies, and when can we hope to see this platform routinely used in the clinical laboratory?

The author's platform *(1)* is divided into serial stages. In the first stage, the cells are passed through a microfluidic chamber where tumor cells are separated from host cells. Here, the cell mixture is exposed to antibodies tagged with magnetic particles. This permits the removal of contaminating immune cells or positive selection of tumor cells. In the second stage, the cancer cells are incubated with a cocktail of antibodies that are labeled with DNA "barcodes." Each barcode is a different 70-nucleotide sequence. The DNA barcodes are attached to the antibodies with a photocleavable linker. During the incubation process, the antibodies bind to the suspended cells. Each cell in the mixture can potentially bind 100 different types of labeled antibodies. In the third stage, the unbound antibodies are washed out, and the barcodes are cleaved away from the bound antibodies. In the final stages, the released barcode tags are detected by use of a second complementary DNA barcode conjugated to a code of 5 different fluorescent markers and counted by use of the NanoString charged-coupled-device camera nCounter system. Overall, the system does not involve an amplification step, but the authors state that the sensitivity approaches the single-cell level.

Ullal et al. *(1)* document the utility of their new platform with cell lines, including the A431 cell line, with and without gefitinib treatment. They selected 88 antibodies that recognize proteins and phosphoproteins involved in a variety of cancer cell functions such as kinase signaling, epithelial–mesenchyme transition, apoptosis, and autophagy. The authors could establish quantitative thresholds for each antibody above immunoglobulin G controls, and the barcode method correlated with flow cytometry results on the same cell types. When the A431 cells were treated with gefitinib, the authors could not detect changes in the phosphorylation of epidermal growth factor receptor, but they could detect changes in phosphorylated-S6 ribosomal protein and phosphorylated-histone H3 *(1)*.

After proof-of-principle in cell lines, the authors explored the use of the platform in clinical specimens. They obtained FNAs from single-pass samples of lung adenocarcinoma. Epithelial cell adhesion molecule–positive cells were sorted and assumed to be tumor cells. The results indicated a discordance between the bulk tumor sample and the FNA measurements. The authors interpreted this discordance as evidence of within-tumor heterogeneity. Moreover, when the barcode system was tested on bulk samples from 6 patients
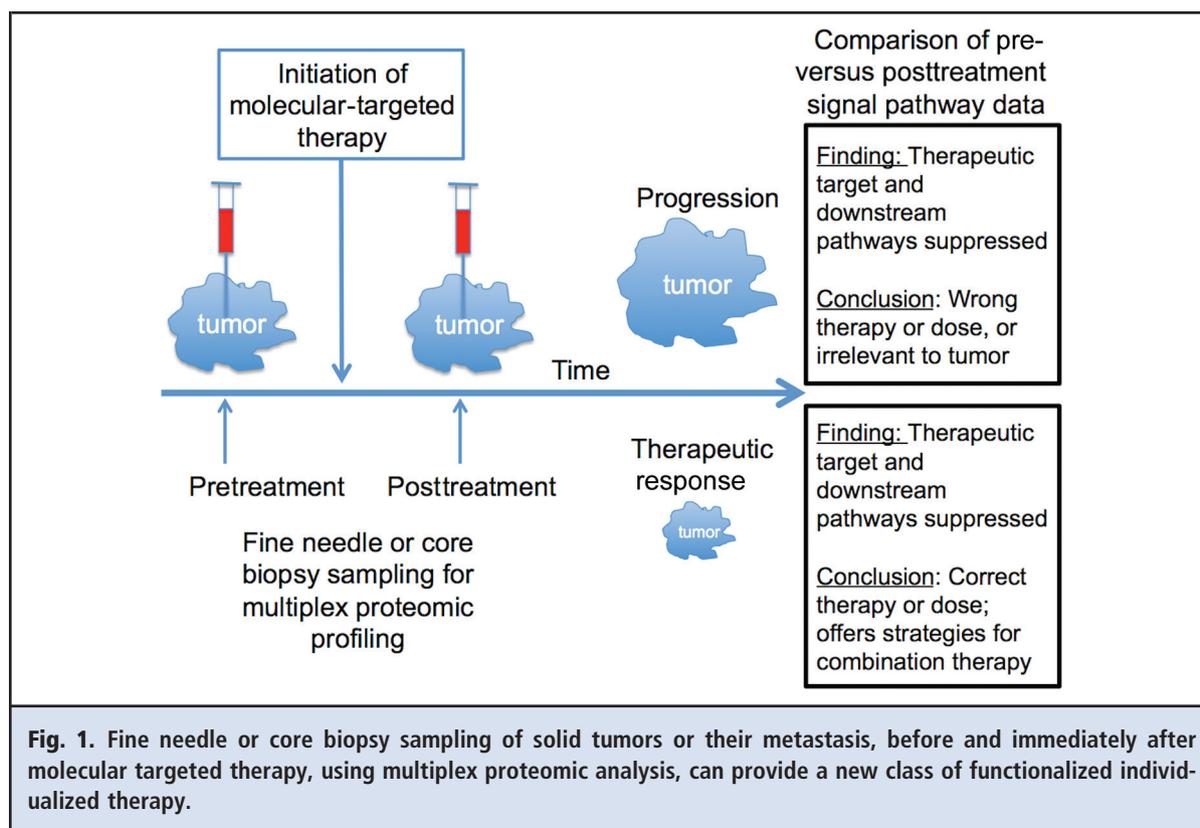
Fig. 1. Fine needle or core biopsy sampling of solid tumors or their metastasis, before and immediately after molecular targeted therapy, using multiplex proteomic analysis, can provide a new class of functionalized individualized therapy.

with lung adenocarcinoma, clear differences were found between each patient. Finally, the authors applied the technology to 4 patients with metastatic cancer being treated with a phosphoinositide 3-kinase inhibitor. Two of the 4 patients were responders. Unsupervised clustering across the marker panel separated the 2 responders. The top marker that correlated with treatment response was methylation of histone H3 at Lys79. The authors concluded that their technology was compatible with clinical applications and showed potential to measure drug response. They envisioned that this platform would be used to "yield mechanistic insights into existing and/or novel therapeutic strategies" and might be applicable to circulating tumor cells.

The technology of Ullal et al. *(1)* is a novel combination of microfluidics, cell sorting, antibody barcode labeling, and nonamplified fluorescent code readout on the nanString pCounter analysis system. Several existing technologies are also being applied to multiplex proteomic analysis of clinical specimens *(2)*. Reverse-phase protein arrays (RPPAs) *(2)* measure hundreds of proteins, phosphoproteins, and modified proteins in tissue cells derived from whole tissue samples, FNAs, or laser capture–enriched tumor cells or stroma from core biopsies and FNAs. The RPPA technology differs

from the barcode method because antibodies are incubated with cell lysates, not whole cells. RPPA uses an avidin-biotin–based amplification step to achieve a sensitivity that can reach low-abundance phosphorylated signal pathway proteins with a detection sensitivity of less then a cell equivalent *(2)*. Mass cytometry *(3)* and Phospho-flow *(4)* are another category of highly sensitive technologies routinely being applied to cell suspensions for multiplex protein analysis. These methods are similar to the barcode technology in that the cell suspension is incubated with a panel of tagged antibodies. The difference is that mass cytometry and Phospho-flow use a different antibody tagging system and a different detection system. These 4 different technologies were all developed with the goal to bring quantitative multiplex proteomics to routine clinical use for individualized patient therapeutic stratification.

A highly notable aspect of the approach of Ullal et al. *(1)* is the use of the technology to interrogate the proteomic signaling architecture before and after therapy in the same patient. Profiling the protein signaling network before and after therapy is an important, and growing, clinical opportunity for companion diagnostics. Current research trials typically sample the tumor (or rarely the metastasis) before treatment exclusively. In contrast, proteomic/phosphoproteomic profiling

before and after therapy in the same patient has a number of advantages. First, with these technologies, it will be possible to immediately judge if the therapy is actually suppressing the intended target. If not, this may mean that the patient has been given the wrong dose or the wrong type of therapy or that the tumor cell circuitry bypassed the target. Second, if the therapeutic target is suppressed, but the tumor is unaffected, then this means that the intended pathway (and therapy) was irrelevant to the tumor. Third, if suppression of the target is associated with a concurrent change in the signaling pathway downstream of the target, or activation of other compensatory signaling pathways, this could point to appropriate combination therapies (Fig. 1). Armed with in vivo functional response profiling data, the clinician can make a decision to change the dose, stop, add, or change therapies.

Despite the novelty of the barcode technology, the routine application of this technology to the clinical laboratory may be many years in the future. Unsupervised heat maps presented by Ullal et al. *(1)* generate hypotheses concerning patient correlations, but they do not provide cutoff values that can be used for ROC curves to determine sensitivity and specificity for individual patients. Sources of preanalytical variability, tumor histologic and clonal heterogeneity, full-process precision, antibody specificity, and labeled antibody access to permeabilized cytoplasmic and nuclear epitopes all have to be addressed and controlled/optimized before we can imagine that this technology could be used routinely in a College of American Pathologists/CLIA-accredited laboratory. Inter- and intraassay full-process precision and accuracy must be tested on future statistically powered cohorts.

FNAs are often dominated by blood cells and host immune/inflammatory cells and contain variable proportions of stromal, epithelial, and vascular cells. The tumor cells, which can constitute a minor population, are frequently clumped or entrained in fibrin. Pretreatment by enzymatic disaggregation of tumor cell clumps, as mentioned by Ullal et al. *(1)*, could perturb the cell proteome, particularly the cell surface receptors, and may eliminate the clumped tumor cells that contain key information. The proportion of the tumor cells to other nontarget cells types is unknown and un-knowable ahead of time. Important drug target signaling proteins, such as protein kinase B, extracellular signal-regulated protein kinase, and mammalian target of rapamycin, are activated and present in nearly every cell type in the body. Indeed, it has been recently shown that the accuracy of the proteomic/phosphoproteomic data obtained from whole ground-up cancer tissue samples may be inferior to that of enriched cells obtained from laser-dissected matched samples *(5)*. How can variability due to specimen cellular heterogeneity be distinguished from within-tumor heterogeneity or analytical variability of the Ullal multistep technology? The act of conducting the FNA causes bleeding and can alter subsequent FNA passes. Moreover, depending on the location and depth of the needle penetration, or the evacuation strength, the yield of tumor cells and the subpopulation they represent can vary from one part of the tumor to the other. Thus it may not be possible to apply the same criteria for within-tumor FNA precision that we apply to a homogeneous blood specimen. In fact, we may have to come up with new ways to qualify the accuracy, precision, and sensitivity of analyte panels measured in FNAs of inherently heterogeneous tumor aspirates.

References

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