Use of Nano-ImmunoAssay to Generate Rapid, Quantitative Nanoscale Proteomic Profiling of the Hypoxia Pathway in Renal Cell Carcinoma Clinical Specimens

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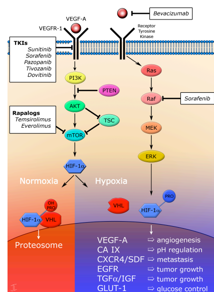
Abstract

Background: Novel inhibitors of the hypoxia pathway [VEGF, PDGF] achieve response rates of 30-57% in renal cell carcinoma (RCC); yet threshold levels of targets and downstream signaling proteins have not been identified as biomarkers to guide treatment.

Methods: To profile hypoxia proteins in RCC clinical specimens, we have developed the use of automated nanoscale immunoassays for charge-based protein separation (NIA, Nanopro1000) and charge-based protein separation (Simple Western, Sally). To decrease the amount of tissue and invasive procedures required to obtain cells for analysis, we optimized assays to profile specimens acquired by fine needle aspiration (FNA).

Results: We used Simple Western to quantify proteins of the MAPK (ERK1, ERK2, pERK1, pERK2, MEK2), PI3K (S6, GSK3b, AKT2, pan-AKT) and STAT pathways (p-STAT5) and loading controls (tubulin, HSP-70) in more than 200 FNA's from solid tumors including RCC. Profiles can be completed overnight after receiving the specimen. Unique to NIA, we also analyzed percent phosphorylation and resolved differences in even a single phosphorylation in FNA specimens, allowing us to group tumors based upon different patterns of phosphorylation and percent phosphorylation.

Conclusions: Rapid and quantitative nanoproteomic profiling in very small amounts of clinical specimen is enabling translational studies for novel diagnostic and predictive biomarkers.



Methods

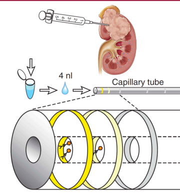


Figure 1. Automated capillary-based system. Lysate in 96-well plate undergoes either size-based separation (Simple Western) or charge-based isoelectric focusing (NIA) in capillary. Protein is fixed to capillary wall, and detected using protein-specific primary antibody. HRP-conjugated secondary gives chemiluminescent signal.

Loading nanogram amounts of total protein gives linear quantification

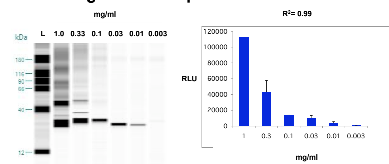


Figure 2. PRDX6 protein was measured in serial dilutions of Hela lysate. Final concentration per well is indicated, in 5 microliters total volume per well. Similar results were obtained for ERK , pERK, and AKT (simple western).

Protein modifications can be quantified in 100 cells

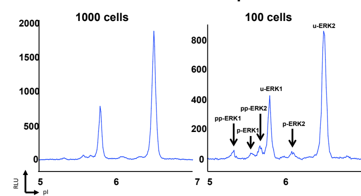


Figure 3. ERK Analysis: pellets of 1000 or 100 cells (RCC cell line). Using a single antibody that recognizes total ERK1/2, simultaneous quantification of mono-phospho- (p), dual-phospho- (pp) and unphosphorylated (u) forms of ERK1 and ERK2 can be resolved using isoelectric separation (NIA).

Results

FNA's are stable on ice for 60 min

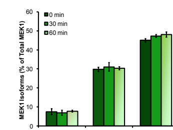


Figure 4. FNA's were transported on ice for 0, 30, or 60 minutes prior to banking (after transport, specimens were snap frozen and stored at -80F until analysis).

20 FNA's were profiled for MAPK/ PI3K/STAT pathways in two overnight runs

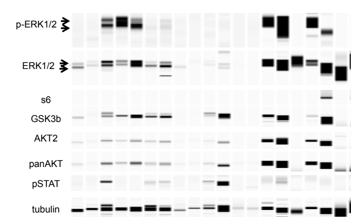


Figure 5. 10 FNA RCC specimens are analyzed per overnight run, requiring 1 hour set-up time and 14 hours unattended instrument time per run.

Baseline ERK1 expression varies widely across tumors Comparison of 200 tumor specimens (log_{10} scale)

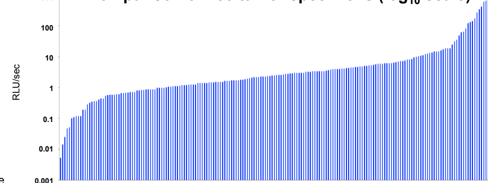


Figure 6. 200 tumor lesions and adjacent tissues were quantified for ERK1 (normalized to tubulin) with size-based separation.

Results

Phosphorylated ERK Distinguishes Tumor From Non-tumor Tissue

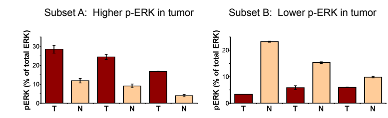


Figure 7. NIA measurements of ERK in paired tumor (T) and adjacent non-tumor (N) specimens from individual patients. NIA analysis reveals higher percent phosphorylation of ERK in a subset of patient tumors compared with adjacent non-tumor samples, whereas a distinct subset has lower ERK phosphorylation in tumor. 3 representative patients in each subset are shown.

Summary

We have developed NIA and Simple Western to profile signaling proteins in a wide range of hematologic and solid tumors, now including FNA's in RCC.

We can profile proteins with high sensitivity (starting with only 100 cells) and resolution (distinguishing single phosphorylations from un-phosphorylated forms of proteins) overnight.

The set-point for hypoxia signaling varies widely in individual patient tumors; paired analysis of patient tumor vs. adjacent non-tumor tissue may lead to better predictive biomarkers.

We are currently developing additional diagnostic assays for RCC-specific proteins and to analyze serial FNA's during treatment.

Acknowledgements

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