Elevations of intracellular kinases AKT1, GSK3β, JNK2 and ERK2 in primary breast cancer tissue quantified with a novel multi-panel of single molecule immunoassays.

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ABSTRACT

The MAPK and PI3K signaling cascades are critical pathways in malignant transformation, tumor progression and multi-drug resistance (MDR) of human cancers. Clinical investigations into the role of phosphorylation of specific kinases are hindered by the lack of ultra-sensitive assay technology with requisite isoform- and phophorylation-state specificity. Improved assays are necessary to determine clinical relevance and potential diagnostic utility with a high degree of specificity. We tested adjacent normal and primary breast cancer tissue lysates with a novel panel of eight immunoassays for quantifying the total and phosphorylated forms of AKT1, GSK3β, JNK2 and ERK2. The ultra-sensitive assay panel was developed for use with the Erenna® Immunoassay System (Singulex), which uses single molecule detection technology. Analytical sensitivity, inter-assay precision, linearity and specificity were determined and compared to corresponding ELISA assays. The novel assay panel was validated in HeLa and Jurkat cell lysates, region of linearity was determined, and phospho-specificity of each assay was confirmed. All assays were validated for use in human plasma, cell culture lysates, and human tissue specimens. Results in matched breast cancer tissue lysates showed that all total kinase concentrations (tAKT1, tGSK3β, tERK2, and tJNK2) were found to be elevated in primary tumors compared to surrounding adjacent normal tissue, however concentrations of phosphorylated forms (pAKT1, pGSK3β, pERK2, and pJNK2) were not. All eight novel assays displayed exceptional linearity (R^2=0.99) and precision (%CV 4.1 - 9.4), and were significantly more sensitive than the corresponding ELISA assay method based upon sample endpoint dilutions. The detection limits of the tAKT1, tGSK3β, pGSK3β, pERK2, tJNK2 and pJNK2 assays were all ≤0.3 pg/mL, while the detection limit of tERK2 was < 2pg/mL. The LLoQ of each assay in the panel was sufficient for quantification from a minimum of approximately 100-200 cells. We show that significant elevations in total kinase concentrations of tAKT1, tGSK3β, tERK2 and tJNK2 can be quantified in primary tumors compared to surrounding adjacent tissue using a panel of isoform- and phosphorylation-specific single molecule immunoassays. We have further demonstrated that assays in this panel can quantify analytes from as little as 100 cells, and exhibit superior analytical performance over ELISA based methods. Thus, these novel assays greatly improve upon pre-existing ELISA technology that is limited by assay sensitivity, providing a new tool for clinical investigations of the MAPK and PI3K signaling cascades.

Introduction

The MAPK and PI3K signaling cascades (**Figure 1**) are critical pathways in malignant transformation, tumor progression and multi-drug resistance (MDR) of human cancers. Receptor tyrosine kinase (RTK) inhibitors, for example, have shown high efficacy as therapeutic drugs. However, many RTK-inhibitors currently at market target multiple receptors, like Sunitinib which targets class III RTKs, and hence elicit downstream secondary effects which often lead to adverse events.

In an effort to minimize such side effects, recent studies focus on investigational drugs that target specific receptors or downstream effectors of one of these signal transduction pathways. However, elucidation of the relative contributions of distinct kinases is made difficult due to many factors, including: prevalence of multiple isoforms, post-translational modifications, synergistic activation and pathway cross-talk.

Clinical investigations into the role of phosphorylation of specific kinases are further hindered by the lack of ultra-sensitive assay technology with requisite isoform- and phophorylation-state specificity. Improved assays are necessary to determine clinical relevance and potential diagnostic utility with a high degree of specificity.

Thus, we have developed a panel of novel phospho- and isoform-specific kinase assays utilizing the Erenna Immunoassay System (**Figure 2**), which utilizes a proprietary single molecule detection system to improve sensitivity compared to traditional ELISA-based methods.

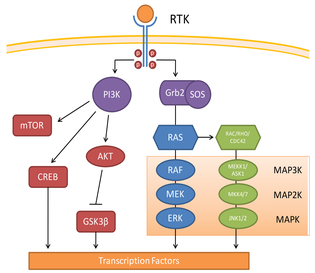

Figure 1. Overview of the PI3-Kinase (PI3K) and Mitogen Activated Kinase (MAPK) signal transduction pathways.

Methods

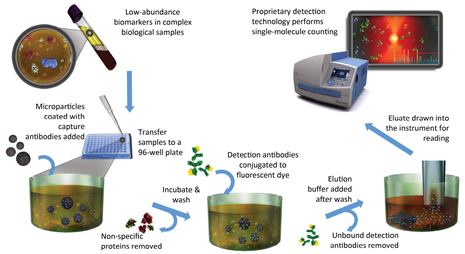

Figure 2. The ultrasensitive Erenna Immunoassay System incorporates a robust digital molecule-counting module together with proprietary background reduction methodology to produce a large dynamic range of 4+ logs, enabling accurate quantification of oncology markers in precious biological samples.

Assay Development: Total- and phospho- immunoassays for AKT1, JNK2, ERK2, and GSK3β were developed. Analytical performance of the developed assays is shown in **Table 1**, with comparisons to analogous ELISA assays from R&D Systems shown in **Figure 3**. Phospho-specificity was tested in cell lysates (**Figure 4**). Jurkat cells were treated with selective inhibitors of the MAPK (LY294002) pathway, or with broad activators of intracellular phosphorylation (Anisomycin or Calyculin Pervanadate). Resulting assays were tested for use with HeLa, Jurkat and NIH/3T3 cells (data not shown).

Clinical Tissue Specimen Testing: Pre-lysed breast cancer tumor biopsy specimens were obtained representing four individuals, with total protein concentrations pre-qualified by the manufacturer (Capital Biosciences, Rockville, MD). Biopsy consisted of three matched tissue types: (1) normal, (2) surrounding primary tumor tissue, and (3) metastatic tumor tissue. Tissue lysates were tested with each assay over several serial dilutions, and the measured value was determined as the mean from a minimum of three sequential linear points each within ±20% linearity from point to point calculations.

Analytical Validation

Table 1. Analytical validation of the Singulex Erenna Kinase immunoassay panel.

Performance Parameter	pAKT1	tAKT1	pJNK2	tJNK2	pGSK3β	tGSK3β	pERK2	tERK2
Sensitivity (LoD10%, pg/mL)	0.08	0.30	0.02	0.2	0.3	0.10	0.15	1.61
LLoQ (pg/mL)	0.40	2.0	0.25	1.0	2.1	0.8	1.1	4.2
Assay Linearity (R^2)	0.9996	0.9999	0.9975	0.9994	0.9995	0.999	0.9993	0.9999
Analytical Precision (Mean CV%)	4.1	7	6.6	6.4	8.8	6.1	9.4	8.8
Inter-assay Precision in HeLa & Jurkat Lysates (%CV Range)	14-15	2-3	9-18	7-15	14-18	11-15	12-14	13-17
Intra-assay Precision in HeLa & Jurkat Lysates (%CV Range)	3-23	3-17	5-20	2-15	1-22	2-6	4-20	10-26
Phospho-Specificity	YES	N/A	YES	N/A	YES	N/A	YES	N/A

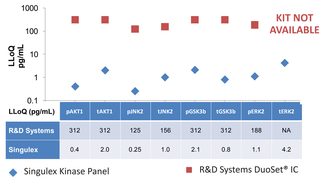

Figure 3. The Singulex Erenna Kinase immunoassay panel offers superior Lower Limit of Quantification (LLoQ) over commercially available ELISA assays from R&D Systems. Performance of R&D Systems' DuoSet kits is defined by the lowest dilution point on the published assay standard curve.

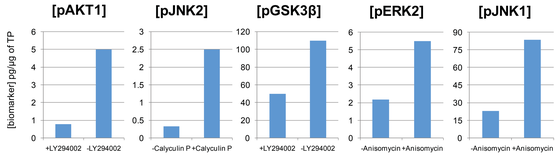

Figure 4. Phospho-specificity of the Singulex phospho-kinase assays tested in Jurkat cells treated with selective inhibitors of the MAPK (LY294002) pathway, or with broad activators of intracellular phosphorylation (Anisomycin or Calyculin Pervanadate).

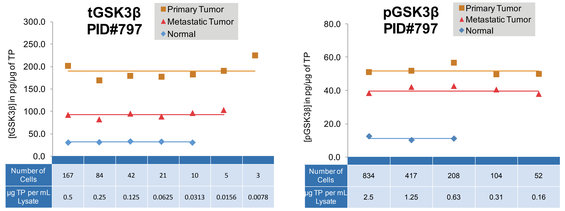

Figure 5. Representative dilutional linearity of clinical tumor tissue lysate specimens tested with the Singulex total- and phospho-GSK3β assays. Horizontal lines represent mean biomarker concentrations from the linear region of matched normal (◆), primary (■), and metastatic (▲) breast cancer tissue lysates.

Results

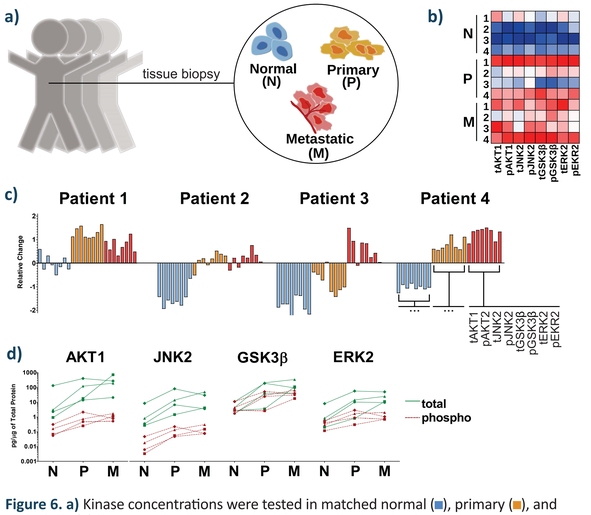

Figure 6. a) Kinase concentrations were tested in matched normal (■), primary (■), and metastatic (■) tissue lysates from four individual patients using the Singulex Kinase Assay Panel. **b)** Heat map representation of eight kinase concentrations across three matched tissue types and **c)** differences observed within and between individual patients are shown. Compared to normal tissue samples, both primary and metastatic breast cancer tissue lysates showed an elevation in kinase concentrations. **d)** Comparison of total- (——) and phospho- (- - -) kinase concentrations measured from normal (N), primary (P) and metastatic (M) breast cancer tissue types observed between individual patients.

CONCLUSION:

- Single molecule counting enables superior sensitivity, enabling development of ultrasensitive, isoform-specific immunoassays that can quantify analytes from a diluted clinical tissue lysate specimen representing as few as 3-10 cells (tGSK3β assay).
- By testing matched specimens from a single individual with the Singulex Kinase Assay Panel, we show that elevations in kinase concentrations can be quantified in primary and metastatic tumors compared to surrounding adjacent normal tissue.
- Results from clinical breast cancer specimens containing metastatic, primary tumor and surrounding normal tissue show that differences between primary and metastatic tissue compared to matched normal tissue may vary between individuals.
- Thus, these novel assays greatly improve upon pre-existing ELISA technology that is limited by assay sensitivity, providing a new tool for clinical investigations of the MAPK and PI3K signaling cascades.

ACKNOWLEDGEMENT: This work is funded by the National Cancer Institute, Contract No. HHSN261200700047C; NCI Control No. N43-CM-2007-0047C; PI: Dr. John Todd

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