Mapping the Activity of Oncogenic Signaling Networks with Phospho-Specific Liquid Tissue®-SRM Mass Spectrometry

Todd Hembrough[1], Sheeno Thyparambil[1], Wei-Li Liao[1], David Krizman[1], Marlene Darfler[1], Kathleen Bengali[1], Joe Abdo[1], Mike Moran[2], Ming Tsao[2], Jon Burrows[1].
[1]Expression Pathology Inc, Rockville MD, USA, [2]University of Toronto, MaRS Centre, Toronto, ON, Canada

Introduction

- Many targeted oncology therapies block the activity of specific tyrosine kinases (e.g. EGFR, Akt, PI3K, etc).
- Standard diagnostic tests measure only expression levels but cannot assess the activation state of these kinase targets.
- We have developed a Liquid Tissue®-SRM technology platform which enables relative and absolute quantification of the phosphorylation status of multiple oncogenic proteins directly in formalin-fixed paraffin-embedded tissue.
- This multiplexed assay format has been validated preclinically on 10 human NSCLC xenograft explants and mapped the activity of the EGFR signaling pathway.
- The assay is currently undergoing clinical validation on 32 FFPE NSCLC tumors from gefitinib treated patients.

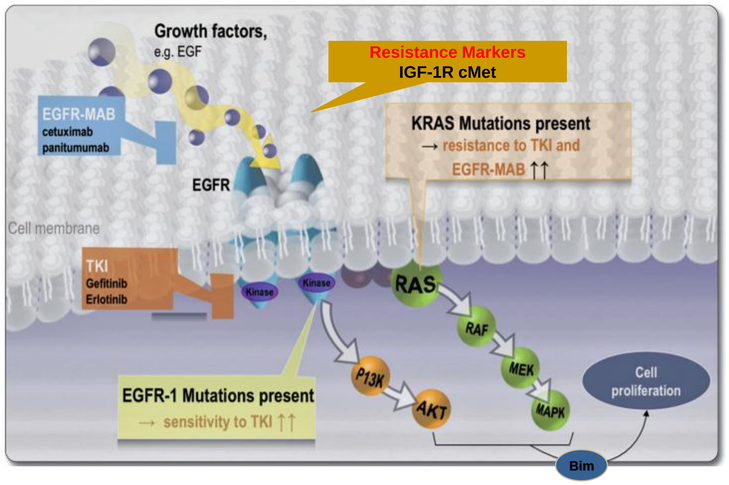
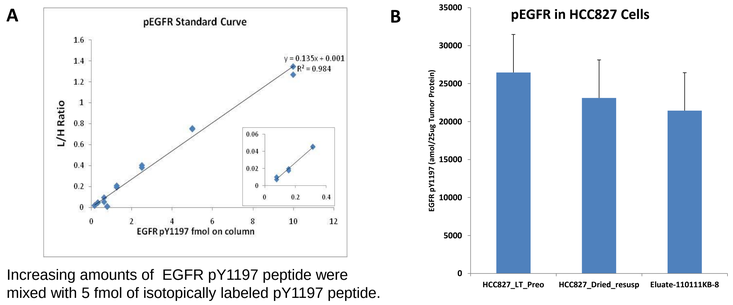


Figure 1. Potential biomarkers of EGFR inhibitor activity in the EGFR signaling pathway.

Development of a Quantitative pEGFR SRM in FFPE Human Tumor Tissue

An assay was developed using a specific EGFR phospho-peptide. A synthetic version of this peptide was used to generate a standard curve. The assay was then used to measure pEGFR in a number of different cancer samples and xenografts. The same approach was used for all the other analytes. These were then combined into a multiplexed assay.



Increasing amounts of EGFR pY1197 peptide were mixed with 5 fmol of isotopically labeled pY1197 peptide.

LOQ<160 amol, LOD=80 amol

Figure 2. Absolute SRM quantitation of Liquid Tissue processed Formalin Fixed HCC827 Cells. **A.** pEGFR heavy:light peptide standard curve. **B.** Quantitation of EGFR p1197 expression phospho-enriched from 50 µg HCC827 tumor cell protein. The HCC827 express a mutated EGFR which is constitutively active.

Time course analysis of EGFR signaling pathway activation in formalin fixed A431 Cells by SRM

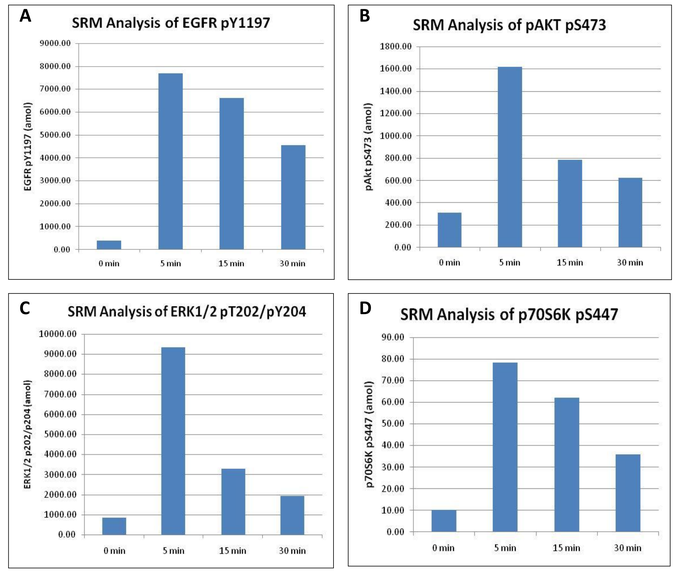


Figure 3. Time Course Analysis of EGF Signaling in Formalin Fixed Cells. Cells were incubated for increasing times with 50 ng/ml EGF after which cells were washed, fixed with formalin and subjected to Liquid Tissue processing. 100 µg of cellular protein was phosphoenriched with TiO_2. SRM analysis of 1/3 of bound material was performed to quantitate EGFR pY1197 (**A**), Akt pS473 (**B**) , ERK pY204 (**C**) and p70S6K pS447 (**D**)

Dose response analysis of EGFR signaling pathway activation in formalin fixed A431 Cells by SRM

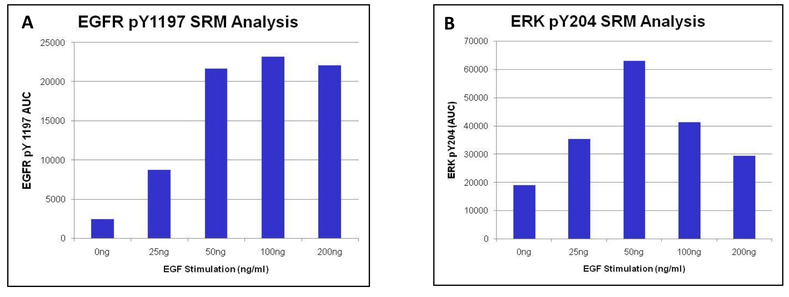


Figure 4. Dose response of EGF signaling in formalin fixed A431 cells. Cells were incubated for 5 minutes with increasing concentrations of EGF after which cells were washed, fixed with formalin and subjected to Liquid Tissue processing. 100 µg of cellular protein was phosphoenriched with TiO_2. SRM analysis of 1/3 of bound material was performed to quantitate EGFR pY1197 (**A**) and ERK pY204 (**B**)

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Phospho-enrichment Optimization

To be of practical use in the clinic, it is essential to minimize the amount of tissue required for analysis. Current pathological techniques, including fine needle aspiration, needle biopsies and core biopsies recover very little tumor for analysis. For this reason, we optimized phospho-enrichment so we can reproducibly quantitate phospho-peptide expression from 25 µg of tumor protein, an amount which can be recovered from a single core tumor biopsy.

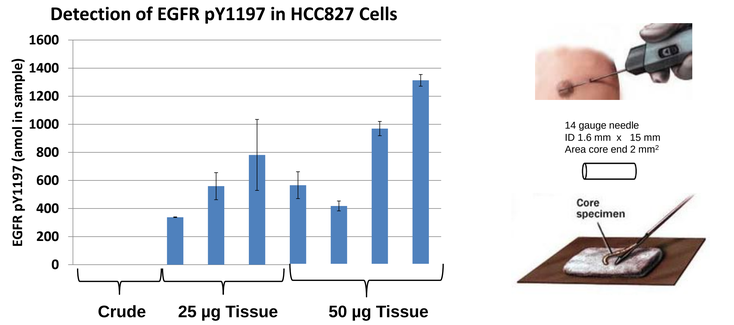

Figure 5. 25 or 50 µg of formalin fixed HCC827 cell protein was Liquid tissue processed, and subjected to optimization of the multiple different experimental parameters. Using the best conditions, we were able to reproducibly quantitate multiple phosphopeptides from 1/3 of a 25 µg tissue sample.

Quantitation of EGFR pY1197 in FFPE NSCLC Tumor Explant Tissue

Ten human NSCLC tumors grown as xenografts. Approximately 50 µg of microdissected tumor tissue was Liquid Tissue processed, and subjected to phospho-enrichment and then SRM analysis of ~10 phosphopeptide targets.

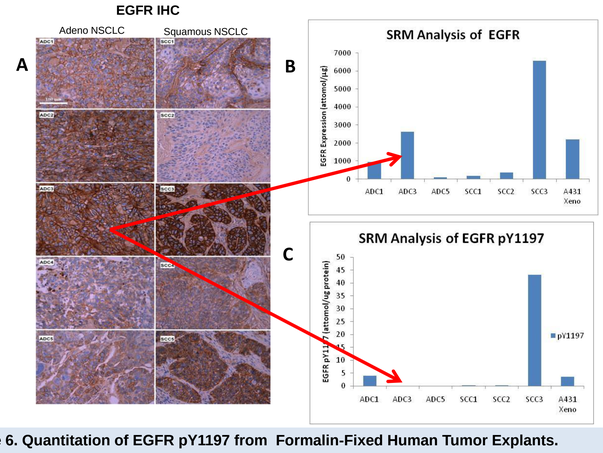

Figure 6. Quantitation of EGFR pY1197 from Formalin-Fixed Human Tumor Explants.

A. Immunohistochemical characterization of EGFR in ten NSCLC explants, five adenocarinoma (ADC1-5) and five squamous carcinoma (SCC1-5). **B.** Quantitation of EGFR pY1197 levels from selected adeno and squamous carcinoma tumors in A. Approximately 50 µg of FFPE tumor protein was used for phosphoenrichment (10 µg for SCC3), and 1/3 of the bound material was subjected to mass spectrometry. The red arrows show one tumor ADC3 where there is a significant discrepancy between total EGFR and phospho-EGFR expression levels.

Conclusion

- The phosphorylation state of multiple components of the EGF receptor signaling pathway were quantitated in formalin-fixed paraffin-embedded (FFPE) tissue sections using a combination of laser microdissection, Liquid Tissue processing, and selected reaction monitoring mass spectrometry.
- The results provide proof of concept for a robust approach to monitor the EGFR signaling pathway and its activation through phosphorylation in FFPE tissues, assuming adequate fixation of the tissues and therefore accurate preservation of phospho-epitopes.
- Optimization of phospho-enrichment methods should enable us to quantitate phospho-pathway activity in very small sources of tumor tissue, such as core biopsies, where immediate fixation of these tissues is possible, and the impact of pre-analytical variables will be minimized.