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Abstract

Introduction: Dysregulated activation of the phosphoinositide 3-kinase (PI3K) pathway is responsible for both tumorigenesis and tumor maintenance in a wide range of human cancers. A growing number of therapeutics target the PI3K pathway, ranging from drugs that prevent receptor dimerization to those acting on various downstream targets to interfere with signal transduction (e.g. PI3K, mTOR, AKT). Relying on PI3K pathway activation in patient tumor samples and correctly identifying the primary causes for PI3K pathway dysregulation will be essential in guiding treatment with targeted therapies.

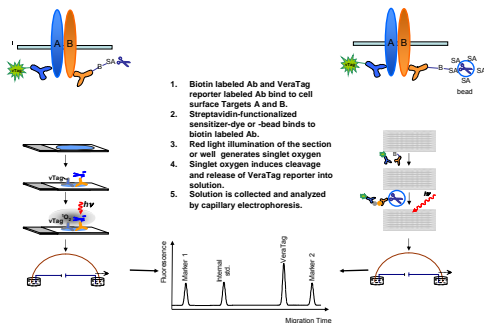
Methods: We developed a suite of assays targeting PI3K pathway proteins using a novel technology that quantitatively and precisely measures proteins, protein complexes, and protein activation in formalin-fixed, paraffin-embedded (FFPE) tissue samples. The technology uses fluorescently tagged antibodies targeted against proteins or phosphoproteins of interest, and can measure complexes of proteins in close association via distance-dependent cleavage of the tags in singlet oxygen-mediated reactions. The panel of activation assays comprises measurements of HER2-3 heterodimers, HER3 phosphorylation ("pHER3" via pan-Tyr), HER3-PI3K complexes and Akt phosphorylation ("pAkt" via Ser473).

Results: PI3K pathway activation was demonstrated and characterized in a series of FFPE breast cancer cell line controls (including low and high HER2 expressing cells) cultured under conditions of both serum starvation and heregulin stimulation. In addition to the expected ligand-induced activation of the PI3K pathway observed in all cells, our results demonstrated a significant level of basal HER2-3 heterodimer formation in high HER2 cell lines, which was associated with HER3 phosphorylation, HER3-PI3K complex formation, and phosphorylation of AKT. These data were confirmed using Co-IP Westerns. We then used our PI3K activation assays to profile a series of 45 breast tumors. In a subset of 18 HER2-positive breast cancers, the most significant linear correlations in expression of activation-related analytes were: HER2 expression with HER2-3 heterodimer (r=0.89), HER3-PI3K with pHER3 (r=0.71) and HER3-PI3K with pAkt (r=0.6). We present these results in the context of whether samples harbored activating mutations in the PI3K-CA gene (i.e., helical or kinase domains of the p110a subunit: E542K/E545K and H1047R, respectively).

Methods- VeraTag assays

VeraTag FFPE assay

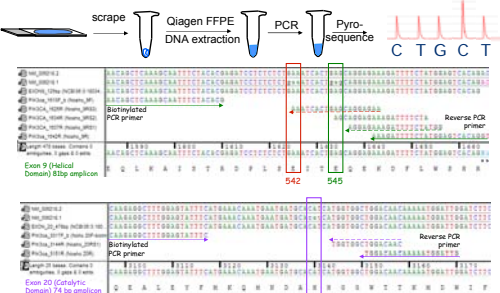
VeraTag lysate assay



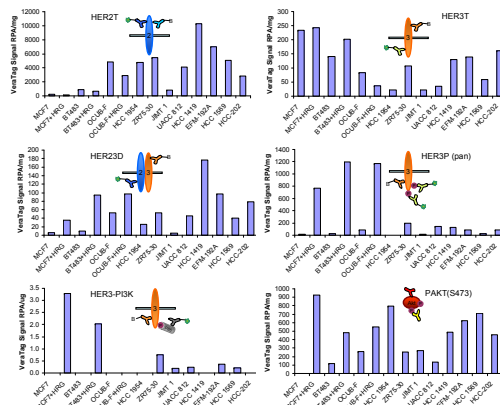
1. Biotin labeled Ab and VeraTag reporter labeled Ab bind to cell surface Targets A and B.
2. Streptavidin-functionalized sensitizer-dye or -bead binds to biotin labeled Ab.
3. Red light illumination of the section or well generates singlet oxygen. Singlet oxygen induces cleavage and release of VeraTag reporter into solution.
4. Solution is collected and analyzed by capillary electrophoresis.

Methods - PIK3CA mutations

Pyrosequencing for PIK3CA E542K, E545K and H1047R (ref: Noshro et al, Neoplasia, 10(6), 534 (2008))



PI3K pathway activation in cell lines



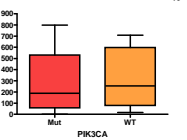
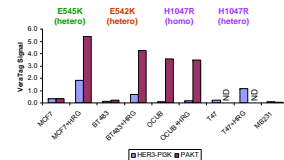
- Cell lines show ligand dependent and independent activation of HER3-PI3K pathway
- Levels of HER2-3 track strongly with levels of HER2
- Ligand independent activation of HER3 is also associated with downstream signaling
- OCUB-F shows high HER3P and PAKT but near undetectable levels of HER3-PI3K complex

PI3K pathway activation in cell lines (cont.)

PIK3CA Mutation	E545K	E542K	H1047R	H1047R	WT	WT	WT	WT	WT	WT	E545K
Cell Line	MCF7	BT483	OCUB-F	HCC1954	ZR-75-30	JMT-1	UAC182.1	HCC1419	EFM-192A	HCC1569	HCC202

- HER3-PI3K levels seem independent of PIK3CA mutation status

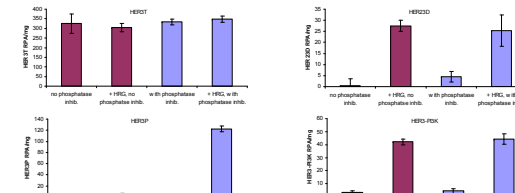
FFPE HER3-PI3K and PAKT assays:



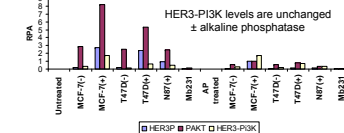
- VeraTag FFPE data shows good correlation with VeraTag lysate assays and Co-IP Western

Stability of HER3-PI3K Measurement

Stability of HER3P and HER3-PI3K complex in cell line lysates in the absence/presence of phosphatase inhibitor:



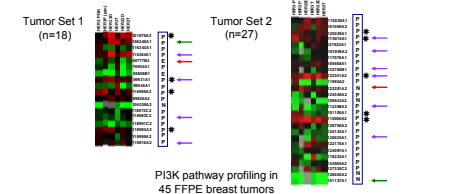
Measurement of HER3P, PAKT and HER3-PI3K in FFPE sections after treatment with phosphatase:



- Experimentally HER3-PI3K complex is a more stable measurement than HER3P

PI3K pathway profiling in FFPE tumors

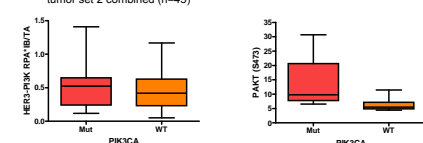
Below median Above median Not determined Activated H3P/H3-PI3K
 E542K E545K H1047R
 P=HER2 positive by HERmark assay
 E=HER2 equivocal by HERmark assay
 N=HER2 negative by HERmark assay



Mutation frequency:

Data Set	Tumors	Total mutants (% of total)	E542K	E545K	H1047R
Tumor Set 1	18	6 (33%)	1	1	4
Tumor Set 2	27	8 (30%)	1	1	6

HER3-PI3K signal in tumor set 1 and tumor set 2 combined (n=45)



- PIK3CA mutation status does not effect HER3-PI3K VeraTag signal

- AKT (S473) phosphorylation measured in matched tumor tissue lysate by VeraTag lysate assay (lysate data not shown)

Summary

- VeraTag assays were developed to monitor HER3 pathway activation in tumor cell lysates and FFPE tumor sections.
- The use of HER2-3 heterodimer as an indicator of pathway activation is somewhat compromised by the existence of less-active, ligand independent complexes.
- In contrast, HER3-PI3K measurements better correlate with HER3 phosphorylation and the HER3-PI3K complex is more resistant to the action of phosphatases than the phosphorylated receptor.
- Mutations in PIK3CA do not appear to correlate with measurable changes in levels of the HER3-PI3K complex.
- PAKT was higher in tumors with PIK3CA mutation versus wild type.