

Abstract

Background: Ligand-bound and phosphorylated ErbB/HER heterodimers are the proposed potent signaling forms of this receptor family, and quantitative measurements of these active receptors may be predictive of response to targeted drugs. Using VeraTag™ technology, we developed and characterized quantitative assays in FFPE tumor cell lines measuring EGF-dependent increases in activated HER receptors. We utilized these assays to determine the prevalence and distribution of activated HER1, HER2, and HER1-HER2 heterodimer receptors in breast and head/neck FFPE tumors.

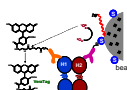
Materials and Methods: Assays for activated HER1 and HER2 receptors in FFPE and cell lysate formats were developed using VeraTag technology, which requires the proximity of an antibody pair for light-dependent release of a fluorescently labeled tag, followed by capillary electrophoresis-based quantitation.

Results: We identified a panel of cell lines that differentially expressed a range of EGF-dependent HER1-HER2 heterodimers using the lysate format of the VeraTag proximity assay. This cell line panel was used to develop FFPE assays that measure a >10-fold range of EGF-dependent HER1-HER2 heterodimer signal. An activated HER2 VeraTag FFPE assay and two activated HER1 VeraTag FFPE assays were also developed, all of which detect up to 100-fold ranges of EGF-dependent phosphotyrosine signal in cell lines. All VeraTag FFPE assays were cross-validated by co-immunoprecipitation. These assays, in addition to our HER1 and HER2 (HERmark™) assays, were implemented to profile 43 HER2-positive FFPE breast tumors. 63% of the samples displayed phosphorylated HER2 at levels that were significantly greater than the corresponding isotype control, across a >20-fold dynamic range of signal. These results were cross-validated by co-immunoprecipitation and western blotting in matched fresh-frozen samples. HER1-HER2 heterodimers were detected in ~10% of HER2-positive tumors, and these also expressed HER1. Additionally, we surveyed 29 head and neck tumors, most of which expressed HER1. Phosphorylated HER1 and HER1-HER2 heterodimers were detected in a subset of the HER1-positive tumors. Further, these VeraTag lysate and FFPE assays were used to characterize the effects of 2C4, erlotinib, and lapatinib on basal and ligand-dependent dimerization of HER1 and HER2 receptors in cell lines.

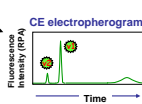
Conclusions: Quantitative VeraTag FFPE assays for HER1, HER2, phosphorylated HER1, phosphorylated HER2 and HER1-HER2 heterodimers were used to profile breast and head/neck tumors. Activated receptors were detected in a subset of tumors that expressed HER1 or HER2, supporting the hypothesis that FFPE assays measuring HER phosphorylation and dimerization status may have utility in patient selection for HER targeted therapies.

Methods

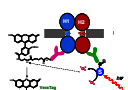
VeraTag lysate assay



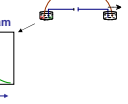
- Ab-biotin and Ab-VeraTag reporter bind to analytes.
- Streptavidin-functionalized sensitizer dye binds to Ab-biotin.
- 670 nm light causes singlet oxygen release.
- Singlet oxygen induces cleavage and release of VeraTag reporter into solution.
- Solution is collected and analyzed by capillary electrophoresis.



VeraTag FFPE assay



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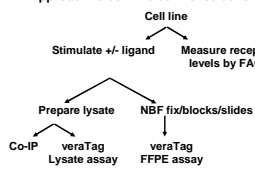


Results: VeraTag Lysate Assays

1. Identification and characterization of HER1-HER2 control cells

Cell line controls spanning a range of HER1-HER2 heterodimer expression were selected by VeraTag lysate assays and confirmed by co-immunoprecipitation. HER1-HER2 heterodimers were induced upon stimulation with EGF, while total HER1 and HER2 protein expression levels were unchanged.

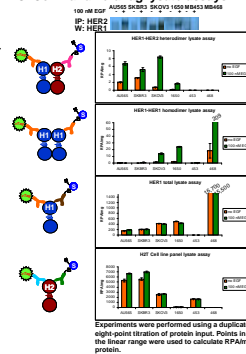
A. Approach to cell line control selection



B. Receptor levels measured by FACS

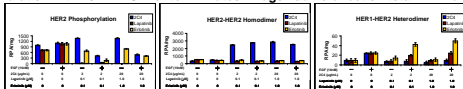
Cell line	HER1	HER2
AU565	204,560	1,447,688
SKBR3	143,599	1,402,832
SKOV3	387,771	657,000
1650	158,872	53,810
MB453	5,316	292,984
MB468	3,389,807	1,209

C. Co-IP and veraTag lysate assays

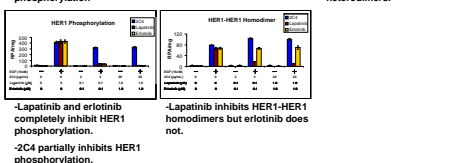


2. 2C4, lapatinib, erlotinib differentially modulate HER1 and HER2

• SKOV3 cells were serum-starved overnight then exposed 2hrs to either 2C4, lapatinib, or erlotinib at two different doses, followed by stimulation or mock-stimulation with 16 nM EGF for ten minutes. Drugs had no effect on total HER levels.



- Lapatinib completely inhibits HER2 phosphorylation.
- 2C4 significantly increases HER2-HER2 homodimer measurements.
- 2C4 inhibits EGF-dependent HER1-HER2 heterodimers.

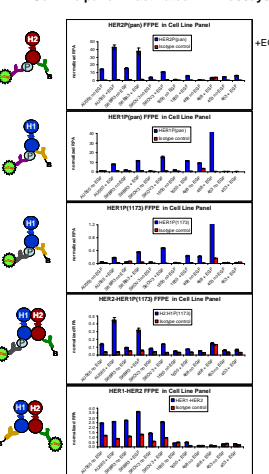


Results: VeraTag FFPE Assays

3. Activated HER1, HER2 and HER1-HER2 in FFPE cell lines

Cell line controls identified by VeraTag lysate assays were used for development of FFPE assays measuring activated HER1, HER2 and HER1-HER2 heterodimers. Assay signals in FFPE track with levels expected from lysate assays. FFPE assays were cross-validated by co-immunoprecipitation.

A. Cell line panel in activated FFPE assays

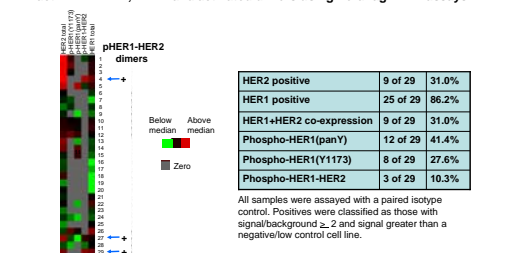


B. Cell line panel in Co-IP/Westerns



4. A subset of HER1 receptors are activated in SCCNH tumors

29 squamous cell carcinoma tumors of the head/neck were surveyed for activated HER1, HER2 and activated dimers using VeraTag FFPE assays.



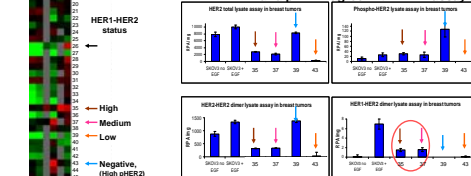
All samples were assayed with a paired isotope control. Positives were classified as those with signal/background ≥ 2 and signal greater than a negative/low/control cell line.

5. A subset of HER2 receptors are activated in breast tumors

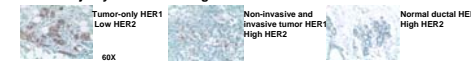
A. Distribution of analyses in the HER1-HER2 axis in 48 breast tumors that were preselected for high HER2 using VeraTag FFPE assays.

HER2 positive	43 of 48	89.6%
Phospho-HER2	27 of 48	56.3%
HER1 positive	13 of 48	27.0%
HER1+HER2	12 of 48	25.0%
HER1-HER2	3 of 48	6.3%
Phospho-HER1-HER2	14 of 48	29.2%

B. Four tumors with matched fresh-frozen blocks were lysed and analyzed by VeraTag lysate assay: HER1-HER2 and pHER2 agree with FFPE assay.



C. The majority of HER1 IHC signal localizes to tumor cells



Conclusions

- VeraTag lysate assays revealed inhibitory and stabilizing effects of 2C4, lapatinib, and erlotinib on the HER1-HER2 axis in SKOV3 cells.
 - 2C4 inhibited EGF-dependent HER1-HER2 heterodimers and phospho-HER1 and -HER2, and stabilized HER2-HER2 homodimers.
 - Lapatinib inhibited phospho-HER1 and -HER2 and HER1-HER1 homodimers, but did not affect HER1-HER2 heterodimers.
 - Erlotinib completely inhibited phospho-HER1, partially inhibited phospho-HER2, stabilized EGF-dependent HER1-HER2 heterodimers, but does not affect HER1-HER1 heterodimers.
- We profiled 48 HER2-positive FFPE breast tumors for analyses in the HER1-HER2 axis:
 - 90% of tumors were HER2-positive; 50% were positive for phospho-HER2
 - Approximately 25% of the HER2-positive tumors also expressed HER1.
 - HER1-HER2 heterodimers were detected in 15-60% of this HER1-expressing subset, depending on detection method, and agreed with 6/30 = 20% HER1 IHC in tumor cells only.
- We profiled nearly 30 FFPE head/neck tumors (SCCHN) for analyses in the HER1-HER2 axis.
 - 90% of tumors expressed HER1; 32-45% were positive for phospho-HER1, depending on detection method.
 - 17% of tumors expressed high levels of HER2; 14% expressed elevated levels.
 - HER1-HER2 heterodimers were detected in 33% of tumors that co-expressed HER1 and HER2.
- VeraTag lysate assays can be used as a tool for understanding the effects of HER-family inhibitors in the preclinical setting, while the combination of our current CLIA-validated FFPE total and HER2 homodimer assays (HERmark™) with our new VeraTag-activated FFPE assays may provide powerful tools to more accurately predict clinical response to both HER1 and HER2 targeted therapies in tumor biopsy tissue.