Profiling of Receptor Tyrosine Kinases (RTK) Activation in Circulating Tumor Cells (CTC) in Metastatic Tumors Using Proximity Mediated Microarray Immunoassay

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ABSTRACT

The abnormal activation of EGFR and HER2 has been linked to various types of cancer progression, and the changes in expression status between primary tumor and CTCs have been reported to occur at a significant frequency. Methods for detecting EGFR/HER2 phosphorylation in serially collected CTCs may provide valuable insight into the overall disease profile shift, and therefore lead to better selection of therapy combination for individual patients. A triple-antibody-enzyme-channeling multiplexed protein microarray platform was developed to detect the phosphorylation on target molecules. It utilizes a unique immuno-complex formation *via* co-localization of two detector enzyme-conjugated-antibodies once target proteins are captured on the microarray-surface. The channeling events between two detector enzymes in proximity enabled profiling of the RTKs with a single-cell level sensitivity. In order to validate the method on clinical samples, CTCs from 27 breast cancer patients with metstatic disease on various therapy regimens were analyzed. The multiplexed-proximity mediated immunoassay successfully detected the amplification and activation of RTKs in CTCs isolated from various cancer patients. CTCs with amplified and activated HER2 were found in 5 out of 17 (29%) breast cancer patients with HER2 negative primary tumors. As CTCs found in metastatic stage represent the most aggressive invading cell population, serial CTC-profiling can lead to better therapy selection/adjustment and disease monitoring.

INTRODUCTION

There have been numerous studies reporting the detection of carcinoma cells in the blood of patients with solid tumors. Detection of circulating tumor cells (CTCs) before initiation of first-line therapy in patients with metastatic breast cancer is highly predictive of progression free survival and overall survival (1-3). In breast cancer, the relationship of HER2 gene status between the primary breast cancer and distant metastasis, based on analysis of tumor tissue, has been described in a number of studies (4-10). Expression of a therapy target in primary tumor tissue may be different from expression at distant tumor sites, and that difference can develop over time. Changes in target expression could affect the efficacy of treatment, and, if reliably known in a timely fashion, could be used to guide therapy. As they are more readily obtained from patients than serial tumor biopsies, CTCs (as well as FNA samples collected from metastatic sites) may be used to monitor these changes. The multiplexed proximity based immuno assay described here is used to profile the expression and activation levels of the ErbB family receptor tyrosine kinases (RTKs) EGFR and HER2 in CTCs.

METHOD

Multiplexed Proximity Assay : The Prometheus Oncology Diagnostic assay is based on a multiplexed protein microarray platform combined with triple-antibody-enzyme channeling signal amplification process. The unique and novel design is provided by the triple-antibody enzyme approach that confers ultra high sensitivity while preserving specificity:

(1) The selected target is captured by target-specific antibodies printed in serial dilutions on a microarray surface. This format requires a co-localization of two additional detector-antibodies linked with enzymes (shown in the Fig.1).

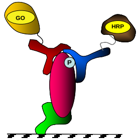


Fig. 1. Proximity Assay Format

(2) The immuno-complex formed by the initial target binding by capture antibodies and the secondary binding of Glucose Oxidase (GO) conjugated antibodies that recognize alternate epitope on the captured target molecules can produce H_2O_2 in the presence of the GO substrate, glucose.

(3) The target specific local influx of H_2O_2 is then utilized by phospho-peptide-specific antibodies conjugated with horseradish peroxides (HRP) that bind to the captured target. Specificity for the detection of phosphorylated targets is greatly increased through the requirement for simultaneous binding of three different antibodies. The detection and quantification of as few as ~2-3 x 10^4 phosphorylation events is routinely achieved by this method bringing its detection to a "single" cell level.

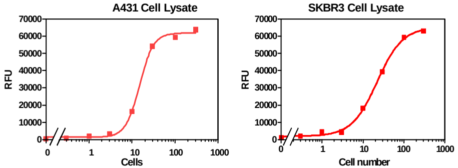
Slide Printing : A contact microarray printer (Genetix) was used to print on 16 pad nitrocellulose FAST slides (Whatman).

Frozen Tissues: Frozen breast cancer tissues were from Caucasian patients with ductal breast carcinoma at stage II or III. Collected tissue samples were lysed and stored at -80°C until the performance of the proximity assay.

CTCs: CTCs were isolated from whole blood of cancer patients by magnetic particles coated with anti-Ep-CAM antibodies using the CTC-Profiler (Veridex). Enriched CTCs were activated, lysed, and stored at -80°C until performance of the assay.

RESULTS

Sensitivity : We detected the activation and expression of EGFR and HER2 at a sensitivity level of a single cell in cell lines A431 and SKBR-3. These cell lines express ~ 1 x 10^6 total RTKs on their cell membrane per cell, although only subsets of the total RTKs get phosphorylated and such phosphorylation is required for pathway activation. Both cell lines peak at approximately 10% of their RTKs activated (~1 x 10^5 phosphorylation events per cell). Our assay format enabled us to detect less than 10^5 activation events with single cell sensitivity as shown in Fig 2.



A431 Cell Lysate

Cells	mean RFU	SD	%CV	S/N ratio
300	63,740.33	121.58	0.19	172.12
100	59,247.00	874.58	1.48	159.98
30	54,043.33	1,031.06	1.91	145.93
10	16,350.33	196.15	1.20	44.15
3	3,165.33	327.07	10.33	8.55
1	2,111.33	342.96	16.24	5.70
0.3	833.00	57.51	6.90	2.25
0	370.33	14.57	3.93	1.00

SKBR3 Cell Lysate

Cells	mean RFU	SD	%CV	S/N ratio
300	62,991.67	506.28	0.80	53.76
100	59,289.00	166.53	0.28	50.60
30	39,317.67	2,151.80	5.47	33.56
10	18,149.00	1,552.85	8.56	15.49
3	4,339.33	147.08	3.39	3.70
1	4,326.67	728.09	16.83	3.69
0.3	1,954.67	41.04	2.10	1.67
0	1,171.67	98.60	8.42	1.00

Figure 2. Assay Sensitivity

Frozen Tissue : To further demonstrate the utility of our assay format, we collected samples from 29 stage II to III frozen breast ductal carcinoma (14 with known HER2-IHC status). The expression and activation of HER2 receptor detected by our assay is concordant with the tumor IHC score (figure 3). We have 4 patients with a high IHC score (3+) in primary tumors, and all 4 have high expression and high activation of HER2. Interestingly, 20% of ER positive patients with HER2 expression (+1 IHC) have fair amount of activated HER2. This could have implications in patients resistant to hormonal therapy.

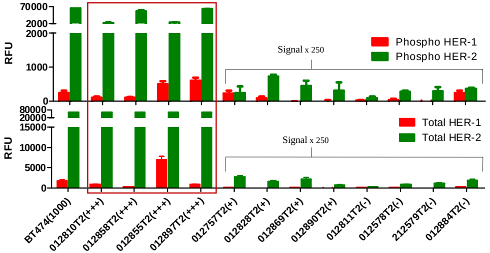


Figure 3. Correlation of IHC and Proximity Assay Results with FNA from Frozen Tissue.

CTC: CTCs were isolated from blood of patients with metastatic cancer. CTCs from whole blood of metastatic cancer patients and 60 healthy volunteers were analyzed for EGFR and HER2 expression and activation. The number of breast cancer patients with negative HER2 expression in the primary tumor and conversion to HER2 positive in CTCs is shown below.

HER2 Negative Primary Tumor (IHC)

Analysis of CTC following Recurrence (N = 17)

HER2 Positive CTCs (N=9)	
P-HER2+ (Activated)	9 (52%)
T-HER2+ (Amplified)	5 (29%)
P/T-HER2+ (Amplified and Activated)	5 (29%)

HER2 Negative CTCs (N=8)	
P-HER2 – (Not-Activated)	8 (48%)

Figure 4. Analysis of CTCs from Patients with Metastatic Breast Cancer.

Limits of Detection: Based on testing of 60 healthy controls, lower limits of detection (LOD) and lower limits of quantitation (LLOQ) were determined for total and phosphorylated HER1 and HER2. Data are reported in computed units (CU) based on calculations from standard curves generated with cell lines expressing known levels of HER1 or HER2 and degree of phosphorylation upon stimulation.

	LOD	LLOQ
EGFR-P	0.69	3.20
HER2-P	0.25	0.51
EGFR-T	53.06	80.0
HER2-T	1.75	3.20

Table I. Limits of Detection

SUMMARY

- A novel technology with unparalleled sensitivity and specificity successfully detected the activation of ErbB RTKs in CTCs isolated from cancer patients.
- Activated EGFR and HER2 were detected with single cell sensitivity.
- Testing of samples from frozen breast cancer tissues showed concordance between reported HER2 status (IHC) and Proximity Assay results.
- Analysis of CTCs from metastatic breast cancer patients showed that
  - 52% of patients with HER2 negative primary tumors had CTCs with activated HER2
  - 29% of patients with HER2 negative primary tumors had CTCs with activated and amplified HER2
- The expression/activation profiling of kinases and other signal transduction pathway molecules on a serial sampling of CTCs will provide valuable information on changes occurring in tumor cells as a function of time and therapies. This method can provide guidance, not only for initial selection of targeted therapeutics, but also in subsequent monitoring for rapidly 'evolving' cancer signatures in each patient.

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