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Clinical Utility of Target Selector™ Circulating Tumor Cell (CTC) Testing in Tumor Marker Gene Amplification and Protein Expression in Metastatic Breast Cancer Management

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Introduction

For breast cancer patients, molecular tumor characterization (including protein expression levels and genetic alterations or amplifications) identifies features that predict drug responsiveness. This information can be used to design personalized therapeutic strategies. Approximately 75% of breast tumors express hormonal receptors for Estrogen (ER) and/or Progesterone (PR) [1,2]; these patients typically respond well to endocrine therapy with or without CD4/6 kinase inhibitors [3]. Another major tumor growth driver of breast cancer is HER2 gene amplification, which is seen in ~20% of breast cancers. These patients can be treated successfully with monoclonal antibodies that block HER2 function [4,5]. Despite benefits to patient survival, molecular data is often difficult to obtain in metastatic settings when patients' health or refusals preclude biopsy, or the tumor metastasizes to a difficult-to-sample region of the body. Additionally, tissue molecular analyses may be inconclusive due to insufficient tissue amounts from biopsies or interference of bone tissue decalcification procedures with immuno-histochemical (IHC) stains. Without understanding the molecular drivers of patients' disease, treatment regimens based on tumor characteristics can neither be appropriately prescribed nor adjusted to tackle evolving properties of progressive disease.

The recent development of "liquid biopsy" technologies that analyze circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) allows physicians to obtain molecular data for guiding individual patient's treatment approaches [6]. CTCs are cells that have shed into the bloodstream from a primary or metastatic tumor, representing an alternative source of tumor material for non-invasive disease assessment [7,8]. Importantly, liquid biopsies provide a systemic representation of existing tumor clones, giving insight into tumor heterogeneity, emergence of new drivers, and the divergence between primary and metastatic tumors [6,9].

Here we describe a patient with recurrent breast cancer, who at one point declined an additional bone biopsy. Biocept's Target Selector™ liquid biopsy [10] (Figure 1) revealed ER expression and HER2 gene amplification in CTCs (Figure 2). Based on these data, the patient was able to receive anti-HER2 therapy earlier, providing a clinical example of the utility of liquid biopsy testing to gather molecular data that was unsuccessful by standard image-guided biopsy. In this case, CTC results of newly found HER2 amplification were paramount towards altering treatment strategies and inclusion of HER2 targeted therapies, which ultimately extended patient survival and quality of life.

Case Presentation

The patient is a 51 year-old female, first diagnosed with stage 2 (T2N1M0) invasive ductal carcinoma in April 2003 at age 37. The primary tumor was strongly ER and PR positive by IHC staining, and the HER2 fluorescence in situ hybridization (FISH) analysis was negative. Subsequently, she completed 4 cycles of dose-dense doxorubicin/cyclophosphamide (AC) and 4 cycles of paclitaxel, followed by luprolide/exemestane.

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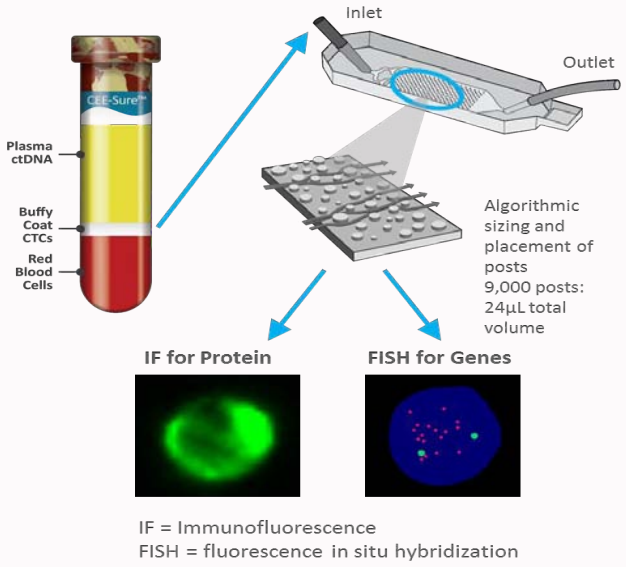

Figure 1: Target Selector™ CTC analysis work flow.
Whole blood is collected into proprietary CEE-Sure Blood Collection Tubes (Biocept, Inc.) Circulating tumor cells (CTCs) are enriched and isolated in Biocept's microfluidic channel for subsequent enumeration, protein expression analysis by immunofluorescence, and analysis for gene amplifications or translocations by fluorescence *in situ* hybridization (FISH).

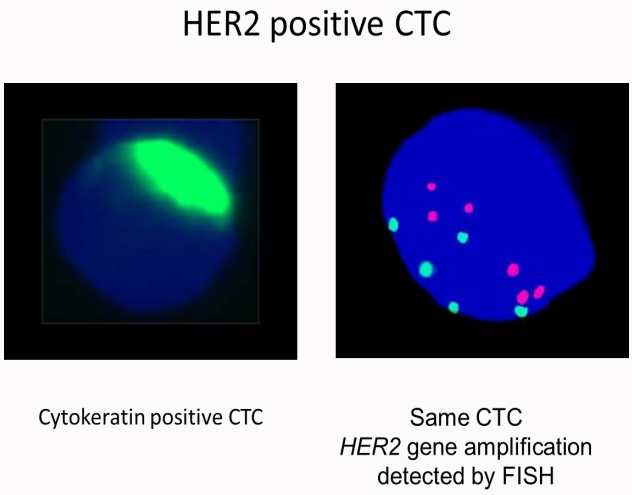

Figure 2: CTC analysis of clinical samples.
Figure 2a: HER2 gene amplification is detected in a cytokeratin positive CTC from a blood sample collected in the recurrent breast cancer case study patient with bone metastases.

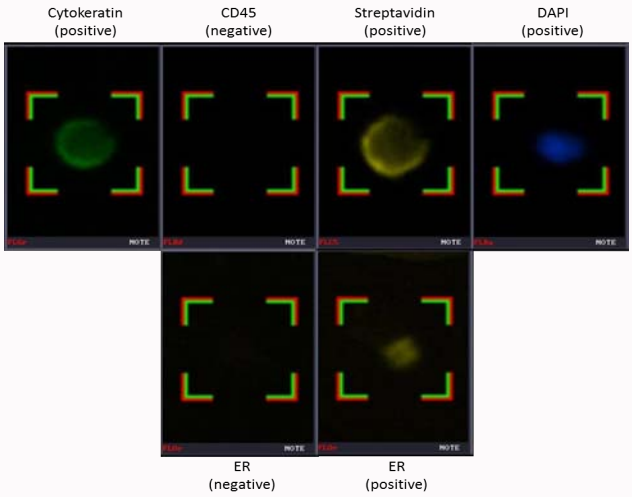

Figure 2b: CTC staining examples utilizing the Target Selector™ platform are shown. CTCs are defined as CD45 negative, DAPI positive cells, and can be either cytokeratin positive or negative. Streptavidin staining confirms the presence of biotin-conjugated secondary antibodies that bind to Biocept's primary antibody cocktail (directed against 10 epitopes commonly expressed by a broad range of cancer cells) [1]. Examples of ER positive and negative cells are depicted on the bottom row of the figure.

In late 2006, imaging revealed multiple osseous metastatic lesions. Her T11 spine bone biopsy confirmed metastatic adenocarcinoma, and the IHC analysis was negative for ER, PR, and HER2. It was noted that the samples were decalcified per standard protocol. She received external beam radiation to her spine followed by docetaxel/capecitabine, continuing afterwards on endocrine therapy and zoledronic acid.

PET/CT scans in August 2009 revealed a new lesion in the right scapula, and the needle biopsy was positive for metastatic carcinoma; ER, PR, and HER2 IHC stains were technically inconclusive. Subsequently, she started fulvestrant therapy.

The patient's osseous disease progressed in 2013 and she received further chemotherapy. Her cancer progressed further in May 2014, and the decision was made to re-evaluate her hormone receptor and HER2 status. A right iliac bone biopsy attempted in August was negative for tumor cells, and the patient refused a repeat biopsy.

Target Selector™ liquid biopsy on her blood specimen from July 10, 2014, showed HER2 amplification by FISH and ER expression. Based on the HER2 positivity in CTCs, she began HER2 directed therapy with chemotherapy. Restaging imaging in January 2015 showed interval decrease of abnormal osseous activity in the numerous osseous metastases since May 2014 and no evidence of visceral involvement.

Restaging scans in October 2015 revealed new lesions in the skull, bilateral femurs, and ribs. Another Biocept liquid biopsy on October 14, 2015 revealed positive ER expression and negative HER2; it is important to note that this was while she was on HER2 therapy. She underwent surgical nail placement of the bilateral femurs, during which bone biopsies were obtained in November 2015, confirming metastatic carcinoma with positive ER and HER2 (3+) expression by IHC. For the first time, metastases were also noted in the lungs and liver, and she was started on trastuzumab emtansine in December.

Imaging in April 2016 showed increased adenopathy in the right supraclavicular region, and aright supraclavicular lymph node biopsy in May showed increased ER and PR expression. The HER2 analyses were equivocal, and therefore a tertiary HERmark® test was performed, resulting in a positive quantitative result of 25.11. The patient's treatment was switched to vinorelbine, and anti-HER2 therapy was continued.

The patient had metastatic progression into the dura and right lower lung lobe in January 2017, while her osseous metastatic sites remained stable. Her restaging scans in June 2017 demonstrated an overall stability of her metastatic disease except for slight increase in left retroperitoneal lymphadenopathy. Her most recent Biocept liquid biopsy from July 2017 revealed one cytokeratin positive cell with negative HER2 expression. As of December 2017, our patient

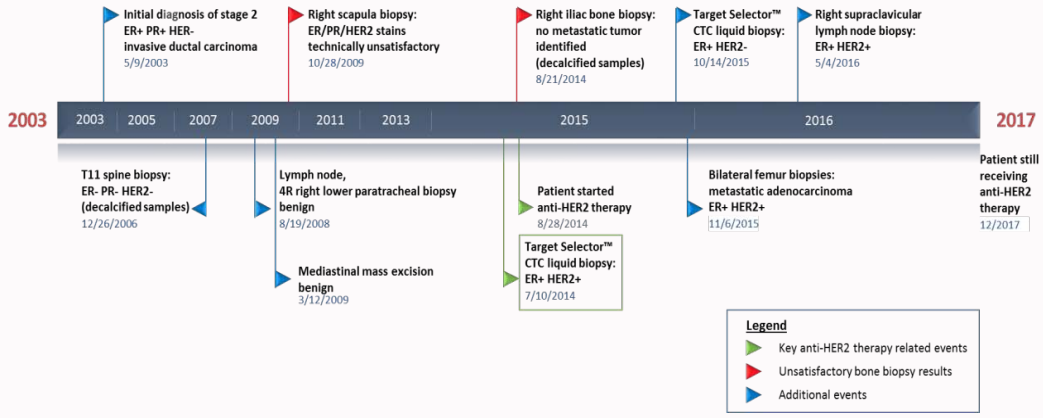

Figure 3: 2003 to 2017 Case study timeline of tumor tissue and CTC results.
Bone tissue testing and liquid biopsy results are depicted for hormonal receptor expression and HER2 (protein or FISH). Key events related to administration of anti-HER2 therapy are highlighted.

Table 1: Chronological History of Tumor Biopsies, showing pathologic results and marker expression.

Date	Test	Findings
5/9/2003	Right breast lumpectomy and sentinel lymph node excisional biopsy	Sentinel lymph nodes: 1/2 lymph nodes with metastatic cancer
		Lumpectomy: infiltrating ductal carcinoma, Nottingham grade 1
		C-erb B2-2+ indeterminate.
		FISH for HER2/neu-1.18, not amplified.
12/26/2006	T11 Spine biopsy	Metastatic adenocarcinoma consistent with breast primary
		Quantitative Image Analysis: ER 0% , PR 0% and Her2/neu staining intensity 0.2-negative
8/19/2008	Lymph Node, 4r Right Lower Para tracheal, Biopsy	Lymphocytic proliferation no evidence of carcinoma
3/12/2009	Mediastinal mass excision	Stromal proliferation consistent with Castleman's Disease, Hyaline Vascular type (CD-HV)
10/28/2009	Core needle biopsy, right scapula	Positive for metastatic carcinoma- Pancytokeratin IHC stain shows positivity in scattered tumor cell within the marrow space.
		ER, PR, and Her2-Neu stains were technically unsatisfactory.
8/21/2014	Right iliac bone core needle biopsy	Thick trabecular / cortical bone only. No marrow or metastatic tumor identified. (Decalcified sections examined.)
11/4/2015	Right and left femur surgical biopsy	Metastatic carcinoma, consistent with clinical history of breast carcinoma. Decalcified sections examined.
		Left femur biopsy-bone with metastatic carcinoma, morphologically consistent with breast primary.
		Immunostains positive for cytokeratin, HER2-3+, ER+
5/4/2016	Right supraclavicular FNA lymph node biopsy	Positive for metastatic adenocarcinoma, consistent with breast primary, GATA3 positive
		Aperio Scan Scope® system for quantitative image analysis: ER 59%, PR 32%, Her2/neu staining intensity 2 (borderline).
		HER2 FISH HER2/CEP17: 1.8 Equivocal HER mark® breast cancer assay 25.11, HER2 positive

continues on anti-HER2 therapy with chemotherapy. Table 1 and Figure 3 summarize key events.

Discussion

Successful treatment of advanced breast cancer is driven by the knowledge of ER/PR and HER2 expression, and biopsy of metastatic sites is recommended to reconfirm the expression of these markers. In one study, discordance in expression measurements for ER, PR and HER2 between primary and recurrent breast tumors occurred in 18.4%, 40.3%, and 13.6% of cases respectively and had a negative effect on prognosis and survival likely due to misuse of targeted therapies [11]. In this study, the discordant biomarker expression was thought to be due to tumor heterogeneity, change in clinical phenotype, or suboptimal reproducibility of measurement methods, particularly IHC.

Here we describe clinical difficulties with obtaining adequate tumor tissue for reconfirmation of ER/PR and HER2 expression. Our patient with a long history of metastatic breast cancer with bone only involvement underwent 4 tumor biopsy procedures to reassess expression of ER/PR and HER2. Her primary tumor initially tested HER2-negative, but after several years of treatment her metastatic tumor was found to be HER2 overexpressed. This finding led to therapy with anti-HER2 agents and almost certainly extended her life expectancy. The change in the breast cancer tumor expression of the HER2 is well-described in the literature [12] and occurs in up to 20% of cases [13]. Difficulties in assessing the patient's bone-only metastases are described, especially when bone biopsy IHC analyses were inconclusive or negative for hormone receptors and HER2, despite initial hormone receptor positivity of the primary tumor. It is well established that pathology processing and decalcification

of tumor tissue from bone biopsies can interfere with IHC stains, leading to inconclusive or negative ER, PR, and HER2 stains. This could explain the patient's negative or inconclusive tumor bone biopsy IHC staining results.

A meta-analysis of 49 studies (6,825 patients) showed that presence of CTCs was significantly associated with shorter survival; the prognostic value of CTCs was significant in both early (DFS: HR, 2.86; OS: HR, 2.78) and metastatic breast cancer (PFS: HR, 1.78; OS: HR, 2.33) [14]. Furthermore, declining numbers of CTCs while on systemic treatments for advanced breast cancer are predictive of response to therapy and improved survival [15-18]. Assessment of CTC tumor markers is available commercially, but the clinical relevance of expression of tumor markers such as HER2 is not well understood [19-21]. A prospective study in metastatic patients with HER2-negative primary tumors showed that 24%of patients had HER2positive CTCs later; and this was associated with significantly shorter progression-free (P = 0.001) and overall survival (P = 0.013) compared with patients without HER2-positive CTCs [22]. Further prospective studies are needed to determine the potential role of HER2-targeted therapies for patients with HER2-positive CTCs and HER2-negative primary tumors.

The first HER2 positive test in this patient's case was obtained by CTC FISH testing. We eventually were able to confirm HER2 overexpression in tumor tissue approximately 16 months later with a surgical tumor sample from her orthopedic surgery, while prior less invasive needle bone biopsies were inconclusive. Based on CTC HER2 positivity, our patient received anti-HER2 therapy sooner than otherwise would have been the case based on tumor tissue testing.

In summary, HER2 is an important target for breast cancer therapy, and anti-HER2 therapy improves survival of advanced breast cancer patients [23,24]. In the metastatic setting, challenges exist with obtaining tumor tissue for re-testing markers. Our case illustrates the clinical applicability of a CTC HER2 test, which provided critical molecular information concerning HER2 tumor status 16 months before the tissue test confirmed HER2 overexpression. We conclude that ultimately our patient derived a clinically meaningful benefit from blood CTC HER2 testing, and the earlier initiation of anti-HER2 therapy extended her life-expectancy and improved her quality of life.

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