Liquid biopsy identification of *ERBB2* amplified and HER2 expressing metastatic breast cancer: comparison and combination of cell and cell-free platforms.

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BACKGROUND

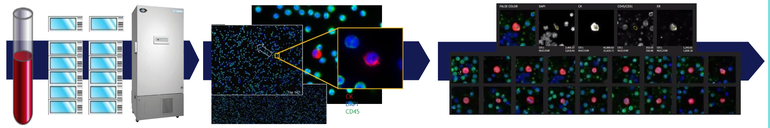
Liquid biopsies are a non-invasive diagnostic approach for detecting circulating tumor cells (CTCs) or circulating tumor DNA (ctDNA) that may provide clinically actionable information for treatment decisions for metastatic breast cancer (MBC) patients when a conventional biopsy is otherwise infeasible. In addition, the development of quantitative, reproducible, and more sensitive HER2 assays is expected to enable the identification of patients with HER2-low MBC that may benefit from novel HER2-targeted therapies. Here we report a comprehensive liquid biopsy platform including: 1) quantitative immunofluorescent HER2 and ER protein expression in CTCs (CTCIF), 2) determination of ERBB2 amplification and the number of Large-scale State transitions (LST) by single-cells CTC genomics (CTCDNA) and 3) ctDNA alterations in plasma.

METHODS

Blood samples from 247 progressing metastatic breast cancer patients were collected for cell & cell-free DNA analysis. Blood was also collected from 25 blood donors (HDs) with no known cancer history as controls. After plasma isolation, nucleated cells were plated, & slides were bio-banked. Immunofluorescent staining & subsequent imaging were performed on replicate slides. CTCs were identified using Epic Sciences digital imaging & machine learning algorithms. Single-cell CTC isolation and sequencing for genomic quantification of large-scale state transitions (LSTs) and *ERBB2* Copy Number Variants (CNV) was used to determine genomic instability and *ERBB2*^{amp} in individual CTCs. Bio-banked plasma was analyzed to detect ctDNA alterations with high clinical relevance (Class IA).

EPIC SCIENCES LIQUID BIOPSY PLATFORM

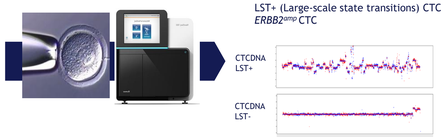
Figure 1. CTC morphology by digital pathology coupled with Immunofluorescent detection & biomarkers expression (HER2$^+$ and ER$^+$ CTCIF)



CK, CD45, DAPI, HER2 and ER

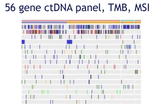
Schematic of Epic Science's CTC collection and detection process: 1) Nucleated cell from blood are plated onto slides and stored in the -80C biorepository; **2)** Slides are stained with CK, CD45, DAPI, HER2 and ER; **3)** Slides are scanned, and CTC candidates detected by multi-parametric digital pathology algorithm.

Figure 2. CTC single cell isolation for low-pass Whole Genome Sequencing (WGS) (LST$^+$ and *ERBB2*^{amp} CTCDNA)



Schematic of Epic Science's CTC single cells genomics: 1) CTC are picked with a microcapillary and isolated for single cells WGS **2)** Large scale transitions (LST) values and *ERBB2*^{amp} Z scores are computed through the bioinformatics pipeline for each CTC candidate.

Figure 3. Plasma isolation & ctDNA analysis for SNVs and CNVs detection



Epic Science's ctDNA pipeline: Plasma is isolated and SNVs Variant Allele Frequencies (VAF) and genomics CNVs relevant for breast cancer are computed through the bioinformatics pipeline for each patient.

RESULTS (^{CTC}DNA AND $_{CT}$DNA GENOMIC, AND ^{CTC}IF PLATFORMS)

Prevalence of CTCs, CTCDNA (LST$^+$), ctDNA (all, and 1A) and *ERBB2*^{amp} by ctDNA and CTCDNA genomics

	CTC$^+$ %	CTCDNA (LST$^+$) %	ctDNA (all$^+$) %#	ctDNA (IA$^+$) %#	ERBB2^{amp} ctDNA(IA$^+$) %#*	ERBB2^{amp} CTCDNA %#^
MBC (n=247)	94 (233/247)	63 (157/247)	62 (112/182)	30 (54/182)	2 (3/182)	15 (37/247)
Healthy donors (n=25)	0 (0/25)	0 (0/25)	0 (0/25)	0 (0/25)	0 (0/25)	0 (0/25)

65 patients had failed QC for ctDNA analysis (or NSQ non-sufficient ctDNA quantity)
*ctDNA LoD ≥ 1.4 "apparent" folds change (VAF/CNV) ^CTCDNA LoD ≥ 2 folds change (single cell level)

Table 1 (Left). No false positive detection in Healthy Donors, suggesting high specificity for each analysis. **Table 1 (Right).** CTCDNA single-cell genomics was more sensitive than ctDNA in detecting *ERBB2*^{amp} in MBC patients (15%, and 2%, respectively).

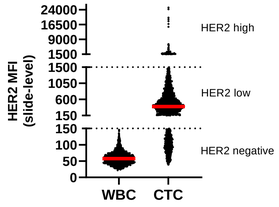
Prevalence of *ERBB2*^{amp} by ctDNA and CTCDNA with low (≤30 VAF) and high (>30 VAF) tumor fraction

	ctDNA available: Yes CTCDNA *ERBB2*^{amp} %	ctDNA available: No CTCDNA *ERBB2*^{amp} %	VAF ≤ 30: ctDNA(1A)$^+$ (*ERBB2*^{amp}) % # *	VAF ≤ 30: CTCDNA (*ERBB2*^{amp}) % µ ^	VAF > 30: ctDNA(1A)$^+$ (*ERBB2*^{amp}) % # *	VAF > 30: CTCDNA (*ERBB2*^{amp}) % µ ^
ERBB2^{amp}	54 (20/37)	46 (17/37)	0 (0/2)	85 (17/20)	100 (2/2)	15 (3/20)

#, µ *ERBB2amp* and VAF detection was present in 2 out of 3 patients by # ctDNA and 20 out of 37 patients by µ CTCDNA
*ctDNA LoD ≥ 1.4 "apparent" folds change (VAF/CNV) ^CTCDNA LoD ≥ 2 folds change (single cell level)

Table 2 (Left). CTCDNA single-cell genomics identified a similar prevalence of *ERBB2amp* patients with (54%) and without (46%) ctDNA available. **Table 2 (Right).** CTCDNA and ctDNA platforms detected *ERBB2amp* in 85% and 0% of patients, respectively, with a variant allele frequency (VAF) ≤ 30, suggesting that the CTCDNA platform can identify *ERBB2amp* among patients with a low ctDNA circulating tumor fraction (VAF ≤ 30), while the ctDNA platform cannot.

Distributions of HER2 and ER protein MFI in WBC and CTC by CTCIF



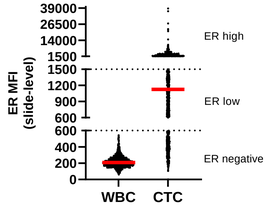


Figure 4. HER2 (left) and ER (right) MFI expression by CTCIF across all patients slides in White Blood Cells (WBC) and CTC identify HER2 (left) and ER (right) negative, and low threshold value, respectively.

Prevalence of HER2$^+$ and ER$^+$ CTC

	CTCIF HER2^{exp} %	CTCIF ER^{exp} %
MBC (n=247)	79 (194/247)	76 (189/247)

Table 3. HER2$^+$ and ER$^+$ CTC by CTCIF were detected in 79% and 76% of MBC patients, respectively.

HER2$^+$CTC MFI in *ERBB2amp*

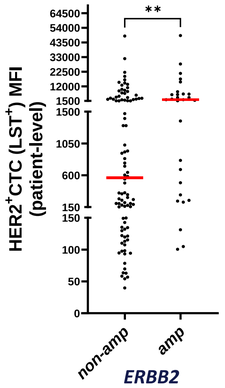


Figure 5. HER2$^+$ (LST$^+$) CTC MFI by CTCIF had a higher level in MBC patients with detectable *ERBB2*^{amp} CTC by CTCDNA compared to those with *ERBB2*^{nonamp} CTC (Median: 2390 MFI vs. 564 MFI, respectively; p<0.01).

Liquid biopsy classification of HER2 status

	HER2 amplified: *ERBB2*^{amp} by CTCDNA or ctDNA %	HER2 expressed HER2 non-amplified: HER2^{exp} by CTCIF and *ERBB2*$^{non\text{-}amp}$ by CTCDNA or ctDNA %	HER2 negative: HER2$^{non\text{-}exp}$ by CTCIF and *ERBB2*$^{non\text{-}amp}$ by CTCDNA or ctDNA %
MBC (n=247)	15 (37/247)	65 (162/247)	20 (48/247)

Table 4. A liquid biopsy classification of HER2 status by combining the three platforms (CTCIF, CTCDNA, and ctDNA) identified that among MBC, 15% were *ERBB2*^{amp}, 65% were HER2^{exp} (HER2$^+$ and *ERBB2*^{nonamp}), and 20% were HER2^{neg} (HER2^{nonexp} and *ERBB2*^{nonamp}).

CONCLUSIONS

1. This liquid biopsy platform integrates comprehensive CTC analysis and ctDNA to report HER2 and ER status with high specificity and sensitivity in MBC patients.
2. CTCDNA single-cell genomics is more sensitive than ctDNA at detecting *ERBB2*^{amp}
3. This platform has potential clinical utility in selecting appropriate treatments for MBC without a tissue biopsy and/or in later lines of therapy among often biologically heterogeneous tumor sites of metastatic disease.