A novel, semi-automated Pipeline for HER2 Quantification on CTCs in breast cancer patients. Is cytopathology of peripheral blood a new diagnostic option?

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Introduction

The Human Epidermal Growth Factor Receptor 2 (HER2) plays a central role in breast cancer (BC). Nowadays, the assessment of HER2 status and the selection of patients eligible for anti-HER2 therapy rely on immunohistochemistry (IHC) and *in situ* hybridization on tissue biopsy, an invasive approach, unable to capture intratumor heterogeneity and dynamic of HER2 expression. Circulating tumor cells (CTCs) offer an alternative material to evaluate HER2 expression in *real-time*, through a simple blood draw. Limitations to the use of CTCs for HER2 assessment derive from limited sensitivity in detection methods and lack of standardization.

To overcome these limits, we developed a semiautomated pipeline combining label-independent CTC enrichment and HER2 expression quantification and we compared it to the gold-standard CellSearch®, in a cohort of patients (pts) with metastatic BC (mBC).

Materials and methods

Blood samples (7.5 mL) were collected in EDTA tubes from pts with mBC enrolled at Weill Cornell Medicine and processed within 1 hour for CTC analysis. CTCs were captured using Parsortix™ and stained for epithelial (EpCAM and cytokeratins [CK]), leukocyte (CD45) and HER2 markers as well as for nuclear staining(**Figure 1.1**) . CTCs were identified as nucleated cells, EPCAM/CK+ and CD45-. For each CTC, 4 color digital images were captured (**Figure 1.2**) and processed with the automated post-processing developed tool: a combination of an open-source image analysis software CellProfiler (**Figure 1.3**) and a custom MATLAB code (**Figure 1.4**) allowing for CTC identification and HER2 expression categorization into high, low and no expression. A second aliquot for each sample was processed in parallel with CellSearch® for comparison.

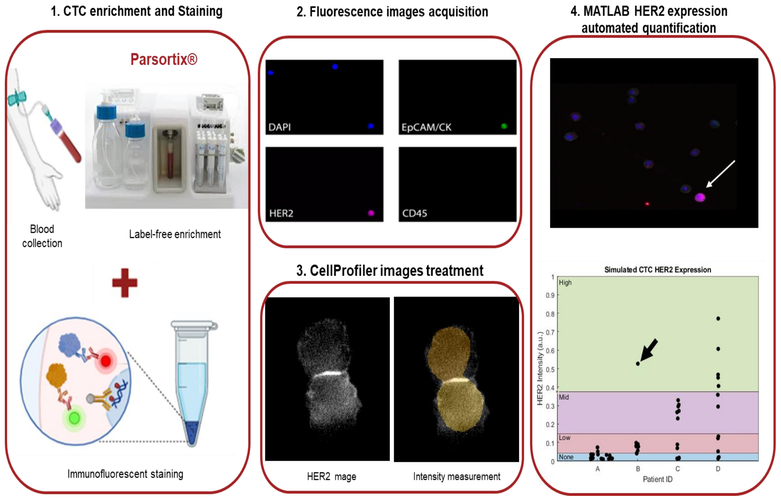


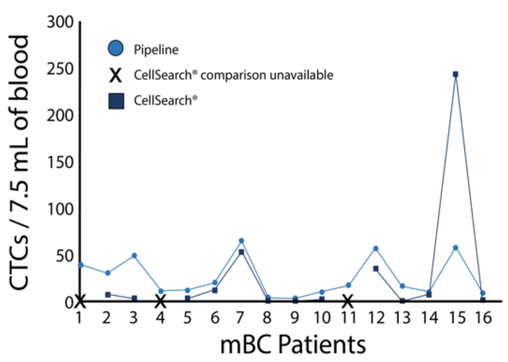
Figure 1. Study experimental design

Results

- A total of 16 whole blood samples were collected from mBC patients (**Table 1**).
- Our pipeline identified CTCs in all the samples, whereas CellSearch® only did in 10/13 of evaluable samples (**Figure 1**).

Table 1. Clinical characteristics of the enrolled patients.

Patients Clinical Data	N	%
Number of Patients	14	-
Mean age at MBC diagnosis (Years)	48.6 (34--69)	
Histological subtype		
IDC	12	86
ILC	2	14
Molecular Subtype		
HR+ HER2-	4	28
HER2+	5	36
HR- HER2-	5	36
HER2 status		
Positive (IHC 3+, 2+ FISH amplified)	5	36
Low (IHC 1+, 2+ FISH not amplified)	6	43
zero (IHC 0)	3	21
IBC		
Yes	5	36
No	9	64
Number of Metastatic sites		
Single	2	14
Multiple	12	86



Patients	CTC count Pipeline	CellSearch®
P1	39	N/A
P2	30	7
P3	49	3
P4	11	N/A
P5	12	3
P6	20	12
P7	65	53
P8	4	0
P9	3	0
P10	10	2
P11	17	N/A
P12	58	35
P13	16	0
P14	10	7
P15	58	245
P16	9	1
	= 411	= 368

Figure 2: Comparative line plot showing CTCs enumeration on 16 mBC patients processed by the developed pipeline (dots) and CellSearch® (squares).

- Our pipeline detected HER2+ CTCs in all the samples, while CellSearch® only did in 4/13 of the evaluable samples. Among all detected CTCs, 38% (158/411) were HER2-positive using our pipeline, whereas only 11% (40/368) identified were HER2-positive using CellSearch® (p<0.0001) (**Figure 3**).

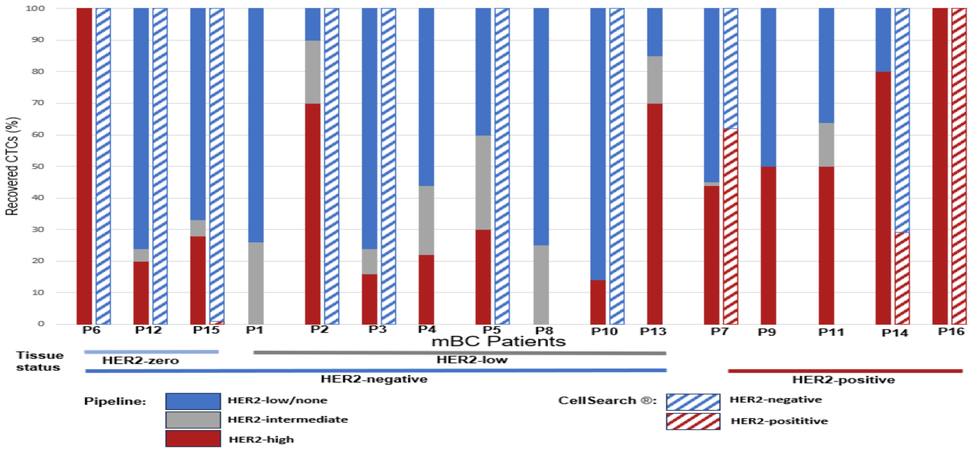


Figure 3. Comparisons of HER2 assessment on 16 mBC patients' CTCs processed in parallel with the developed pipeline and CellSearch®. CTCs were input into their corresponding HER2 expression categories (high, intermediate, or none/low)..

- A higher percentage of HER2-positive CTCs was found in the HER2-positive BC cohort compared to the HER2-negative BC cohort (= 55% (57/104) versus 33% (101/307), respectively) (p = 0.0017) (**Figure 4**).

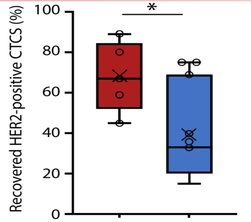


Figure 4: Comparative box plots showing HER2-positive CTCs enumeration across the HER2 positive and the HER2 negative patients by using our developed pipeline

- Our pipeline identified 83 cells with low/negative EPCAM/CK expression. These cells were CD45-negative and 12% of the cases presented a HER2 expression (**Figure 5A**).
- We identified CD45-negative, EpCAM/CK positive CTC clusters, ranging from 2–10 cells per cluster in 11/16 of the 16 processed samples (69%) with our pipeline. Some of the clusters were also expressing HER2 (**Figure 5B**).

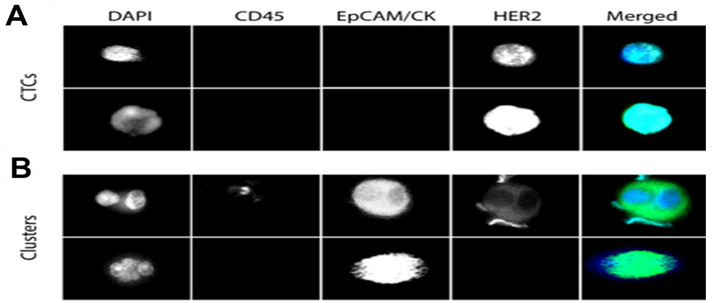


Figure 5: (A) Fluorescence images of EpCAM/CK-, HER2-high and EpCAM/CK-, HER2-intermediate CTCs recovered respectively on patient P3 and P9 indicating the pipeline' ability to recover and detect non-epithelial CTCs. (B) Fluorescence images of CD45-, EpCAM/CK+, HER2+ and CD45-, EpCAM/CK+, HER2- CTC cluster recovered from patient P3 and P15, respectively.

Conclusions

- **The current study shows the feasibility of a *real time* HER2 assessment on CTCs enriched from BC pts.**
- The developed pipeline was able to count and identify HER2-positive CTCs with higher efficiency than the gold-standard CellSearch® overall and interestingly, also in the IHC HER2-negative subgroup. This is a preliminary analysis that should be confirmed in larger cohorts.
- HER2 marker is a therapeutic target and an accurate and real-time assessment of the HER2 status could be used to better guide treatment in a larger cohort of patient with advanced breast cancer with any detectable HER2 expression that can be effectively treated with antibody-drug conjugates.

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