Interrogating HER2 status in Circulating Tumor Cells isolated using the Parsortix® System from Metastatic Breast Cancer patients

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Introduction

Diagnosis and treatment of breast cancer patients is guided by evaluation of human epidermal growth factor receptor 2 (HER2) status in tissue biopsies. Extracting tissue biopsies is invasive, with little opportunity for repeat testing. However, monitoring Circulating Tumour Cells (CTC) isolated from blood can be made possible through minimally invasive liquid biopsy techniques. While many CTC capture systems are epitope-dependant and primarily rely on epithelial markers, limiting potential of detection of mesenchymal and epithelial-to-mesenchymal transitioning (EMT) CTCs, ANGLE's Parsortix® instrument is an epitope-independent microfluidic device that isolates and harvests CTCs from blood based on size and deformability. In this study, ANGLE's Parsortix® instrument was combined with a downstream Immunofluorescence (IF) assay for HER2 protein identification on CTCs isolated from blood of Metastatic Breast Cancer (MBC) patients collected at multiple timepoints.

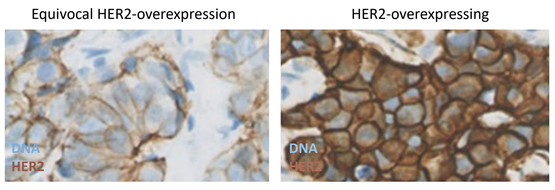

Figure 1. Determination of HER2 status in a tissue section of a solid breast tumour by immunohistochemistry. Figure obtained from van den Ende NS, Smid M, Timmermans A, et al. HER2-low breast cancer shows a lower immune response compared to HER2-negative cases. Sci Rep 12, 12974 (2022).

Workflow

Performance was established using Healthy Volunteer's (HV) blood samples spiked with either HER2-overexpressing or HER2-negative breast cancer cell lines (analytical performance) and using blood samples from MBC patients. In both cases (**Figure 2**):

- Peripheral blood was drawn into Streck Cell-Free DNA tubes and stored for up to 144 hours from collection before processing.
- Blood samples were processed on Parsortix® instruments, a microfluidic device capable of capturing and harvesting CTCs from bodily fluids based on cell size and lack of deformability, employing a separation cassette (GEN3P6.5) comprising a series of steps leading to a smaller critical gap. Most of the common blood cells and components pass across the critical gap, while CTC are retained in the separation cassette due to their size and rigidity.
- Harvested CTCs were cytospun on ANGLE's CellKeep™ slides to maximize the retention.
- Slides were stained using ANGLE's Portrait® HER2 IF staining panel, comprising a nuclear dye (Hoechst) and antibodies against epithelial markers (FITC), mesenchymal markers (Cy7), HER2 (Cy3) and blood lineage markers (Cy5), including antigens expressed by blood cells such as lymphocytes, macrophages, granulocytes, monocytes, fibroblasts, and cells of megakaryoblastic potential.
- Stained slides were imaged using a BioView Allegro Plus imaging system.

Research use only (RUO). Not for use in Diagnostic Procedures.

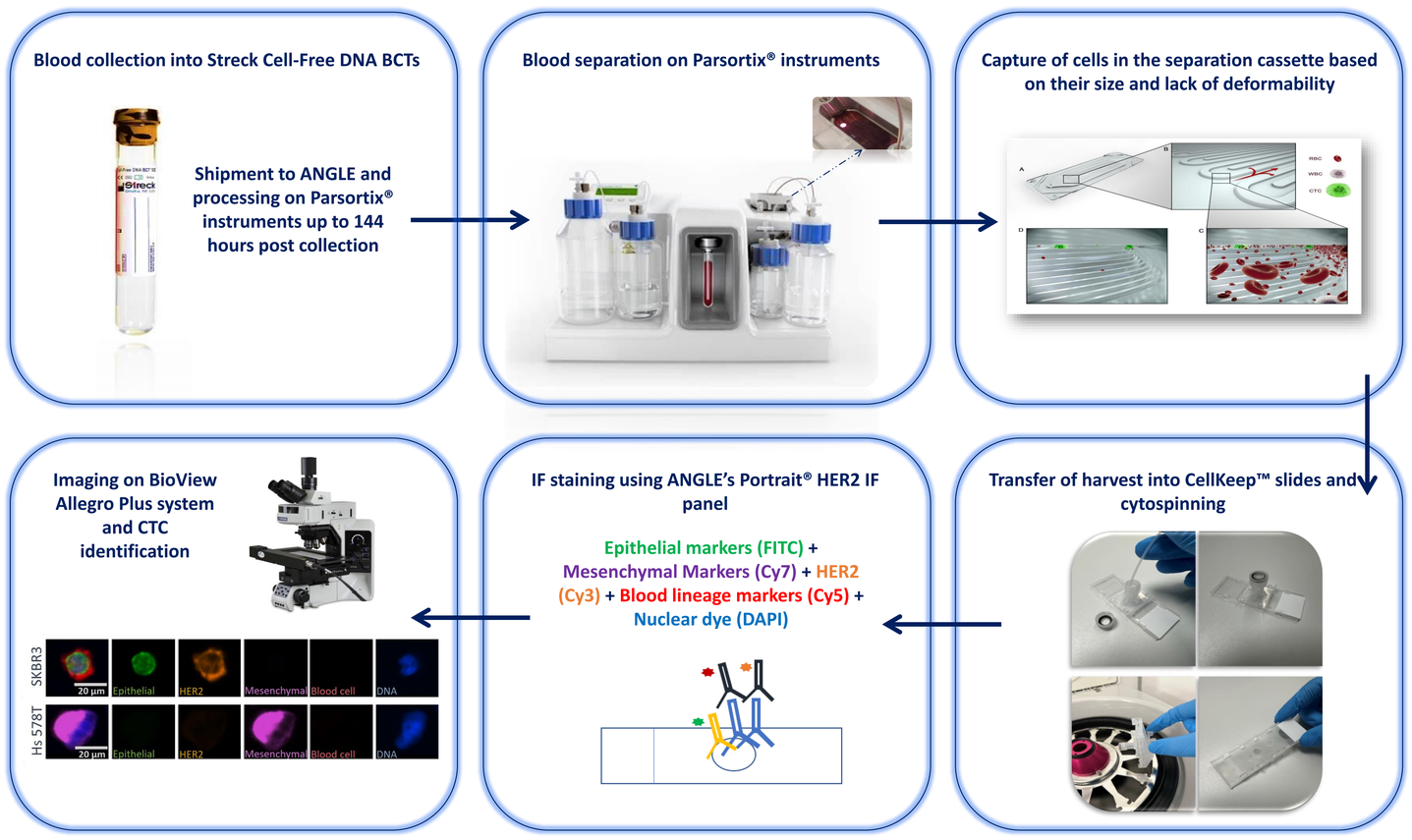

Figure 2. Schematic representation of the assay workflow.

Analytical Results

Analytical performance of the assay was evaluated using contrived samples from six healthy volunteers, spiked with cultured breast cancer cell lines (SKBR3, HCC1954, MCF-7, Hs 578T or T47D).

- Evaluation of HER2 signal in circulating cancer cells from contrived samples showed that mean HER2 Mean Fluorescence Intensity (MFI) signal in HER2-positive cells (SKBR3 and HCC1954; mean MFI 139 and 133, respectively) was significantly higher than that of HER2-negative cells (MCF-7, Hs 578T and T47D; mean MFI 16, 10 and 16, respectively) (**Figure 3.A, B**).
- Analytical specificity and sensitivity of HER2, epithelial markers, mesenchymal markers and blood lineage markers in the assay were over 95% (**Figure 4**).

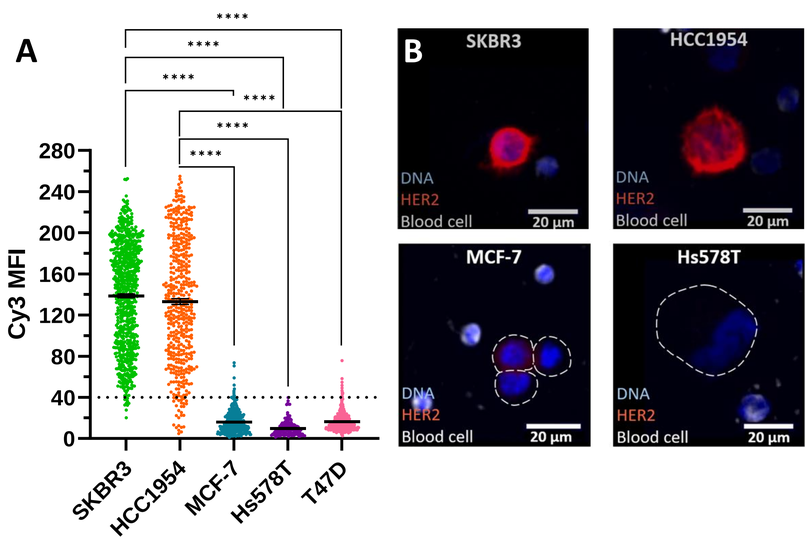

Figure 3. Evaluation of HER2 protein in HER2-positive and HER2-negative cancer cells from contrived samples separated on Parsortix® instruments. (A) Dot plot showing the mean ± standard error of mean (SEM) of HER2 MFI in each cancer cell line tested. Data was statistically compared by running a one-way ANOVA followed by Tukey's multiple comparisons test, *****p* ≤ 0.0001. **(B)** Representative images of cancer cell lines tested where HER2 is shown in red (Cy3), blood cell lineage markers in white (Cy5), and DNA in blue (DAPI): SKBR3 (top-left), HCC1954 (top-right), MCF-7 (bottom-left), Hs578T (bottom-right).

	HER2	Epithelial Markers	Mesenchymal Markers	Blood Lineage Markers
Specificity	97.3%	100%	99.5%	99%
Sensitivity	96.9%	97.2%	99%	99%

Figure 4. Specificity and Sensitivity of Portrait® HER2 assay. Analytical specificity (percentage of harvested cells known to not express marker(s) of interest which were negative after IF staining) and analytical sensitivity (percentage of harvested cells known to express marker(s) of interest which were positive after IF staining) of the Portrait® HER2 assay, evaluated using SKBR3, HCC1954, MCF-7, Hs 578T and T47D cancer cell lines and blood lineage cells.

Patients' Results

Samples from a total of 31 MBC patients were processed in this study (as per **Workflow** section). From each patient, two to six blood draws were obtained, with at least one month between subsequent draws.

Of the 31 MBC patients, seven had HER2-positive primary tumour and were receiving HER2 targeted therapy (Group 1), while 24 patients had HER2-negative primary tumour and were not receiving HER2 targeted therapy (Group 2).

Donors were regarded as EMT in **Figure 5B** if they presented EMT CTCs and/or both Mesenchymal and Epithelial CTCs.

Group 1 donors were regarded as discordant if they did not present any HER2+ CTCs, while Group 2 donors were regarded as discordant if they presented at least one HER2+ CTC.

- Donor CTC positivity was consistent between Group 1 (85.7%) and Group 2 (87.5%) (**Figure 5.A**).
- Percentage of donors with HER2+ CTCs was comparable between Group 1 (33.3%) and Group 2 (28.6%) (**Figure 5.A**). Overall discordance between HER2 status in the tissue and in the CTCs was 37%, with discordance in Group 1 being over double that of Group 2, suggesting that the HER2 targeted therapy received by Group 1 patients may influence HER2 protein expression in CTCs (**Figure 5.A**).
- Most HER2+ CTCs observed were Epithelial or EMT cells.
- Notably, five donors from Group 2 (tissue HER2-negative) passed away within 4 months of presenting clusters of Epithelial CTCs in a blood draw. Of these five donors, three presented clusters of Epithelial HER2+ CTCs.

A

		Group 1	Group 2
Total Donors		7	24
Donors with ≥1 CTC in at least 1 draw		6/7 (85.7%)	21/24 (87.5%)
#CTCs	Range in CTC+ samples	1 - 79	1 - 393
	Mean of CTC+ samples	11	35
	Median of CTC+ samples	3	8
Donors with ≥1 CTC cluster in at least 1 draw		3/6 (50%)	16/21 (76.2%)
#Clusters (range)		1 - 3	1 - 61
#CTCs in clusters (range)		2 - 70	1 - 62

B

		Group 1	Group 2
% CTC+ donors with HER2+ CTCs		2/6 (33.3%)	6/21 (28.6%)
Donor phenotype	Mesenchymal & HER2-	2/6 (33.3%)	10/21 (47.6%)
	Epithelial & HER2+	0 (0%)	3/21 (14.3%)
	Epithelial & HER2-	1/6 (16.6%)	1/21 (4.8%)
	EMT & HER2+	2/6 (33.3%)	3/21 (14.3%)
	EMT & HER2-	1/6 (16.6%)	4/21 (19.1%)
Discordance between HER2 status in tissue and CTCs		4/6 (66.6%)	6/21 (28.6%)
		Overall discordance 10/27 (37%)	

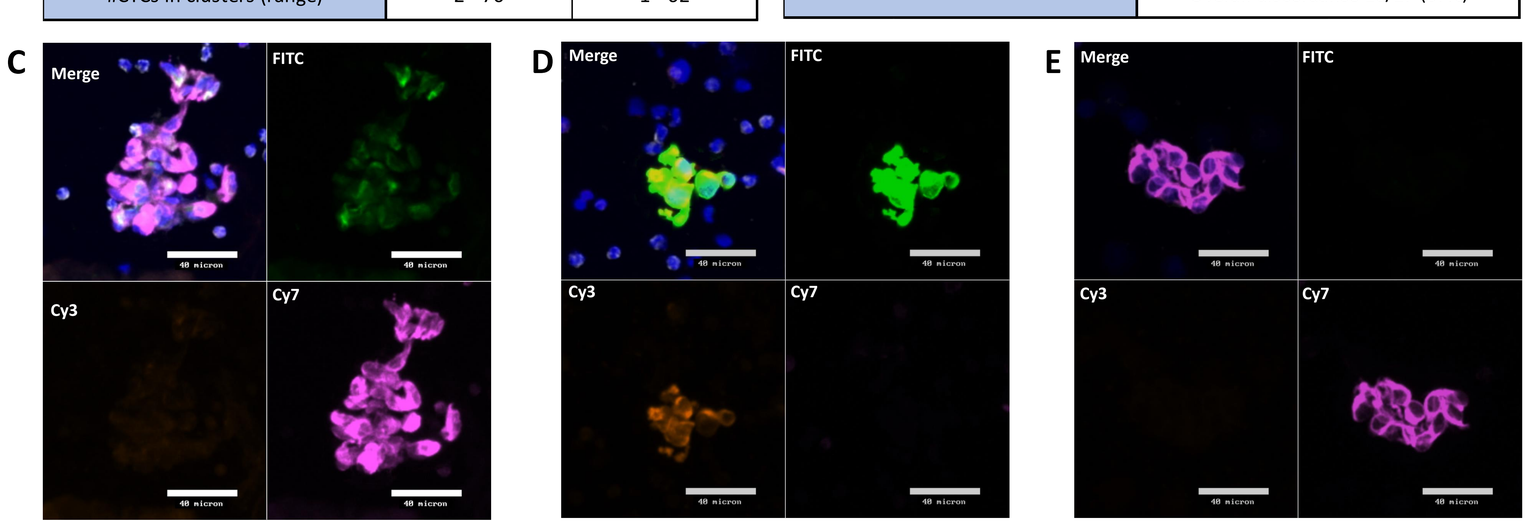

Figure 5. CTC identification and phenotyping. (A) Table showing number of donors included in each cohort, number and percentage (%) of donors with ≥1 CTC, range, mean and median of CTCs (excluding negative donors), number and percentage of donors with ≥1 CTC cluster, range of CTC clusters per donor, range of number of CTCs per cluster. **(B)** Table showing % of donors with HER2+ CTCs, donors' CTC phenotype and discordance between HER2 status in the tissue and in the CTCs. **(C, D, E)** Representative images of a cluster of EMT HER2- CTCs (C), a cluster of Epithelial HER2+ CTCs (D), and a cluster of Mesenchymal HER2- CTCs (E). Merge colors: Epithelial markers (FITC) in green, Mesenchymal markers (Cy7) in magenta, Blood lineage markers (Cy5) in white, HER2 (Cy3) in orange, Nuclear dye (DAPI) in blue.

Conclusions

This study demonstrated the ability of the Parsortix® instrument and ANGLE's Portrait® HER2 IF staining panel to effectively isolate and characterise CTCs of a range of phenotypes from MBC patients and the importance of using epitope-independent methods to capture CTCs, as 81.5% of CTC+ donors presented mesenchymal or EMT CTCs, which may be missed or incorrectly phenotyped by an epithelial marker reliant capture system. Overall, 37% of donors in this study presented CTCs with HER2 status not matching that of the primary tumour. The presence of HER2+ CTCs in patients with HER2- primary tumour highlights the potential of liquid biopsy to be used for more up to date and dynamic disease analysis than tissue biopsy alone, allowing monitoring of MBC disease progression and more accurate stratification of patients that may benefit HER2 targeted therapy.