Analytical validation of ANGLE's combined IF and FISH assay for assessment of HER2 in circulating tumor cells.

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Introduction

Evaluation of HER2 status in tissue biopsies through immunohistochemistry (IHC) and, in equivocal cases, fluorescence in-situ hybridization (FISH) is routinely performed to guide diagnosis and treatment decisions for breast cancer (BC) patients. Tissue biopsy is an invasive procedure for which re-examination is difficult and not commonly performed. Information obtained from an initial tissue biopsy may become outdated over time as the tumor evolves. Conversely, liquid biopsy is minimally invasive, with opportunity for recurrent sample collection, allowing monitoring of circulating tumor cells (CTCs) from which accurate, up to date information on an individual's disease can be garnered. This study aimed to validate a Research Use Only assay to concomitantly interrogate HER2 protein overexpression and gene amplification on the same CTCs in each sample. This dual analysis is important as previously it has been shown that protein overexpression and gene amplification do not always correlate[1].

Workflow

Analytical performance of the assay was established using contrived samples consisting of HER2+ (SKBR3) and HER2- (Hs 578T (protein negative) and MCF-7 (amplification negative)) cultured cancer cells spiked into healthy volunteer (HV) blood samples, drawn in Streck Cell-Free DNA tubes. Spiked blood samples were subjected to ANGLE's HER2 IF/FISH assay as described in **Figure 1.**

The analytical performance of the assay was assessed through determining the sensitivity, specificity, linearity, repeatability, reproducibility (inter-donor, operators, runs and reagents) and possible interference of endogenous substances.

After validation of the assay, the same workflow was applied to samples obtained from HER2+ and HER2- metastatic BC (MBC) patients as part of an ongoing clinical study. HER2 testing was performed at the time of tissue biopsy; in some cases, multiple years before the first blood draw. Blood (15 mL) was drawn from patients at up to six regular intervals to monitor CTCs over time.

CTCs were considered to overexpress HER2 protein if the Mean Fluorescence Intensity (MFI) value was above the minimum positivity threshold and considered to present *HER2* gene amplification if the ratio of *HER2* foci to CEN17 foci was ≥ 2.

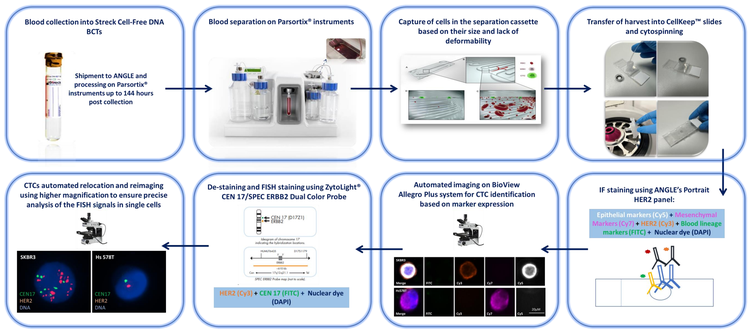

Figure 1. Schematic representation of the assay workflow. Peripheral blood was drawn into Streck Cell-Free DNA tubes and stored for up to 144 hours from collection before processing. Blood samples (2 × 7.5 mL) were processed on the Parsortix® instrument, a microfluidic device capable of capturing and harvesting CTCs from bodily fluids based on cell size and lack of deformability. Harvested CTCs were spun on ANGLE's CellKeep™ slides to maximize the retention. Slides were stained using ANGLE's IF-based HER2 assay, comprising a nuclear dye (Hoechst) and antibodies against epithelial markers (Cy5), mesenchymal markers (Cy7), HER2 (Cy3) and blood lineage markers (FITC) such as antigens expressed by blood cells (lymphocytes, macrophages, granulocytes, monocytes, fibroblasts and cells of megakaryoblastic potential). Stained slides were imaged and then de-stained to be subjected to *HER2* FISH using a commercially available kit (ZytoLight® CEN 17/SPEC ERBB2 Dual Color Probe). All slides were imaged twice using a BioView Allegro Plus system, a platform equipped with artificial intelligence for automated imaging, CTC candidate identification and reporting. First, an automated scan was performed on IF-stained samples to identify CTCs based on marker expression and morphology profiles. Second, an automated relocation of the CTCs based on IF results and reimaging using higher magnification to ensure precise analysis of the FISH signals in single cells.

References

1. Koo JS, Jung W, Yang WI. HER-2 protein overexpressing breast cancer without gene amplification shows higher hormone receptor expression than HER-2 protein overexpressing breast cancer with gene amplification. *Int. J. Surg. Pathol.*19:425–432 (2011).

Analytical results

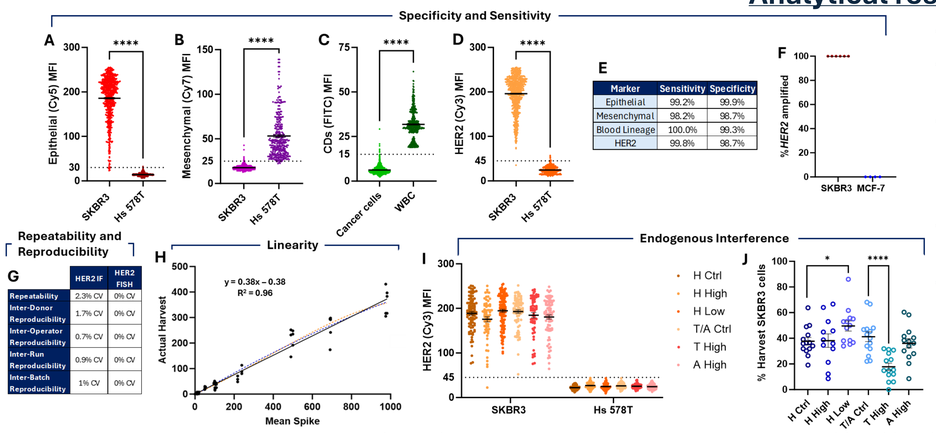

Figure 2. Analytical validation of ANGLE's HER2 IF and FISH assay. (A, B, C, D) Dot plots show raw MFI of cancer cells and white blood cells in the **(A)** Cy5, **(B)** Cy7, **(C)** FITC and **(D)** Cy3 channels representing epithelial, mesenchymal, blood lineage and HER2 markers, respectively. Dotted lines represent MFI thresholds for each IF channel. **(E)** Table shows percentage sensitivity and specificity of epithelial, mesenchymal, blood lineage and HER2 markers, determined as percentage of expected positive and negative cells with MFI above or below threshold in graphs **A, B, C** and **D**. **(F)** Dot plot shows percentage HER2 positivity of SKBR3 (*HER2* amplified) and MCF-7 (*HER2* non-amplified) cells by FISH based on ratio of *HER2* to CEN17 foci. **(G)** Table shows percentage coefficient of variation (%CV) of the percentage positivity of SKBR3 cells in the Cy3 channel measuring repeatability and inter-donor, inter-operator, inter-run and inter reagent batch reproducibility of HER2 IF staining, and %CV of percentage positivity of SKBR3 cells measuring repeatability and reproducibility of HER2 FISH staining. **(H)** Graph shows mean number of SKBR3 cells spiked (x axis) plotted against actual number of SKBR3 cells harvested from each blood sample after IF staining with the HER2 assay. First (black), second (blue) and third (orange) order polynomial trendlines are indicated on the graph. **(I & J)** Dot plot shows mean ± SEM of HER2 MFI value in Cy3 channel of SKBR3 and Hs 578T cells **(I)** and percentage harvest of SKBR3 cells **(J)** from blood samples in the H Ctrl (non-adjusted blood), H High (hematocrit increased), H Low (hematocrit reduced), T/A Ctrl (non-adjusted blood), T High (triglycerides increased) and A High (albumin increased) conditions. One-way ANOVAs applied to each cell line on each graph (*p<0.05, ****p<0.0001).

- Sensitivity and specificity of all markers in the IF assay were >98% (**Figure 2.A/B/C/D/E**).
- Sensitivity and specificity of the FISH assay, based on HER2 positivity rate of SKBR3 and MCF-7 cells, were 100% (**Figure 2.F**).
- Assay was repeatable and reproducible across donors, operators, runs and reagents with qualitative and quantitative performance of the assay consistent across all metrics (**Figure 2.G**). All SKBR3 cells observed across all repeatability and reproducibility studies were *HER2* amplified.
- The assay produced linear results through IF staining across the range of 0 – 1000 cells (R^2 = 0.956) with minimal degree of non-linearity (**Figure 2.H**).
- High or low hematocrit levels, high triglyceride levels, or high albumin levels in blood did not affect qualitative performance of the IF (**Figure 2.I**) or FISH components of the assay. However, low levels of hematocrit resulted in higher percentage harvest of SKBR3 cells, whilst higher triglyceride levels results in a significantly lower percentage harvest of SKBR3 cells (**Figure 2.J**). Assay quantitative performance may be negatively affected by high triglyceride levels in blood.

Patients' results

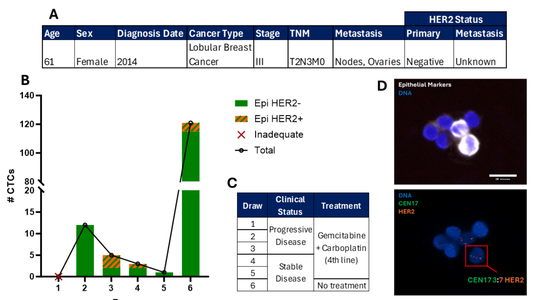

Figure 3. Patient case study 1. (A) Table shows patient's age, sex, diagnosis date, cancer type, stage and TNM classification, metastases locations and HER2 status of primary tumor and metastatic sites. **(B)** Number and IF phenotype of CTCs observed in each blood draw, processed as per workflow in **Figure 1.** **(C)** Table shows clinical status and treatment received by MBC patient at each blood draw. **(D)** Cluster of six epithelial (HER2-) CTCs imaged post-IF (top; Nuclear stain [DAPI] in blue, Epithelial markers [Cy5] in white) and post-FISH (bottom; Nuclear stain [DAPI] in blue, CEN17 control foci [FITC] in green, *HER2* foci [Cy3] in orange). Red box indicates *HER2* amplified CTC, CEN17:*HER2* foci ratio 3:7.

- Total CTC count dramatically increased at 6th draw after treatment was stopped (115 epithelial HER2-, 6 epithelial HER2+ CTCs) **(Figure 3.B/C).**
- HER2+ CTCs were observed in three of five successfully processed blood draws (**Figure 3.B**).
- *HER2* amplification was observed in one epithelial HER2+ CTC at draw 3 and draw 6, 10 epithelial HER2- CTC at draw 6.
- CTCs were observed to have differing *HER2* amplification status within individual clusters (**Figure 3.D**).
- Donor was previously determined HER2 negative through IHC of biopsy, however, results of liquid biopsy testing and staining with ANGLE's HER2 assay show HER2 positivity in CTCs.

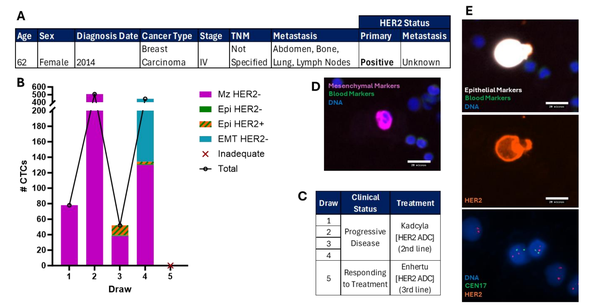

Figure 4. Patient case study 2. (A) Table shows patient's age, sex, diagnosis date, cancer type, stage and TNM classification, metastases locations and HER2 status of primary tumor and metastatic sites. **(B)** Number and IF phenotype of CTCs observed in each blood draw, processed as per workflow in **Figure 1.** **(C)** Table shows clinical status and treatment received by MBC patient at each blood draw. **(D)** Single mesenchymal (HER2-) CTC imaged post-IF (Nuclear stain [DAPI] in blue, Blood lineage markers [FITC] in green, Mesenchymal markers [Cy7] in magenta). **(E)** Cluster of two epithelial HER2+ CTCs imaged post-IF (top; Nuclear stain [DAPI] in blue, Epithelial markers [Cy5] in white, Blood lineage markers [FITC] in green) (middle; HER2 [Cy3] in orange) and post-FISH (bottom; Nuclear stain [DAPI] in blue, CEN17 control foci [FITC] in green, *HER2* foci [Cy3] in orange). CTCs were *HER2* non-amplified, CEN17:*HER2* foci ratio 2:2.

- *HER2* amplification observed in 36 mesenchymal CTCs at draw 2 (**Figure 4.B**) before first observation of HER2 protein in CTCs at draw 3.
- *HER2* amplification was not observed in CTCs with overexpression of HER2 protein.
- Blood samples at draw 5 failed to separate through Parsortix (**Figure 4.B**), exceeding the allowed timeframe for separations as part of the clinical study.
- **Patient died** approximately 8 months after first observation of epithelial HER2+ CTCs in draw 3 (**Figure 4.B**), despite the clinical status of the patient changing to "Responding to treatment" at the 5th draw.

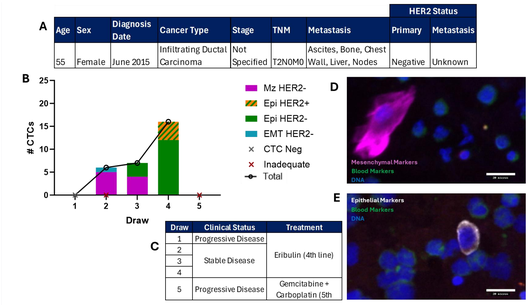

Figure 5. Patient case study 3. (A) Table shows patient's age, sex, diagnosis date, cancer type, stage and TNM classification, metastases locations and HER2 status of primary tumor and metastatic sites. **(B)** Number and IF phenotype of CTCs observed in each blood draw, processed as per workflow in **Figure 1.** **(C)** Table shows clinical status and treatment received by MBC patient at each blood draw. **(D)** Single mesenchymal (HER2-) CTC imaged post-IF (Nuclear stain [DAPI] in blue, Blood lineage markers [FITC] in green, Mesenchymal markers [Cy7] in magenta). **(E)** Single epithelial HER2- CTC imaged post-IF (Nuclear stain [DAPI] in blue, Epithelial markers [Cy5] in white, Blood lineage markers [FITC] in green. All CTCs observed in samples of this donor were *HER2* non-amplified.

- *HER2* amplification was not observed in any CTCs at any blood draw .
- Staining of Parsortix separated samples using ANGLE's HER2 assay showed transition in CTC phenotypes across draws from EMT + mesenchymal, to epithelial + mesenchymal, to epithelial only phenotype with concomitant detection of HER2+ CTC (**Figure 5.B**).
- These results show a change in HER2 status of CTCs over time, not consistent with the status at initial diagnosis.
- Donor was alive at the point of last follow up and a 6th blood draw is expected to be received.

Conclusions

This study outlines the analytical validation of ANGLE's combined IF and FISH assay able to identify CTCs based on IF staining of epithelial, mesenchymal and HER2 proteins and examine amplification of *HER2* gene through FISH staining. The application of this combined HER2 assay to CTCs isolated from blood samples of MBC patient samples demonstrated its ability to provide critical up to date information about changing HER2 status of patients which would otherwise be unavailable through tissue biopsy alone. Additionally, the low concordance of HER2 protein overexpression (only observed in epithelial CTCs in this cohort) and gene amplification within MBC patient samples of this study aligns with previously reported data[1] and outlines the advantage of the dual analysis made possible by the combined IF and FISH assay.