Detection of DNA Damage in Circulating Tumor Cells Isolated using the Parsortix® system

Amina Mezni1, Rachel Downes2, Matthew Hardy1, Marioicristina Ciccioli1, Steven Gross2, Anne-Sophie Palhes-Jimenez2

1ANGLE Europe Limited, 2 Occam Court, Surrey Research Park, Guildford, Surrey GU2 7QR United Kingdom
2ANGLE North America Inc., 5100 Campus Drive, Suite 120, Plymouth Meeting, PA 19462

Introduction

Phospho-pKAP1 (pKAP1) and gamma-H2AX (γ-H2AX) are predictive biomarkers used to assess DNA damage response (DDR) and repair in cancer cells. Expression of these markers has been associated with DNA double strand breaks in many cancers, with the potential to measure the efficacy of DNA-targeting cancer therapies (Figure 1). Liquid biopsy, including Circulating Tumor Cells (CTCs), offers repeated, real-time and minimally invasive samples for monitoring of DNA damage therapies and disease progression. ANGLE has developed a Research Use Only (RUO) workflow for the identification of DNA Damage on epithelial and mesenchymal CTCs. This was achieved by combining the Parsortix® technology, an epoxide-independent microfluidic device that isolates and harvests CTCs from blood based on their size and deformability, with an ANGLE immunofluorescence (IF) assay for the detection of Epithelial, Mesenchymal and transitioning CTCs and DNA Damage status, by targeting pPhospho-pKAP1 and γ-H2AX. In this study, these assays were assessed as well, as the feasibility of detecting DDR signals in CTCs isolated from cancer patient samples. Research Use Only. Not for use in diagnostic procedures.

Workflow

- Blood samples from 36 healthy volunteers collected into Streck Cell-Free Blood Collection tubes (BCTs) and spiked with DNA-Damage induced H226 or MCF7 cells were used for analytical verification. Samples were processed as per workflow in Figure 2 and used to assess analytical sensitivity, specificity, concentration response and linearity of the assay.
- Clinical samples from 24 Triple Negative Breast Cancer (TNBC) patients, on a variety of treatment programmes, including platinum treatment, were collected into Streck Cell-Free DNA BCTs and processed as shown in Figure 2. Blood samples were processed at 96 hours post draw and stained using the ANGLE IF Epithelial-to-Mesenchymal transitioning (EMT) assay combined with DNA damage markers.

 Cancer Cell lines treated with Etosopse for 3 hours
 Cells detached by fixed in 4% Formaldehyde Cells spiked from healthy volunteers collected into Streck Cell-Free DNA tubes
 Collection and shipment of TNBC patients blood samples into Streck Cell-Free DNA tubes
 Processing on Parsortix® PLS System Capture of cells in the separation cassette based on their size and deformability
 Blood separation on Parsortix® System and or Parsortix® PLS System up to 120 hours post collection
 Harvest and cytoprint onto a cytology slide
 Immunizing using ANGLE IF assay:
 Staining using ANGLE IF assay:
 Epithelial markers (IF) + Mesenchymal markers (CTCs + Blood damage marker (Cy3): H2AX, γH2AX) + Nuclear dye (DAPI)

Figure 2. Schematic representation of the assay workflow. For analytical samples, peripheral blood (7.5 mL) was drawn into Streck Cell-Free DNA BCTs from healthy volunteers and processed on Parsortix® systems at 120 hours post collection. Cell lines expressing epithelial and/or mesenchymal (EMT) markers were spiked for DNA damage, fixed with 4% formaldehyde and spiked into healthy volunteer blood sample. Donor samples from 24 TNBC patients (12 for each assay) and processed on Parsortix® systems at 96-hours post collection. Cells captured in the Parsortix® GENS Cell Separation Cassette due to their size and lower compressibility compared to other blood components are harvested, cytospun onto slides, and immunofluorescence stained for detection of epithelial and mesenchymal CTCs and DNA damage. Slides were imaged using a BioView Allegro Plus imaging system. CTCs were then identified for the presence of DNA damage signal, γ-H2AX positivity was identified mostly by the presence of a distinct nuclear focus and, occasionally, diffuse nuclear signal, while pKAP1 positivity was identified by the presence of a distinct diffuse nuclear signal.

Analytical Results

- Concentration response testing, to assess sensitivity of DDR assays at varied levels of DNA damage, demonstrated a proportional correlation between expression of DDR markers in treated cancer cells and increasing concentrations of Etosopse, as well as the ability to detect low levels of DNA damage (Figure 3 A-B).
- High sensitivity and specificity of DDR assays demonstrated in treated and untreated cancer cell models (Figure 3 C-D).
- In both DDR assays, a linear relationship was found between the number of harvested and spiked cancer cells over a range of 0-500 (Figure 3 E-F).

Figure 3. Analytical performance of the γ-H2AX and pKAP1 assays. Histograms show the mean and SEM of increasing (A) γ-H2AX foc expression and (B) pKAP1 expression in three replicate samples spiked with fixed cancer cells treated with increasing concentrations of Etosopse; summary table shows the mean sensitivity and specificity (C) for the γ-H2AX assay and (D) the pKAP1 assay from six replicate samples collected with fixed Etosopse treated or untreated cancer cells; graph shows linear regression analysis of (E) the γ-H2AX assay on eight replicate samples and (F) the pKAP1 assay on six replicate samples spiked with fixed Etosopse treated cancer cells over a range of 0-500 cells.

- 67-75% of the TNBC patients showed at least one CTC, with a range of 0-507 CTCs and a mostly mesenchymal phenotype (Figure 4).
- 75% of the TNBC patients showed at least one CTC cluster, with a range in clusters number per donor of 0-79 and a range in size of cluster of 2-68 CTCs (Figure 4).
- 4/9 (44%) CTC-positive donors processed with the γ-H2AX assay showed either nuclear foci or nuclear diffuse signal (Figure 4).
- 3/8 (38%) CTC-positive donors stained with the pKAP1 assay showed diffuse nuclear or peripheral signal (Figure 4).

Figure 4. Detection of CTCs and DNA damage in TNBC samples. (A) Summary table shows number of donors included for each assay (N) and percentage (D) with δ±1 CTC, range, mean and median of CTC captured across donors, donor CTCs phenotypes, number and percentage of donors with δ±1 CTC cluster, range of clusters per donor, range of CTCs per cluster, N and % of donors with δ±1 CTC with DNA damage; (B) dot plot median ±95% Confidence Interval (CI) of the total number of CTCs identified in each donor for two 7.5 mL BCTs with each assay, representative images of: (C) a cluster of transitioning CTCs (Left), a cluster of mesenchymal CTCs (Middle) and a large cluster of mesenchymal CTCs (Right); (D) pKAP1 nuclear signal in mesenchymal CTC cluster; (E) γ-H2AX nuclear foci signal in a single mesenchymal CTC, Colors: FITC (apoptotic markers in green), Cy5 (mesenchymal markers in Magenta), Cy3 (blood lineage markers in white), DAPI (nuclear staining in blue), Cy5 (DNA damage markers) in orange.

Conclusions

- Analytical verification of the assays demonstrated linear, highly sensitive and highly specific results for both target markers. The concentration response data indicates a high sensitivity for detecting lower levels of DNA damage.
- ANGLE IF assays can detect CTCs in TNBC patient samples with a positivity rate observed in this study of 67-75% and a range 0-507 CTCs.
- ANGLE demonstrated the ability to detect both γ-H2AX and pKAP1 in CTCs isolated from patient samples and processed through the Parsortix® system (DNA damage rate of 44% and 38% respectively). Applied to a clinical setting, this workflow would allow for minimally invasive monitoring of DNA damage targeting therapies.