Saliva Preparation for Rare Tumor Cell Analysis Workflow

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Abstract

Circulating tumor cells (CTCs) are a valuable cancer diagnostic tool due to their easy access in bodily fluids and their role in metastasis (Micalizzi et al. 2017). While CTCs have been extensively studied in some solid tumor cancers, their study in head and neck squamous cell carcinoma (HNSCC) presents unique challenges due to their low prevalence, heterogeneous morphology, and reduced EpCAM expression (Lindgren et al. 2017). This study investigates the use of saliva as a liquid biopsy for capturing CTCs and other rare cells in HNSCC, focusing on overcoming these challenges using a size-based enrichment method. The approach may provide a better method for detecting and characterizing CTCs in HNSCC, potentially enabling earlier detection of HNSCC CTCs and more effective monitoring of treatment response.

Introduction

CTCs serve as a valuable biomarker in liquid biopsy (LBx) owing to their detectability in accessible bodily fluids, including blood, urine, and saliva, and their well-established role in metastatic progression (Micalizzi et al. 2017). CTC analysis provides critical insights into cancer mechanisms, and CTCs have been extensively studied in various solid tumor types, including breast, colorectal, and lung cancers, for precision treatments (Mendelaar 2021, Alix-Panabières and Pantel 2021).

However, CTC detection and characterization in HNSCC have proven more complex. Challenges such as low CTC yield, heterogeneous cell morphology, and reduced EpCAM expression limit the effectiveness of standard enrichment methods (Lindgren et al. 2017).

This study demonstrates that saliva-based liquid biopsy can effectively capture CTCs and other rare cells in HNSCC by leveraging a more direct source of CTCs, owing to their proximity to tumor sites. Additionally, size-based enrichment shows promising results in overcoming challenges such as morphology variability, CTC heterogeneity, and variable EpCAM expression. Together, these factors position this approach as a promising strategy for overcoming the challenges of CTC analysis in HNSCC.

Methods

Instruments

The Genesis Cell Isolation System (Bio-Rad Laboratories, Inc., catalog #12019822) was utilized for cell isolation. Samples were processed for centrifugation using the Sorvall ST 8 Small Benchtop Centrifuge (Thermo Fisher Scientific Inc., #75007200) and the Centrifuge 5810R, 15-amp version (Thermo Fisher, Eppendorf SE, #022625501). The BioTek Lionheart LX Automated Microscope (Agilent) was used for imaging, equipped with optics for 4x magnification and the following filters: tetramethylrhodamine (TRITC, 554 LED 1225012 Rev B), phycoerythrin (PE, 465 LED 1225001 Rev N), 4',6-diamidino-2-phenylindole (DAPI, 377/447 1225100 Rev N), green fluorescent protein (GFP, 469/525 1225101 Rev O), cyanine 5 (Cy5, 628/685 1225105 Rev O), and acridine orange (AO, 469/647 1225109 Rev L). The imaging system was operated using BioTek Gen5 Software, version 3.15 (Agilent).

HNSCC Panel

The antibodies listed in Table 1 were used for the HNSCC panel. Each antibody was diluted from its stock solution as recommended in each assay protocol.

Table 1. Antibodies for head and neck cancer panel.

Antibody	Channel	Catalog #	Company
Pan Cytokeratin Monoclonal Antibody (AE1/AE3)	AF488	53-9003-82	Thermo Fisher Scientific Inc.
Vimentin (V9)	AF546	sc-6260	Santa Cruz Biotechnology, Inc.
Human E-Cadherin Alexa Fluor 700-Conjugated Antibody	AF700	FAB18381N-100UG	Bio-Techne
Mouse Anti-Human CD45	AF647	MCA87A647	Bio-Rad Laboratories, Inc.
DAPI	UV	sc-3598	Santa Cruz
Human PD-L1/B7-H1 PE-Conjugated Antibody	PE	FAB1561P	Bio-Techne

AF, Alexa Fluor; DAPI, 4',6-diamidino-2-phenylindole; PD-L1, programmed death-ligand 1; PE, phycoerythrin; UV, ultraviolet.

Saliva Collection

For samples, 3–5 ml of saliva was collected in a 50 ml Falcon tube and immediately placed on ice until processing within 2 hours. Patients were instructed to allow saliva to accumulate on the floor of the mouth for 5 min before spitting into the collection tube.

Saliva Procedure

Each saliva sample was first diluted with 1x phosphate buffered saline (PBS) to a final volume of 5 ml. An additional 5 ml of 1x PBS was added, bringing the total volume to 10 ml, and the sample was gently mixed by pipetting up and down 10 times. The sample was then centrifuged at 2,000 rpm for 5 min (brake set to 9), and the supernatant was carefully discarded without disturbing the pellet. The pellet was resuspended in 20 ml of 1x PBS and vortexed thoroughly to ensure a uniform mixture with no clumps. For analysis, a 1:20 dilution was prepared by mixing 1 ml of the resuspended cell mixture with 19 ml of 1x PBS. Some samples may require only a 1:10 dilution, depending upon initial sample viscosity. Finally, the sample was split using 10 ml for enumeration, and the remaining 10 ml was stored at 4°C for enrichment.

CTC Enumeration Using the Genesis System and Celselect Slides™ 2.0

CTC enumeration was performed using the Genesis Cell Isolation System (Bio-Rad, #12019822) with the Celselect Slides Enumeration Direct Stain Kit 2.0 (Bio-Rad, #17009841). Depending on the staining method, either the Celselect Slides Enumeration Direct Stain Kit 2.0 (bulletin 3339) or Celselect Slides Enumeration Indirect Stain Kit 2.0 (bulletin 3338) can be followed.

In brief, the Genesis Cell Isolation System first underwent a self-test to ensure optimal functioning. The system was then primed and assembled according to the manufacturer's instructions. The antibodies outlined in the HNSCC Panel section were incorporated into the reagent's cartridge before loading. The prepared saliva sample at its final dilution concentration was loaded directly into the sample funnel without further dilution with PBS. Cells were then captured on the Celselect Slides with the Genesis System. After cell capture, analysis was performed using the BioTek Lionheart LX Automated Microscope with the appropriate optical setup and fluorescent filters as noted in the Instruments section. Images were captured and analyzed using BioTek Gen5 Software, version 3.15, providing a detailed enumeration of the captured rare cells.

CTC Enrichment Using the Genesis System and Celselect Slides 2.0

CTC enrichment was performed using the Genesis Cell Isolation System (Bio-Rad) with the Celselect Slides Enrichment Kit 2.0 (Bio-Rad, #17009455), following kit guidelines. In brief, the Genesis Cell Isolation System first underwent a self-test to ensure proper functionality. The system was then primed and assembled according to the manufacturer's instructions. The sample prepared in the Saliva Procedure section was loaded directly into the sample funnel without further dilution with PBS. After CTCs were captured on Celselect Slides using the Genesis System, the cell populations were captured from the Celselect Slides by reverse flow, as described in the system's guidelines.

Results

CTC numbers from a patient with HNSCC, collected at progressive stages of the disease (S1–S4), are listed in Table 2. Captured cells were enumerated using Gen5 Software on the BioTek LionHeart LX Automated Microscope. CTC count increased as samples were collected longitudinally. Specifically, a noticeable increase in CTC numbers was observed from the early to later stages of the disease, with the highest counts observed at S4 (Table 2.)

CTCs were identified by positive staining for DAPI, pan-cytokeratin, and vimentin, negative staining for CD45, and circularity (aspect ratio) analysis. Staining for E-cadherin and programmed death-ligand 1 (PD-L1) showed variability across the captured CTC population (Figures 1A and B), indicating heterogeneity within the CTC population. White blood cells (WBCs) displayed strong CD45 and DAPI staining but lacked tumor-specific markers, as expected, demonstrating the ability to distinguish CTCs from WBCs in saliva clinical samples (Figure 1).

Table 2: Saliva enumeration results.

Sample Number	CTC Number
S1	1,169
S2	1,355
S3	1,529
S4	2,870

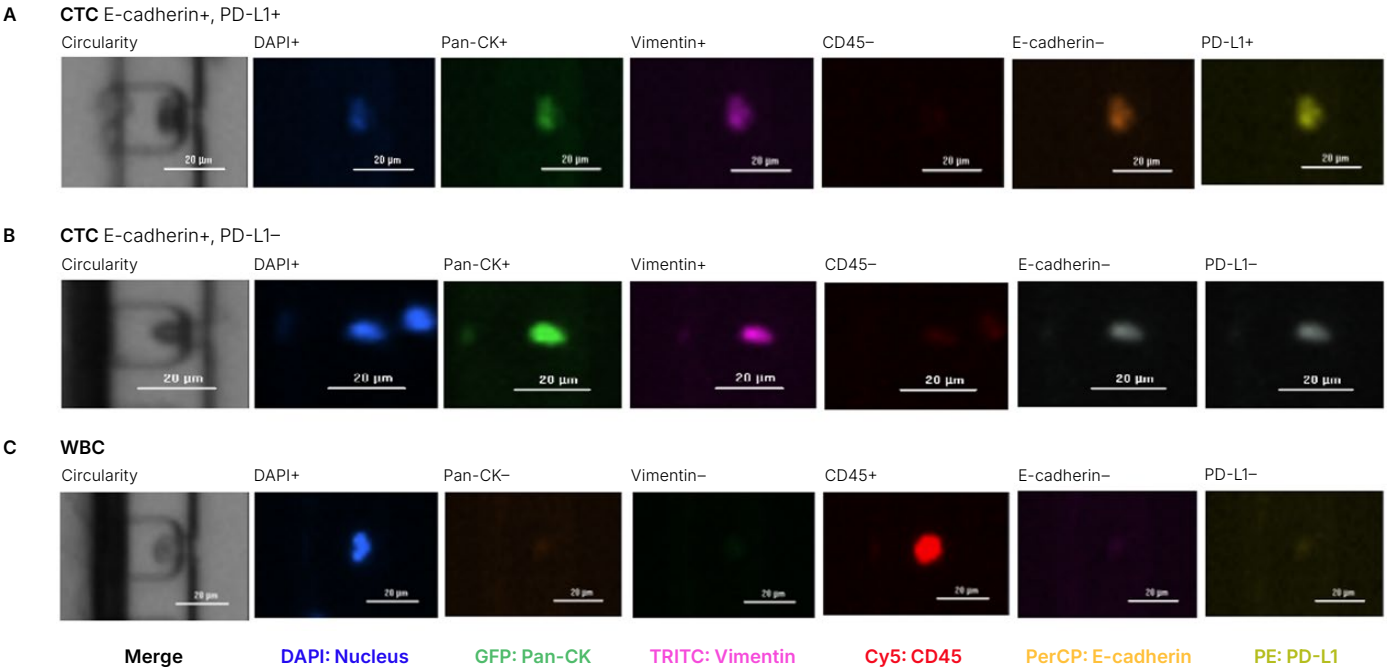

Fig. 1. Staining and imaging of captured cells from a patient with metastatic head and neck squamous cell carcinoma (HNSCC). 3 ml of saliva was processed following the standard enumeration protocol and then stained to identify circulating tumor cells (CTCs) and white blood cells (WBCs). Potential CTCs (**A**, **B**) display variable staining for numerous markers (E-cadherin, PD-L1), indicating heterogeneity in the population, while maintaining consistent staining for DAPI, pan-cytokeratin, and vimentin. Example images for CTCs and WBC: **A**, CTC: DAPI+, pan-CK+, vimentin+, CD45–, E-cadherin+, PD-L1+; **B**, CTC: DAPI+, pan-CK+, vimentin+, CD45–, E-cadherin–, PD-L1–; **C**, WBC: DAPI+, pan-CK–, vimentin–, CD45+, E-cadherin–, PD-L1–. Cy5, cyanine 5; DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein; pan-CK, pan-cytokeratin; PD-L1, programmed death-ligand 1; PE, phycoerythrin; perCP, peridinin chlorophyll protein; TRITC, tetramethylrhodamine.

Discussion

The protocol outlined above describes an easy-to-use, efficient method for enriching and enumerating CTCs shed from individuals with HNSCC, utilizing saliva as a liquid biopsy CTC source. Saliva offers a convenient alternative to traditional liquid biopsy samples, providing easier access to larger volumes of material.

Preliminary data indicate that CTC counts per milliliter in saliva are higher than typically observed in other liquid biopsy samples, such as blood and urine, from individuals with HNSCC (Gao et al. 2021), which, combined with the larger volumes of saliva available, make saliva an easily accessible and effective source to study HNSCC. Using size-based selection with the Celselect Slides method compares favorably to other established liquid biopsy CTC capture methods that rely on surface markers such as EpCAM which are less effective for isolating CTCs in HNSCC.

Differential staining of established CTC markers (DAPI, pan-cytokeratin, and vimentin) compared to markers for WBCs allows for the differential enumeration of HNSCC-associated CTCs and residual WBCs typically found in saliva. Additional markers for cell type or disease state, such as PD-L1 and E-cadherin, can be incorporated into the enumeration protocol to identify further CTC subtypes.

Some evidence suggests that the size selection of rare cells using the Genesis System facilitates the capture of exosomes and extracellular vesicles in parallel to CTCs. Therefore, downstream analysis of enriched samples may require additional methods to discriminate and identify these individual subcellular components of saliva separately from CTCs. Suitable downstream methods include Droplet Digital™ PCR (ddPCR™), reverse transcription quantitative PCR (RT-qPCR), and next-generation sequencing (NGS), as outlined in bulletins 3686, 3599, and 3555 (Bio-Rad).

CTC monitoring is an established liquid biopsy method for several cancer types, including HNSCC. However, CTC detection can be time-consuming and impacted by cell surface marker heterogeneity in HNSCC (Pantel, 2019). The above method describes a simple and relatively rapid method using size-based separation of CTCs from other cell types in saliva. This method could be applied for early detection of HNSCC or longitudinal monitoring of treatment response or disease progression.

References

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