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Abstract:

Background: Circulating tumour cells (CTCs) detected in patient blood samples are relevant as diagnostic and prognostic markers offering insights into tumour behaviour and guiding treatment of cancer at an individualised level. The aim of this study is to ascertain the feasibility of detecting CTCs in oral squamous cell carcinoma (OSCC) using two different methods so as to determine the optimal method for the study of this cancer.

Methods: Comparison of the numbers of CTCs, circulating tumour micro-emboli (CTMs) and circulating tumour endothelial cells (CTECs) was undertaken in forty clinical samples of oral squamous cell carcinoma (OSCC) determined by filtration (ISET®) and in-situ fluorescent immunostaining (i-FISH, Cytelligen®) immunostaining and in-situ hybridisation.

Results: i-FISH detected CTCs in 80% of samples compared to 40% of samples analysed by microfiltration. i-FISH detected CTCs in a further 40% of samples in which microfiltration did not detect CTCs. No CTC clusters were detected by microfiltration while i-FISH detected CTM in 12.5% of samples. i-FISH analysis detected CTECs in 20/40 samples.

Conclusion: These results highlight significant differences in detection of CTCs, CTM and CTECs between i-FISH and microfiltration when applied to OSCC samples, suggesting that technologies capable of detecting circulating aneuploidic cells more accurately detect CTCs. i-FISH also detected CTM and CTEC not detected using ISET®. With proven prognostic relevance in adenocarcinomas, accurate enumeration of CTCs, CTMs and CTECs may be a clinically useful tool in the management of OSCC and may aid in the reduction of false negative diagnoses.

Keywords: circulating tumour cells, oral cancer, ISET, Fluorescence In Situ Hybridization

Introduction

Biomarkers are currently utilised as aids in diagnosis, prognostication and monitoring of many patients with cancer. In addition to readily available biochemical parameters, malignant cells and cellular remnants (eg: circulating tumour DNA and RNA) present in peripheral blood samples are being investigated as cancer biomarkers [1]. In this regard, circulating malignant cells (CMCs) have the advantage of delivering viable cancerous cells for analysis. CMCs include single circulating tumour cells (CTCs), circulating tumour micrometastases (CTMs) and circulating tumour endothelial cells (CTECs).

Considerable evidence has established CMCs as an independent marker in several forms of cancer for which there are many ongoing CMC-based clinical trials which results are pending [2]. Most evidence pertaining to CMC is based on analysis of adenocarcinomas and comparatively little data is available relating to oral squamous cell carcinoma (OSCC) [3].

Many methods of CTC detection and enumeration are currently available. Most techniques are based on the presence of epithelial cell adhesion molecule (EpCAM) expressed on the cell membrane to identify malignant cells as EpCAM is not expressed on bloodbourne cells. The only method of CTC detection certified by the US Food and Drug Administration (FDA) is CellSearch® which requires a cell to be EpCAM +ve, cytokeratin +ve (CK) and CD45-ve to be included as a CTC. There are however a number of significant limitations with such methods. Over reporting may occur due to antibody cross-reactions or when white blood cells (WBC) that have ingested fragments of EpCAM+ve CTCs are reported as false positives [4,5]. Under-reporting may result when EpCAM-ve CTCs escape detection, for example when clusters of CTCs are counted as one CTC or failure to detect those CTCs undergoing epithelial-mesenchymal transition (EMT) which do not express EpCAM [6].

To avoid such limitations, filtration methods are based on the premise that CTCs are larger than normal blood cells [6]. A micro-filtration technique, namely Isolation by Size of Epithelial Tumour cells (ISET®) method retains objects larger than the 8 μm pore size which are then stained and examined for the cytological characteristics of malignancy, which renders this method dependent on a cytopathologist [7,8]. While overcoming the issues of CTC detection by epitope labelling, filtration methods are also subject to inaccuracies. Under-reporting may occur when

pores become blocked leading to incorrect reporting of larger cellular structures as CTCs or when small CTCs pass through the pores of the filters [9].

More recently, it has become possible to detect malignant circulating cells on DNA aneuploidy - the hallmark of cancer - using in-situ hybridisation [10,11]. One such method, Cytelligen®, uses negative selection in that WBCs are selected for and discarded, leaving a sample rich in tumour-derived cells that is then subjected to fluorescent immunostaining (SE-FiSH or i-FISH) to identify aneuploidy and non-synonomous single nucleotide variants (SNVs). Cells with aneuploidy of chromosome 8 are further categorised using fluorescent immunostaining enabling sub-typing into CTCs, circulating tumour micro-emboli (CTM, also called clusters) and circulating tumour endothelial cells (CTECs) [10,12].

Of available studies reporting CMCs in oral OSCC, most have used epitope labelling (EpCAM) with only very limited information (one case report [13], and one case series of five samples [14]) documenting the efficacy of microfiltration of this pathology. No previous study have utilised i-FISH to detect CTCs in patients with OSCC, nor previous comparison of filtration with i-FISH in patients with OSCC.

With the future aim of determining the relevance of CMCs in OSCC, this study aimed to firstly determine the feasibility of CMC detection using microfiltration and SE-i-FISH methods and secondly, by comparison of the numbers and types of CMCs detected by each method to determine the optimal method to be employed in future studies.

Methods and Materials

Patients

Following ethical approval granted by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (Reference number UW 18-611), from September 2020 to February 2021, ten patients with histologically confirmed oral cavity cancer treated at Queen Mary Hospital, Hong Kong were prospectively tested for the presence of circulating tumour cells (CTCs) at four different timepoints; at baseline (just before receipt of anaesthesia for surgical resection of tumour), during surgery at the point when the surgeon declares the tumour has been resected, and on post-operative days one and seven. All patients gave written informed consent in order to participate in the study.

Sample collection and CTC enumeration

Ten millilitres (ml) of peripheral blood was collected in EDTA tubes to isolate CTCs by Isolation by Size of Tumour Cells (ISET®) following manufacturer's instructions [7,8]. Blood samples (10 ml) were processed within 4 hours of collection, diluted 1:10 in an erythrocyte-lysis buffer and filtered on the ISET® device. The filter module consisted of a membrane with 10 wells making it possible to process 10ml of blood from each patient. After filtration, membranes were washed with phosphate buffered saline, disassembled from the filtration module, allowed to air-dry overnight and stored at −20 °C until staining. The filter was thawed and then stained using haematoxylin and eosin (H&E, five minutes with each stain and rinsing in between). To avoid blood contamination by epithelial cells of the skin, all blood samples were obtained after the first 4ml of blood was discarded.

Images obtained by the ISET® system were reviewed by a scientist (SWC) and a cytopathologist (from Rarecells Diagnostics) who had received the appropriate training in image interpretation□ [2] for the definition of CTCs.

A further 10 ml of peripheral blood was collected in ACD solution B anticoagulant tubes to isolate CTCs, circulating tumour cell clusters also known as circulating tumour cell microemboli (CTMs), and circulating tumour derived endothelial cells (CTECs) by i-FISH following manufacturer's instructions. Briefly, 7.5 ml of blood was centrifuged at 800× *g* for 7 min and the

supernatant containing plasma and platelets was removed and discarded. The pellet was then mixed with separation matrix (Cytelligen, San Diego, CA, USA) and further centrifuged at 450× *g* for 7 min. Immuno-magnetic beads[labelled as needed] (Cytelligen) were added to the supernatant and incubated at room temperature for 20 min to remove the remaining blood cells by centrifugation and magnetic separation (Cytelligen). After two more centrifugation cycles, the pellet were labelled with CD31 [3], CD45 and vimentin□ [4] antibodies. Ten µL chromosome enumeration probes 8 reagent (CEP-8, Abbott, Downer's Grove, IL, USA) was added to the cells and stained with 4′,6-diamidino-2-phenylindole (DAPI [5], Cytelligen) and then observed under a fluorescence microscope. Blood was processed for CTC enumeration within 48 hours of blood taking. White blood cells were stained CD45+, DAPI+ but CTCs were stained CD45− DAPI+. Cells expressing vimentin is an indication of epithelial-mesenchymal transition. Cells expressing CD31+ are indicative of being tumour derived endothelial cells.

Results

The demographic and clinico-pathologic information of the ten patients are given in Table 1. There were nine males and one female in this group, with a mean (SD) age of 68.8 (8.52) years, all of whom were still alive at the project census. Only one patient had a history of cancer (not in the oral cavity). The number of CTC, CTM, and CTEC enumerated using i-FISH as well as the CTCs enumerated using ISET® at each timepoint are given in Table 2. The total number of CTCs isolated using ISET® is consistently lower than the number of CTCs isolated using i-FISH. At baseline, a total of 39 CTCs were found in the ten patients using i-FISH versus nine CTCs using ISET®, at the tumour-out timepoint, a total of 50 CTCs were isolated using i-FISH versus 14 using ISET®, at post-surgical day 1, 64 CTCs were isolated using i-FISH versus 16 using ISET® while at post-surgical day 7, 119 CTCs were isolated using i-FISH versus 30 using ISET® (figure 1). Only around 25% of the CTCs could be isolated from a sample using ISET® compared to when using i-FISH.

Discussion

This prospective comparison study of the number of CTCs enumerated by the ISET® and i-FISH methods for ten patients with OSCC demonstrates considerable differences.

In this study using forty clinical samples of OSCC, chromosomal aneuploidy via i-FISH detected more CTCs, CTMs and CTECs than ISET® microfiltration. Since all of these circulating malignant cells are crucial in cancer biology, their accurate detection is necessary to enable valid assessment of the prognostic relevance of these findings in the clinical setting.

Circulating tumour cells

Both methods were able to detect CTCs in this series of patients with OSCC however there was considerable difference in number and type of cells detected.

ISET® detected CTCs in 40% of the samples which compares favourably to the 12.5% detected by the FDA approved EpCAM-dependant CellSearch® method in a similar patient population [15]. All the samples reported positive for CTCs by ISET® were also reported positive by Cytelligen® (concordant). However, i-FISH analysis detected CTCs in a further 40% of samples reported as devoid of CTCs by ISET® analysis (discordant). This means that ISET® analysis alone would not have detected CTCs in 40% of the test population. This signifies the i-FISH method has greater sensitivity in detecting CTCs than ISET®.

Although filtration methods offer advantages overcoming the limitations of detecting EpCAM-ve CTCs faced by epitope labelling methods, the results of this study reveal a significant shortfall in the detection of CTCs using the ISET® filtration method compared to detection using i-FISH [8]. This difference may be due to the detection of aneuploidic CTCs smaller than the 8 μm pores of the ISET® filter [6]. This population of small CTCs is likely to include cells undergoing epithelial mesenchymal transition (EMT) which tend to be small and thus may escape detection by filtration and additionally confirmed by the mesenchymal marker vimentin□ [2] used in the i-FISH Cytelligen method [16]. Although the presence of small CTCs has prognostic significance in gastric carcinoma, the significance of such smaller CTCs in OSCC is currently unreported [3,17].

While various biomarkers have gained routine use in management of a number of cancers, there is an absence of clinically useful biomarkers for OSCC capable of detecting early disease, relapse or disease progression. Current methods of detection and monitoring for OSCC require the

presence of macroscopic disease and despite regular surveillance, cancer recurrence occurs in apparently disease free patients months or years after treatment [18]. And yet, in OSCC, as well as in HNSCC more generally, the presence of CTCs has been found to be extremely specific for locoregional relapse and overall survival [15,19,20]. Early detection of CTCs and particularly detection of CTMs may portend the establishment of macroscopic tumour deposits prior to detection by existing imaging techniques [21,22].

These findings accentuate both the significance of CTC detection in OSCC and that the accurate detection of CTCs is imperative to unravel their potential role in patient management.

Circulating Tumour Micro-emboli

With an estimated metastatic potential 23- to 50-fold that of a single CTC and ability to establish independent viable colonies, CTMs are viewed as the harbingers of metastatic disease [23].

In this study no CTMs were detected using ISET® while Cytelligen® i-FISH analysis detected CTMs in 12.5% (5/40) of samples. This discrepancy is significant and surprising since ISET® microfiltration has been reported to detect CTMs. Most of these reports have analysed samples arising from either cell lines or patients with adenocarcinoma, so the difference may be due to differences in the physical characteristics of the CTC clusters in OSCC compared to those in adenocarcinoma.

Because of central role CTMs have in disease progression, the efficacy of CTC detection methods to detect CTMs must be assessed when comparing methods and a method's detection of CTMs is pivotal when considering potential clinical applications of the results provided. The discrepancy between the detection of CTMs between these two methods is a significant finding of this study.

Circulating Tumour Endothelial Cells

While mature circulating endothelial cells (CECs) are present in healthy donors and those with vascular damage, the presence of aneuploidy and CD31 forms the distinct subset of circulating tumour cells (CTECs) [24,25]. By protecting CTCs from ankoisis□ [4] CTECs facilitate metastasis [26]. CTECs have been found to be present in patients with cancer, including those with HNSCC [26].

CTECs were detected in 20/40 of samples following i-FISH analysis while it was not possible to detect CTECs using ISET® analysis. As a result of their rarity, the detection of CTECs presents similar challenges to the detection of CTCs [12]. These findings support earlier work demonstrating that tumour vascularisation is an important parameter in HNSCC biology [27]. CTECs are also involved in the process of metastasis. In a range of cancers, including HNSCC, detection of CTECs is indicative of progressive disease and is associated with poorer prognosis [28,29]. Changes in CTECs have been used to assess efficiency and guide chemotherapy dosing in advanced HNSCC [30].

Methodological Differences

There are major methodological differences between ISET® and i-FISH. With regard to sampling time, it is suggested that samples are processed using ISET® within four hours of blood-draw, whereas blood can be retained at room temperature for up to 48 hours before processing using i-FISH. Therefore, the i-FISH method allows for batch processing whereas samples must be processed nearly immediately upon sample collection when the ISET® method is used.

The ISET® method presents as an apparently simple method of CTC acquisition requiring mixing the blood sample with the commercial buffer to lyse red blood cells before applying to the filtration unit. However, several difficulties may occur during processing. Optimally, the blood will be filtered within a minute. However, processing of samples collected intra-operatively filtration was difficult. This may be a consequence of subtle changes in coagulation due to peri-operative management. Once the filter has dried, it can be stored at -20°C and batched for staining with H&E, or with immunohistochemical methods for the identification of CTCs. Identification of the stained cells via a light microscope is an extremely time consuming process and requires a trained scientist or cytopathologist. Attempts at applying articifical intelligence to read and recognise cells on this filter have not yielded promising results (authors' unpublished data) due to the myriad of debris which will exist on the filter, rendering the recognition of cells more difficult.

Although the i-FISH method is more flexible with regard to the time of processing after blood-draw, it is a time consuming and manually demanding method requiring many steps and

much training. The advantage of the i-FISH method is also why some clinicians choose not to use this method. Since the cells are stained using fluorescent dyes, slides can be scanned and cells counted automatically without humn intervention. However, because the cells are stained fluorescently, cellular components cannot be identified. Since cells on the ISET® filter are simply stained with H&E, it is possible for the cytopathologist and the clinician to clearly identify cellular structures and use personal discretion in the identification of CTCs instead of relying solely on cell surface protein expression or aneuploidy.

Conclusion

These results confirm that while both microfiltration and i-FISH methods can detect CMCs, detection of CMCs based on aneuploidy reveals the presence CMCs in greater numbers than microfiltration. Furthermore, sample analysis via i-FISH also enables detection sub-classification to enumerate both CTMs and CTECs. Overall, this study found that in clinical OSCC samples, i-FISH (Cytelligen®) analysis provided more detailed data than microfiltration (ISET®) for detecting CTCs, CTM and CTECs and appears to be the preferred method of CMC detection in this population.

Table 1: Patient Characteristics

Patient code	Sex	Age	Location	Overall Stage	Tumour Grading	Depth of Invasion (cm)	No. of metastatic lymph nodes
P-0A	M	72	Hard palate	Unknown	Unknown	0.81	No neck dissection
P-0B	F	78	Posterior tongue	Unknown	Unknown	Unknown	4
P-0C	M	68	Retromolar area	Unknown	Moderately differentiated	0.15	No neck dissection
P-0D	M	67	Soft palate	Unknown	Poorly differentiated	0.4	4
P-0E	M	66	Overlapping sites	4	Well differentiated	0.275	0
P-0F	M	77	Anterior tongue	4	Moderately differentiated	2.25	2
P-0G	M	80	Maxillary gingiva	Unknown	Moderately differentiated	Unknown	0
P-0H	M	55	Buccal mucosa	1	Well differentiated	0.22	0
P-0I	M	56	Posterior tongue	4	Unknown	0.28	1
P-0J	M	69	Hard palate	4	No tumour; Benign	0.24	0

Table 2: Circulating tumour cell numbers compared

Patient code	*i-FISH (baseline)*			*i-FISH (tumour-out)*			*i-FISH (post-op day 1)*			*i-FISH (post-op day 7)*			*ISET (baseline)*	*ISET (tumour-*	*ISET (post-op day 1)*	*ISET (post-op day 7)*
	CTC	*CTM*	*CTEC*	*CTC*	*CTM*	*CTEC*	*CTC*	*CTM*	*CTEC*	*CTC*	*CTM*	*CTEC*	*CTC*	*CTC*	*CTC*	*CTC*
P-0A	*8*	*0*	*0*	*5*	*0*	*0*	*15*	*2*	*0*	*15*	*0*	*3*	*4*	*0*	*5*	*3*
P-0B	*7*	*0*	*0*	*10*	*0*	*0*	*11*	*0*	*1*	*0*	*0*	*0*	*2*	*4*	*4*	*0*
P-0C	*5*	*0*	*1*	*14*	*0*	*12*	*2*	*0*	*0*	*10*	*0*	*1*	*0*	*5*	*0*	*4*
P-0D	*12*	*3*	*5*	*10*	*1*	*9*	*16*	*0*	*6*	*49*	*2*	*16*	*3*	*5*	*4*	*13*
P-0E	*1*	*0*	*0*	*0*	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
P-0F	*0*	*0*	*0*	*2*	*0*	*0*	*2*	*0*	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*
P-0G	*3*	*0*	*0*	*4*	*0*	*2*	*11*	*0*	*3*	*9*	*0*	*2*	*0*	*0*	*3*	*2*
P-0H	*2*	*0*	*1*	*4*	*0*	*0*	*4*	*0*	*1*	*1*	*0*	*0*	*0*	*0*	*0*	*0*
P-0I	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*3*	*8*	*1*	*0*	*0*	*0*	*0*	*3*
P-0J	*0*	*0*	*1*	*1*	*0*	*2*	*3*	*0*	*2*	*26*	*0*	*4*	*0*	*0*	*0*	*5*

CTC: Circulating tumour cells

CTM: Circulating tumour microemboli

CTEC: Circulating tumour derived endothelial cells

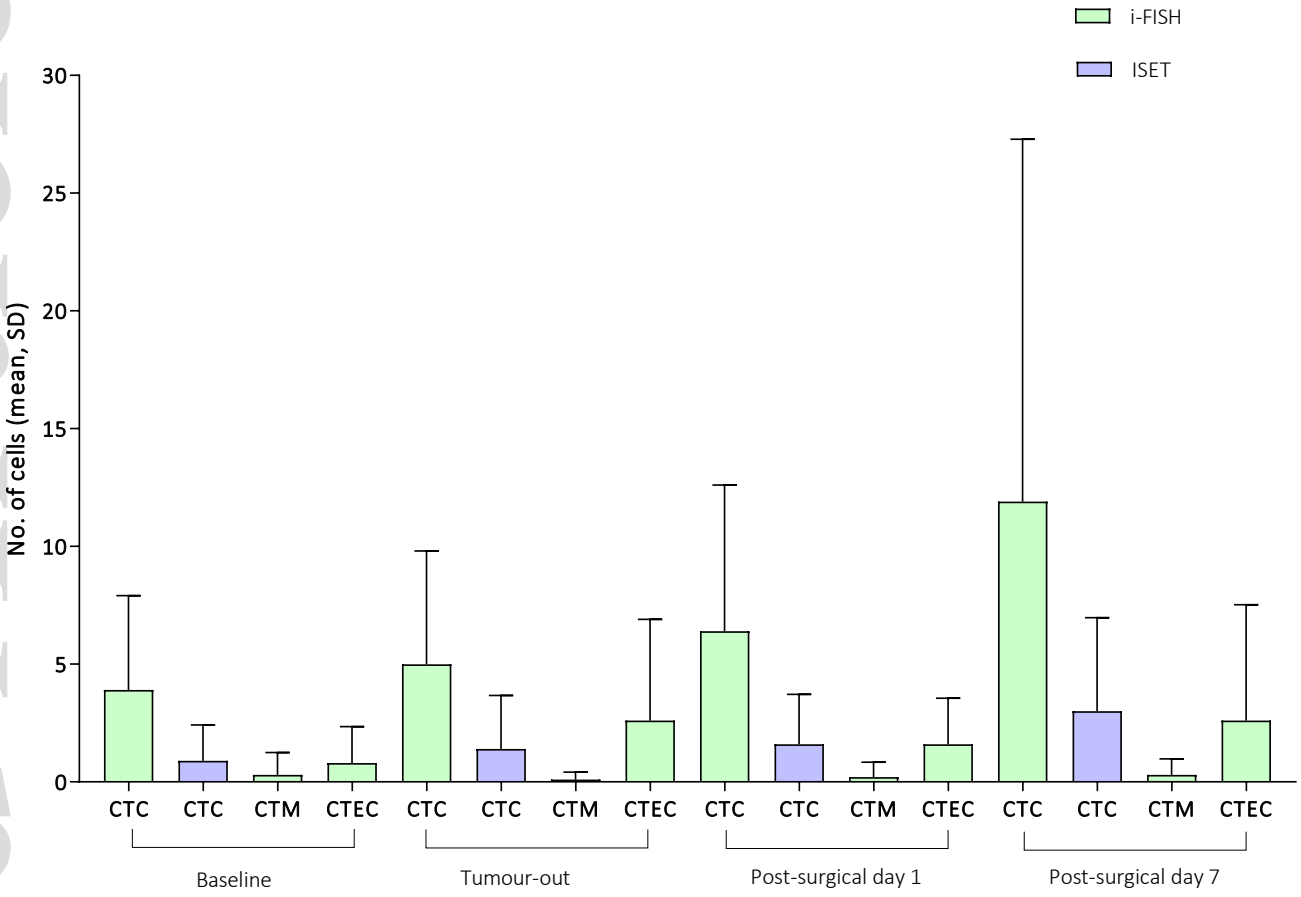

Figure 1: Comparison between CTC numbers enumerated using i-FISH and ISET and comparison of cells found in blood samples at different timepoints

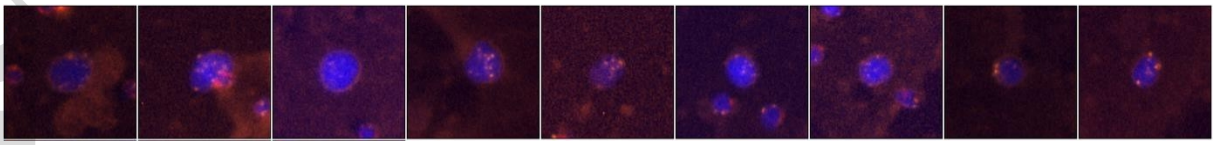

Figure 2: Images of CTCs isolated using i-FISH stained with CEP8 (red) and DAPI (blue)

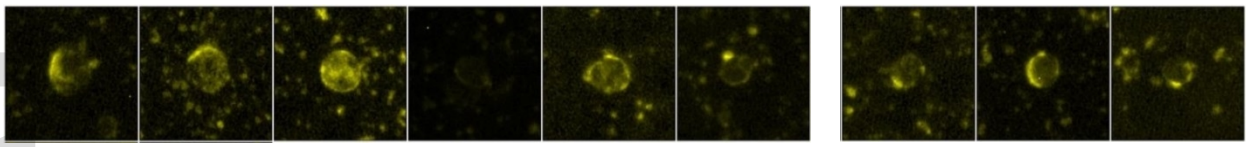

Figure 3: Images of CTECs using i-FISH stained CD31+ (yellow)

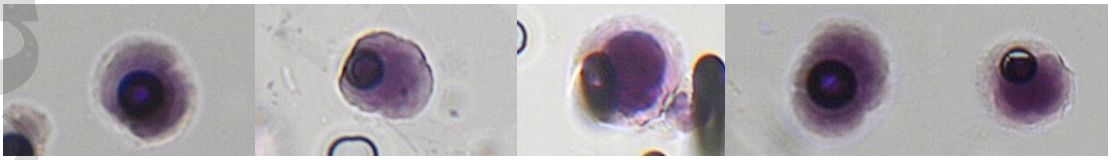

Figure 4: Images of CTCs using ISET stained with H and E

List of Abbreviations

ACD: Acid citrate dextrose

CTC: Circulating tumour cells

CTM: Circulating tumour microemboli

CTEC: Circulating tumour derived endothelial cells

CD: Cluster of Differentiation

EDTA: Ethylenediaminetetraacetic acid

H and E: Haematoxylin and eosin

Declarations

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