

Detection of Circulating Tumor Cells in Patients with Breast, Prostate, Pancreatic, Colon and Melanoma Cancer: A Blinded Comparative Study Using Healthy Donors

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Abstract

Cancer is a diverse disease characterized by abnormal cell growth and the ability to invade or spread to other parts of the body. Because the yearly cancer rate is increasing, an important area for cancer researchers is to improve the ability to detect and treat cancer early. The current study analyzes the potential of flow cytometry to be used to detect circulating tumor cells (CTCs) in patients with various cancer types and stages. CTCs are cells that have detached from the primary tumor and entered the blood stream in the process of metastasizing to other organs. To determine the accuracy of flow cytometry in detecting CTCs, a comparative study was performed on healthy donors. In this study, blood samples from patients with breast, prostate, pancreatic, colon and skin cancer were analyzed and compared with healthy donors. The data were collected and analyzed statistically with receiver operating characteristic curve analysis. The results indicate that CTCs can be detected in over 83% of the cancer patients and therefore may be a promising method for diagnosing cancer.

Keywords

Circulating Tumor Cell, Cancer Detection, Diagnosis, Flow Cytometry

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1. Introduction

Cancer is associated with high rates of mortality and morbidity worldwide and is the third leading cause of death after cardiovascular and infectious diseases [1]. Established metastatic lesions are the major cause of death in cancer patients, rather than the primary tumor. Scientists have made significant efforts to understand, treat cancer and detect it early; however, the specific mechanisms involved in carcinogenesis and metastasis are not fully understood [2]. It is known that metastasis involves tumor cells invading surrounding tissues and entering the blood or lymphatics [3]. Circulating tumor cells (CTCs) were first observed in 1869 by Thomas Ashworth and have since been of great importance in delineating the metastatic spread of carcinomas. These cells have detached from the primary tumor and flow into the blood or lymphatic circulation creating a secondary tumor [4]. The number of CTCs could be a predictive marker for cancer progression. However, as these cells are rare in cell population, their detection is important [5]. Researchers have developed several assays to detect CTCs for cancer diagnosis. One platform consists of EpCAM antibodies attached to magnetic beads, so the cells are isolated with a magnetic field. Another platform is based in gene expression profiling of CTCs, so the isolation is performed by targeting specific genes. The use of herringbone-chip, an enhanced platform for CTC isolation is also an alternative method for isolation. It is based on the increase of interactions between CTCs and the chip surface which is coated with EpCAM antibodies [6]-[8]. There is a controversy among the scientific community about the effectiveness of each method. The purpose of this study was to evaluate an alternative fluidic-based method to identify CTCs in the blood of cancer patients and to validate its reliability by also examining healthy individuals.

2. Materials and Methods

2.1. Sample Collection

Blood samples from 30 healthy individuals and 30 patients were collected in sterilized 50 ml falcon tubes (Corning, NY, USA) containing 7 ml 0.02M EDTA (Duchefa Biochemie B.V, Haarlem, The Netherlands) as an anticoagulant. Healthy individuals were identified as healthy or with non-malignant disease by their physicians. The cancer types and stages of the samples used include: Breast cancer, two patients with stage I, two patients with stage II, four patients with stage III and two patients with stage IV; prostate cancer, one patient with stage I, two patients with stage IV and two patients with a non-applicable stage; colon, one patient with stage III, two patients with stage IV and two patients with a non-applicable stage; pancreatic, four patients with stage IV and one with stage III; melanoma, three patients with stage III and two with a non-applicable stage. The quantity of blood collected was 20 ml in both healthy and cancer patients. The samples were cultivated on a roller for 30 minutes and then sent to the laboratory for analysis. The transit of the samples to the laboratory did not exceed 72 hours. The study was performed from January to April of 2015. The samples that were used were collected randomly among cancer and healthy samples, based only on the type of the cancer as well as the stage of the disease and then were analyzed blindly. The breast cancer samples were collected from women with an average age of 58 years old, while the prostate samples from men between 52 - 72 years old. The samples were received both from American and European countries.

2.2. Samples Stability

The period between transportation and analysis did not affect the experimental analysis. To ensure this, blood samples were collected from five healthy donors and placed in five different 50 ml falcon tubes. The tubes were then stored at 4°C, which is the temperature of transportation package. Each day, starting from 0 h, every sample from each donor, was tested by using molecular and cellular assays. In a time window of 0, 24, 48, 72 and 96 h of storage, the gene expression of many genes correlated with cell cycle, apoptosis, cytoskeleton, stemness, cytokeratins, growth factors, signaling transduction pathways etc were tested. The same procedure was performed with flow cytometry to study the protein level. Finally, the number of CTCs was measured for every sample each day. The data were analyzed and there was not observed any statistically significant difference among the different time periods. Concerning the above experimental data, the transit period, did not affect the analysis of the present study.

2.3. Ethical Approval

This study is not a clinical trial and does not include intervention in patients. All procedures were conducted according to the standards of Safety, Bioethics and Validation. The study was reviewed and approved by Bioethical Committee of Research Genetic Cancer Centre Group. Each patient has consented writing for use of his sample in the present study. The patients retained the right to withdraw their sample until the date when the sample was received at the laboratory and tested.

2.4. Sample Preparation

2 ml of blood was mixed with 2 ml of fetal bovine serum (Gibco, California, USA) in 15-ml centrifuge tubes (Corning, New York, USA) to regain the cells' shape. The samples were then centrifuged at 1200 rpm for 10 minutes at room temperature and the supernatant was discarded. 100 μ l of sample was transferred to BD Falcon™ Round-Bottom Tubes (12 \times 75 mm; BD Biosciences, San Jose, CA) for flow cytometry analysis. Different cancer types required different amounts of staining panels; breast, colon, pancreatic and melanoma cancer samples required four tubes and prostate cancer samples required five tubes. **Table 1** shows the panel of antibodies used for each tube.

2.5. Antibodies

The following mouse anti-human antibodies were used: anti-cytokeratin (Pan-reactive) monoclonal antibody (Sigma, Taufkirchen, Germany) stained with a Cy5.5[®] Conjugation Kit (Abcam, Cambridge, UK), CD227 FITC (AbD Serotec, Oxford, UK), CD63 FITC (Becton Dickinson, New Jersey, USA), CD31 RPE (AbD Serotec, Oxford, UK) and CD45 PE-Cy7 (Invitrogen, California, USA). Human PSMA/FOLH1 Phycoerythrin MAb (RnD Systems, Abingdon, UK) and Human HGF R/c-MET Phycoerythrin MAb (RnD Systems, Abingdon, UK) were also used. All antibodies were used according to the manufacturer's instructions. The antibodies used and their characteristics are presented in **Table 2**.

2.6. Staining Procedure

Because intracellular antibodies were used, the cells required fixation and permeabilization. The samples were therefore stained using LEUCOPERM™ (AbD Serotec, Oxford, UK) according to the manufacturer's instructions. The only variation from the manufacturer's protocol included a decrease in the incubation time of the samples with Reagent A from 15 to 5 minutes because it caused cell decomposition. After staining, the samples were kept at 4°C until analysis.

2.7. Instrumentation-Data Analysis

The instrument used to analyze the samples was a BD Accuri C6 cytometer, using two lasers (blue and red; BD

Table 1. Illustration of number of tubes stained per type of cancer.

Number	Tubes	Breast	Prostate	Melanoma	All other types
1	Unstained-unfixed	√	√	√	√
2	Unstained-fixed	√	√	√	√
3	CD31-CD45-pan CK		√		√
4	cMET-CD45-pan CK		√		√
5	PSMA-CD45-pan CK		√		
6	CD227-CD31-CD45-pan CK	√			
7	CD227-cMet-CD45-pan CK	√			
8	CD63-CD31-CD45-pan CK			√	
9	CD63-cMet-CD45-pan CK			√	

Biosciences, San Jose, CA). 50,000 events were recorded from each sample. To avoid bias, the researcher responsible for sample analysis was unaware of the samples' origin (cancerous or healthy). Analysis was performed using CFlow Plus software. Samples with low forward and side scatters were considered inappropriate and were not included in the study. Non-hematological cells were gated out using a CD45 negative selection [9]. The endothelial cells were then removed using a CD31 negative gating selection [10]. Tumor cells were identified by Pan-CK positive selection [11]. Finally, the metastatic breast, prostate and melanoma cells were analyzed for expression of c-MET, CD227, prostate-specific membrane antigen (PSMA) and CD63 [12]-[15]. Unstained and fixed samples were used as negative controls. **Figure 1** and **Figure 2** represent the analysis of one healthy patient and one patient with breast cancer.

2.8. Statistical Analysis

Prior to statistical analysis, the identity of the samples was revealed to the analyzing researcher. The data were analyzed using receiver operating characteristic (ROC) curve analysis with SPSS software [16]. The analysis was performed by one researcher to maintain accuracy. A significant p value was defined as <0.05.

3. Results

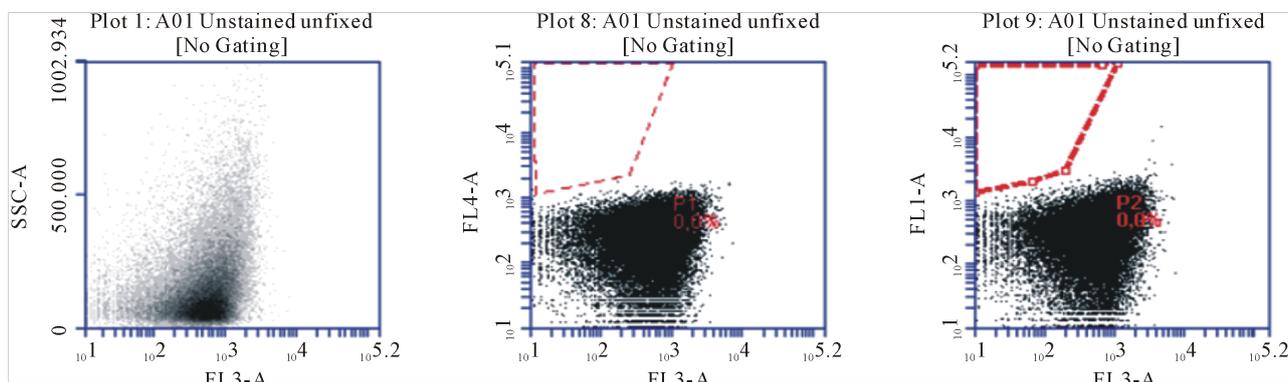
The samples were initially classified as either 30 positive or 30 negative based on whether they were cancerous or healthy, respectively. Flow cytometric analysis revealed that 31 samples contained CTCs and 29 did not. The data are summarized in **Table 3**. The results of the present study show 0.839 area under the curve, a percentage indicating that there is 83.9% probability for a randomly selected cancerous case to be regarded with greater suspicion than a healthy one. Of the healthy donor samples, only five were positive for CTCs, which suggests that the analysis is highly sensitivity (86.2%). In general, the sensitivity refers to the probability that a test result will be positive when the CTCs are present, while the specificity presents the probability that a test result will be negative when the CTCs are not present. The results of the present study, illustrated in **Figure 3**, show a specificity (true negative rate) of 83.9% (71.3% - 91.6%) and sensitivity (true positive rate) 86.2% (72.7% - 94.4%) when analyzing all cancer types ($p < 0.05$).

Table 2. Specific antibodies used per type of cancer.

Antibody	Feature
CD45	Pan Leucocyte marker
Pan-Cytokeratin	Tumor marker
CD227	Breast, lung cancer marker
CD63	Melanoma marker
CD31	Endothelial cell marker
c-Met	Metastasis marker
PSMA	Prostate cancer marker

Table 3. Summarized results of patients as true positive and false positive. "Tested" refers to the outcome from the flow cytometry experiments, while "Real" represents the data from patients' medical forms.

		Tested		Total
		Positive (Cancerous)	Negative (Healthy)	
Real	Positive (Cancerous)	26	4	30
	Negative (Healthy)	5	25	30
	Total	31	29	



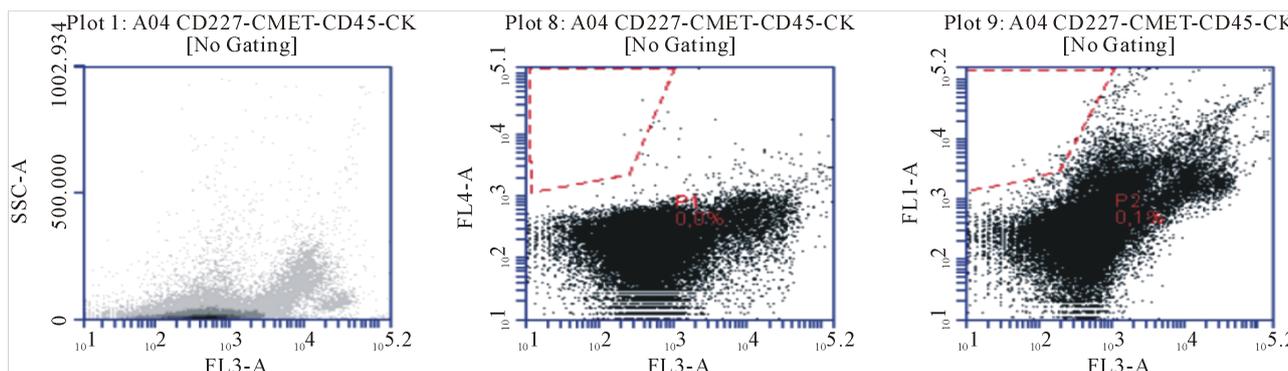
(a)

<input checked="" type="checkbox"/> Plot 1: A01 Unstained unfixed	Count	Volume (μL)	% of This Plot	% of All	Mean FL3-A	Mean SSC-A	CV FL3-A	CV SSC-A	Median FL3-A	Median SSC-A
All	50.000	1	100.00%	100.00%	571.33	143113.16	91.66%	87.57%		

<input checked="" type="checkbox"/> Plot 8: A01 Unstained unfixed	Count	Volume (μL)	% of This Plot	% of All	Mean FL3-A	Mean FL4-A	CV FL3-A	CV FL4-A	Median FL3-A	Median FL4-A
All	50.000	1	100.00%	100.00%	571.33	233.53	91.66%	94.24%		
P1	0	1	0.00%	0.00%	0.00	0.00	0.00%	0.00%		

<input checked="" type="checkbox"/> Plot 9: A01 Unstained unfixed	Count	Volume (μL)	% of This Plot	% of All	Mean FL3-A	Mean FL1-A	CV FL3-A	CV FL1-A	Median FL3-A	Median FL1-A
All	50.000	1	100.00%	100.00%	571.33	301.93	91.66%	100.99%		
P2	0	1	0.00%	0.00%	0.00	0.00	0.00%	0.00%		

(b)



(c)

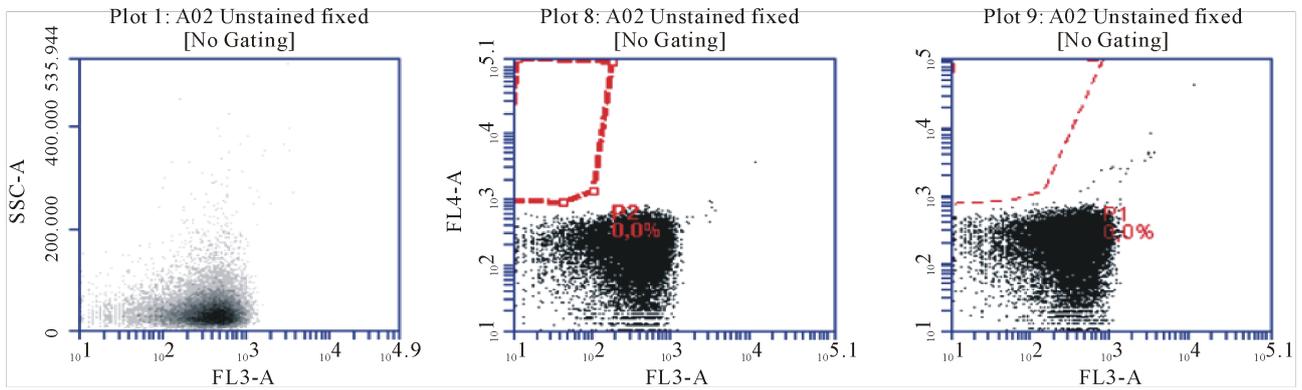
<input checked="" type="checkbox"/> Plot 1: A04 CD227-CMET-CD45-CK	Count	Volume (μL)	% of This Plot	% of All	Mean FL3-A	Mean SSC-A	CV FL3-A	CV SSC-A	Median FL3-A	Median SSC-A
All	50.000	37	100.00%	100.00%	2258.12	31516.49	1174.85%	521.79%		

<input checked="" type="checkbox"/> Plot 8: A04 CD227-CMET-CD45-CK	Count	Volume (μL)	% of This Plot	% of All	Mean FL3-A	Mean FL4-A	CV FL3-A	CV FL4-A	Median FL3-A	Median FL4-A
All	50.000	37	100.00%	100.00%	2258.12	538.89	1174.85%	4653.15%		
P1	3	37	0.01%	0.01%	323.33	18940.33	23.34%	61.33%		

<input checked="" type="checkbox"/> Plot 9: A04 CD227-CMET-CD45-CK	Count	Volume (μL)	% of This Plot	% of All	Mean FL3-A	Mean FL1-A	CV FL3-A	CV FL1-A	Median FL3-A	Median FL1-A
All	50.000	37	100.00%	100.00%	2258.12	1776.47	1174.85%	2844.81%		
P2	34	37	0.07%	0.07%	211.35	9381.38	64.28%	129.35%		

(d)

Figure 1. Flow cytometric analysis of a breast cancer sample. Unstained fixed cells (a) and stained cells (c). The first plot analyzes cell granularity and CD45 expression. The second plot indicates the gate used to identify PanCytokeratin (FL4) positive and CD45 (FL3) negative cells. The final plot demonstrates the gate used to identify CD227 (FL1) positive and CD45 negative cells. (b) Statistical analysis of the unstained cells using the above gates. (d) Statistical analysis of the stained cells using the above gates.



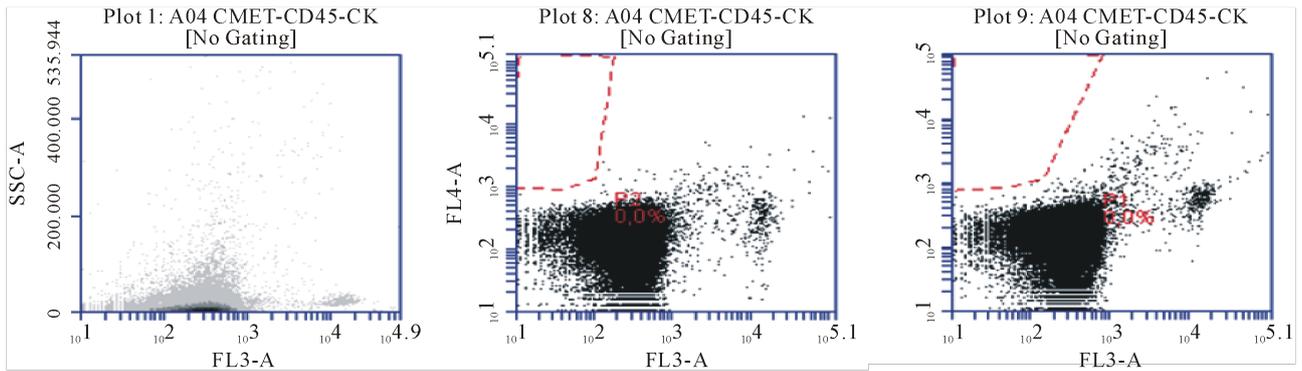
(a)

<input checked="" type="checkbox"/> Plot 1: A02 Unstained unfixed	Count	Volume (μL)	% of This Plot	% of All	Mean FL3-A	Mean SSC-A	CV FL3-A	CV SSC-A	Median FL3-A	Median SSC-A
All	20.000	3	100.00%	100.00%	381.10	40658.24	68.34%	138.63%		

<input checked="" type="checkbox"/> Plot 8: A02 Unstained unfixed	Count	Volume (μL)	% of This Plot	% of All	Mean FL3-A	Mean FL4-A	CV FL3-A	CV FL4-A	Median FL3-A	Median FL4-A
All	20.000	3	100.00%	100.00%	381.10	182.64	68.34%	73.24%		
P2	0	3	0.00%	0.00%	0.00	0.00	0.00%	0.00%		

<input checked="" type="checkbox"/> Plot 9: A02 Unstained unfixed	Count	Volume (μL)	% of This Plot	% of All	Mean FL3-A	Mean FL1-A	CV FL3-A	CV FL1-A	Median FL3-A	Median FL1-A
All	20.000	3	100.00%	100.00%	381.10	207.86	68.34%	163.54%		
P1	0	3	0.00%	0.00%	0.00	0.00	0.00%	0.00%		

(b)



(c)

<input checked="" type="checkbox"/> Plot 1: A04 CMET-CD45-CK	Count	Volume (μL)	% of This Plot	% of All	Mean FL3-A	Mean SSC-A	CV FL3-A	CV SSC-A	Median FL3-A	Median SSC-A
All	50.000	15	100.00%	100.00%	470.35	13337.40	1468.75%	925.32%		

<input checked="" type="checkbox"/> Plot 8: A04 CMET-CD45-CK	Count	Volume (μL)	% of This Plot	% of All	Mean FL3-A	Mean FL4-A	CV FL3-A	CV FL4-A	Median FL3-A	Median FL4-A
All	50.000	15	100.00%	100.00%	470.35	521.27	1468.75%	5702.90%		
P2	0	15	0.00%	0.00%	0.00	0.00	0.00%	0.00%		

<input checked="" type="checkbox"/> Plot 9: A04 CMET-CD45-CK	Count	Volume (μL)	% of This Plot	% of All	Mean FL3-A	Mean FL1-A	CV FL3-A	CV FL1-A	Median FL3-A	Median FL1-A
All	50.000	15	100.00%	100.00%	470.35	191.34	1468.75%	1423.76%		
P1	0	15	0.00%	0.00%	0.00	0.00	0.00%	0.00%		

(d)

Figure 2. Flow cytometric analysis of a healthy sample. Unstained fixed cells (a) and stained cells (c). The first plot analyzes cell granularity and CD45 expression. The second plot indicates the gate used to identify PanCytokeratin (FL4) positive and CD45 (FL3) negative cells. The final plot demonstrates the gate used to identify CD227 (FL1) positive and CD45 negative cells. (b) Statistical analysis of the unstained cells using the above gates. (d) Statistical analysis of the stained cells using the above gates.

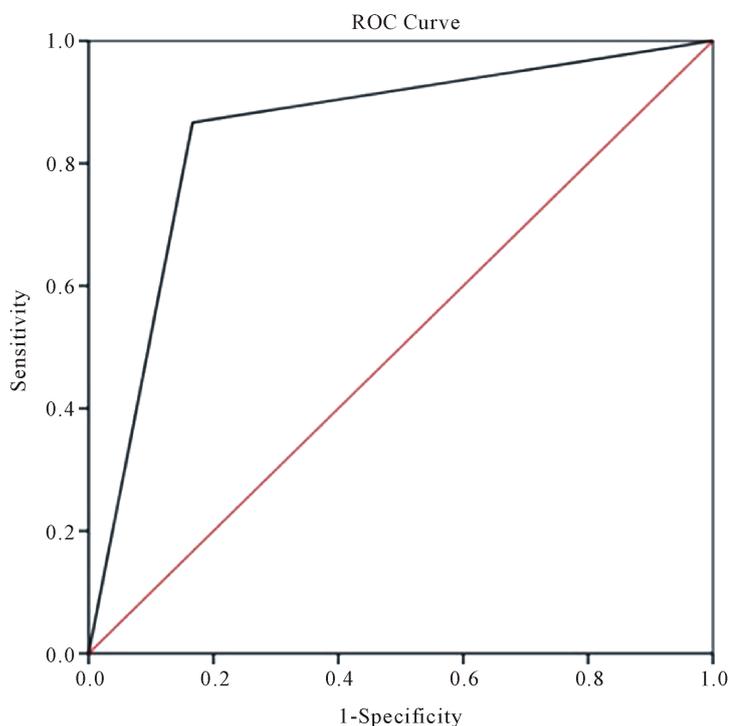


Figure 3. ROC curve analysis of samples. The present figure represents the true positive rate (sensitivity) together with the false positive rate (1-specificity). The black line indicates the change of the above parameters. The point which is nearest to the upper-left corner indicates the distinguish percentage of the method, which means the ability of the method to distinct the patients from normal samples. The confidence interval was set to 95% both for sensitivity and specificity.

4. Discussion

Cancer cases are increasing at an exponential rate every year. Because of the frequency of new cancer cases each year, it is essential for scientists to develop techniques to diagnose patients early. Preferably, the developed techniques will identify cancer cells in body liquids so that more invasive strategies, such as biopsies, are not required. Blood is one such liquid that can easily be obtained to identify the presence of cancer [17] [18].

Several markers associated with cancer are analyzed in the blood, such as CA 15-3 for breast cancer, CA 19-9 for pancreatic, stomach and bile duct cancer, CA-125 for ovarian cancer, prostate-specific antigen for prostate cancer and carcinoembryonic antigen for colorectal cancer [19]. However, these markers have limitations, the most important being their tendency to be detectable only when the tumor-burden is severe [20]. For that reason, evaluating alternative methods to detect carcinogenesis at its early stages and provide reliable and accurate results is essential.

CTCs are cells that have entered the blood after being detached from the primary tumor [21]. These cells are believed to play a crucial role in metastasis and cancer progression [22]-[24]. Therefore, detecting and monitoring CTCs is useful for prognosis and predicting disease progression and response to treatment, and several different methods have been developed to identify CTCs [25]-[27].

Because CTCs are rare in the circulation (1 in 10^9 blood cells), methods applied to assess them face many challenges [8]. Current techniques to isolate and enumerate CTCs do so based on their physical properties or morphological features. Other technologies use PCR-based methods or antibodies directed against epithelial cell adhesion molecules (EpCAM) on the cell surface of CTCs [4]. However, the above methods have limitations. Specifically, the cytomorphology of CTCs is not consistent, the PCR-based method does not enable the cells to be further analyzed and the antibody bead-based assays are limited by the availability of specific antibodies.

The only Food and Drug Administration-approved method for detecting CTCs is the CellSearch system,

which is currently considered the “gold standard”. This platform is a magnetic-bead-based assay that uses EpCAM antibodies to enrich the epithelial cells from the blood, as CTCs demonstrate epithelial origin [28]-[30]. Briefly, CTCs are magnetically separated from other cells, are stained with cytokeratin monoclonal antibodies cells and identification of contaminating leukocytes follows. Then the nuclei of CTCs are stained with DAPI and the cells are enumerated. The drawback of this method is that a proportion of CTCs may be lost during enrichment due to lack of EpCAM expression (false negatives) [31].

The aim of this study was to improve the recovery and performance of CTC detection and enumeration in compare with previous study whose sensitivity varies from 26% to 57%. The main difference between the present study and the recommended gold standard is that blood samples were not spiked with tumor cells, in order to determine the specificity [6] [25]. With the new method, only the tumor cells already in the blood of cancer patients were detected. Another significant difference is that the present study was blind, which means that sample encoding was performed by one scientist and the researcher responsible for data analysis was unaware of whether the sample was from a healthy patient. Finally, in the present study a number of biomarker specific for different types of cancer (CD227 for breast cancer, CD63 for melanomas, PSMA for prostate cancer and c-MET for metastatic cells) were used to make the analysis specific for each cancer type. EpCAM is the most common marker used for the identification of tumor cells; however, a number of other epitopes are currently used in tumor detection [32]. Mucins cover the surface of epithelial cells in several organs and protect the body from infection. Overexpression of the glycoprotein CD227 (Mucin-1 or CA 15-3) is often associated with colon, breast, ovarian, lung and pancreatic cancers [33]. CD63 is a member of the transmembrane 4 superfamily, also known as the tetraspanin family. These proteins mediate signal transduction events that play a role in the regulation of cell development, activation, growth and motility. CD63 has been shown to regulate melanoma cell growth and motility [34] [35]. PSMA is an enzyme that, in humans, is encoded by the FOLH1 (folate hydrolase 1) gene. In prostate cancer it is the second most upregulated gene, with an increase of 8 - 12-fold higher than the noncancerous prostate. Because of this high expression, therapies and imaging strategies are being developed to target FOLH1 [36]. c-Met, also called hepatocyte growth factor receptor, is normally expressed by epithelial cells. Abnormal c-MET activation in cancer correlates with poor prognosis and triggers tumor proliferation, neovascularization and metastases. Therefore, c-Met-positive cells are of interest [37]. The expression of cytokeratins by cells is organ and tissue specific. Moreover, the cytokeratin profile tends to remain intact when an epithelial cell undergoes a malignant transformation. Therefore, analyzing the cytokeratin profile of tumor cells is helpful for tumor characterization [26].

5. Conclusion

In conclusion, this study demonstrates that it is possible to detect CTCs with higher sensitivity (86.2%) and specificity (83.9%) compared with routine clinical methodologies. The parameters may vary depending on the antibody panel used; however, using flow cytometry to identify CTCs has proven to be efficient. These results suggest that further studies are required to improve the accuracy by which CTCs and CTC subtypes can be identified by flow cytometry and thereby improve our ability to detect and follow the progression of cancer.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Abbreviation List

CTCs: Circulating tumor cells
ROC: Receiver operating characteristic
EpCAM: Epithelial cell adhesion molecule
EDTA: Ethylenediaminetetraacetic acid
Cy5.5: Cyanine 5.5
FITC: Fluorescein isothiocyanate
RPE: R Phycoerythrin
PE-Cy 7: Phycoerythrin cyanine 7
CD: Cluster of differentiation
PSMA: Prostate-specific membrane antigen
CA: Cancer antigen
DAPI: 4',6-diamidino-2-phenylindole
FOLH1: Folate hydrolase 1