Using a combination of gangliosides and cell surface vimentin as surface biomarkers for isolating osteosarcoma cells in microfluidic devices

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A B S T R A C T

Background: Osteosarcoma (OS) is the most common primary bone tumor and the third leading cause of pediatric cancer deaths. Liquid biopsies are an alternative to current diagnostic imaging modalities that can be used to monitor treatment efficacy and the development of metastases. This study addresses the use of novel biomarkers to detect circulating osteosarcoma cells.
Procedures: Flow cytometry was used to evaluate the relative expression of epithelial cell adhesion molecule (EpCAM), ganglioside 2 and 3 (GD2/3), and cell surface vimentin (CSV) on a panel of OS cell lines. A microfluidic device was used to affirm the efficacy of GD2/3 and CSV to capture CTCs. Once captured, CTCs on the device are enumerated and the capture efficiency for each marker is measured. Patient samples were captured using the LFAM chip.
Results: We report the evaluation of GD2, GD3, and CSV as markers for OS cell capture in cell lines and in patient samples. The results of our capture studies correlate with our flow cytometry data and have shown a low capture efficiency of OS cells using EpCAM antibodies, while showing a moderate capture efficiency of OS cells using the GD2, GD3, and CSV antibodies independently. The combination of biomarkers demonstrate a high capture efficiency of approximately 80%. This is further supported by the detection of 1–1.5 CTCs per mL of blood using GD2 + CSV in OS patient samples.
Conclusions: The combination of GD2 + CSV significantly increased the capture efficacy of OS cells. The detection of CTCs through routine blood sampling may be used clinically for earlier detection of metastases and monitoring the therapeutic effect of treatments in metastatic osteosarcomas.

1. Introduction

Osteosarcoma (OS) is the most common bone malignancy that primarily affects the pediatric population [1]. Although the integration of chemotherapy and surgery for localized OS has improved long-term survival of patients, the 5-year survival for metastatic OS has remained stagnant. OS is particularly lethal, as approximately 30–40% of pediatric patients diagnosed with OS will succumb to the disease [2]. Currently, there is no screening method for OS and most tumors are discovered incidentally or when tumors become symptomatic [3]. A key reason for the poor survival of OS is due to its high metastatic incidence. Current detection of metastasis is done with radiographic imaging or positron emission tomography (PET), which expose patients to radiation and can only detect tumors once they reach a 7 mm diameter size [2,4]. Of patients diagnosed with OS, 15–20% present with lung metastases upon diagnosis and another 40% will develop metastasis at a later stage. suggesting that most patients have occult metastases upon diagnosis [5]. Therefore, there is a clear need for more sensitive methods to detect metastatic lesions in patients with OS to improve overall outcomes.

Circulating tumor cell (CTC) detection has the ability to shed light into the "black box" of cases where metastasis is present despite a lack of radiological evidence. CTCs are hypothetically detectable in peripheral blood the moment metastasis begins and therefore can aid in the early detection of metastases or monitoring of therapeutic efficacy. Immunoaffinity isolation of CTCs for the purpose of detection has been demonstrated in a number of carcinomas [6–9]. Currently, the only FDA approved CTC device is CellSearch®, which utilizes epithelial cell adhesion molecule (EpCAM) as a cell surface marker for CTCs [6,10]. To date, the majority of the studies on CTCs have focused on carcinomas. Little is known about sarcomas, which are less common than carcinomas, but generally have a poorer prognosis and higher metastatic incidence. Therefore, there is a need for the evaluation of CTCs in sarcomas.

Due to the biological differences between epithelial-derived carcinomas and mesenchymal-derived sarcomas, EpCAM may not be an appropriate marker for CTC detection in OS [11]. Therefore, novel biomarkers should be explored to determine markers that are suitable for CTC detection in OS. We evaluated the use of ganglioside 2 (GD2), ganglioside 3 (GD3), and cell surface vimentin (CSV), as potential cell surface markers for CTC detection in OS.

1.1. Gangliosides

Gangliosides are sialic acid-containing glycosphingolipids that are primarily involved in nervous system development and expressed in the non-differentiating nervous tissue [12]. GD2 and GD3 are normally expressed in adult tissue of the brain, spinal cord, peripheral nerves, melanocytes, and mesenchymal cells [13–15]. While typically associated with neuronal cells, GD2 and GD3 are detectable in a variety of cancer types including neuroblastoma [16–18], melanoma [19–22], T-cell leukemias [23–24], lung [25–27], and breast cancers [28–31] and are used as therapeutic targets in clinical trials [32–36]. The differentiation of tumor associated glycolipids from normal tissue, in principle enables the selective detection and therapeutic targeting of cancer cells [18,37]. Based on multiple criteria, the National Cancer Institute (NCI) program for prioritization of cancer antigens ranked GD2 12th and GD3 40th among a list of top cancer antigens. [38]

In OS cell lines, increased GD2 and GD3 expression is associated with increased tumorigenicity [39]. While both GD2 and GD3 are expressed across OS cell lines and tumor samples, there is a larger body of evidence to support the clinical use of GD2 as a biomarker in OS [40–41]. In addition to cell lines, GD2 and GD3 also are highly expressed in human OS tissue [39–42]. Immunohistochemistry (IHC) staining has demonstrated high GD2 and GD3 expression in both primary and recurrent OS tumor samples [39–40,42]. In one study of 41 patient tumor samples, Roth et al. found that 100% of patient samples had positive GD2 expression via IHC [40]. In samples from patients with recurrent tumors, GD2 and GD3 were partially conserved. In the same study by Roth et al., GD2 expression was found to be increased upon recurrence, however this study did not match primary and recurrent samples [40]. In matched OS samples, GD2 expression was found to decrease, though it was still expressed in most tumors, while GD3 expression was maintained with recurrence [39]. As a potential marker, the presence of GD2 and GD3 in both primary and recurrent tumors is appealing, since it suggests that CTCs in OS might still be detectable after clonal selection.

Since CTCs are released from primary tumors to form secondary tumors, this conserved expression between the two tumor types suggests that CTCs might express GD2 as well. Finally, GD2 and GD3 transfection induced expression in cells, are associated with an increase in metastatic potential due to increasing cell motility, invasion, and decreasing cellular adhesion [41].

1.2. Cell surface vimentin

Vimentin is an intermediate filament typically found in connective tissue, mesenchymal cells, vascular smooth muscle cells, cardiomyocytes, neutrophils, and monocyte derived macrophages [43–44]. While typically thought of as a cytosolic protein, evidence suggests that it can also be expressed on surface of some cells [44–45]. Vimentin is involved in various pathways important to metastasis including cell adhesion and migration, fibroblast and lymphocyte migration, and arterial resistance [43]. Increased vimentin expression has been documented in various carcinomas and sarcomas, including OS [44–45]. In epithelial cancers, the presence of vimentin might be reflective of epithelial to mesenchymal transition [43–45]. While typically intracellular, Satelli et al. demonstrated via flow cytometry that CSV is expressed on sarcoma cell lines [45]. Furthermore, CSVs specificity for metastatic OS cells suggests that it may be suitable for detection [45]. Currently, Satelli et al. have developed an antibody (monoclonal antibody 84–1, Abnova) for CSV and have demonstrated its ability to capture CTCs in animal models and patients [45].

2. Materials and methods

Reagents: Dulbecco's phosphate buffered saline (DPBS) with 0.49 mM magnesium chloride and 0.9 mM calcium chloride, DPBS containing 1% bovine serum albumin (BSA), DPBS without calcium chloride and magnesium chloride, sylgard 184 reagents, ethanol, and Tween 20 were purchased from Thermo Fisher Scientific (Hampton, NH).

Cell lines: Hu09 cells (CVCL-01298, human OS) [46] were obtained from Dr. Lin Ren at the National Cancer Institute (NCI). CAL72 (CVL-1113, human OS) [47] cells were obtained from the Université Nice Sophia Antipolis. OS156 cells (human OS) were obtained from Dr. Parker Gibbs (Department of Orthopedics and Sports Medicine, UF). MG63 (CRL-1427, human OS), BxPC3 (CRL-1687, human pancreatic adenocarcinoma), and CCRF-CEM (CCL-119, human acute lymphoblastic leukemia cells) cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). All cell lines were STR analyzed and authenticated. Cells were cultured in the appropriate medium (RPMI 1640, F12/Ham's, or Dulbecco's Modified Eagle Medium (DMEM)) (ATCC) supplemented with 5 or 10% fetal bovine serum (FBS; GIBCO of Thermo Fisher). All cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. All cell line experiments were done within 10 generations.

Antibodies: Human BD Fc Block (BD Pharmigen Product No. 564220), FITC mouse anti-CD326 (EpCAM) (BioLegend, clone: 9C4, Product No.: 324204), Biotin Anti-CD326 (EpCAM) (eBiosciences, clone: 1B7 catalog # 13–9326-82), FITC mouse anti-human disialoganglioside GD2 (BD Pharmingen Product No. 563,439 clone: 14.2 Ga), Mouse anti-human disialoganglioside GD2 (BD Pharmigen Product No.: 554,272 clone: 14.G2a), Mouse anti- human disialoganglioside GD3 (BD Pharmigen Product No. 554274, clone MB3.6), and CSV (Abnova, Product number: H00007431-M08 clone: 84–1). Goat anti-Mouse IgG (H + L) highly cross-absorbed secondary antibody, Alexa Fluor 594 (Invitrogen, Product No. A-11032).

Flow cytometry: Cultured cells were dissociated using an enzyme free dissociation buffer (Gibco, cat no.: 13151014). Cell lines for flow cytometry were prepared as a solution of 10 [6] in 100 μL in PBS buffer. Cells were incubated with FITC (EpCAM and GD2) or AF594 (GD3 and CSV) antibody for 30 min at 4 °C. Cells were washed three times in 200 μL of flow buffer. All washes were performed at 400 g for 5 min. Samples were analyzed using a BD

Fortessa flow cytometer to evaluate the cell surface expression of EpCAM. Up to 10,000 events were recorded for each sample.

Microfluidic Device Cell capture: Microfluidic Device Cell capture: Antibodies were biotinylated using the EZ-link sulfo-NHS-LC-Biotin kit (Catalog No. PI21935). Cells used for cell line and blood spiking experiments were pre-stained by incubating cells with Vybrant DiD for 20 min. After staining, cells were washed by centrifuging at 200g for 5 min, the supernatant was discarded, and cells were resuspended in 1 mL of DPBS. Washing was repeated three times, and cells were resuspended in 1% BSA. Cells were then serially diluted to the necessary concentration for cell line or spiked blood experiments. A previously described [8,48] geometrically enhanced microfluidic (GEM) device containing herringbone-based micromixers was functionalized with anti-EpCAM, GD2, GD3, CSV, GD2/GD3 or GD2/CSV antibody on the same day prior to cell capture experiments using a protocol described as follows. The GEM chip was selected for cell lines capture as it primarily captures cells by immunoaffinity and is therefore best suited to demonstrate the effect different antibodies have on OS cell isolation. Previous work with the GEM chip establishes its capture efficiency at > 90% and a purity of > 80% when capturing antibody positive cells from blood samples [8]. 150 μL of 1 mg/mL avidin (Thermo Fisher Scientific) was incubated in the device for ≥ 15 min to allow physical absorption of avidin onto the channel surfaces. The device was then washed with 300 μL of DPBS. Afterward, 100 μL of 20 μg/mL of biotinylated antibody or antibody combination was introduced into the device and incubated for ≥ 15 min. Finally, devices were washed and allowed to incubate with 300 μL of 1% BSA for one hour to reduce nonspecific binding. All tubes used for serial dilution were incubated for one hour with 1.2 mL of 1% BSA.

Cell solutions were passed through the previously functionalized device at 1 μL/s, followed by a DPBS wash at 2 μL/s by using a syringe pump. A tiny magnetic bar was placed inside a 1-mL syringe over a stir plate to keep the cells in suspension as the cell solution or blood samples were pumped through the device. To determine the number of cells captured in the microfluidic device an Olympus IX71 microscope (Olympus America, Melville, NY) was used for imaging and the cells were counted manually by a trained observer.

Spiked Blood Capture: Both antibody combinations (GD2 + GD3 and GD2 + CSV) were tested in blood spiking experiments. Either 10, 100, or 1000 OS cells were diluted in 1 mL of 1% BSA by serial dilution before being added to 1 mL of whole blood. 1% BSA was used for serial dilution to reduce any potential cell loss between dilution steps. The cells captured using the GEM device were counted and their numbers were compared with the number of cells introduced (Fig. 4). To establish a false positive rate for spiked blood experiments, 5 μL of Vybrant DiD was added to 1 mL of DPBS containing no cells which was centrifuged and "washed" identically to cells and "resuspended" in 1% BSA. After adding 1 mL of cell free 1% BSA to 1 mL of blood, the sample was passaged and counted at the same exposure as cell spiked samples. The experiment was repeated three times, no CTCs were detected in the non-spiked blood samples.

Patient Sample Collection: This study was approved by the University of Florida Internal Review Board. All study participants provided written informed consent. Samples were provided from two patients to demonstrate proof of concept. Patient-001 was diagnosed with right distal femur osteosarcoma treated with high dose methotrexate and underwent a rotationplasty, sample was collected status post chemotherapy and surgery. Patient-005 was diagnoses with right femoral osteosarcoma treated with doxorubicin, cisplatin and underwent a radial resection of the right femur. At the time of sample collection, neither patient had evidence of metastasis on chest X-ray. Blood samples (10 mL) were collected

in BD Vacutainer tubes with sodium heparin. Blood was diluted with an equal volume of DPBS creating a 2X dilution of whole blood. 4 mL of diluted blood was passed through a lateral filter array microfluidic (LFAM) device at 1 μL/sec followed by a washing step with DPBS at 2 μL/sec. The LFAM device uses a combination of size and immunoaffinity based isolation to capture CTCs [49]. CTCs captured then were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton-X. To distinguish captured CTCs from WBCs, a triple immunohistochemistry cocktail of 4′,6-diamidino-2-phenylindole (DAPI), anti-cytokeratin-FITC (anti-CK-FITC), anti-CD45-PE was incubated in devices with captured cells for 45 min. CTCs were defined as cells that were DAPI+, CK +, and CD45-.

In addition to OS patients, 3 healthy volunteer samples were used to control for false positives. 2 mL of healthy patient blood was diluted 2X with DPBS, passed through devices internally coated with GD2 + CSV, and stained identically to OS patient samples. Afterwards, LFAM devices were counted for false positive CTCs.

Statistical analysis: EpCAM, GD2, GD3, CSV, and antibody combinations were compared using a one-way analysis of variance (ANOVA) with Bonferroni correction for Hu09 and MG63 individually. For either cell line, a p-value < 0.025 demonstrated a significant difference in cell capture efficiency and post-hoc Tukey tests were done to determine which cell surface markers resulted in significantly different capture efficiency.

3. Results

EpCAM Marker Expression and Cell Capture in OS cell lines: EpCAM cell surface expression was evaluated using flow cytometry. All OS cell lines had few EpCAM expressing cells (HU09, 0.45%; OS156, 0.33%; CAL72, 0.5%; MG63, 0.26%) (Fig. 1; Table 1). Previous studies showed that OS cell lines express a little to no EpCAM on their surface; a result consistent with our findings [11]. The low number of EpCAM expressing cells seen by flow cytometry translated to an ineffective cell capture / cell detection using the GEM device (Fig. 2).

GD2, GD3, and CSV Marker Expression in OS cell lines: While previous studies have shown that GD2 and GD3 are expressed in the majority of OS tumors [39–40,42], there is little information on the number of cells expressing these markers within tumors or on CTCs. GD3, GD2, and CSV are expressed on variable numbers of cells on our OS cell lines (Fig. 1, Table 1). Using flow cytometry, we evaluated the cell surface expression of GD2, GD3, and CSV on the previously mentioned cell lines. GD2, GD3, and CSV are expressed on the surface of more cells in our panel of cell lines (GD2: HU09,80.75%; OS156,60.2%; CAL72,12%; MG63,74.9%; GD3: HU09,0.92%; OS156,26.3%; 7CAL72,5.8%; MG63,43.7%; CSV: HU09,20%; OS156,94%; CAL72,38%; MG63,77%) than EpCAM. GD3 had the lowest percent of expressing cells of the three markers, with a maximum expression of 44% for MG63. GD2 showed a moderate to high number of expressing cells across most cell lines, with the exception of CAL72 (12%). Similarly, CSV showed a high variance in expressing cells, being present on a few Hu09 (20%) and CAL72 (38%) cells, but having the highest presence on MG63 (77%).

3.1. Cell capture of Hu09 and MG63

Hu09 and MG63 were used for capture in cell line studies. MG63 was selected as a model cell line because as it demonstrated the highest per-cell expression of GD2, GD3, and CSV. Hu09 was selected as a heterogenous case as few cells expressed GD3, some cells expressed CSV, and most cells expressed GD2. Ability to capture cells from both cell lines were assessed with individual
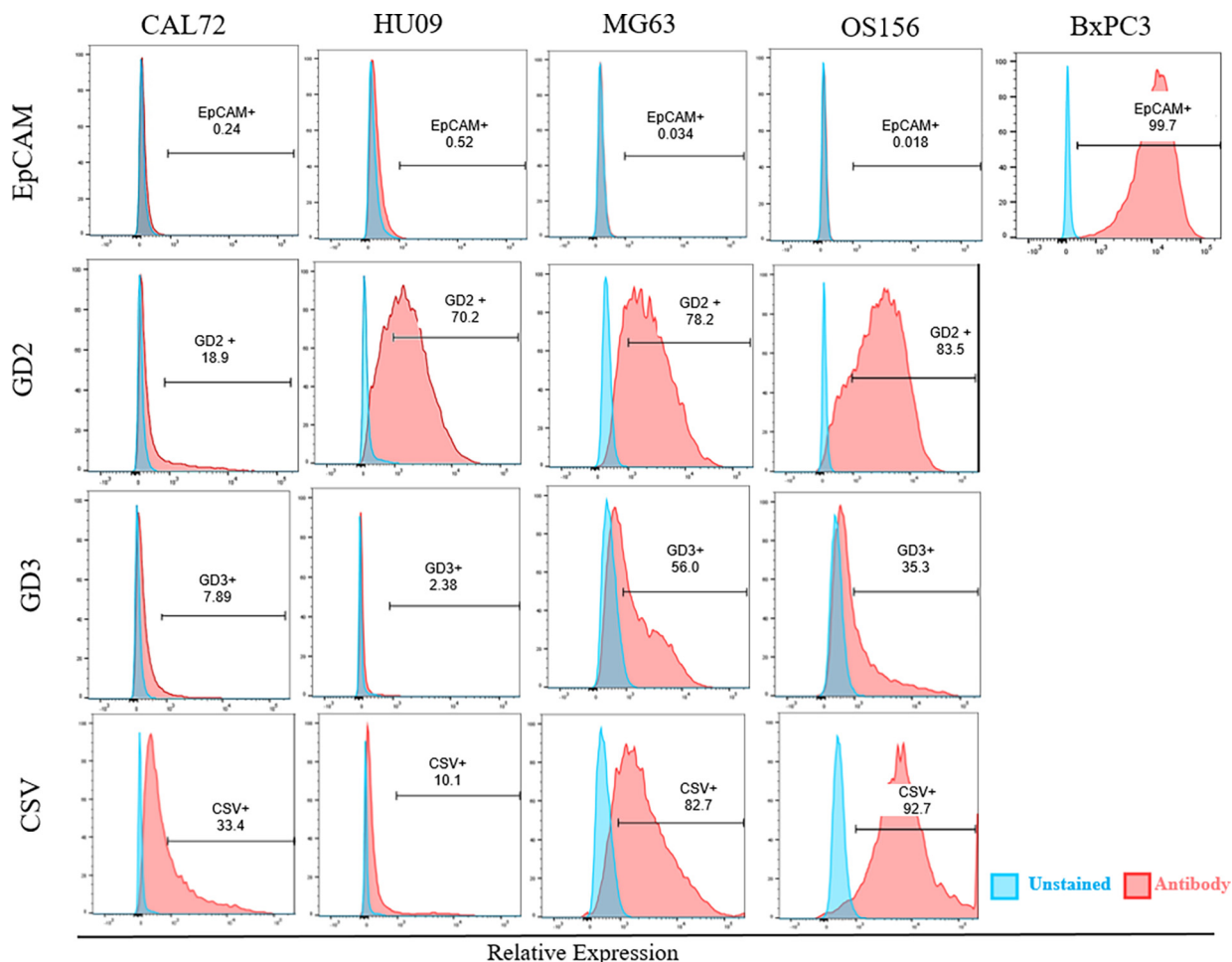
Fig. 1. EpCAM, GD2, GD3, and CSV expression in the OS panel (CAL72, MG63, HU09, OS156) detected via flow cytometry using a FITC (EpCAM and GD2) and AF594 (GD3 and CSV) labeled antibody. The BxPC3 cell line was used as a positive antibody control.

Table 1

Average surface marker expression of EpCAM, GD2, GD3, and CSV for OS cell lines OS156, CAL72, Hu09, and MG63. All cell lines show a low EpCAM expression (<1%) while showing various degrees of GD2, GD3, and CSV expression. Average expressions are from $\geq$3 independent experiments.

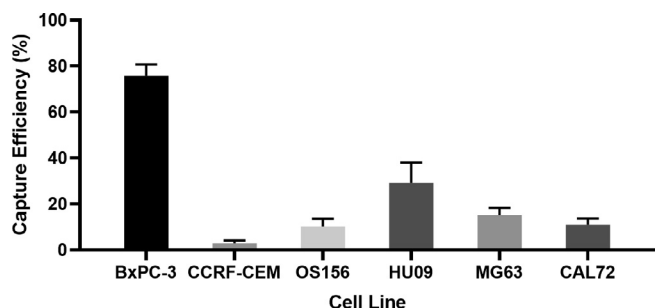| OS cell line	EPCAM	GD2	GD3	CSV
BxPC-3	97.4±2.36%			
OS156	0.33±0.4%	60.2±29.7%	26.3±8.9%	94.1±5.3%
CAL72	0.50±0.4%	12.0±7.3%	5.8±1.7%	37.9±13.8%
HU09	0.45±0.5%	80.8±18.7%	0.92±1.0%	20.1±15.7%
MG63	0.26±0.5%	74.9±18.9%	43.7±6.0%	77.2±24.2%

Fig. 2. Cell Capture of OS cell lines using the EpCAM antibody. Cell capture efficiency for OS cell line show a low detection rate in comparison to the pancreatic cell line Bx-PC3 (positive control). The CCRF-CEM cell line is used as a negative control.

antibodies (EpCAM, GD2, GD3, or CSV) and antibody combinations (GD2 + GD3, GD2 + CSV). Both Hu09 (p = 0.013, Fig. 3**A**) and MG63 (p = 0.013, Fig. 3**B**) showed a significantly different capture efficiency depending on which marker was used (p < 0.0001, Fig. 3**B**). GD3 showed the lowest capture efficiency for both cell lines (19.02% for Hu09, 49.1% for MG63), but both values were high relative to the number of cells expressing these cell surface markers as determined by flow cytometry. GD2 showed a higher cell capture efficiency than EpCAM and GD3 for both Hu09 (60.2%) and MG63 (60%). For MG63, CSV showed a high capture (74.2%), which was comparable to EpCAM in positive controls and significantly higher than MG63 capture with EpCAM (p = 0.006).

For individual antibodies, our cell capture studies partially mirrored our flow cytometry data (Supplemental Fig. 1). In general, EpCAM was the least favorable marker for OS cell capture, while GD2 and CSV were the best antibodies for cell capture using an individual antibody. For MG63, the difference in capture efficiency
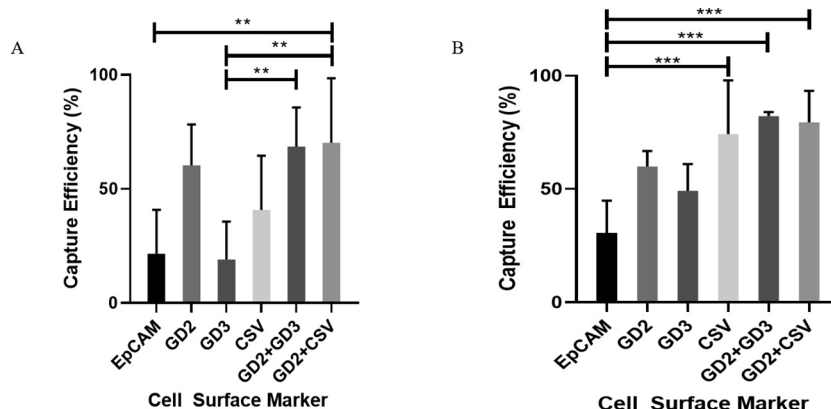
Fig. 3. Cell capture efficiency of EpCAM, GD2, GD3, and CSV using the GEM device in the Hu09 (A) and MG63 (B) cell lines. Results are the means and standard deviations of three independent experiments (**) p < 0.05 (***) p < 0.01.

between CSV and EpCAM was significant (p = 0.004) and the difference between EpCAM and GD2 trended towards significance (p = 0.07). For Hu09, this failure to demonstrate significance between individual markers was due to high standard deviation in the capture of Hu09 cells across different cell surface markers, however there is a noted increase in cell capture efficiency using GD2 or CSV antibodies.

For cell surface marker combinations, GD2 combined with GD3 (GD2 + GD3) as well as GD2 combined with CSV (GD2 + CSV) were used to evaluate which combination would provide the highest cell capture. GD2 + CSV was selected, as every cell line except for CAL72 showed a high number of GD2 or CSV expressing cells (Supplemental Table 1). For Hu09, GD2 + CSV combined had a significantly increased capture efficiency compared to EpCAM (p = 0.028) and GD3 (p = 0.019).Additionally, GD2 + GD3 had a significantly increased capture efficiency compared to GD3 (p = 0.044) which trended toward significance when compared to EpCAM (p = 0.07). For MG63, GD2 + GD3 (p = 0.001) and GD2 + CSV (p = 0.003) was significantly different compared to MG63 capture by EpCAM. Interestingly, CSV (74% ± 23.7%) alone showed comparable capture to GD2 + CSV (79% ± 13.9%) for MG63 cells. The noted heterogeneous per-cell expression for different cell lines supports the use of multiplexing GD2 + CSV for efficient cell capture across different OS cell lines.

Cell Capture is Maintained in Spiked Blood Samples: In order to evaluate the function of the GEM device for OS cells, we used spiked blood samples to demonstrate that capture can be maintained in whole blood samples using GD2 + GD3 and GD2 + CSV. While GD2 or GD3 expression in WBCs has been reported in T-cell leukemia [23–24,50], there is no reported expression of GD2 or GD3 on normal WBCs. Similarly, flow cytometry demonstrates that CSV is not expressed on the surface of WBCs [45]. There is also no reported expression of GD2, GD3, or CSV in red blood cells [45].

For GD2 + GD3, cell capture efficiency ranged from 54.5% at 100 cells to 85.7% at 10 cells. Despite having a lower capture efficiency in buffer solutions, GD2 + GD3 demonstrated comparable capture efficiency in blood samples spiked with MG63 cells as compared to GD2 + CSV. For GD2 + CSV, the capture efficiency was 64.6% at 10 cells, but decreased with increasing cell concentration to 26% at 1000 cells. For both GD2 + GD3 and GD2 + CSV the highest capture efficiency was observed at 10 cells. Several studies have demonstrated that CTCs are typically present in the blood at 1–10 cells/mL [6,8,51–52], suggesting that we retain highly accurate capture at the most physiologically relevant concentration of tumor cells. In healthy blood samples, no false positives were detectable (ie: there were no CTCs detected in healthy blood samples).

Table 2
The number of COCs identified per mL of blood in two OS patients.

Patient ID	CTCs/mL of whole blood	Status
UF-PEDS-001	1	No metastases
UF-PEDS-005	1.5	No metastases

Circulating tumor cells in Patient Samples: To demonstrate that these antibodies translate to patient samples, we captured CTCs from two samples collected from patients with OS. CTCs were detected using the LFAM device. CTCs were observed in both patients (Table 2), demonstrating, as a proof of concept, that our antibodies are capable of isolating CTCs from OS patient blood samples. No CTCs were observed in healthy patient samples.

4. Discussion

Collectively, our results suggest that GD2, GD3, and CSV may be better cell surface markers than EpCAM for immunoaffinity CTC isolation of osteosarcoma cells. The high heterogeneity across different OS cell lines suggests that combinations of markers may improve the capture efficiency of CTCs by targeting different subpopulations of OS cells. Both GD2 (57%±31%) and CSV (57%+34%) showed the best per-cell expression across all OS cell lines and with the exception of CAL72, and complemented each other on which cell lines had a high number of cell surface marker expressing cells, suggesting that the combination of these antibodies would capture CTCs present in a wider range of OS patients.

Our capture of Hu09 and MG63 cells demonstrate that combined antibodies (GD2 + GD3 or GD2 + CSV) had a greater capture efficiency compared to the individual antibodies alone (Fig. 3). Large standard deviations in the capture efficiency of our cell line capture made it difficult to demonstrate a significant difference for any one or more combination of antibody and cell line. Despite this, the consistently high capture of our combined antibody combinations allowed us to demonstrate a significant difference in capture from EpCAM. In the case of Hu09 cells, both antibody combinations demonstrated increase capture when compared to GD3 alone. Without prior knowledge of which cell surface markers are expressed on OS CTCs, multiplexing antibodies should enhance the ability to capture OS cells expressing different antigens. While most of the capture of Hu09 cells is likely driven by GD2, other OS cell lines such as OS156 would have more of their capture driven by CSV. Since in patient samples we have no prior knowledge of marker expression on CTCs, multiplexing clearly would be advantageous. Therefore, combinations of GD2 + GD3 or GD2 + CSV are
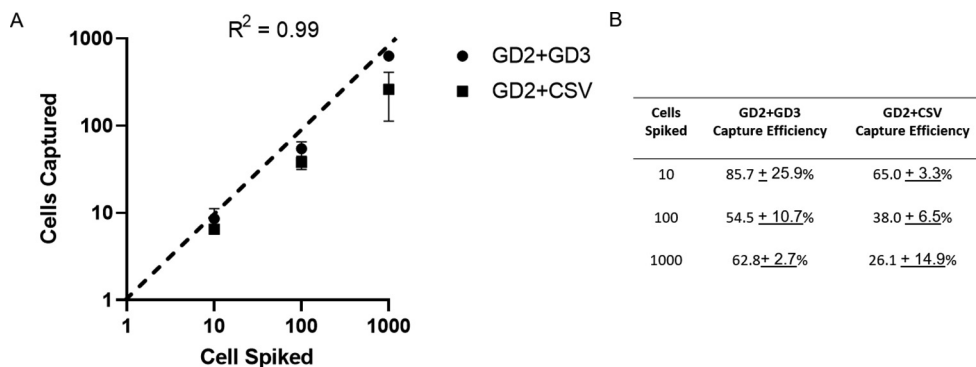
Fig. 4. Cell capture is maintained in spiked blood samples using the MG63 cell line(A). A tabular representation of the capture efficacy for each cell line at each concentration (B). At expected CTC concentrations (10 CTCs per mL), cell capture ranges from 70 to 80% with the combination of GD2 + GD3 as well as GD2 + CSV.

better suited for capturing various OS cells compared to individual antibodies.

Our spiked blood experiments (Fig. 4) served to demonstrate that we could still maintain cell capture when a large number of contaminating cells are present. Typically, only 1–10 CTCs are present among billions of red and white blood cells [6,8,51–52]. Therefore, it is necessary that antibodies used for cell capture should not bind to non-CTCs. For both GD2 + GD3 and GD2 + CSV, we showed a linear relationship between the number of MG63 cells spiked into blood and the number of cells captured. The retained linearity of the cell capture at different cell concentrations demonstrates that (i) these antibodies are capable of capturing MG63 cells in blood and (ii) there is a high specificity of these antibodies for CTCs in comparison to other components of whole blood such as red blood cells, white blood cells and platelets.

Finally, our two patient samples demonstrate that the combination GD2 + CSV antibodies is capable of capturing CTCs from patient blood samples. While the low sample size of patients prohibits us from drawing statistical conclusions between cancer stage or prognosis and CTC counts, we were still able to demonstrate that capture of OS CTCs is possible using these antibodies.

The low sample size of patients in our study, as well as the difference in capture and staining methods, make it difficult to compare our patient results to those of other studies. Size based methods, such as those utilized by Hayashi et al [53], are capable of capturing CTCs regardless of cell surface marker expression. Despite the theoretically improved capture of size-based methods, Hayashi et al reports OS CTCs to be present in concentrations as low as the concentration of CTCs we observe.

One study of note is Wu et al which demonstrated that epithelial CTCs are expressed by OS patients which seemingly contradicts our observation that EpCAM is poorly expressed on OS CTCs. Wu et al [54] used a size-based isolation method, CanPatrol™, to isolate CTCs, followed by antibody staining to determine epithelial versus mesenchymal CTCs. Epithelial CTCs were determined using a combination of staining antibodies including Anti-EpCAM, Anti-CK 8, 18, and 19. We used Anti-CK 7 and 8, but not EpCAM, to stain for CTCs since CTCs are defined as CK⁺. It's possible that the epithelial CTCs observed by Wu et al are CK⁺, which would not contradict our observation that EpCAM is poorly expressed in OS. When accounting for the larger blood volume utilized by Wu et al (10 mL), our CTC/mL for our two patients fall within the range of epithelial CTCs observed by Wu et al. This suggests some consistency between our observations, while implying that including vimentin staining post capture might increase the number of CTCs we can detect. Despite this, while difficult to compare, both Hayashi et al and Wu et al suggest that our patient CTC/mL is within a similar range. Additional OS patient samples are required to further compare our methods efficacy to theirs.

While we have demonstrated GD2 and CSV as strong markers for OS CTC isolation, we recognize that this study is not comprehensive and alternative OS CTC markers may exist. Two targets of note are insulin-like growth factor receptor 2 (IGF-2R) and human epidermal growth factor receptor 2 (HER2) [55–57]. IGF-2R has been shown to be overexpressed across OS cell lines [55], suggesting that it would have good coverage across multiple cell lines. HER2 is of note as recent evidence suggests it can be used as a target for OS treatment [56–57]. Further studies are needed to demonstrate which antibody combinations results in the best coverage for OS CTC capture.

5. Conclusions

Metastatic OS is a highly lethal disease partially due to the frequent presence of occult metastasis leading to relapse. Currently, there is no method to detect occult metastases or predict relapse for OS. In some clinical cases of carcinomas there exists evidence that CTC detection may predict relapse in patients with no radiologic or clinical signs of metastatic disease [58–59]. EpCAM has traditionally been used in CTC detection. However, our findings in OS models, demonstrate that EpCAM should not be used as a universal CTC marker. In OS, EpCAM expression is low; therefore, alternative cell surface markers should be evaluated for both prognostic and diagnostic studies.

This study reports the use of GD2, GD3, and CSV in comparison to EpCAM for the detection of CTCs in OS. We demonstrated that GD2, GD3, and CSV, either individually or collectively serve as potential biomarkers for CTC detection in OS. Several studies have demonstrated GD2, GD3, and CSV to be present on the surface of both primary and metastatic OS tumors [39–40,44–45,60], however few studies have attempted to use CSV for the capture of CTCs or utilize the combination of markers for CTC capture. In addition to diagnostic value, GD2 therapies have been proposed to be used clinically to selectively target tumor cells in patients [42,61]. Consequently, the use of GD2 for cell capture could also identify patients that may benefit from the addition of an anti-GD2 therapy in addition to standard of care.

We have demonstrated that GD2 and CSV are expressed on the majority of cells in a panel of OS cell lines and that the combination of these markers can be successfully applied to capture OS cells from both cell solutions and patient blood. The limitation of this work is the small number of patient samples evaluated. However, we did demonstrate as proof as concept that GD2/CSV can be used to detect CTCs in patients with OS. Further optimization and capture from samples collected from patients with OS will be required to fully validate these markers for CTCs.

CRediT authorship contribution statement

Henrietta O. Fasanya: Conceptualization, Investigation, Resources, Data curation, Formal analysis, Funding acquisition, Writing - original draft, Writing - review & editing. **Pablo J. Dopico:** Conceptualization, Investigation, Resources, Data curation, Formal analysis, Supervision, Funding acquisition, Writing - original draft, Writing - review & editing. **Zachary Yeager:** Investigation, Writing - review & editing. **Z. Hugh Fan:** Conceptualization, Supervision, Writing - review & editing. **Dietmar W. Siemann:** Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbo.2021.100357.

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