Universal Marker and Detection Tool for Human Sarcoma Circulating Tumor Cells

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ABSTRACT

To date, no specific marker exists for the detection of circulating tumor cell from different types of sarcomas, though tools are available for detection of circulating tumor cell (CTC) in peripheral blood of cancer patients for epithelial cancers. Here we report cell-surface vimentin (CSV) as an exclusive marker on sarcoma CTC regardless of the tissue origin of the sarcoma as detected by a novel monoclonal antibody. Utilizing CSV as a probe we isolated and enumerated sarcoma CTC with high sensitivity and specificity from the blood of patients bearing different types of sarcoma, validating their phenotype by single cell genomic amplification, mutation detection and fluorescence in situ hybridization. Our results establish the first universal and specific CTC marker described for enumerating CTC from different types of sarcoma, thereby providing a key prognosis tool to monitor cancer metastasis and relapse.
Introduction

Sarcoma constitute ~10% of different cancer types (1). These are a rare group of malignant tumors that develop in the soft tissue and bone. There are several kinds of sarcomas, with soft tissue sarcomas that occur in fat, nerves, blood vessels, muscles and deep skin tissues, while osteosarcomas occur in the bone and Ewing sarcomas are associated with bone and soft tissue. Despite the low incidence of these tumors, their occurrence is more common in adolescents and young adults in comparison to other cancers thus causing a loss of substantial years to the treatment of this disease and affects the quality of life. One way to detect the early spread of the localized disease to distant organs is to detect the circulating tumor cells (CTC) from the peripheral blood of the patients. CTC are rare cells that detach themselves from primary tumor and enter into blood stream, from where they are carried to distant organs to metastasize. These CTC are considered to be the seeds of metastases (2) and are emerging as promising targets for early detection and monitoring therapeutic efficacy of anti-cancer drugs (3). At present the primary markers for detection of CTC are EpCAM and cytokeratins that can be used to detect CTC from epithelial cancers only (4) and lack the capability to detect CTC from sarcoma tumors since these are mesenchymal in origin and do not express epithelial specific markers. Although there are new technologies that are enriching the CTC based on size and density of CTC (5), none of these studies are applied for CTC enumeration from sarcoma patients. CTC have been isolated and identified in a wide range of malignancies and it has been well demonstrated that CTC are associated with metastasis and play a key role in cancer progression and relapse (6), however due to the limitations of existing epithelial markers of CTC and the absence of a specific marker for detecting sarcoma CTC, the research in this direction remains hampered.
Therefore, identification of a new marker that can be useful in the enumeration of CTC from sarcoma patients will provide valuable information for patient care.

Vimentin over expression is frequently associated with different cancers (reviewed in (7)) and single cell profiling of CTC isolated from cancer patients indicates the overexpression of vimentin transcript (8); however, intracellular expression of vimentin in normal mesenchymal cells including most of the white blood cells (WBC), limits its usage as a CTC marker. We and others have previously reported the detection of CSV in cancer cells (7, 9, 10), however it remains unknown if CSV can serve as a marker for detecting CTC from blood of cancer patients. Here for the first time, we report the discovery of CSV as a universal sarcoma CTC marker by using a monoclonal antibody 84-1 that was generated for detection of CSV on CTC. The data reported here holds great promise for the detection and enumeration of CTC from patient bearing any given type of sarcoma tumor irrespective of the origin, thus making CSV a universal sarcoma CTC marker.
Materials and Methods

Cell lines

HUVEC cells were obtained from Dr. Lee Ellis (MD Anderson Cancer Center). LM7, SAOS-2, K7, K7M3, LM-8 and DUNN cells were kindly provided by Dr. Eugenie S Kleinerman (MD Anderson Cancer Center). HOS, MG-263, OS-D, OS-O, LM7-GFP and OS-25 cells were kindly provided by Dr. Dennis Hughes (MD Anderson Cancer Center). Primary cell cultures from Osteosarcoma patients were kindly provided by Dr. Dina Lev (MD Anderson Cancer Center). HUVEC, HFOB and SAOS-2 cell lines were obtained directly from American Type Culture Collection (ATCC) (Manassas, VA, USA). Authenticity for LM7, K7, K7M3, LM-8, HOS, MG-263, OS-D, OS-O, SKNBE-2, LM7-GFP and OS-25 cells were validated using STR DNA Fingerprinting before experimentation at characterized cell line core facility, MD Anderson Cancer Center.

Blood collection and processing:

Human blood samples for CTC analysis were obtained after informed consent, per IRB protocol at MD Anderson Cancer Center. Healthy blood samples were obtained from Gulf Coast Blood Center. A maximum of 8 ml of blood were obtained at any given blood draw, using CPT Vacutainer tubes (BD Bioscience). Single nucleated cells were isolated as per manufacturer recommendation. Cells were then washed in PBS and used for further analysis.

Spiking Assay

For sensitivity assay demonstration, ~2, ~5, and ~10 Calcein AM labeled (EMD Bioscience) LM7 cells were spiked in to 10 million PBMC containing sample in triplicate. For specificity
demonstration, ~5 cells of Calcein AM labeled LM7 cells were spiked into 10, 20 and 25 million PBMC containing samples in triplicates. All cells used for spiking experiments were subjected to 84-1+ve selection a day before the analysis to increase the fraction of 84-1+ve cells. For cell counting, cells were harvested in culture medium and then serially diluted to achieve the required counts, which were then confirmed in a series of 5 μl spots under a microscope. Under circumstances where in lower/higher numbers of cells were observed, calculations were performed to mix and obtain the necessary counts of cells required to be spiked. Spiking experiments were performed in triplicates to ensure the sensitivity and specificity of the method. For negative controls, CSV negative cells were spiked into blood and analyzed for positive selection.

84-1 positive cell selection

First, CD45 positive cells were depleted using EasySep™ Human CD45 Depletion Kit (Stem cell technologies) according to manufacturer’s recommendation. To minimize nonspecific binding, antibody against human Fc receptor was added to the cocktail. Second, the CD45-ve cell fraction was subjected to 84-1 positive selection. Briefly, cells were labeled with 84-1 antibody and later mouse IgG binding microbeads (Miltenyi Biotec) were added to the mixture. 84-1 +ve cells were then extracted using the magnetic column from Miltenyi Biotec according to manufacturer’s recommendation.

Antibody production

Recombinant human vimentin (rhVim) with 6-His (R&D Systems) was used as an antigen for antibody production. Anti-vimentin titer was determined using ELISA assay. Briefly, rhVim was
used as solid antigen in ELISA. Serum at serial two-fold dilutions was incubated for 2h in ELISA wells coated with rhVim. After washes, and incubation with peroxidase-coupled anti-mouse IgG antibody a color reaction was performed and analyzed with ortho-phenylene-diamine (OPD). Antibodies that showed higher O.D. at lower dilutions was considered for further screening. Antibodies selected for screening were incubated with osteosarcoma cell lines with and without vimentin on the cell-surface. Antibodies that have very high affinity for CSV were selected using flow cytometry and analyzed further. 84-1 was the best clone available to detect cell surface vimentin with high affinity and sensitivity. This antibody was then further characterized for vimentin binding by ELISA, western blotting, immunoprecipitation, immunocytochemistry and immunohistochemistry. Also, from the screening analysis, we identified 12-1, a different mAb that also showed binding affinity to vimentin, however, this antibody was used only for validation purposes.

*Mutation analysis*

Whole genome amplification from small numbers of CTC cells or single cells were performed using the REPLI-g Mini Kit (QIAGEN, Valencia, CA).

*Flow cytometry*

Indirect method of detection was used to detect 84-1 binding. Data acquisition was performed using Attune Flow cytometer (Applied Biosystems) and data analysis was performed using FlowJo software (Treestar).

*Sodium orthovanadate treatment*
LM8 cells plated on 8-well chamber slides and WBC in suspension were subjected to Sodium orthovanadate (SOV) (100 μM) or PBS as a control.

*Fluorescence in situ hybridization (FISH)*

Cells were exposed to hypotonic treatment (0.075 M KCl) for 20 minutes at room temperature. Cells were fixed in a methanol and acetic acid (3:1 by volume) mixture for 15 minutes, and washed three times in the fixative. The slides were air-dried and stored at -20 °C. FISH was performed on these slides using K-RAS and MDM2 FISH probes (Agilent Technologies Inc., California, USA) according to the manufacturer’s protocol with slight modifications. Images were captured using a Nikon 80i microscope with a UV source using DAPI, FITC and Spectrum orange filters.

*Microscopy image capture and analysis*

All microscopic image capture and analysis was performed using Zeiss LSM 510 confocal microscope (Zeiss).

*Statistical analysis*

Data reported here are as mean ± standard error of the mean as noted. Differences were considered significant at the 95% confidence level ($p < 0.05$). Data analysis was performed using Graphpad Prism software.

More detailed materials and methods can be found in the supplementary material.
Results & Discussion

To date, detection of cell-surface vimentin (CSV) on cancer cells has been shown by only using vimentin specific peptide (9) or virus particles (11), since none of the commercial antibodies bind to CSV (Supplementary Fig. 1A). To generate CSV specific antibody that binds only to cancer cells while excluding the normal cells, a cell differential expression screening strategy was used (Fig. 1A). Briefly, we generated a large number of hybridoma clones against full length human vimentin (NCBI: NP_003371.2) and screened vimentin binding antibodies for CSV binding utilizing flow cytometry by targeting CSV on human metastatic osteosarcoma (LM7) cell line, which showed positive binding for CSV specific peptide [7] (Supplementary Fig. 1B). For negative binding, normal cell lines human fetal osteoblast (HFOB) and NCM-356 (normal colon) were utilized. Utilizing this strategy, we identified a monoclonal antibody, or a CSV binding IgG thereof, from hybridoma 84-1. The specificity of 84-1 towards vimentin was further confirmed using western blotting, immunoprecipitation and immunofluorescence (Supplementary Fig. 1 C, D, and E). Furthermore, other sarcoma cell lines were evaluated for CSV expression using flow cytometry (Supplementary table 1).

Cell-surface screening for different cancer and normal cell lines using flow cytometry indicated the presence of vimentin only on the surface of cancer cells (Fig. 1B). Importantly, 84-1 did not show any binding towards macrophages, endothelial cells, neutrophils, platelets, and apoptotic T lymphocytes that are abundantly present in the blood stream (Supplementary Fig. 1F), indicating the specificity of 84-1 toward cancer CSV. Immunocytochemistry analysis of human metastatic osteosarcoma cells LM7 indicates the presence of vimentin only on the surface of cancer cells (Fig. 1C) that co-localizes with Wheat-germ Agglutinin (WGA), a cell-surface marker. An
analysis of the primary cancer cell lines generated from human osteosarcoma patient samples (Supplementary Fig. 1G) shows expression of CSV restricted to metastatic cancer cells while primary cancer cells were negative. These results indicate that CSV expression is associated with metastatic phenotype of the cells.

Based on the detection of CSV in a range of sarcoma cell lines, we hypothesized that CSV could serve as a marker to detect sarcoma CTC. To test this hypothesis, we performed a blood spiking assay using labeled LM7 cells. Known concentrations of Calcein AM labeled cells were spiked into blood and after CD45 depletion and 84-1 positive selection, the cells recovered were subjected to immunofluorescence staining (schematic representation in Fig. 2A). From micrographs it is evident that single labeled cell isolated from whole blood using 84-1 antibody is detectable utilizing fluorescence microscopy (Fig. 2B). Since sensitivity (limit of detection) and specificity (no background/ unwanted cells) of detection are important parameters for using an antibody for CTC enumeration, we evaluated these parameters in this spiking assay using 84-1+ve LM7 cells. Linear regression of the number of detected tumor cells versus the number of tumor cells spiked yielded a correlation coefficient ($R^2$) of 0.976 (Fig.2C) and indicated that the recovery rates were >60% with ~100% specificity (Supplementary Fig. 1H). We also performed spiking assay by spiking higher numbers of cells that yielded similar results (Supplementary Fig. 1I). Furthermore, the isolated tumor cells were negative for CD45 staining, a leukocyte marker (Fig. 2D). As control, normal HFOB cells were spiked (100 cells) and were not detectable by 84-1 antibody. These spiking assays were further corroborated by in vivo studies wherein we utilized LM8 cells for osteosarcoma mouse model. These mice were monitored for changes in CTC over a period of time (Supplementary Fig. 2A) and at the end of the study; the cells were isolated and confirmed for CSV+, CD45- staining (Supplementary Fig. 2B) and at the end of the
study, the CTC were isolated and cultured in-vitro (Supplementary Fig. 2C). We also utilized a spontaneous tumor model of p53-mutated mice that showed the presence of CTC in different types of sarcoma tumors that developed spontaneously over a period of time (Supplementary Fig. 2D). Furthermore, we tested for CTC in the blood of canine with spontaneous sarcoma tumors and 84-1 antibody was able to detect CTC in this model (Supplementary Fig. 2E). These results indicate a high sensitivity and specificity of 84-1 antibody towards spiked cancer cells as well as CTC that are derived from spontaneous mice and canine sarcoma models.

Next, we tested human blood samples from healthy volunteers and sarcomas that include osteosarcoma, Ewing sarcoma (EWS), angiosarcoma (AGS), leiomyosarcoma (LMS) and undifferentiated pleomorphic sarcoma (UPS) (Supplementary Table. 2). No CTC were detectable in blood samples from healthy donors. Collectively, an increase in CTC count was observed in patients who were diagnosed with metastasis in comparison to patients with localized disease only at the time of first presentation of the clinical disease (Fig. 3A). Also, patients who have undergone prior chemotherapy had lower CTC counts. The CTC isolated from sarcomas were further characterized by either single cell mutation analysis or Fluorescence in situ hybridization (FISH) using specific probes or by specific markers. Angiosarcoma derived CTC were tested for mutations in TP53 and FLT4 genes that were detectable in primary tumors, however only TP53 mutation was detectable in CTC (Fig. 3B) indicating the heterogeneous nature of CTC (12). Osteosarcoma derived CTC were evaluated for MDM-2 and KRAS amplification using FISH and from results it is evident that there is increasing amplification of these genes in patient OS7 when compared to patient OS8 that did not show any amplification (Fig. 3C). OS7 patient was diagnosed with metastasis in lung, while OS8 had localized osteosarcoma. Since MDM-2 and KRAS have been previously shown to be amplified in metastatic osteosarcoma patients (13) (14)
and from our results, amplification in CTC is associated with metastasis, it is tempting to speculate that the detection of this amplification in CTC can predict the onset of metastatic lesions at distant sites and also can prove to be an important tool to predict the therapeutic efficacy of the anti-cancer drugs. Furthermore, the isolated 84-1+ CD45− CTC were validated by utilizing specific markers for a given tumor; CD99 was used as a marker for Ewing sarcoma (15), α-SMA for leiomyosarcoma and CD31 for angiosarcoma (Fig. 3D). To our knowledge, this is the first study to enumerate and validate CTC from different types of sarcomas using a single specific marker.

Although, the mechanism of cell-surface transport of vimentin remains unclear, previous reports have suggested the translocation of vimentin to cell-surface is phosphorylation dependent (reviewed in (7)). We hypothesized that inhibiting specific phosphatases can enhance phosphorylation of vimentin, thereby increasing vimentin translocation to cell surface. To test this hypothesis we used Sodium Orthovanadate (SOV), an inhibitor of tyrosine phosphatases. SOV increases CSV expression in cancer cells (Fig. 4A, middle panel); however there is no change in the CSV of normal leukocytes (Fig. 4A lower panel). Further, we tested the translocation of vimentin to the surface of the 84-1+ CD45− CTC isolated from osteosarcoma patient sample by treating with SOV or control PBS and the results indicated a considerable amount of vimentin on the surface of the cancer cells treated with SOV when compared to control treated sample as detectable by fluorescence microscopy (Fig. 4B). Also, we stained the CTC isolated from blood of patient with GIST tumor (with and without SOV treatment) for CD31 marker to prove that these are not endothelial cells (Supplementary Fig. S3). SOV can therefore be used as a CSV boosting agent thus increasing the yield of CTC, a major requirement in the detection of CTC.
CTC detection using CSV as a marker provides several advantages. First, since there are no literature reports for a universal specific marker to detect sarcoma tumor-derived CTC in the, this CSV marker together with 84-1 antibody fills this gap in the field of CTC detection. Second, isolation of viable CTC for further molecular characterization, not just from human but also from canine and mouse models highlights its usage as both preclinical and clinical research tool. Importantly, this is the first proof-of-principle study with small number of sarcoma patients analyzed and this study will be followed up with larger validation studies with clinical endpoints such as relapse and survival. In summary, isolation of CSV positive CTC will provide an understanding about the metastatic precursor subpopulation and also help in providing novel diagnostics; treatment and prognostic options based on therapeutic monitoring in sarcoma patients and will play a potential role in clinical decision-making.

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References


Figure Legends

**Fig. 1** Isolation and analysis of CSV-specific 84-1 monoclonal antibody: (A) Schematic representation of screening antibodies for CSV specific binding. Pools of monoclonal antibodies from different hybridoma supernatants were analyzed for CSV detection using flow cytometry and antibodies that bound only cancer cells were selected and characterized for vimentin binding using immunocytochemistry (IFC) and western blotting. (B) Immunological assessment of CSV expression in different cancer and normal cell lines using flow cytometry: CSV is detectable only in the cancer cell lines SKNBE-2 (neuroblastoma), RH-41 (rhabdomyosarcoma) and LM7 (osteosarcoma) cells, while the non-epithelial normal cells HUVEC, HFOB and PBMCs were negative for CSV expression. Isotype controls were used for negative controls. (C) Cell surface staining analysis for CSV in LM7 (osteosarcoma) cancer cell line using confocal microscopy: cells were stained for CSV (green), WGA (red) and nuclear stain DRAQ5 (blue). 84-1 co-localizing with WGA indicates cell surface vimentin. Scales indicate 10 μm.

**Fig. 2** Spiking assay: (A) Schematic representation of 84-1 mediated CTC detection, isolation, enumeration and analysis. (B) Detection of LM7 cells labeled with tracker dye Calcein-AM (CAM) (green) that were spiked into 7.5 mL blood using fluorescence microscopy. (C) Regression analysis of capture efficiency for different cell numbers of LM7 cells spiked in human blood. (D) Micrographs of cells isolated LM7 cells from blood were co-stained using antibodies against CSV (green), CD45 (red) and a nuclear stain DRAQ5 (blue). Scale indicates 10 μm.

**Fig. 3** Enumeration and characterization of CTC: (A) Enumeration of CSV positive CTCs from healthy and sarcoma cancer patient blood specimens. Green: healthy, blue: primary tumors.
only, red: metastatic tumors. (B) Single cell mutational analysis of TP53 in angiosarcoma derived CTC indicates mutation in TP53 gene. (C) Representative case of Osteosarcoma CTC characterized by fluorescence in situ hybridization (FISH). Results indicate KRAS (green) and MDM2 (red) amplification in OS7 sample. Nucleus is stained with DAPI (blue). Sample OS8 and Control leukocyte showed basal signal. Scale indicates 5 μm. (D) Analysis of 84-1+ CD45− CTC isolated from Ewing Sarcoma (EWS), Leiomyosarcoma (LMS) and Angiosarcoma (AGS) samples. EWS CTC were validated using CD99, LMS CTC using α-SMA and AGS CTC using CD31. Scale indicates 10 μm.

**Fig. 4 Enhancing vimentin transport to cell surface:** (A) Effect of Sodium orthovanadate (SOV) on LM8 (mouse metastatic osteosarcoma cells) and white blood cell (WBC). LM8 and WBCs were treated with SOV and analyzed for the expression of CSV using confocal microscopy. Cells were stained for CSV (green), cell surface marker WGA (red) and nuclear stain DRAQ5 (blue). It is evident that treatment of cancer cells with SOV for 15 minutes enhances the CSV expression when compared to that of the untreated cells. There was no effect of SOV observed on WBC. (B) Analysis of SOV effect on patient derived CTC. CTC isolated were subjected to SOV treatment and stained for vimentin using 84-1 (green) and nuclear stain DRAQ5 (blue). SOV treatment enhanced the expression of CSV in CTC. Scale indicates 10 μm.
Figure 4

A

B
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