AACR, San Diego, April 5-9, 2014 **Poster No. 3082**

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Isolation and Identification of Disseminated Tumor Cells from Bone Marrow

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INTRODUCTION

- Disseminated tumor cells (DTCs) are detected in the bone marrow (BM) of up to 40% of breast cancer patients at the time of diagnosis and are an independent prognostic factor for recurrent disease development.
- DTCs can persist for years and remain a predictor for disease recurrence.
- DTCs are rare cells and present at a level of 1-20 per million nucleated BM cells.
- Two important applications for DTC detection are the monitoring of therapeutic efficacy in those patients without radiographic or clinical evidence of disease and identifying therapeutic targets in those patients with minimal residual disease.
- Present techniques for detection of DTCs are often laborious, and insensitive due to the molecular heterogeneity of the DTCs.
- Robust, reproducible assays for detection of DTCs are needed for characterization, staging, and monitoring therapeutic response.
- Conventional antibody-based enrichment methods may not capture a large percentage of DTCs.
- We explored the use of microfiltration, an antibody-independent method, to improve detection efficiency of low level DTCs in bone marrow.

MATERIALS & METHODS

- BM aspirates were collected from normal volunteers or patients with breast cancer.
- For spiking experiments, SKBR3 breast cancer cells were added at various concentrations to normal BM. A total of 14 million nucleated BM cells were in each specimen.
- BM was then diluted 1:10 with PBS.
- 7mL of the diluted sample was placed in CellSave™ tubes and shipped at ambient temperature to Creatv for processing. Specimens were processed within 24 h of collection.
- Specimens were pre-filtered using a 70-µm mesh sieve to remove large particles.
- The effluent was filtered through CellSieve™ microfilters, which have 7-µm diameter pores in a uniform array, of 160,000 pores in a 9 mm diameter area (**Fig. 1**).

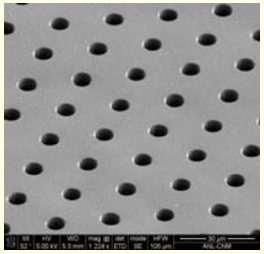

Figure 1. SEM image of CellSieve™ microfilters with 7-µm diameter pores in a uniform array.

- Filter-captured cells were post-fixed, permeabilized, and stained with DAPI and fluorescent antibodies specific to cytokeratins (CK) 8, 18, and 19 (FITC), EpCAM (PE), and CD45 (Cy5, leucocyte marker).
- For quantitation, SKBR3 cells on the entire filter membrane were analyzed to determine the average and standard deviation (Sample #2 and 3); five 1% area images were analyzed when high numbers of SKBR3 cells were input (Sample #4).
- Contamination was calculated by subtracting the total DAPI count from the DTC count.

RESULTS

Recovery efficiency of cancer cells by using CellSieve™ microfiltration.

- SKBR3 cells were spiked into normal BM samples at 1, 10 and 100 cells per 1 million BM cells.
- 14 million nucleated BM cells were processed by microfiltration per sample - Recovery efficiency was estimated by counting the tumor cells based on DAPI and antibody staining (**Fig. 2**, **Table 1**).
- Control BM specimens with no added tumor cells demonstrated only rare staining cells (range 0-4 per sample) likely due to background or epithelial cell contamination.

Table 1. Recovery efficiency of SKBR3 cells from bone marrow.

Sample	SKBR3-Input	SKBR3-Recovered	Recovery (%)	Average (%)	SD (%)
1	0	0	NA		
2	14	12	85.71	87.38	4.19
3	140	129	92.14		
4	1,400	1,180	84.29		

Identification of the filter-captured SKBR3 tumor cells by fluorescent antibody staining.

- On-filter antibody staining was performed to distinguish tumor cells from white blood cells (**Fig. 3**). The filter-captured SKBR3 tumor cells stained with DAPI (blue), CKs (green), EpCAM (orange) but not for CD45 (red). Normal blood cells in BM stained with DAPI and CD45.

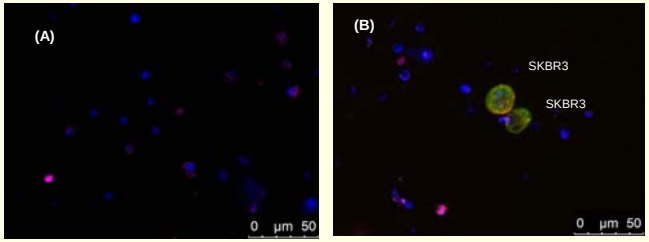

Figure 2 (above). Images of the filter-captured cells. (A) Bone marrow control (no-spiking). (B) Bone marrow sample spiked with SKBR3 cells. Arrows indicate SKBR3 cells.

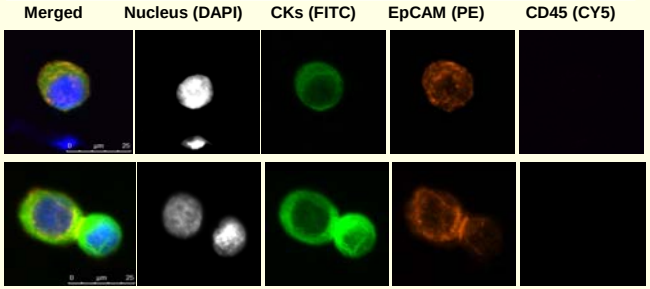

Figure 3. Patterns of antibody staining of SKBR3 on the filter membrane. Nuclei are shown as blue in the merged images.

Enrichment for breast cancer cells from BM by microfiltration

- Residual mononucleated cells from 14 million BM cells ranged from 26,000 to 41,000.
- Fold enrichment ranged from 340-570 with an average of approximately 430. (**Table 2**)

Table 2. Residual BM MNC with filtration

Sample*	Residual Mononucleated Cells	Fold Enrichment
2	40,800	343
3	36,200	386
4	24,200	400
Average	31,900	438
SD	7,900	

*corresponds to samples in Table 1

Analysis of BM from a patient with metastatic breast cancer.

- Identification of DTCs from patient specimens was based on nuclear features, CK+, EpCAM+/-, CD45-, and increased nuclear-cytoplasmic ratio (**Fig. 4 A, B**). Normal BM cells could be distinguished from DTCs nuclear-cytoplasmic ratios and staining by CD45 (**Fig. 4 C**).

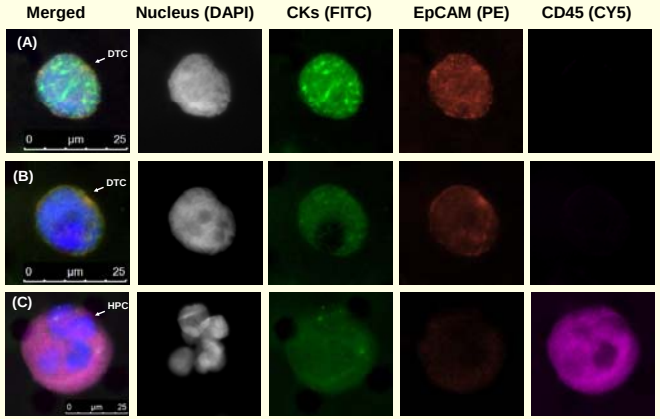

Figure 4. Characterization of the filter-captured cells from patient's bone marrow samples by fluorescent antibody staining. (A, B), Filter-captured DTCs; (C), Hematopoietic precursor cells (HPC). Nuclei are shown as blue in the merged images.

CONCLUSIONS

- Breast cancer cells can be recovered with high efficiency (87%) from bone marrow using microfiltration.
- Recovered breast cancer cells could be further characterized by fluorescent antibody staining.
- Enrichment of breast cancer cells from BM mononucleated cells was approximately 350-400 fold.
- Incorporation of microfiltration may facilitate detection of DTCs from bone marrow.

REFERENCES

1. Adams, DL, Zhu, P, Makarova, OV, Martin, SS, Charpentier, M, Chumsri, S, Li,, S, Amstutz, P and Tang, C-M, The systematic study of circulating tumor cell isolation using lithographic microfilters, RSC Adv., 2014, 4, 4334.