CHANG GUNG UNIVERSITY

A negative selection system *PowerMag* for enhanced detection of EpCAM positive and negative circulating tumor cells: implication in disease progression monitoring

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Abstract

Circulating tumor cell (CTC) is a good indicator for cancer progression and treatment response. A long-lasting issue of leukocyte contamination has hindered the simultaneous isolation of CTCs that are either positive or negative for the epithelial cell markers and correlate the clinical status of these cell populations. In this study, a *PowerMag* system was designed to enhance leukocyte depletion and CTCs isolation. The recovered cells were characterized by immunofluorescence staining using anti-EpCAM and anti-CD45 antibodies. *PowerMag* is efficient in removal of $CD45^+$-leukocytes with the recovery rates reaching 77-82% and 46-62% when the tests were performed in leukocyte cell suspension and whole blood, respectively. The blood samples from healthy control (n=27) and from the patients with colorectal cancer (CRC, n=24) and head and neck squamous cell carcinoma (HNSCC, n=28) collected at baseline were analyzed. At baseline, the numbers for both cell types, $EpCAM^+CD45^-$ and $EpCAM^-CD45^-$ cells, were significantly increased in these patients when compared to healthy control. After chemotherapy, the cell numbers for $EpCAM^+CD45^-$ and $EpCAM^-CD45^-$ were decreased more prominently in CRC and HNSCC patients, respectively. Together, *PowerMag* is effective in leukocyte depletion and holds great promise for analyzing CTCs status of cancer patients.

Methods

■**Cancer cell spiking :** Pre-staining PC3 cell with calcein red/orange, then add the quantified cell into whole blood contained anticoagulant.

■**Normal cell depletion :** Using RBC lysis buffer to remove most of the RBC and processing WBC depletion by CD45 depletion cocktail with *PowerMag* column.

■**Immunofluorescence staining :** After blood sample process with *PowerMag* system, stain the cells with CD45-PE for WBC, EpCAM + AF-488 for CTCs and Hoechst for total nucleated cells.

Schematic representation of the *PowerMag* system

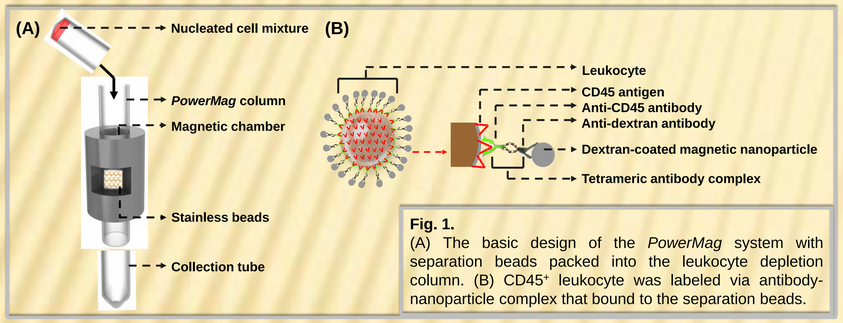

Fig. 1.
(A) The basic design of the *PowerMag* system with separation beads packed into the leukocyte depletion column. (B) $CD45^+$ leukocyte was labeled via antibody-nanoparticle complex that bound to the separation beads.

Evaluation of cancer cell recovery rate by spiking test

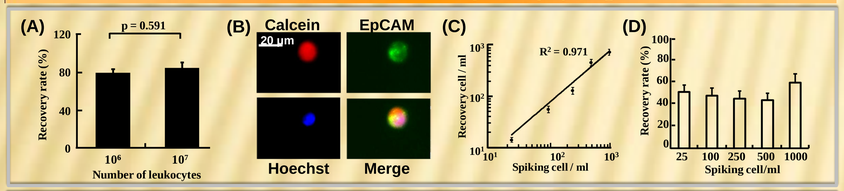

Fig. 2.
(A) One hundred PC3 cancer cells were pre-labeled with calcein red/orange dye and were spiked into cell suspension containing 10^6 and 10^7 leukocytes, respectively. After enrichment by *PowerMag* system, the number of PC3 cells was counted and the percentage of cell recovery was determined. The data represent the mean ± SE of 5 and 10 independent experiments for spiking into 10^6 and 10^7 leukocytes, respectively. (B-D) Pre-labeled PC3 cells were spiked into the whole blood of healthy volunteer to compose whole blood samples with different PC3 cell concentrations (25, 100, 250, 500 and 1,000 cells/ml). After processing by *PowerMag*, the collected cells were analyzed by immunofluorescence staining using anti-EpCAM antibody followed by Alexa Fluor 488-conjugated donkey anti-mouse secondary antibody. PC3 cells were identified by positive staining of calcein red/orange dye, Alexa Fluor 488 and Hoechst (panel B). The number and the percentage of cell recovery were determined (panel C and D). The data represent the mean ± SE for 3-4 independent experiments.

The number of PC3 cancer cells recovered from spiking test

Table 1.

Spiking test	1	2	3	4	5	6	7	8	9	10	
No. of cell spiked	No. of cell recovery										Mean ± SD
1	1	0	1	0	0	1	1	1	0	1	-
5	2	5	2	3	3	3	3	3	3	3	3.0 ± 0.3
10	7	6	8	6	8	6	5	6	7	8	6.7 ± 0.3

Cell population and proliferative capability of the nucleated cells enriched by *PowerMag*

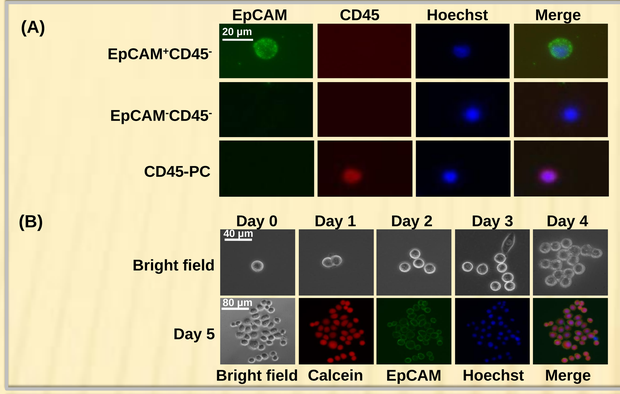

Fig. 3.
(A) The PC3 cells were spiked into the whole blood of healthy volunteer and were recovered from the whole blood using the *PowerMag* system. The recovered cell suspension was subject to immunofluorescence staining using the anti-EpCAM (green) and anti-CD45 (red) antibodies. Leukocytes were used as a positive control (PC) for immunostaining of anti-CD45 antibody. Two major cell populations were defined according to the immunofluorescence staining patterns. (B) The PC3 cells that were recovered after *PowerMag* CTCs isolation scheme were cultured for 1-5 days. A representative PC3 cell colony at the indicated days of culture was shown (upper panel). At day 5 of culture, the cells were labeled with the calcein red/orange dye and were subject to immunofluorescence staining using the anti-EpCAM antibody and Hoechst staining dye.

Basic characteristics for the cancer patients subject to CTCs analyses

Table 2.

Patient characteristics	CRC[a]	HNSCC[a]
Patients number	24	28
Median age (range), years	61.5 (38-82)	56 (41-80)
Sex (Male/Female)	13/11	26/2
Stage III/IV without distant metastasis[b]	4	21
Stage IV with distant metastasis	20	7

[a]CRC, colorectal carcinoma; HNSCC: head and neck squamous cell carcinoma.

[b]According to American Joint Committee on Cancer (AJCC), 7th edition, 2010. In HNSCC, stage IVa or IVb means locally advanced stage (often extended regional lymph node involvement without distant metastasis.

The CTCs status for the healthy control and cancer patients

Table 3.

Type	Condition	$EpCAM^+$ Median	$EpCAM^+$ Mean	$EpCAM^+$ ≥5	$Markers^-$ Median	$Markers^-$ Mean	$Markers^-$ ≥650	$EpCAM^+$≥5 or $Markers^-$≥650
Normal	Control (n = 27)	1.3	1.6	0.00%	182	250.8	11.10%	11.10%
CRC	Baseline (n = 24)	56.3	461.7	83.30%	755.9	1411.2	61.10%	95.80%
	Follow up (n = 22)	7.2	17.7	54.50%	519	1019.3	45.50%	77.30%
	Change (%)	**-87.3**	**-96.2**	-	**-24.2**	**-25.7**	-	-
HNSCC	Baseline (n = 28)	24.8	109.4	100.00%	889.4	1347.3	60.70%	100.00%
	Follow up (n = 19)	21	76.3	94.70%	371	760.7	47.40%	94.70%
	Change (%)	**-15.2**	**-30.2**	-	**-58.3**	**-43.5**	-	-

The population and cell size of CTCs isolated from healthy donor and cancer patients by *PowerMag*

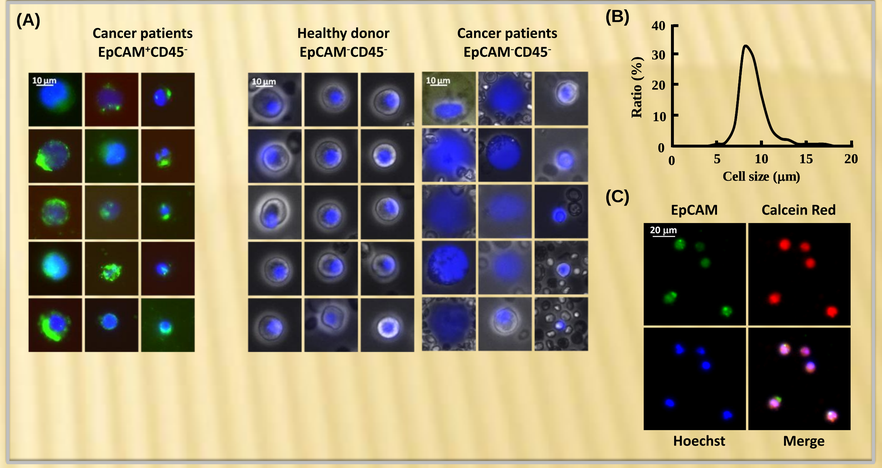

Fig. 4.
(A) The peripheral blood of healthy donor and cancer patients was subject to *PowerMag* analysis. Enriched nucleated cells were subject to immunofluorescence staining by anti-EpCAM and anti-CD45 (red) antibodies. Two major cell populations, $EpCAM^+CD45^-$ ($EpCAM^+$-CTCs) and $EpCAM^-CD45^-$, were defined. Fifteen representative images for each cell population were shown. Note the heterogeneous distribution of cell size for $EpCAM^-CD45^-$ cells isolated from cancer patients. (B) The diameter for $EpCAM^+$-CTCs cells (n = 150) was determined and the cell size distribution was plotted. (C) The $EpCAM^+CD45^-$ ($EpCAM^+$-CTCs) band $EpCAM^-CD45^-$ cells isolated from cancer patient were subject to vital staining with calcein red/orange dye. A representative field was shown.

CTCs tests for healthy donors and cancer patients

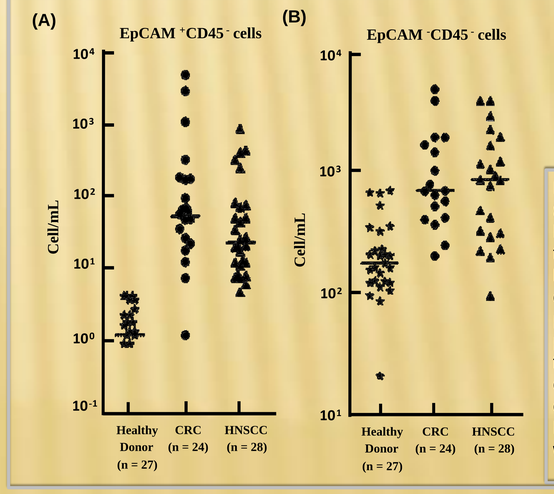

Fig. 5.
(A) and (B) Peripheral blood from healthy donors (n=27) were analyzed to define the reference values for EpCAM+CD45- and EpCAM-CD45- cells. In addition, CTCs tests were performed by *PowerMag* using the peripheral blood collected at baseline from CRC (n=24) and HNSCC (n=28) cancer patients. The cell number for each test and the medium value for healthy control, CRC and HNSCC were plotted.

Conclusion

PowerMag system is effective for CTCs isolation and has the benefits of low cost per test. As a whole, *PowerMag* holds great promise as a platform for CTCs isolation and detetion. By this system, both EpCAM+-CTCs and marker negative cells can be monitored simultaneously that offers benefits in understanding the status of cancer progression and treatment response of cancer patients.