Clinical Implications of Circulating Tumor Cells of Breast Cancer Patients: Role of Epithelial-Mesenchymal Plasticity

Seung Il Kim, M.D., Ph.D.

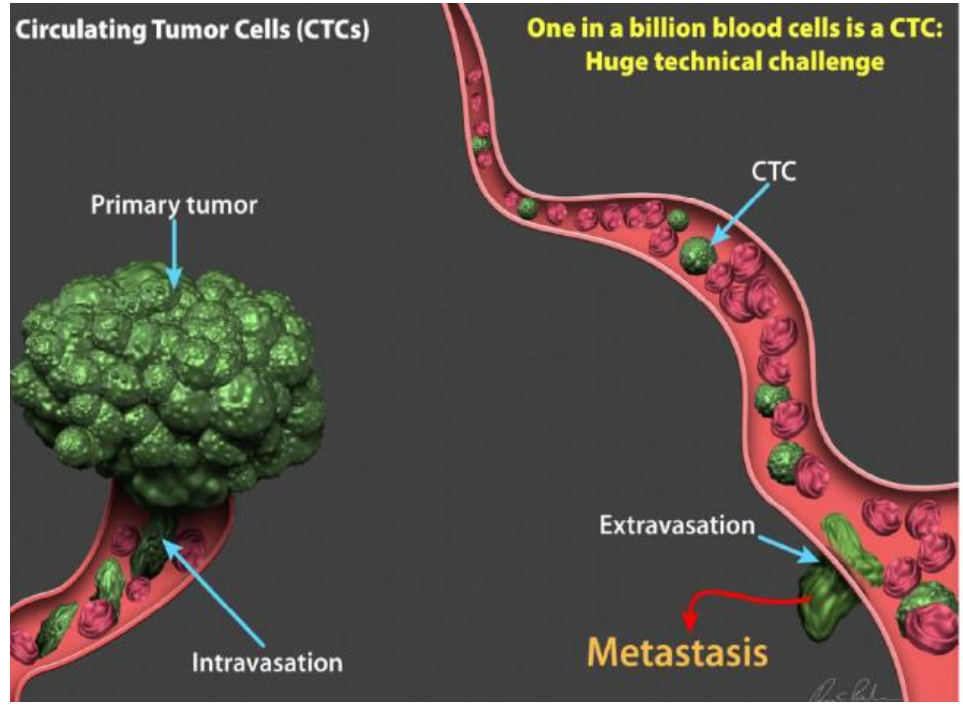
Department of Surgery, Yonsei University College of Medicine

Breast Cancer Center, Severance Cancer Hospital

What is the Circulating Tumor cells (CTCs)?

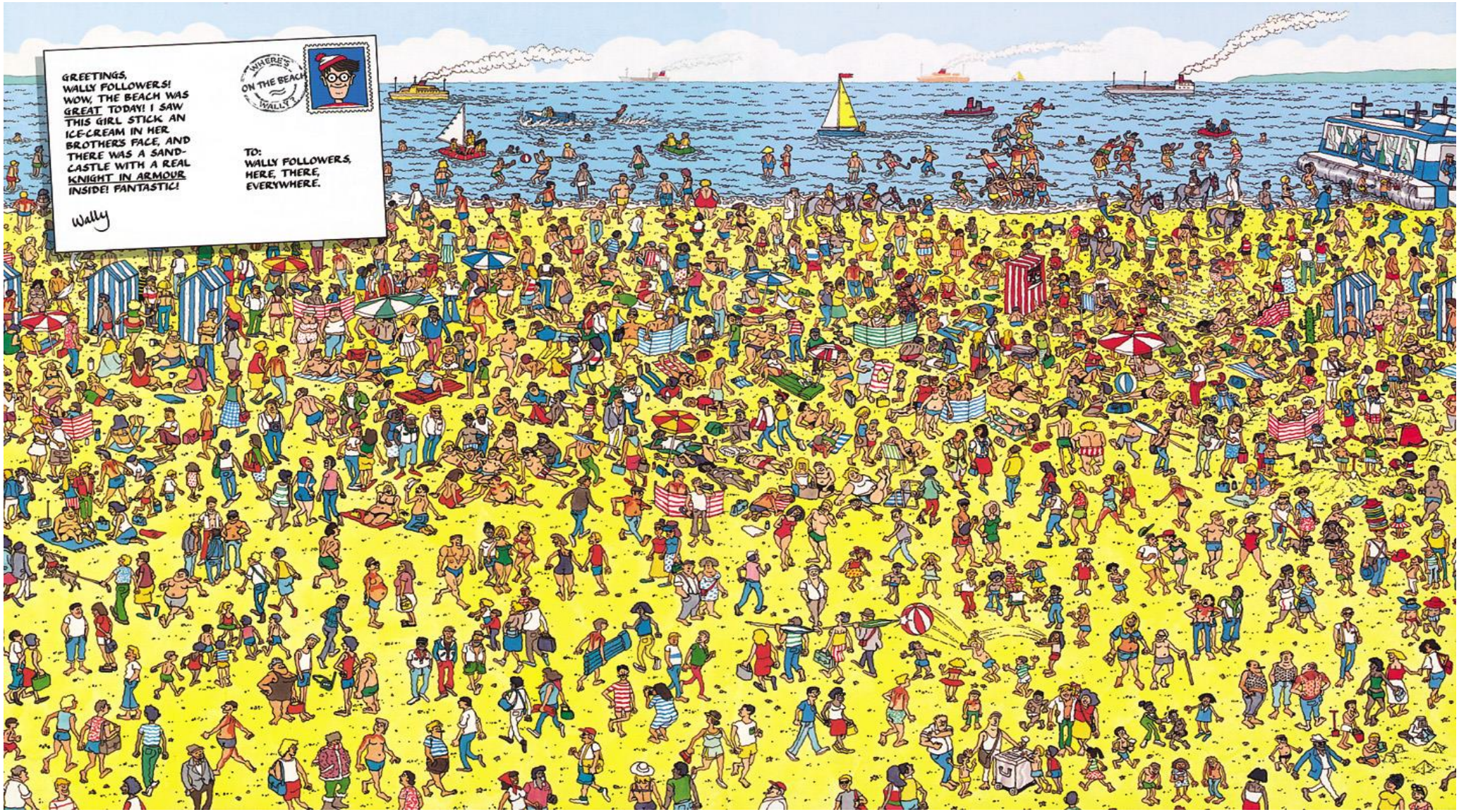
✓Identification of tumor like cells in the peripheral blood of cancer patients
: described as early as 1869 by Ashworth
- cells in peripheral blood with phenotype of cancer

(Ashworth T. Australian Med J 1869;14:146.)



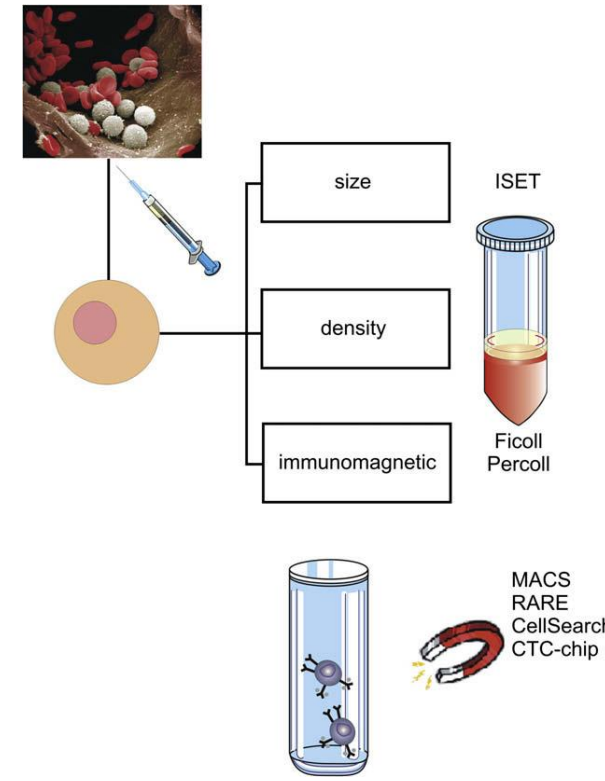
(Shannon Stott, et al.)

Detection of CTCs
- Where's Wally?



Methods for CTCs Detection

1. Enrichment
2. Identification



Enrichment

(1) physical properties

- ***size***: ISET (Isolation by Size of Epithelial Tumor cells) assay

- ***density***: Ficoll density gradient centrifugation

(2) biologic properties: *specific protein expression (EpCAM)*

- ***immunomagnetic techniques***
  : AdnaTest (AdnaGen AG, Langenhagen, Germany),
  : CellSearch (Veridex, Raritan, NJ)

- ***microfluidic platform***
  : CTC-chip
  : CTC-iChip

(Mostert B, et al. Cancer Treat Rev 2009;35:463-74.)

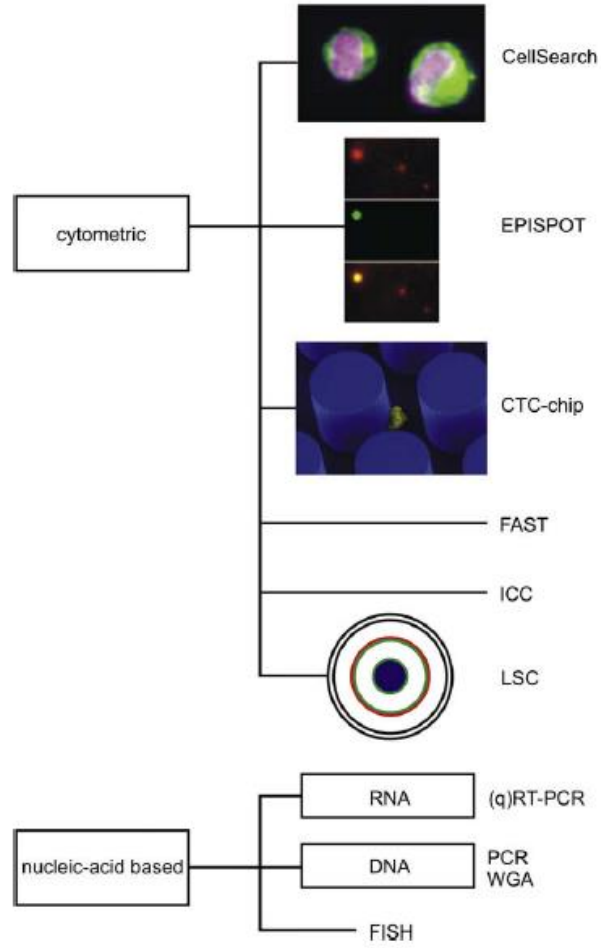
CTC Identification

✓**Cytometric**

- antibodies targeting epithelial antigens
  : breast cancer- *cytokeratin*, mammaglobin
- preservation of cell

✓**Nucleic-acid based**

- RT-PCR
  : amplify and identify tumor-associated RNA *(cytokeratin 19)*



(Mostert B, et al. Cancer Treat Rev 2009;35:463-74.)

CTCs detection systems

Morphological based approaches
- ISET
- Density gradient separation (Oncoquick)

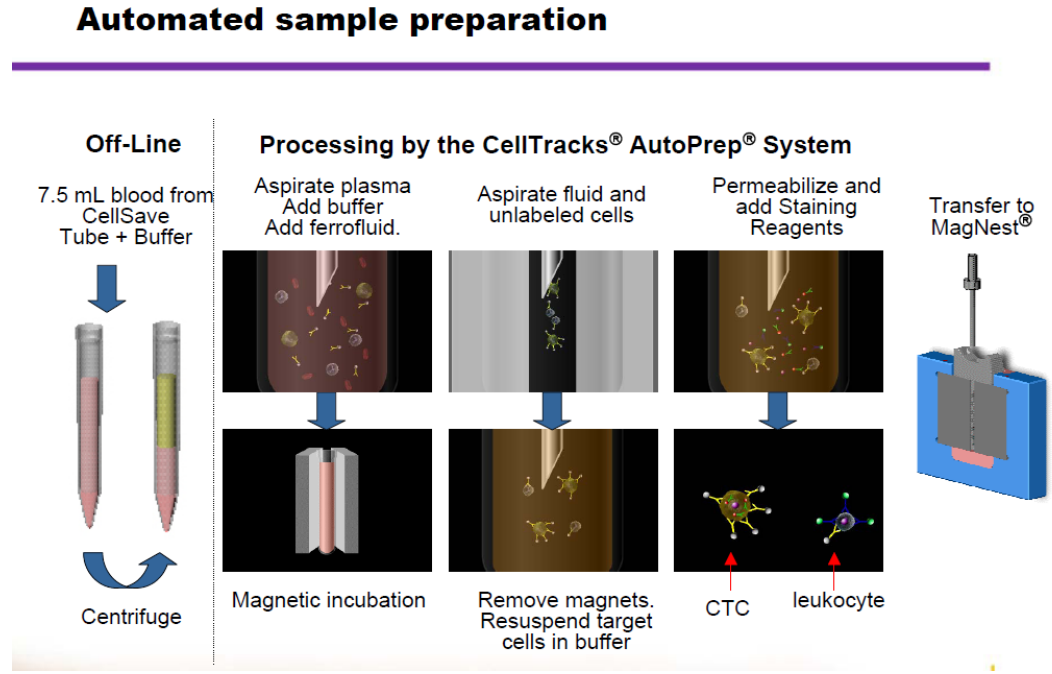
Immunological based approaches
- **CellSearch®**
- Adnatest
- **CTC-Chip, CTC-iChip**

Other approaches
- CAM assay
- EPISPOT
- LSC

CellSearch®

✓**CellSearch assay (Veridex, New Jersey, USA)**
- automated Assays
  : combining enrichment/identification
- separation of CTCs from the plasma
  : captured using antibody against *EpCAM*
- pan-CK antibody/anti-CD45 antibody

✓**Definition of CTCs**
- *expressing CK/ but lacking CD45*

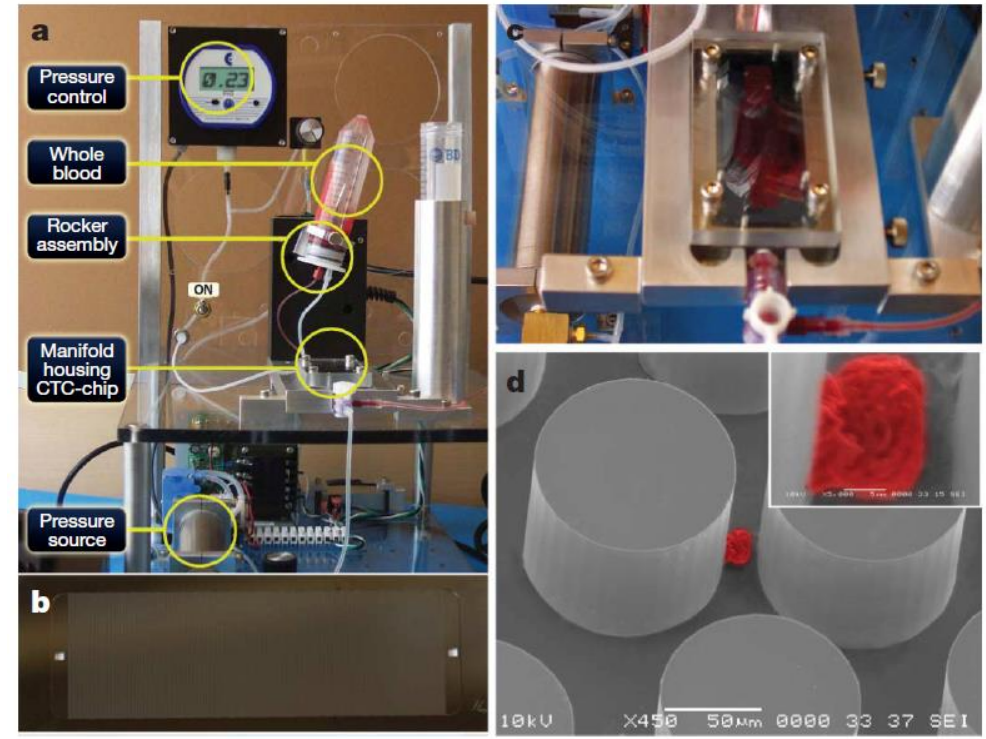


CTC-Chip

✓**CTC-Chip**
- microfluidic platform
- flows peripheral blood through an array of microposts
  : *coated with anti-EpCAM*

✓**Highly sensitive method**
- isolate CTCs in 99% of blood samples
  (metastatic lung, prostate, breast, colorectal cancers)

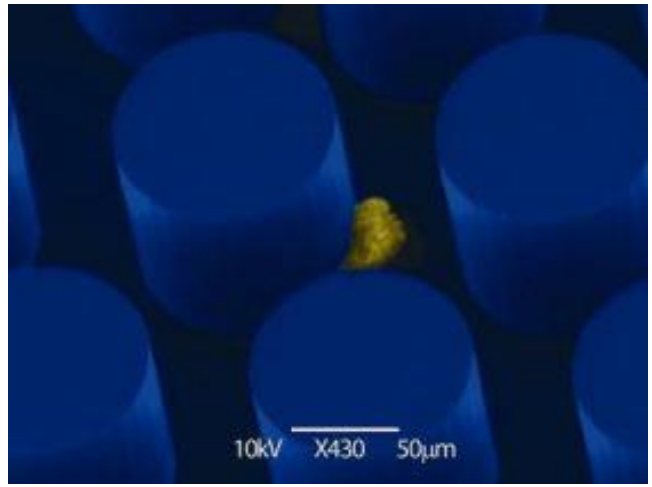
✓**Advantage**
- single step directly from whole blood
  : *without preparatory procedures*
  (centrifugation, washing, or incubation)

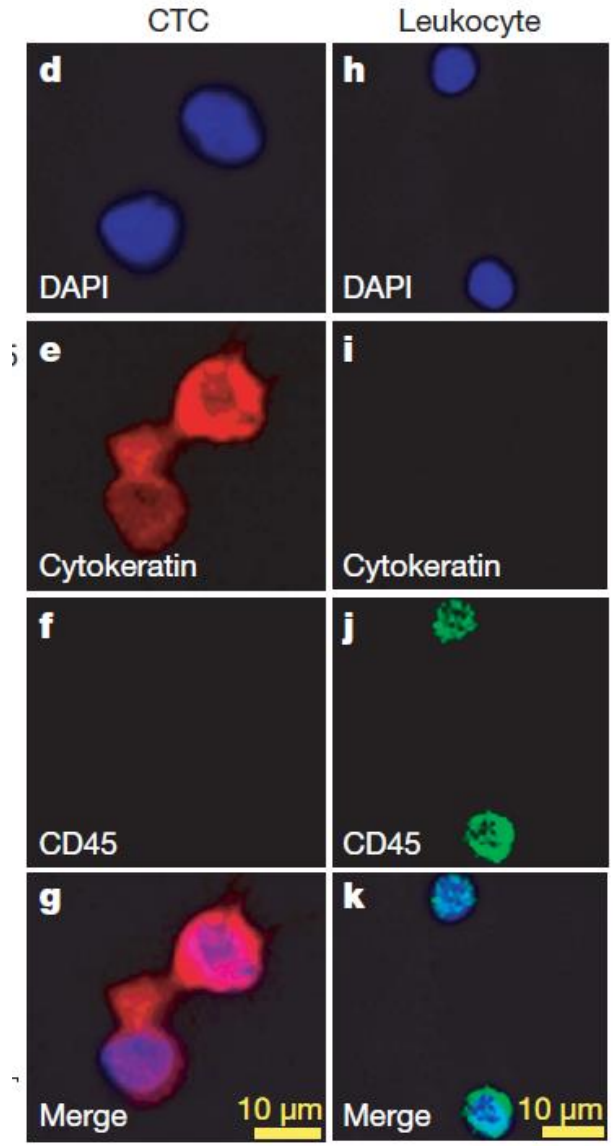


(Nagrath S, et al. Nature 2007;450:1235-9.)

CTCs characterization- *CTCchip*

CTCs capture





(Nagrath S, et al. Nature 2007;450:1235-9.)

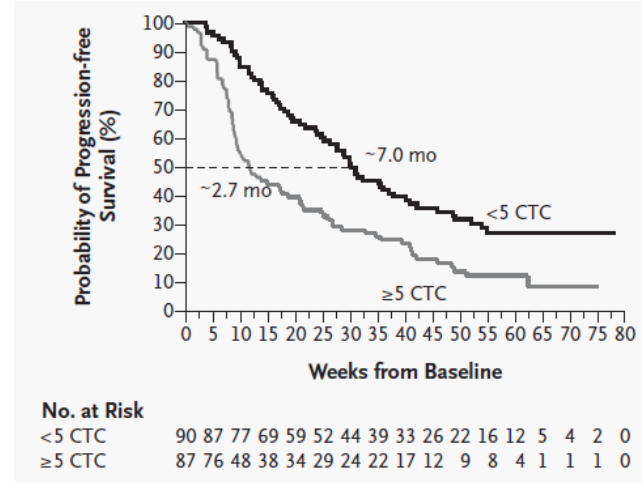
CTCs- Clinical Evidence

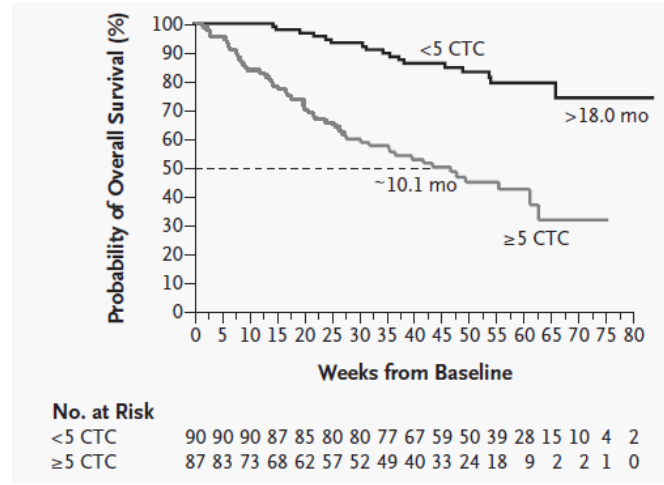
Metastatic breast cancer (MBC)

✓**Metastatic Breast Cancer**
- evaluated the number of CTCs
  : at the time of metastasis

✓**Number of CTCs before initiation of therapy**
- 5 or more CTCs per 7.5 ml blood at baseline
  : shorter median PFS time
  (2.7 months vs. 7.0 months; p < .001)
  : shorter OS time
  (10.1 months vs. 18 months; p .001)
- ***independent predictor of PFS and OS***

CTCs as a prognostic model !!





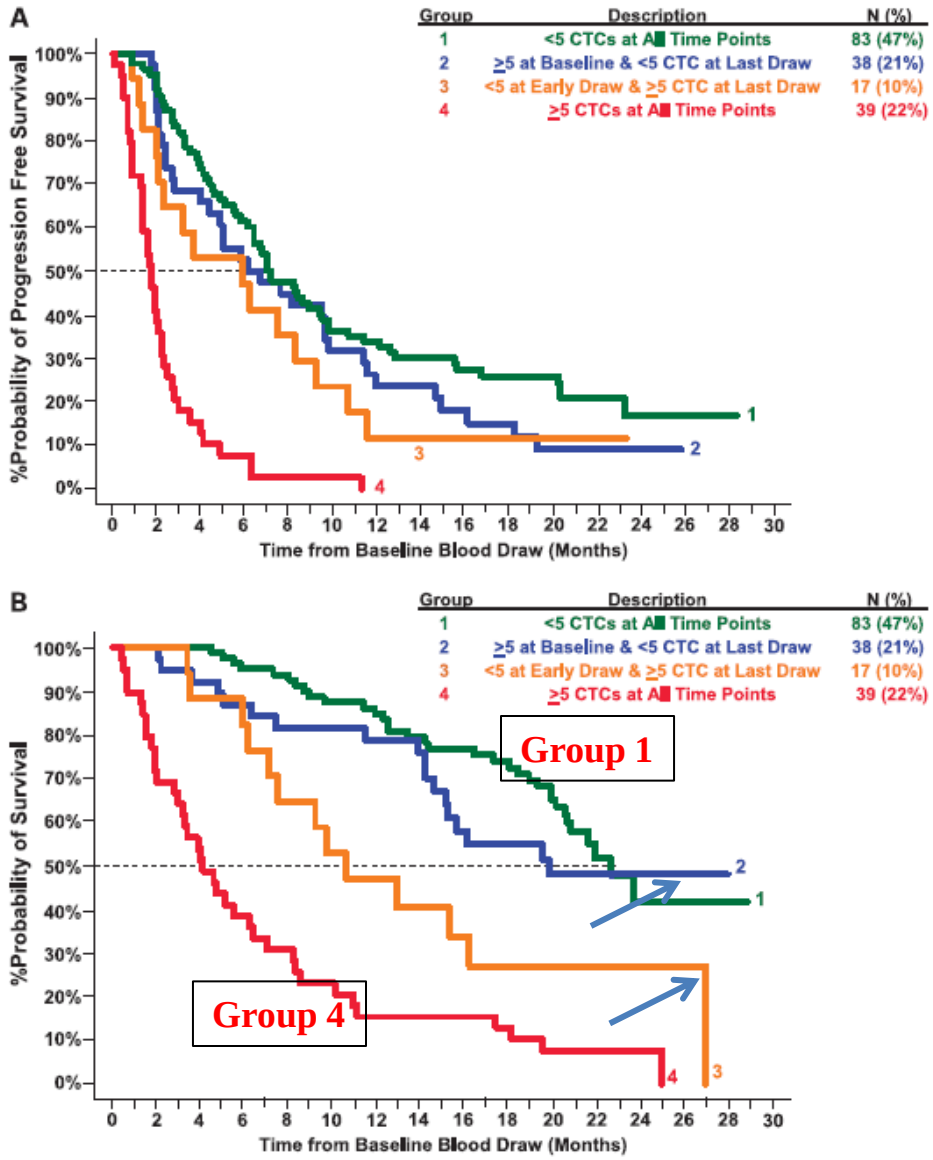
(Cristofanilli M, et al. N Engl J Med 2004; 351:781-791.)

Stage IV

By presence of CTC >5

- Stage IV-A ?

- Stage IV-B ?



(Hayes DF, et al. Clin Cancer Res 2006;12:4218-4224.)

✓**group 1** 5↓ → 5 ↓
- patients with <5 CTCs at all blood draw time points

✓**group 2** 5 ↑ → 5 ↓
- patients with >5 CTCs before the initiation of therapy but who had decreased to <5 CTCs

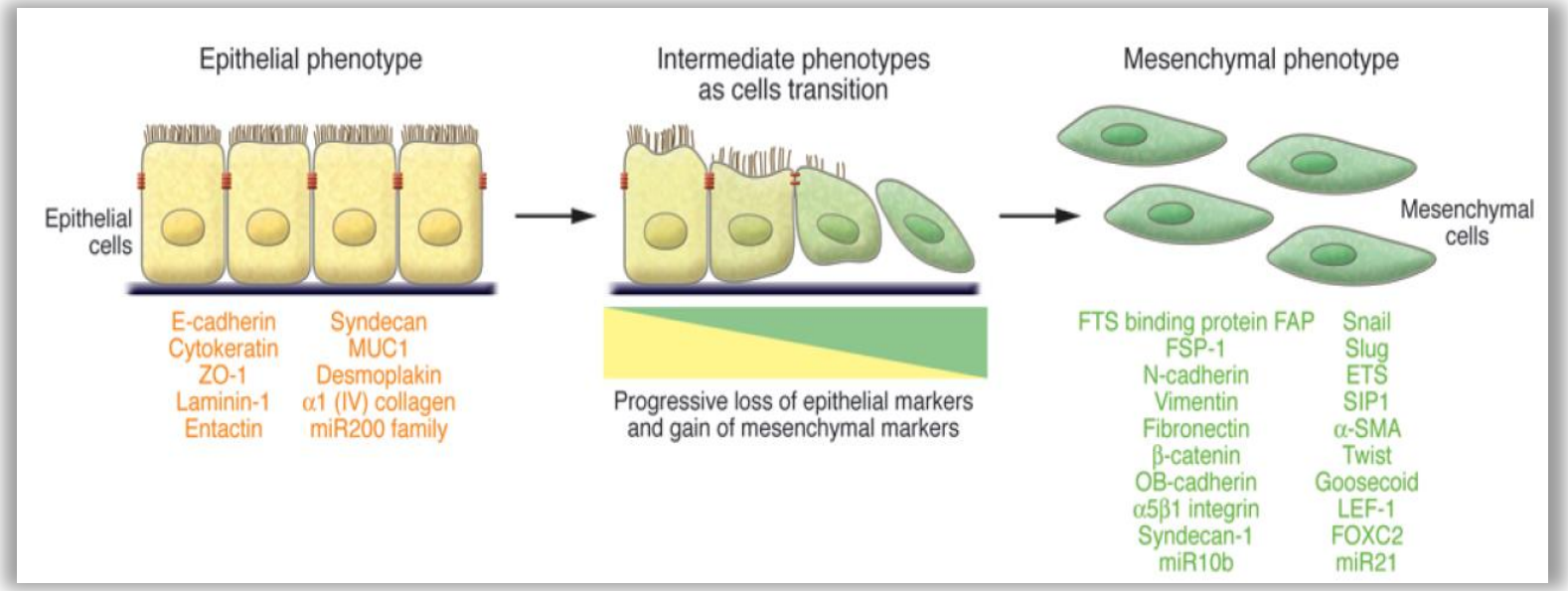
✓**group 3** 5 ↓ → 5 ↑
- patients with <5 CTCs at baseline, increased to >5 CTCs

✓**group 4** 5 ↑ → 5 ↑
- patients with >5 CTCs at all blood draw time points.

CTCs as a predictive model !!

Epithelial to Mesenchymal Transition

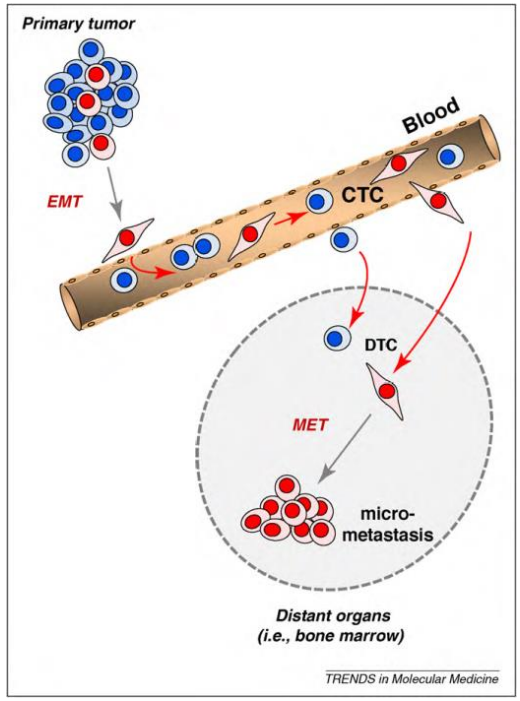
- The epithelial-to-mesenchymal transition (EMT) plays a crucial role in the formation of the body plan and in the differentiation of multiple tissues and organs
- EMT promote carcinoma progression through a variety of mechanisms
- EMT endows cells with **migratory and invasive properties**, induces stem cell properties, prevents apoptosis
- The **mesenchymal state** is associated with the capacity of cells to migrate to distant organs and maintain stemness, allowing development and the initiation of metastasis

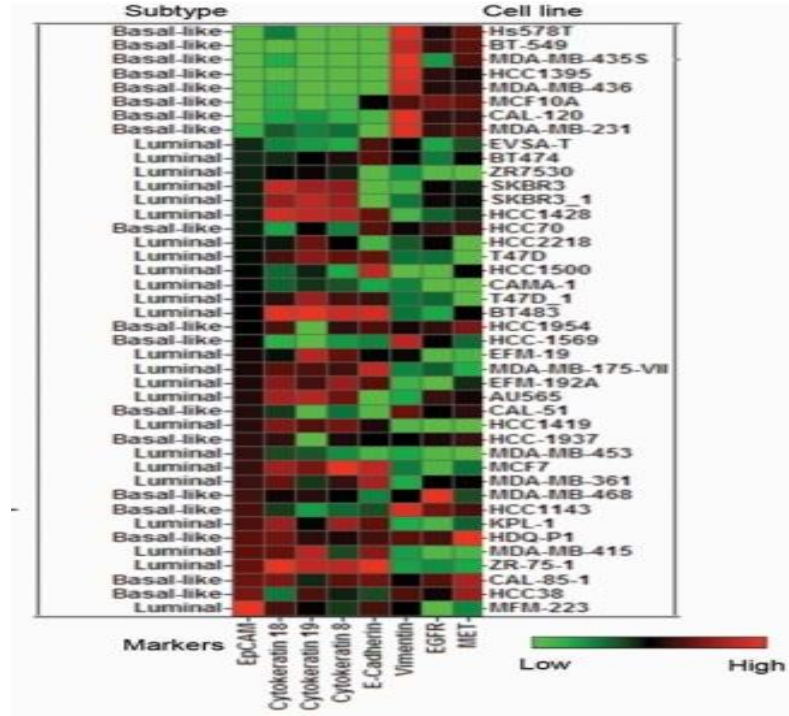


Major drawback of EpCAM-based enrichment

EpCAM

- is not expressed by all epithelial cancers
- heterogeneously expressed even by highly expressing tumors





EpCAM negative CTCs?

Limitation of using EpCAM Abs

Current methods detect only EpCAM positive cell

CTC may loose their epithelial surface markers

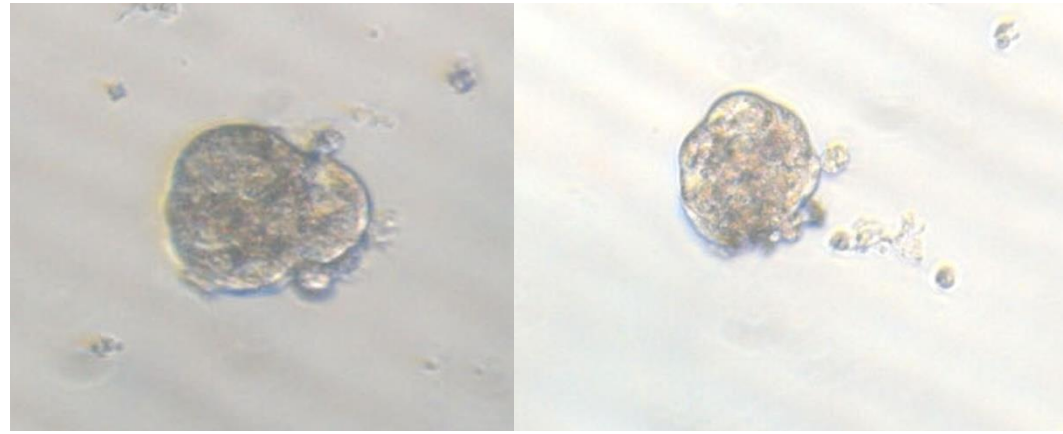
Need another method which can detect both EpCAM positive and EpCAM negative CTC

Mammosphere culture

- has been utilized to enrich for cancer populations of stem cells (CSCs),

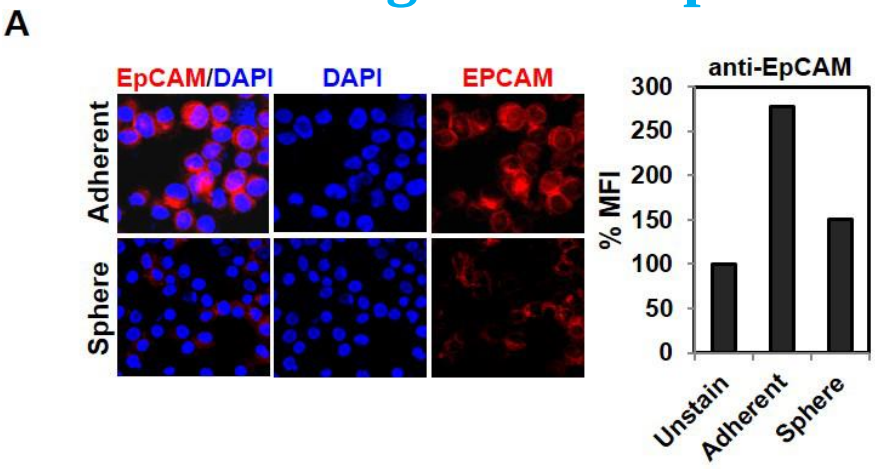
- as well as to initiate EMT

- We thus established a cell model system for mammosphere-induced EMT

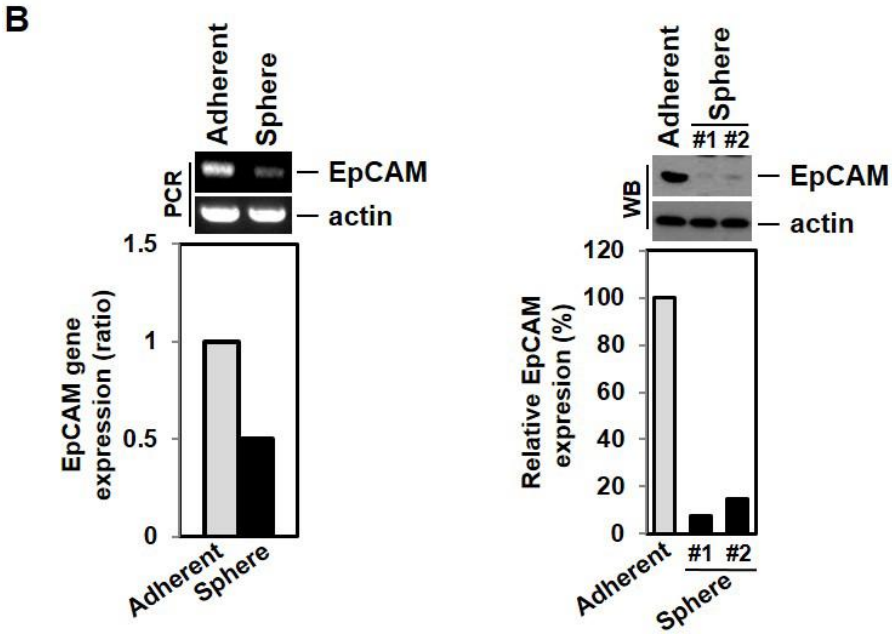


Mamosphere from patients with breast cancer

Down-regulation of EpCAM expression by EMT induction : using mammosphere culture system



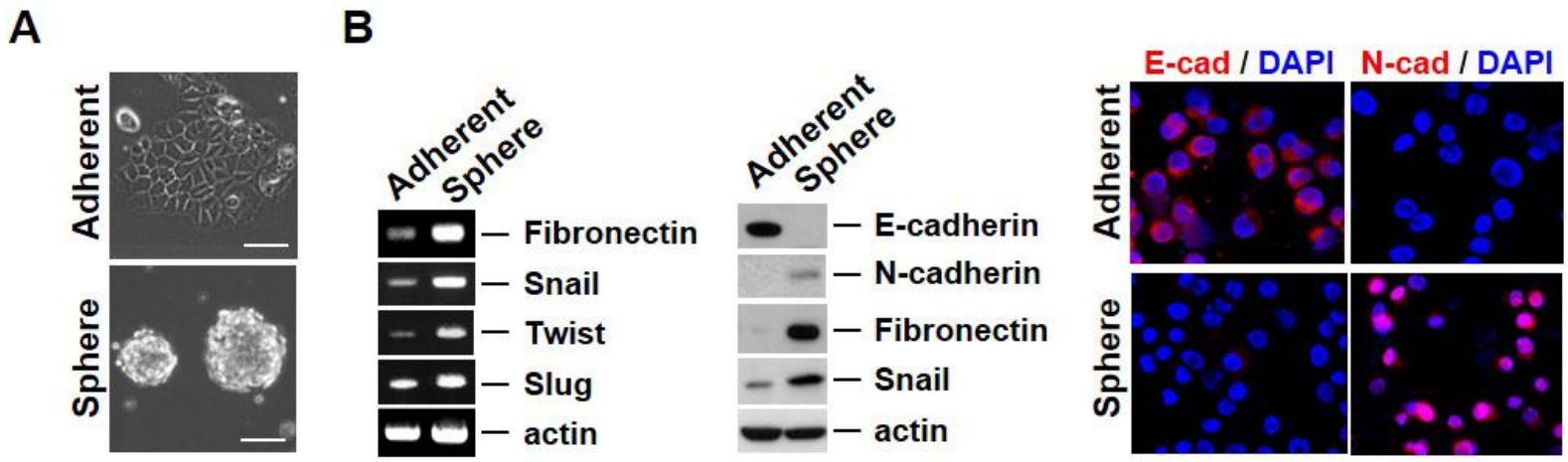
Low expression of EpCAM cell surface marker expression in mammosphere-cultured cells.
- MCF-7 and sphere cultured cells were stained with EpCAM antibody
- analyzed by fluorescence microscope and FACS analysis.



EMT phenotype
- decreased expression levels of the EpCAM gene and protein

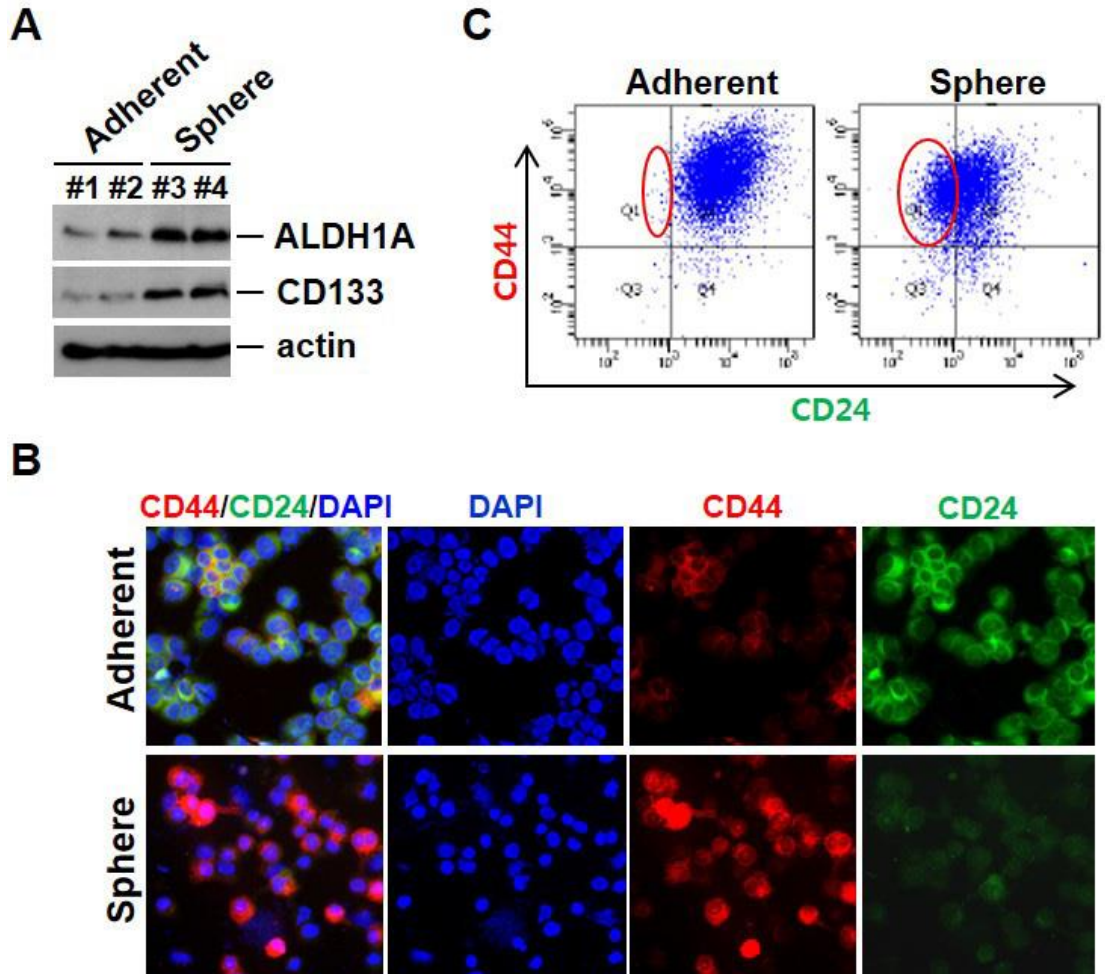
(Seung Il Kim, et al. Oncotarget. 2016 Mar 22. [Epub ahead of print])

Mammosphere cultured MCF-7 cells acquire EMT phenotypes



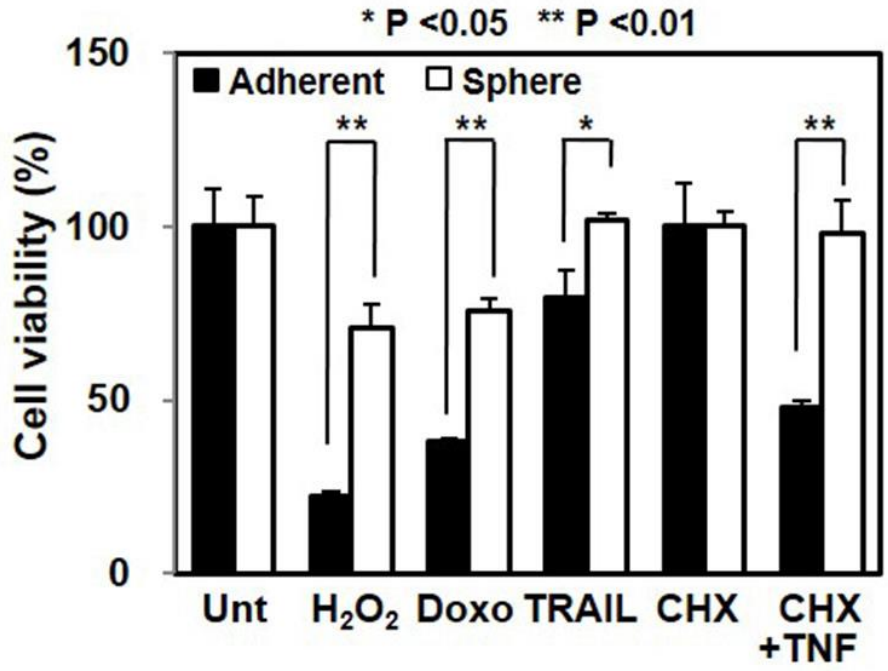
(Seung Il Kim, et al. Oncotarget. 2016 Mar 22. [Epub ahead of print])

Cancer stem-like cells can arise as a result of EMT



(Seung Il Kim, et al. Oncotarget. 2016 Mar 22. [Epub ahead of print])

Chemoresistance is associated with cancer stem cell-like properties and EMT



(Seung Il Kim, et al. Oncotarget. 2016 Mar 22. [Epub ahead of print])

Experience of Yonsei University, Severance Hospital

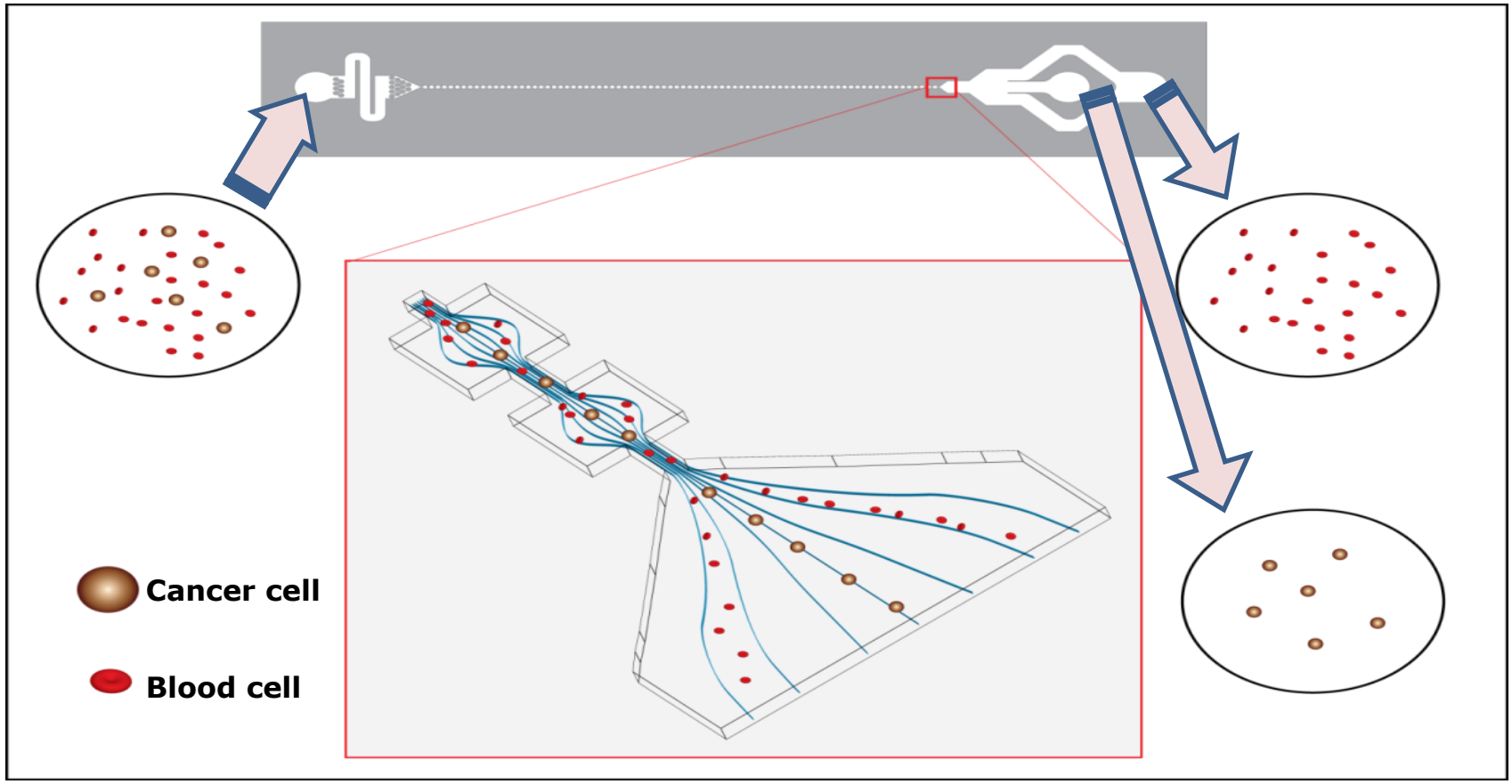
Multi-orifice flow fractionation (MOFF)

: Cytometric identification

Cytometric - based CTC separation

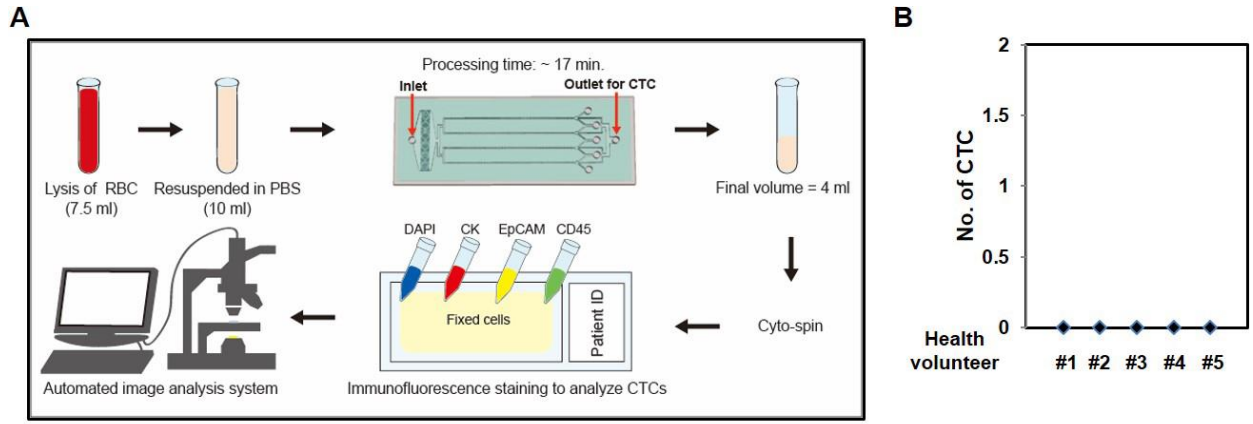
Multi-orifice flow fractionation (MOFF)
: microfluidic device- separation of CTCs based on the physical properties of cells
: hydrodynamic separation- high throughput filtration of blood cells

Microchannel Design



Collaboration with Prof. Hyo-Il Jung. Ph.D.
Biochip Lab, department of mechanical engineering, Yonsei University

CTC isolation using a p-MOFF chip



Overview of MOFF System

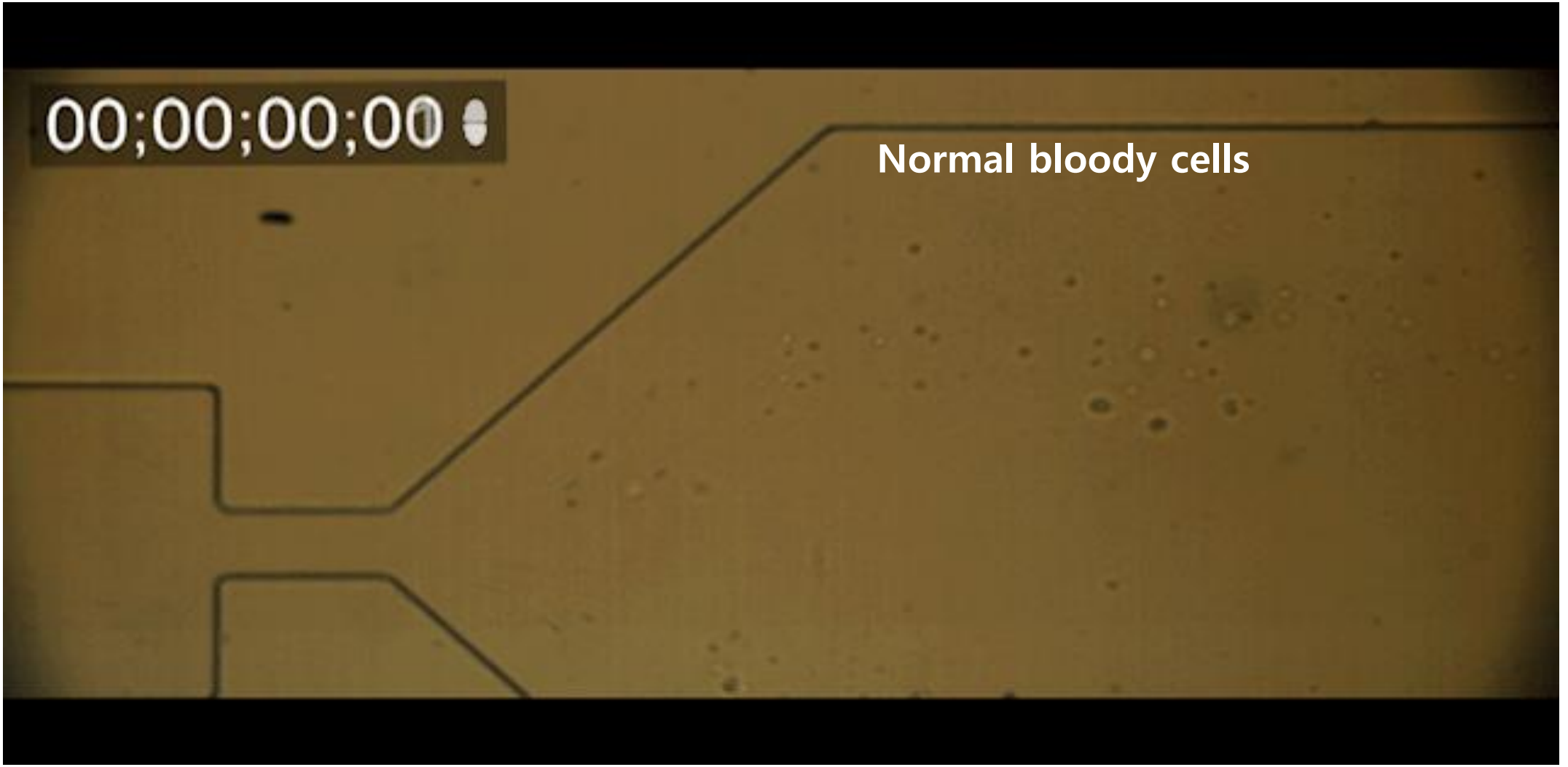
Light source

Image capture program

Syringe pump

CCD

Microscope


00;00;00;00
Normal bloody cells

EpCAM negative CTCs

✓**MOFF**

: without using EpCAM Enrichment

: detection of **EpCAM negative CTCs**

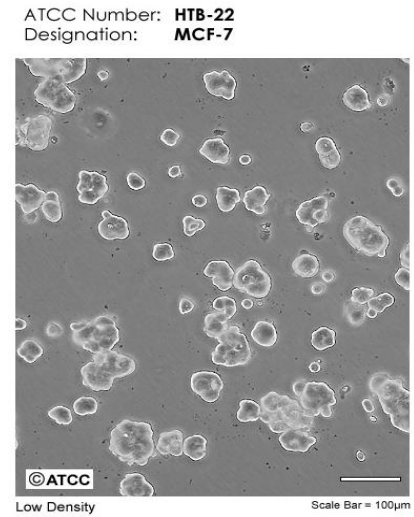
Expression of EpCAM on human breast cancer carcinoma cell lines

Cell line	Ep-CAM expression[a]
MT-3	671.2 (±123.1)
ZR-751	298.2 (±98.2)
MCF7	222.1 (±13.7)
MDA-MB-453	180.3 (±30.7)
BT20	139.5 (±27.0)
SKBR3	125.5 (±31.6)
MaTu	123.9 (±34.2)
BT474	122.0 (±40.0)
MDA-MB-231	11.7 (±0.6)
KATO III	893.1 (±166.7)

(British Journal of Cancer 2005;92:342-349.)

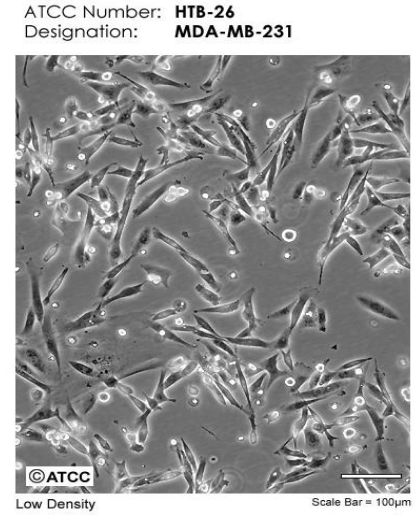
MOFF System
- recovery rate of *EpCAM (+)* & *EpCAM (-)* cell lines

✓**Separation of EpCAM positive**
- MCF 7
- **93.75%**



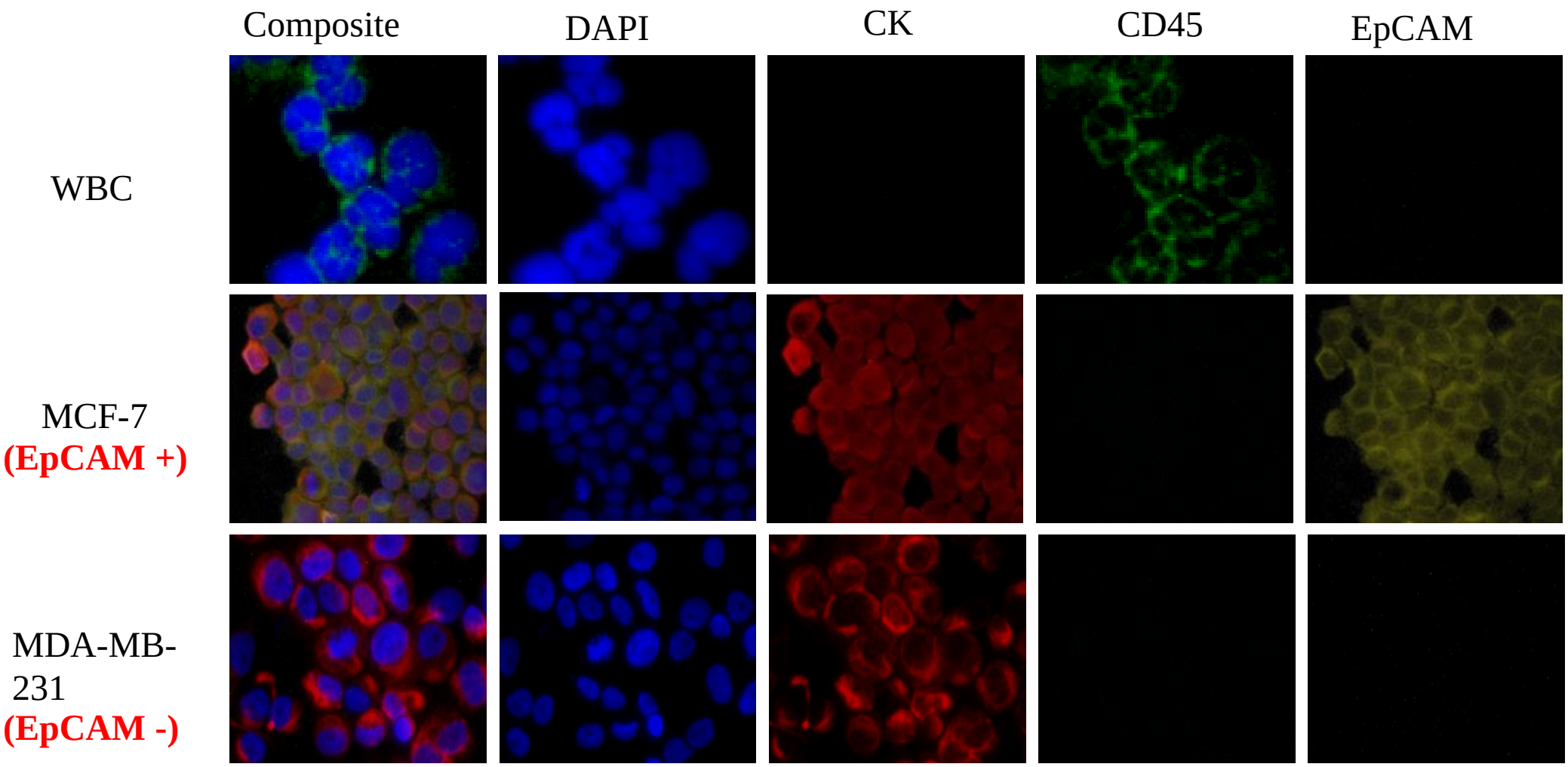
	Outlet	Waste
Concentration	1.476×10^5(/ml)	6.550×10^3(/ml)
Flow rate	240 ul/min	360 ul/min
Recovery	93.75%	6.25%

✓**Separation of EpCAM negative**
- MDA-MB-231
- **91.60%**

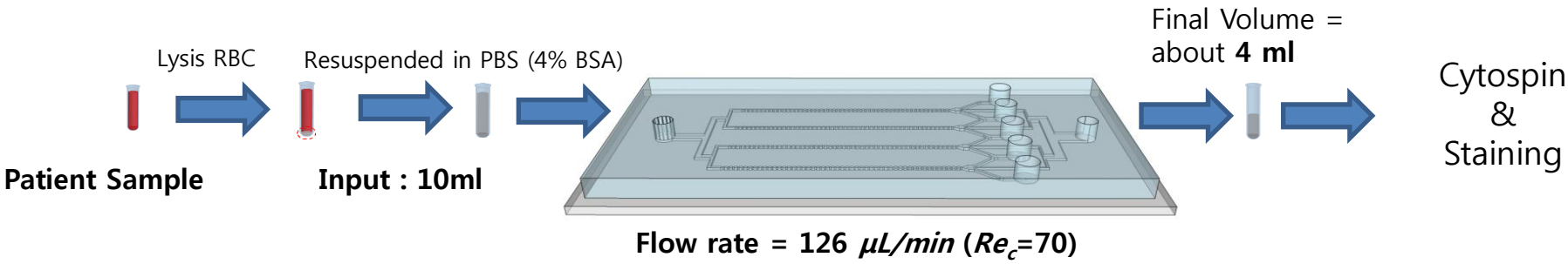


	Outlet	Waste
Concentration	1.873×10^5(/ml)	1.145×10^4(/ml)
Flow rate	240 ul/min	360 ul/min
Recovery	91.60%	8.4%

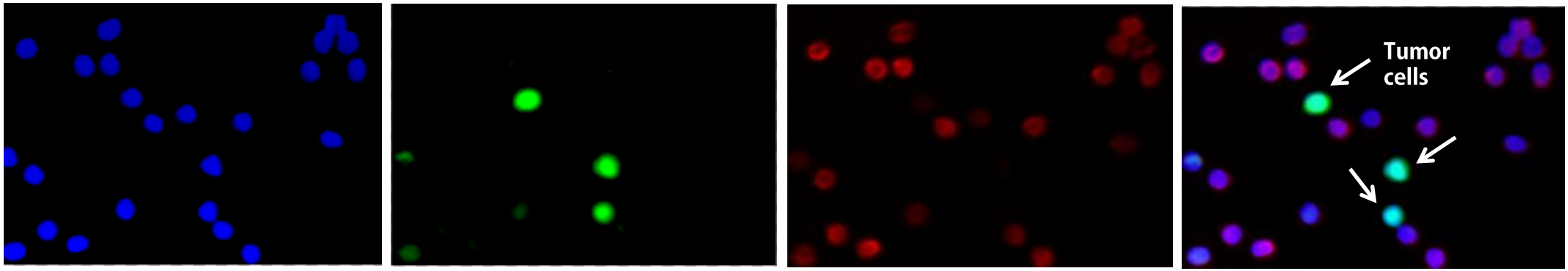
Detection of EpCAM (+)/EpCAM (-) Cells



Experimental protocol



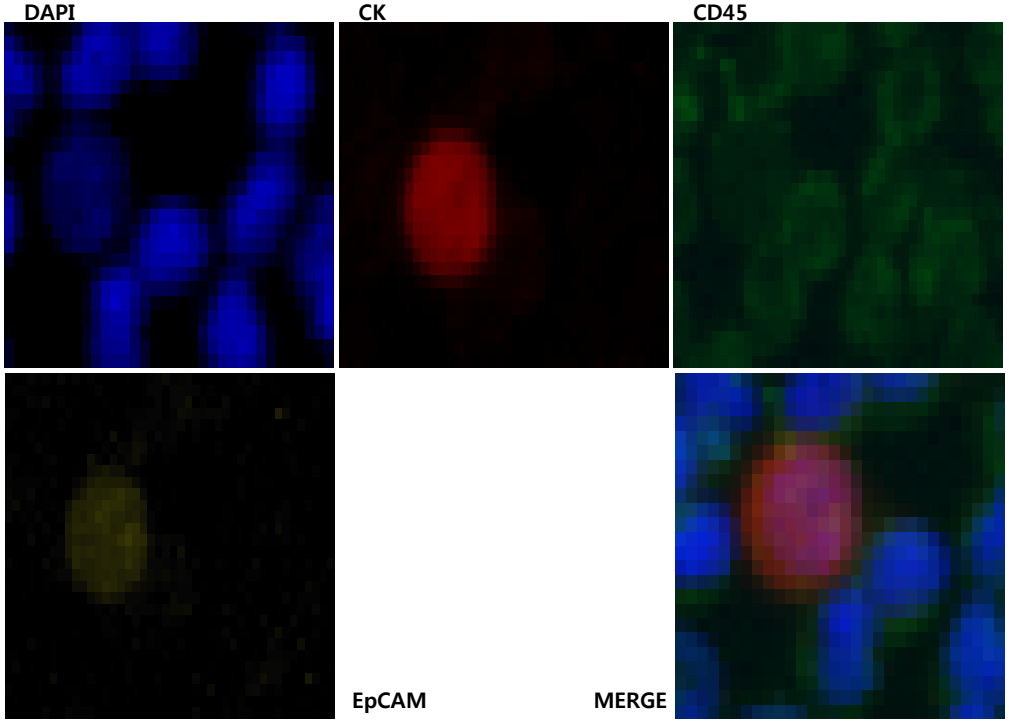
After separate the metastasis patient blood



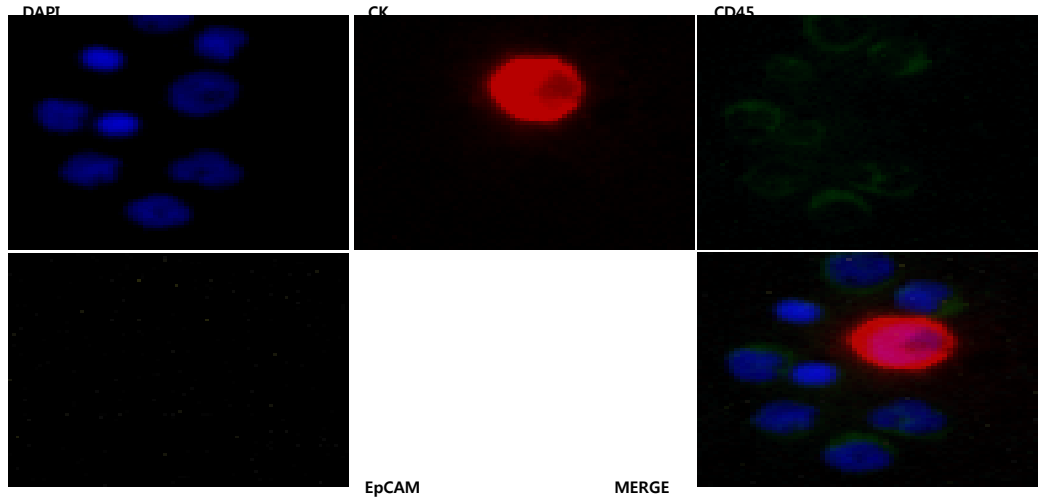
DAPI : Cell DNA
EpCAM : MCF-7 membrane
CD45 : White blood cell membrane

EpCAM positive and Negative CTCs in real patients with MBC

(A) EpCAM positive tumor cell

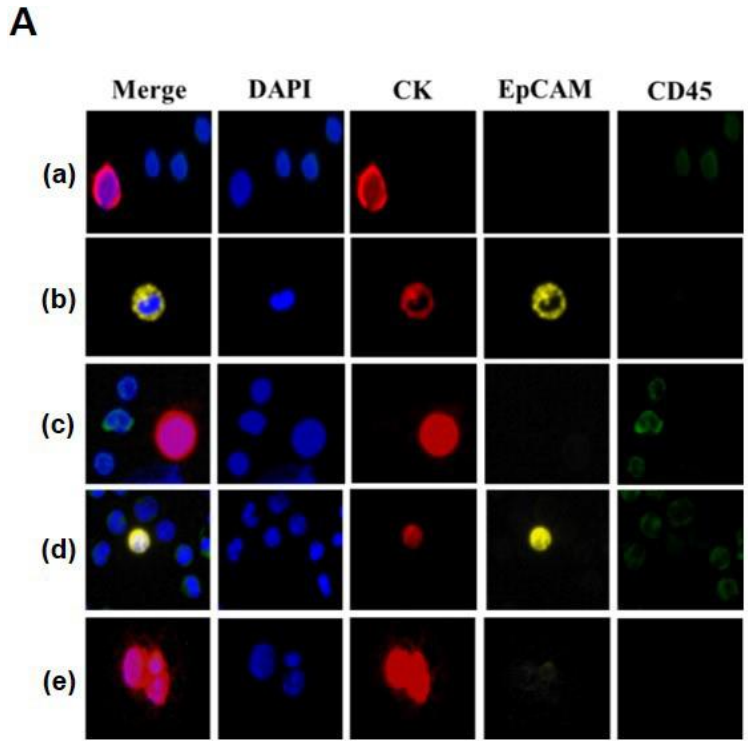


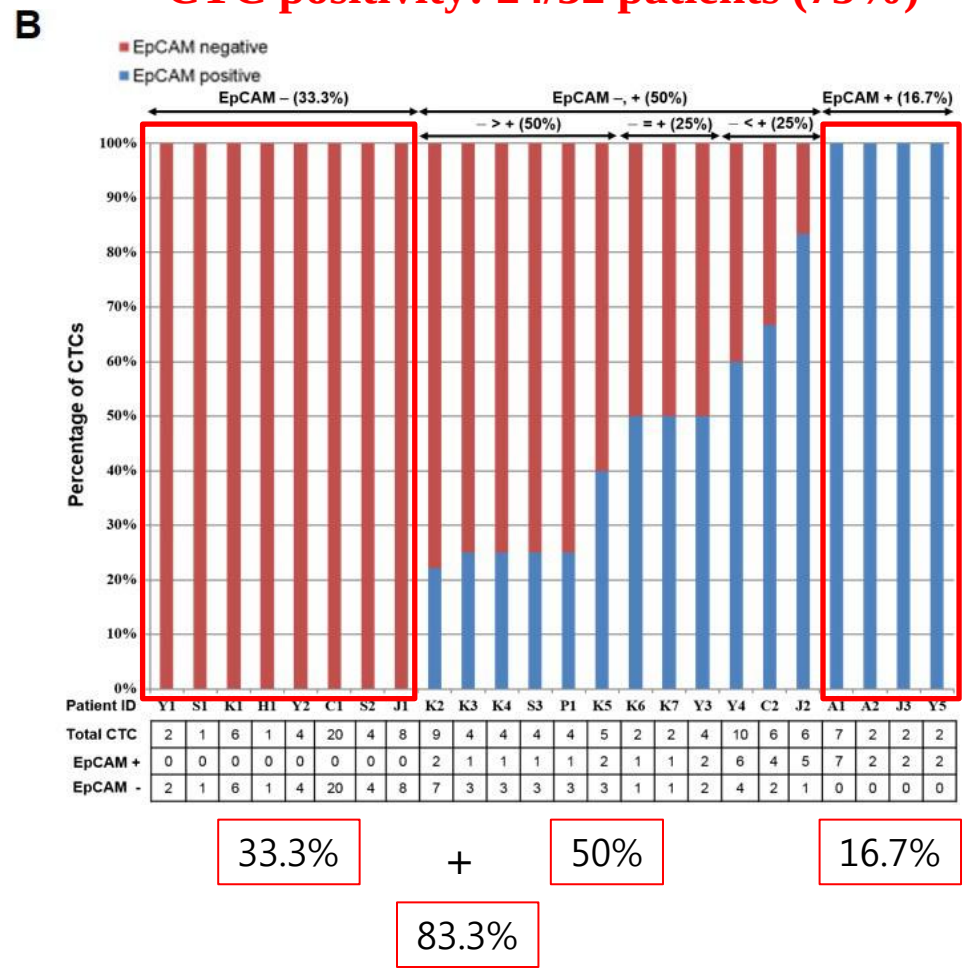
(B) EpCAM negative tumor cells



Isolation of CTCs from metastatic breast cancer patients using the p-MOFF chip.

CTC positivity: 24/32 patients (75%)





Patient ID	Y1	S1	K1	H1	Y2	C1	S2	J1	K2	K3	K4	S3	P1	K5	K6	K7	Y3	Y4	C2	J2	A1	A2	J3	Y5
Total CTC	2	1	6	1	4	20	4	8	9	4	4	4	4	5	2	2	4	10	6	6	7	2	2	2
EpCAM +	0	0	0	0	0	0	0	0	2	1	1	1	1	2	1	1	2	6	4	5	7	2	2	2
EpCAM -	2	1	6	1	4	20	4	8	7	3	3	3	3	3	1	1	2	4	2	1	0	0	0	0

33.3% + 50% 16.7%

83.3%

MOFF test using blood of Volunteers

No. of Sample : 10

Result : 0/10

	한O주	김O영	김O나	강O진	이O하
No. CTC	N/T	N/T	N/T	N/T	N/T
	이O현	제O연	현O아	최O지	이O현
No. CTC	N/T	N/T	N/T	N/T	N/T

Experience of Yonsei University, Severance Hospital

Nucleic acid based techniques

: Real-time PCR for 5 matkers

- **EpCAM, CK 19, Ki 67, HER2, hTERT**

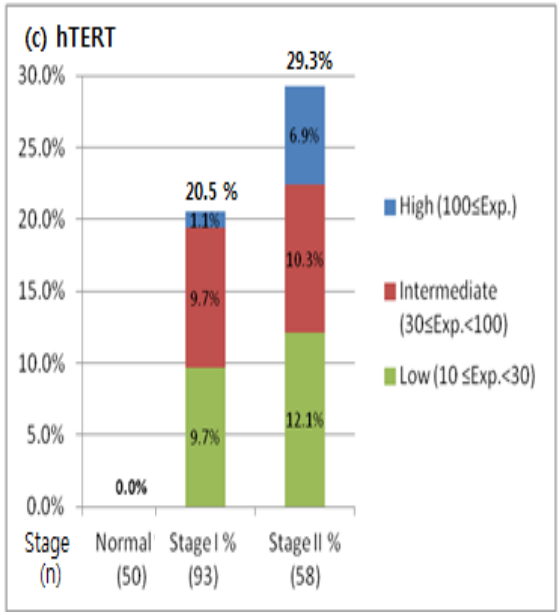
Collaboration with Prof. Hyeyoung Lee, Ph.D.
-Department of Biomedical Laboratory Science, College of Health Sciences, Yonsei University

Prospectively test 5-marker system

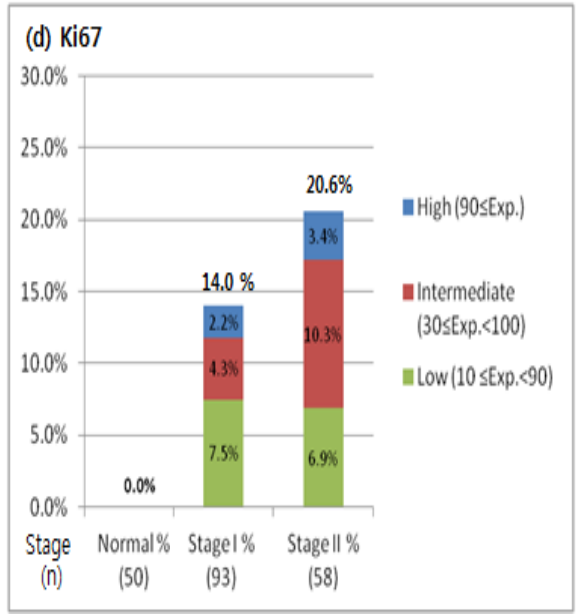
	Patients	Samples
Clinical Patients	Adjuvant-363 Neoadjuvant-90 Metastasis-39 Unknown-6	Adjuvant-908 Neoadjuvant-318 Metastasis-94 Unknown-6
	Total – 498 patients	Total – 1326 blood
Healthy volunteer	Female 350 Male 67	417
	Total – 417	

Detection rate of CTCs according to Stage

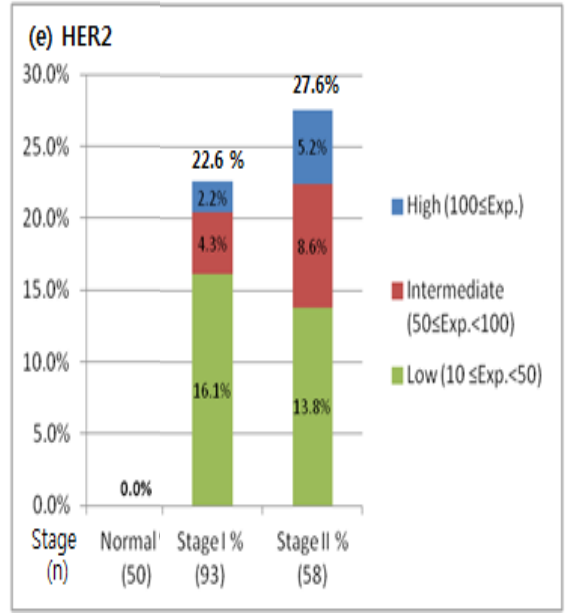
hTERT



Ki67



HER2



Correlation coefficient with CTC markers and tumor status

		Histo_Grade
CTC_Markers	Correlation coefficient	*0.168**
	p-value	*0.038*
	N	154

Survival Data- Pending!

Target of CTC biomarkers

Function	Biomarker	
Epithelial marker	**EpCAM**	**CK-19**
Breast cancer specific marker	**HER2**	
Proliferation marker	**Ki-67**	**hTERT**
Epithelial to Mesenchymal marker	**Vimentin**	**Slug**
	FOXA2	**RUNX1**
Metastasis marker	**NPTN**	**CD146**

CTC biomarker EMT analysis

Breast cancer patient	n (%)
EMT marker positive	**126 (100)**
CTC Epithelial marker (+)	20 (15.9)
CTC Epithelial marker (-)	**106 (84.1)**
EMT marker negative	**302 (100)**
Total	154

Conclusion

dissemination of circulating tumor cells (CTCs)
- requires the Epithelial-to- Mesenchymal transition (EMT),
- lose their epithelial characteristics
- acquire more mesenchymal-like phenotypes

Current isolation of CTCs relies on expression of EpCAM
- may underestimate CTC number and potentially miss critical subpopulations

EMT-induced breast cancer cells maintained in prolonged mammosphere culture conditions
- possess increased EMT markers and cancer stem cell markers
- EpCAM expression is dramatically decreased in these cells

Label-free microfluidic flow fractionation device data
- 16.7%: only EpCAM-positive CTCs
- 50%: both EpCAM-negative and EpCAM-positive CTCs
- 33.3%: only EpCAM-negative CTCs,

Further characterization of CTCs, including low-EpCAM populations
- improve understanding CTC biology and ultimately improving cancer treatment.

Acknowledgements

Prof. Hyo-Il Jung (Ph.D.)
-School of Mechanical Engineering, Yonsei University

Prof. Hyeyoung Lee (Ph.D.)
-Department of Biomedical Laboratory Science, College of Health Sciences, Yonsei University

Prof. You-Sun Kim (Ph.D.)
-Department of Biochemistry, Ajou University of Medicine

Prof. Joohyuk Sohn (M.D./Ph.D.)
-Department of Medical Oncology, Yonsei University College of Medicine

Kyung-A Hyun (Ph.D.)
-BioNano Health Guard Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB)

보건복지부
MINISTRY OF HEALTH & WELFARE

-National R&D Program for Cancer Control
-Translational Research
-Korea Research-driven Hospitals

미래창조과학부
Ministry of Science, ICT and Future Planning

-Basic Science Research Program (Key Joint Research Program)

교육과학기술부
MINISTRY OF EDUCATION, SCIENCE AND TECHNOLOGY

-Basic Science Research Program (Formerly General Researcher Program)

Thanks for Your Attention