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

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)

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)

CTCs characterization - CTCchip

CTCs capture

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 !!

Stage IV

By presence of CTC >5

- *Stage IV-A*?

- *Stage IV-B*?

**Group 1**
- patients with <5 CTCs at all blood draw time points

**Group 2**
- patients with >5 CTCs before the initiation of therapy but who had decreased to <5 CTCs

**Group 3**
- patients with <5 CTCs at baseline, increased to >5 CTCs

**Group 4**
- 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 lose 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
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

A

Lysis of RBC (7.5 ml) → Resuspended in PBS (10 ml) → Automated image analysis system

Processing time: ~ 17 min.

Inlet → Outlet for CTC → Final volume = 4 ml → Cyto-spin

Immunofluorescence staining to analyze CTCs

B

No. of CTC

0 0.5 1 1.5 2

Health volunteer #1 #2 #3 #4 #5
Overview of MOFF System

Experimental setup

Syringe pump

CCD

Microscope

Image capture program

Light source

Micro channel
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

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

(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%

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

<table>
<thead>
<tr>
<th></th>
<th>Outlet</th>
<th>Waste</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Concentration</strong></td>
<td>1.476x10⁵(/ml)</td>
<td>6.550x10³(/ml)</td>
</tr>
<tr>
<td><strong>Flow rate</strong></td>
<td>240 ul/min</td>
<td>360 ul/min</td>
</tr>
<tr>
<td><strong>Recovery</strong></td>
<td>93.75%</td>
<td>6.25%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Outlet</th>
<th>Waste</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Concentration</strong></td>
<td>1.873x10⁵(/ml)</td>
<td>1.145x10⁴(/ml)</td>
</tr>
<tr>
<td><strong>Flow rate</strong></td>
<td>240 ul/min</td>
<td>360 ul/min</td>
</tr>
<tr>
<td><strong>Recovery</strong></td>
<td>91.60%</td>
<td>8.4%</td>
</tr>
</tbody>
</table>
Detection of EpCAM (+)/EpCAM (-) Cells
Experimental protocol

Patient Sample → Lysis RBC → Resuspended in PBS (4% BSA) → Final Volume = about 4 ml → Cytospin & Staining

Flow rate = 126 μL/min ($Re_c=70$)

After separate the metastasis patient blood

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

Tumor cells
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%)
MOFF test using blood of Volunteers

No. of Sample : 10

Result : 0/10

<table>
<thead>
<tr>
<th></th>
<th>한O주</th>
<th>김O영</th>
<th>김O나</th>
<th>강O진</th>
<th>이O하</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. CTC</td>
<td>N/T</td>
<td>N/T</td>
<td>N/T</td>
<td>N/T</td>
<td>N/T</td>
</tr>
<tr>
<td>이O현</td>
<td>재O연</td>
<td>현O아</td>
<td>최O지</td>
<td>이O현</td>
<td></td>
</tr>
<tr>
<td>No. CTC</td>
<td>N/T</td>
<td>N/T</td>
<td>N/T</td>
<td>N/T</td>
<td>N/T</td>
</tr>
</tbody>
</table>
Experience of Yonsei University, Severance Hospital

Nucleic acid based techniques

: Real-time PCR for 5 markers

- 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**

<table>
<thead>
<tr>
<th>Clinical Patients</th>
<th>Patients</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adjuvant-363</td>
<td>Adjuvant-908</td>
<td></td>
</tr>
<tr>
<td>Neoadjuvant-90</td>
<td>Neoadjuvant-318</td>
<td></td>
</tr>
<tr>
<td>Metastasis-39</td>
<td>Metastasis-94</td>
<td></td>
</tr>
<tr>
<td>Unknown-6</td>
<td>Unknown-6</td>
<td></td>
</tr>
<tr>
<td><strong>Total – 498 patients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy volunteer</td>
<td>Female 350</td>
<td>Male 67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>417</td>
</tr>
<tr>
<td><strong>Total – 417</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Detection rate of CTCs according to Stage

### hTERT

- Stage I % (93): 20.5%
- Stage II % (58): 29.3%
- High (100 ≤ Exp.): 0.0%
- Intermediate (30 ≤ Exp. < 100): 8.7%
- Low (10 ≤ Exp. < 30): 11.3%

### Ki67

- Stage I % (93): 14.0%
- Stage II % (58): 20.6%
- High (90 ≤ Exp.): 0.0%
- Intermediate (30 ≤ Exp. < 100): 12.2%
- Low (10 ≤ Exp. < 30): 7.5%

### HER2

- Stage I % (93): 22.6%
- Stage II % (58): 27.6%
- High (100 ≤ Exp.): 0.0%
- Intermediate (50 ≤ Exp. < 100): 4.3%
- Low (10 ≤ Exp. < 50): 16.1%

#### Correlation coefficient with CTC markers and tumor status

<table>
<thead>
<tr>
<th>Histo_Grade</th>
<th>Correlation coefficient</th>
<th>p-value</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>154</td>
</tr>
<tr>
<td></td>
<td>0.168*</td>
<td>0.038</td>
<td></td>
</tr>
</tbody>
</table>

Survival Data - Pending!
<table>
<thead>
<tr>
<th>Function</th>
<th>Biomarker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial marker</td>
<td>EpCAM</td>
</tr>
<tr>
<td></td>
<td>CK-19</td>
</tr>
<tr>
<td>Breast cancer specific marker</td>
<td>HER2</td>
</tr>
<tr>
<td>Proliferation marker</td>
<td>Ki-67</td>
</tr>
<tr>
<td></td>
<td>hTERT</td>
</tr>
<tr>
<td>Epithelial to Mesenchymal marker</td>
<td>Vimentin</td>
</tr>
<tr>
<td></td>
<td>Slug</td>
</tr>
<tr>
<td></td>
<td>FOXA2</td>
</tr>
<tr>
<td></td>
<td>RUNX1</td>
</tr>
<tr>
<td>Metastasis marker</td>
<td>NPTN</td>
</tr>
<tr>
<td></td>
<td>CD146</td>
</tr>
<tr>
<td>Breast cancer patient</td>
<td>n (%)</td>
</tr>
<tr>
<td>-----------------------</td>
<td>-------</td>
</tr>
<tr>
<td>EMT marker positive</td>
<td>126 (100)</td>
</tr>
<tr>
<td>CTC Epithelial marker (+)</td>
<td>20 (15.9)</td>
</tr>
<tr>
<td>CTC Epithelial marker (-)</td>
<td>106 (84.1)</td>
</tr>
<tr>
<td>EMT marker negative</td>
<td>302 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>154</td>
</tr>
</tbody>
</table>
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-II 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)

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

-Basic Science Research Program (Key Joint Research Program)

-Basic Science Research Program (Formerly General Researcher Program)