Malini Harigopal, MD
Assistant Professor
Department of Pathology
&
Kevin Schofield, CT(ASCP)
Manager
Department of Cytology

Yale School of Medicine
New Haven, CT
We have no financial disclosures.
Basic Introduction to Circulating Tumor cells (CTCs) & The CellSearch™ System
Objectives

- Evaluate the use of CTCs and CellSearch (Veridex, Raritan, NJ) in the management of patients with cancer
- Discuss the cytopathologist’s role in measuring CTCs
- Describe the integration of CTC assessment into the practice of cytopathology
- Identify and evaluate CTCs in peripheral blood
• Circulating tumor cells (CTCs): cancer cells shed from either the primary tumor or its metastases
  - Epithelial cells derived from solid tumors
  - Metastatic disease is responsible for most cancer deaths (>90%).
History of CTCs

- Tumor cells were first identified in the blood stream of patients in (1869) by Thomas Ashworth

- Engel, 1955: cancer cells in the peripheral blood of pts with various types of cancer.

- Hematologists, Cytologists & Surgeons: background in Papanicolou & Romanowsky stains: morphologic criteria for cancer cells
Slide Seminar The Circulating Cancer Cell Cooperative (CCCC): NCI Identification of CTC: Morphologic criteria
The Circulating Cancer Cell Cooperative 1962:

- Morphology, techniques and patient selection
- Conclusion: “More extensive well-controlled studies, improved techniques, sharper criteria for recognition of tumor cells are required.”

Immunofluorescence technique by Coons et al: labeling of antibodies with fluorochromes improved the specificity of detection of CTC.

Value to cytologic diagnosis of CTC
Circulating Tumor Cells, Disease Progression, and Survival in Metastatic Breast Cancer

Massimo Cristofanilli, M.D., G. Thomas Budd, M.D., Matthew J. Ellis, M.B., Ph.D., Alison Stopeck, M.D., Jeri Matera, R.S., R.Ph., M. Craig Miller, R.S., James M. Reuben, Ph.D., Gerald V. Doyle, D.D.S., W. Jeffrey Allard, Ph.D., Leon W.M.M. Terstappen, M.D., Ph.D., and Daniel F. Hayes, M.D.
CTC: Rare in healthy women and in patients with benign breast disease (<1 per 7.5ml blood).

Monitoring CTC (counts) can predict prognosis in many solid tumors, breast, prostate and colorectal cancers.

Measuring changes in CTC counts help monitor patient outcome.

Molecular characterization of CTCs (HER2, EGFR) help select patient’s targeted therapy and limit metastases.
The role of CTCs in blood is still under active investigation, their biological significance/therapeutic relevance is debated.

Identification, enumeration and molecular characterization of CTCs could expand our understanding of the biology of metastases.

Several strategies have been used for CTC enumeration.
Techniques for CTC Enumeration

Antibody-based cytometric assays (intact tumor cells)
- IF based technology with monoclonal antibodies to epithelial specific antigens EpCAM, CK
- Epithelial tumors are detected

Molecular (nucleic acid-based assays): PCR/RT-PCR techniques
- DNA or mRNA: transcripts for EpCam, CK; highly sensitive but lacks specificity

Enrichment: isolate CTCs
Techniques for CTC Enumeration

- CellSearch system (Immunocon & Veridex, LLC): immunomagnetic/ immunofluorescent based technology to capture of epithelial cells (EpCAM) by ferrofluid
- CTC-chip: Microposts/columns coated with EpCAM ab on silicon chip
- FAST (Fiber-optic Array Scanning Technology): digital microscopy to scan labeled cells, 300,000 cells/min
- Oncoquick: density gradient
- MACS (Magnetic Activated Sorting system): immunobeads capture of epithelial cells
- Microfilter Device: ISET (isolation by size), polycarbonate filter with pore size 8um. Live CTC capture measuring telomerase activity
- The Adna-Test Breast Cancer, RARE, Epispot
CellSearch™ System

• Automated, standardized technology for CTC detection
• Based Immunomagnetic and immunofluorescence
• CellSearch system validated in multiple clinical trails
• FDA approved for CTC detection
System Overview

- Instruments (CellTrack Auto autoprep system)
- Specimen collection, processing and Quality control
- Enumeration of CTCs for predicting progression-free and overall survival in patients with metastatic breast, colorectal and prostate cancer
- Clinical trial background and conclusions
- Interpretation of Results
- Limitations
CellSearch™ System

- **Sample preparation system**
  - Cell Search Epithelial kit (Veridex Corporation, Warren NJ)
    - Anti Epcam antibodies:
      - Anticytokeratin antibodies conjugated to phycoerythrin (PE) 8,18 & 19
      - Antibody to CD45 conjugated to allophycocyanin (APC): WBC,
      - Nuclear dye (DAPI, 4'6-diamidino-2-phenylindole)
  - Controls: Breast cancer cell line (SKBr3)
  - CellTracks AutoPrep system: Automated

- **Sample evaluation**
  - CellSpotter Analyser (Veridex, immunocon): CTC Identification and enumeration.

- **Interpretation of images**: operators (cytotec & pathologist)
Aspirate fluid and un-labeled cells

Permeabilize and add Staining Reagents

Aspirate plasma
Add buffer
Add ferrofluid.

Processing by the CellTracks™ AutoPrep System

Magnetic incubation

Remove magnets. Re-suspend target cells in buffer

CTC
leukocyte

Transfer to MagNest™

Off-Line

7.5 ml blood from CellSave™ Tube + Buffer

Centrifuge

Immunomagnetic CTC Selection
Immunomagnetic Labeling and Immunofluorescent Identification of Cells

Circulating Tumor Cell
Reproducibility of CTC Counts in Duplicate MCRC Samples (n=1627) with Average of <3 or ≥3 CTC per 7.5 mL of blood.

Note: There may be more than one point superimposed over another. For example, on this plot, there are 975 instances (60%) where both tubes had 0 CTC, 116 instances (7%) where Tube 1 had 0 CTC and Tube 2 had 1 CTC, and another 109 instances (7%) where Tube 1 had 1 CTC and Tube 2 had 0 CTC.

R² = 0.96
Frequency of CTCs: CellSearch™ System

Figure 1. Frequency of CTC in Controls (Subjects without Cancer) and Patients with Metastatic Breast\(^1\) (MBC), Metastatic Colorectal\(^2\) (MCRC) or Metastatic Prostate Cancer\(^3\) (MPC) before Initiation of a new line of Therapy (Baseline) and ~2-5 weeks After the Initiation of Therapy.

\[^1\]MBC reference population information on page 7 of the clinical IFU.
\[^2\]MCRC reference population information on page 27 of the clinical IFU.
\[^3\]MPC reference population information on page 46 of the clinical IFU.
Clinical Trial

3 Prospective multi-institutional clinical trials assessed the performance of the CellSearch™ Assay

- Metastatic Breast Cancer (MBC) > 5 cutoff
- Metastatic Colorectal Cancer (MCRC) >3 cutoff
- Metastatic Prostate Cancer (MPC) >5 cutoff
- Selection of CTC cutoff : Prospectively identified in patients in a training set and confirmed in a validation set
Circulating tumor cells, Disease Progression, and Survival in Metastatic Breast Cancer, Cristofanilli et al, Sem Oncol. 2006

MBC Clinical Trial Design

177 MBC (metastatic breast cancer)(20 centers)
- Measurable disease, any type or line of therapy (first line, chemo Rx)
- (67%ER/PR+, HER2 52%)
- 145 healthy and 200 pts with benign disease
- Imaging and CTC analysis (prior to initiation of therapy)
  CTC performed, 1 follow-up(~ 4 weeks) and 12 weeks
Duration of CTC: 6 months or until progression
Clinical follow up: 50 months

Imaging and clinical progression of disease at 12 weeks*

*Circulating tumor cells, disease Progression,and Survival in Metastatic Breast Cancer, Cristofanilli et al, NEJM 2004
Predictive Value: OS of MBC Patients with <5 or >5 CTC at Baseline (N=177)

Logrank p < 0.0001

Cox Hazards Ratio = 2.4
chi-square = 19.54
(p-value < 0.0001)

CTC / 7.5mL at Baseline N (%) Median OS in Months (95% C.I.)
<5 CTC 89 (50%) 21.9 (20.1 to 28.6)
>5 CTC 88 (50%) 10.9 ( 7.0 to 15.2)


FOR INTERNAL AND EXTERNAL USE
Predictive Value: OS of MBC Patients with <5 or ≥5 CTC at different times of Follow-Up

Predictive Value: PFS of MBC Patients with <5 or ≥5 CTC at Baseline (N=177)

Cox Hazards Ratio = 1.9
chi-square = 14.44
(p-value = 0.0001)

%Probability of Progression Free Survival

Logrank p = 0.0001

A Reduction in CTC Below 5 After the Initiation of Therapy Predicts Longer OS whereas an Increase in CTC Count to 5 or above Predicts a Shorter OS

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>N (%)</th>
<th>Median OS in Months (95% C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;5 CTCs at All Time Points</td>
<td>83 (47%)</td>
<td>22.6 (20.4 to &gt;45)</td>
</tr>
<tr>
<td>2</td>
<td>&gt;5 at Baseline &amp; &lt;5 CTC at Last Draw</td>
<td>38 (21%)</td>
<td>19.8 (14.6 to 31.6)</td>
</tr>
<tr>
<td>3</td>
<td>&lt;5 at Early Draw &amp; &gt;5 CTC at Last Draw</td>
<td>17 (10%)</td>
<td>10.6 (6.1 to 16.2)</td>
</tr>
<tr>
<td>4</td>
<td>&gt;5 CTCs at All Time Points</td>
<td>39 (22%)</td>
<td>4.1 (2.8 to 6.4)</td>
</tr>
</tbody>
</table>

Curve Logrank Comparison p-Value*
1 vs. 2 0.2023
1 vs. 3 0.0017
1 vs. 4 <0.0001
2 vs. 3 0.1025
2 vs. 4 <0.0001
3 vs. 4 0.0045

MBC, MCRC, & MPC
Median Overall Survival Comparison (in months)

<table>
<thead>
<tr>
<th></th>
<th>MBC</th>
<th>MCRC</th>
<th>MPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;cut-off at all time points</td>
<td>22.6</td>
<td>18.6</td>
<td>&gt;26</td>
</tr>
<tr>
<td>≥cut-off at all time points</td>
<td>4.1</td>
<td>3.9</td>
<td>6.8</td>
</tr>
<tr>
<td>≥cut-off at baseline and &lt;cut-off at final draw</td>
<td>19.8</td>
<td>11.7</td>
<td>21.3</td>
</tr>
<tr>
<td>&lt;cut-off at early draw and ≥cut-off at final draw</td>
<td>10.6</td>
<td>7.1</td>
<td>9.3</td>
</tr>
</tbody>
</table>
# MBC, MCRC, and MPC Summary & Conclusion

## MBC
- 177 patients
- Cut-off = \( \geq 5 \) CTC
- Patients with \( \geq 5 \) CTC at baseline = 50% (88/177 evaluable patients)
- Should be used for serial monitoring
- Predicts PFS and OS
- Combination of CTC and imaging may provide the most accurate assessment of patient prognosis

## MCRC
- 430 patients
- Cut-off = \( \geq 3 \) CTC
- Patients with \( \geq 3 \) CTC at baseline = 26% (108/413 evaluable patients)
- Should be used for serial monitoring
- Predicts PFS and OS
- Combination of CTC and imaging may provide the most accurate assessment of patient prognosis

## MPC
- 231 patients
- Cut-off = \( \geq 5 \) CTC
- Patients with \( \geq 5 \) CTC at baseline = 57% (125/219 evaluable patients)
- Should be used for serial monitoring
- Predicts PFS and OS
- Combination of CTC and PSA may provide the most accurate assessment of patient prognosis
Results should be used in conjunction with diagnostic tests (lab, imaging), physical exam and medical history.

Not proven to affect overall health outcomes in patients with metastatic carcinoma.

Potential for monitoring patients.

Insufficient evidence as a marker of disease progression.
Yale CTC Experience

CellSearch (Veridex device) 2006
• >1000 CTC tests
• Clinicians (Oncologists): breast, colorectal and lung cancers
• Guide treatment, research use
• CTCs investigated for HER2/neu protein expression in breast cancer patient’s
Interpretation

- Pathologist and cytotechnologist (certified by Veridex)
- CTC are defined as:
  - Nucleated cells lacking CD45 and express CK (8, 18 & 19).
  - Morphology (round or oval with a nucleus within the cytoplasm).
  - Size (4um)
  - Heterogeneity (morphology and size).
Tumor Cell

Cytoplasm  |  Nucleus  |  Cell Membrane  |  Composite
---|---|---|---
CK-PE pos  |  DAPI pos  |  CD45-APC neg  |  Tumor Cell

Leukocyte nucleus + CD45+ Membrane = Tumor Cell
Leukocytes (CK-PE-/DAPI+/CD45-APC+)

- CK-PE staining in this example is not associated with a nucleus, so it is not a tumor cell.
- The nucleic acid staining is associated with staining in the CD45-APC channel, which means that this cell is a leukocyte.
- No boxes should be checked.
**Tumor Cell and Leukocyte in Same Frame**

<table>
<thead>
<tr>
<th>Comp</th>
<th>CD45-APC</th>
<th>CK-PE</th>
<th>DAPI</th>
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<tbody>
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<td><img src="image1.png" alt="Image" /></td>
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- A single frame may contain more than one cell
- In the examples above, note that the DAPI channel presents two clearly identifiable nuclei
  - one nucleus corresponds to a CK-PE + cell
  - one nucleus corresponds to a CD45-APC + cell
  - the composite box should be checked in both instances to count the tumor cell
### Tumor Cells with Dim PE and Bright DAPI

<table>
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- If the CK-PE image is dim, the DAPI image may appear larger than the CK-PE image
  - Carefully examine CK-PE
  - Dim region in CK-PE is part of the entire cell

Note CD45-APC channel in first example:

A leukocyte is also visible, but no nucleus is visible in the DAPI channel for the leukocyte.
Tumor Cells: Cytoplasmic Image in APC

<table>
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- Dimmer, irregular staining (bleed thru) in CD45-APC channel
- Very bright CK-PE channel

- If image shows a very bright CK-PE image and a dim, irregular or jagged, membrane pattern staining in the CD45-APC channel and all other tumor cell criteria are present, classify the cell as a tumor cell
**Typical Tumor Cells**

<table>
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</table>

- **CK+, bright or dim**

**Tumor cells - nucleus more than 50% in**

| ![Image](image5.jpg) | ![Image](image6.jpg) | ![Image](image7.jpg) | ![Image](image8.jpg) |

- **CK+, bright or dim**

**Tumor cells, nuclear shape in APC**

| ![Image](image9.jpg) | ![Image](image10.jpg) | ![Image](image11.jpg) | ![Image](image12.jpg) |

- **Very bright CK+**

**Tumor cells, cytoplasmic shape in APC**

| ![Image](image13.jpg) | ![Image](image14.jpg) | ![Image](image15.jpg) | ![Image](image16.jpg) |

- **Very bright CK+**
**Suspicious Objects**

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<tr>
<td>![Image]</td>
<td>![Image]</td>
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</tbody>
</table>

"Detached Nuclei" Suspicious Cells

- Cytoplasm area does not surround the nucleus
- Nucleus appears to overlap the cytoplasm

Note: If many images in the sample display this appearance, it is also possible that the microscope stage has malfunctioned.

Suspicious objects should not be counted as tumor cells because their significance has not been established.
### Suspicious Objects

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**“Speckled” or “Punctate” Suspicious Cells**

- delineated nuclear image
- irregular, speckled cytoplasmic staining

**Note:** Suspicious objects should not be counted as tumor cells because their significance has not been established.
## Not Classified Cells

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### Computer Noise

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- Caused by over-amplification of the CK-PE or DAPI signals
- Easily recognized as non-cellular events.
### Squamous Cells

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- Easily identified by their low nuclear to cytoplasmic ratio
- “Corn flake” cytoplasmic appearance
- Very large, polygonal cells with round nuclei
# Cell Interpretation Practice

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<td><img src="image27.png" alt="Image" /></td>
<td><img src="image28.png" alt="Image" /></td>
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</table>
**Research Mode**

**PE+/DAPI+/APC-/FITC+**

Click the composite box and the FITC box to count the target cell as positive for the additional marker.

<table>
<thead>
<tr>
<th>Comp</th>
<th>APC</th>
<th>FITC</th>
<th>PE</th>
<th>DAPI</th>
</tr>
</thead>
</table>
Cell Analysis

CTC's
CK-PE+/DAPI+/CD45-APC-

CD45-APC
CTCs
## PATIENT REPORT

**Facility:** Yale University  
430 Congress St  
New Haven CT.  

**Sample ID:** YA1-R4  
**Volume:** 7.5 mL  

**Patient ID:**  

**Cartridge ID:** 540008  
**Scan #:** 1  

### Instruments and Operators

**CellTracks® Analyzer II**  
**Serial #:** CT0607028  
**Scan Operator ID:** rv  
**Scan Date/Time:** 09/03/2009 07:59 AM  
**First Reviewer ID:** rv  
**Review Date/Time:** 09/03/2009 09:17 AM  
**Last Reviewer ID:** ghl  
**Review Date/Time:** 09/10/2009 05:40 AM  

**CellTracks® AutoPrep® System**  
**Serial #:** AP06060618  
**Operator ID:** rapa  
**Prop Date:** 09/02/2009  
**Prop Time:** 03:12 PM  
**Sample Position:** 4  
**Draw Date:**  
**Draw Time:**  

### Batch Information

**Reagent Kit**  
**Kit ID:** CellSearch™ CTC  
**Kit Lot:** 0079  
**Expiration:** 05/20/2010  

### Results

<table>
<thead>
<tr>
<th>CTC</th>
<th>CK-PRC</th>
<th>CK-PRC (+/−)</th>
<th>Unassigned</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td></td>
<td>0</td>
<td>49</td>
</tr>
</tbody>
</table>

### Comments

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**Report Authorization:** ___________________________  
**Date:** ___________________________
CTC detection in peripheral blood in clinical practice

• Low frequency (rare): 1 CTC among million red blood cells
• Standardized methods with high degree of reproducibility
• Currently, most data on the prognostic value, available for breast, prostate and colon cancers.
• Multicenter analysis and validation is needed to confirm clinical significance.
Summary

CellSearch™ System

- Valuable tool for monitoring cancer patient status and outcome. FDA approved.
- Employs immunomagnetic-enrichment based protocols focused on CTC number as the indicator of patient status or outcome.
- Multi center trial: The number of CTCs was a significant independent predictor of OS and PFS in patients with MBC, MCRC and MPC
- American Society of Clinical Oncology (ASCO): recommendation 2007: CTC test should not be used to make diagnostic or treatment decisions in patients with MBC
Future Potential and Applications: CTCs

- Guide prognosis: Metastatic and early stage cancer patients
- Measure response to anticancer Rx: predictive biomarker
- Select patients for adjuvant chemotherapy
- Detect recurrent disease
- ‘Real time biopsy’: Surrogate for Tumor biology
- Molecular characterization: Discover and identify new targets for therapeutic manipulation
Conclusion

• CTC level (< 5): Favorable, this may imply a good response to treatment.
• Caution is warranted because of the lower sensitivity of the CTC test.
• Radiologic disease progression should not be ignored on the basis of a favorable CTC level.
• Favorable CTC level with overt radiologic progression may still suggest a better outcome.
The CellSearch System (Veridex)

- Morphology skills highly similar to those of the Cytopathologist
  - Interpretation and Enumeration of CTCs.
  - Protein expression patterns of CTC (ER, PR, HER2, EGFR), additional prognostic information.

- Cytopathology lab with trained cytotechnologists and cytopathologists
  - Natural location for this technology in the healthcare delivery system.
Acknowledgements:

Yale University School of Medicine, Dept of Pathology
- David L. Rimm
- David Chhieng
- Diane Kowalski
- Lab Manager: Kevin Schofield
- Cytotechnologists: Brett Minger, Philip Galullo, Kristina Gordy, Rupa Vyas

Veridex
  Brian Zuchelkowski
  Vera Gibson
History

- Breast Group CEC/CTC Enumeration Study
What does it cost?

Lab cost around $175 per test
CPT Codes:
88346 x 3 (immunofluorescent study) = $380 x 3 = $1140
88361 x 2 (morphometric analysis, IHC) = $505 x 2 = $1010
88313 x 1 (special stain) = $210
Charge $2360 per test
Total Costs

Medicare Reimbursement Avg: $777.53
Labor/Overhead: $386.00
Labor/Overhead + Cost per test = $386.00 + $175.00 = $561.00
Tests Requirements

- High Complexity Tests
- Pathologist and cytotechnologist (certified by Veridex)
- Cell Interpretation Proficiency Assessment
- PT Test Requirement