111 Circulating Tumor Cells (CTCs) in Cytology

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AMERICAN SOCIETY FOR CLINICAL PATHOLOGY
33 W. Monroe, Ste. 1600
Chicago, IL 60603
This session will provide an introduction to CTCs. A brief history and background of CTCs will be presented, followed by a review of current technology, supporting literature and a demonstration of how the technology has been implemented in the presenter's laboratory. Key points of the session will include: instrumentation (CellTrack Autoprep system), enumeration of CTCs for predicting progression-free and overall survival in metastatic breast cancer patients, specimen collection, quality control, interpretation of results, limitations, clinical trial conclusions, and novel methods for CTC identification.

- To integrate the methods of CTC detection into the practice of cytopathology.
- To be able to identify and evaluate CTCs in peripheral blood using CellSearch System.
- To recognize the potential role of the cytopathologist in the process of measuring CTCs.

FACULTY:

Malini Harigopal MD

Entire Pathology Team
New Techniques and Technologies
New Techniques & Technologies
1.0 CME/CMLE Credit

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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

Definition

- Circulating tumor cells (CTCs): cancer cells shed from either the primary tumor or its metastases
  - Epithelial cells derived from solid tumors
  - Metastases are responsible for most cancer deaths.

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: labeling of antibodies with fluorochromes improved the specificity of detection of CTC.
• Value to cytologic diagnosis of CTC
Utility CTC Measurement

- 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 enable treatment to be tailored and limit metastases.

Utility CTC Measurement

- The role of CTC in blood is still under active investigation, biological significance/therapeutic relevance (debated).
- Identification, enumeration and molecular characterization of CTCs could expand the understanding of the biology of metastases.
- Several strategies have been used for CTC enumeration.
- Nucleic-acid-based (PCR) and antibody-based cytometric assays (intact cells).
Techniques for CTC Enumeration

- CellSearch system (Veridex, Warren, NJ): capture of epithelial cells (EpCAM) by ferrofluid
- CTC-chip: microfluid platform
- FAST (fiber-optic array scanning technology)
- Oncoquick: cellular density
- MACS: capture of epithelial cells by immunobeads
- ISET: cellular size

CellSearch™ System

- CellSearch System (Veridex, Warren NJ): Technology
- Automated, standardized technology for CTC detection
- Based immunofluorescence
- CellSearch system validated in clinical trials
- 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:
    - Anti-cytokeratin 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)
Immunomagnetic Labeling and Immunofluorescent Identification of Cells

Circulating Tumor Cell

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.

Table 1 CTC Counts

<table>
<thead>
<tr>
<th>Tube 1 CTC Count</th>
<th>Tube 2 CTC Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Frequency of CTCs: CellSearch™ System

Figure 1: Frequency of CTC in Cancers (Subjects without Cancer) and Patients with Metastatic Breast (MBC), Metastatic Colorectal (MCRC) or Metastatic Prostate Cancer (MPC) before initiation of a new line of Therapy (Breast) and 6.6 years after the initiation of Therapy.
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

Metastatic Breast Cancer (MBC) cutoff ≥ 5 CTC

Circulating tumor cells, Disease Progression, and Survival in Metastatic Breast Cancer, Cristofanilli et al, Sem Oncol. 2006


MBC Clinical Trial Design

- Measurable disease, any type or line of therapy (first line, chemo Rx)
- 177 MBC (metastatic breast cancer)(20 centers)
  (67%ER/PR+, HER2 52%)
  CTC analysis (performed in 7 centers)
- 145 healthy and 200 pts with benign disease
- Imaging and CTC analysis (prior to initiation of therapy)
- CTC performed, 1 follow-up (~ 4 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)
- Median OS 21.9 Months

CTC / 7.5mL                          Median OS in at Baseline        N (%)       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 USEMKG-1866, Rev. 1

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)
- Median PFS 7.0 Months

CTC / 7.5mL                          Median PFS in at Baseline        N (%)        Months (95% C.I.)
<5 CTC         89 (50%)       7.0  (5.6 to 8.9)
>5 CTC         88 (50%)       2.7  (2.1 to 4.4)

FOR INTERNAL AND EXTERNAL USEMKG-1866, Rev. 1

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

- Logrank p = 0.0001
- Median OS in Group                         Description                                N (%)        Months (95% C.I.)
1                 <5 CTCs at All Time Points                83 (47%)      22.6  (20.4 to >45)
2        >5 at Baseline & <5 CTC at Last Draw       38 (21%)      19.8  (14.6 to 31.6)
3     <5 at Early Draw & >5 CTC at Last Draw      17 (10%)      10.6  (  6.1 to 16.2)
4                 >5 CTCs at All Time Points                39 (22%)       4.1  (  2.8 to   6.4)

FOR INTERNAL AND EXTERNAL USEMKG-1866, Rev. 1
Predictive Value: OS of MBC Patients with <5 or ≥5 CTC at different times of Follow-Up


Metastatic Colorectal Cancers (MCRC) Cut off ≥ 3 CTCs


MCRC Clinical Trial Design
- Measurable disease, 1st or 2nd line of therapy
  - 3rd line, only if anti-EGFR (chemo Rx)
- 430 MCRC (metastatic colorectal cancer)
  - CTC analysis (performed in 4 centers)
- 158 healthy and 55 pts with benign disease
- Imaging and CTC analysis (prior to initiation of therapy)
- Frequency of CTC: 4 weeks
- Duration of CTC: 12 months or until clinical progression (imaging, clin eval)
- Clinical follow up: 36 months
Predictive Value: OS of MCRC Patients with <3 or ≥3 CTC at Baseline (N=413)

- Cox Hazard Ratio = 2.5
- chi-square = 31.48 (p-value < 0.0001)

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>N (%)</th>
<th>Median OS (95% C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;3 CTC at All Draws</td>
<td>303 (70%)</td>
<td>18.6 (15.9 to 22.5)</td>
</tr>
<tr>
<td>2</td>
<td>&gt;3 CTC at BL &amp; &lt;3 CTC at Last Draw</td>
<td>74 (17%)</td>
<td>11.7 (9.4 to 18.7)</td>
</tr>
<tr>
<td>3</td>
<td>&lt;3 CTC at Early Draw &amp; &gt;3 CTC at Last Draw</td>
<td>29 (7%)</td>
<td>7.1 (6.3 to 10.8)</td>
</tr>
<tr>
<td>4</td>
<td>&gt;3 CTC at All Draws</td>
<td>24 (6%)</td>
<td>3.9 (2.5 to 5.4)</td>
</tr>
</tbody>
</table>

Predictive Value: PFS of MCRC Patients with <3 or ≥3 CTC at Baseline (N=413)

- Cox Hazard Ratio = 1.6
- chi-square = 12.19 (p-value = 0.0002)

A Reduction in CTC Below 3 After the Initiation of Therapy Predicts Longer OS whereas an Increase in CTC Count to 3 or above Predicts a Shorter OS
Predictive Value: OS of MCRC Patients with <3 or ≥3 CTC at different times of Follow-Up

<table>
<thead>
<tr>
<th>Time from Blood Draw (Months)</th>
<th>&lt;3 CTC at:</th>
<th>≥3 CTC at:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- 2 Weeks (n=316)</td>
<td>1- 2 Weeks (n=41)</td>
<td></td>
</tr>
<tr>
<td>3- 5 Weeks (n=292)</td>
<td>3- 5 Weeks (n=41)</td>
<td></td>
</tr>
<tr>
<td>6-12 Weeks (n=285)</td>
<td>6-12 Weeks (n=25)</td>
<td></td>
</tr>
<tr>
<td>13-20 Weeks (n=172)</td>
<td>13-20 Weeks (n=21)</td>
<td></td>
</tr>
</tbody>
</table>

%Probability

Metastatic Prostate Cancers (MPC)-K073338 Cut off ≥ 5 CTCs


MPC Clinical Trial Design

- 231 MPC (metastatic prostate cancers), two consecutive increases in serum PSA, androgen independent, hormone resistant prostate cancer
- Bone metastases pos (90%), non-measurable disease 62%
- Baseline CTC count prior to initiation of new line of chemotherapy
- Frequency of CTC: 2-4 weeks
- Duration of CTC: 18 months or until progression
- Clinical follow-up: 36 months.
- Disease progression (PSA, imaging and clinical signs and symptoms).
Predictive Value: OS of MPC Patients with <5 or ≥5 CTC at Baseline (N=219)

<table>
<thead>
<tr>
<th>CTC Count</th>
<th>N (%)</th>
<th>Median OS (95% C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;5 CTC</td>
<td>94 (43%)</td>
<td>21.7 (21.3 to ------)</td>
</tr>
<tr>
<td>&gt;5 CTC</td>
<td>125 (57%)</td>
<td>11.5 (  9.3 to 13.7)</td>
</tr>
</tbody>
</table>

Logrank p < 0.0001

Predictive Value: PFS of MPC Patients with <5 or ≥5 CTC at Baseline (N=219)

<table>
<thead>
<tr>
<th>CTC Count</th>
<th>N (%)</th>
<th>Median PFS (95% C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;5 CTC</td>
<td>94 (43%)</td>
<td>5.8 (5.0 to 7.9)</td>
</tr>
<tr>
<td>&gt;5 CTC</td>
<td>125 (57%)</td>
<td>4.2 (3.1 to 4.9)</td>
</tr>
</tbody>
</table>

Cox Hazard Ratio = 1.6
chi-square = 11.03
(p value = 0.0009)

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 in MPC Patients
**Predictive Value: OS of MPC Patients with <5 or ≥5 CTC at different times of Follow-Up**

<table>
<thead>
<tr>
<th>Time from Blood Draw (Months)</th>
<th>&lt;5 CTC at:</th>
<th>≥5 CTC at:</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-5 Weeks (n=123)</td>
<td>2-5 Weeks (n=110)</td>
<td></td>
</tr>
<tr>
<td>6-8 Weeks (n=100)</td>
<td>6-8 Weeks (n=99)</td>
<td></td>
</tr>
<tr>
<td>9-12 Weeks (n=99)</td>
<td>9-12 Weeks (n=99)</td>
<td></td>
</tr>
<tr>
<td>13-20 Weeks (n=99)</td>
<td>13-20 Weeks (n=99)</td>
<td></td>
</tr>
</tbody>
</table>

**Summary & Conclusion**

- **MBC, MCRC, and MPC**
  - **MBC**
    - 177 patients
    - Cut-off = ≥5 CTC
    - Patients with ≥5 CTC at baseline = 26% (98/177 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 = ≥3 CTC
    - Patients with ≥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 = ≥5 CTC
    - Patients with ≥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

**Median Overall Survival Comparison**

<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>&lt;cut-off at all time points</td>
<td>4.1</td>
<td>3.9</td>
<td>6.8</td>
</tr>
<tr>
<td>&lt;cut-off at baseline and ≥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>
Summary

• 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 CD 45 and expressing cytokeratin (8, 18 & 19).
  - Morphology (round or oval with a nucleus within the cytoplasm).
  - Size (4um)
  - Heterogeneity (morphology and size).
Tumor Cell and Leukocyte in Same Frame

- 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

- 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

- 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.

10/8/2011
**Suspicious Objects**

<table>
<thead>
<tr>
<th>Comp</th>
<th>CD56-APC</th>
<th>CK-PE</th>
<th>DAPI</th>
</tr>
</thead>
<tbody>
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</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**

<table>
<thead>
<tr>
<th>Comp</th>
<th>CD56-APC</th>
<th>CK-PE</th>
<th>DAPI</th>
</tr>
</thead>
<tbody>
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</tbody>
</table>

**“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.

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**Not Classified Cells**

<table>
<thead>
<tr>
<th>Comp</th>
<th>CD56-APC</th>
<th>CK-PE</th>
<th>DAPI</th>
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<tbody>
<tr>
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Computer Noise

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

Squamous Cells

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

Cell Interpretation Practice
Cell Analysis

CTCs
CK-PE+/DAPI+/CD45-APC-

Gallery of images HER2 + CTCs
CTC detection in peripheral blood in clinical practice
- Low frequency (rare)
- 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

- 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

Conclusions

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.
History

• Breast Group CEC/CTC Enumeration Study

What does it cost?

<table>
<thead>
<tr>
<th>Code</th>
<th>Description</th>
<th>Fee</th>
</tr>
</thead>
<tbody>
<tr>
<td>88346</td>
<td>Immunofluorescent study</td>
<td>$380</td>
</tr>
<tr>
<td>88361</td>
<td>Morphometric analysis, IHC</td>
<td>$505</td>
</tr>
<tr>
<td>88313</td>
<td>Special stain</td>
<td>$210</td>
</tr>
</tbody>
</table>

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

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