Klaus Pantel

Circulating tumor cells (CTC) as prognostic/monitoring tool in epithelial solid tumors
Detection of CTC in the peripheral blood

June 2012: > 400 registered clinical trials with CTC as biomarkers

The technical challenge:
Finding one tumor cell in $10^6 - 10^8$ normal blood cells
2012: > 50 different CTC assays!

Pantel et al., Nat. Rev. Cancer 2008
New approach: In vivo capture of CTC

Insertion into patient’s vein at the doctor’s office
30 minutes exposure time in a vein

Decision  Result  Diagnostics

ERA-NET TRANSCAN: CTC-SCAN Project
High-risk Prostate Cancer (stage M0)
Gilupi Detector vs. CellSearch vs. EPISPOT
Partners: Germany, France, Greece, Poland, Austria
CTC Identification Methods

Real-time RT-PCR

Cytokeratins as standard CTC markers
BUT differential expression of individual CKs
(Joosse/Pantel et al., Clin Cancer Res 2012)

Tumor cell

nucleic acids

DNA

mRNA

intra-cytoplasmic proteins

membrane proteins

secreted proteins by VAILABLE cells

Immunocytochemistry

EPISPOT assay

Alix-Panabières et al., Clin Cancer Res, 2008
Design of robust automated systems for reproducible CTC detection
CellSearch™ System (FDA-cleared)

Enrichment of CTC with anti-EpCAM ferro fluids

Cristofanilli et al., NEJM, 2004
Riethdorf et al., CCR, 2007 & 2010
DeBono et al, CCR, 2008
Cohen et al, JCO, 2008
Krebs et al, JCO, 2012

MagNest™

CellTracks™ Analyzer II w/ Linux operating system
## CellSearch™ System: Images of Tumor Cells

<table>
<thead>
<tr>
<th>Cytoplasm</th>
<th>Nucleus</th>
<th>Cell Membrane</th>
<th>Composite</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK-PE pos</td>
<td>DAPI pos</td>
<td>CD45-APC neg</td>
<td>Tumor Cell</td>
</tr>
</tbody>
</table>

![Images of Tumor Cells](image-url)
Automated multiplex q-RT-PCR: Lab-in-a-cartridge

**Reaction chamber:**
1. mRNA-capturing
2. c-DNA synthesis
3. PCR

**Lysis chamber:**
- binding of Biotin-Oligos to mRNA

**Array:**
- Signal monitoring of real time PCR

**Processing Fluid**
- dry reagents RT, TAQ, Primer, Reporter

**Sample inlet**
- Waste

Alere Technologies GmbH Jena
Prognostic value of CTC
Prognostic value of CTC counts for survival in cancer patients with advanced disease

Breast Cancer
Christofanilli, NEJM, 2004

Colorectal Cancer
Cohen, JCO, 2008

Prostate Cancer
De Bono, Clin Can Res, 2008

→ FDA clearance
Multivariate Analysis for DFS for different CTC cut-offs

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard Ratio adjusted for treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 vs. ≥ 1</td>
</tr>
<tr>
<td></td>
<td>0, 1 vs. ≥ 2</td>
</tr>
<tr>
<td></td>
<td>0-4 vs. ≥ 5</td>
</tr>
<tr>
<td>CTCs in blood pos/neg</td>
<td>1.878 *</td>
</tr>
<tr>
<td>Hormone receptor status pos/neg</td>
<td>2.073 *</td>
</tr>
<tr>
<td>Lymph Node Involvement pos/neg</td>
<td>1.698 *</td>
</tr>
<tr>
<td>Grading G1 vs. G2-3</td>
<td>2.961 *</td>
</tr>
<tr>
<td>Tumor size T1 vs. T2-4</td>
<td>1.629 *</td>
</tr>
</tbody>
</table>

* P < 0.05

Rack, Janni et al, unpublished
Meta-Analysis of 49 studies comprising 6815 breast cancer patients

Progression-free survival

CTC detection: ICC & RT-PCR

Overall survival

Distant Metastases (M)

M0  No clinical or radiographic evidence of distant metastases

cM0(i+)  No clinical or radiographic evidence of distant metastases, but deposits of molecularly or microscopically detected tumor cells in circulating blood, bone marrow, or other nonregional nodal tissue that are no larger than 0.2 mm in a patient without symptoms or signs of metastases

M1  Distant detectable metastases as determined by classic clinical and radiographic means and/or histologically proven larger than 0.2 mm
Challenges for CTC Detection

- Circulation of non-malignant epithelial cells (specificity problem)
- Epithelial-Mesenchymal Transition of CTC (sensitivity problem)
Detection of epithelial cells (CK+/CD45-) in blood of patients with benign (inflammatory) colon diseases

<table>
<thead>
<tr>
<th></th>
<th>EPISPOT CTC+</th>
<th>CellSearch CTC+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>10/53 (18.9%)</td>
<td>6/53 (11.3%)</td>
</tr>
<tr>
<td>Range</td>
<td>1-41</td>
<td>3-37</td>
</tr>
</tbody>
</table>

After 3 years of follow-up, none of these patients have been diagnosed with colorectal cancer or another epithelial cancer.
Epithelial-Mesenchymal Plasticity of CTC

Epithelial phenotype

EpCam, CK

Mesenchymal phenotype

Vimentin

<table>
<thead>
<tr>
<th>Epithelial phenotype</th>
<th>Epithelial phenotype with minor mesenchymal features</th>
<th>Semi-mesenchymal phenotype</th>
<th>Mesenchymal phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial markers strongly expressed</td>
<td>Epithelial markers moderately expressed</td>
<td>Epithelial markers weakly expressed</td>
<td>No epithelial markers</td>
</tr>
<tr>
<td>No mesenchymal markers</td>
<td>Mesenchymal markers weakly expressed</td>
<td>Mesenchymal markers moderately expressed</td>
<td>Mesenchymal markers strongly expressed</td>
</tr>
<tr>
<td>Detection by standard CTC technology</td>
<td>Detection by standard CTC technology</td>
<td>Limited detection by standard CTC technology</td>
<td>No detection by standard CTC technology</td>
</tr>
</tbody>
</table>

Bednarz-Knoll, Alix-Panabières & Pantel Cancer & Met Rev 2012
EMT in prostate cancer: BRCA1 gene loss in vimentin-positive CTC

FISH

Immunocytochemistry

CK

Vimentin

FISH: BRCA1 CEP17

CK-pos. CTC

VIM-pos. CTC: EMT!

Bednarz/Pantel/Brandt et al., Clin Cancer Res 2010
Expression profile of CTCs in breast cancer

Yu et al, Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. Science, Febr. 2013

Yokobori, Mimori, Pantel, Mori et al. Plastin-3 as new CTC marker not downregulated during EMT, Cancer Res. Febr. 2013

Significance of PLS3 in peripheral blood from CRC Cases

Overall survival
(Dukes A-D)

Disease-free survival
(Dukes A-C)

Overall survival
(Dukes B)

Disease-free survival
(Dukes B)
Tumor cell dissemination and tumor cell plasticity
Bednarz-Knoll, Alix-Panabières & Pantel
*Cancer & Met Rev* 2012

**Early diagnostic of progression:** CTC isolation

**Primary tumor**

**EMT**

**Barrier:** primary site - blood

**CTCs:** (semi-)mesenchymal phenotype

**EMT**

**Barrier:** blood - secondary site

**CTCs:** semi-epithelial/mesenchymal phenotype

**MET**

**Barrier:** secondary site - blood

**CTCs:** epithelial phenotype

**Micro- and overt metastasis**

**Barrier:** secondary site - blood

**CTCs:** epithelial or (semi-)mesenchymal phenotype

**Late diagnostic of progression:** standard imaging methods

**Early diagnostic of relapse:** CTC isolation

**Barrier:** secondary site - blood

**Micro- and overt metastasis**

**Barrier:** secondary site - blood

**CTCs:** (semi-)mesenchymal phenotype

**Epithelial phenotype**

**Blood vessel lumen**
Perspective:

Molecular Characterization of CTC

("real-time liquid biopsy")
CTC as Liquid Biopsy for metastatic cells

Metastasis evolve many years after primary tumor resection and can harbor unique genomic alterations.

Biopsy of metastases is an invasive and sometimes dangerous procedure.

Can the molecular characterization of CTC reveal representative information on metastatic cells located at different sites?

Detection of therapeutic targets on CTC: HER2 oncogene in breast cancer

**CTC without HER2 gene amplification**

**CTC with HER2 gene amplification**

**SK-BR-3**

Red: CK

ICC Green: HER2 FISH

Red: Cen17 FISH

**CB11 A0485 FISH**

**Composite CK**

**DAPI**

**CD45**

**HER2**

**MCF-7**

**BT20**

**T47D**

**MDA-MB-453**

**SK-BR-3**

**BT474**

<table>
<thead>
<tr>
<th></th>
<th>Composite</th>
<th>CK</th>
<th>DAPI</th>
<th>CD45</th>
<th>HER2</th>
<th>CB11</th>
<th>A0485</th>
<th>FISH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF-7</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td>0</td>
<td><img src="image6.png" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>BT20</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td>1+</td>
<td><img src="image6.png" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>T47D</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td>1+</td>
<td><img src="image6.png" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td>2+</td>
<td><img src="image6.png" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>SK-BR-3</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td>3+</td>
<td><img src="image6.png" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>BT474</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td>3+</td>
<td><img src="image6.png" alt="Image" /></td>
<td></td>
</tr>
</tbody>
</table>

**Detect-III study:** Anti-HER2 therapy (lapatinib) in metastatic breast cancer patients with HER2-negative primary tumors and HER2-positive CTC

**Discordance between HER2 status of primary tumor and CTC**

Riethdorf/Pantel *et al.*, *Clinical Cancer Res* 2010 - Fehm/Pantel *et al.*, *Breast Cancer Res Treat* 2010

Genomic Characterization of single CTC

CTC detection

CTC isolation

WGA +
- Mutation analysis
- CGH (conv./array)
- NextGen Sequencing
Detection of mutations in genes relevant for resistance of targeted therapies (eg, EGFR inhibition)

<table>
<thead>
<tr>
<th>Patient</th>
<th>No. Of CTCs</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P53</td>
<td></td>
<td></td>
<td>KRAS</td>
<td></td>
<td></td>
<td>BRAF</td>
<td></td>
<td>PIK3CA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>WT</td>
<td>MUT</td>
<td>n.a.</td>
<td>WT</td>
<td>MUT</td>
<td>n.a.</td>
<td>WT</td>
<td>MUT</td>
<td>n.a.</td>
<td>WT</td>
</tr>
<tr>
<td>1</td>
<td>38</td>
<td>18</td>
<td>2</td>
<td>18</td>
<td>13</td>
<td>7</td>
<td>18</td>
<td>14</td>
<td>-</td>
<td>24</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>10</td>
<td>-</td>
<td>4</td>
<td>9</td>
<td>-</td>
<td>5</td>
<td>9</td>
<td>-</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>5</td>
<td>-</td>
<td>1</td>
<td>5</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>9</td>
<td>2</td>
<td>-</td>
<td>7</td>
<td>-</td>
<td>4</td>
<td>2</td>
<td>-</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td>7</td>
<td>15</td>
<td>6</td>
<td>21</td>
<td>-</td>
<td>7</td>
<td>20</td>
<td>-</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>6</td>
<td>-</td>
<td>4</td>
<td>5</td>
<td>-</td>
<td>5</td>
<td>4</td>
<td>-</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>1</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>13</td>
<td>2</td>
<td>4</td>
<td>7</td>
<td>7</td>
<td>-</td>
<td>6</td>
<td>7</td>
<td>-</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>12</td>
<td>5</td>
<td>3</td>
<td>-</td>
<td>2</td>
<td>3</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>13</td>
<td>3</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>141</td>
<td>70</td>
<td>23</td>
<td>48</td>
<td>81</td>
<td>8</td>
<td>52</td>
<td>68</td>
<td>1</td>
<td>72</td>
<td>64</td>
</tr>
</tbody>
</table>

NextGen Sequencing (NGS) of single SKBR3 breast cancer cells

Single cell 1

Single cell 2

Pool-NGS

Pool-aCGH

Chromosome 8

Chromosome 17

Single cell vs pooled DNA

\[ r^2 = 0.91 \]

Simon Joosse et al, unpublished
Analysis of CTC, primary tumors and metastases from patients with colorectal cancer

DNA isolation and amplification

arrayCGH

sequencing

...and many other bioinformatic tools

Collaboration with Michael Speicher’s group, Graz; unpublished
Distribution of mutations in primary tumor, metastases and CTC

### Patient #6
- **APC** [p.R332X]
- **KRAS** [p.G12V]
- **PIK3CA** [p.E542K]
- **TP53** [p.R141C]

### Patient #26
- **APC** [p.R332X]
- **KRAAS** [p.G12V]
- **PIK3CA** [p.E542K]
- **TP53** [p.R141C]

**Table: Mutations and Clinical Significance**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Point mutation</th>
<th>Point mutations primary tumor</th>
<th>Point mutations cerebellar metastasis</th>
<th>Potentially clinically significant</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>p.R332X</td>
<td>p.R332X</td>
<td>p.R332X</td>
<td>No</td>
</tr>
</tbody>
</table>

**Table: Copy Number Variations**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Point mutation</th>
<th>Copy number primary tumor (log2)</th>
<th>Copy number cerebellar metastasis (log2)</th>
<th>Copy number CTCs (Abs.)</th>
<th>Potentially clinically significant</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>Ø</td>
<td>-0.5 (loss)</td>
<td>-0.5 (loss)</td>
<td>2 (loss)</td>
<td>No</td>
</tr>
<tr>
<td>CDK8</td>
<td>Ø</td>
<td>0 (balanced)</td>
<td>0 (balanced)</td>
<td>7 (gain)</td>
<td>CDK-inhibitors</td>
</tr>
</tbody>
</table>
Detection of subclonal single-nucleotide variants in tumor cell populations by deep sequencing

17/20 “private CTC mutations“ were identified as subclonal variants in the primary tumor, metastasis, and plasma DNA with mutated frequencies ranging from 0.02 to 0.46.

Collaboration with Michael Speicher’s group, Graz (Heitzer et al, Cancer Res., 2013)
Tumor-associated circulating cell-free nucleic acids in blood

Micrometastasis

CTC

Tumor DNA

KRAS Mutations:
Diaz et al, Nature 2012
Heitzer et al, IJC 2013

microRNAs:
Roth et al, BCR 2010 & PLoSONE 2012

Schwarzenbach/Hoon/Pantel, Nat Rev Cancer, 2011
RESULTS:

• Enrichment of DNA between 85-230bp in 65.6% of patients and in healthy controls

• 34.4% of patients showed a second peak in the range of 240bp to 400bp

• Patients with biphasic plasma DNA size distributions had significantly higher plasma DNA concentrations ($P<0.0001$)

• Significant correlation between the presence of a biphasic peak and CTC occurrence (1.5 CTCs vs. 52 CTCs, $P<0.0001$)

(Heitzer et al., Int J Cancer 2013)
Aims of Research on Circulating Tumor Cells

- Estimation of the risk for metastatic relapse or metastatic progression (prognostic information)
- Stratification & real-time monitoring of therapies
- Identification of therapeutic targets and resistance mechanisms (biological therapies)
- Understanding the biology of metastatic development
Tumor cell dissemination and cancer dormancy
(Uhr & Pantel, PNAS 2011)

Experimental findings:
- **Reseeding of the primary tumor:**
Recirculation of breast cancer cells from the bone marrow to the primary site
(J. Massague’s group, Kim et al, Cell 2009)

- **Escape from dormancy:**
VCAM1 promotes osteoclast differentiation & activation & attracts osteoclast progenitors
(Y. Kang’s group, Lu/Pantel et al Cancer Cell 2011)

**Cancer micrometastases**
Klaus Pantel, Catherine Alix-Panabières and Sabine Riethdorf
Center of Experimental Medicine
Institute of Tumor Biology - Klaus Pantel

Grant Support:
- Sabine Riethdorf/Christin Gasch
- Heidi Schwarzenbach
- Harriet Wikman/Michaela Wrage
- Katharina Effenberger
- Juliane Hannemann/Simon Joosse
- Kai Bartkowiak

DFG
BMBF
EU / ERC
Dt. Krebshilfe
Sander-Stiftung
Roggenbuck-Stiftung
Micrometastasis Research Network at UCCH/UKE
EU-Consortium-DISMAL

Start: November 2005  Coordinator: Klaus Pantel

Free University of Amsterdam Medical Center (The Netherlands)

Imperial College London (United Kingdom)

University Medical Center Hamburg-Eppendorf (Germany)

University of Utrecht (The Netherlands)

Lapeyronie Hospital, Montpellier, (France)

ERC Advanced Investigator Grant „DISSECT“
(2011-2016, PI: K. Pantel)

SME 1 Applied Imaging, (United Kingdom)

SME 2 TILL Photonics (Germany)

SME 3 Agendia, (The Netherlands)

Netherlands Cancer Institute (The Netherlands)

Netherlands Cancer Research Center, (Germany)

German Cancer Research Center, (Germany)

Heinrich-Pette-Institut (Germany)

Radium Hospital Oslo, (Norway)

Leiden University Medical Center (The Netherlands),

Imperial College London (United Kingdom),

University of Utrecht (The Netherlands),

Free University of Amsterdam Medical Center (The Netherlands)

University Medical Center Hamburg-Eppendorf (Germany)
9th International Symposium on Minimal Residual Cancer
September 24-27, 2013
Pullman Paris Bercy, France

Organizers
Jean-Yves Pierga
MD, PhD, Institut Curie,
Paris Descartes University, France

Catherine Alix-Panabières
Ph.D, University Medical Centre Montpellier,
UM1, Montpellier, France

Klaus Pantel
MD, Ph.D, University Medical Centre
Hamburg-Eppendorf, Hamburg, Germany

www.ismrc2013.com