Institute for Tumor Biology
Professor Klaus Pantel

CTC-DTC Research: State of the Art & Perspectives
Tumor Cell Dissemination
Key Step in Tumor Progression

Primary Tumor → Lymph-node metastasis

Hematogenous dissemination → CTC → DTC → Distant metastases

Izbicki/Pantel et al., NEJM, 1997
De Boer et al., NEJM, 2009
Detection of CTC in the peripheral blood

September 2013:

> 400 registered clinical trials with CTC as biomarkers

> 13,000 publications in PubMed

Advantages over DTC detection:

• Less invasive than BM sampling

• Pool of DTC from multiple distant sites
• Metastasis biology
• Novel CTC assays
• CTC in clinical studies
• Molecular characterization of CTC
• CTC and other circulating markers
Metastasis Biology

Cancer Cell
Perspective

Tumor Cell Dissemination: Emerging Biological Insights from Animal Models and Cancer Patients

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http://dx.doi.org/10.1016/j.ccr.2013.04.017
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-Panabères and Sabine Riethdorf*
Detection of DTC in bone marrow

Bone marrow aspirates taken from the upper iliac crest

Immunocytochemistry:
Cytokeratin staining with mAB A45-B/B3

2 x 10^6 MNC per patient

Breast Cancer: 199/552 (36%)
(Braun, Pantel et al. NEJM, 2000 & 2005)

Prostate Cancer: 86/193 (44.6%)
(Koellermann/Pantel et al. JCO 2008)

Nonmalignant disease: 2/191 (1%)

• DTC detection correlates with metastatic AND locoregional relapse
  • Most DTC are Ki67- and have CD44+/CD24- phenotype
  • DTC detection might be useful for stratification of bone-directed anti-cancer therapies (e.g., bisphosphonates, RANKL Abs)
    • Bisphosphonate treatment reduces DTC counts and prevents metastatic & locoregional relapse
Cancer Dormancy: Research questions

• Do all cancer patients have dormant tumor cells?
• Can host factors induce or break dormancy? Stress? Inflammation?
• Are there preferred reservoirs of dormant cells (e.g., bone marrow)?
• Does the immune system play a role in dormancy?
• What is the effect of current therapies on dormant cells or dormancy?
• What signaling pathways or events reactivate dormant cells?
• Do dormant cells have properties of cancer stem cells?
• How does genetic background affect dormancy?

Uhr & Pantel PNAS 2011; Kang & Pantel, Cancer Cell 2013
Novel CTC Assays
CTC Enrichment Methods

2013: > 50 different CTC assays!

The technical challenge:
Finding one tumor cell in $10^6 \div 10^8$ normal blood cells

CTC Identification Methods

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

Real-time RT-PCR

nucleic acids

mRNA

DNA

intra-cytoplasmic proteins

membrane proteins

secreted proteins by VIABLE cells

Alix-Panabieres et al., Clin Cancer Res, 2008

Immunocytochemistry

EPISPOT assay
Design of robust automated systems for reproducible CTC detection
CellSearch® System (FDA-cleared)

CellSave® Preservative Tube

MagNest™

Cell tracks™ autoprep system

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

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>

- **Cytoplasm**: CellSearch® technology uses labels for specific markers.
- **Nucleus**: Positives (pos) and negatives (neg).
- **Cell Membrane**: Positives (pos) and negatives (neg).

**Images**:
- **Leukocyte nucleus**: Green
- **CD45+ Membrane**: Blue
- **Leukocyte Tumor Cell**: Yellow
- **Tumor Cell**: Yellow with green

This diagram illustrates the analysis process for identifying and characterizing tumor cells.
Automated multiplex q-RT-PCR: Lab-in-a-cartridge

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

Array:
Signal monitoring of real time PCR

Lysis chamber:
Binding of Biotin-Oligos to mRNA

Processing Fluid:
dry reagents RT, TAQ, Primer, Reporter

sample inlet

Waste
Challenge of CTC detection:
Epithelial-Mesenchymal Transition (EMT) of carcinoma cells
Tumor cell dissemination, plasticity and EMT
(Bednarz-Knoll et al CMR 2012; Kang & Pantel, Cancer Cell 2013)

Dormancy
> 10 years

Early diagnostic of progression:
CTC isolation

CTCs: (semi-)mesenchymal phenotype

CTCs: semi-epithelial/mesenchymal phenotype

CTCs: epithelial phenotype

Barrier: primary site - blood

Barrier: blood - secondary site

Late diagnostics of progression:
standard imaging methods

Dormancy
> 10 years

Early diagnostic of relapse:
CTC isolation

CTC isolation

Secondary dissemination

Micro- and overt metastasis

Barrier: secondary site - blood

Epithelial phenotype

(semi-)mesenchymal phenotype

Blood vessel lumen
Epithelial-Mesenchymal Plasticity of CTC

EpCAM, CK

<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
Direct link between EMT and gain of stem cell properties and chemotherapy resistance (Mani/Weinberg, et al., Cell, 2008;)

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

Epithelial-Mesenchymal Transition in DTC line BC-M1

2-D DIGE

Western Blot

MDA-468 BC-M1

MW (kDa)

55
55
55
55
55
55
55
55
55
55
55
55

CK7
CK8
CK17
CK18
CK19
Vimentin

α-Tubulin

Willipinski-Stapelfeldt et al. Clin Cancer Res, 2005
CTC in Clinical Studies
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
Meta-Analysis of 49 studies comprising 6815 breast cancer patients

Progression-free survival

CTC detection: ICC & RT-PCR

Overall survival

Real-time monitoring of CTC (surrogate marker)

Urgent need for biomarkers to tailor systemic therapy in individual cancer patients, such as the blood glucose test for directing insulin treatment of diabetes.
Abiraterone Phase III Study with CTC as secondary end point in patients with metastatic prostate cancer

Planned Patients
- 1195 patients with progressive mCRPC
- Failed 1 or 2 chemotherapy regimens

Randomized 2:1

Abiraterone 1000 mg daily
Prednisone 5 mg BID
n = 797

Placebo daily
Prednisone 5 mg BID
n = 398

Efficacy end points (ITT)
Primary end point:
- OS (25% improvement; HR 0.8)
Secondary/tertiary end points:
TTP, rPFS, PSA response
- CTC enumeration

HR, hazard ratio; ITT, intent to treat;

H. Scher, ASCO 2011
Detection of CTC in early stage cancer patients (low CTC counts):

Is the ability to release cancer cells into the circulation relevant for the development of distant metastases?
### Multivariate Analysis for DFS for different CTC cut-offs

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard Ratio adjusted for treatment</th>
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<tbody>
<tr>
<td></td>
<td>0 vs. ≥ 1</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
Prognostic value of CTC in urinary bladder cancer

Survival outcomes: Independent prognostic factor
Median Follow-up: 18 months

DFS HR: 4.6
CSS HR: 5.2

Rink, Pantel, Riethdorf et al., Eur Urol 2012
Molecular Characterization of CTC
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

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

<table>
<thead>
<tr>
<th>B</th>
<th>Composite</th>
<th>CK</th>
<th>DAPI</th>
<th>CD45</th>
<th>HER2</th>
<th>C</th>
<th>CB11</th>
<th>A0485</th>
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<td>MDA-MB-453</td>
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<td>SK-BR-3</td>
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</table>

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
Ignatiadis/Sotiriou et al., PlosONE 2011 - Ignatiadis/Pantel et al., SABCS 2011
Heterogeneity of ER status in CTCs of breast cancer patients with ER-positive primary tumors

Babayan, Joosse, Pantel et al., PLOS ONE 2013

ER-negative CTCs may survive endocrine therapy
Genomic Characterization of single CTC

CTC detection

CTC isolation

WGA +
- Mutation analysis
- CGH (conv./array)
- NextGen Sequencing
Deep targeted sequencing revealed that 17 of 20 private CTC mutations were also present in subclones of the primary tumor and metastases.
CTC and Other Circulating Markers
Real-Time Liquid Biopsy in Cancer Patients: Fact or Fiction?

Klaus Pantel¹ and Catherine Alix-Panabières²,³

Potential utility of CTC and ctDNA analyses:
- Estimation of the risk for metastatic relapse or metastatic progression.
- Stratification and real-time monitoring of therapies.
- Identification of therapeutic targets and resistance mechanisms.
- Understanding metastatic development in patients with cancer.
# Real-Time Liquid Biopsy in Cancer Patients: Fact or Fiction?

Klaus Pantel¹ and Catherine Alix-Panabières²,³

<table>
<thead>
<tr>
<th>Targets</th>
<th>CTCs</th>
<th>ctDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origins</td>
<td>Selected viable tumor cells leaving actively the primary tumor and/or metastases</td>
<td>Necrotic and apoptotic tumor cells</td>
</tr>
<tr>
<td>Definition</td>
<td>Tumor cells as a real-time liquid biopsy of the tumor and/or metastases</td>
<td>Fragmented genomes released from dying tumor cells of the primary tumor and/or metastases and/or CTC</td>
</tr>
<tr>
<td>Analytes</td>
<td>DNA, RNA (mRNA/microRNA), and protein functional studies (<em>in vitro, in vivo</em>)</td>
<td>DNA</td>
</tr>
<tr>
<td>Technologies</td>
<td>Immunocytologic and molecular assays (including next-generation sequencing), cell culture, and xenotransplantation</td>
<td>Molecular DNA assays (including next-generation sequencing)</td>
</tr>
</tbody>
</table>
Correlation **CTC & Circulating Tumor DNA:**

**Prostate Cancer:** Schwarzenbach, Alix-Panabieres, Pantel et al., Clin Cancer Res 2009; **Breast cancer:** Dawson et al, NEJM, 2013; **Colon Cancer:** Heitzer, Pantel et al, Int J Cancer, 2013

Correlation **CTC & Circulating microRNA:**

**Breast Cancer:** Madhavan, Pantel et al Clin Cancer Res 2012

**BUT: ctDNA is released from apoptotic/necrotic cells**

Summary: Aims of Research on DTC & CTC

- 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
Grant Support:

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Micrometastasis Research Network at UCCH/UKE

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Klinik und Poliklinik für Neurochirurgie

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II. Medizinische Klinik und Poliklinik

Institut für Tumorbiologie

Klinik und Poliklinik für Urologie
Catherine Alix-Panabieres, Montpellier:  
Prix cancer Gallet et Breton 2012

Klaus Pantel, Hamburg:  
ERC Advanced Investigator Grant  
D ISSECT (2011-2016)  
ERA-NET TRANSCAN: CTC-SCAN  
Project (2013 - 2016)
9th International Symposium on Minimal Residual Cancer

September 24-27, 2013
Pullman Paris Bercy, France

Organizers

Jean-Yves Pierga
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Paris Descartes University, France

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www.ismrc2013.com