Circulating Tumor Cells in Solid Cancers: Enrichment, Detection & Characterization

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Real-Time Liquid Biopsy

Cellules tumorales circulantes : biopsie liquide du cancer
Circulating Tumor Cells: Liquid Biopsy
Catherine Alix-Panabières1,2,3 and Klaus Pantel4

Clinical Chemistry 2013
The technical challenge:
Finding one tumor cell in $10^6$ – $10^8$ normal blood cells
Detection of CTC/DTC...
...identifying a needle in a hay-stack

One tumor cell

RARE EVENT!

CTC

Millions of hematopoietic cells

RIGHT EVENT!
1 mL of blood

Ficoll gradient

Magnetic sorting

Serum: tumor markers

Blood cells

- Red blood cells: $5 \times 10^9$
- Platelets: $2 \times 10^8$
- Leucocytes: $8 \times 10^6$
  - Polynuclear cells
  - Lymphocytes
  - Monocytes
- Rare cells < $10^4$
  - Dendritic cells
  - Immature hematopoietic cells
+ Tumor cells?
CTC Enrichment

Alix-Panabières & Pantel
Nat. Rev. Cancer
2014
5. RosetteSep (StemCell Technology)

Rosette with an unwanted cell and erythrocytes formed by antibodies complexes.
**RosetteSep** (StemCell Technology)

Unwanted cells are crosslinked to RBCs

Rossetted (unwanted) cells pellet over Ficoll™
1. Add RosetteSep cocktail to sample
2. Incubate 20 min at R.T.
3. Dilute with buffer
4. Layer over the density medium
5. Spin 20 min, 1200 xg
6. Collect enriched cells in the interface
7. Wash cells with Buffer

CD45- cells

CTC detection
Technical Advance

Isolation by Size of Epithelial Tumor Cells

A New Method for the Immunomorphological and Molecular Characterization of Circulating Tumor Cells

Pores diameter = 8\mu m
Parsortix System

→ Size and deformability of CTCs
CTCs are caught on a step that criss-crosses the microscope slide sized cassette.
« Circulating Tumor Cell » in PubMed
→ 20,330 articles in NOVEMBER 2017
Pre-Analytic Steps
- Collection tubes
- Blood volume
- Storage
- Delivery date
- Blood sample preparation

Analytic performance
- Robustness
- Reproducibility
- Sensitivity
- Specificity

Clinical Relevance!
Detection of CTCs

Alix-Panabières & Pantel, Nat Rev Cancer 2014
CellSearch™ System

- MagNest™
- 7.5 mL of blood
- Cleared by US-FDA!
- Epithelial Cell Kit
- CellSpotter analyzer

10 en France
Enrichissement des CTC avec des Acs anti-EpCAM

- **Tumor Cell**
- **White Blood Cell**
- **Red Blood Cell**
- **Platelet**

<table>
<thead>
<tr>
<th>Method</th>
<th>Tumor Cell</th>
<th>White Blood Cell</th>
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<tr>
<td>anti-EPCAM Ferro Fluids</td>
<td>+</td>
<td>-</td>
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<td>anti-cytokeratins</td>
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<tr>
<td>anti-CD 45</td>
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</table>

**CTC**

**M1 COLON CANCER**

1 CTC 1 Leucocyte

- Large
- Median
- Small
Clusters

$M_1$ COLON CANCER
Biology of CTCs
Alix-Panabières & Pantel, Nat Rev Cancer, 2014
Epithelial-Mesenchymal Plasticity of CTCs

EpCAM, CK

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>
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<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
Epithelial-mesenchymal plasticity in circulating tumor cells

Catherine Alix-Panabières¹² · Sonja Mader³ · Klaus Pantel³

Biology of CTCs and their epithelial-to-mesenchymal plasticity

Technologies for enrichment and detection of CTCs with high epithelial-mesenchymal plasticity

Clinical studies on the relevance of CTCs with high epithelial-mesenchymal plasticity
Epithelial-to-Mesenchymal Plasticity & CTCs

2017
Alix-Panabières, Mader, Pantel
Molecular characterization of CTCs

DNA - RNAs/microRNAs - PROTEINS

- Therapeutic targets
- Resistance mechanisms
Silicon Biosystems Patented Technology: Moving Dielectrophoretic (DEP) Cages

Non-uniform electric field generated by the chip electrodes (cross section)
Cell trapping by DEP cages
cage-move
Detection and recovery of circulating colon cancer cells using a dielectrophoresis-based device: KRAS mutation status in pure CTCs.

Fabbri et al., Cancer Lett 2013
Peeters et al., Br J Cancer, 2013

1. Inject, trap and image all cells
2. Move all cells of interest into Parking chamber
3. Move separately to Recovery chamber and flush

Analysis at the molecular level of pure CTC, avoiding lymphocyte contamination
Individual Cell Sorting with DEPArray™ A300K
Genomic Characterization of single CTCs

CTC detection & isolation

DEPArray System

WGA +
- Mutation analysis
- CGH (conv./array)
- NextGen Sequencing

WTA

Functional TESTS
Tumor cells

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

Her2pos-CTC in patients with Her2neg primary tumor

Riethdorf et al, Clin Cancer Res, 2010
Primary tumor: Overexpression of Her2 - only a very low number of cells are analyzed in routine for the Her2 statut determination.

Her2 Amplification pre-exist in the primary tumor

Riethdorf et al, Clin Cancer Res, 2010
Characterisation of CTC - HER2 STATUT

Micrometastatic cells: Gain of Her2 clone selection Her2^{pos}-CTC

(Meng et al. PNAS, 2004)

Acquisition of Her2 amplification during invasion
Frequent expression of PD-L1 on circulating breast cancer cells

Martine Mazel, William Jacot, Klaus Pantel, Kai Bartkowiak, Delphine Topart,
Laure Cayrefourcq, Delphine Rossille, Thierry Maudelonde, Thierry Fest,
Catherine Alix-Panabières

Molecular Oncology
PD-L1 expressed in tumors functions as a key component of the cancer-immunity cycle by preventing the immune system from destroying cancer cells.

Overexpression of PD-L1 on both tumor cells & tumor-infiltrating immune cells has been observed in multiple cancer types, making PD-L1 a potential target in cancer research.

Immune checkpoint regulators (such as PD-L1) have become exciting new therapeutic targets leading to long lasting remissions in patients with advanced malignancies...
Antibodies targeting the PD-1/PD-L1 checkpoint have shown dynamic and durable tumor regressions suggesting a rebalancing of the host–tumor interaction.
PD-L1 is frequently expressed on CTCs (> 60% of patients) in metastatic breast cancer patients.
Detection of CTCs expressing PD-L1 means that they:

- may have the capacity to escape the immune system,
- are potential targets for anti-PD-L1 therapies.
Detection of CTCs

Alix-Panabières & Pantel, Nat Rev Cancer 2014
Functional Characterization of CTC

Primary tumor → Local relapse

Blood

Distant tissue (e.g. bone marrow)

Recirculation

DTC

Tumor cell dormancy

Metastasis

Micrometastasis

Escape

Viable

Apoptotic

NATURE REVIEWS CLINICAL ONCOLOGY Pantel & Alix-Panabières VOLUME 6 JUNE 2009 339
Detection of Functional CTC/DTC

CELL CULTURE needed!

EPISPOT assay

secreted proteins by VIABLE cells
Expert Review of Molecular Diagnostics
September 2015

Enrichment, detection & characterization of Viable CTC
EPISPOT procedure
Biology comprehension using the EPISPOT assay
Prognostic relevance of viable CTCs
Ongoing clinical trials using the EPISPOT assay

August 2017
Clinical Applications in Cancer Patients
Clinical Applications of Circulating Tumor Cells and Circulating Tumor DNA as Liquid Biopsy

Catherine Alix-Panabieres L3 and Klaus Pantel4

Noninvasive blood sample
CTCs
ctDNA
Real-time liquid biopsy

Screening and early detection of cancer
- EGFR mt on ctDNA (NSCLC)
- CTC counts (NSCLC)

Stratification and therapeutic intervention
- HER2 or ER expression on CTCs (BC)
- CTC counts (BC) - METABREAST trial

Real-time monitoring of therapy
- CTC counts (BC)
- KRAS mt on ctDNA (CRC)
- AR mt on ctDNA (PC)

Therapeutic targets and resistance mechanisms
- KRAS mt (CRC)
- EGFR mt (NSCLC)
- Lack of ER expression (BC)
- AR mt or ARV7 expression (PC)

Risk for metastatic relapse (prognosis)
- CTC counts in solid tumors (e.g., breast, prostate, colorectal, lung, bladder cancers)
- KRAS mt on ctDNA (CRC)

Personalized Treatment

Cancer Discovery 2016
- **Clinical validity**: How well the test relates to the clinical outcome of interest (such as survival or response to therapy).

- **Clinical utility**: Whether the results of the test provide information that contributes to and improves current optimal management of the patient’s disease.
Monitoring of CTCs

Can *early* changes in CTC counts predict the *efficacy* of therapeutic interventions (e.g., chemotherapy, hormonal therapy)?
Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data

17 centres provided data for 1944 eligible patients from 20 studies

Meta-analysis on raw data.
CTCs vs. conventional tumor markers (PFS, p values) in metastatic breast cancer patients receiving chemotherapy

<table>
<thead>
<tr>
<th>Model used as reference</th>
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<td></td>
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<td>CTCBL</td>
<td>CA15-3BL</td>
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<td>CP + CTCBL</td>
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<tr>
<td>CP + CTCBL + CTC3-5</td>
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<td>.13</td>
<td>.08</td>
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<tr>
<td>CP + CTCBL + CTC6-8</td>
<td></td>
<td>.21</td>
<td>.58</td>
<td></td>
</tr>
</tbody>
</table>

Bidard, Pierga, Pantel et al., Lancet Oncology 2014, European Pooled Analysis of CTCs in metastatic BC (n=1944)
Level of evidence 1

Demonstration of clinical validity

CTC count (CellSearch) is a prognostic biomarker before and during therapy in stage IV breast cancer.

CEA and CA15.3 levels at baseline & during therapy did NOT increase the prediction performance of the model

⇒ CTC >> serum markers for prognosis assessment
A randomized study to assess « Circulating Tumor Cells » count at baseline as a tool to choose between HormonoT vs ChemoT in HR+ M+ breast cancer patients.

In PROGRESS

Clinical Utility!
M+ HR+ breast cancer patients before any treatment who can be treated either by Hormone T or chemo T.

Randomization stratified on center & PS and metastasis-free interval

Baseline CTC count blinded

Baseline CTC count disclosed

Standard arm N=497

Clinician choice

favorable

Hormone T.

unfavorable

Chemo T.

CTC arm N=497

CTC-driven decision

<5 CTC/7.5ml

Hormone T.

≥5 CTC/7.5ml

Chemo T.

Tumor evaluation until progression

Endpoints

Medical: PFS (non-inferiority), quality of life, toxicity, OS

Economics: differential costs per life year without disease progression, global costs
RESULTS
Treatment allocation & CTC-induced changes in 530 patients

<table>
<thead>
<tr>
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<th>Arm following physician-driven choice</th>
<th>Arm following CTC-driven choice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A priori choice of physician</strong></td>
<td>HT: N=185, 70%</td>
<td>CT: N=82, 30%</td>
</tr>
<tr>
<td>CTC count</td>
<td>Not disclosed to physicians</td>
<td>&lt;5 CTC: N=122, 68%</td>
</tr>
<tr>
<td>Treatment received</td>
<td>HT: N=186, 70%</td>
<td>CT: N=81, 30%</td>
</tr>
</tbody>
</table>

**CTC count**
- confirmed the *a priori* choice in 62% of pts (157/253)
- induced a change of therapy in 38% of pts (95/253)
Identification of Metastasis-Competent CTCs
In vitro expansion of breast cancer CTCs

- **2013**
  - Identification of a population of blood circulating tumor cells from breast cancer patients that initiates metastasis in a xenograft assay
  - Irène Baccelli, Andreas Schneeweiss, Sabine Riethdorf, Albrecht Stenzinger, Anja Schillert, Vanessa Vogel, Corinna Klein, Massimo Saini, Tobias Bäuerle, Markus Wallwiener, Tim Holland-Letz, Thomas Höfner, Martin Sprick, Martina Scharpf, Frederik Manné, Hans Peter Sinn, Klaus Pantel, Wilko Weichert & Andreas Trumpp
  - *Nature Biotechnology*

- **2014**
  - In vitro expansion of breast cancer CTCs
  - *Cell*

In vitro expansion of lung cancer CTCs

- **April 2013**
  - Ex vivo culture of circulating breast tumor cells for individualized testing of drug susceptibility
  - Min Yu et al.
  - *Science*

- **July 2014**
  - In vitro expansion of prostate cancer CTCs

In vitro expansion of prostate cancer CTCs

- **September 2014**
  - Organoid Cultures Derived from Patients with Advanced Prostate Cancer
  - Dong Guo, Dan Qian, Philip J. Iaconetta, Wenjing Yang, Xiaoyong Li, Xiaoyang Li, Robert J. Metcalfe, Dominik G. Rothwell, Francesca Tranquilli, Radhika Polasa, Deborah J. Bent, Kathy J. Simpson, Karen Morris, Stuart D. Pegg, Elisa N. Nova, Alan Grayson, David J. Bird, Paul Kelly, Becky Bola, Matthew G. Krebs, Jenny Auton, Maddox Ayers, Suzanne Faustner, Ianrey Priest, Louise Carter, Catherine Tate, Crispin Miller, Fiona Blackhall, Paul Brady & Caroline Dyer

- **December 2014**
  - Expansion of CTCs from early stage lung cancer patients using a microfluidic co-culture model

CTC Expansion - History
Ex vivo expansion of colon cancer CTCs
Cell line from human circulating colon cancer cells - Establishment

Laure Cayrefourcq

N=165

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<th>CTCs</th>
<th>Patients</th>
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<tr>
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<tr>
<td>&gt;100</td>
<td>3 (4.2%)</td>
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<tr>
<td><strong>Total</strong></td>
<td><strong>71</strong></td>
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CTC detection

CTC culture

1 permanent cell line ‘CTC-MCC-41’

Cayrefourcq et al., Cancer Research 2015
<table>
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<tr>
<th>Événement</th>
<th>Cadre</th>
<th>DAPI/CK-PE</th>
<th>CK-PE</th>
<th>DAPI</th>
<th>CD45-APC</th>
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1 Leucocyte 1 CTC

M<sub>1</sub> COLON CANCER
Clusters

M₁ COLON CANCER
Capture of Viable Circulating Tumor Cells in the Liver of Colorectal Cancer Patients

Eric Denève,¹ Sabine Riethdorf,² Jeanne Ramos,³ David Nocca,¹ Amandine Coffy,⁴ Jean-Pierre Daurès,⁴ Thierry Maudelonde,⁵ Jean-Michel Fabre,¹ Klaus Pantel,² and Catherine Alix-Panabières⁴,⁵,⁶*

N=75
- 60 M₀
- 15 M₁
CTCs Patients

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70.4% of N=165 patients had circulating cancer cells (CTCs).

1 permanent cell line CTC-MCC-41
CTC-MCC-41 Cell line from human circulating colon cancer cells - Establishment

Cayrefourcq et al., Cancer Research 2015
Cell line from human circulating colon cancer cells - Genomic Characterization
Cell line from human circulating colon cancer cells - Transcriptomic Characterization

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<th>ALDH-1</th>
<th>CD133</th>
<th>VEGF</th>
<th>cMel</th>
<th>OPG</th>
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- **Epithelial**
- **Mesenchymal**
- **Stem cell**
- **Oncogene**

**Osteomimetism**

**Angiogenesis**

*Marianna Alunni, Karin Görner*
Cell line from human circulating colon cancer cells - Proteomic Characterization

<table>
<thead>
<tr>
<th>Markers</th>
<th>Cytometry</th>
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Cayrefourcq et al., Cancer Research 2015
### Cell line from human circulating colon cancer cells - Functional Characterization

#### EPISPOT assays

<table>
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<tr>
<th>Markers</th>
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<th>CTC-MCC line</th>
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**Epithelial Protein**

**Stem Cell Growth Factor**

**Angiogenesis Factor**

**Osteomimetism Signature**
Cell line from human circulating colon cancer cells - Functional Characterization

After 6hrs

A

Basal medium

Complete medium

CTC line supernatant

Cayrefourcq et al., Cancer Research 2015
Cell line from human circulating colon cancer cells - Comparison Primary tumor/Metastasis/Xenografts in immunodeficient mice

**Colon cancer patient**

<table>
<thead>
<tr>
<th>Primary Tumor</th>
<th>Lymph node metastasis</th>
<th>SCID mice</th>
<th>Xenografts</th>
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<tbody>
<tr>
<td><em>(A)</em></td>
<td><em>(C)</em></td>
<td><em>(E)</em></td>
<td><em>(F)</em></td>
</tr>
</tbody>
</table>

**hu CK20**

**KRAS**

- WT
- V600E

**BRAF**

- WT
- V600E

Cayrefourcq *et al.*, Cancer Research 2015
- Stable for >4 years
- Shares important features of the tumor cells in the patient with colon cancer (primary tumor/metastasis: KRAS wt; BRAF mutated)
- Showed an intermediate epithelial/mesenchymal phenotype (EM Plasticity) with stem cell-like characteristics (e.g., growth as microspheres and expression of cancer stem cell markers)
- Induced \textit{in vitro} angiogenesis
- Induced tumors in immunodeficient mice.

We have established a cell line with important properties relevant for the development and progression of metastatic disease.
The establishment of this first colon cancer CTC line allows now:

- a wealth for functional studies on the biology of special CTCs
- *in vitro* and *in vivo* drug testing.
Molecular Portrait of Metastasis-Competent Circulating Tumor Cells in Colon Cancer Reveals the Crucial Role of Genes Regulating Energy Metabolism and DNA Repair

Catherine Alix-Panabières,1,2* Laure Cayrefourcq,1,2 Thibault Mazar,3,4 Thierry Maudelonde,5,2 Eric Assenat,6 and Said Assou7,8
Colorectal adenocarcinoma

Non-invasive peripheral blood sample → CTCs (Circulating Tumor Cells) → CTC-MCC-41

B

Primary tumor cell → HT-29
Samples can be divided in two distinct groups based on their gene expression profiles.
CTC-MCC-41 line displays a very specific transcription program.

A hierarchical clustering analysis mapped the samples in two main major clusters.
Venn diagram representing the number of genes in each comparison & overlaps between the three main comparison groups.
Comprehensive outlook on the molecular events involved in colon cancer progression and provides potential CTC biomarkers that may help developing new therapies to specifically target CTCs with stem cell properties that cause metastases and tumor relapse in patients with colon cancer.
Metastatic Colon Cancer patient


S. Assou

Next steps...
Other Circulating biomarkers

- ctDNA
- cfmiRNA
- exosomes
Clinical prospects of liquid biopsies

Nature Biomedical Engineering

Catherine Alix-Panabières and Klaus Pantel

Primary tumour or metastatic lesion

Apopotic or necrotic cells

ctDNA

Exosomes

Leukocytes

Erythrocytes

Blood sample

Bloodstream

CTC

ctDNA

Exosomes

Liquid biopsy

Personalized treatment

CTCs

tDNA

Exosomes?
32 partners:
- 6 EFPIA companies (lead Bayer, co-lead Menarini)
- 17 academic/clinical sites
- 6 SMEs
- 2 non-profit organizations
- 1 non-SME/non-EFPIA
Thank you for your attention!