R.G.C.C. International GmbH

PERSONALIZE MEDICINE IN ONCOLOGY
SIMILARITIES AND DIFFERENCES BETWEEN CTC AND CANCER CELLS:
MECHANISMS AND PATHWAYS

AUTHOR-PRESENTER: DR. IOANNIS PAPASOTIRIOU
SCIENTIFIC BACKGROUND

(HOW CANCER IS PROGRESSING
AND
WHICH IS THE RESPONSIBLE ENTITY WHICH CONNECTS THE DOTS)
Cancer Hallmarks

- Self-sufficiency in growth signals
- Evading apoptosis
- Insensitivity to anti-growth signals
- Sustained angiogenesis
- Tissue invasion & metastasis
- Limitless replicative potential

CARCINOGENESIS STEPS

INITIATION
- Viral interference
- Chemical interference
- Radiation influence

PROMOTION
- Cell cycle instability
- DNA repair aberration
- Apoptosis instability

PROGRESS
- Invasion
- Neo-angiogenesis
- EMT and MET

VOGELSTEIN MODEL OF DEVELOPING COLON CANCER

normal epithelium \[\xrightarrow{\text{loss of } APC}\] hyperplastic epithelium \[\xrightarrow{\text{DNA hypomethylation}}\] early \[\rightarrow\] intermediate \[\rightarrow\] late adenomas \[\rightarrow\] carcinoma \[\rightarrow\] invasion & metastasis

- loss of $K-ras$
- loss of 18q TSG
- loss of $p53$
Tumor heterogeneity and plasticity: a critical problem
Present therapeutic concept
Cancer therapy plan (so far)

Cancer patient

staging

Curable?

Palliation-terminal care

no

Down staging?

Resectable?

radiotherapy

Adjuvant chemo

Radiotherapy

Radiotherapy+Ctx

dermotherapy

chemotherapy

De Vita et al 2012
Cancer therapy plan (so far)

- After therapy
  - MRD?
    - Second line of therapy
    - High risk of relapse?
      - Watch and wait or Follow up
      - Intensive follow-up
Rate of success

• For Adjuvant chemotherapy the success rate for the 5 major types of malignancy varies from 2.1% to 2.3% in 5 years.
  Royal North Shore Hospital Clin Oncol (R Coll Radiol) 2005 Jun;17(4):294

• For curative stage of disease the success rate varies between 5 to 7.5% for the same 5 types of malignancies.
Dead-End in empirical treatment

Cancer patient

staging

Curable?

Is actual the disease local?

Is the disease extended as micro-colonies

Will the patient respond to radiotherapy or chemotherapy?

Are there any CSCs?

Palliation-terminal care

no

Down staging?

Resectable?

Radiotherapy

Radiotherapy+Ctx

Adjuvant chemo

chemotherapy

Radiotherapy

Is the disease in exponential phase of growth
Dead-End in empirical treatment

Is there a recommended 2\textsuperscript{nd} line of therapy?

After therapy

MRD?

High risk of relapse?

Watch and wait or Follow up

Second line of therapy

Intensive follow-up

Is there a more sensitive method to detect the risk or disseminate disease?
Reasons and causes

1. Lack of sensitive methods to detect the MRD
2. Lack to discriminate the actual important cell from the irrelevant
3. Lack to detect and control the genetic instability of malignant cells.
4. Lack to distinguish which cells may shift to the driving entity and which may not.
Examples

Non small cell lung carcinoma

Squamous cell type
- PLATINUM DERIVATIVES + GEMCITABINE (GEMZAR)

Adenocarcinoma
- PLATINUM DERIVATIVES + VINCA ALCALOID
Examples

Non small cell lung carcinoma

ERCC1+

PLATINUM DERIVATIVES
NON SENSITIVE

ERCC1-

PLATINUM DERIVATIVES
SENSITIVE
Examples

Non small cell lung carcinoma

RRM1+

GEMCITABINE
NON SENSITIVE

RRM1-

GEMCITABINE SENSITIVE
Examples

Lung carcinoma

Adenocarcinoma

ERCC1+
- VINORELBINE + GEMCITABINE

ERCC1-
- CDDP + VINORELBINE

ERCC1+

ERCC1-

SCC

ERCC1+

ERCC1-

RRM1+  RRM1-  RRM1-  RRM1+

RRM1+
- VINORELBINE + TAXOTERE

RRM1-
- GEMCITABINE + TAXOTERE

RRM1-
- CDDP + GEMCITABINE

RRM1+
- CDDP + TAXOTERE
Response rates

- Empirical
- Personalized

- Palliation
- Respond 3
- Respond 2
- Respond
Analyzing the proper sample

- Facts that are well established and proved:
  1. A tumor consist from the cancer cells and the stroma
  2. The stroma cells composed from fibroblast, lymphocytes, endothelial cells etc
  3. The cancer population is heterogeneous and composed from subpopulations with different features and aggressive behavior.
  4. One of the subpopulation is the progenitor of a tumor and the generator of metastases. This population is known as Cancer Stem Cell like cells
  5. This subpopulation has the ability to invade the surrounding organs, enter the circulation (blood vessel or lymphatics) and engraft to distant organs in order to generate metastases and relapses.
Tumor physiology

Physical translocation from primary tumor to distant organ

A. Acquisition of invasive phenotype

B. Local invasion cells invade into surrounding stroma, then intravasate to enter hematogenous circulation

Colonization

C. CTCs transit to distant organ

D. CTCs extravasate and invade into the parenchyma of foreign tissue

E. Survival at secondary site

F. Adaptation and proliferation to form metastases

Legend:
- Differentiated cancer cell
- Transitioning cancer cell
- Cancer stem cell
- Stromal cell
- Inflammatory cell
Tumor physiology
(heterogeneity-pleomorphy)
Tumor physiology
(heterogeneity-pleomorphism)
Comparison between primary and metastases

• GENOMIC LEVEL

The gene mutations signature is similar between primary and metastatic tumors.
Comparison between primary and metastases

- **EPGENETIC AND BIOMARKERS LEVEL**

The gene expression profile alters between primary and metastatic tumors.

The median age at the time of the diagnosis of brain metastasis was 53 years old (range, 30 to 76 years). The median time to brain metastasis from the time of breast cancer diagnosis was 2.9 years (range, 0 to 23.1 years). Seven of the patients had received no systemic therapy prior to brain tumor resection. Among 22 patients who had a prior history of receiving systemic therapy, eight had received taxotere-containing chemotherapy.

The proportion of ER-, PgR-, HER2/neu-positive tumors in 24 primary lesions were 12.5%, 8.3%, 37.5%, respectively. The proportion of ER-, PgR-, HER2/neu-, and CK5/6-positive tumors among the brain metastases were 13.8%, 6.9%, 37.0%, and 24.1%, respectively. The immunohistochemical profiles including ER, PgR, and HER2/neu of the primary tumor and the brain metastasis differed in 7 patients (29.2%, N=7/24) [see Figure 2]. Among eight patients who had been previously treated with trastuzumab, two had HER2/neu negative brain metastases.

**Future Tasks**

The results of the above-described study suggest that distant metastases are not necessarily biologically similar to the primary tumors. The difference of the biomarker expression between the primary tumors and brain metastases may be due to modification by systemic treatments or change along with disease progression.

Re-assessment of the immunohistochemical status of the brain metastasis, if possible, may be useful to optimize treatment in the future. Although biomarker studies of brain metastases are very difficult to carry out because only a limited number of patients undergo surgery for brain metastases, in order to develop biologically rational treatments, further studies to elucidate the mechanism and biology of brain metastases are warranted.

**References**


58 | Connection 2008
Comparison between primary and metastases

Epigenetic and Biomarkers Level

The gene expression profile alters between primary and metastatic tumors.

Abstract

OBJECTIVES: The expression of predictive markers including the estrogen (ER) and progesterone receptor (PR) expression can change during the course of the disease. Therefore, reassessment of these markers at the time of disease progression might help to optimize treatment decisions. Metastatic tissue may be difficult to obtain for repeated analysis. In this context, characterization of circulating tumor cells (CTCs) could be of relevance. It was the purpose of the present study (1) to reevaluate the ER/PR expression by CTCs and (2) to compare the hormone receptor status expression profile of CTCs with the primary tumor.

METHODS: We evaluated 193 blood samples from metastatic breast cancer patients at the time of first diagnosis of metastatic disease or disease progression. All samples underwent immunomagnetic enrichment using the AdnaTest BreastCancerSelect (AdnaGen AG, Germany) within 4h after blood withdrawal followed by RNA isolation and subsequent gene expression analysis by reverse transcription and Multiplex-PCR in separated tumor cells using the AdnaTest BreastCancerDetect. CTCs were analyzed for the three breast cancer-associated markers: EpCAM, Muc-1, Her-2 and actin as an internal PCR control. Expression of the ER and PR was assessed in an additional RT-PCR. The analysis of PCR products was performed by capillary electrophoresis on the Agilent Bioanalyzer 2100.

RESULTS: The overall detection rate for CTCs was 45% (87/193 patients) with the expression rates of 71% for EpCAM (62/87 patients), 73% for MUC1 (64/87 patients), 48% for HER2 (42/87 patients), 19% for ER (17/87 patients) and 10% for PR (9/87 patients), respectively. Comparisons with the primary tumor were only performed in CTC+ patients (n=87). In 48/62 (77%) patients with ER+ tumors, CTCs were ER- and 46/53 (87%) patients with PR+ tumors did not express PR on CTCs. Primary tumors and CTCs displayed a concordant ER and PR status in only 41% (p=0.260) and 45% (p=0.274) of cases, respectively.

CONCLUSION: Most of the CTCs were ER/PR-negative despite the presence of an ER/PR-positive primary tumor. The predictive value of hormone receptor status expression profile of CTCs for palliative endocrine therapy has to be prospectively evaluated. STATEMENT: We recently demonstrated in more than 400 primary breast cancer patients that the expression profile between CTCs and the primary tumor with regard to ER/PR/HER2 positivity differs. The concordance rate between ER, PR and HER2 status of CTCs and the primary tumor was 29%, 25% and 53%, respectively (Fehm T et al., Breast Cancer Res Aug 10 2009, 11(4) pR59). Based on these results we studied blood samples of 193 metastatic breast cancer patients participating in the German DETECT study (1) to reevaluate the ER/PR expression by CTCs and (2) to compare the hormone receptor status expression profile of CTCs with the primary. As already shown for primary breast cancer, most of the CTCs were ER/PR-negative despite the presence of an ER/PR-positive primary tumor. In the metastatic setting the phenotype of CTC reflects the phenotype of metastatic disease. Therefore palliative treatment selected based on the expression profile may not be effective since the phenotype has changed during disease progression. To our knowledge, this study is one of the biggest to compare hormonal receptor expression on CTC and the primary tumor. We hope that our manuscript is suitable for publication in Gynecologic Oncology.
Possible scenario

Patient A presents with localized breast cancer and has surgical resection of the primary tumor. Pathological examination shows this tumor to be HER2⁻.

2 years later, Patient A shows signs of metastatic disease disseminated to the lung and liver.

Potential Outcomes without CTC Analysis

- Based on the pathological characteristics of the primary tumor (HER2⁻), Patient A is treated with chemotherapy, but not with Herceptin.
- Patient A fails chemotherapy and is now considered end-stage/palliative.

Potential Outcomes with CTC Analysis and Characterization

- Blood is collected from Patient A and CTCs are assessed and found to overexpress HER2. Patient A is treated with Herceptin.
- Patient A demonstrates treatment response and is now considered to have stable disease.
Do we use a right non invasive diagnostic for prevention?

**Methods**
- X ray
- MRI
- PET/CT or PET/MRI
- CT
- U/S (Echo)

**Limitations**

Minimal invasive method: BIOPSY
Are we focused to a wrong type of cancer cells?

• The tumor consist from in-homogenous population of cancer cells
• Few sub-clones are able to metastasize and generate metastases
• The CTCs are cancer cells that have perform in majority the EMT
• CTCs are still in-homogenous but with bigger proportion of cancer cells with metastatic features
• CSCs are a subset of CTCs that may generate relapses
The reason of heterogeneity and plasticity of the disease lead us to the personalized approach as therapeutic concept
ANALYSIS ON SEVERAL LEVELS

- GENOMIC
- EPIGENETIC
- PROTEOMIC

Multiple platforms need to be applied in order to stratify patients and develop therapeutic options under more personalized perspective.
ANALYSIS ON SEVERAL LEVELS

- GENOMIC
- EPIGENETIC
- PROTEOMIC

Multiple platform need to be applied in order to stratify patients and develop therapeutic options under more personalized perspective.
ANALYSIS ON SEVERAL LEVELS

- GENOMIC
- EPIGENETIC
- PROTEOMIC

MULTIPLE PLATFORM NEED TO BE APPLIED IN ORDER TO STRATIFY PATIENTS AND DEVELOP THERAPEUTIC OPTIONS UNDER MORE PERSONALIZED PERSPECTIVE
Empirical vs Personalized treatment
Pros & Cons

How personalized treatment rise the last years.

1. Need of pharma industry of select the patients where their product will be successful.
2. Need to medical practitioners to identify candidates that will develop severe side effects from a medication.

How Personalize treatment is feasible:

1. Identify each case metabolic abilities (normal, accumulator, rapid metabolizer)-Pharmacokinetic
2. Identify for each case the cellular genetic and protein profile of their abnormal cells that causes the disease.
## Empirical vs Personalized treatment

### Pros & Cons

<table>
<thead>
<tr>
<th></th>
<th>Empirical lines of therapy</th>
<th>Personalized plan of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Advantages</strong></td>
<td>• Low cost (in short term) • Fast applicable • Applicable to masses • No need of higher training of physician in PK and PD</td>
<td>• High rate of success • Avoid of side effect &amp; toxicities • Long term cost effective • Shortening of hospital admission and residence of a patient</td>
</tr>
<tr>
<td><strong>Disadvantages</strong></td>
<td>• High rate of failure • No participation of each case individuality • After last recommended line, no other option</td>
<td>• Higher cost than empirical • Need previously a series of analysis to be performed</td>
</tr>
</tbody>
</table>
CTCs & CSCs
PRACTICAL ISSUES WHAT PROHIBIT THE “JUMP” FROM BENCH TO BED -TRANSLATIONAL MEDICINE-

(CLINICAL REALITY)
How can be found a needle in a hay-stack?

Average No of CTCs in blood sample is 10-30cell/50,000 events (RBC and platelets have been subtracted)
What we need to preserve during detection and isolation of CTCs

1. High purity of CTCs
2. Viable CTCs
3. Isolate all subsets of CTCs
4. Detect the disease relevant CTCs
5. Detect CTCs subset with stemness properties
6. Pin point the subclasses of CTCs with plasticity properties (EMT-MET)
CHOOSING THE RIGHT METHOD

POSITIVE SELECTION
CHOOSING THE RIGHT METHOD

BEAD BASED METHOD

Undesired cells + Desired cells

The CellSearch system technology

CTC identification:
- CK +
- EpCAM +
- DAPI +
- CD45 -

CTC
Leukocytes

Magnet cartridge

1. Add EasySep™ selection cocktail to single cell suspension
2. Labeled cell suspension
   Incubate
3. Add EasySep™ magnetic particles
   Incubate
4. Place tube in magnet:
   **Negative Selection:** desired cells are decanted into new tube.
   **Positive Selection:** desired cells remain in tube.
   Pour off and collect cells accordingly.
CHOOSING THE RIGHT METHOD

MICROSCOPY BASED METHOD

- FIXATION OF THE SAMPLE
- POSITIVE SELECTION METHOD
- DAMAGE OF SAMPLE DURING PROCESS
CHOOSING THE RIGHT METHOD

GRADIENT BASED METHOD

A. Density Gradient Separation
   - Blood Or BM
   - Ficoll-Hypaque Gradient
   - Plasma
   - Enriched MNC fraction
   - Erythrocytes
   - Granulocytes

B. Immunomagnetic Separation
   - Molecules bind to specific antigens
   - Separation by magnetic field

C. Separation by Size
   - Cells are separated based on size

CHOOSING THE RIGHT METHOD

PCR BASED METHODS

BreastSelect beads → Incubation → Separation → Cell lysis

Separation of magnetic beads → RT-PCR

Blood cells
Tumor cells
Antibody- or oligo (dT)$_{25}$ coated magnetic beads
More things to consider

• qPCR (all genome)

  mRNA extraction
  \[\downarrow\]
  Convert to cDNA
  \[\downarrow\]
  Preamplification of cDNA with random primers
  \[\downarrow\]
  Final amplification of cDNA
More things to consider

- qPCR or real time PCR (all genome expression amplification)
More things to consider

- qPCR or real time PCR (all genome expression amplification)
More things to consider

- qPCR or real time PCR (all genome expression amplification)
More things to consider

- qPCR or real time PCR (all genome expression amplification)
More things to consider

- qPCR or real time PCR (all genome expression amplification)
More things to consider

• We may consider to epithelial carcinomas that a common marker will allow us to detect CTCs (like CKs or EpCam). Is this correct?
More things to consider

- We may consider to epithelial carcinomas that a common marker will allow us to detect CTCs (like CKs or EpCam). Is this correct?
More things to consider

- We may consider to epithelial carcinomas that a common marker will allow us to detect CTCs (like CKs or EpCam). Is this correct?
FLOW CYTOMETRY and CTCs

- Using parameters like FS, SS and fluorescence we can detect multiple antigens inside each cell.
- There are two approaches to detect CTCs: positive selection and negative selection.
- FC can provide information about quantity and quality of CTCs.
## CHOOSING THE RIGHT METHOD

### COMPARATIVE METHODS

<table>
<thead>
<tr>
<th>Method of Isolation</th>
<th>Beads Based Method</th>
<th>PCR Based Method</th>
<th>R.G.C.C. Ltd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Magnetic Beads (antibodies with iron particles)</td>
<td>PCR based method which need to destroy the cells in order to identify one marker (mainly panCK or EpCAM)</td>
<td>Flow cytometric sorting with interrogation in droplets in ratio of droplet per cell (1:1)</td>
</tr>
<tr>
<td>Purity of CTCs</td>
<td>Enrichment method and not isolation method</td>
<td>There are no cells any more</td>
<td>Purity is higher than 97 to 99% (isolation method)</td>
</tr>
<tr>
<td>Viability of the isolated cells</td>
<td>70-85%</td>
<td>No cells</td>
<td>Viability &gt;99%</td>
</tr>
<tr>
<td>Quality of CTCs for further analysis</td>
<td>Inappropriate for further molecular analysis due to lymphocyte contamination</td>
<td>Limited for further molecular analysis</td>
<td>Appropriate for further molecular analysis since there is no noise</td>
</tr>
<tr>
<td>Selection of CTCs</td>
<td>Based mainly in positive selection of CTCs in a few number of markers</td>
<td>Based on positive selection</td>
<td>Based on negative and positive selection in order to identify and secondly immunophenotyping CTCs</td>
</tr>
<tr>
<td>Further abilities</td>
<td>Identification of heterogeneity of CTCs</td>
<td>Method to enumerate CTCs and identify only very limited features of CTCs</td>
<td>Method which allows to perform gene expression assays and determine features vital for therapy scheduling</td>
</tr>
<tr>
<td>Additional features</td>
<td>Method only to enumerate CTCs</td>
<td>Method to enumerate CTCs and identify only very limited features of CTCs</td>
<td>Method which allows to perform gene expression assays and determine features vital for therapy scheduling</td>
</tr>
</tbody>
</table>
Heterogeneity of CTCs (EMT-MET)

Primary Tumor

Blood Vessel

Basement Membrane

Endothelial Cells

Intravasation into systemic circulation (blood or lymphatic vessels)

Extravasation at site of future metastases

Metastatic Tumor
Heterogeneity of CTCs (EMT-MET)
Heterogeneity of CTCs (EMT-MET)
CTCs & CANCER STEM CELL LIKE OR TUMOR INITIATING CELLS
SELF – RENEWAL (CCSs)

http://njms.umdnj.edu/gsbs/stemcell/scofthemonth/scofthemonth2/braincancerstemcellsci.htm
SELF – RENEWAL (CCSs)

http://njms.umdnj.edu/gsbs/stemcell/scofthemonth/scofthemonth2/braincancerstemcellsci.htm
Phosphorusindac (OXT-328) selectively targets breast cancer stem cells in vitro and in human breast cancer xenografts

Caihua Zhu, Ka-Wing Cheng, Nongtai Ouyang, Liqun Huang, Yu Sun, Panayiotis P. Constantinides, and Basil Rigas

1Division of Cancer Prevention, Department of Medicine, Stony Brook University, Stony Brook, New York
2Medicon Pharmaceuticals, Inc., Stony Brook, New York

Abstract
Pharmacological targeting of breast cancer stem cells (CSCs) is highly promising for the treatment of breast cancer, as the small population of CSCs appears responsible for tumor initiation and progression and also for resistance to conventional treatment. Here we report that the novel phosphorusindac (OXT-328, PS) selectively and effectively eliminates breast CSCs both in vitro and in vivo. PS reduced cell proliferation and induced apoptosis in various breast CSCs. Breast CSCs are resistant to conventional cancer drugs but are sensitive to PS. Long-term treatment with PS of mixtures of cultured breast CSCs with breast cancer cells preferentially eliminated the CSCs. PS impaired the ability of CSCs to form mammospheres and markedly suppressed the expression of CSC-related genes. More importantly, PS presented by half (p<0.05) the formation of tumors initiated by CSCs in immunodeficient mice, and inhibited by 83% (p<0.05) the growth of already formed breast cancer xenografts, reducing the proportion of CSCs in them. PS suppressed the Wnt/β-catenin pathway by stimulating the degradation of β-catenin and its relocalization to the cell membrane, and also blocked the epithelial-mesenchymal transition (EMT) and the generation of breast CSCs. These results indicate that PS has a strong inhibitory effect against breast cancer, acting at least in part, by targeting CSCs through a signaling mechanism involving Wnt signaling.

Keywords
CSCs; phosphorusindac; Wnt/β-catenin; EMT; breast cancer
RELEVANCE BETWEEN TUMOR INITIATING CELLS AND TUMORIGENICITY


Silencing hypoxia inducible factor-2α gene by small interference RNA inhibits the growth of mammosphere cells in nude mice under hypoxic microenvironment.

[Article in Chinese]
Qu HB¹, Fan YM, Han ML, Zeng N, Zhu ZK, Liu H, Xie J, Wu CY, Tang WX.

Abstract
OBJECTIVE: To explore the effects of silencing hypoxia inducible factor-2α (HIF-2α) by small interference RNA on the growth of mammosphere cells in nude mice under hypoxic microenvironment.

METHODS: The empty and interference vectors were transfected into MCF-7 cell. Then G418 was added to screen the positive cells to obtain stable cell line. The empty and interference vectors were inoculated subcutaneously into left and right back near hind limb of nude mice. The volume and weight of tumors were calculated respectively. The expressions of HIF-2α, CD44, OCT-4 and KLF-4 protein in xenograft tumor tissues were detected by Western blot.

RESULTS: The expression vector of HIF-2α-αiRNA was successfully established. The formation of mammosphere was lowered by silencing HIF-2α gene expression. In contrast to empty vector group cell, there were obvious decreases in the volumes and weights of tumors in interference group (P < 0.05). The expression of HIF-2α protein of interference group (0.42 ± 0.01) was much lower than that of the empty vector group (0.89 ± 0.03, P < 0.05). The expression of CD44 protein of interference group (0.21 ± 0.01) was much lower than the empty vector group (0.73 ± 0.03, P < 0.05), the expression of OCT-4 protein of interference group (0.42 ± 0.01) was much lower than the empty vector group (0.88 ± 0.03, P < 0.05) and the expression of KLF-4 protein of interference group (0.34 ± 0.01) was much lower than the empty vector group (0.72 ± 0.03, P < 0.05).

CONCLUSION: Silencing HIF-2α gene can effectively inhibit the growth of breast cancer stem cells in nude mice under hypoxic microenvironment. Its mechanism may be through inhibited capacity for self-renewal and proliferation of breast cancer stem cells in vivo through the down-regulated expressions of markers associated with stem cells. HIF-2α is expected to become a new target for gene therapy of breast cancer.
A mammosphere formation RNAi screen reveals that ATG4A promotes a breast cancer stem-like phenotype.

Wolf J, Dewi DL, Fredebohm J, Müller-Decker K, Flechtenmacher C, Hoheisel JD, Boettcher M.

Abstract

INTRODUCTION: Breast cancer stem cells are suspected to be responsible for tumour recurrence, metastasis formation as well as chemoresistance. Consequently, great efforts have been made to understand the molecular mechanisms underlying cancer stem cell maintenance. In order to study these rare cells in-vitro, they are typically enriched via mammosphere culture. Here we developed a mammosphere-based negative selection shRNAi screening system suitable to analyse the involvement of thousands of genes in the survival of cells with cancer stem cell properties.

METHODS: We describe a sub-population expressing the stem-like marker CD44(+)CD24(-/low) in SUM149 that were enriched in mammospheres. To identify genes functionally involved in the maintenance of the sub-population with cancer stem cell properties, we targeted over 5000 genes by RNAi and tested their ability to grow as mammospheres. The identified candidate ATG4A was validated in mammosphere and soft agar colony formation assays. Further, we evaluated the influence of ATG4A expression on the sub-population expressing the stem-like marker CD44(+)CD24(low). Next, the tumorigenic potential of SUM149 after up- or down-regulation of ATG4A was examined by xenograft experiments.

RESULTS: Using this method, Jak-STAT as well as cytokine signalling were identified to be involved in mammosphere formation. Furthermore, the autophagy regulator ATG4A was found to be essential for the maintenance of a sub-population with cancer stem cell properties and to regulate breast cancer cell tumourigenicity in vivo.

CONCLUSION: In summary, we present a high-throughput screening system to identify genes involved in cancer stem cell maintenance and demonstrate its utility by means of ATG4A.
Can these cells be expanded?
In vitro culture and characterization of human lung cancer circulating tumor cells isolated by size exclusion from an orthotopic nude-mouse model expressing fluorescent protein.

Košťová K1, Zhang Y, Hoffman RM, Bobek V.

Abstract
In the present study, we demonstrate an animal model and recently introduced size-based exclusion method for circulating tumor cells (CTCs) isolation. The methodology enables subsequent in vitro CTC-culture and characterization. Human lung cancer cell line H460, expressing red fluorescent protein (1480-RFP), was orthotopically implanted in nude mice. CTCs were isolated by a size-based filtration method and successfully cultured in vitro on the separating membrane (MetaCell®), analyzed by means of time-lapse imaging. The cultured CTCs were heterogeneous in size and morphology even though they originated from a single tumor. The outer CTC-membranes were blebbing in general. Abnormal mitosis resulting in three daughter cells was frequently observed. The expression of RFP ensured that the CTCs originated from lung tumor. These readily isolatable, identifiable and cultivable CTCs can be used to characterize individual patient cancers and for screening of more effective treatment.

PMID: 25141982 [PubMed - in process]
Cancer therapy. Ex vivo culture of circulating breast tumor cells for individualized testing of drug susceptibility.


Author information

Abstract

Circulating tumor cells (CTCs) are present at low concentrations in the peripheral blood of patients with solid tumors. It has been proposed that the isolation, ex vivo culture, and characterization of CTCs may provide an opportunity to noninvasively monitor the changing patterns of drug susceptibility in individual patients as their tumors acquire new mutations. In a proof-of-concept study, we established CTC cultures from six patients with estrogen receptor-positive breast cancer. Three of five CTC lines tested were tumorigenic in mice. Genome sequencing of the CTC lines revealed preexisting mutations in the PIK3CA gene and newly acquired mutations in the estrogen receptor gene (ESR1), PIK3CA gene, and fibroblast growth factor receptor gene (FOGF1), among others. Drug sensitivity testing of CTC lines with multiple mutations revealed potential new therapeutic targets. With optimization of CTC culture conditions, this strategy may help identify the best therapies for individual cancer patients over the course of their disease.

Copyright © 2014, American Association for the Advancement of Science.

PMID: 25013076 [PubMed - indexed for MEDLINE]
CTCs expansion

• Proper culture media
• Treat the cells as Cell with stemness properties
• Require of the proper environmental conditions
• Require proper supplements (StemPro base media etc)
Cultivation of CSCs

• Isolation from blood or tissue samples
• Flasks which are treated with specialized extra cellular matrix which provides the foundation for three-dimensional (3D) culture studies
• Appropriate growth medium containing growth factors and ingredients
Since cancer cells are genetically unstable how is it possible to expand them without deviating severely from clinical reality?

**Short Tandem Repeats (STRs)**

Short Tandem Repeats are short sequences of DNA (2-16 base pairs), that are repeated numerous times. The repeated sequences are directly adjacent to each other and typically are in the non-coding “intron” region. The polymorphisms in STRs are due to the different number of copies of the repeat element that can occur in a population of individuals. By identifying the repeats of a specific location in the genome, can be created a genetic profile of an individual STRs loci amplified with polymerase chain reaction (PCR), without the problem of differential amplification.
TECHNICAL ISSUE 2

• How stable the sample is during transportation under the parameter of time?

Short Tandem Repeats (STRs)

Samples have been tested on epigenetics as well as according to immunophenotype using the following techniques:
1. Real time PCR

![Transportation Stability Test Sample 1](chart.png)
TECHNICAL ISSUE 2

• How stable the sample is during transportation under the parameter of time?

**Short Tandem Repeats (STRs)**

Samples have been tested on epigenetic as well as according to immunophenotype using the following techniques:

1. Real time PCR

![Transportation Stability Test Sample 2](chart.png)
TECHNICAL ISSUE 2

• How stable the sample is during transportation under the parameter of time?

Short Tandem Repeats (STRs)

Samples have been tested on epigenetic as well as according to immunophenotype using the following techniques:

1. Flow cytometry
Sample 1 Day 0 (sample collection)
Gate 1: lymphocytes
Gate 2: CD31 negative cells
Gate 3: CK positive cells
Gate 4: cMet positive cells
Sample 1 Day 1

<table>
<thead>
<tr>
<th>Overlay #</th>
<th>FCS Filename</th>
<th>Gate</th>
<th># of Events</th>
<th>X Geometric Mean</th>
<th>Y Geometric Mean</th>
<th>% of Gated Cells</th>
<th>% of All Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Z0031654.LMD</td>
<td>None</td>
<td>50889</td>
<td>42.85</td>
<td>0.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>1</td>
<td>Z0031654.LMD</td>
<td>Gate 1</td>
<td>12953</td>
<td>213.69</td>
<td>36.56</td>
<td>25.45</td>
<td>25.45</td>
</tr>
<tr>
<td>1</td>
<td>Z0031654.LMD</td>
<td>Gate 2</td>
<td>5627</td>
<td>213.34</td>
<td>34.34</td>
<td>11.06</td>
<td>11.06</td>
</tr>
<tr>
<td>1</td>
<td>Z0031654.LMD</td>
<td>Gate 3</td>
<td>1</td>
<td>248.05</td>
<td>37.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>1</td>
<td>Z0031654.LMD</td>
<td>Gate 4</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>
## Overlay # | FCS Filename | Gate | # of Events | X Geometric Mean | Y Geometric Mean | % of Gated Cells | % of All Cells
--- | --- | --- | --- | --- | --- | --- | ---
1 | Z0031696.LMD | None | 56573 | 38,43 | 0,0 | 100,0 | 100,0
1 | Z0031696.LMD | Gate 1 | 13561 | 181,42 | 35,14 | 23,97 | 23,97
1 | Z0031696.LMD | Gate 2 | 6424 | 184,41 | 34,3 | 11,36 | 11,36
1 | Z0031696.LMD | Gate 3 | 0 | 0,0 | 0,0 | 0,0 | 0,0
1 | Z0031696.LMD | Gate 4 | 0 | 0,0 | 0,0 | 0,0 | 0,0
Sample 1 Day 4

<table>
<thead>
<tr>
<th>Overlay #</th>
<th>FCS Filename</th>
<th>Gate</th>
<th># of Events</th>
<th>X Geometric Mean</th>
<th>Y Geometric Mean</th>
<th>% of Gated Cells</th>
<th>% of All Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Z0031710.LMD</td>
<td>None</td>
<td>58035</td>
<td>45,72</td>
<td>0,0</td>
<td>100,0</td>
<td>100,0</td>
</tr>
<tr>
<td>1</td>
<td>Z0031710.LMD</td>
<td>Gate 1</td>
<td>13910</td>
<td>169,33</td>
<td>35,48</td>
<td>23,97</td>
<td>23,97</td>
</tr>
<tr>
<td>1</td>
<td>Z0031710.LMD</td>
<td>Gate 2</td>
<td>6839</td>
<td>176,43</td>
<td>34,92</td>
<td>11,78</td>
<td>11,78</td>
</tr>
<tr>
<td>1</td>
<td>Z0031710.LMD</td>
<td>Gate 3</td>
<td>7</td>
<td>191,1</td>
<td>38,12</td>
<td>0,01</td>
<td>0,01</td>
</tr>
<tr>
<td>1</td>
<td>Z0031710.LMD</td>
<td>Gate 4</td>
<td>0</td>
<td>0,0</td>
<td>0,0</td>
<td>0,0</td>
<td>0,0</td>
</tr>
</tbody>
</table>
Sample 2 Day 0 (sample collection)
Gate 1: Lymphocytes
Gate 2: CD31 negative cells
Gate 3: CK positive cells
Gate 4: cMet positive cells

<table>
<thead>
<tr>
<th>Overlay #</th>
<th>FCS Filename</th>
<th>Gate</th>
<th># of Events</th>
<th>X Geometric Mean</th>
<th>Y Geometric Mean</th>
<th>% of Gated Cells</th>
<th>% of All Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Z0031646.LMD</td>
<td>None</td>
<td>45557</td>
<td>7,06</td>
<td>5,39</td>
<td>100,0</td>
<td>87,6</td>
</tr>
<tr>
<td>1</td>
<td>Z0031646.LMD</td>
<td>Gate 1</td>
<td>20981</td>
<td>10,12</td>
<td>4,17</td>
<td>46,05</td>
<td>40,34</td>
</tr>
<tr>
<td>1</td>
<td>Z0031646.LMD</td>
<td>Gate 2</td>
<td>45557</td>
<td>7,06</td>
<td>5,39</td>
<td>100,0</td>
<td>87,6</td>
</tr>
<tr>
<td>1</td>
<td>Z0031646.LMD</td>
<td>Gate 3</td>
<td>13</td>
<td>11,9</td>
<td>36,22</td>
<td>0,03</td>
<td>0,02</td>
</tr>
<tr>
<td>1</td>
<td>Z0031646.LMD</td>
<td>Gate 4</td>
<td>0</td>
<td>0,0</td>
<td>0,0</td>
<td>0,0</td>
<td>0,0</td>
</tr>
<tr>
<td>Overlay #</td>
<td>FCS Filename</td>
<td>Gate</td>
<td># of Events</td>
<td>X Geometric Mean</td>
<td>Y Geometric Mean</td>
<td>% of Gated Cells</td>
<td>% of All Cells</td>
</tr>
<tr>
<td>-----------</td>
<td>----------------</td>
<td>--------</td>
<td>-------------</td>
<td>------------------</td>
<td>------------------</td>
<td>------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>1</td>
<td>Z0031658.LMD</td>
<td>None</td>
<td>8769</td>
<td>10,0</td>
<td>4,31</td>
<td>100,0</td>
<td>17,39</td>
</tr>
<tr>
<td>1</td>
<td>Z0031658.LMD</td>
<td>Gate 1</td>
<td>8748</td>
<td>10,0</td>
<td>4,3</td>
<td>99,76</td>
<td>17,35</td>
</tr>
<tr>
<td>1</td>
<td>Z0031658.LMD</td>
<td>Gate 2</td>
<td>8769</td>
<td>10,0</td>
<td>4,31</td>
<td>100,0</td>
<td>17,39</td>
</tr>
<tr>
<td>1</td>
<td>Z0031658.LMD</td>
<td>Gate 3</td>
<td>2</td>
<td>13,22</td>
<td>35,71</td>
<td>0,02</td>
<td>0,0</td>
</tr>
<tr>
<td>1</td>
<td>Z0031658.LMD</td>
<td>Gate 4</td>
<td>0</td>
<td>0,0</td>
<td>0,0</td>
<td>0,0</td>
<td>0,0</td>
</tr>
<tr>
<td>Overlay #</td>
<td>FCS Filename</td>
<td>Gate</td>
<td># of Events</td>
<td>X Geometric Mean</td>
<td>Y Geometric Mean</td>
<td>% of Gated Cells</td>
<td>% of All Cells</td>
</tr>
<tr>
<td>-----------</td>
<td>------------------</td>
<td>---------</td>
<td>-------------</td>
<td>------------------</td>
<td>------------------</td>
<td>------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>1</td>
<td>Z0031684.LMD</td>
<td>None</td>
<td>50963</td>
<td>55,81</td>
<td>84,1</td>
<td>100,0</td>
<td>100,0</td>
</tr>
<tr>
<td>1</td>
<td>Z0031684.LMD</td>
<td>Gate 1</td>
<td>19926</td>
<td>178,95</td>
<td>36,43</td>
<td>39,1</td>
<td>39,1</td>
</tr>
<tr>
<td>1</td>
<td>Z0031684.LMD</td>
<td>Gate 2</td>
<td>9093</td>
<td>177,43</td>
<td>36,07</td>
<td>17,84</td>
<td>17,84</td>
</tr>
<tr>
<td>1</td>
<td>Z0031684.LMD</td>
<td>Gate 3</td>
<td>3</td>
<td>164,0</td>
<td>42,22</td>
<td>0,01</td>
<td>0,01</td>
</tr>
<tr>
<td>1</td>
<td>Z0031684.LMD</td>
<td>Gate 4</td>
<td>0</td>
<td>0,0</td>
<td>0,0</td>
<td>0,0</td>
<td>0,0</td>
</tr>
</tbody>
</table>
Sample 2 Day 3

<table>
<thead>
<tr>
<th>Overlay #</th>
<th>FCS Filename</th>
<th>Gate</th>
<th># of Events</th>
<th>X Geometric Mean</th>
<th>Y Geometric Mean</th>
<th>% of Gated Cells</th>
<th>% of All Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Z0031700.LMD</td>
<td>None</td>
<td>50336</td>
<td>51,85</td>
<td>0,0</td>
<td>100,0</td>
<td>100,0</td>
</tr>
<tr>
<td>1</td>
<td>Z0031700.LMD</td>
<td>Gate 1</td>
<td>18283</td>
<td>167,1</td>
<td>34,88</td>
<td>36,32</td>
<td>36,32</td>
</tr>
<tr>
<td>1</td>
<td>Z0031700.LMD</td>
<td>Gate 2</td>
<td>8419</td>
<td>166,04</td>
<td>34,88</td>
<td>16,73</td>
<td>16,73</td>
</tr>
<tr>
<td>1</td>
<td>Z0031700.LMD</td>
<td>Gate 3</td>
<td>5</td>
<td>127,26</td>
<td>38,07</td>
<td>0,01</td>
<td>0,01</td>
</tr>
<tr>
<td>1</td>
<td>Z0031700.LMD</td>
<td>Gate 4</td>
<td>0</td>
<td>0,0</td>
<td>0,0</td>
<td>0,0</td>
<td>0,0</td>
</tr>
</tbody>
</table>
Sample 2 Day 4

<table>
<thead>
<tr>
<th>Overlay #</th>
<th>FCS Filename</th>
<th>Gate</th>
<th># of Events</th>
<th>X Geometric Mean</th>
<th>Y Geometric Mean</th>
<th>% of Gated Cells</th>
<th>% of All Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Z0031714.LMD</td>
<td>None</td>
<td>50295</td>
<td>47,43</td>
<td>0,0</td>
<td>100,0</td>
<td>100,0</td>
</tr>
<tr>
<td>1</td>
<td>Z0031714.LMD</td>
<td>Gate 1</td>
<td>17596</td>
<td>169,61</td>
<td>36,25</td>
<td>34,99</td>
<td>34,99</td>
</tr>
<tr>
<td>1</td>
<td>Z0031714.LMD</td>
<td>Gate 2</td>
<td>8150</td>
<td>170,23</td>
<td>36,47</td>
<td>16,2</td>
<td>16,2</td>
</tr>
<tr>
<td>1</td>
<td>Z0031714.LMD</td>
<td>Gate 3</td>
<td>3</td>
<td>204,12</td>
<td>68,94</td>
<td>0,01</td>
<td>0,01</td>
</tr>
<tr>
<td>1</td>
<td>Z0031714.LMD</td>
<td>Gate 4</td>
<td>0</td>
<td>0,0</td>
<td>0,0</td>
<td>0,0</td>
<td>0,0</td>
</tr>
</tbody>
</table>
STEMNESS PHENOTYPE

Growth curve analysis

Useful model in order to study the cancer cell’s growth rate over a period of time

A typical growth curve

Growth curve analysis for pancreatic cancer cells.
Comparison of the Growth Curves of Cancer Cells and Cancer Stem Cells

Maria Toloudi, Eleni Ioannou, Marina Chatziioannou, Panagiotis Apostolou, Christos Kiriotes, Stella Manta, Dimitrios Konriotis, and Ioannis Papasotiropoulos

Abstract: A fundamental problem in cancer research is identification of the cells responsible for tumor formation. The latest field of cancer research has revealed the existence and role of cancer stem cells (CSCs). These findings support the idea that malignancies originate from a small fraction of cancer cells that show self-renewal and multi- or pluripotency. Identification of this CSC population has important implications for the management of cancer patients, including diagnostic and predictive laboratory assays as well as novel therapeutic strategies that specifically target CSCs. In this study, we investigated the growth rates of CSC populations for comparison with cancer cell lines. To construct the growth curves, blood-derived CSCs were isolated from patients with breast, colon, or lung cancer and cultured in vitro. Quantitative real-time PCR was then performed to identify CSCs in the samples. We found that CSCs did not follow the common pattern of a typical growth curve of mammalian cells in contrast to the cancer cell lines. This observation of rapidly growing CSCs indicates their involvement in tumor formation.

Keywords: Cancer stem cells, growth curves, Nanog, Oct3/4, Sox2.

Correlation between Cancer Stem Cells and Circulating Tumor Cells and Their Value
Maria Toloudi, Panagiotis Apostolou, Marina Chatziioannou, Ioannis Papasotiropoulos
Research Genetic Cancer Center (R.G.C.C. Ltd.), Filotas, Greece

Case Rep Oncol 2011;4:44-54
CSCs molecular markers

- **Nanog gene**: Pluripotency promoting gene, self renewal, block differentiation, embryonic stem cell marker
- **Oct3/4 gene**: (Octamer-binding protein 3/4) Key regulator of self renewal, embryonic stem cell marker, pluripotent marker
- **Nestin & Sox-2 gene**: Pluripotent and early neural stem cell marker
Cancer Stem Cells Stemness Transcription Factors Expression Correlates with Breast Cancer Disease Stage

Panagiotis Apostolou¹, Maria Toloudi¹, Marina Chatziioannou¹, Eleni Ioannou¹ and Ioannis Papasotiriou*¹

¹Research Genetic Cancer Centre Ltd (R.G.C.C. Ltd), 115 M.Alexandrou Str, 53070 Filotas, Florina, Greece

Abstract: Cancer stem cell-like cells (CSCs) are cancer cells that have the ability of self-renewal and differentiation into multiple malignant cell types (hierarchy). Thus, can cause relapses and metastasis. CSCs’ phenotype is defined by special transcription factors such as Nanog, Oct3/4, Sox2, Nestin, and CD34. The present study aims to determine the change in gene expression of the above markers in correlation with the stage of the disease in breast cancer patients. Initially, whole blood samples from patients with breast cancer were collected, followed by the isolation and culture of circulating tumor cells (CTCs). This was followed by the quantification of CSCs from the above cultures. CSCs’ molecular analysis was performed with qPCR, with the use of gene specific primers. At the same time of the analysis, the clinical assessments of the patients were requested from their physicians. The results indicated a linear relationship between the gene expression of stemness markers and the stage of the disease, as well as specific expression patterns by stage. It seems that these genes have an important role in the progression of the disease, thus they might be target for new treatment approaches.

Keywords: Breast cancer, cancer stem cells, Nanog, Sox2, Oct3/4, CD34, Nestin.
What the stemness driving molecule induce
Intrinsic regulation of stemness (spontaneous dedifferentiation)

1. Internal induction of transcription factors line Sox-Oct-4 (epigenetic factors)

2. Transposition factors (L1 retrotransposones non LTR is related with stemness phenotype and CSCs)
How CSCs can appeared in a culture
CSCs and clinical reality

There is an obvious trend of increasing expression of CSCs markers inside the population of CTCs in linear relation with stage and progress of the disease.
Correlation between Cancer Stem Cells and Circulating Tumor Cells and Their Value

Maria Toloudi, Panagiotis Apostolou, Marina Chatziioannou, Ioannis Papasotiriou

Research Genetic Cancer Center (R.G.C.C. Ltd.), Filotas, Greece

Case Rep Oncol 2011;4:44-54
Cancer Stem Cells Stemness Transcription Factors Expression Correlates with Breast Cancer Disease Stage

Panagiotis Apostolou¹, Maria Toloudi¹, Marina Chatziioannou¹, Eleni Ioannou¹ and Ioannis Papasotiriou*,¹

¹Research Genetic Cancer Centre Ltd (R.G.C.C. Ltd), 115 M.Alexandrou Str, 53070 Filotas, Florina, Greece

Abstract: Cancer stem cell-like cells (CSCs) are cancer cells that have the ability of self-renewal and differentiation into multiple malignant cell types (hierarchy). Thus, can cause relapses and metastasis. CSCs’ phenotype is defined by special transcription factors such as Nanog, Oct3/4, Sox2, Nestin, and CD34. The present study aims to determine the change in gene expression of the above markers in correlation with the stage of the disease in breast cancer patients. Initially, whole blood samples from patients with breast cancer were collected, followed by the isolation and culture of circulating tumor cells (CTCs). This was followed by the quantification of CSCs from the above cultures. CSCs’ molecular analysis was performed with qPCR, with the use of gene specific primers. At the same time of the analysis, the clinical assessments of the patients were requested from their physicians. The results indicated a linear relationship between the gene expression of stemness markers and the stage of the disease, as well as specific expression patterns by stage. It seems that these genes have an important role in the progression of the disease, thus they might be target for new treatment approaches.

Keywords: Breast cancer, cancer stem cells, Nanog, Sox2, Oct3/4, CD34, Nestin.
How epigenetics implicated in CSCs phenotype development

• The stemness phenotype is triggered and “be revived”

• Mechanism of reactivation:
  – Methylations/hypemethylation of genes that involved in stemness cascade (nanog, Sox-2, Oct-3/4, twist, snail etc)
  – Genetic rearrangment/instabiity (transpositions etc)
  – Gene expression regulation though micro-RNA
CSCs-EMT and MET
How epigenetics implicated in CSCs phenotype development

• The stemness phenotype is triggered and “be revived’

• Mechanism of reactivation:
  – Methylations/hypemethylation of genes that involved in stemness cascade (nanog, Sox-2,Oct-3/4, twist, snail etc)
  – Genetic rearrangment/instabiity (transpositions etc)
  – Gene expression regulation though micro-RNA
DNA hypermethylation & hyperacetylation

- Involves enzymes which methylate/acetylate parts of genome and shift the expression rate of genes.
- The field is already under extensive research.
- Hypermethylation is not clearly related with stemness (multiple other co-founders are involved).
- New agents have been generated as inhibitors of hypemethylation/hyperacetylation of DNA (Vorinostat-HDAC inhibitor).
Micro-RNA (RNAi) and CSCs phenotype

- Several patterns have been identified, but very few related with stemness.
- So far there is no drugable target for these patterns and mechanisms.
Transposable genetic elements

• Transposon (cut paste)-Class II
  – Transposase
  – Resolvase
  – Gene of interest

• Retrotransposon (copy paste)-Class I
  – LTR retrotransposon
  – Non LTR
    • LINE (they include reverse transcriptase)
    • SINE
Relation between transposable genetic elements and cancer

- Responsible to resistance phenotype development (ERCC-1 and platinum derivatives resistance)
- Relation between SETmar and topoisomerases II inhibitor resistance in breast carcinoma
- Microsatellite instability (MLH1, MSH) and resistant phenotype in colon cancer
- Responsible for genetic instability and rearrangements in all genome
Transposable genetic elements and CSCs phenotype

- Expression of L1 in CSCs
- Expression of SETMAR in CSCs
- Relation between METnase in CSCs and MET transition process
- Resistance of CSCs and transposase activity
Experimental evidence (Knock down METnase)

Percentage Knock Down Metnase by 53,51%
Experimental evidence (Knock down METnase)
Not all transposases are present in cells with “Stemness” properties
<table>
<thead>
<tr>
<th>Gene</th>
<th>siRNA</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Notch-1</td>
<td>Sense</td>
<td>UAGUAGGGGAAGAUAUCUUU</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>AGAUGAUCUUCCCCCUACUAUU</td>
</tr>
<tr>
<td>Notch-2</td>
<td>Sense</td>
<td>UAAAUUUGGAUGGACUGAUU</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>UCAGUCCUAUCCAAAUUUAUU</td>
</tr>
<tr>
<td>Notch-3</td>
<td>Sense</td>
<td>UUAUUGGCUCCAUUUUUUGAUU</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>UCAAAAAUGGAGCCAAUAAUU</td>
</tr>
<tr>
<td>Notch-4</td>
<td>Sense</td>
<td>UAAAUGCGAUAGCAGUGGUU</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CCACUGCUAUCGCUAAUUUAU</td>
</tr>
</tbody>
</table>

**Changes in gene expression**

![Graph showing changes in gene expression](image.jpg)
# Experiments

## Notch Knockdown

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Notch-1</th>
<th>Notch-2</th>
<th>Notch-3</th>
<th>Notch-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon CSCS</td>
<td>92%</td>
<td>95%</td>
<td>92%</td>
<td>88%</td>
</tr>
</tbody>
</table>

---

**The effect of Notch receptors in stemness**

![Graph showing the effect of Notch receptors in stemness](image)

---

![Images of cell lines](image)
Experiments (controversies)

Another pathway that may trigger the stemness is the heterodimer AP-1 which is formed by c-FOS and c-JUN.

FOS KnockDown (63%)

- Nanog
- Oct3/4
- Sox2

FOS KnockDown (85%)

- Nanog
- Oct3/4
- Sox2
Experiments (intrinsic pathways)

Possible involvement of MET process as well as plasticity.
Outcome of the experiments

• Microenvironment plays an important role in stemness development
• During progression many types of CSCs may be generated
• There are several pathways with multiple “cross talking” to promote stemness phenotype
Experiments II (Sox-2 or 17 & Oct-4)
Experiment II continue

- Leading compound for leucin Zipper transcription factor Sox-Oct-4 inhibitor

Vogt et al. 2002
Extrinsic mechanism promoting the progression of stemness

TMX2: A potential biomarker or a predictive marker in breast cancer?

Subcategory:
Genomic and Epigenomic Biomarkers

Category:
Tumor Biology

Meeting:
2014 ASCO Annual Meeting

Session Type and Session Title:
This abstract will not be presented at the 2014 ASCO Annual Meeting but has been published in conjunction with the meeting.

Abstract Number:
e22077

Citation:
J Clin Oncol 32, 2014 (suppl; abstr e22077)

Author(s):
Ioannis Papasotirou, Panagiotis Apostolou, Maria Toloudi, Marina Chatziioannou, Georgia Mimikakou, Eleni Kourtidou, Ioanna Vlachou; Research Genetic Cancer Centre, Ltd, Filotas, Greece
REALITY vs LAB BENCH

• We must not forget that clinical reality is different than a controlled environment of a lab.
• Complexity of cancer may generate options of escape in clinical practice.
• PD and PK may play equally important role.
• Toxicity may also generate barriers to an option.
LIMITATIONS-BARRIERS

• Clinically
  1. CTCs are still inhomogenous
  2. Shifting of CTCs to CSCs phenotype (plasticity).
  3. Relevant subpopulation that drives the disease
  4. In advance stage even the CSCs are heterogeneous and which CSCs initiate relapse.
  5. Still many factors that shift CTCs to CSCs and backwards are unknown

• Research field
  1. Plasticity of the CTCs and CSCs
  2. Multiple mechanism that drives the genetic instability
  3. Dependency of stemness from multiple pathways.
  4. What triggers the CSCs to exponential phase of growth, is still unknown.
POINT THAT NEED TO BE CONSIDERED

- Cancer Stem cell like (Tumor initiating cells) model is not applicable in all types of malignancies (melanoma for example)
Is it possible to analyze the gene expression profile of CTCs?
Data derived from micro arrays

- The information are normalized through a series of endogenous genes (housekeeping).
- The hybridization is always take place with orthotopic use of appropriate normal cell of tissue of origin.
- The non parametric analysis (ANOVA) may allow reveal relevance between genes, types of cancer or even repeatable patterns.
Micro-Arrays analysis

• TODAY IT IS POSSIBLE TO DETECT ALL GENOME TRANSCRIPTOMICS WITH 12K SPOT SLIDES UP TO 90K SPOTSLIDES (hyperdense micro-array slide)
Analysis of epigenetic data
Experiments
(expression patterns in CSCs)

- Hybridization image Cy5-cDNA with Cy5 reference mRNA
Chemosensitivity testing

• All assays started from the need to define and point out recommended agents with anti-cancer activity

• It combines molecular as well as cellular approach which all validated by a viability assays (there is not always linear relation between gene expression profile, protein production and cellular activity)
Golden rule: Always double platform

- It is known that gene expression profile has not always linear relation with protein expression rate (post transcription processes, glucosilation etc).

- A drug to have an effect needs to be distributed to the intracellular area (membrane permeability).

- In order to confirm the information that the expression micro-array generates, the cancer cells are exposed to the active substance of each drug and the cytotoxicity/viability is explored.
Steps of the process

1. Identify and isolate the CTCs
2. Identify and expand CSCs
3. Gene expression profile (micro-arrays)
4. Viability assays after exposure to active substance
5. Combine and compare the data between the two platforms

RESULTS
Should we start thinking broadening our way of treating cancer?
THEORETICAL PLAN OF TREATMENT

TREATMENT PLAN = INFORMATION + APPLICATION

DIAGNOSTIC

- FOLLOW-UP
- EXTENSION OF THE DISEASE
- IMMUNOSTATUS
- EFFICACY OF AGENTS
- ABILITY TO PD & PK

TREATMENT APPLICATION

- PROLIFERATIVE CANCER CELLS
- CANCER STEM CELL
- PROTECTIVE EFFECT AND SENSITIZING
Proposed algorithm of treatment

- TUMOR
  - CANCER CELLS
    - PROLIFERATIVE CANCER CELLS
      - CONVENTIONAL CHEMOTHERAPY AGENTS
        - CTX-TKIs-MoAb
    - CANCER STEM CELL (TUMOR INITIATING CELLS)
      - NEW AGENTS AGAINST CSCs
  - STROMA (MICROENVIRONMENT)

INTERACT
AT THE END

- Parameters of clinical value: RR, OS, DFS
FUTURE PERSPECTIVES

• Identify patterns of mechanism on CTCs
• Understand plasticity
• Pin point “drugable” targets
• Design tailor made therapies based on markers and molecular patterns
• Change the therapeutic concept based on understanding cancer biology
Meta-Analysis of the Prognostic Value of Circulating Tumor Cells in Breast Cancer
CONCLUSION

• Personalized treatment becomes the optimal strategy for effective cancer treatment
• CTCs have more similarities with the metastatic points as well as with the primary.
• CTCs are heterogenous population but their proportion in cancer stem cells is higher than the primary tumor.
• CSCs is the responsible type of cancer cells that have the ability to regrow a tumor.
• CTCs can become the proper sample to detect and isolate the CSCs.
• Today there are proper methodologies and techniques to analyze the CTCs and the CSCs in order to harvest clinically relevant data.
IN SUMMARY

• CTCs & CSCs HAVE SIGNIFICANT DIAGNOSTIC AND PREDICTIVE VALUE BUT...

1. A COMBINATION OF ASSAYS ARE REQUIRED FOR DETECTING THE RARE EVENT OF CTCs

2. AN ACCURATE SERIES OF METHODS ARE REQUIRED TO DETECT THE CLINICALY RELEVANT SUBCLONES OF CANCER CELLS.

3. MORE RELEVANT INFORMATION OF PD BUT ALSO PK ARE REQUIRED.

4. MORE FLEXIBLE TEST ARE REQUIRED TO FOLLOW UP CANCER PATIENTS.
QUESTIONS?

questions@rgcc-international.com
THANK YOU FOR YOUR TIME. 
Visit our web site 
www.rgcc-international.com

Since the data and information is large and further questions and definition may generated, we strongly recommend to obtain the DVD presentation of RGCC International GmbH where more information and definition are there in order to help therapist to understand what is feasible in laboratory reality and what is applicable to clinical use.

In case also of additional questions please do not hesitate to come in direct contact with RGCC International GmbH for any inquiry. The previous direct email address is specifically for this purpose.