

Expression of stem cell and epithelial-mesenchymal transition markers in metastatic breast cancer patients with circulating tumor cells



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Abstract

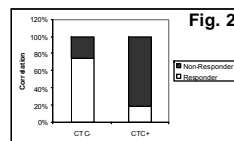
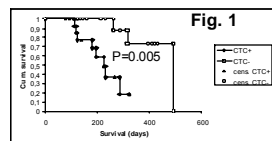
Background: Stem cell like tumor cells have been suggested to be the active source of metastatic spread in primary tumors. Furthermore, these cells may undergo phenotypic changes, known as epithelial-mesenchymal transition (EMT), which allows them to travel to the site of metastasis formation without getting affected by conventional treatment. Assuming that metastasis requires a dissemination of tumor stem cells or tumor cells showing EMT, it was the purpose of this study to evaluate the correlation of the expression of stem cell and epithelial-mesenchymal transition markers and the presence of circulating tumor cells (CTC) in blood samples of 28 metastatic breast cancer patients.

Materials and Methods: 5 ml blood was analyzed for EpCAM, MUC-1 and HER-2 transcripts with the *AdnaTest BreastCancer* (AdnaGen AG). The recovered c-DNA was additionally multiplex tested for three EMT markers [Twist, Akt2, PI3K (TAP)] and separately for the tumor stem-cell marker ALDH1. The analytical sensitivity was determined by the detection of a low number of target cells (5 IGROV cells spiked into 5 ml blood of healthy donors) using the *AdnaTest BreastCancer* procedure. The identification of EMT markers was considered positive if at least one marker was detected in the sample. Healthy donor samples without spiked tumor cells were used to determine the specificity of the test.

Results: Applying an amplicon cut-off value of 0.2 ng/μl for Akt2, 0.15 ng/μl for Twist, 0.25 ng/μl for PI3K and 0.15 ng/μl for ALDH1, 97% of 30 healthy donor samples investigated were negative for TAP and 95% for ALDH1 transcripts. The spiking experiments revealed 80% recovery of the IGROV cells. CTC were detected in 12/28 (43%) cancer samples. All samples were further examined for tumor stem cell or TAP markers. In the CTC (+) group 50% were positive for at least one of the TAP markers and 42% for ALDH1. In the CTC (-) group the percentages were 19% and 12%, respectively. The expression of TAP and/or stem cell markers in CTC was compared with the clinical follow up results. In CTC (+) patients diagnosed as non-responders the expression of TAP and ALDH1 was found in 5/12 (40%) samples. In the CTC (+) responder group only one sample expressed TAP and no positive case was found for ALDH1. In the CTC (-) non-responder group 1/14 was positive for TAP and ALDH1. 2/14 (14%) of CTC (-) responders were positive for TAP and 1/14 for ALDH1, respectively.

Conclusion: The strong correlation between the expression of stem cell and EMT markers and the presence of CTC indicate that a major proportion of CTC found in the blood of metastatic breast cancer patients shows EMT and tumor stem cell characteristics. These markers may serve as an indicator for therapy resistant tumor cell populations and, therefore, for an inferior prognosis.

Background



We recently demonstrated that the presence or disappearance of CTC during the time course of individual treatment is a predictor of therapy response in metastatic breast cancer (Fig. 1).

Breast cancer patients, CTC positive either before or during therapy, showed a correlation between presence, persistence or early disappearance of CTC with therapy response in 78% of all cases (Fig. 2) [5].

Objectives

- To evaluate the expression of stem cell and EMT markers in blood of metastatic breast cancer patients.
- To correlate these markers with the presence of CTC.
- To correlate these findings with response to treatment.

The Targets

TWIST: Twist is described to bind to E-box elements on the AKT2 promoter and to enhance its transcriptional activity and thus is likely to be related to the EMT phenomenon in cancer cells.

PI3K/Akt: PI3K activates the Akt1 and Akt2 Ser/Thr kinase. Activated Akt is responsible for proliferation and has anti-apoptotic function. Two iso-forms of the Akt protein are described: (a) Akt1, which if up-regulated in tumors stimulates proliferation and (b) Akt2, which seems to be involved in cell survival.

ALDH1: Normal and cancer human mammary epithelial cells with increased ALDH1 activity have stem/progenitor cell properties. Over-expression of ALDH1 in tissue of primary breast tumors correlates significantly with poor prognosis. In breast carcinomas, high ALDH1 activity identifies the tumorigenic cell fraction, capable of self-renewal and of generating tumors that recapitulate the heterogeneity of the parental tumor [6].

Methods / Patients

(A) The AdnaTest BreastCancer [1-4] This test enables the immunomagnetic enrichment of tumor cells via epithelial and tumor associated antigens. Two antibodies against the epithelial antigen MUC1 and one antibody against the epithelial glycoprotein GA733-2 (EpCAM) are conjugated to magnetic beads (Dynabeads) for the labelling of tumor cells in peripheral blood. The labeled cells are extracted by a magnetic particle concentrator (MPC). Subsequently, mRNA isolation from lysed, enriched cells is performed with the Dynabeads mRNA DIRECT™ Micro Kit (Dyna Biotech GmbH, Hamburg, Germany). Reverse transcription results in cDNA, which is the template for tumor cell detection and characterization by multiplex RT-PCR for three tumor-associated transcripts: HER2, MUC1 and GA733-2.

(B) The AdnaTest TumorStemCell / (C) The AdnaTest EMT Both tests require the enrichment of CTC from 5ml blood using the AdnaTest BreastCancerSelect (modified by the use of special washing buffers to reduce leukocyte cross-reactions) prior to the singleplex PCR assay to analyze ALDH1 and the multiplex PCR assay to analyze Twist, PI3Kα, Akt2 and Aktin as an internal control. **Evaluation of data:** Visualization of all PCR fragments was carried out with a 2100 Bioanalyzer using the DNA 1000 LabChips (Agilent Technologies) and the Expert Software Package (version B.02.03.SI307). The test is considered positive if a PCR fragment of at least one tumor associated transcript is clearly detected. Peaks with a concentration of > 0.15 ng/μl are positive for the transcripts GA733-2, MUC1 and HER2.

Patients: Blood samples of 28 patients were analyzed during follow-up of their individual therapies. **The eligibility criteria** were as follows: age ≥ 18 years; patients with measurable or evaluable metastatic breast cancer; predicted life expectancy ≥ 2 months; ECOG scores for performance status of 0-2; no severe uncontrolled co-morbidities or medical conditions; no second malignancies. Patients had either a relapse of breast cancer diagnosed years before and were to start chemotherapy or a documented progressive breast cancer before receiving a new endocrine, chemo- or experimental therapy. Prior adjuvant treatment, radiation or any other treatment of metastatic disease were permitted. **Response criteria:** Before starting a new treatment, patients underwent an evaluation of metastatic sites by ultrasound, x-ray or computer tomography. Blood samples were collected for laboratory evaluations, including CEA and Ca 15-3. Re-evaluations of disease status were done by the same techniques every 8-12 weeks, depending on the treatment schedule, until the loss or death of a patient.

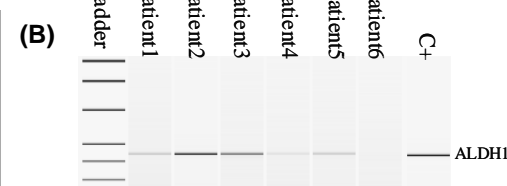
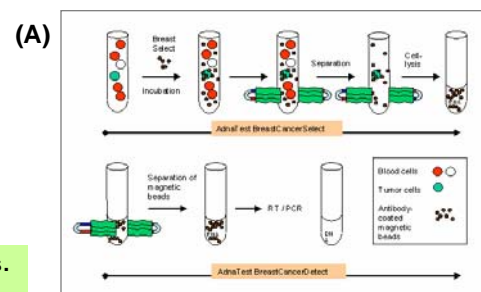


Fig. Singleplex determination of the tumor stem cell marker ALDH1 in CTC enriched from 5 ml blood of metastatic breast cancer patients. This marker seems to be differentially expressed in patient samples indicating tumor stem cell characteristics of CTC.

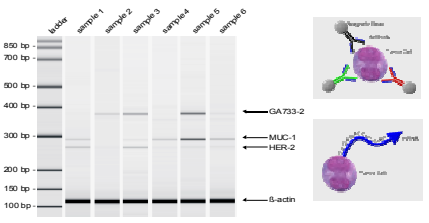


Fig. Multiplex determination of CTC markers GA733-2, MUC-1, and HER2 in CTC enriched from 5 ml blood of metastatic breast cancer patients.

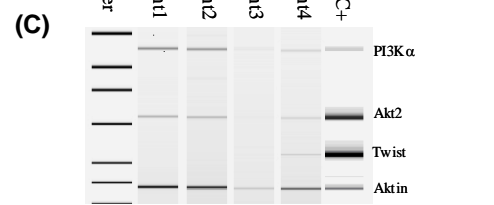


Fig. Multiplex determination of EMT markers Twist, PI3Kα and Akt2 in CTC enriched from 5 ml blood of metastatic breast cancer patients. These markers seem to be differentially expressed in these samples indicating EMT characteristics of CTC.

Results

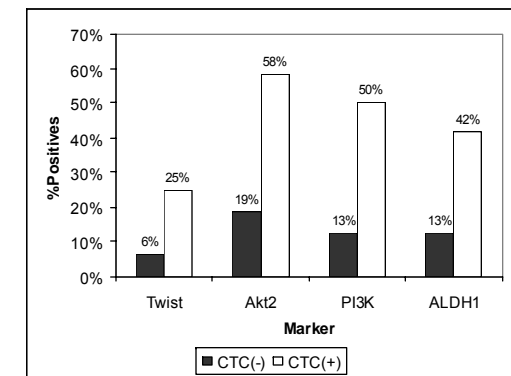


Fig. 3 Expression of ALDH1 and EMT markers in metastatic breast cancer patients undergoing chemo-, hormonal- or trastuzumab therapy.

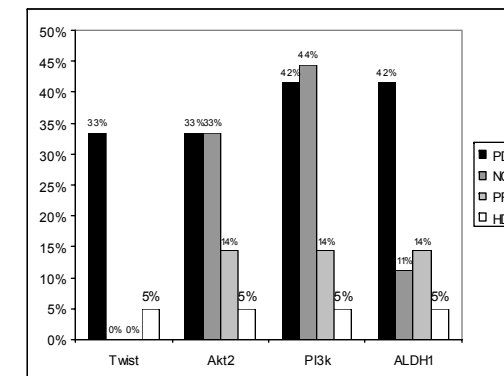


Fig. 4. Correlation between ALDH1 and EMT markers with therapy response (PD: progressive disease; NC: no change; PR: partial remission; HD: Healthy Donors.)

Conclusions

CTC might often display tumor stem cell characteristics highlighting their role in metastasis formation. The ALDH1 over-expression allows to analyze the impact of this phenotype change with regards to prognosis, therapy failure and metastasis formation.

EMT characteristics are detectable in CTC analyzed in metastatic breast cancer samples, giving a hint for the negative prognostic impact of such cells due to the EMT switch that leads to decreased apoptosis and the development of chemo-resistance.

Both tests allow for the first time a deep insight into tumor biology of CTC in breast cancer and its function in therapy failure and metastasis formation.

References

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