AdnaTest TumorStemCell and *AdnaTest EMT*

Tools for breast cancer research

The biology of circulating tumor cells (CTC) can be assessed with various molecular biological methods (RT-PCR, real-time PCR, micro-chip technology). The determination of over-expressed pharmaceutical target genes (estrogen receptor, progesterone receptor, EGFR, HER2) might be helpful in the context of personalized strategies in cancer treatment. However, the global determination of signal transduction pathways that are altered in the cancer cell metabolism may lead to a better understanding how tumor cells are enabled to escape the immune system and to build up resistance against drug treatment. Recent key findings in primary tumor tissue suggest that the metastatic potential of a tumor is based on the presence of a low number of stem cell like tumor cells that have been identified to be the active source of metastatic spread (Wicha 2006). Cancer stem cells detected in primary tumor tissue were found to be relevant for clinical outcome after treatment. Consequently, one can assume that such tumor stem cells are disseminated from the primary tumor into the circulation and escape therapy due to their stem cell properties until they reach their homing organ where they act as seed for metastasis formation (Pantel *et al.* 2008).Cancer stem cells, enriched from the primary tumor, were found to stain positive for CD44 stem cell marker but negative for CD24. More recently also ALDH1 was found to be a specific marker for cancer stem cells (Ginestier *et al.* 2007). There is certain evidence that the circulating tumor cells might be identified partly as cancer stem cells due to similarities such as increased resistance to chemotherapy and decreased proliferation during circulation. Similar findings were reported for disseminated tumor cells in bone marrow where tumor cells with a stem cell like phenotype were demonstrated (Balic *et al.* 2006). Corresponding experimental results for CTC are still outstanding.

Furthermore, in addition to the cancer stem cell concept, it was hypothesized that tumor cells spread into the circulation may undergo phenotypic changes, known as epithelial-mesenchymal transition (EMT). These cells have reduced apoptosis and are drug resistant. This allows them to travel to the site of metastasis formation and prevents them from getting affected by conventional treatment.. EMT is known to occur in embryonic development where epithelial cells must escape structural constraints imposed by tissue architecture. They achieve this by adopting a phenotype more amenable to cell movement. The progression of carcinomas to invasive and metastatic disease shows high similarities to this process. Previous epithelial tumor cells that may convert into mesenchymal phenotype could, therefore, escape the primary tumor tissue and develop resistance against conventional therapy regimens, like anti-hormone treatment, since they lost the relevant therapeutic targets during that transformation (own findings). On the other hand it might also be possible, that the expression of potential therapeutic targets, like the HER2-receptor, is induced in such cells, even if the primary tumor was found negative for these targets (Fehm *et al.* 2007).

Due to the fact, that metastazation requires a dissemination of tumor stem cells or tumor cells showing EMT, it seems likely that such cells should be detectable amongst the CTC found in the circulation of cancer patients. The detection and characterisation of CTC that show an EMT or stem cell like metabolism could be a powerful diagnostic tool for the early determination of therapy failure or the potential risk of resistance to a given therapeutic intervention. To address this AdnaGen developed 2 research kits for the detection of EMT markers PI3Kα, Akt2 and Twist and for the analysis of the tumor stem cell marker ALDH1, respectively.

The Targets

TWIST

Basic helix-loop-helix (bHLH) transcription factors have been implicated in cell lineage determination and differentiation. The protein encoded by this gene is a bHLH transcription factor and shares similarity with another bHLH transcription factor, Dermo1. The strongest expression of this mRNA is in placental tissue; in adults, mesodermally derived tissues express this mRNA preferentially. Twist is described to bind to E-box elements on Akt2 promoter and enhanced 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 (inhibition of caspase 9 activity). There are several possibilities how the Pi3K/Akt (mTOR) signal cascade can be deregulated in cancer. One possibility is the loss of PTEN activity, which is a regulator of Pi3K that leads to a permanent activation of the pathway. Furthermore, Pi3K mutations as well as over-expression are described. Since the Pi3K/Akt pathway is also activated *via* tyrosine kinases it may be affected in case of EGFR over-expression/mutation and HER2 over-expression. Another activator is the IGF receptor which is, for instance, described in breast cancer to potentially shortcut ER dependency for proliferation if over-expressed. Several iso-forms Of Pi3K are described whereby in cancer mainly the PI3Kα (p110α) isoform is of importance. Of the Akt protein (protein kinase b) two iso-forms are described: (a) Akt1, which if up-regulated in tumors stimulates proliferation and (b) Akt2, which seems to be involved in cell survival.
Drugs affecting the pI3K/Akt pathway are LY294002 and LY294002 geldamnamycin hetero-dimers developed to block Pi3K activity or 17-allyl-geldanamycin, which affects Akt activity and thus enhances apoptotic effects of cytotoxic agents.

ALDH1

Utilizing *in vitro* and **in vivo** experimental systems, it was shown (Ginestier *et al* 2007) that normal and cancer human mammary epithelial cells with increased aldehyde dehydrogenase 1 activity (ALDH1) have stem/progenitor 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.

The *AdnaTest EMT*

For research use use with the *AdnaTest BreastCancer*

The test requires the enrichment of CTC from 5ml blood followed by reverse transcription cDNA synthesis using the *AdnaTests BreastCancerSelect/Detect* (modified by the use of special washing buffers to reduce leukocyte cross-reactions) prior to the multiplex PCR assay to analyse Twist, PI3Kα, Akt2 and Actin as an internal control. The Kit contains modified wash buffer for the *AdnaTest BreastCancerSelect* tumor cell enrichment procedure, primer-mix to amplify c-DNA target fragments of Twist, PI3Kα, Akt2 and actin, positive control samples. The Kit is designed for 12 EMT marker determinations.

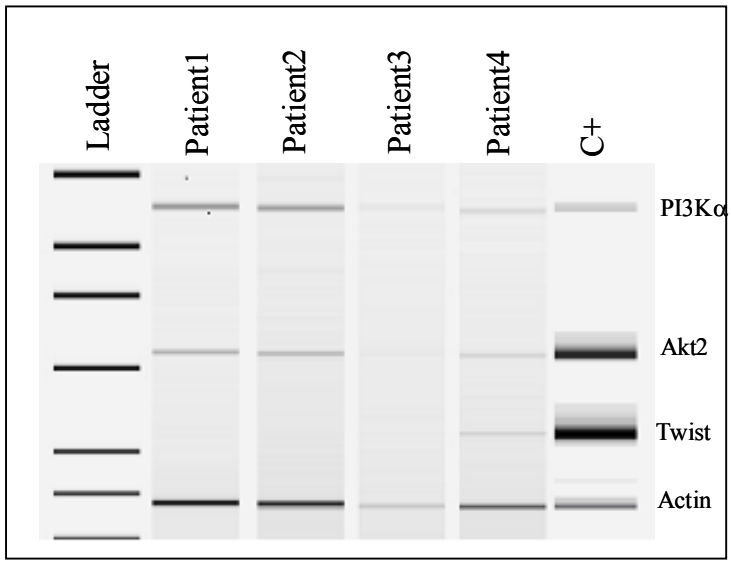

Fig.1 Multiplex determination of EMT markers Twist, PI3Kα and Akt2 in CTC enriched from 5 ml of metastatic breast cancer patients blood using the *AdnaTest BreastCancerSelect*. It could be shown, that the markers seem to be differentially expressed in these samples indicating EMT characteristics of CTC.

EMT characteristics are detectable in CTC analysed in metastatic breast cancer samples (Fig.1), 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.

The *AdnaTest EMT* allows for the first time a deep insight into tumor biology of CTC in breast cancer and its function in therapy failure and metastasis formation.

The *AdnaTest TumorStemCell*

For research use use with the *AdnaTest BreastCancer*

The test requires 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 multiplex PCR assay to analyse ALDH1 and actin as an internal control. The Kit contains modified wash buffer for the *AdnaTest BreastCancerSelect* cell enrichment procedure, primer-mix to amplify c-DNA target fragments of ALDH1 and actin, positive control samples. The Kit is designed for 12 tumor stem cell marker determinations.

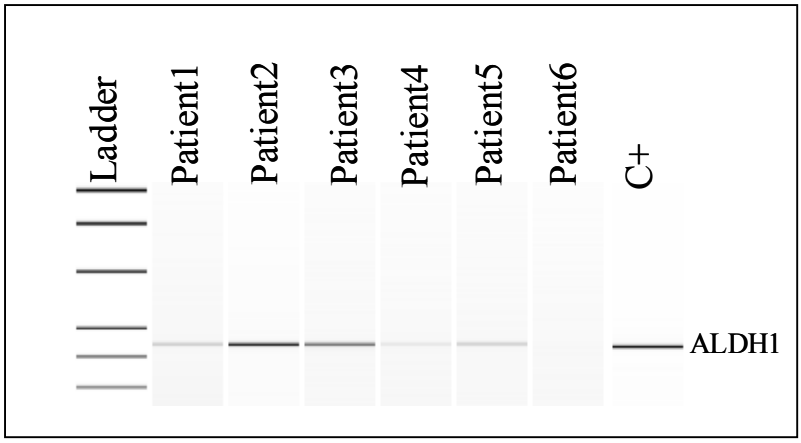

Fig.2 Singleplex determination of the tumor stem cell marker ALDH1 in CTC enriched from 5 ml of metastatic breast cancer patients blood using the *AdnaTest BreastCancerSelect/Detect*. It could be shown, that the marker seems to be differentially expressed in patient samples indicating tumor stem cell characteristics of CTC.

When CTC from metastatic breast cancer patients were analysed by RT-PCR for ALDH1 an over-expression was detected in a substantial amount of samples (Fig. 2). This indicates that CTC might often display tumor stem cell characteristics highlighting their role in metastasis formation. The ALDH1 over-expression allows to analyse the impact of this phenotype changes with regards to prognosis, therapy failure and metastasis formation.

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