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Review

Hormone receptor status, erbB2 expression and cancer stem cell characteristics of circulating tumor cells in breast cancer patients

Brigitte Rack[1], Carolin Bock[1], Ulrich Andergassen[1] and Sophie Doisneau-Sixou[1,2]

[1]Tumorbiology Laboratory, Clinic for Gynaecology and Obstetrics, Campus Innenstadt, Ludwig-Maximilians University, Munich, Germany and [2]Paul Sabatier University, Toulouse III, Faculty of Pharmaceutical Sciences, Toulouse, France

Summary. The most important predictor for disease-free and overall survival of breast cancer patients is the presence of axillary lymph node metastasis. For surveillance during recurrence-free follow-up or in metastatic disease no marker is available at the moment. Several trials have shown the prognostic relevance of circulating tumor cells (CTC) in early and metastatic breast cancers. Indeed, only CTC that exhibit specific molecular characteristics including stem cell characteristics, could be able to create new metastasis. Hormone therapy or anti-erbB2 therapies are prescribed according to the hormone (ERα/PR expression) and erbB2 status of the initial tumor. Nonetheless, it appears that the CTC, and consequently the metastatic cells, may have a very different hormone and erbB2 status. An optimal individualized treatment could then be obtained by characterizing ERα and erbB2 status in the CTC and comparing it to the primary tumor.

Key words: Estrogen receptor alpha, erbB2, Circulating tumor cells, Breast cancer, Cancer stem cells

Introduction

Breast cancer mortality has declined over the last 10 years with the improvement of screening, diagnosis and therapies. It nevertheless remains the most common cancer and cause of death for women worldwide (Levi et al., 2005). It is a heterogeneous disease and patient outcome varies significantly according to subtypes based on prognostic features. The most important predictor of disease-free survival (DFS) and overall survival (OS) for breast cancer is the presence of axillary lymph node metastasis and more than 90% of cancer deaths result from the development of haematogenously disseminated metastasis. Based on recent data, prognosis of patients is thought to depend mainly on tumor biology. Evolving technologies allow us now to collect increasingly large amounts of molecular data from tumors and to establish a new classification (Perou et al., 2000) and gene signatures of progression and metastasis (van de Vijver et al., 2002; Paik et al., 2004; Wang et al., 2005), for review (Rodenhiser et al., 2011). Gene expression profiling can further classify invasive ductal carcinomas (80% of the breast cancers besides 10-15% of invasive lobular carcinoma) into five subtypes: luminal A, luminal B, erbB2, basal and normal-like (Perou et al., 2000). The two breast cancer subtypes with bad outcomes are basal-like and erbB2 breast tumors. While many prognostic and predictive tools are available at primary diagnosis (Mammaprint, Oncotype DX, uPA/PAI-1), no marker is available during recurrence-free follow-up or in metastatic disease (Kantelhardt et al., 2011; Rodenhiser et al., 2011).

The detection of breast cancer is now earlier and earlier, and survival has been improved thanks to surgery improvements and adjuvant therapy. 70 to 80% of breast cancers are rapidly classified as “hormone responsive” because the primary tumor cells express the estrogen receptor alpha (ERα) and/or progesterone receptor (PR). The reference treatment was first tamoxifen, a selective estrogen receptor modulator (SERM) that is an ERα antagonist in breast cells, but is now completed with the pure ERα antagonist (fulvestrant) and aromatase inhibitors (AI) (Lin et al., 2010). 20 to 30% of ERα/PR

Offprint requests to: Prof. S. Doisneau-Sixou, Tumorbiologisches Labor der Klinik und Poliklinik für Frauenheilkunde und Geburtshilfe, Campus Innenstadt, Klinikum der Ludwig-Maximilians-Universität, Maistraße 11, 80337 München, Deuschland. e-mail: sophie.doisneausixou@med.uni-muenchen.de

negative tumors often overexpress the transmembrane receptor erbB2 (epidermal growth factor receptor 2, also named HER2/neu) and exhibit aggressive tumor behaviour and resistance to cytotoxic and endocrine therapy (Slamon et al., 1989; Konecny et al., 2003). Patients benefit from a targeted anti-erbB2 therapy (trastuzumab, Herceptin®) although new strategies for response prediction and follow-up are still of high clinical relevance (Slamon et al., 1989), for reviews (Di Cosimo and Baselga, 2008, 2010). Besides, less than 5% of tumors are called triple negative, and analyses have shown that 80-90% of those are basal-like tumors. Triple negative tumors are known to be very aggressive and are treated by chemotherapy without specific targeted therapy. In any case, distant metastases are still the leading cause of deaths for breast cancer patients.

Necessity of markers of sensitivity/resistance for hormone therapy (HT) and other targeted therapies, including erbB2

HT is an effective treatment strategy for early-stage breast cancer patients without indication for chemotherapy and even more for metastatic patients. Around one third of the metastatic patients will benefit from it. In cases with distinct molecular targets, specific antibody based therapies, such as trastuzumab, are used as being most effective. Nonetheless, hormone therapy, as well as targeted anti-erbB2 therapy, is not always efficient and primary or secondary resistance systematically occurs. It is crucial to consider that hormone receptor status may change during disease progression.

Clear molecular differences have been reported between the primary tumor and the related metastasis (Raemaekers et al., 1984; Lower et al., 2005; Gomez-Fernandez et al., 2008; Liedtke et al., 2009; Aitken et al., 2010). Assessment of ERα and PR status is routinely performed by immunohistochemistry of the primary tumor to determine patient eligibility for adjuvant or palliative hormone treatments in breast cancer patients. In parallel, patients are selected for erbB2-targeted therapies using either immunohistochemistry or gene amplification by fluorescence *in situ* hybridization (FISH) on the primary tumor too. It is assumed that the primary tumor and metastatic disease share the same characteristics. But the progression of the disease may only be possible if new biological characteristics are acquired by few cells. Only those selected cells reach the blood circulation and elicit the metastatic process (Chambers et al., 2000). Moreover, those characteristics may be impaired during the treatment itself. The hormone and erbB2 status of the primary tumor may not be the best therapy response marker of metastatic cells and the identification of new therapy markers is essential (Dalenc et al., 2010). The material support of the markers may be selected in between the primary tumor and the metastases or micrometastases.

Circulating tumor cells issued from breast cancers

Circulating tumor cells (CTC) and disseminated tumor cells (DTC) in breast cancers

In solid tumors, most patients will not die from their primary tumor, but from distant metastases which may develop even years after treatment of the primary tumor. In breast cancer, for example, about one-third of axillary node-negative patients will develop local or distant metastases during the further course of their disease, even if there was no evidence of tumor spread beyond the breast at the time of primary diagnosis (De Vita, 1989; Rosner and Lane, 1993). We saw that the most important predictor of DFS and OS for breast cancer is still today the presence of axillary lymph node metastasis. Nonetheless, it appears that the presence of disseminated tumor cells (DTC) in bone marrow samples and of circulating tumor cells (CTC) in the blood can be a much earlier progression marker, and this not only in metastatic diseases. Indeed some of these CTC will be able to invade and create new metastasis.

Metastases are probably caused by occult haematogenous spreading of tumor cells early during the disease. Several studies support the hypothesis that isolated tumor cells (i.e. DTC or minimal residual diseases (MRD)) in bone marrow of cancer patients can be regarded as precursors of clinically manifest distant metastases (Cote et al., 1991; Harbeck et al., 1994; Diel et al., 1996; Landys et al., 1998; Mansi et al., 1999; Braun et al., 2000; Gebauer et al., 2001; Gerber et al., 2001). Thus, early detection of MRD in bone marrow has the potential of accurate risk stratification for subsequent therapy decisions before metastases development. While data on DTC in the bone marrow show good sensitivity and prognostic value of these cells in all stages of the disease, the bone marrow aspiration is an invasive procedure and is not widely used among clinicians. In contrast, peripheral blood would be an ideal source for the detection of tumor cells due to its easy sampling procedure and accessibility at any time of the disease, providing the equivalent of multiple serial biopsies.

CTC are defined as tumor cells circulating in the peripheral blood of patients, issued from either the primary tumors or the metastases. The dissemination of tumour cells to the blood is frequent after vascular invasion of the primary tumour and increases the risk for haematogenous metastases (McCulloch et al., 1995). However, the prognostic relevance of CTC in the peripheral blood of breast cancer patients is still under investigation (Pantel et al., 1999; Kostler et al., 2000). CTC detected in breast cancer patients are significantly associated with a worse outcome, for both localized and metastatic tumors (Cristofanilli et al., 2004, 2005; Rack et al., 2008; Daskalaki et al., 2009; Xenidis et al., 2009; Giordano et al., 2012; Giuliano et al., 2011; Hayashi et al., 2011).

Detection of CTC in early-stage and metastatic breast cancer patients

Many techniques have been developed in the last 20 years to detect, isolate and characterize CTC in cancer patients and especially in breast cancer patients. The major requisites are sensitivity, specificity and reproductivity (Ring et al., 2005; Tao et al., 2011). The main methods developed today are individual cytometric analysis using specific monoclonal antibodies against epithelial cells or reverse transcriptase polymerase chain reaction (RT-PCR) to amplify epithelial specific genes (Tao et al., 2011). In any case an initial enrichment step is necessary, with for example a size filtration, laser scanning cytometry (LSC) or the use of antibody-coated magnetic beads, as developed for the CellSearch System® (Veridex, New Jersey, US). This technique is the only one approved by the FDA (US Food and Drug Administration) at the moment and was used in the recent GeparQuattro and SUCCESS clinical trials, where CTC were prospectively monitored in neo-adjuvant and adjuvant therapy. It relies on the immunocapture of CTC using antibodies against the epithelial cell adhesion molecule (EpCam), expressed on the cell membrane of many epithelial tumor cells. This step is followed by proper nuclear staining (with DAPI, 4',6-diamino-2-phenylindole) and immunostaining of cytokeratins, which confirm the epithelial character, and of CD45 (positive only for leucocytes). Various studies demonstrated the relevance of this technique as the presence of elevated CTC levels negatively correlates with prognosis in patients with metastatic breast, prostate and colon cancers (Cristofanilli et al., 2004; Cohen et al., 2008; de Bono et al., 2008; De Giorgi et al., 2009; Liu et al., 2009). CTC detection has been proven so far in 10 to 20% of patients with early stage breast cancer (Pierga et al., 2008; Xenidis et al., 2009; Krishnamurthy et al., 2010; Rack et al., 2010; Riethdorf et al., 2010; Molloy et al., 2011) and in 40 to 80% of patients with advanced breast cancers (Cristofanilli et al., 2004; Budd et al., 2006; Molloy et al., 2011). The percentages considerably differ according to the sensitivity of each technique and need to be strictly defined in each case. CTC detection is associated with a worse outcome in breast cancer patients with both localized and metastatic breast cancers. CTC are now proven to be an independent predictor of OS and DFS for metastatic patients (Cristofanilli et al., 2004, 2005). CTC detection is proposed also as a potential marker of response to systemic chemotherapy (Pachmann et al., 2008). A cut-off of ≥5 CTC/7.5 mL whole blood cells has been accepted as being optimal to identify metastatic breast cancer patients with worse prognosis. In patients with early-stage breast cancer, much fewer studies have been performed but the prognostic significance of CTC could be assessed (Rack et al., 2008; Daskalaki et al., 2009; Muller and Pantel, 2009; Xenidis et al., 2009). For early stage breast cancer a lower cut-off of ≥1 CTC/7.5 mL whole blood cells has already been used (Riethdorf et al., 2010; Molloy et al., 2011). We recently suggested that using an even less stringent cut-off of ≥1 CTC/23 mL whole blood cells, early-stage patients (10% of stages I and II breast cancers reach the cut-off) do have a modest but statistically significant worse prognosis (Rack et al., 2010). Because of the lower number of CTC for those patients, techniques have to be exceptionally sensitive and specific. Any cut-off should be standardized for each approved assay.

It is noteworthy that EpCam expression may be down-regulated in cells displaying properties of epithelial mesenchymal transition (EMT), as occurs with increased metastatic ability (Bonnomet et al., 2010). Indeed, EpCam-based CellSearch System® was shown to be unable to detect a subtype of breast cancer, especially normal/basal genotype (triple negative phenotype) which has very low levels of EpCAM expression (Sieuwerts et al., 2009). As CellSearch System® may then underestimate the number of EpCam-expressing cells and most certainly of the CTC cells, other techniques are developed using, for example, immunocapture, size or migration differences (for review, (Hayes and Smerage, 2010)). Alternatively, reverse transcriptase polymerase chain reaction (RT-PCR) is widely evaluated as a sensitive approach to detect and estimate specific epithelial transcripts which should be specific of cancer cells and absent from normal haematopoietic cells, especially in breast cancers (Lee et al., 1987; Negrin and Blume, 1991). The AdnaTest BreastCancer® is a RT-PCR based technique that detects 3 tumor-associated transcripts after immunomagnetic enrichment of tumor cells. It has already be widely used in studies of CTC (Cristofanilli et al., 2004; Riethdorf et al., 2007; Fehm et al., 2009; Liedtke et al., 2009; Aktas et al., 2011) and was recently compared for erbB2 detection to the CellSearch System® in a large prospective multicentric study (Fehm et al., 2010). Overall agreement between the 2 techniques was 64%, which is low considering that the 2 techniques should detect erbB2 positive cells. Nonetheless, the cut-off has still to be defined, as the determination of erbB2 status in CTC has been based on an immunofluorescence staining score (Meng et al., 2004; Riethdorf et al., 2010). Another comparison of the CellSearch System® and AdnaTest BreastCancer® was performed with an 80% concordance rate using a lower cut-off of 2 CTC/7.5 mL for the CellSearch System® (Van der Auwera et al., 2010).

Extensive studies have only been possible with the requisite high sensitivity and reproductivity to detect and characterize even very small numbers of CTC in peripheral blood. Moreover, the global cost of the assays may be considered, as a CellSearch System® analysis may be 24 times more expensive than a quantitative multi PCR assay (600 US$ versus 25 US$ per sample (Kaiser, 2010; Molloy et al., 2011). As recently suggested (Wicha and Hayes, 2011), the lack of specificity may come not only from the technique but also from the biology of the tumors themselves. The

only presence of CTC then may not be the only criteria to consider but also their characterization in terms of markers such as ERα/PR, erbB2, EGFR, insulin-like growth factor receptor-1 (IGFR-1), urokinase-type plasminogen activator (uPA) (Hayes and Smerage, 2010) and stem cell markers which will be discussed below.

In any case, the individual analysis of the CTC of the protein levels of those markers is expected to be more informative than a global analysis that may mask relevant information. It can be now introduced as real-time biopsy to reassess predictive markers over the progression of the disease (Cristofanilli et al., 2004, 2005; Tewes et al., 2009; Swaby and Cristofanilli, 2011).

Hormonal status of circulating tumor cells

Evolution of the predictive markers during disease progression

Early markers of response to treatments are major issues to consider in cancer therapies, and especially hormone therapy. Hormone therapy or anti-erbB2 therapies are determined according to the hormone (ERα/PR expression) and erbB2 status of the initial tumor, but it appears that the CTC and consequently the metastatic cells may have a very different hormone and erbB2 status (for review (Arslan et al., 2011)). Unfortunately, the reassessment of the markers is not always performed because of technical limitations, specifically the location of the metastatic sites and the less reliable immunostaining of hormone receptors observed in needle-aspiration samples (Gong et al., 2004). An objective response rate of 30 to 40% is observed for hormone therapy of metastatic treatments. Since endocrine and growth factor signalings, including erbB2 and IGFR, are involved in invasion and metastasis processes, any change of those pathways may promote metastasis development and treatment resistance (Maynadier et al., 2008). Some of the CTC will be then able to invade and create new metastases. The emerging idea today is that only CTC that exhibit specific molecular characteristics, including specific stem cell markers, can drive metastasis development.

The comparison between primary breast tumors and related metastases showed 3 to 40% discrepancy in ERα/PR expression (Gomez-Fernandez et al., 2008; Liedtke et al., 2009; Aitken et al., 2010) and 7 to 26% in erbB2 expression (Tanner et al., 2001; Vincent-Salomon et al., 2007). A primary tumor and related nodes comparison was even able to demonstrate a shift to triple negativity of ERα/PR and erbB2 for 23% of the analyzed patients (Aitken et al., 2010). This common change from receptor positivity to triple receptor negativity has been confirmed with recurrent disease (Liedtke et al., 2009). Analyses of the discordances clearly proved the common loss of ERα/PR between the primary tumor and the metastases, which can explain the resistance to hormone therapy and poor outcome of some of the so-called «ERα/PR positive» patients. Regarding early stage breast cancers, much fewer studies could analyze the evolution of receptor expression between the primary tumors and DTC or micro-metastases, because of obvious methodological limitations.

Specific evolution of the predictive markers in CTC

The German laboratories of T. Fehm in Tübingen, B. Aktas in Essen and other collaborators (including ours) already produced some exciting literature in the last few years on the evolution of ERα, PR and erbB2 between the primary tumors and/or the CTC and DTC. They first established in a large cohort of 254 primary breast cancer patients that primary tumor and DTC from the bone marrow display only 28% of concordance for ERα status, with only 12 of 88 patients with ERα positive tumors that also had ERα positive DTC (Fehm et al., 2008). In this study, an original double immuno-fluorescence staining procedure was used to visualize and quantify ERα levels and cytokeratin presence on cytospins prepared from patient bone marrow aspirates. This technique visualizes the expression of the markers at the protein level, for each individual cell. They then studied a cohort of 431 primary breast cancer patients the concordance rates for ERα, PR and erbB2 status between the primary tumors, the CTC (in 58/431, i.e. 13% of patients) and the DTC (in 107/414, i.e. 24% of patients) from bone marrow using a RT-PCR approach (AdnaTest BreastCancer® (Fehm et al., 2009). The primary tumors and CTC have a concordance rate of 29, 25 and 53% respectively for ERα, PR and erbB2 status. The following changes were observed between the primary tumors and the CTC: a decrease from 78 to 25% of positivity for ERα, from 71 to 4% for PR and an increase from 16 to 38% for erbB2. Interestingly, the proportion of triple receptor negative rose from 15% for the primary tumors to 50% for the CTC, suggesting that CTC mostly derive from triple negative tumors. Besides, a weak concordance was observed between the hormone status of CTC from peripheral blood and DTC from bone marrow, and the DTC are less related to the biology of the primary tumor than the CTC. This implies that the clinical follow-up of these early stage breast cancers will be correlated to the assessment of the CTC analysis to elucidate their prognostic significance. The previous results have been extended from primary breast cancer patients to 193 metastatic patients with the same AdnaTest BreastCancer® (Aktas et al., 2011). The overall detection rate for CTC was 45% (87 patients) with expression rates of 19, 10 and 48% for respectively ERα, PR and erbB2. 77 and 87% of the primary tumors lost ERα and PR expression respectively. 45% of the CTC were triple negative (versus 17% in the related primary tumors), 32% only erbB2 positive (versus 8%) and 23% ERα and/or PR positive (versus 75%). We could then confirm that a high proportion of the CTC were ERα/PR negative despite the presence of ERα/PR positive primary tumors. An additional fourth study

extended the results to 254 metastatic patients to detect erbB2 in CTC by comparing the 2 reference techniques which are the CellSearch System® and the AdnaTest BreastCancer® (Fehm et al., 2010). Using the CellSearch System®, 50% of the patients were CTC positive (≥5 CTC / 7.5 mL blood) with 41% of erbB2 positivity (≥1 CTC / 7.5 mL blood). Using the AdnaTest BreastCancer®, 39% patients were CTC positive with 47% of erbB2 positivity. In both analyses, the rate of breast cancer patients with erbB2 negative tumors that shifted to erbB2 positive CTC was significant (32 and 49% respectively), with an identical 50% rate for patients whose erbB2 determinations were concordant (Somlo et al., 2011) with both techniques.

An interesting new approach used a fiber-optic array scanning technology (FAST) to detect cytokeratin presence, nuclear staining, absence of CK45 and expression of erbB2 and ERα in the primary tumors and CTC, for 26 patients with metastatic or locally advanced/inflammatory breast cancer. Considering metastatic patients only, 33% of ERα positive tumors lost ERα expression in the CTC and 23% of erbB2 negative tumors gained erbB2 expression. Surprisingly, 60% of ERα negative tumors gained ERα expression in the CTC and 60% of erbB2 positive tumors lost erbB2 expression. All these changes in receptor status were significantly increased between metastatic patients and locally/advanced inflammatory breast cancer patients. These results (with a very limited number of cases for some percentages) are slightly different but converging with the results already published. If the protein expression at the level of each individual CTC is analyzed with a sensitive original technique, the detection limits may be lower and bring new insights to the result interpretations. Indeed, cells with normal/basal genotype (triple negative phenotype) express very low levels of EpCAM and may not be detected with the CellSearch® technique (Sieuwerts et al., 2009).

CTC as predictive markers of therapy response

CTC have been recently analyzed in a cohort of 235 metastatic breast cancer patients demonstrating that with a median follow-up of 18 months, the CTC count was confirmed to be a robust prognostic marker in the overall population. Conversely, in patients with erbB2 overexpressing tumors receiving trastuzumab or lapatinib, the baseline CTC count was not prognostic (Giuliano et al., 2011). Nonetheless, in patients with erbB2 normal tumors, a baseline CTC count ≥5/7.5 mL identified subjects who benefitted from more aggressive treatments, including combination chemotherapy and chemotherapy plus bevacizumab. If the CTC count can help in patient stratification and therapeutic selection, we think that erbB2 expression in CTC could be even more relevant. A study of 52 metastatic breast cancer patients has recently been published with a median follow-up of 21 months, showing that erbB2 positivity of the CTC at first follow-up may be a prognostic factor in terms of DFS and OS (Hayashi et al., 2011).

Altogether, the literature shows that CTC and DTC clearly express less ERα/PR and more erbB2 than the primary tumor cells, and this may explain some of the resistance to hormone therapy or anti-erbB2 treatment. An optimal individualized treatment could then be obtained by characterizing ERα/PR and erbB2 status in the CTC and comparing it to that of the primary tumor. The predictive value of the hormone receptor and erbB2 profile of CTC for both adjuvant and palliative targeted therapies has to be evaluated further. Most reports study only mRNA expression of potential markers in CTC and DTC, but it is of great importance, for ERα and PR, to study the effective protein expression in CTC cells, in the same way as it is performed by immunohistochemistry for the primary tumors at the time of the diagnosis. A major point is now to define a gold standard technique in the clinical CTC study settings.

Relevance of cancer stem cell markers

Cancer stem cells (CSC) and their markers

It is now widely accepted that cancers are composed of heterogeneous populations of cells. Tumors classified as hormone receptor positive may contain different proportions of ERα and PR positive cells, of responsive and less responsive cells to the treatments, and of spreadable and less spreadable cells (for review, (Visvader, 2009)). This is considered as intratumoral heterogeneity, where cancer stem cells (CSC) can specifically support therapy resistance and metastasis. Solid tumor stem cell biology is now in the forefront of clinical oncology because of the identification and analysis of new identified stem cell markers that are putative biomarkers for prognostic and therapy choice. Here again, development of such biomarkers in clinics needs robust, reproducible and validated technologies (review in (Woodward and Sulman, 2008)).

Stem cell are present in small proportions in acute myelogenous leukaemia, as well as in many solid tumors (for reviews, (Visvader and Lindeman, 2008; Cheng et al., 2011)), including breast cancers (Al-Hajj et al., 2003). They are multipotent single cells capable of recapitulating the heterogeneity of the tumor from which it was derived. They are able to proliferate, self-renew and differentiate into the various cell types seen in the bulk of the tumor. They are then considered as tumor-initiating cells even though they may need the complex interplay relationships with the microenvironment, e.g. the stroma cells. Stem cell markers are surface markers that should reliably identify those characteristics. For some solid tumors, a specific pattern of cell surface markers (also called progenitor markers) is emerging, without consideration of the stroma influence. The following antigenic pattern has been repeatedly used and often identified in the CSC issued from breast cancers: $CD44^{+}/CD24^{low}/ESA^{+}$ (epithelial specific antigen) and lack of specific lineage markers: Lin^{-}. A generally

observed trend is the negative correlation between $CD44^+/CD24^{low}$ and erbB2 expression. The comparison of a gene expression analysis of $CD44^+/CD24^{low}$ cells from mice xenografts with cells from normal mammary epithelium yielded a gene signature that predicted DFS and OS in breast cancer (Liu et al., 2007). Those encouraging results were difficult to extend to human tumors (Abraham et al., 2005; Woodward and Sulman, 2008). A major issue is the heterogeneity of CSC themselves in one single tumor and among different breast tumor types. The profiling of 9 known solid CSC markers clearly demonstrated the multiple lineage of human breast CSC originating from 8 breast cancer cell lines and 19 clinical specimens (Hwang-Verslues et al., 2009). The widely varying marker expression in the tumors strongly suggests that the initially emphasized markers identify the highly tumorigenic cells but should be completed by other markers. Basal-like breast cancers are clinically associated with the triple negative cells and they are composed mainly of cells expressing the CSC markers $CD44^+$ and cytokeratin 5/6 (Fulford et al., 2007; Polyak, 2007; Shipitsin et al., 2007). Inversely, $CD44^+/CD24^{low}$ cells are common in basal-like tumors and strongly associated with Brca1 hereditary breast cancers, but not every basal breast tumor contains $CD44^+/CD24^{low}$ cells (Honeth et al., 2008). Moreover, the presence of $CD44^+/CD24^{low}$ cells does not correlate with clinical outcome (Abraham et al., 2005; Shipitsin et al., 2007).

Since these markers are not universal, they are now being completed and/or replaced by other additional markers such as ALDH1, CD133 and PROCR which are already recognized as CSC markers for other solid tumors (Ginestier et al., 2007; Shipitsin et al., 2007; Wright et al., 2008). Aldehyde dehydrogenase (ALDH1), a detoxifying enzyme responsible for the oxidation of intracellular aldehydes, was increased in a subpopulation of both normal and cancer human mammary epithelial cells that exhibit stem cell properties (Ginestier et al., 2007). Although the ALDH1 phenotype correlated with the clinical outcome, only 30% of breast tumors contained $ALDH1^+$ cells and no association with a particular molecular subtype of cancer could be observed.

CD133 (Prominin-1 or AC133) is a pentaspan transmembrane glycoprotein initially considered as a marker of hematopoietic stem cells (Miraglia et al., 1997). It then appeared to select a population of tumor initiating, treatment resistant cells in cells derived from high grade glioma, but is now a known marker of CSC in several tumors originating in brain, blood, liver and prostate (Vercauteren and Sutherland, 2001; Al-Hajj et al., 2003; Singh et al., 2004; Collins et al., 2005; Yin et al., 2007). It was identified from breast cancer stem cells isolated from cell lines generated from $Brca1^{2exon11}/P53^{+/2}$ mouse mammary tumors (Wright et al., 2008). It was recently shown to be overexpressed in 43.3% (29/67) of triple negative tumors and that this expression correlates with tumor size, clinical stage and lymphatic metastasis, OS and DFS (Zhao et al., 2011).

Cancer stem cell characteristics of CTC

CSC are considered as relatively resistant to both chemotherapies and radiotherapies and may then largely contribute to treatment failure and relapse (Li et al., 2008; Creighton et al., 2009; Wicha and Hayes, 2011). Moreover, they exhibit invasive and metastatic features in cell models (Charafe-Jauffret et al., 2009). In metastatic breast cancer patients, stem cell markers such as $CD44^+/CD24^{low}$ (Aktas et al., 2009) or ALDH1 (Aktas et al., 2009; Fehm et al., 2009) have been shown to be frequently overexpressed in CTC. ALDH1 overexpression was found in approximatively 70% of the blood samples also positive for CTC (Fehm et al., 2009).

Altogether, these data suggest that, like the micrometastases, CTC may be naturally enriched in CSC, supporting the invasive ability of the primary tumors (Balic et al., 2006). The key element of CTC analysis would then be the circulating tumor stem cells (CTSC). This suggests that the number of total CTC of patients should be completed by the assessment of their expression of most relevant stem cell markers.

Among the validated markers, $CD44^+$ and $ALDH1^+$ are expressed in haematopoietic stem cells and can only be used for potential CTSC if an efficient elimination of the leukocytes has been initially performed. A negative selection was performed in the 2 cited studies (Aktas et al., 2009; Fehm et al., 2009), using antibodies against CD45, and a haematopoietic specific antigen.

As described above, CD133 is expressed in a number of solid tumor stem cells. It has recently been reported and discussed a mRNA detection of CD133, cytokeratin and carcinoembryonic antigen (CEA) in CTC from 315 colorectal cancer patients (Iinuma et al., 2011). The $CD133/CK/CEA^+$ patients exhibited a worse OS and DFS than the $CD133/CK/CEA^-$ patients. This result was strictly limited to the less favourable Dukes' stage B patients and to stage C cancer patients. This study suggests that the stem cell character of the CTC may be associated to both a poor prognosis and resistance to therapy.

This is a very relevant hypothesis that has to be considered and evaluated in the near future, both for colorectal and breast cancer patients, and both for palliative and adjuvant therapies. The molecular signature of the small population of CTSC with highly tumorigenic activity is crucial. The analysis should be at a single cell level of the CTC to avoid haematopoietic stem cell contamination, without relying only on EpCam selection. It should focus on the determination of the real protein expression of the markers and not only of their transcript levels.

Conclusion

In future, clinical studies should not only answer questions about the therapeutic efficacy of certain therapy regimes, but should also enhance the understanding of tumor biology. Clinical studies can serve as a perfect basis for tumor biological analyses

with optimized statistics and documentation.

Ambitious joined translational programs have then been designed, which include some promising novel prognostic and predictive markers.

While data on DTC in the bone marrow shows good sensitivity and prognostic value of these cells in all stages of the disease, bone marrow aspiration is an invasive procedure. In contrast, peripheral blood would be an ideal source for the detection of tumor cells due to its easy sampling procedure and accessibility at any time of the disease. However, more data are necessary on the detection and prognostic relevance of CTC in peripheral blood.

As an attempt to meet the crucial need for identification of the sub-population of patients that will benefit from more individualized therapies, rapidly evolving therapies should allow a molecular profiling of the tumors and/or of the CTC. The literature clearly shows now that reliance on the phenotype of the primary tumor can be misleading, as ERα, PR and erbB2 status are often differentially expressed on CTC compared to the primary tumor. The hormone receptor status of CTC is a very challenging issue if it allows adjusting the choice of anti-hormone therapies (Nolvadex®, Fulvestrant®, anti-aromatase) and anti-erbB2 therapy (Herceptin®) for each patient. CTC may then appear as new prognosis and treatment marker for both metastatic and adjuvant breast cancers.

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