Circulating Tumour Cells in Breast Cancer – Review

Cirkulujúce nádorové bunky u rakoviny prsníka – prehľad

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Summary
Disseminated malignancies are responsible for the majority of cancer-related deaths. During the metastatic process, circulating tumour cells (CTCs) are generated. The presence of CTCs, epithelial cells found in the peripheral blood, is an essential step in establishing distant metastases. Circulating epithelial cells have the morphology of malignant cells and their number in the blood correlates with tumour burden. To identify CTCs in peripheral blood, two major approaches are used involving additional antibodies and nucleic acid-based techniques. Tumour cells with HER-2 overexpression are frequently resistant to cytotoxic drugs and radiotherapy. Wider clinical application of the detection of minimal residual disease is partly limited by the lack of standardized methods for detection. Recent studies suggest that in addition to the prognostic significance of tumour cells, determination of CTCs may be important in therapy monitoring or as potential targets for targeted therapy. Persistence of minimal residual disease after primary treatment may be an indication for extensive adjuvant treatment in order to prevent relapse of the disease. Detection of CTCs and the use of prognostic markers such as HER-2 overexpression may help us to better understand the biology and clinical significance of the presence of CTCs in breast cancer patients.

Key words
metastatic breast cancer – circulating tumour cells – minimal residual disease

Súhrn
Diseminované malignity sú zodpovedné za väčšinu úmrtí na rakovinu. Cirkulujúce nádorové bunky (circulating tumor cells – CTC) sú generované počas metastatického procesu. Prítomnosť CTC, epiteliálnych buniek nachádzajúcich sa v periférnej krvi, je povinný krok rozvoja vzdialených metastáz. Cirkulujúce epitelové bunky majú morfológiu malígnych buniek a ich počet v krvi koreluje s rozsahom nádorovej choroby. Na detekciu CTC v periférnej krvi sú využívané dva hlavné prístupy:1) metóda využívajúca reakciu s protilátkou a 2) na detekcii nukleových kyselín založené techniky. Nádorové bunky s HER-2 overexpresiou sú často rezistentné na cytotoxickú liečbu a rádioterapiu. Širšie klinické použitie detekcie minimálnej zvyškovej choroby je čiastočne obmedzené nedostatkom štandardizovaných metód detekcie. Nedávne štúdie naznačujú, že okrem prognostického významu nádorových buniek môže byť ich stanovenie dôležité pri liečbe a následnom sledovaní pacienta, alebo ako potenciálne ciele pre cielenú terapiu. Perzistencia minimálnej reziduálnej/zvyškovej choroby po primárnej liečbe môže byť indíciou na podanie rozsiahlej adjuvantnej liečby, aby sa zabránilo relapsu ochorenia. Detekcia CTC a využívanie prognostických markerov, ako je HER-2 overexpresia, môže napomôcť lepšie pochopiť biológiu a klinický význam prítomnosti CTC u pacientov s karcinómom prsníka.

Kľúčové slová
metastatická rakovina prsníka – cirkulujúce nádorové bunky – minimálna reziduálna/zvyšková choroba

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Introduction

Minimal residual disease is defined as the persistence of tumor cells that remain in the body after so-called curative resection of the primary tumor. These cells may lead to a relapse of the disease months or even years after primary treatment. Correlate of minimal residual disease in the biological material is the presence of tumor cells, which can be detected using histogenetic markers in mesenchymal organs (for example bone marrow, blood and lymph nodes). The presence of these cells in the bone marrow and lymph nodes is generally associated with shortened overall survival in malignant diseases [1]. Patients with cancer at an early stage with good prognostic features, often suffer relapse of the disease despite succesfull primary treatment. The cause of early relapse is the subject of many studies. Disseminated malignancies are responsible for the majority of cancer related deaths [2]. Approximately 25% of breast cancer patients without lymph node metastases develop systemic relapse. It is assumed that metastases are caused by spread of tumor cells in a relatively early stage of the disease [3]. Micrometastases are stroma containing organized group of cells, infiltrating into the capillary system from the primary tumor. As micrometastases are groups of cells labeled by size 0,2–2,0 mm in greatest dimension, they are detecable in lymph nodes by using molecular-diagnostic methods [4]. Several studies support the hypothesis that minimal residual disease may be considered a precursor of clinically overt distant metastasis of solid tumors [6]. Genetic variability and genome instability of the precursors of distant metastases in a state of minimal residual disease has been demonstrated by extensive clinical studies. It seems that the selection of clonally expanding cells forming metastases takes place after the start of the process of dissemination. This story is the reason that modalities of adjuvant therapy are often confronted with an extremely large reservoir of genetically variable cells, which may become resistant cells in the course of therapy [6]. A growing body of evidence supports the notion that hematogenous dissemination of breast cancer occurs independently of lymphatic spread of disease, however current clinical practice does not involve routine analyses of circulating tumor cells (CTCs) or disseminated cells [7]. During the metastatic process, CTCs are generated. The presence of CTCs, epithelial cells found in the peripheral blood, is a mandatory step in establishing distant metastases [2]. Disseminated tumor cells (DTCs) are cells which originate from the primary tumor and persist in an isolated form in the bone marrow. Bone marrow is a well vascularisated tissue, an ideal incubator in which disseminated cells may stay in a dormant stage, or stage of development [8]. To date, there is no clear correlation between CTCs from peripheral blood and disseminated tumour cells (DTCs) in bone marrow. However, the evidence of DTCs in bone marrow is an independent prognostic factor in patients with carcinoma of breast [9–10], lungs [11] and colon and rectum [12]. Peripheral blood is one of the most important diagnostic specimens and appears an ideal source for monitoring CTCs. Peripheral blood sampling can be done at frequent intervals [13]. Prognostic value of CTC detection in peripheral blood is demonstrated in patients with metastatic disease [14].

Detection methods

Many recently published studies have documented that both CTCs within the blood and DTCs in bone marrow can be identified using a variety of techniques [7]. Detection and characterization of tumor cells is due to their low concentration in the bone marrow and peripheral blood, the frequency of about 1×10^{-5} to 1×10^{-6}, a relatively complicated process. Furthermore, there are several cell contaminants present in tissues (stem cells and other epithelial cells in bone marrow) [15]. There is a heterogeneous group of malignant cells in breast tumors with varying degrees of expression of tumor specific markers. Therefore, CTCs found in one patient, must not express the particular type of marker, which is detected by chosen detection method. The ideal marker for identifying tumor cells should be universal and uniformly expressed in all cells of malignant breast tumors. Yet been identified, no marker, meets these criteria. In order to increase the detection rate there must be a suitable multimarker method [16]. The methods for identifying CTCs should distinguish between epithelial and other (mainly hematopoetic) cells [13]. As a first step of detection process, enrichment (increasing the number of tumor cells) of the sample is necessary [15]. Molecular studies of CTCs require efficient pre-enrichment steps to obtain a pure population of target cells for further characterization [17]. There are three main enrichment methods – density gradient separation, filtration and imunomagnetic separation [15]. To identify CTCs in peripheral blood, two major approaches are used involving additional antibodies and nucleic acid based techniques [13]. These methods differ in their sensitivity and specificity [16]. The principle of imunocytochemistry using methods (imunocytochemistry, flow cytometry and laser scanning cytometry) is detection of epithelial epitopes and cell surface structures or structures inside the cell, using antibodies that do not react with mesenchymal cells. Metastatic tumor cells exhibit a remarkable heterogenenity in the expression of tumor-associated cell surface molecules, which may affect the effectiveness of this method in the determination of CTCs. Cytometry is now becoming a standard diagnostic method. Cell populations can be studied either by the method of flow cytometry or using a laser scanning cytometry (LSC). The analysis principle of these two methods is essentially the same. While in flow cytometry a suspension of cells is used, LSC allows investigation of cells after immobilization on a conventional microscope slide as well as investigation of in paraffin embedded biopsy samples of tissue [18]. The most suitable method for visualization of CTCs, applied after pre-enrichment, is LSC. LSC has been used for morphological and cytometric analysis of the cells since the beginning of the 90s [18]. The use of LSC for analysing cells on membrane made it possible to simplify the preparation of CTCs and to use cytometric analysis of isola-

ted cells [19]. A new method of intravital flow cytometry allows noninvasive monitoring of circulating tumor cells in vivo during their circulation in the peripheral circulation. Theoretically, non-invasive monitoring in real time can improve detection impressionability allowing analysis of significantly larger volumes of blood, potentially even the entire blood volume. To date, however, the analysis demonstrated success only in the ex vivo labeling of tumor cells prior to intravenous administration. No method for in vivo labeling and quantification of CTCs in clinical use has not yet been developed [20]. Molecular detection methods detect circulating DNA using determination of specific stretches of DNA, or using recognition of messenger RNA (mRNA) and the detection of specific gene expression. Polymerase chain reaction (PCR) method and reverse transcriptase polymerase chain reaction (RT-PCR) and its variations are often used in this case [5]. A significant correlation between the presence of circulating DNA in peripheral blood and poor prognosis was described in breast cancer [21]. DNA of tumor cells can be amplified by PCR, which can prove the presence of even a very small number of tumor cells with a tumor DNA in a heterogeneous population of cells. However, DNA in human tissues is relatively stable, and moreover, fraction of circulating tumor DNA varies between patients. Therefore, detection and quantification of circulating tumor DNA may not be indicative with regard to the content of viable tumor cells, and also the extent of metastatic disease [16]. Excellent analytical impressionability of RT-PCR allows the detection of tumor-associated mRNA expression in single tumor cells from 10^6 to 10^8 blood leukocytes. The development of new technological processes allows to obtain results within a few hours [4].

Pitfalls of detection

Apoptoic cells

CTCs are very unefficient in causing metastases [13]. Not all cancer cells detected in peripheral blood and bone marrow, carry metastatic potential, ability to form metastases retain only viable cells [22]. Many CTCs are apoptoic cells which are detectable but cannot cause spread of the disease [13]. It is difficult, but using new experimental methods possible to differentiate cells that die because of apoptosis, necrosis or other processes, from cells, which hold the metastatic potential. M30 assay and M65 assay are relatively new methods, so called sandvich ELISA tests, which allow the determination of circulating forms of protein – cytokeratin 18 (CK18) in plasma or serum of patients with breast cancer. They are proposed as suitable biomarkers of different mechanisms of cell death [23]. In the process of detection of tumor cells in the peripheral blood or in the bone marrow, the question arises how to prove that these cells are tumor cells and not normal epithelial cells in healthy tissues. Circulating epithelial cells have the morphology of malignant cells and their number in the blood correlates with tumor burden. The evidence that these cells are malignant, is essential for a definitive interpretation of the data and decisions that affect the choice of treatment regimen [22].

CTCs that escape conventional detection

Epithelial-mesenchymal transition (EMT) is defined by the loss of epithelial characteristics and the acquisition of a mesenchymal phenotype [24]. For epithelial malignancies, the epithelial-mesenchymal transition (EMT) is considered to be the crucial event in the metastatic process, which involves the disruption of epithelial cell homeostasis and the acquisition of a migratory mesenchymal phenotype allowing these cells to travel to the site of metastasis formation without being affected by conventional treatment [25–26]. These cells have reduced apoptosis and are drug resistant. A recent report suggests that a direct link might exist between the EMT and the acquisition of stem cell properties [27]. CTCs might be identified partly as cancer stem cells because of similarities such as increased resistance to chemotherapy and decreased proliferation during circulation. Similar findings were reported for DTCs in bone marrow, where tumor cells with a stem cell-like phenotype were demonstrated [28]. Corresponding experimental results for CTCs are still outstanding but preliminary data, presented at the annual meeting of the American Association for Cancer Research 2008, identified a breast cancer stem cell-like phenotype in blood samples of patients with breast cancer [29–30]. In carcinoma cells, EMT can be associated with increased aggressiveness, and invasive and metastatic potential. Sarrio and collegues assessed the occurence of EMT in human breast tumors, using a tissue microarray-based immunohistochemical methods in invasive breast carcinomas and carcinosarcomas using 28 different markers. Up-regulation of EMT markers (vimentin, smooth-muscle-actin, N-cadherin, and cadherin-11) and overexpression of proteins involved in extracellular matrix remodeling and invasion (SPARC, laminin, and fascin), together with reduction of characteristic epithelial markers (E-cadherin and cytokeratins), was preferentially observed in breast tumors with the „basal-like phenotype." In summary, their data indicate that EMT likely occurs in tumors with the basal-like fenotype, a specific genetic context and suggest the relation to the high agressiveness and metastatic spread of these tumors [24]. According to data presented by investigators from the University of Texas M. D. Anderson Cancer Center, at the annual CTRC-AACR San Antonio Breast Cancer Symposium, 2009, USA., CTCs that undergo epithelial-mesenchymal transition (EMT), resulting in a loss of epithelial markers, may escape conventional epithelial-markers associated detection methods. It is assumed that cells with EMT phenotype are involved in tumor dissemination and represent tumor initiating cells. Data shown during presentation suggest that currently used detection methods may underestimate the most important subpopulation of CTCs, which are involved in tumour dissemination in metastatic breast cancer. During retrospective evaluation of the outcome of breast cancer patients, three subgroups of patients with poor prognosis were observed. Patients with brain metastasis, triple negative tumors or inflammatory breast cancer despite of poor prognosis had low or undetectable

CTCs using conventional detection methods. Scientists suspect that these low or undectable levels of CTCs were due to existence of a subpopulation of cancer cells that underwent the process of EMT. During the process the cells lose their epithelial receptors. They cannot be no longer detected by detection assays as they use epithelial receptors to identify the cells. Cells without epithelial receptors become resistant to chemotherapy and radiotherapy. Man can hypothesize that CTCs after the process of EMT are responsible for tumor dissemination. Novel detection method capable of identifying these special cells in peripheral blood of breast cancer patients was introduced by researches from M. D. Anderson Cancer Center. In their prospective study they isolated CTCs from peripheral blood of breast cancer patients in all stages of disease (including patients with poor prognosis) using magnetic beads coated with monoclonal antibodies. Following the process of isolation using a PCR they managed to isolate RNA and detect genes involved in EMT. EMT genes were more commonly overexpressed in patients who had triple-negative breast cancer or in by neoadjuvant chemotherapy pretreated patients who have developed resistence to therapy. This new detection method could bring an important prognostic information and could be useful for monitoring treatment efficacy. A confirmatory study in metastatic breast cancer patients to confirm their findings, was also initiated. Ongoing research at The University of Texas M. D. Anderson Cancer Center and the National Cancer Institute in Bratislava aimes the detection of CTCs with tumour-initiating properties, and identification of potential therapeutic targets for CTCs. Identification of therapeutic targets could help to eradicate the micrometastatic disease in breast cancer and epithelial tumors [31].

Clinical use

Surgical treatment

Surgical treatment of malignant breast tumors is performed with the intent to eliminate as much as possible of the tumor mass. Subsequently applied adjuvant systemic therapy should eliminate malignant cells, which were not removed surgically [32]. It was shown that routinely used surgical treatment increases the number of CTCs, which persist in the bloodstream for a long time. Camara [32] presented a new knowledge about the detection of disseminated cells before, during and after surgical intervention. Tumor cells were present in the peripheral blood of patients already before surgery. Increase in their number occurred despite the R0 resection of the primary tumor and the level of epithelial cells was stable even after long time, regardless of the size of the tumor, despite the application of adjuvant therapy. Part of the circulating cell survives long in bloodstream. It is assumed, that these are cells capable of aggressive growth, which contribute to formation of metastases. This hypothesis was confirmed by monitoring of relapsed patients, who had rising concentrations of tumor cells [16]. Even regressive tumors exclude tumor cells the circulation to the same extent as progressive tumors. Most of these cells, however, undergoes apoptosis induced by cytostatic therapy. Theoretically, if complete remission of solid tumor indicates the eradication of disease, in patients with complete remission should be no detectable tumor cells, and they should not develop metastatic disease. Since the primary tumor is removed at surgery, one of the sources of disseminated metastatic cells is the bone marrow, as a reservoir of malignant cellls. Disseminated tumor cells have the ability to live in the peripheral circulation, despite the loss of intercellular matrix and are apparently resistant to systemic therapy, thus fulfilling the two basic conditions for metastatic cascade.

Neoadjuvant and adjuvant therapy

Determination of the status of bone marrow after neoadjuvant therapy may identify patients who are at high risk of developing metastatic disease. Moreover, bone marrow examination for the presence of persistent tumor cells, could also help to determine the subgroup of patients who require secondary treatment. Secondary treatment, for example on the basis of targeted agents and the antibody-based strategies, can help to complete the eradication of minimal residual disease [22]. Cancer can relapse after long time from primary diagnosis and assessment of primary tumor removal. The presence of circulating tumor cells in patients in long-term remission, which are considered to be cured, can represent new knowledge and provides new insight into the mechanism of the process of metastasis. Some of the detected cells survive in an organism in dormant status and can acquire aggression after a long latency time [15]. In some patients, disseminated tumor cells are not eliminated by administration of chemotherapeutic agents. The reason may be that only a minority of these cells is in the growth phase of the cell cycle. Majority of these cells are dormant cells, remaining in G0 phase of cell cycle, which is demonstrable even by negativity of Ki67 as a proliferation marker [2]. Pachman [33] presented, that in patients treated with neoadjuvant chemotherapy, already the first cycle of chemotherapy had an effect on the number of CTCs in peripheral blood. Response of CTCs to therapy was the same as the response of primary tumor cells, thereby confirming the assumption that these cells in untreated patients come from the primary tumor. Correlation between reduction in CTCs and ultimately reducing the size of the tumor indicates, that monitoring the number of CTCs during first cycles of therapy, may help to predict the adequate response to treatment. Thus, toxicity which could accompany inefective treatment, can be avoided. The authors also mention, that by determination of prognosis would not be appropriate to take into account the total number of tumor cells in the blood of the patient, but rather the behavior of these cells (increase/decrease in the number) in the course of therapy [33].

Targeted therapy

The HER-2 gene is amplified in 20–25% of invasive breast cancer. Survival rates are worse in patients whose tumors carry the HER-2 gene amplification [34]. Tumor cells with HER-2 overexpression are frequently resistant to cytotoxic

drugs and radiotherapy [35]. HER-2 status is often assessed in primary breast tumors, and this information is used to make therapeutic decisions in the metastatic setting. The concordance between HER-2 expression in primary tumors and distant metastases is ranging between 80 and 94%. It is thought that a clinical disconcordance may be the result of clonal selection or the upregulation of HER-2 during the metastatic process [34]. The HER-2 status of distant metastases is often not evaluated [35]. HER-2 gene amplification can be accurately measured in individual cells and this can be used in determining the HER-2 status of circulating tumor cells [34]. Analysis of HER-2 status of CTCs may help to identify high-risk patients who could benefit from targeted anti-HER-2 therapy [35]. Also, the presence of HER-2 positive CTCs in women with non-metastatic disease could be a specific indicator of the presence of hidden metastases. CTCs could present a suitable marker for micrometastatic disease detectable long time before macroscopic detection of metastatic process. Their detection may influence therapeutic decisions by the addition of targeted therapy in the adjuvant administration. Determination of HER-2 status of tumor cells in real time and repeatedly during the course of the disease may help to monitor the disease because of the fact that HER-2 status may change as a result of the ongoing genetic changes in circulating tumor cells [36]. Targeted therapy using trastuzumab following detection of HER-2/neu amplification on CTCs represents one of new therapeutic modalities [31].

Metastatic breast cancer

The incidence of metastases in patients with negative lymph nodes at the time of diagnosis points to the possibility that cancer cells appear to circumvent the lymph nodes and diseminnate directly hematogenous to distant organs [36]. Early detection of minimal residual disease has the potential to improve the risk stratification in subsequent therapeutic decisions, or even in adaptation, eventually adding conventional or targeted therapies to eradicate these cells before the development of distant metastases. The results indicate poor prognosis in patients with present CTCs at the time of the primary diagnosis and primary treatment [36]. As CTCs are an important predictor of survival in metastatic breast cancer patients, the presence of an increased number of these cells is associated with poor prognosis [31]. The detection of CTCs in patients with metastatic breast cancer, who are about to start a new line of treatment, has been shown to predict progression-free survival and overall survival and treatment benefit. Therefore, the measurment of these cells has a potential as a surrogate marker for monitoring anti-angiogenic treatment and drug activity, and could help determine the optimal dose of drugs used in medical oncology [37].

Conclusion

Wider clinical application of detection of minimal residual disease is partly limited by the lack of standardized methods for detection. Furthermore, clinical studies, which would demonstrate a significant benefit of real therapeutic interventions based on evidence of tumor cells in bone marrow and peripheral blood are still lacking. Recent studies suggest that in addition to prognostic significance of tumor cells, determination of CTCs may be important in therapy monitoring or as potential targets for targeted therapy. Persistence of minimal residual disease after primary treatment may be an indication for extensive adjuvant treatment , in order to prevent relapse of disease. Detection of CTCs and the use of prognostic markers such as HER-2 expression may help to better understand the biology and clinical significance of the presence of CTCs in breast cancer patients [36]. Very provocative theory is that the persistent subpopulation of tumor cells could be seen as cancer stem cells. They are dormant cells, but also retain the ability to grow in spite of the applied treatment. Identification and characterization of this cell subsets may, in the future, help to better understand the mechanism of tumor cell growth and metastatic disease. Moreover, the development of new drugs, based on the results of pre-clinical and clinical studies, can help to optimize treatment strategies, if the assumption of direct role of CTCs in metastatic process is confirmed [22]. In general, new markers like for example presence of DTCs in bone marrow and CTCs in peripheral blood may have a role as prognostic factors indicating a possible further development of the disease, or predictive factors which have a role with respect to the expected response to therapy. Moreover, new markers themselves can serve as targets of a new biological treatment. While it will be possible to introduce these new markers in the management of patients, it is required to meet certain specified criteria, especially in methods of detection [5]. Methods for determination must be standardized and must have guaranteed quality. The clinical relevance must be proven by independent clinical studies. In addition, a new marker has to be clinically usable as part of therapeutic decision independently of the existing markers.

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