Detection of circulating tumor cells in non-small cell lung cancer.

Abstract
In recent years, new insights into cancer organization have changed its definition from a single- towards a multi-organ disease. It is nowadays characterized by a primary tumor site with secondary spread to distant organs. The treatment for NSCLC in specific, which is a prominent cause of cancer-related deaths, consists of surgical resection of the tumor. However, patients who are cleared from the primary cancer are not guaranteed to stay cancer-free for life and distant organ metastasis and minimal residual disease comprise serious threats. The identification and characterization of circulating tumor cells (CTCs) could play an important role for diagnostic and treatment purposes. CTCs are shed from either the primary tumor or its metastases and can be detected in the peripheral blood. Identification of CTCs in blood samples provides a low invasive technique compared to the obtainment of biopsies, which is nowadays the common way to detect tumors in combination with microscopic techniques and scans. Identification is based on $EpCAM^{+}$, $cytokeratin^{+}$, $CD45^{-}$ and presence of a $DAPI^{+}$ nucleus. Here an overview is given of methods and studies in which CTCs have been detected in NSCLC patients. With that the implications of CTC detection for diagnostic and therapeutic purposes are discussed. A number of methods to detect and characterize CTCs are being explored but only one method is currently validated and approved for use in clinical studies. This CellSearch (Veridex) platform uses an epithelial marker to enrich tumor cells from epithelial origin, followed by detection and enumeration using immunohistochemistry. Different studies show that $CK19^{+}$-cells are differently present before, during and after treatment and the relation between $CK19^{+}$-cells and several parameters related to survival. It turned out that the CTCs present in the bloodstream were similar to the primary tumor cells present relatively late in the course of the metastasis. Detection rates however largely differ (from several to thousands of CTC) between detection techniques and control groups vary in different studies, which makes solid conclusions difficult. CTCs could be used as reliable diagnostic and prognostic tool (maybe for screening) in the future, but for now it should be combined with conventional methods to achieve maximal effect.

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Table of contents

Lung cancer

Cancer has always been considered a single-organ disease defined by a distinct localization and cellular composition. In recent years however, new insights into cancer organization have changed its definition towards a multi-organ disease. It is nowadays characterized by a primary tumor site with secondary spread to distant organs. Also, the idea about the composition of tumors has changed from a uniform, homogenous lump of cells to a heterogeneous array of different cell types. Even tumor cells with the same histological appearance might differ completely regarding their genetic constitution. This phenomenon may account for the different responses to treatment and therapy observed in cancer patients (Walker 2008). Currently, the cancer stem cell (CSC), which is characterized in the cancer stem cell theory, seems the most important cell type in lung tumors. Although some controversy remains, this theory is widely accepted in the scientific world of today. It states that a small population of specific lung cancer stem cells (lCSCs) is the main disruptor in cancer. These lCSCs resemble normal stem cells in their capacity for self-renewal, production of differentiated progeny and capacity to form secondary tumors, but are deregulated. Although many potential markers have been studied, lung cancer stem cells have not been identified yet (Kitamura et al. 2009).

Lung cancer is the most prominent cause of cancer-related deaths (9773 over 2007 (IKCnet)) in the Netherlands. Two major types of lung cancer have been defined, with corresponding histological properties. Small cell lung cancer (SCLC) is characterized by rapid growth and an early ability to spread to organs different than the original tumor site. This makes it one of the most aggressive tumors known, with limited effect of surgical treatment. The reaction to chemo- and radiotherapy is however substantial, which makes this the basic treatment for SCLC. The long-term survival rate is consequently high, considering the aggressive nature of the cancer (Perez-Gracia et al. 2007). On the other hand, non-small cell lung cancer (NSCLC) is characterized by adeno-, squamous or large cell carcinoma and is the most prevalent type (80% of lung cancer cases). It grows fairly slow, causing tumors to be detected in a reasonably early stage; usually before metastasis and spread to the lymph nodes (Walker 2008).

When looking at the onset of lung cancer several environmental factors, e.g. asbestos, arsenic and air pollution, have been shown to induce lung cancer in variable extent (Alberg, Samet 2003);(Tang et al. 2010). Moreover, the positive correlation and causal relationship between tobacco smoke and lung cancer has been shown many times in literature (Tang et al. 2010;Takahashi et al. 2010;Pavlovska et al. 2009). For instance, Ozlu et al. (2005) showed a 20-40 times increase in risk for lifelong smokers compared to non-smokers. With that, individually determined protein levels are adjusted in lung cancer; CyclophilinA for instance is upregulated. This protein regulates the correct maintenance of denatured proteins and protects against environmental stressors, which may reflect the reason for the high expression in cancer (Lee 2010).

Diagnosis and treatment

To decide which type of treatment is appropriate for a specific case of cancer, oncologists use the tumor-node-metastasis (TNM) classification. The classification is based on a process called staging: the determination of the phase in which the tumor occurs. This universally applied diagnostic system defines the tumor into one of five stages, depending on size and potential spread to surrounding lymph nodes (Watanabe 2003). On the basis of this staging process, an appropriate treatment method is chosen and executed. The treatment for early-stage NSCLC consists primarily of surgical resection of the tumor. This technique is effective because of the reasonably slow growth in early stages of the disease. In later stages or after complications, the treatment could be supplemented with adjuvant chemo- or radiotherapy (Walker 2008). Even though the treatment options for chemotherapy have drastically increased, a plateau has been reached in the response to traditional chemotherapy. Because of this development, new strategies concerning chemotherapy use are under investigation. Specific inhibitors (*Erlotinib*) or antibodies (*Cetuximab*) directed against epidermal growth factor receptor (EGFR) inactivate its tyrosine kinase domain or inhibit the binding of EGF to its receptor. This receptor activates the Ras/Raf/MAPK, PI3K/Akt and Jak/STAT pathway and leads to promotion of cancer cell proliferation and tumor invasion among other events (see fig.1) (Inamura et al. 2010). Inhibition of the EGFR will lead to reduced stimulation of these three signaling routes, which is particularly effective in mutations that excessively active them. To increase the effectiveness of cancer demoting activity, treatments with different chemotherapeutic agents are combined to achieve maximal results (Triano, Deshpande & Gettinger 2010).

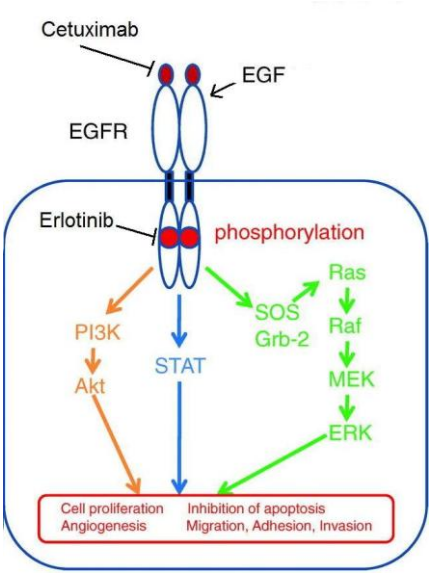

Fig.1 The molecular pathway of EGFR activation leading to cell proliferation and tumor invasion. It is shown that the activation of EGFR leads to cell proliferation, angiogenesis and inhibition of apoptosis through a series of steps. This whole signal transduction cascade is initiated via the binding of EGF to its receptor. Cetuximab binds to the EGFR which inhibits EGF binding and Erlotinib binds to the intracellular phosphorylation site with inhibited signal transduction as a result. This will cause the three signaling routes to be inhibited, which will reduce cell proliferation and tumor invasion, just as therapy intends. Figure adapted from (Inamura et al. 2010).

Yet it turned out that patients who are cleared from the primary cancer are not guaranteed to stay cancer-free for life. Next to possible metastasis after long periods of time, minimal residual disease (MRD) is a serious "hidden" problem. MRD comprises the presence of tumor cells in cancer patients after surgical removal of the primary tumor. This effect has been shown through the frequent metastatic relapse (40%) of patients after

surgical resection of their primary tumor (Pantel, Brakenhoff & Brandt 2008). It was therefore suspected that tumor material was not completely removed and it was still present in some extent after primary surgery. This false-negative for tumor removal is a consequence of the detection threshold of current routine diagnostic procedures used for tumor staging. Tumors consist of billions of cells by the time they become visible on current visualizing methods. When treatment reduces the tumor to a level which is below the sensitivity threshold of the diagnostic equipment, the tumor is undetectable by tumor staging methods and therefore the patient is wrongfully diagnosed as cancer-free (Hosch, Scheunemann & Izbicki 2001).

Metastasis and seeding

Even though treatment options have enhanced, the five-year-survival rate of NSCLC lies surprisingly low at only 15% (Walker 2008). This poor prognosis is caused by the occurrence of metastases to several organs by the time the tumor treatment is effective (Sone, Yano 2007). Since these distant metastases are the most frequent cause of deaths after surgical resection, the identification of metastatic sites could have great prognostic value. Especially the fast metastases to liver, brain, bone and adrenal glands seem responsible for poor long-term survival (Walker 2008, Passlick 2001). Hung et al. (2010) showed actual percentages in 106 NSCLC-patients with single-organ metastasis: 32.1% to bone, 29.2% to brain, 20.8% back to lung, 13.2% to liver and 0.9% to adrenal gland. Of these metastases however, only bone and lung metastases were significantly associated with post-recurrence survival.

Until recently it was thought that metastasis only occurred during advanced-staged cancer, but new work suggests that dissemination of primary cancer cells to several organs might be an event also present in early stages (Passlick 2001). Although the metastatic pattern of different cancer types varies greatly, the cellular and molecular mechanism are mostly similar (see fig. 2). First the primary tumor needs the formation of new blood vessels from existing ones to retain adequate blood flow while increasing the number of tumor cells. This process of angiogenesis occurs through production of pro-angiogenic factors like vascular epithelial growth factor (VEGF), basic fibroblast growth factor (FGF), hypoxia-inducible factor 1 and angiopoietins (Schoettler, Brahn 2009). Once these vessels have been formed, the tumor has the possibility to escape its primary site of residence and spread through the circulatory system. For this, the tumor cells need to pass the basal layer around the blood vessel (tumor border) and migrate into the bloodstream, known as a process called intravasation. With that, the metastatic cells must survive in the bloodstream, without support of surrounding cells, until arrest in a secondary organ. With this arrest, a small part of the cells will leave the circulation and enter the surrounding tissue in the process of extravasation. In this new site the cells must maintain an environment for growth and survival through the development of newly formed blood vessels and micrometastasis (metastasis to minuscule to detect). Since all these steps represent a tremendous selective pressure on the original metastatic cells, only a small portion of them will eventually start secondary metastasis (Chambers, Groom & MacDonald 2002).

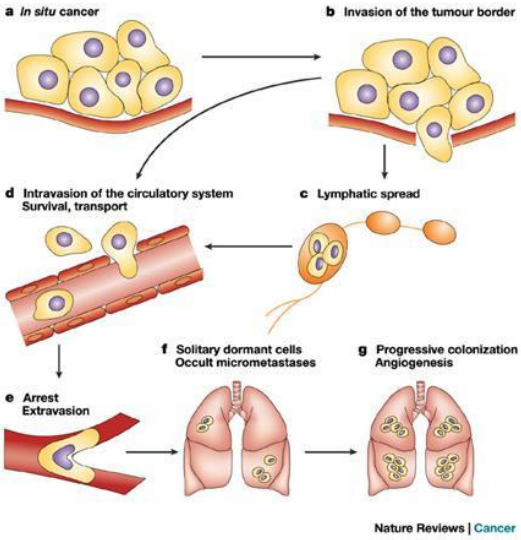

Fig. 2 Visualization of the step-by-step metastatic process with angiogenesis at the primary tumor site, intravasation after passage of the tumor border, lymphatic spread through the circulation, extravasation to the secondary tumor site and angiogenesis to remain viable (Steeg 2003).

Kim et al. (Kim et al. 2009) focused on the phenomenon described as tumor self-seeding. Instead of metastasizing to distant organs, the tumor cells released from the primary tumor reinfiltrate and colonize their primary tumor site (As (Hung et al. 2010) showed in 20.8% of cases). The leaky state of blood vessels does not only facilitate the passage of cells into the bloodstream but also the passage back into the tumor tissue. This process is enhanced because tumor cells don't need further adaption to thrive at their primary site, which makes infiltration relatively easy. However, the tumor cells that have survived a dangerous phase in the bloodstream are probably more aggressive than "homed" tumor cells, potentially transitioning the tumor to a more aggressive nature. This theory is strengthened by the observation that metastatic tumors are more efficient as seeders than the primary ones. On the other hand, the repopulation of primary tumors reduces the amount of tumor cells in circulation, which might reflect a decreased chance for distant metastasis.

Enrichment and detection

In case of NSCLC, staging often takes place using Computed Tomography (CT), Positron Emission Tomography (PET) and recently Transbronchial Needle Aspiration (TBNA) confirmed by lung biopsies (Ceron, Michieletto & Zamperlin 2009). Since these techniques are invasive to the patient, great interest goes into development of more patient-friendly diagnostic measurements. This came when Pantel et al. (2007) proved that metastatic cells derived from many cancer types commonly home in the bone marrow. When these cells reach the bone marrow and home in anticipation of metastatic potential, they are called disseminated tumor cells (DTC) (Pantel, Brakenhoff & Brandt 2008). Aspiration of small amounts of bone marrow fluid is enough to diagnose presence of DTC, though the procedure is painful. Pantel et al. also put their discovery into perspective by adding that the bone marrow is an organ easy to access. Aspiration of bone marrow is a relatively low invasive technique compared to the obtainment of fluids from lung or breast tissue. The tumor cells might therefore be able to home in all distant organs, but the current techniques just don't offer us the chance to detect them there.

Following the breakthrough of DTC, Tanaka et al. (2009) found that the amount of circulating tumor cells (CTC) was significantly increased in lung cancer patients compared to nonmalignant patients. These cells are shed from either the primary tumor or its metastases which circulate in the peripheral blood of patients (Mostert et al. 2009). This contains a great alternative for bioptic diagnosis, since collecting blood is a pretty low-invasive procedure. With that they found that CTC numbers were significantly increased with tumor progression, which opens possibilities for diagnosis of minimal residual disease and metastasis. Since the metastatic process is characterized by spread of tumor cells through the circulation, diagnosis of this process could be of great diagnostic value.

To find the origin of disseminated and circulating tumor cells, Braun et al. constructed a study (2000) in which aspiration of bone marrow was performed on 552 early-stage cancer patients after complete resection and 191 patients with non-malignant disease. The aspirated cells were analyzed for expression of cytokeratin (histological marker specific for epithelial cells) to find the original location of disseminated cells. It turned out that cytokeratin-positive cells were present in 36% of cancer patients, compared to 1% in nonmalignant cases. This shows that the bone marrow of cancer patients is much more invaded by epithelial cells (derived from the primary tumor and not native to the bone marrow) than that of nonmalignant cases. It suggests that disseminated cells are of epithelial origin and relocate to the bone marrow and maybe other organs. Sawabata et al. (2007) studied blood samples that were collected during and after thoracotomy to resect primary lung tumor. It turned out that just prior treatment, only one of nine subjects showed presence of CTC, while after treatment three of them did. This indicates that CTCs in peripheral blood may be caused by the manipulation performed in surgical resection of the primary lung tumor. This suggests that the cure of the primary tumor creates the possibility for secondary tumors to arise, which is of course problematic. With that, Braun et al. (2000) showed the presence of micrometastasis in the bone marrow was significantly associated with increased overt metastasis in other organs and cancer-related deaths after a follow-up period of four years ($P<0.001$). This paved the way for the use of DTCs as a predictive tool in the prognosis of metastatic cancer.

Now that the potential of DTC as diagnostic and prognostic tool has been established, the comparison between DTC and CTC was studied. Daskalaki et al. (2009) showed the correlation between CK19 mRNA-positive CTCs and CK19 mRNA-positive DTCs in early-stage breast cancer. They analyzed the detection rate of CTC (55.2%) and DTC (57.6%) before and after (52.4% resp. 51.2%) chemotherapy. From this finding it can be concluded that CTCs are not inferior to DTC as a diagnostic tool for monitoring minimal residual disease. Seen that this study has been conducted on breast cancer material, the same experiment should be done with lung cancer to find according results. Since determination of CTCs is much less invasive than that of DTC, this would be a good alternative method to detect cancer types in the clinic.

CTC appear in the peripheral blood in a concentration of only several cells/ml or even one CTC per million peripheral blood mononuclear cells (PBMC), which makes detection and characterization challenging. Several techniques have been developed over the years, but little prove sufficient sensitivity, specificity and reproducibility. To achieve better results, a two-step method has been developed: enrichment from peripheral blood as the first step, followed by detection and enumeration using varying methods.

Especially the Cellsearch system, CTC-chip and immunostaining are widely applied and yield promising results regarding detection and identification of CTCs. Common strategy in enrichment is the application of anti-EpCAM (membrane-embedded glycoprotein epithelial cell adhesion molecule, (Sebastian et al. 2007)) antibodies to select for epithelial cells, followed by depletion of differentiated hematopoietic cells using CD45 (Tanaka et al. 2009) and staining with DAPI (4,6-diamidino-2-phenylindole). The aim of this is to specifically enrich CTCs from peripheral whole blood, which can be enumerated and analyzed. To improve sensitivity and reproducibility, ongoing modifications have been made to these methods; from the application of different CTC-stains to detection of varying markers. Since many manufacturers are trying to find the perfect test, not all can be described here. Fig. 3 shows the tree of techniques used in detection of CTC in peripheral blood and the biological foundation. The techniques highlighted in the red square will be discussed in the following section.

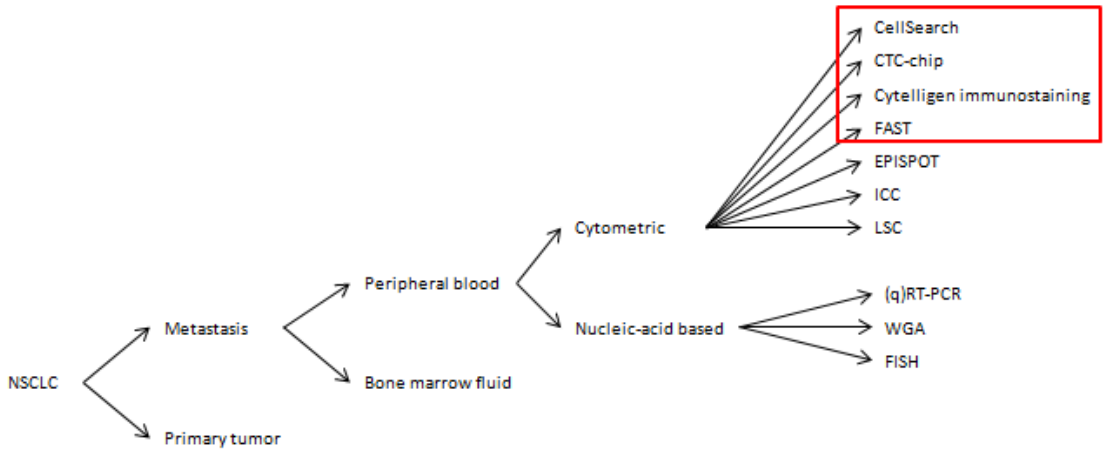

Fig. 3 Tree of techniques used in detection of CTC in peripheral blood and the biological foundation. Abbreviations: EPISPOT; EPIthelial immunoSPOT, FAST; Fiber-optic Array Scanning Technology, ICC; immunocytochemistry; LSC; Laser Scan Cytometer, NSCLC; Non-Small cell lung cancer, (q)RT-PCR; (quantative) Reverse Transctriptase- Polymerase Chain Reaction, WGA; Whole Genome Amplification, FISH: Fluorescent In Situ Hybridization (Based on schedule of (Mostert et al. 2009).

Tanaka et al. (2009) used CellSearch platform for CTC enumeration. This technique uses ferroparticles coupled to anti-EpCAM antibodies to select for epithelial cells and extract them using immunomagnetism. With this, antibodies directed against CD45 are added to exclude white blood cells. Then staining with DAPI and CK-PE is applied to exclusively detect CTC using microscopic techniques. It is shown that the Cellsearch system is capable of detecting micrometastasis, which are not detectable with current routine diagnostic techniques. Nagrath et al. (2007) used a CTC-chip which consists of an array of 78.000 microspots with EpCAM antibodies. The blood sample flows through this array, in which CTCs are captured by the anti-EpCAM. After a washing step CTCs are identified using DAPI-stain, anti-CD45 and anti-KERSMCR (rhodamine-conjugated anti-cytokeratin). As it turns out, this technique is unique in its ability to sort CTCs directly from whole blood in a single step. With that, the isolated cells remain viable, which opens windows for further experiments on survival of CTCs in the circulation. Another study by Wu et al. (2009) took a completely other approach; instead of selecting for specific CTC, they applied a concept of negative enrichment called Cytelligen immunostaining. In this, they enrich CTC from peripheral blood by

depleting the red blood cells through cell lysis, white blood cells by anti-CD45 antibodies and serum proteins. CTCs are fixed to a substrate and analyzed using FAST fluorescence scanner. This technique proves potential clinical use through rapid, real-time evaluation of chemotherapy and monitoring of recurrent disease. The Fiber-optic array scanning technology (FAST) as applied by Hsieh et al. (2006) is exclusive in its ability to measure and stain cells multiple times. Cells are incubated in monoclonal anti-pan cytokeratin antibody directed against several human cytokeratins and mounted to a substrate. Then secondary antibodies are applied in combination with DAPI-stain after which an ion laser excites labeled cells. A fluorescence microscope is used to identify CTCs in high resolution, which could provide vital clues in sample identification. And because this technique identifies cells using internal cytokeratins, it can detect CTCs in even the most undifferentiated epithelial tumors.

Technique	**Most important enrichment steps**	**#/ml(mean ± SD, range, Vol)**	**Study (et al.)**
CellSearch system (Veridex LLC)	- ferroparticles coupled to anti-EpCAM to select epithelial cells - CD45 antibody to exclude leukocytes - DAPI and CK-PE stain to select CTCs - Analysis using fluorescence microscope	1.1±1.4 7.5 ml	(Tanaka et al. 2009)
CTC-chip	- Anti-EpCAM to select CTCs Identification using: - DAPI stain for DNA content - Anti-CD45 to exclude haematologic cells - Anti-KERSMCR to select epithelial cells	155±236 2.7 ml tested	(Nagrath et al. 2007)
- Cytelligen immunostaining approach	- Lysis to exclude red blood cells - Anti-CD45 coated with magnetic beads to exclude leukocytes - Monoclonal antibodies antiCK18 (Alexa Flora 594) and antiCK19 (Alexa Flora 488) to select CTC - Analysis using fluorescent microscope	Range: 0-80 7.5 ml	(Wu et al. 2009)
Fiber-optic array scanning technology (FAST)	- Lysis to exclude red blood cells - Monoclonal anti-pan cytokeratin antibody to select cytokeratins 1, 4, 5, 6, 8, 10, 13, 18 and 19-positive cells - DAPI-stain and Alexa Fluor 488 and Alexa Fluor 555 conjugated goat anti-mouse antibody to select CTC - Analysis using fluorescent microscope	Range: 0-659 10 ml	(Hsieh et al. 2006)

Table 1 overview of new, widely applied CTC enrichment and detection techniques used in specific studies. Abbreviations: CD; cluster of differentiation, CK; cytokeratin, CTC; circulating tumor cell, DAPI; 4,6-diamidino-2-phenylindole, EpCAM; Epithelial cell adhesion molecule, KERSMCR; rhodamine-conjugated anti-cytokeratin.

Characterization of CTC

Before further clinical application, first the appearance of CTCs should be established, so that they can be distinguished from other types of cells. Marrinucci et al. (2009) found interesting results in their evaluation of the cytomorphology of CTC from one patient with stage IIIB lung adenocarcinoma. CTCs were identified by fluorescent imaging using cytokeratin-positivity (red), CD45-negativity (green) and possession of a 4′,6-diamidino-2-phenylindole (DAPI)–positive nucleus (blue) using fiber-optic array scanning technology (FAST). Looking at fig. 4 it became clear that CTCs are larger than the white blood cells surrounding them, which are characterized by CD45 (green) positivity. They also have large amounts of cytoplasm compared to the nucleus, resulting in low nucleus:cytoplasm ratios. With that, groups of cells (clusters) are observed, just like CTCs with irregular shapes.

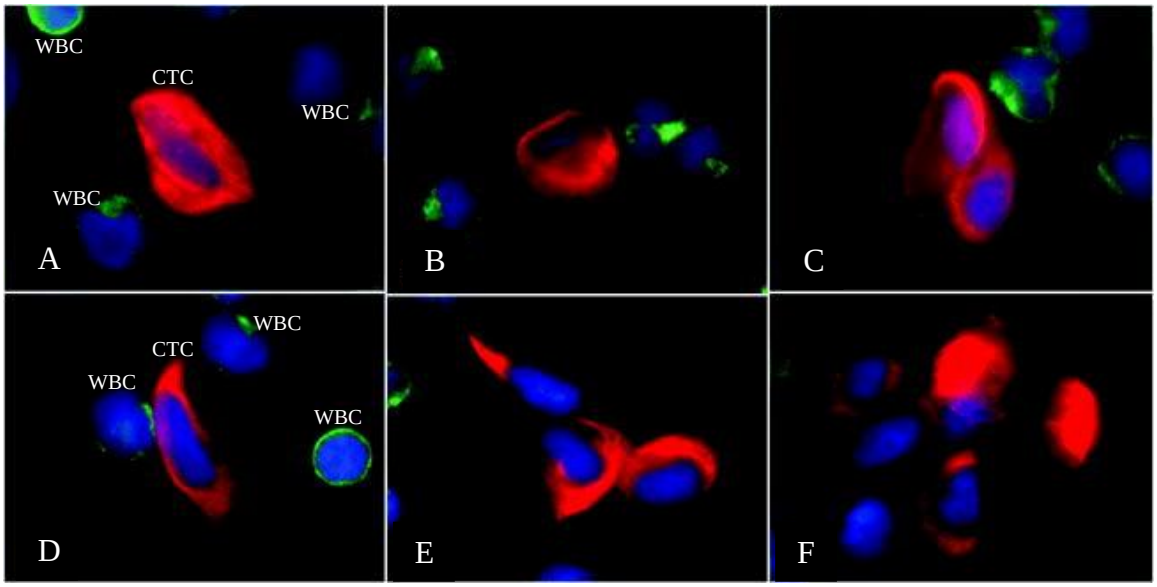

Fig. 4 Fluorescent images of CTC identified by cytokeratin-positivity (red), CD45-negativity (green) and a DAPI- positive nucleus (blue) using fiber-optic array scanning technology (FAST). CTC are larger than white blood cells (WBC) (Figure 4A and B), show low nucleus:cytoplasm ratios (Figure 4A and B), irregular shapes (Figure 4C and D) and the formation of clusters (Figure 4C, E, and F). (Hsieh et al. 2006, Marrinucci et al. 2009)

These cells were then compared to tumor cells retrieved from the primary tumor four years earlier regarding their physical representation and markers using again FAST detection.

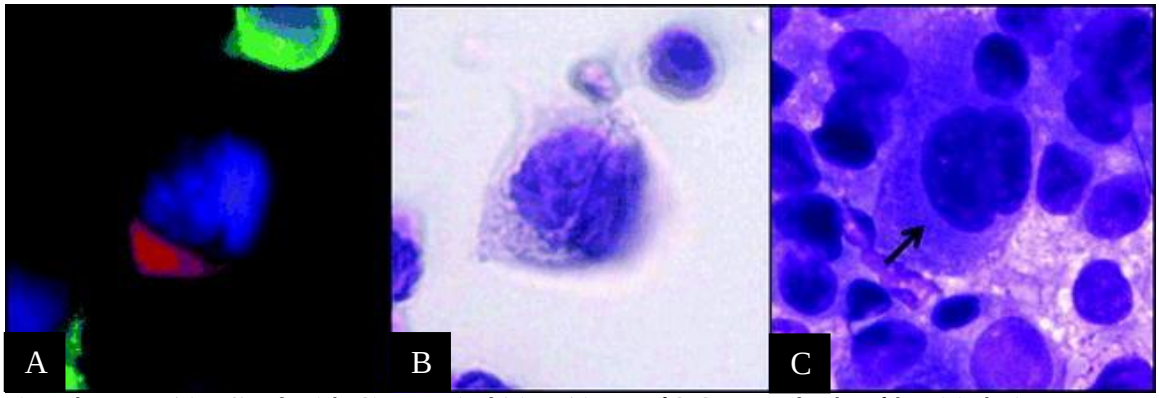

Fig. 5 Fluorescent (Fig. 5A) and Wright-Giemsa–stained (Fig. 5B) images of CTC compared to that of the original primary tumor from four years prior (Fig. 5C) using FAST. Comparable large size, low nucleus:cytoplasm ratio and round-to-oval nuclear shape are visible in all three images (Marrinucci et al. 2009).

It turned out that the CTCs present in the bloodstream were similar to the primary tumor cells present relatively late in the course of the metastasis. As can be seen in fig. 5, the large size, low nucleus:cytoplasm ratio and round-to-oval nuclear shape are present in both the circulating sample and that of the primary tumor. Marrinucci et al. propose an idea in which the circulating cells resemble the original population of tumor cells in the primary tumor. They therefore reject the established theory that the tumor cells that circulate in the bloodstream are a distinct subset of tumor stem cells. No evidence has however been found on the metastatic capacity of these cells, they might be solely passive cells shed from the tumor. Complicating factor in all of these evaluations is the little amount of knowledge about the appearance of tumor cells in the circulation. The

appearance in the primary tumor is reasonably well documented, but the travel through the bloodstream changes the cells significantly. Bonnomet et al. (2010) described the epithelial-to-mesenchymal transition (EMT); epithelial characteristics are reduced and expression of mesenchymal markers is increased. This proposes new challenges in the identification since the used EpCAM antibody does not recognize mesenchymal cells. Specific mesenchymal markers like vimentin, N-cadherin and MMPs should be implemented into detection strategies to cover the variety of tumor phenotypes. Characterization of these tumor cells in the bloodstream and other body fluids therefore remains a challenge in the field for coming years.

Predictive diagnosis and prognosis

Now that we have a temperate morphological idea about the appearance of CTC, the hunt for diagnostic and prognostic characterization using exact marker identification and genetic constitution is under investigation. As mentioned before CD45 and EpCAM are used to select for CTCs, in combination with cytokeratin. Especially cytokeratin-19 (CK19) is interesting since it is abundantly expressed in the majority of epithelial tumor cells, but not in lymph-node lymphocytes, peripheral blood and bone marrow cells (Datta et al. 1994). It therefore appears to be a sensitive marker for tumor detection and is widely used for the diagnosis of malignant epithelial cells in blood, bone marrow and lymph nodes.

Looking at detection rates, Sher et al. (2005) studied marker-genes that were differentially expressed in peripheral blood from 54 NSCLC patients and 24 controls. They found that 41% of all CTC in the patients were detected using CK19 as marker, though this detection rate increased to 72% using complementary markers PGP9.5, HSFIB1 and *TRIM28*. Based on the findings of Sher, Liu et al (2008) analyzed the combined expression of additional markers in 134 lung cancer patients, 106 patients with benign pulmonary disease and 80 healthy controls. It turned out that combined detection using CK19 and pre-proGRP (a hallmark of neoplastic transformation epithelium) showed positive discrimination between lung cancer and benign disease in 67.2% of cases. Addition of TSA-9 (tumor-specific antigen 9) increased this detection to a percentage of 84.3, which makes the detection using combinations of markers an attractive option.

When looking at CK19 expression on CTCs in combination with treatment, Yoon et al. (2010) investigated the clinical relevance of CTC in NSCLC-patients who underwent surgical resection. It turned out that CK19 mRNA-expressing CTCs were detected in 42.6% of patients before surgery and 25.0% after it. A trend towards correlation between CK19-positivity and shorter disease progression-free survival is visible, though not significant. Chen et al. (2007) studied the presence of CTCs in patients with NSCLC before and after chemoradiation with curative intent. They showed that CK19 mRNA in CTC was present for 66% of cases before chemotreatment, while present in only 33% after treatment. Although CK19 mRNA-level before treatment were only correlated to stage of the tumor (spread), after treatment it was correlated to histological type, overall survival, progression-free survival and again stage.

Studying the operation of tumors itself, Ge et al. (2006) considered the dissemination of malignant cells in the circulation pre-, intra- and postoperative of surgical manoeuvre or resection of NSCLC. It turned out that CK19 mRNA-positive

CTCs were significantly higher during surgery than before and after surgery, and all healthy volunteers were negative for this marker. They therefore conclude that CK19 is not the best marker during operations, due to the transient increase in expression. Important is the discovery that release of tumor cells into the bloodstream could be promoted by surgical resection, but that the ligation of the pulmonary vein partly prevent this.

CK19 has also been shown to contain prognostic and diagnostic potential in other cancer types, especially those of epithelial origin. Daskalaki et al. (2009) showed the diagnostic capability of CK19 mRNA-positive CTCs and CK19 mRNA-positive DTCs in early-stage breast cancer. Already in 1998, Yeh et al. (Yeh et al. 1998) showed that the median survival of gastric cancer patients with detectable cell-free CK19 mRNA in their circulation was 2.5 months lower than that of patients without it (1 resp. 3.5 months). This indicates that CK19 detection is associated with poor prognosis of gastric cancer, and that is could be used as prognostic tool in gastric cancer patients. Hoffmann et al. (2007) studied the detection of CK19 in blood, bone marrow and peritoneal lavage in patients with adenocarcinoma in the pancreas. It turned out that detection rate of CK19 is correlated with tumor stage and differentiation and a trend towards shorter survival. In this pancreatic tumor case, CK19 in combination with other tumor markers is a proper tool to distinct between benign and malignant pancreatic disease.

Serum DNA detection

After all, there might be another factor suitable for diagnosis of tumor and/or metastasis. Hodgson et al. (2010) showed that there is a scientific link between the detection of circulating genetic material from a tumor and from a fetus. Namely, circulating tumor and fetal cells (2-20/20ml blood) are both rarely found in the peripheral blood of the patient resp. mother during pregnancy. Yet it turns out that the cell-free fetal DNA is easily obtainable, which makes it a good candidate for screening. In cancer patients, the concentration circulating DNA is estimated around 4 ng/ml, which is a fourfold increase compared to healthy individuals. The exact mechanism behind this increase is not known but apoptotic cells might be responsible; the induced stress causes the cells to release their (genetic) components into the surrounding bloodstream by apoptosis. It is striking to see that the chromosomal and microsatellite aberrations, abnormal promoter hypermethylation and point mutations observed in serum DNA are in many instances the same as in the original tumor. This suggest that there is a good correlation between the serum DNA and the DNA of the primary tumor, at least for the BRAF mutation in advanced melanoma (Board et al. 2009). Another study by Paci (2009) confirmed that the concentration circulating hTERT DNA was four times increased in NSCLC patients compared to control subjects. They also showed that the concentration of circulating DNA was an important risk factor for the presence of disease and prognosis.

Concluding remarks and future prospects

Since lung cancer is a major problem in healthcare today, many efforts are conducted to find easy and reliable methods for its diagnosis and prognosis. A novel technique is based on the diagnostic and prognostic value of CTCs in the peripheral blood. Only a small volume of blood is enough to measure and quantify the CTC count of a patient with primary tumor and/or metastasis. Since these cells are rare, only one CTC

per million PBMC, sensitive methods have been developed to enrich and detect them accurately. Although actual CTC count differs greatly between the investigated techniques (see table 1), it is clear that CTCs are present in peripheral blood at least in some extent. It makes it however difficult to draw pronounced conclusions about CTC count in relation to the used technique. With that, the patient populations in the discussed studies differ, which results in an impossible comparison between techniques. It might seem that the more CTC are detected, the better the technique, but this is not automatically true. Metastatic patients probably have more CTCs than patients with primary lung cancer, which would result in higher CTC counts, but does not say anything about sensitivity and reliability of the technique. The same goes for advanced patient compared to early stage cancer, since it is shown that CTC amount increases with disease time.

Although Cellsearch is the only CTC enumeration technique approved by the FDA (Food and Drug Administration; USA) at the moment, it does not seem to contain groundbreaking innovations. It uses anti-EpCAM, anti-CD45 and DAPI-stain like the CTC-chip and a fluorescence microscope like Cytelligen and FAST. The technique has however been validated and reproduced in across different laboratories, backing it up as a accurate and precise technique. It has also been shown capable of detecting micrometastasis, which are not detectable with current routine diagnosis. This opens great possibilities for diagnosis of lung cancer that was otherwise missed by current techniques. But even this approved technique has a detection rate of only 30.6%: you will miss two out of three CTCs. For this to improve, detection methods should give out constant CTC counts in constant situations, preferably the same disease. This process of validation is important in the progress towards techniques with maximal sensitivity and specificity. Most scientists are aware of the fact that detection methods are not perfect and keep trying to find the best technique. As a first step, a (literature) study comparing several detection methods concerning the same patient populations should be carried out. In this way, the statistical parameters for different detection methods could be determined and true comparisons will be possible. The most solid technique to further investigate seems the CTC-chip. Enrichment and sorting in one step with viable cells as a result is a good basis to further develop this method. Especially the survival of cells in this procedure opens possibilities to culture them outside the body and research special characteristics. Combined with the speed and high resolution of FAST this could provide a serious new method. It is not surprising that there is discussion about the use of one appropriate technique. The field of CTC is reasonably young, and it is unreal to expect perfect techniques at this time point.

Further development of detection methods should be conducted to monitor CTC, possibly in combination with circulating epithelial tumor cells (CEC), primary tumor cells and cell-free DNA. If it turns out that metastatic cells indeed resemble cells of the primary tumor, this opens great possibilities for treatment. You may find information about the primary tumor before this has actually presented itself with current detection techniques. Also during resection of a patients' tumor, specific tumor cells in a biopsy could be characterized. Tailored treatment strategies against this primary tumor could then also be applied to the circulating cells. One major problem in this strategy is however the changing appearance of CTC in the bloodstream, of which our knowledge is limited. Minimal changes during their journey through the circulation might make CTC

insensitive and evade the applied treatment. In our current understanding, the CTC are characterized by presence of EpCAM, round-to-oval morphology with a visible nucleus, cytokeratin-positivity and CD45-negativity. It is hard to imagine that these four criteria are sufficient to detect all and only CTC from the total pool of varying cell types in the blood though. New target markers should therefore be studied, for instance epithelial markers like E-cadherin or occludin. With that, one should keep in mind that most studies use EpCAM to select for circulating cells in the peripheral blood. But as shown earlier, EMT transition is an important variable in cancer dissemination, so alternative phenotypical markers should also be targeted.

The mentioned studies suggest that CK19 is a proper marker to identify CTC, but specific additional markers per cancer type enhance the detection. Chemotreatment reduced the presence of CTC in the circulation, and since we assume that treatment reduces tumor load, both parameters are probably linked. In the reversed line of reasoning, increased levels of CTCs would indicate a higher tumor load and further progressed lung cancer. In this way, the number of CTCs could be used as clear-cut diagnostic value for tumor presence and progression. The lower the CTC count, the closer you are to healthy controls, which don't have any CTCs. Great advantage with CTC is the lowered threshold for detection. Since detection techniques can detect one CTC per million blood cells, MRD will become less of a problem. If it turns out that CTC are present in the early phase of tumor origination (including metastasis) this could provide vital insights into the origination of cancer. Potentially, obtainment of small amounts of peripheral blood provides enough information to be used in screening of former cancer patients. When CTC count in the peripheral blood gets higher than a certain threshold, new therapy should be started to prevent further growth of the tumor. In this way, CTC can play an diagnostic role in the clinic. This could drastically cut back the number of lung cancer patients and clear it as number one cancer-related death cause. When looking at the prediction for future recurrence, CTCs could play an important prognostic role. CTC-positivity after chemoradiation indicates presence of tumor cells and could be used as quantity to predict treatment outcome. High CTC counts will visualize the need for extensive systemic treatment and close follow-up to decrease the chance for distant metastasis and recurrence. Also, the prognostic aspect of CTCs will consist of prediction of long-term survival. The more CTCs present in the sample, the bigger the cancer load and the smaller the chances for survival. The number of CTCs after treatment could predict the tumor progression and chance for metastasis.

One remark about the integration of CTCs into the CSC theory, which seems challenging. Although these CTC probably have metastatic potential and can colonize distant organs, they are differentiated cells of epithelial origin. In this process of differentiation, they would have lost their capacity for self-renewal and are not stem cells anymore. There is however some evidence that a subpopulation of CTCs has a stem cell progenitor phenotype (Theodoropoulos et al. 2010). Both CTCs with epithelial characteristic and CSC originate from normal stem cells. The exact properties of CSC are not known, so possibly they still contain epithelial characteristics without being differentiated. In this way, the CTC could be fitted into the CSC theory as being an subpopulation of undifferentiated CSC with self-renewal capacity. This does however complicate the whole theory about metastasis. How could undifferentiated CSC be demonstrated? Which type of tumor cell should preferably be characterized first? And

which parameter proves the most reliable in the clinic? All questions that should be answered before any true understanding of metastasis and diagnosis is possible.

In my opinion, CTC detection and enumeration could not be used as single technique in the determination of lung cancer and metastasis at this point. The lack of solid validation for different techniques, the absence of a single detection method in the clinic and the incomplete knowledge about changing appearance in the circulation need further attention. For now CTC enumeration and current conventional techniques, though also not perfect, should be combined to achieve maximal result. However, new promising techniques are under development, and will be improved in the near future to become reliable and reproducible in the clinic. In this way, CTC count could be used both as a diagnostic, as well as a prognostic tool.

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