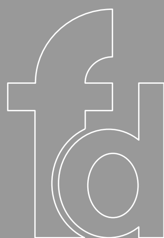

Detection of disseminated tumor cells: strategies and diagnostic implications

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Disseminated cells from primary solid tumors are considered to be the cause of metastases formation and relapse of disease. Consequently, their detection is of high importance for staging, prognosis and decisions about adjuvant therapy. Residual disease is conventionally detected by histological evaluation of biopsy specimens. Continuing efforts to increase the sensitivity to identify occult tumor cells in lymph node, bone marrow and blood have led to the development of various strategies. This review will discuss histological, immunological and molecular approaches to detect micrometastases from solid tumors.

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The first important step in diagnosis and consequently effective treatment of cancer is to distinguish between tumor and normal or benign cells. In solid tissue mass, malignant cells can be pathologically detected by histology, staining and immunohistochemistry, whereas the identification of single disseminated tumor cells in the hematogenic or lymphogenic circulation can present a complex challenge. Metastatic tumor cells are considered to be the source of disease relapse after surgery. Therefore, the detection of residual disease is the most important prognostic indicator for decisions concerning adjuvant and follow-up treatment.

Several approaches to detect disseminated epithelial cancer cells are currently being used or evaluated. However, methods combining high sensitivity with reliable specificity are still needed. For a strategy to be clinically useful, two challenging requirements have to be met. Firstly, the likelihood of losing or missing tumor cells dispersed among a large excess amount of normal cells has to be minimized. Secondly, metastatic cancer cells have to be dependably distinguished from normal somatic cells.

Detection strategies

Histological

Conventional histopathologic methods use staining procedures in combination with cytologic evaluation to identify tumor cells. Hematoxylin and eosin (H&E) staining is routinely used to localize tumor cells in cross-sections of formalin-fixed and paraffin-embedded lymph nodes. Microscopic assessment is subjective and the identification of atypical cells in contrast to nonrelevant suspicious cells – e.g., activated endothelial cells – has to be performed by experienced cytopathologists. The application of conventional histological methods is restricted in bone marrow and blood analysis, since cytologic assessment is complicated due to the presence of multinucleated cells. For this reason, immunologic technologies that provide higher sensitivity and specificity have been applied.

Immunological

Immunohistochemistry significantly increases sensitivity in the detection of occult nodal metastases. Specific monoclonal antibodies are used to label tumor cells that can consequently be visualized by color reactions [1]. Immunocytochemistry can detect tumor cells in cell smears or cytospins from bone marrow or blood. However, background expression of epithelial antigens, mucins and cytokeratins in hematopoietic tissues can result in false-positive results and thus microscopic assessment and the choice of antibody used for detection

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are crucial for specific tumor cell identification [2]. The lack of methodological standardization is thought to be the reasons for discrepancies in immunocytochemical data from different studies [3,4]. Cell smears are often used but do not allow a reproducible, quantitative transfer of cells to slide surfaces in comparison to cytocentrifugation. Up to 1 million cells can be placed on a single slide and detection sensitivity of one tumor cell in 2 x 10^6 mononuclear cells has been reported [5]. If only two slides are analyzed, the test result would depend on one positive event. Therefore, a higher number of slides has to be considered for analysis, in order to increase test reliability.

Moreover, different staining techniques can result in specificity variations. Hematopoietic cells can be directly reactive to alkaline phosphatase [6] or produce endogenous peroxidase [3], consequently resulting in false-positive staining in alkaline phosphatase-based or peroxidase-based methods. Immunocytochemical analysis is laborious and microscopic interpretation is influenced by subjectivity. The development of automated screening systems could provide more standardization with higher throughput in the future [7,8].

Histological and immunological staining protocols are currently used as standard procedures to detect metastatic tumor cells. However, the sensitivity to detect micrometastases by these technologies has been questioned due to the limited amount of screened cells and tissue. In order to increase sensitivity, nucleic acid-based approaches have been suggested and pursued.

Molecular

Over the past decade, various molecular approaches have been developed to detect disseminated tumor cells [9,10]. Nucleic acids can be amplified by polymerase chain reaction (PCR) with extraordinary sensitivity. Accordingly, nucleic acids from rare disseminated tumor cells (e.g., one tumor cell in 10^7 white blood cells) can be detected. Technical optimization and appropriate controls are necessary to obtain comparable sensitivity levels, achieve reproducibility and to avoid cross-contaminations and consequent false-positive results. Amplification conditions (annealing temperature, additives and buffer concentrations) and primer sequences in particular have to be carefully designed and consistency has to be monitored. The presence of pseudogenes or borderline target transcription in non-neoplastic cells has to be considered to achieve reliable assay specificity. If tissue-specific markers are used for detection, the introduction of cells in the circulation after tumor surgery can produce false-positive results. The availability of closed-tube real-time PCR technology offers new advantages including reduced contamination risk, direct quantification and most importantly high-throughput due to minimized downstream processing and rapid cycle programs.

The detection of genetic changes for diagnostic evaluations is currently only routinely applied in hematologic malignancies. Several DNA and RNA markers have been evaluated for PCR-based methods to detect disseminated cells from solid tumors.

DNA markers

Genomic mutations in proto-oncogenes (e.g., k-ras) or tumor suppressor genes (e.g., p53) have been reported to be useful to identify tumor cells for lymph node analysis in colorectal and non-small cell lung cancer [11,12]. Two labor-intensive approaches have been used to detect tumor genomic mutations by PCR, mutant allele-specific amplification (MASA) and oligonucleotide-mediated mismatch ligation. The methylation status of certain genes (e.g., p16 [13], glutathione-*S*-transferase P1 [14]), detectable by methylation-specific PCR (MSP), or the presence of carcinogenic virus sequences (e.g., human papillomavirus [15]) are also described as promising tumor cell markers.

Changes in DNA repeat sequences, so-called microsatellite instabilities, which are caused by mutations of DNA mismatch repair genes, are also useful to detect allele alterations in neoplastic cells. The detection of circulating microsatellite DNA markers in plasma has been shown to be able to provide prognostic information [16]. Circulating DNA material can be released by decaying tumor cells and features high stability in comparison to RNA. Therefore, the presence of DNA markers amplified by PCR does not necessarily confirm the presence of viable circulating tumor cells but is indicative of tumor burden.

Fluorescence *in situ* hybridization (FISH) can be used to directly identify circulating tumor cells [17]. FISH technology can detect specific aberrations in chromosomes that accumulate in neoplastic cells (i.e., loss of heterozygosity, gene rearrangements, translocations and gene amplifications, e.g., HER2/neu in breast cancer). But in comparison to PCR technology, FISH is labor-intensive and low in sensitivity.

In contrast to hematological tumor cell detection, the main approach to develop molecular diagnostic assays for solid tumors has focused on RNA markers due to a lack of universally applicable DNA markers so far.

RNA markers

In general, RNA-based methodology is more labor-intensive than DNA protocols. Special treatments are necessary since RNA molecules are very prone to decay once released from the cell and mRNA has to be reverse transcribed into cDNA (RT-PCR) prior to amplification. On the other hand, only viable circulating tumor cells will be detected by RNA markers in their actual location. The successful detection of circulating RNA in plasma, however, has been reported [18]. In contrast to immunological strategies, the detection of specific RNA transcripts by RT-PCR does not allow actual quantification of tumor cells due to variable, unknown expression levels.

Transcripts of tumor-associated (carcinoembryonic antigen [CEA]) [19,20], melanoma-associated antigen (MAGE) [21], β-subunit of human chorionic gonadotropin (β-hCG) [22,23], mucin (MUC-1) [24] and tissue-specific genes (prostate-specific antigen [PSA] and prostate-specific membrane antigen [PSMA] for prostatic carcinomas [25], cytokeratin 19 and 20 for epithelial tumors [26–28], tyrosinase for melanoma [29,30], mammaglobin for breast cancers [31–33]) have been used successfully to detect disseminated cancer cells. Moreover, unique transcripts

from fusion genes [34] or from virus sequences [35] can be used to distinguish neoplastic from normal cells. The detection of enzyme activity and of the RNA component of telomerase - a ribonucleoprotein extending chromosomal ends – by PCR-based assays has been demonstrated as useful in diagnosing and staging carcinoma cells [36,37].

Universal tumor markers with overexpression in a majority of the heterogeneous cancer population have not been characterized and limited specificity has been demonstrated for epithelial markers [38–42]. Recently, the combination of mRNA markers has been suggested to improve sensitivity in the detection of disseminated tumor cells in melanoma, breast cancer and gastric carcinoma [21,32,33,43–46].

Sample types

The choice of the appropriate detection strategy depends also on the tissue type to be analyzed. Historically, the detection of disseminated tumor cells is most important in pathological staging of lymph node specimens. In the last few years bone marrow analysis has also been demonstrated to provide additional prognostic information. Promising detection strategies for circulating tumor cells in blood are also being evaluated. Other body fluids and lavages can potentially be used to detect disseminated tumor cells in different tumors.

Lymph nodes

The status of axillary nodes is considered the most important prognostic indicator for breast cancer. In other epithelial tumors, lymph node metastases are also considered a feature of poor prognosis. However, considering that significant numbers of lymph node-negative patients develop metastatic disease, the reliability of current staging procedures to detect disseminated tumor cells should be questioned. An increase in sensitivity to detect metastatic disease in regional lymph nodes can be achieved by serial sectioning and histopathologic examination of an extensive number of sections. This approach is time-consuming, which hampers its routine application. Immunohistochemical staining against cytokeratins can significantly increase the sensitivity to detect lymph node micrometastases in comparison to H&E staining [1,47]. However, cytokeratin antibodies can cause nonspecific staining since they are also expressed in nonepithelial cells (for example reticulum cells). Therefore, a subjective, morphological assessment of the identified cells is still required. Histologic examination can also distinguish between single isolated tumor cells trapped in the lymph node tissue and metastatic tumor cell clumps [48].

RT-PCR has been reported to be able to provide higher sensitivity to detect disseminated tumor cells in comparison to conventional lymph node staging approaches in melanoma, breast, colon, non-small cell lung and cervical cancer [12,49–52]. The establishment of high-throughput protocols utilizing highly sensitive PCR strategies could provide a platform for intra-operative sentinel lymph node analysis. Sentinel lymph node biopsy (SLNB) – extensively evaluated in melanoma and breast cancer – can provide prognostic values with minimal associated morbidity in contrast to complete lymph node dissection [53]. SLNB implements mapping of the one or two lymph nodes that primarily drain the tumor and therefore are most likely to harbor metastatic disease (the sentinel nodes). This strategy might gain importance in the future since tumor sizes at the point of diagnosis are decreasing and clinical data, particularly in breast cancer, demonstrates that lymphatic spread and nodal metastases size directly correlate with size of the primary tumor. Unnecessary axillary dissection and its subsequent morbidity could be avoided by prescreening sentinel lymph nodes with highly sensitive detection methods for micrometastases. Currently, sentinel lymph nodes are mostly analyzed by immunohistochemistry. PCR has successfully been used to increase detection sensitivity for tumor cells in lymph nodes [54,55] but application on a routine basis is still hampered by the availability of specific markers and by lack of compatibility of PCR protocols with routinely used methods.

Bone marrow

Whereas lymphogenic spread is still the most important prognostic indicator for cancer staging, hematogenic dissemination of tumor cells has been gaining increasing interest. In particular, the detection of micrometastases in bone marrow has been suggested to provide additional prognostic information for predicting recurrence and survival in breast, colorectal, prostate, non-small cell lung and pancreatic cancer [5,56–60]. Distant occult metastases are often located in bone marrow, in particular for epithelial tumors that tend to have skeletal metastases, hence important prognostic information could be gained by analyzing this mesenchymal organ for minimal residual disease [61]. Aspirations can be obtained from the patient's iliac crest or rib during surgery and can be evaluated by immunocytochemical- and PCR-based methods [62]. Analysis of minimal residual disease in bone marrow also provides high potential for the assessment of adjuvant therapy efficacy. Monitoring the eradication of tumor cells in bone marrow could be an important tool to evaluate chemotherapy resistance and therapy strategies.

Strong correlations between the presence of bone marrow micrometastases and poor survival have been reported in breast cancer independently from lymph node metastases [5,63]. However, due to differing published results of various studies, the prognostic impact of epithelial cells in bone marrow is still under discussion [3,64]. Pathological evaluation of bone marrow can be hampered by the presence of multinucleated cells and epithelial marker expressing hematopoietic cells. Therefore, the discrepancies in clinical studies could be explained by the diversity of immunocytological antibodies used for detection and the need for standardized methodological approaches. Increased sensitivity of PCR-based assays to detect tumor cells in bone marrow in comparison to labor-intensive immunocytochemistry have been demonstrated [19]. However, the lack of specific universal nucleic markers is still limiting the application of PCR technology in tumor cell detection [65].

Blood

Peripheral blood is historically one of the most important diagnostic specimens. It is routinely used to assess the overall health and immune system status of patients. Circulating tumor markers have been monitored in serum for the last several years to provide indicative values about metastatic (CEA, CA 125) or emerging primary disease (PSA, CA 125). These serum markers can be good indicators for tumor load but may fail to provide information about minimal residual disease. On a research scale several approaches to identify circulating tumor cells in blood are currently developed and optimized. The ability to detect one cancer cell in 10^7 peripheral blood mononuclear cells (corresponding to 10 ml blood) has been reported frequently. These sensitivity levels have been evaluated by *in vitro* spiking experiments using cancer cell lines. Metastatic tumor cells *in vivo*, however, might not (or at a significantly lower level) express the tested markers due to tumor heterogeneity. Therapy at the time of the sample draw can also interfere with the ability to detect the presence of circulating tumor cells. Hormonal treatment could downregulate the expression of the tumor marker of interest (e.g., antiandrogen effect on PSA expression). In addition, sequential sampling might be necessary to improve tumor cell detection since shedding into the circulation could occur intermittently [66]. Several reports suggest that the detection of circulating tumor cells in the blood has prognostic implications in cancers of the gastrointestinal tract (CK20 [26], mucins [67]), in prostate cancer (PSA [68]), in melanoma (tyrosinase [69,70]) and in breast cancer (mammaglobin [31], MUC1 [24]). On the other hand, there have been studies that could not confirm prognostic significance of disseminated tumor cells in peripheral blood [71] but long-term follow-ups are necessary.

Due to the small ratio of circulating tumor cells *versus* a high background of blood cells, the chosen technical approach is crucial for sensitivity and specificity of the assay. Several technologies for blood processing have been pursued to detect circulating micrometastases.

Straight forward protocols analyzing whole blood directly have been applied most frequently. Nevertheless, with these approaches, assay sensitivity is often sacrificed since tumor cells are dispersed in an exceeding background of blood cells. Loss of specificity can occur if the expression of tumor markers is detectable in nonmalignant cells (e.g., cytokeratins [72,73]).

Cell enrichment and volume reduction can be achieved by isolating mononuclear cells from blood using either red blood cell lysis or gradient separation techniques. However, both approaches can result in loss of tumor cells due to technical variations. Tumor cells can be lost during the red cell lysis process or in the granulocyte fraction of density gradients. Recently the development of special gradients has been reported to optimize tumor cell separation in particular (OncoQuick®, Hexal Gentech/Greiner Bio-One) but no clinical data has been demonstrated so far.

Several advanced tumor cell enrichment protocols can be used to increase the sensitivity of tumor cell detection in peripheral blood. Positive selection of epithelial tumor cells is often performed by immunomagnetic beads coated with the monoclonal antibody Ber-EP4, targeting two epithelial cell surface glycoproteins of 34 and 39 kD [74]. This approach has been successfully used to detect circulating tumor cells by RT-PCR in various cancers [24,37]. However, due to heterogeneity not every tumor expresses this specific epithelial surface antigen [75]. Consequently, tumor cells could escape this positive selection procedure. To demonstrate successful enrichment of tumor cells, it would be necessary to analyze the noncaptured cell fractions in these studies. Immunomagnetic enrichment of tumor cells might also be improved by targeting multiple antigens and using antibody cocktails, e.g., Ber-EP4, EpCam, HER2/neu and Muc-1 in breast cancer but specific studies have to be performed for different tumor types.

Negative selection approaches utilizing immunomagnetic beads (e.g., AntiCD45-conjugated Dynabeads™) can improve sensitivity and the ability to analyze larger sample sizes [76,77]. Depletion technologies using tetrameric antibody complexes to link leukocytes to red blood cells followed by density gradient separation promise high consistency and effortless protocols (RosetteSep™; StemCell Technologies). The loss of tumor cells due to absence of targeted capture antigens is minimized by using negative selection approaches. However, the available protocols do not completely eradicate the presence of hematopoietic cells. Therefore, it is crucial for the development of these molecular diagnostic assays to choose nucleic acid markers that are not expressed in normal hematological tissue.

Other tissues

The detection of disseminated tumor cells in tissues and specimens other than lymph nodes, blood and bone marrow could provide valuable prognostic information in certain tumor types. For example, the detection of disseminated tumor cells in the peritoneal cavity lavages of pancreatic cancer patients by immunocytology is suggested as a prognostic factor [60]. For lung cancer new molecular approaches have been reported to detect exfoliated cancer cells in sputum [78,79]. The increased sensitivity and specificity in the detection of urothelial cancer cells in urine by using multiprobe FISH assays was reported recently [80,81]. Molecular approaches have also been demonstrated in initial studies to be able to detect DNA mutations originating from colorectal cancer cells in stool samples [82]. Nipple aspirate or ductal lavage fluids could provide new possibilities to monitor and detect breast cancer [83,84].

Summary & conclusions

An important goal for many scientists involved in oncology research is the identification of specific and sensitive tumor markers. The standard markers for immunohistochemistry in tissues are still cytokeratins (e.g., K19, K20). For high-throughput screening, circulating protein markers that are secreted or shed from the surface of tumor cells are desirable. Carcinoembryonic antigen in colorectal cancer, CA 15-3 and HER-2/neu oncoprotein in breast cancer, PSA in prostate cancer and CA 125 in ovarian cancer all give an indication of present tumor

mass and are used to monitor therapy or recurrence of disease. Histological and immunohistochemical approaches are routinely implemented to identify nodal metastases for staging purposes. The high rate of disease recurrence in node-negative patients raises the question if current protocols provide sufficient sensitivity and if other tissues (bone marrow, blood) have to be examined to discover occult micrometastases. Molecular strategies detecting nucleic acid markers are of high interest due to their exquisite sensitivity. PCR-based techniques specifically amplify DNA sequences and provide a highly sensitive diagnostic platform minimizing the amount of starting material needed. Several genetic alterations acquired by neoplastic cells can be used for their identification. Cancer-specific transcribed gene products have been used to detect the presence of a few tumor cells. In contrast to DNA detection, however, RNA detection requires special treatment of clinical specimens to protect RNA material from degradation and reverse transcription prior to PCR amplification. Despite very promising studies, the success of PCR-based tests still seems to be hampered by the lack of specific markers with sufficient coverage in the tumor population and the required tissue processing protocols, which are often not compatible with established pathological assays.

In the past few years the detection of minimal residual disease in bone marrow has been shown to be able to provide a valuable new prognostic tool. Standardizations of protocols and procedures are needed in order to compare different studies and to evaluate new diagnostic approaches. Statistically significant data still has to be generated in order to answer the question whether detection of circulating tumor cells in the blood can predict relapse and survival. Technical considerations about blood processing and chosen tumor markers are needed to achieve necessary sensitivity and specificity for clinically relevant studies.

Expert opinion

Can circulating tumor cells be used to detect early disease or is shedding and metastasis only occurring in advanced stages? Is the detection of micrometastic cells sufficient to improve treatment and clinical outcome?

These are justified questions and the answers will emerge with the development of reliable, standardized detection systems for minimal residual disease and their statistical evaluation. Technical advances have to be pursued in different tissue types to increase detection sensitivity. The establishment of specific detection strategies that use and find the appropriate markers is required for different tumor types, but also for different cancer subsets. Breast cancer is a good example of the heterogeneity of malignant diseases and demonstrates the inability of a single marker to detect all malignancies. The application of several, complementing markers might be necessary to successfully establish acceptable detection sensitivity throughout tumor populations. The design and implementation of multimarker assays requires careful technical considerations including innovative detection strategies (e.g., multicolor approaches) and particular emphasis on consistent specificity.

The clinical application of new technologies that promise high sensitivity for the detection of circulating cancer cells still has to be conclusively demonstrated. Therefore, a standardization of protocols is required and most importantly highly specific tumor markers that detect heterogenous tumor populations are needed.

Five-year view

If reliable detection of circulating tumor cells is accomplished, metastatic cells can be characterized, not only by single or multiple tumor markers, but molecular fingerprints could be established in the future. Differential display, microarray, bioinformatics and recently proteomics technologies are leading to an expansive discovery of potential tumor markers in the last several years. Various chip technologies and real-time PCR with multiple reporters could aid in the categorization of multiple markers and allow the discovery of their diagnostic potential. The detection and monitoring of residual cancer cells and their expressed markers could be an important step to assess their metastatic potential and to support the development of tailored immunotherapic approaches. Standard cytotoxic chemotherapies are targeting cells in the proliferative phase of the cell cycle but micrometastatic tumor cells could be in a dormant stage and therefore remain unaffected by current treatments. Individualized immunotherapies targeting tumor-specific antigens could become powerful approaches in the future. A big challenge will be to establish the clinical usefulness of the growing number of newly discovered markers and pattern recognition. Mathematical tools, such as neural networks and genetic algorithms are being developed to address these issues.

Key issues

- Histopathologic technologies are conventionally used to identify disseminated, tumor cells in lymph nodes; microscopic assessments are labor-intensive, subjective and feature low sensitivity.
- Immunohistochemistry increases sensitivity to detect nodal metastases.
- Immunocytochemistry is applied to detect disseminated tumor cells in bone marrow and blood specimens.
- Specificity discrepancies in immunological approaches are caused by heterogeneity of antibodies and techniques.
- Molecular approaches (polymerase chain reaction) offer high sensitivity, high throughput and low evaluation subjectivity in contrast to microscopic evaluation.
- Several DNA and RNA markers have been evaluated but are still hampered by either background expression in normal tissues or tumor heterogeneity.
- The development of multimarker assays and the discovery of new tumor markers are pursued.

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