

DETECTION OF DISSEMINATED CANCER CELLS IN BLOOD

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16.1 Introduction

The spreading or dissemination of cancer cells from the primary tumour to distant parts of the body is the most important factor affecting the disease progression and outcome in carcinoma patients. Once distant metastases occur, cancer is usually not curable anymore and the medical intervention is restricted to palliative treatment. Haematogenous spread of cancer is a major clinical challenge in oncology and has a fundamental influence on the disease outcome.

The process of metastases formation has been referred to as a cascade event. First, cancer cells with multiple genetic abnormalities start to grow unregulated and lose adherence to each other. By their ability to stimulate angiogenesis they prepare their entry into the blood and lymph circulation. Shed cancer cells circulate through the body until they adhere to the vascular endothelium and eventually leave the circulation to form metastases. In spite of important advances in diagnosis and treatment, for instance in breast cancer, 50 % of patients with apparently localized disease and even 30 % of patients without lymph node involvement will develop distant metastases within 5 years. It seems likely that cancer cells disseminate soon after onset of the disease.

The clinical staging of carcinoma patients is based on tumour characteristics like size, invasive growth and presence of metastases in regional lymph nodes at the time of primary surgery. However, the stratification of patients into prognostic subgroups based on these characteristics is too inaccurate to predict the individual patient outcome. The early spread of metastatic cells is easily overlooked in the traditional tumour staging. Therefore, it has been hypothesized that the detection of disseminated tumour cells in blood would allow an improvement in the prediction of disease

progression and outcome, because they represent a crucial part of the process of metastases formation.

Metastases are currently diagnosed by clinical manifestations, by *in-vivo* imaging technologies and by monitoring of serum tumour markers. However, imaging methods are not able to detect very small metastases and serum tumour markers mainly correlate to tumour mass but miss disseminated tumour cells at the onset of metastases formation.

It is assumed that the detection of the dissemination of cancer cells at an early stage offers a better chance to treat the cancer with higher efficacy and to prevent overt metastases. Furthermore, the detection of disseminated cancer cells in blood after surgical resection of the primary tumour followed by regular monitoring during after-care might be of clinical value by making an earlier prediction of relapse possible in comparison to the measurement of serum tumour marker levels.

Growing awareness regarding the importance of disseminated tumour cells in disease progression and recent progress in the development of highly sensitive *in-vitro* methods spurred many experimental and clinical investigations. The most recent investigations tend to confirm the clinical importance of disseminated cancer cells.

16.2 Methodological Approaches

The detection of disseminated tumour cells in blood or bone marrow requires highly sensitive and specific techniques enabling the detection of one tumour cell amongst 10⁶-10⁷ nucleated haematopoietic cells. Immunocytological and molecular nucleic acid detection techniques like PCR or RT-PCR are the most widely used. Each technique makes use of special characteristics of tumour cells for their identification.

Tumour cell detection by immunochemical methods depends on the ability of antibodies to distinguish between cells of different tissue origin, for instance epithelial cells from nucleated haematopoietic cells. The most widely used antigens on epithelial cells are the cyto keratins, which are uniformly expressed in these cells. Labelled antibodies bound to cytokeratins are then analysed by either fluorescence microscopy, flow cytometry or often by immunocytochemistry (ICC) with peroxidase or alkaline phosphatase staining techniques.

16.3 Immunocytochemistry

Immunocytochemical detection of disseminated tumour cells in bone marrow has been extensively performed and most of the clinical data published to date have been obtained with immunocytochemical investigations in bone marrow. The

immunocytochemistry can be combined with fluorescence *in-situ* hybridization (FISH) and additional morphological analysis. However, the detection of cells containing cytokeratins requires to make their cell membrane permeable for the labelled antibodies, which causes cell death and, hence, makes the discrimination between viable, apoptotic or dead tumour cells impossible. Obviously, only viable cancer cells are able to generate metastases.

Antibody-based detection techniques yield frequently non-specific results, since cytokeratins are sometimes expressed in haematopoietic cells. Antibody binding to Fc-receptor positive cells might also lead to false positive results. Depending on the antibody used a false positive detection rate of 1-3% can be expected. Another shortcoming of the ICC technology is its limited sensitivity, which allows the detection of one tumour cell amongst 10^6 mononuclear cells, which might not be sensitive enough to detect tumour cells reliably when they are in fact present.

Immunocytological screening is labour-intensive and time-consuming making the manual techniques too expensive to be used in routine diagnosis. This is why successful attempts have been made to automate these procedures.

16.4 PCR and RT-PCR

Molecular detection technologies like PCR and RT-PCR (reverse transcriptase PCR) are used to analyse nucleic acids for differences between carcinoma and haematopoietic cells. These technologies have also been used extensively in recent years for the detection of disseminated tumour cells in bone marrow and blood.

A major drawback of the PCR analysis is, that only a few tumour types show characteristic genomic alterations. Mutations can only be detected when these occur in few specified codons of a gene or when the mutation is already known. Carcinomas, however, are generally characterised by an enormous heterogeneity regarding a constant mutation pattern or their expression profile, which restricts the application of PCR analysis.

Another strategy to detect occult tumour cells is the analysis of tissue specific mRNA, a procedure known as RT-PCR. Malignant cells often continue to express markers that are characteristic of or specific for the normal tissue, from which the tumour has originated or with which the tumour shares the histotype. The appearance of these tissue-specific mRNAs at body sites where these transcripts are normally not present indicates tumour spread. Because of the instability of mRNA once released by the cells, the detection of mRNA in peripheral blood depends on the presence of viable tumour cells.

A successful RT-PCR assay for the detection of disseminated tumour cells in blood must meet several requirements among which the following are the most important:

- Specificity
- Sensitivity
- Reproducibility

The assay must have enough sensitivity to enable the detection of scarce disseminated tumour cells amongst an abundance of nucleated blood cells. Although the number of haematogenous disseminated tumour cells in breast cancer patients have been reported to be in a range of 0 up to 8,000 cells per ml blood, it is estimated that generally one tumour cell is found amongst 10^6 - 10^7 nucleated blood cells depending on the stage of the disease.

However, the numbers reported in the literature vary widely most likely because of reasons as described in the following.

The sensitivity of the assay is influenced by the expression level of a tumour marker and by the detection method. A prerequisite for highly sensitive tumour cell detection is a high over-expression of the marker in cancer cells as compared to "normal" blood cells. Furthermore, cancer is characterized by an extensive heterogeneity regarding the DNA mutation pattern and expression profiles of tumour markers. It is unlikely that all disseminated tumour cells express the same tumour markers. Therefore it is advisable to use more than one tumour marker in a test system to enhance the sensitivity.

Since PCR and RT-PCR allow the detection of even single molecules the major problem in detecting disseminated tumour cells is rather more the specificity than sensitivity. The specificity is influenced by several factors among which contamination through carryover, illegitimate (permissive) transcription and expression of tumour markers by "normal" cells in the sample and reliability are the most important.

Contamination problems can be kept in check by the inclusion of appropriate controls in the test system.

The illegitimate transcription is the main problem. This phenomenon is caused by the leakiness of promoters, i.e. it can be expected that any promoter could be activated by ubiquitous transcription factors, which leads to an estimated expression frequency of one tumour marker gene transcript in 500-1,000 non-tumour cells. This kind of expression is called illegitimate since the respective proteins are not formed and, therefore, are irrelevant for cellular functions. Thus, when the sample contains a large number of "normal" cells there might be enough "tumour" transcripts to be detected by the sensitive RT-PCR assay. Also a small subset of the "normal" cell population might express tumour-associated transcripts and antigens and, hence, cause false positive results, albeit it should be noted that false positivity reflects only the clinical but not the analytical outcome.

A reliable test should have a good reproducibility. Therefore, it is remarkable that in most studies on disseminated tumour cells this was not tested for. Inferior reproducibility might be caused by low transcript levels (low number of cells and/or low expression of transcripts in the sample) and usually encountered at expression levels close to the detection limit.

16.5 Improvement of Specificity

A RT-PCR assay must be sensitive enough to detect a very small number of tumour cells amongst an abundance of nucleated blood cells. At the same time it has to be specific enough not to detect illegitimate transcripts, which might yield false positive results (see above). This causes a dilemma, as normally specificity decreases, when sensitivity is increased, and *vice versa*. Therefore, depending on the markers used special experimental modalities like pre-analytical cell fractionation techniques, the use of several tumour markers or a quantification of the tumour marker transcripts have to be taken into account to ensure assay reliability.

Pre-analytical tumour-cell enrichment techniques can be based on tumour-cell density (differential centrifugation), on surface antigen expression (immunosorbent, positive selection) and on the depletion of unwanted cells (negative selection) to enrich the cell fraction to be analysed. The sensitivity of the RT-PCR assay is enhanced as the relation of target cells against background cells increases. At the

same time the specificity is enhanced as the number of unwanted cells, some of which might express illegitimate transcripts, is potentially reduced to a level beyond detection.

Density gradient centrifugation enables tumour cell enrichment in the mononuclear cell fraction of nucleated blood cells. However, loss of tumour cells has been reported in density gradient centrifugation through ficoll/Hypaque as only low tumour-cell detection sensitivity resulted. It has been observed that tumour cells sediment also in the granulocyte fraction. New density gradient fractionation media, e.g. OncoQuick, have been developed especially for the enrichment of disseminated tumour cells.

Immunosorbents enrich cell fractions by solid phase antibody binding to cell surface antigens. The antibodies can be coupled to small magnetic beads. The bead-cell complexes are extracted from the cell suspension by a magnetic field force. The attached cells are analysed afterwards. Again, because of the heterogeneous expression of tumour cell associated antigens a carefully selected panel of antibodies should be chosen as tumour cell recovery rates would improve as compared to single antibody beads. Antibodies binding to epithelial cell surface antigens like EpCAM or MUC1 are a preferred choice. Leukocyte depletion by immunosorbents directed against the CD45 antigen has also been tried.

Heterogeneity or absence of tumour marker expression may cause false negative results. Therefore, it has been proposed to assess several tumour cell markers in one blood sample to enhance the detection sensitivity. *AdnaGen AG* has developed test combinations for the detection of disseminated tumour cells by analysing several tumour cell associated markers selected for different tumour entities. Disseminated tumour cells are selected by a panel of monoclonal antibodies coupled to magnetic beads (Dyna). The enriched cell fraction is analysed in a multiplex RT-PCR for several tumour markers. 2 tumour cells from different breast and colorectal cancer cell lines could be detected in 5 ml blood from healthy donors in spiking experiments. Thus, 1 tumour cell can be reliably detected amongst an abundance of 7.5×10^6 - 2.5×10^7 leukocytes. When blood of cancer patients was analysed by multiplex RT-PCR analysis, tumour cell heterogeneity regarding the expression profile of tumour-associated markers was confirmed.

RT-PCR assays with several tumour-associated markers have also been performed by others and were shown to be superior in comparison to the assessment of single markers.

Specificity might also be enhanced by quantitative RT-PCR of tumour marker transcripts setting a definite cut-off value of tumour marker transcript numbers beyond which transcripts can be considered to be illegitimate and above which as tumour cell derived. Dependent on the chosen tumour marker many investigators claim reliable tumour cell detection by real-time RT-PCR.

16.6 Discussion

New technologies for the sensitive and specific detection of disseminated tumour cells in blood have evolved in recent years. Amongst these RT-PCR has gained wide acceptance as a highly sensitive method to detect disseminated tumour cells in different body compartments like blood, bone marrow and lymph nodes. The sample blood is of special interest since haematogenous spread is

the major route for tumour cells on their way to form metastases in distant parts of the body.

In addition, drawing blood is less invasive than taking bone marrow and makes sequential sampling during disease-monitoring feasible.

Since disseminated tumour cells have a limited life span, whilst in the blood circulation, their presence reflects active cancer growth, shedding of tumour cells and, thus, an ongoing metastatic process.

Different molecular tumour-associated markers have been used in recent years with different results regarding sensitivity and specificity. It can be anticipated that modern gene discovery technology will provide novel markers yielding even more reliable results. Gene expression profiling of carcinoma tissues has resulted in the definition of a variety of genes up-regulated in breast, colon and prostate cancer tissues, which might add to the quality and reliability of RT-PCR assays to detect disseminated tumour cells.

In recent studies the clinical relevance of disseminated tumour cells in the blood of cancer patients has been highlighted whereas some results argued against this. Reflecting on these controversial opinions several factors have to be considered, which include: sampling time, sampling frequency, surgical manipulation, medication etc.

Furthermore, the detection technologies used have to be standardised better in order to allow comparison of sensitivity and specificity of tumour cell detection. As pointed out already, a combination approach of pre-analytical tumour cell enrichment with the assessment of multiple tumour markers by RT-PCR can provide a very sensitive assay, which renders some currently used tumour markers specific enough to detect minute quantities of disseminated tumour cells in peripheral blood.

One can expect that the application of such an optimised and standardised RT-PCR assay of cancer patients in follow-up studies will provide important and valuable clinical information in the near future. Clinically valuable information refers to several aspects in the course of cancerous disease:

- The detection of disseminated tumour cells in blood in newly diagnosed cancer patients might help to select patients for neo-adjuvant or adjuvant chemotherapy.
- The reappearance of tumour cells in blood after tumour resection might be interpreted as a reawakening tumour activity and thus point to treatment failure at a potentially earlier time point than would be possible with conventional aftercare.
- The reduction of disseminated tumour cells reflects the efficacy of the chosen therapy.

Routine monitoring of cancer patients might ensue. Consequently, the therapeutic regimen might be changed or reconsidered, which might result in an increase of the 5-year survival rate of cancer patients.

16.7 Conclusion

Disseminated tumour cells in blood are most likely to be detected during tumour progression, which offers, besides a prognostic value, also an independent means to evaluate therapeutic efficacy

and early recognition of tumour progression with higher sensitivity as has been possible before with conventional aftercare.

Because of the important clinical implications utmost care has to be taken when designing a test system for the detection of disseminated tumour cells in blood. The application of the RT-PCR technology combined with a pre-analytical enrichment of the tumour cells provides the required sensitivity and specificity as long as the chosen antibodies and RNA markers are selected for high expression in tumour cells but low expression levels in "normal" blood cells.

The heterogeneous expression of tumour-associated antigens and RNAs has to be accounted for by, for instance, assembling antibody cocktails and performing a multiplex RT-PCR analysis.

The development of better controlled and standardized test systems will help to establish this important new diagnostic tool in the clinical routine.

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