Novel approaches to the analysis of circulating tumour cells

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Abstract
Introduction
Circulating tumour cells present an important marker of the progress of several cancer diseases including breast and colorectal cancer, and enable interesting prognosis and diagnostic options that can complement convenient diagnostic techniques based on several imaging methods. Based on its relevance, the analysis of such kinds of cells is within the scope of many research and clinical institutes; however, it is still a difficult task. The presented critical review aims at classical as well as novel techniques for the detection and analysis of circulating tumour cells. Classical approaches present a golden standard and are commonly used in many clinical facilities. Alternatively, these techniques are suffering from many drawbacks and disadvantages and hence new options and strategies are being developed. These new approaches include microfluidic devices and detection techniques based on the current possibilities, allowing higher selectivity and speed with considerably lower costs.

Conclusion
Analysis of circulating tumour cells presents a novel option for cancer diagnostics and prognosis. Results from such tests can be helpful in further personalisation of cancer treatment and thus help improve the patient’s quality of life. However, tests for the analysis of circulating tumour cells are still not very easy and their successful application still needs trained personnel with considerable praxis and experience.

Introduction
Cancer is one of the most dangerous diseases nowadays. Its early diagnosis and careful study of its development can considerably increase the quality of life of patients. A demand exists for research and development of methods aimed at studying as well as describing the cancer disease, and many scientists are considering this theme for their study. The current methods used in the treatment and diagnosis of cancer include PET scans, MRI, CT mammography and other more or less classical methods. Nonetheless, sensitivity of information of the obtained data could sometimes considerably limit the overall procedure of diagnosis and hence other possibilities of cancer diagnosis and prognosis are being developed and studied too. The key diagnostic and prognostic markers can also help in the detection of early tumour cell dissemination. Many research teams have therefore aimed their research scope on the development of sensitive assays allowing the specific detection of single tumour cells. An emerging approach to detect metastasis is the analysis of circulating tumour cells (CTCs), which have recently showed promise in filling gaps left by previously mentioned imaging techniques. CTCs are tumour cells that originate in primary tumour sites or metastasis. These cells circulate in a patient’s blood stream and are usually not found in a healthy population.

The challenge of circulating tumour cells detection is related to the requirement of high selectivity and ultra trace limits of detection of the developed methods. It is well-known that the invasion can start very early during tumour development, identification and respective counting of CTCs, when their absolute numbers are very limited (few CTC/CTM per 10 ml of blood, which means few CTC/CTM mixed with approximately 100 million leukocytes and 50 billion erythrocytes) and could have considerable information for the diagnostics. The specificity itself has a similar importance. A proper identification of CTCs and their absolute distinguishment from non-tumour epithelial cells as well as from normal human leukocytes is demanding, because false negative as well as false positive results can rapidly and ultimately change the clinical options, change the life expectancy and thus decrease quality of life of the patient. Many previous reviews have been done earlier regarding the detection and analysis of circulating tumour cells. This particular review covers the most modern methods of analysis and successful detection of circulating tumour cells using modern approaches with a focus on novel approaches based on spectral techniques.

Discussion
The author has referenced some of his own studies in this review. The protocols of these studies have been approved by the relevant ethics committees related to the institution in which they were performed.
Detection of circulating tumour cells using classical approaches

The methods aimed at detection and analysis of circulating tumour cells can be, in their principles, divided into direct and indirect. Indirect methods are aiming at the detection of organ-specific markers, without any information regarding the tumorous nature of the targeted cells. These methods include immuno-mediated or molecular methods.

Indirect methods

This kind of detection is based on immuno-labelling of cells which are previously enriched using immuno-based enrichment approaches or using their physical properties (size, morphology, density). The magnetic separation and respective isolation, and enrichment of the cells is based on an interaction of the cells of interest with a magnetic bead of different size (macro-iron, colloidal iron, ferrofluid). Due to the lack of knowledge of a specific tumour marker, the surface of the beads is covered by antibodies and selectors specific to epithelial antigens, such as EpCAM, some cytokeratins (CK) and others. The main drawback of such isolation is the rate of false positive results, which is caused mainly by an interaction of antibodies with non-tumour cells. For example, there exists a high ratio of CK positive cells in normal control, which ranges between 0% and 20%, where most of these cells are leukocytes. However, leukocytes are not the only affected cells, other cells including plasma cells, macrophages and others can be present in the enriched material. Moreover, some of these cells cannot be easily distinguished from the circulating tumour cells based on their morphology. To increase the specificity of such methods, histochemical labelling is usually performed. The attracted cells are fixed and labelled using usually fluorescent nuclear dye, e.g. DAPI, fluorescent antibody to CD45 or fluorescent antibodies to intracellular cytokeratins such as CK 8, 18 and 19, which are highly specific to epithelial cells. However, these markers are selective to epithelial cells and non-tumour epithelial cells and could be present in some cases as well. The total number of the circulating tumour cells can then be significantly shifted to more positive values and lead to radical problems and errors in diagnostics, prognosis, life expectancy, medication and therapy. Moreover, it was found that EpCAM is present only in 70% of 134 tumours of different histologic types. Similar problems and consequent drawbacks can be found for other types of antibodies as well. The bottom line is that this particular approach cannot be taken as the one and only solution in the analysis and detection of circulating tumour cells due to the previously mentioned drawbacks, especially due to lack of suitable selectivity to circulating tumour cells and unavailable options to distinguish between tumour and non-tumour cells.

Other approaches of indirect methods are based on molecular principles. These methods include detection of genes specific to epithelial cells using RT-PCR. The basic principle consists of several steps covering cell isolation, enrichment, RNA extraction, synthesis of complementary DNA, amplification and product analysis using analytic approaches such as SDS-Page or less commonly used capillary electrophoresis or/and mass spectrometry. The important aspect of such approaches lies in their ultra-high sensitivity (ultra-trace limits of detections of respective products). These methods are able to successfully detect circulating tumour cells at the concentration levels of 1 tumour cell to 1x10⁷ of normal cells. This superior sensitivity is unexpectedly balanced by missing options of quantitative analysis (counting of the cells). Another limitation to the method is over-complexity and limited speed of testing. However, even these parameters can be changed by further research and development on the methods. There is still one point, which limits this solution in a considerable way – lack of proper RNA markers. The present markers are selective to epithelial cells and hence false positive results can be found in cases where the patient’s blood contains non-tumour epithelial cells.

Direct methods

Direct methods include approaches based on a direct evaluation of circulating tumour cells by their careful cytopathological analysis or by an in-depth study of their genome in order to provide information concerning the nature of the cells (tumour vs. non-tumour cells). The genome analysis includes mainly FISH, comparative genomic hybridisation and mutation analysis. However, genome tests are applied especially for the CTC characterisation, where quantity is not required. Alternatively, FISH tests can provide false negative results or shift the determined numbers to more negative values due to the fact that FISH test is not always able to label all circulating tumour cells. Other types of tests, namely, comparative genomic hybridisation (CGH) and mutation analysis are very expensive and are hence limited only to some special cases.

Methods of the circulating tumour cells enrichment

The basic approaches derived from classical analytical tools are commonly used in cell enrichment. These solutions cover filtration, where peripheral blood leukocytes such as lymphocytes are filtered out from the sample using polycarbonate membrane with diameters ranging in orders of micrometres, usually 8 micrometres. Circulating tumour cells remain on the filter due to their average size, say between 29 and 37 micrometres for breast cancer cells.
Detection and analysis of circulating tumour cells using microfluidic devices

Detection of circulating tumour cells using novel analytical approaches opens the door to faster and more reliable diagnostics. Modern methods of miniaturisation and nanotechnology have evolved to the level where even very small and cost-effective solutions can sometimes replace old classical methods. One of the solutions can be microfluidics. A majority of microfluidic devices aimed at circulating tumour cells filtration utilise some kind of affinity-based model. This model is based on a formation of non-covalent bonds between an antigen expressed on a cell membrane and an antibody immobilised on a wall of the respective microfluidic channel. These non-covalent forces are able to hold the target cancer cells, while the rest of the samples, including other types of cells without a sufficient number of interactions with the antibodies, are washed out from the channel by a flow. The captured cells can then be easily gathered using a stronger shear force, specific enzymes or with the use of various physical parameters. One of the first microfluidic devices (CTC-chip by Negrath) was based on the use of EpCAM molecules. It contains an array of several tens of thousands of hot spots modified with this antibody with a total surface area of almost 1000 mm². However, the efficiency of such a device plays an essential role in its possibility to succeed on the market and find a place in clinical practice. In this case, the efficiency can be improved, e.g. by an increase of surface interactions between CTCs and a wall of the microfluidic channel. This has already been shown by Wang et al., who had utilised current knowledge in nanotechnology and developed a 3D nanostructured substrate and thus enhanced local interactions between antibodies and cellular surface. The value of a microfluidic device can be consequently enhanced using various modifications and integrations of additional functions. One of these functions can be, for instance, a biochemical analysis or cell culture. Sivaganam et al. introduced a microfluidic device with a possibility of in situ cell culture of MFC-7 breast cancer cells. He was able to reach a yield of 85 ± 10%. Obtained cells were afterwards released using air bubbles and cultivated. Over 90% of the detached cells were viable and able to be further used.

One of the main problems in microfluidic devices is their limitation to a sample volume. Clinical samples are usually 1.0 or more mL, and the capacity of an average microfluidic device is in order of microlitres. Moreover, the whole sample has to be processed with a very high level of specificity and efficiency. For this reason, several high-throughput sampling units were developed. Adam et al. developed a polymer based on a microsampling unit capable of rapid isolation of CTCs from sample volumes exceeding 1 mL. His device consisted of 51 channels covalently coated with EpCAM antibodies and was able to process 1 mL sample within 1 minute.

Another type of rare cell isolation is utilisation of physical mechanisms which can effectively discriminate cells of interest from other cells and substances presented in a sample. One of these physical forces can be an electrical force. An illustrated example of the utilisation of such a force is the use of DEP-base rare cell isolation. DEP is the effect where the cell is induced to move by an application of a non-uniform electrical field due to a polarisation effect. These forces are determined by dielectric properties of the cell membranes. The effect can be positive or negative, where cells move in a direction of high electric field when their DEP is positive, where this is caused by a higher polarisation of cells compared to a suspension medium. The main advantages of this label-free separation are that the cells are viable and can easily be seen after the separation, and also variability of the mechanism which enables a large specificity for target rare cells based on their dielectric phenotype.

Analysis of circulating tumour cells using Raman spectroscopy

Raman spectroscopy is a powerful analytical technique, which can be potentially used as a solution for many analytical cases. Its implementation as a tool of CTCs analysis has been tested by many authors, where its modification, Surface enhanced Raman Scattering, has been usually employed. Pioneering works were done by Sha et al. where magnetic beads with silver coating were used as effective SERS substrates allowing a magnetic separation with an efficient SERS detection. This work used EpCAM and anti-HER 2 antibodies. Raman technology was also consequently applied in the detection of circulating tumour cells after their isolation using the eletrophoretic microfluidic device. This case was based on the utilisation of Coherent Anti-Stokes Raman Scattering (CARS). One of the main researchers working in this field was Neugebauer, who published several works covering cell analysis using Raman spectroscopy. He has published several papers covering the use of Raman spectroscopy as a suitable detector for CTC analysing microfluidic devices and imaging of circulating tumour cells. His initial works were followed by others, who improved and enhanced the possibilities of this utilisation. Ranc et al. published a work, where he developed a method for direct analysis of CTCs samples on cyto-spins. Wang used SERS technique to analyse samples of 19 patients using a different selector, epidermal growth factor peptide. His work improves the selectivity as well as reliability of this approach. The utilisation of Raman spectroscopy in the field of cell...
analysis was recently summarised in the review written by Vendrell et al.\textsuperscript{33}

Conclusion

Analysis of circulating tumour cells presents a novel option for cancer diagnosis and prognosis. Results from such tests can be helpful in further personalisation of cancer treatment and thus help improve the patient’s quality of life. However, tests for the analysis of circulating tumour cells are still not very easy and their successful application still needs trained personnel with considerable praxis and experience. Development of methods able to simplify the whole procedure is thus demanding and many research teams are focused in this direction. Development of novel strategies based on current technologies and trends enhances and broadens possibilities for a better and faster cancer diagnosis and treatment. Microfluidic devices play an important role and could present a leading trend in such applications. Their proper and efficient hyphenation with currently developed detection techniques such as surface enhanced Raman spectroscopy could even more enhance their application potential. Modern techniques should be able to process many samples in a short period of time with an ultra high accuracy and precision. However, the first requirement can be achieved relatively easily, the second one still presents a challenge.

Abbreviations list

CARS, Coherent Anti-Stokes Raman Scattering; CGH, comparative genomic hybridisation; CK, cytokeratins; CTCs, circulating tumour cells.

References