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PROTEOMIC STRATEGY FOR DETECTION OF CIRCULATING TUMOR CELL SURFACE ANTIGENS

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INTRODUCTION

Tumor cells are often found in the peripheral blood of cancer patients, even those with localised cancer or who have had a primary tumor removed. Such cells are called circulating tumor cells (CTC) and may derive either from the primary tumor or from a metastasis. Presence of CTC in blood has been shown to correlate with metastasis, poor prognosis and poor treatment response and is considered important for the metastatic process. CTCs are thus a promising target for early diagnosis and characterisation of metastatic cancer, as well as for planning treatment and following patient response. Since CTCs may be heterogeneous and as rare as one cell per 10^5–10^7 mononuclear cells, enrichment and detection presents a challenge [1]. The aim of this report is to discuss a strategy for finding CTC-specific antigens that may be used for accurate CTC enrichment and detection.

What kills cancer patients is usually metastasis. Often, clinical signs appear only when the tumor has been existing for years or even decades and may already be widely metastasised throughout the body. In such cases, treatment may be inefficient with risk of resistance development, since the continuous long-term genetic selection, together with the metastatic process, may create highly aggressive, genetically unstable and different phenotypes. The first steps in metastasis involve tumor cells detaching from their tissue of origin and travelling through the bloodstream to metastatic niches in other organs, where they may lay dormant for decades as micro-metastases until symptoms appear [2]. It is necessary to diagnose cancer as early as possible in the metastatic process, and detection of CTCs offers a way of detecting metastasis long before the end of dormancy and presence of symptoms.

Several different techniques for enrichment and detection of CTCs exist, based on differences between cancer cells and leukocytes in gene expression, antigen expression or physical characteristics.

Measuring expression of tumor specific genes or mutations in peripheral blood cells by qPCR may be used as a proxy for CTC detection. However, this approach alone still requires enrichment before analysis. Also, it requires cell lysis, making further CTC enumeration, characterisation and culturing impossible [1].

Antigen-based methods rely on a few markers that have long been known to be overexpressed in cancer. The cells may be captured with antibodies or aptamers toward the markers, or labelled for subsequent cytometry analysis. Common CTC markers are cytokeratins (CK) and epithelial cell adhesion molecule (EpCAM). Antigen-based methods may be used for enrichment, detection and enumeration of CTCs, and do not require cell lysis. The problems with these approaches are related to the markers. CK may be expressed in leukocytes, while EpCAM is not expressed at all in a range of cancers, such as sarcomas, and loss of EpCAM is a component in epithelial-mesenchymal transistion (EMT), a step in metastatic progression. This may lead to false negative CTC detection results in progressed cancer [1].

Physical methods are based on a continuously distributed physical cell characteristic, such as size, hardness, optical or acoustic characteristics. These characteristics affect particle flow and may be used for cell separation. As of now, there have been few trials of such methods for actual CTC detection and counting. Because of CTC heterogeneity, and the fact that the continuous distribution will always lead to compromises between purity and yield, they are probably best suited for enrichment followed by antigen-based detection [3].

Thus, to address CTC heterogeneity and allow simultaneous enrichment and detection of viable cells, reliable cell surface markers of CTCs are needed. These markers may be used both to enrich blood samples for CTCs and to detect CTCs enriched by other methods. The aim of this report is to discuss an unbiased strategy to obtain such markers.

PROTOCOL

If a CTC detection system is based on use of cultured cells, this system may not hold in clinical applications, as differences exist between cultured cells and patient cells. For example, sorting CD45+EpCAM- cells by FACS detected CTCs *in vitro* but not *in vivo* [4]. Thus, clinical samples are necessary to obtain reliable CTC surface markers. The first step is therefore to collect blood from a number of patients representing healthy controls, as well as a number of different cancers of both epithelial and stromal origin.

After centrifugation, RBC lysis and immunomagnetic CD45 depletion, a method successfully used

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Abbreviations: CK – cytokeratin; CTC – circulating tumor cell; EMT – epithelial-mesenchymal transition; EpCAM – epithelial cell adhesion molecule; ESI – electrospray ionization; LC-MS/MS – liquid chromatography- tandem mass spectrometry; m/z – mass to charge ratio; RBC – red blood cell; WBC – white blood cell.

to enrich non-EpCAM CTCs in an unbiased way [5], only non-blood cells will remain. The cells will be exposed to a biotinylation reagent, adding a covalently linked biotin tag to all surface proteins. After lysis, biotin-bound proteins will be captured on avidin-coated resins. After cleavage of a biotin linker, for example by disulfide bridge reduction, proteins are eluted. This method has been shown to enrich surface membrane proteins specifically and with high purity [6]. Enriched proteins will thus represent the membrane-bound fraction, and will be subject to digestion into short peptides, followed by mass spectrometry. The same process will be performed on the CD45-depleted WBC fraction. Thus, four groups will be analysed by MS for every cancer type; healthy non-WBC, cancer non-WBC and the CD45+ leukocyte fractions from both groups.

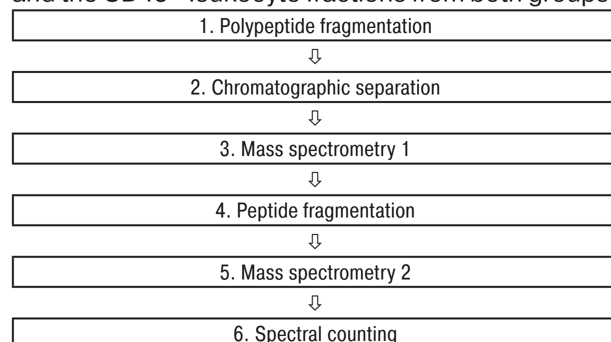

Fig. 1. LC-MS/MS shotgun proteomics. 1. Proteins are digested into shorter peptides, for example with trypsin and CNBr. 2. Peptides are separated through multiple chromatographic steps. In the Mudpit approach, strong cation exchange and reverse-phase chromatography separate peptides by charge and hydrophobicity. 3. Peptides eluted from chromatography column are ionised by ESI and enter the mass spectrometer, where mass to charge ratios (m/z) of the peptides are measured, giving peptide masses. 4. The peptides are further fragmented in the mass spectrometer. A number of different fragmentation methods exist, such as collision-induced dissociation. 5. Peptide fragment m/z are measured, giving sequence information for the fragmented peptide. 6. Numbers of fragmentation spectra representing the same peptide are compared between groups, giving relative quantification of peptide amounts between the groups (Pictures from Wikimedia Commons).

Study of membrane proteins is difficult because the hydrophobic transmembrane domains make proteins difficult to separate, transmembrane peptides are difficult to solubilise and these peptides have few sites for tryptic digestion. The separation problem can be somewhat alleviated in 2D approaches by using for example 16-BAC or blue-native PAGE separation in the first dimension, and in LC approaches by using multi-dimension separations, such as Mudpit. Solubility can be addressed with surfactants or organic modifiers, while digestion is improved by combining tryptic digestion with chemical approaches such as CNBr-dependent fragmentation. LC-MS-electrospray (ESI) approaches have high throughput and a proven track record in membrane protein study [7] and will be used in the proposed protocol.

With Mudpit LC-MS/MS, the peptide mixture is first passed through strong cation exchange and reverse phase chromatography, separating the peptides according to charge and hydrophobicity. As the peptides elute, they are ionised by ESI and injected into the mass spectrometer, where their mass-to charge ratio is determined. Thereafter, peptides are further fractionated and the resulting fractions of each peptide analysed in a second mass analyser, creating MS/MS spectra with peptide sequence information. The peptide masses are searched against public databases to identify the proteins they represent [8]. The spectral counts, numbers of MS/MS spectra for each peptide, are compared between the groups, giving relative inter-group peptide levels in a label-free manner [9].

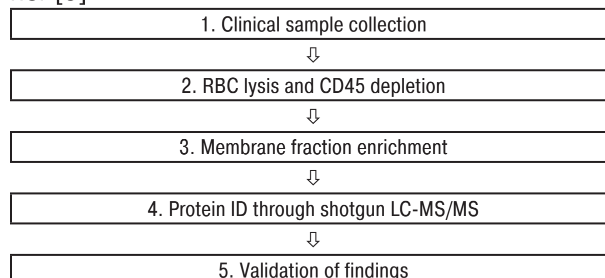

Fig. 2. General approach of the protocol. 1. Clinical blood samples are collected. RBCs are lysed with a special buffer. The samples are incubated with anti-CD45 beads and centrifuged, whereafter the CD45 bound WBC fraction is removed. 2. Cells are exposed to a biotinylation reagent, which adds biotin tags to surface protein amines. Cells are lysed, and biotin-bound proteins are captured on avidin-coated resins. 3. Proteins are eluted from the resins and subjected to shotgun LC-MS/MS, as described above.

With the expression levels of peptides represented as MS/MS spectral counts, the levels will be compared between groups. The proteins that are highly overexpressed in the cancer group as opposed to healthy controls or leukocytes are those that may be considered for use as CTC markers. The marker panel thus created will finally have to be validated. First, the surface expression of the markers will be validated by immunofluorescence. Those actually present on the cell surface will then be validated against another panel of blood samples. Validated markers will be used for detection of cancer.

The general protocol is as follows:

- Clinical blood samples are subject to RBC lysis.
- Centrifugation with anti-CD45 beads depletes WBCs.
- Cell fractions are biotinylated.
- Cell lysis, biotin capture by avidin and elution of bound proteins.
- Membrane protein identification through peptide identification by LC-MS/MS.
- Protein levels in CTCs, WBCs and controls are compared by spectral counts.
- Validation of surface expression of proteins through immunofluorescence.
- Validation of markers on new clinical samples.

DISCUSSION

The aim of the proposed protocol is to outline a strategy to discover specific and sensitive CTC surface antigens. This would improve detection of CTCs by antigen-based methods for early diagnosis of can-

cer. The described strategy subjects blood samples from a range of cancers to membrane enrichment and LC-MS/MS, and compares the proteome of the non-leukocyte membrane fraction of cancer patients to the membrane fractions of healthy control non-leukocytes and leukocytes from both groups.

The technical steps in this strategy have already been shown to work successfully. Negative enrichment by CD45 depletion yields higher CTC recovery rates than positive EpCAM enrichment [5]. Membrane protein enrichment through amine biotinylation is not limited to capturing glycosylated proteins, as the cell surface capture method, and in comparison with colloidal silica beads, only proteins present on the outside of the membrane will be captured, reducing sample complexity. Also, the biotin may be used as a mass tag in MS, increasing confidence in that detected peptides were present on the cell surface [10]. LC-MS/MS has been successfully used in the identification of proteins from such membrane fractions [7].

The result of this strategy will be a panel of cell surface markers, which will be superior to current CTC markers because of the unbiased approach to their identification. The markers may be used in devices for simultaneous enrichment, detection and enumeration of viable CTCs. Based on the importance of CTCs in metastasis, their early intravasation and their correlation with clinical outcomes, better CTC detection will be useful in oncological practice. Accurate CTC markers will allow early diagnosis and in-depth characterisation of cancer, as well as treatment personalisation based on CTC phenotype and response monitoring through counting CTCs after treatment.

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