Detection and Cultivation of Circulating Tumor Cells in Malignant Pleural Mesothelioma

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Abstract. Malignant pleural mesothelioma (MPM) is an aggressive disease with very poor prognosis which tends to affect older patients. Progress in the management of this group of patients has been limited by the rarity of the disease and hence, difficulty in conducting randomized trials. The vast majority of cancer deaths occur due to metastasis of the primary tumor to distant sites via circulating tumor cells (CTCs) in the circulation. CTCs are extremely rare and limits in technology used to capture these cells hamper our complete understanding over the metastatic process. In the present study we present a new method for detection and cultivation of CTCs isolated from peripheral blood of MPM patients.

Patients and Methods: Patients with diagnosed MPM were enrolled into this study. Results: A size-based separation method for viable CTC enrichment from unclothed peripheral blood has been introduced; MetaCell. The size-based enrichment process was based on filtration of peripheral blood (PB) through porous polycarbonate membrane. The separated CTCs are cultured on the membrane in vitro under standard cancer cell culture conditions and observed by an inverted microscope. Conclusion: The reported methodology allows for quick and easy enrichment of CTCs and their cultivation. The cultivated cells can be used for next specification of gene expression and histological/biological specificity of concrete mesothelioma.

Malignant mesothelioma (MM) is a rare neoplasm that usually develops after exposure to asbestos and particularly involves the pleural cavity. It has a poor prognosis with aggressive local invasion and metastatic spread. Malignant pleural mesothelioma (MPM) although rare, is more common than other types of mesothelioma.

MPM is an aggressive disease with very poor prognosis and tends to affect older patients. Progress in the management of this group of patients has been limited by the rarity of the disease and hence, difficulty in conducting randomized trials.

The management of patients with MM is complicated, first of all because the tumor is notoriously difficult to diagnose. The onset of symptoms is often insidious and non-specific. Therefore, an accurate diagnosis is important for appropriated therapeutic intervention and for proper epidemiological records. Because mesothelioma is fairly well-associated with asbestos, and exposure is usual in the workplace, it is hypothesized that monitoring a high-risk population might detect patients at an earlier, more treatable stage and result in prolonged survival over the present median 12 months from the start of therapy. Thus, focus has been put on finding tumor markers in the blood and other biological fluids that can be used in association with radiography for the non-invasive detection of MM.

Solid cancer is an extremely complex disease composed of various molecular alterations and phenotypic changes. The vast majority of cancer deaths occur due to metastasis of the primary tumor to distant sites via circulating tumor cells (CTCs). CTCs are extremely rare and current technology limitations in capturing these cells hamper our complete understanding over the metastatic process.

In the present study we present a new method for detection and cultivation of circulating tumor cells isolated from peripheral blood of MPM patients.

Patients and Methods

Patients. Five patients diagnosed with pleural malignant mesothelioma were enrolled in the study. All patients were candidates for surgery. Following informed consent, clinical data
were collected from all participating patients. The patient’s characteristics are shown in Table I. Peripheral blood was collected prior to surgery. For each patient, approximately 8 ml of venous blood were drawn from the antecubital veins into S-Monovette tubes (Sarstedt AG & Co., Numbrecht, Germany) containing 1,6 mg EDTA/ml blood as an anti-coagulant. The samples were processed at room temperature using an isolation procedure completed within 24 h after the blood draw. The Ethics committee of all participating Universities and Hospitals approved of the study protocol according to the Declaration of Helsinki.

**CTCs enrichment and culture.** Recently, a new size-based separation method for viable CTC enrichment from unclothed peripheral blood has been introduced, MetaCell® (MetaCell s.r.o., Ostrava, Czech Republic). The size-based enrichment process is based on the filtration of peripheral blood (PB) through porous polycarbonate membrane (pores with diameter of 8 μm). The minimum and maximum volume of the filtered PB may be adjusted up to 50 ml of fluid. Eight ml PB from patients suffering with MPM was transferred into the filtration tube.

The successive blood transfer in several steps is preferred to prevent the blood clotting on the membrane filter. The PB filter flow is supported by capillary action of the absorbent touching the membrane filter. Afterwards the membrane filter kept in a plastic ring is transferred into the 6-well cultivation plate, 4 ml RPMI media are added to the filter top and CTCs are cultured on the membrane in vitro under standard cancer cell culture conditions (37°C, 5% atmosphere of CO₂) and observed by an inverted microscope. The CTCs are grown in the FBS-enriched RPMI medium (10%) for a period of 10-14 days, minimum. Grown cells are analyzed by means of histochemistry (May-Grünwald staining) (Figure 1).

Next, the enriched CTC fraction can be transferred from the membrane and cultured directly on the plastic surface or microscopic slide. Microscopic slide culture is preferred if the immunohistochemistry/immunofluorescence analysis is planned. If an intermediate CTC – analysis is awaited, the CTC-fraction is transferred in PBS (1,5 ml) to the cytospin slide. The slide is then dried for 24 h and histochemically-analyzed.

**Cytological analysis.** Stained membranes were examined using light microscopy in two steps: (i) screening at x20 magnification to locate cells, (ii) observation at x40 for detailed cytomorphological analysis. Isolated cells and/or clusters of cells of interest were selected, digitized, and examined by an experienced researcher. CTCs were defined as cells presenting at least three of the following criteria: (i) cytomegaly (cell size more than 20 μm) (ii) nuclear size equal or larger than 10 μm (iii) eccentric round nucleus (iv) presence of a visible cytoplasm (v) normal nuclear-to-cytoplasmatic ratio. (vi) prominent nucleoli (1-3) (vii) bi- or multi-nucleated stage.

### Results

We demonstrated a successful use of a new tool for CTCs isolation from peripheral blood in patients diagnosed with mesothelioma. The circulating mesothelioma cells were detected in 4 of the tested patients (80%). Circulating mesothelioma cells isolated from PB captured on the membrane were cultured successfully *in vitro* for 10 days minimum. In the two studied cases the captured cells proliferated rapidly. After the cultivation, the cells were stained by May–Grünwald to enable for detection of subcellular compartments e.g. nucleus and nucleoli. The cells exhibited evident cytomegaly (size of more than 20-30 μm) with usually a round, relatively uniform nucleus, which was located eccentrically (Figure 1). The nuclei exhibited a prominent nucleolus. The nuclear–cytoplasmatic ratio was relatively normal, obviously due to the fact, that the cultured cells grow into the space on the membrane and they have a bigger cytoplasm. Generally giant mesothelioma cells with prominent nucleoli were detected, some of the cells were bi-nucleated (Figure 1C and D). Mesothelioma cells cultured on the membrane remain loosely (no contacts with neighboring cells) attached. An eccentrically-located nucleus could also represent a sign of an epitheloid mesothelioma cells.

### Discussion

Potential biomarkers might be helpful in managing three clinical aspects of MMs: Early diagnosis, prognosis and treatment outcome prediction. A large number of biomarkers have been assessed in biological fluids and tumor tissues for their prognostic value in MM. In summary, standard markers such as hyaluronic acid, various cytokeratin fragments (CYFRA21-1, TPA) and other cancer antigens (CA15-3, CA-125 or CA19-9 or CEA) are not sensitive or specific enough and cannot be used in clinical practice.

Several small, clinical studies have evaluated the following three new biomarkers: soluble mesothelin-related peptide (SMRP), megakaryocyte potentiating factor (MPF) and osteopontin (1, 2). Mesothelin is a differentiation antigen on mesothelial cells highly expressed in mesothelioma (3). High levels of SMRP have been found to be associated not only with MM but with ovarian (4) and pancreatic cancer (5) as well. Megakaryocyte potentiating factor (MPF) originates from the same precursor protein of mesothelin; it is potentially more sensitive. Osteopontin (OPN) has been described as a promising biomarker for the early detection of MM (6). OPN is a glycoprotein which mediates cell-matrix interactions and cell signaling by binding with integrin and CD44 receptors.
Due to limitations of single biomarkers in terms of sensitivity and specificity much effort has been focused on the use of a biomarker combination that can distinguish between asymptomatic asbestos-exposed subjects and early-stage MM patients.

Often it is possible to confirm a MM diagnosis based on pleural fluid cytology using immunohistochemistry including CEA, BerEP4 and TTF1. Unfortunately, cytological yield in suspected mesothelioma is poor with a sensitivity of only 32% (7). The cytopathological examination should be not limited only on pleural fluid cytology. Zielinski et al. diagnosed positive malignant cells from intra-abdominal lavage in more than 50% patients with pleural malignant mesothelioma (8).

So, the detection and enrichment of mesothelioma cells from circulation or lavage/pleural effusion is an important point in the diagnostic procedure. Monitoring of the amount of these CTCs should be a marker of an effective treatment.

CTCs are a potential diagnostic tool or a surrogate marker of disease progression in human tumors and their role in MM has not been yet set. At present, limited data from two different groups are available on MM and CTCs. Wendell et al. analyzed a small case series and demonstrated that CTCs can be effectively enumerated in MM patients (9). Tanaka’s research group published a study (10, 11) in which patients with suspicion or diagnosis of MM were analyzed. CTCs in peripheral blood were captured and quantitatively evaluated with the ‘CellSearch’ system without knowledge of clinical characteristics of each case. Among 170 eligible cases, 137 were finally diagnosed as having MPM and 33 as non-malignant diseases. CTC test had a positive result in 38% of MPM cases and was also positive in 9% of non-malignant cases. CTC count was significantly higher in MPM than in non-malignant cases.

The main deficit of the studies described above is the application of technology with probably low sensitivity to detect CTCs in MPM. The authors used detection system designed for capturing CTCs of epithelial origin with an antibody against epithelial cell adhesion molecule (EpCAM).
Authors of one of the study reported a previously positive expression of EpCAM only in 50% of patients with malignant pleural mesothelioma (10). Such detection of CTCs by immunocytochemistry has one major drawback: the potential to miss cells not expressing the intended target antigens. As recently shown, this may occur due to epithelial–mesenchymal transition (EMT) and expression of mesenchymal markers by epithelial CTCs (12). This issue also represents a potential major limitation both for affinity-based enrichment and detection of CTCs.

EMT is a physiopathological process by which epithelial cells acquire mesenchymal shape and properties. In cancer, epithelial–mesenchymal transition is involved in many mechanisms, especially invasion and motility, but also in resistance to apoptosis, senescence, immunotolerance, immunosuppression, drug resistance, and the acquisition of stem cell-like properties (13, 14). MPM is histologically-characterized by the concomitant presence of epithelioid and sarcomatoid features, the latter being associated to worse prognosis, thus suggesting a role of epithelial–mesenchymal transition in this dual phenotype. Several authors have reported the association between MPM subtype and patient prognosis (15, 16). Indeed, a purely epithelioid histology involved the longest survival, a purely sarcomatoid histology the worst, and a bi-phasic pattern an intermediate survival (17-19). EMT has a significant part in the morphological features of malignant mesothelioma.

The molecular markers of EMT include a reduced expression of E-cadherin, cytokeratins, b-catenin and an increased expression of Snail, Slug, Twist, ZEB1, ZEB2, N-cadherin, vimentin, smooth muscle actin and matrix metalloproteinases (20, 21). The proteins involved in EMT showed a different expression profile for the three different malignant mesothelioma sub-types. The E-cadherin, epithelioid malignant mesotheliomas expressed b-catenin and cytokeratin-5 and -6 (22, 23) The bi-phasic and sarcomatoid malignant mesothelioma variants lost these proteins and gained a mesenchymal phenotype by increasing the amount of N-cadherin on the cell surface, which results in weaker cell adhesion; expressing vimentin, smooth muscle actin in the cytoskeleton, achieving a resistance to de-formation and motility; and producing metalloproteinases-2 and -9 to degrade the basement membrane (20, 21).

Despite the large number and wide variety of CTCs enrichment platforms, a major challenge for many immunological platforms, especially those based on expression of cell surface markers, is that one limits the detectable CTC pool.

While epithelial antigens like EpCAM are widely expressed on most epithelial malignancies, many of the platforms that rely on these markers for CTC enrichment will, by definition, not be able to enrich for CTCs which have down-regulated or lost epithelial markers, as we discussed previously for EMT. CTCs can lose expression of key epithelial markers through mutation, genomic deletion, clonal selection, or reversion to a more mesenchymal state.

The majority of the aforementioned clinical trials have focused on the clinical utility of CTCs enumeration, and many of these trials have focused only on CTCs expressing epithelial markers. Yet, as discussed previously there is emerging evidence that EMT plays a significant role in the establishment of metastases. Similarly, there is increasing interest in characterizing CTCs as single-cell.

In recent years, the field has expanded beyond enumeration and a large proportion of current work is focused on better characterizing CTC sub-populations including CTC clusters and mesenchymal and stem-like CTCs in order to investigate tumor biology and mechanisms of metastasis. Culture of CTCs both in vitro and in animal models can, therefore, allow for a better understanding of the functional capacity and metastatic potential of CTCs.

Determination of sensitivity to chemotherapy and other new agents is another potential application for CTCs grown in culture. Whether treatment of CTCs with novel therapeutics either in vitro or in animal models will fully-recapitulate the responses observed clinically requires for more study; the pay-off to patients and clinicians however, is potentially very large, and more studies of this nature are eagerly anticipated.

The reported methodology allows for quick and easy enrichment of CTCs and their cultivation. The cultivated cells can be used for specification of gene expression and histological/biological specificity of concrete mesothelioma.

References


