3 Liquid Biopsy for Uveal Melanoma

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Abstract: Despite improvements in the treatment of uveal melanoma (UM), prevention and therapy of systemic metastasis remain unsolved. Current prognostic indicators, either alone or in combination, may predict the pattern of progression and outcome. However, metastasis-related death has been recorded in patients initially diagnosed with early-stage cancer and in other patients many years after initial tumor removal. The mechanisms leading to the extravasation, dissemination, and colonization of organs by UM cells are still unknown but crucial for future therapies. The detection and characterization of circulating melanoma cells (CMCs) can aid in the diagnosis, prognosis, and disease monitoring of UM patients. Furthermore, CMCs provide additional information that cannot be acquired by studying the primary tumor alone. "Liquid biopsy" therefore has substantial potential to serve as an additional tool in the care of UM patients. CMCs can be characterized for the presence of key prognostic factors, such as monosomy-3, and used as a prognostic tool particularly in patients undergoing eye-preserving therapy and where no tumor biopsy is collected. The isolated cells can be further studied *in vitro* to better understand the mechanisms of dissemination and proliferation in the liver. The detection of circulating tumor cells has reached a prominent role in the treatment monitoring of various cancers. Analysis of the CMC in UM patients may assume a similar, leading role in the near future for the early identification of patients at high risk of metastatic disease.

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INTRODUCTION

Circulating tumor cells (CTCs) are rare, genetically and phenotypically heterogeneous cells found in the peripheral blood of cancer patients. Their presence correlates with an unfavorable prognosis and a reduced progression-free interval, and various studies have shown that the monitoring of CTC allows for the evaluation of treatment efficacy in different tumors (1–4). It is assumed that the CTCs are predecessors of a metastatic settlement and thus the main element of the metastatic process itself. For a number of tumors, their detection, often referred to as "liquid biopsy," is already integrated into the daily clinical routine within the framework of tumor staging, follow-up, monitoring, and therapy selection (5–7). This diagnostic tool therefore offers the opportunity to incorporate individualized medicine into everyday clinical practice using a minimally invasive procedure (8). This overview presents the currently available data regarding the potentials and limitations of the different techniques used for the detection of circulating melanoma cells (CMCs) in choroidal tumors.

TUMOR CELL DISSEMINATION AND SYSTEMIC CIRCULATION IN UVEAL MELANOMA

Uveal melanoma (UM) is the most common intraocular tumor in adults. Despite advances in diagnostics and local tumor control, up to 50% of patients develop metastases irrespective of the type of treatment. When the primary tumor is diagnosed, metastases are found in less than 2% of the cases. In contrast, metastatic disease mostly develops within 5 to 10 years and up to 40 years after the successful control of the primary tumor and after the enucleation of the tumor-bearing eye (9, 10). It is therefore assumed that the dissemination of the tumor cells occurs long before the primary tumor has been diagnosed. Tumor doubling time calculations further support this view by suggesting that the metastatic dissemination precedes initial diagnosis and treatment (11). When metastatic disease arises, therapies are limited and the mean survival time is less than 12 months (10). It is therefore essential to reduce the risk of tumor cell dissemination by detecting the cancer at its earliest stage.

Malignant melanoma of the uvea disseminates purely hematogenously, unless it perforates the sclera and infiltrates the conjunctival lymphatics. It is not known at which stage the uveal nevi cells transform into melanoma cells, when they are able to disseminate, or which characteristics are necessary to acquire this ability. However, much attention has been paid to "intratumoral vessels and vascular-like structures" that have been later shown to reflect fibrovascular septa rather than microvasculature (12, 13). The presence of these extravascular matrix patterns, which is termed "vasculogenic mimicry," is associated with death from metastatic

melanoma (14, 15) and the microvascular density itself is prognostically relevant (14, 16). In some cases, tumor cells were found in intra-tumoral blood vessels and identified as a factor for unfavorable outcome (17).

Nevertheless, it is not known why metastatic disease affects predominantly (>90%) the liver, why the cells fall in a dormant status, or which signals finally induce a colonialization of the organ, resulting in the development of clinically detectable metastases (18). Neither the examination of the primary cancer and/or of its metastases nor the *in vitro* and *in vivo* studies have been able to elucidate these crucial steps so far. Currently, it is assumed that the tumor cells undergo a gradual transition with a stepwise increase in malignancy. The isolation and characterization of CMCs have the potential to provide further information to these aspects. Various techniques have been used for the detection of CMC.

TECHNIQUES TO DETECT CTC AND CMC

CTCs were observed for the first time in the blood sample of a man with metastatic cancer in 1869 by Thomas Ashworth, who postulated that "cells identical with those of the cancer itself being seen in the blood may tend to throw some light upon the mode of origin of multiple tumors existing in the same person" (19). However, it took more than 100 years before the importance of CTC was recognized in modern cancer research. This was made possible with the evolvement of a sensitive magnetic separation technology employing ferrofluids (colloidal magnetic nanoparticles) (20).

Since then, further improvements have been made in order to enrich and distinguish the rare CTC from normal cells in the peripheral blood. Capture and enrichment are performed based on the specific morphological and phenotypical characteristics of the tumor cells, such as their size, density, and specific protein expression. However, CTC can also be detected indirectly by amplifying the transcripts of tumor-related proteins by reverse transcriptase polymerase chain reaction (RT-PCR).

This latter technique was indeed the first approach used for the indirect detection of CMC in the lysates of venous blood cells from UM patients, who had no clinical evidence of systemic metastasis (21). Amplification of the transcripts for melanoma-associated proteins, such as tyrosinase and MelanA/Mart1, by RT-PCR was then adopted by several other groups. However, the results disclosed a high variability for the detection rate, with values ranging between 0 and 97% (21–25). This is probably based on the fact that the amplification of pseudogenes and illegitimate transcripts can lead to false-positive results and an overestimation of the CMC number. Furthermore, naturally occurring degradation, release of transcripts, and molecular changes of the disseminated cells may have a substantial impact on the results (25, 26). Finally, this technique does not allow any statement about the characteristics of the CMC, as no cells are available to be examined. This method is therefore not suitable for a direct detection of the CMC themselves, but rather yields an indirect evidence on the basis of a surrogate marker.

In contrast, the detection of intact tumor cells provides substantial advantages. The cells can be isolated with different techniques depending on tumor cell size,

density, and expression of surface antigens. One method is based on filtering blood samples through a membrane (8 µm pore size) by means of a controlled vacuum aspiration. The cells that are retained on the membrane can then be identified by immunostaining (27). However, tumor cells that are smaller than the pore size used would be lost, leading to a low sensitivity of this capture assay. Since tumor cells have a similar density to the mononuclear blood cells, density-based methods also have the disadvantage of poor sensitivity as well as the possible loss of tumor cells and lack of tumor specificity.

More specific approaches are based on the immunomagnetic recognition and extraction with tumor-specific antibodies. For example, the commercially available, FDA-approved CELLSEARCH® CTC Control Kit enables the detection of CTC based on their epithelial surface antigens. This system employs ferrofluid-coupled antibodies directed against characteristic surface molecules of the epithelial cells to be examined. The labeled cells are then separated from the blood cells in a magnetic field. The corresponding immunohistochemical markers are used for further discrimination and identification (28). The method combines a negative selection to exclude other mononuclear cells. However, false-positive results may arise from the identification of normal cells expressing the same antigen, and false-negative results may result from CTC depleted of the epithelial antigens. Our experience with a similar commercially available system using an epithelial marker led to a very high rate of false-positive results in the blood samples from healthy volunteers.

Two different groups used the CellTracks Circulating Melanoma Cell Kit on the CELLSEARCH® System to isolate CMC with antibodies directed against CD146 (Mel-CAM) from UM patients (29, 30). The isolated cells were then immunostained with antibodies directed against the melanoma-associated antigen (HMW-MAA). Both groups examined patients who had already developed metastatic disease. Bidard et al. analyzed the venous blood samples of 40 patients and could detect CMC in 30% (n = 12) of the patients (29). Using the same system, Terai et al. found CMC in 52% of the venous specimens (number of cells: median 1, range: 0–8 cells) and in 100% of the arterial blood samples (number of cells: median 5, fluctuation width: 1–168) (30). The latter group has postulated that the tumor cells may become apoptotic and fragmented while circulating in the peripheral blood. This theory, however, remains to be investigated. In fact, it contradicts the general assumption that tumor cells probably enter the systemic circulation or leave the organ of origin through the venous pathway, and any cell that circulates would have access to both the venous and the arterial arm unless it seeds within an organ or tissue.

Other methods to detect CTC include fiber-optic array scanning, microfluidics, and a photoacoustic flow cytometry. Fiber-optic array scanning technology (FAST) applies laser-printing techniques. Laser-printing optics are used to excite 300,000 cells per second, and emission is collected in an extremely wide field of view, enabling a 500-fold speed-up over automated digital microscopy with comparable sensitivity and superior specificity (31).

The microfluid-based positive enrichment technologies are based on microfabrication that refer to the ability to create structures at or below the cellular length scale. Microfluidic devices allow precise control of fluid flow, which is important because the efficiency of cell capture depends highly on cell–antibody contacts that can be controlled through fluid flow velocity and direction (32).

Photoacoustic methods are based on nonradiative conversion of absorbed light energy into heat accompanied by acoustic waves. Specifically, absorption of laser radiation by a single cell leads to a temperature increase in endogenous and exogenous structures. The temperature distribution is transformed into a refraction distribution that can be detected (33, 34). However, these techniques have not been used yet for the detection of CMC in UM patients (31–34).

Our efforts on the isolation and characterization of CMC started more than a decade ago. Because of its obvious advantages, such as the possibility of *in vitro* expansion (35), we concentrated on the immunomagnetic enrichment of intact melanoma cells from the peripheral blood. Our first experience was in collaboration with a group using the system established primarily for cutaneous melanoma. CMCs were isolated from the venous blood by a monoclonal antibody (clone 9.2.27) directed against the melanoma-associated chondroitin sulfate proteoglycan (MCSP). This antigen does not have a uniform expression profile in UM cell lines, but the protein can be detected in up to 95% of UM samples (36). Using this method, we found CMC (median: 2.5 cells/50 ml blood) in 19% (n = 10 of 52) of the UM patients without clinically detectable metastases (37). The presence of tumor cells in peripheral blood was associated with ciliary body invasion (odds ratio [OR], 20.0; 95% CI, 3.0–131.7), advanced local tumor stage (OR, 6.7; 95% CI, 1.8–25.4), and anterior tumor localization (OR, 4.0; 95% CI, 1.2–12.7), which are all established prognostic factors for UM progression. However, the detection frequency was considerably below the expected rate of metastatic disease. Therefore, the usage of a single antibody does not seem to have sufficient sensitivity.

In order to increase the sensitivity of the immunomagnetic cell enrichment, we evolved the technique using two antibodies (NKI/beteb and NKI/C3) directed against the melanoma-associated glycoprotein as described before (35). In preclinical experiments, we were able to demonstrate a detection sensitivity of 2 UM cells/10 ml of blood. In a prospective study, the detection frequency was 93.5% (in 29 out of 31 patients with UM), with a median CMC density of 3.5 cells/10 ml of blood (range: 0–12.8 cells). Furthermore, no CMCs were detected in a control group of 10 age-matched healthy volunteers, suggesting a high specificity of this assay. Melanoma cells (high NKI/C3 or MCSP expression, no CD45 expression) could be clearly distinguished from the neighboring leukocytes (high CD45 expression) by double immunostaining against the leukocyte antigen CD45 and the melanoma markers NKI/C3 or MCSP. In addition, the morphology of the nucleus provided further discerning features (38).

The high release rate of melanoma cells into the circulatory system suggests that metastasis develops early in the course of UM. Since the CMC could be detected in almost all UM patients (25, 30, 38), it can also be assumed that the metastatic disease, which arises in approximately 50% of UM cases, is not solely dependent on the dissemination of tumor cells into the circulation but also on the ability of the CMC to survive and proliferate primarily in the liver (39, 40). One of the most important risk factors associated with an increased risk of metastasis is the loss of a copy of chromosome-3 (monosomy-3) in the primary UM cells. In addition, monosomy-3-positive cells could be detected in the liver metastases of UM at over-proportionally high rates (41–45). We therefore developed a special fluorescence in situ hybridization (FISH) assay combined with immunocytochemistry to determine the copy number of chromosome-3 in intact CMC, which

specifically expressed melanoma marker proteins (Figure 1). Using this novel Immuno-FISH method, we could detect monosomy-3 in the CMC of nearly 58% of the UM patients who were positive for these cells. In addition, all patients who subsequently developed metastases had monosomy-3-positive CMC at the first diagnosis (46). Our findings therefore suggest that monosomy-3 is not the cause of tumor cell release into the systemic circulation but likely plays a crucial role in the colonialization of the liver.

We could also observe a high degree of concordance between the monosomy-3 status of the CMC and the primary tumor in the samples (n = 10) from 11 UM patients (46). The detection of monosomy-3 in the CMC can therefore be used as a noninvasive and repeatable approach to gain insight into the molecular characteristics of the primary tumor, compared with the biopsy of the primary tumor.

PRESENT POTENTIAL OF CMC DETECTION

Currently, the detection of CTC is not performed routinely in the management of UM patients. Only a few centers have analyzed and shown the presence of CMC in UM patients. The results so far suggest that the release of melanoma cells into the circulatory system occurs early and probably in all cases, although the metastatic disease is expected to occur only in half of the patients. As shown by us in the past (47) and by others recently (48), the presence of CMC also does not show a change following invasive procedures and provides little help in the prognostication of patients with clinically evident metastatic disease.

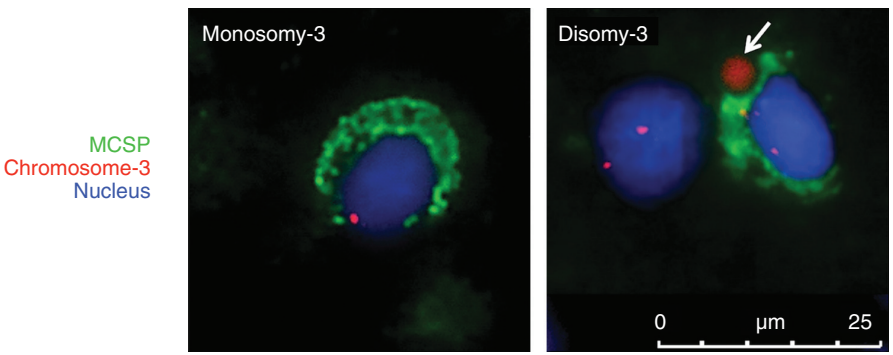

Figure 1 Detection of chromosome-3 in the CMC specifically labeled for a melanoma marker protein. CMCs were isolated from the blood sample of a patient (female, 48 years), who was diagnosed with primary UM without metastases. The cytospin of isolated cells was processed directly for the Immuno-FISH assay to label chromosome-3 (with the centromeric probe CEP3, red) in the tumor cells specifically expressing the MCSP (green). The nuclei were counterstained in blue with DAPI. Cells expressing a single or a double signal for CEP3 were classified as having monosomy-3 (left panel). In contrast, CEP3 signals separated by a distance of more than twice the diameter of a single spot were considered as disomy-3 (right panel). Arrow indicates an immunobead on a CMC with disomy-3, which could be clearly distinguished from the adjacent, MCSP-negative leukocyte. All images were acquired at an original magnification of 400×. This patient developed liver metastases within 2 years following the CMC analysis and enucleation.

Determining the mere presence of CMC is therefore not useful in making any prognostic statements (39).

However, the analysis of CMC can be an additional strong tool that deserves further attention for the management of patients with conspicuous nevi. Currently, the major factors that define the decision-making process in the treatment of these patients include the clinical criteria and regular controls to rule out further growth. A biopsy may also be considered if the lesion appears highly suspicious (49). However, arriving at the decision of such an invasive and potentially complicated procedure can be challenging, depending on the localization and size of the lesion, the function of the affected and the partner eye, the age, and the psyche of the patient (50–52). In addition, any manipulation carries the risk of local and iatrogenic systemic dissemination of the tumor cells, and the histopathological analysis does not always enable a conclusive finding depending on the quality and quantity of the investigated sample. In such cases, a positive CMC result may aid in decision-making for confirmatory biopsy (Figure 2). In addition, this "liquid biopsy" can be repeated as often as necessary as opposed to the conventional one.

Our Immuno-FISH assay for the detection of monosomy-3 in the CMC provides a valuable prognostic tool without the need for disrupting the integrity of the primary tumor (46), particularly when an eye-preserving therapy is indicated. Currently, when the clinical evidence is not sufficient to distinguish a nevus from a small UM, we use a conventional biopsy to confirm the CMC findings before further treatment is planned. With accumulating evidence, however, the conventional biopsy may be completely replaced by "liquid biopsy" in the future.

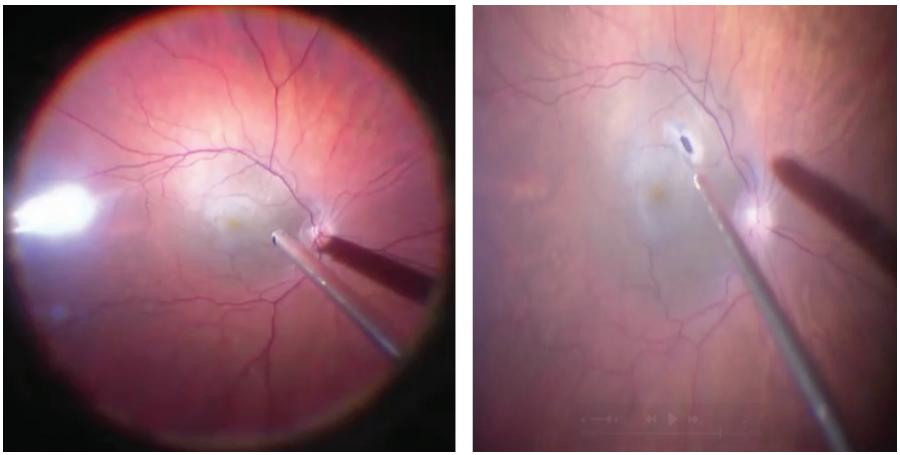

Figure 2 Intraoperative fundus photography of a patient with a pigmented choroidal lesion. The thickness of the lesion was <2 mm, no subretinal fluid was detected in SD-OCT, and no orange pigment obvious or detectable with autofluorescence and no growth has been reported so far. The patient was positive for CMCs. Since therapy would have affected central vision, biopsy was performed. Frameshot at the beginning of pars plana vitrectomy (left panel). Frameshot after biopsy was performed close to the vascular arcade (right panel).

FUTURE POTENTIAL

The currently available findings indicate that analyzing not the mere presence but the monosomy-3 status of CMC possesses a great prognostic potential for gaining important information on the risk of metastasis and the further course of the disease. This approach can also possibly aid in the evaluation of treatment efficacy and selection of particular therapy methods in the future. However, 5–20% of UM patients can develop metastases despite the absence of monosomy-3 in the primary tumor. In such cases, the deletion of individual genes on chromosome-3 or an isodisomy, which is present in approximately 6% of the UMs, can be regarded as additional factors influencing the risk of metastasis (53). A further aberration associated with a reduced survival time is the presence of an additional copy of the chromosome-8q in the primary UM (9, 54–56). A more comprehensive genetic analysis of CMC can therefore aid in enhancing the prognostic relevance of these cells.

A further increase in sensitivity could be achieved by the additional determination of mutations in the genes encoding the G-alpha proteins GNAQ or GNA11. Bidard et al. has developed assays to detect the most recurrent, UM-specific GNAQ and GNA11 point mutations in circulating tumor DNA (29). The group of Metz et al. could also determine the GNAQ or GNA11 mutations in the cell-free DNA derived from the venous blood of UM patients. However, mutations in the region of Q209 could be detected in only 9 out of 22 metastatic patients (40.9%) with the latter approach (57).

Further studies must therefore be undertaken to determine whether the presence of these mutations, which are also found in 55% of blue nevi (57), can be useful in diagnosis and aid in therapeutic planning with medications that are directed against the signaling cascades downstream of the G-alpha proteins, such as the mitogen-activated protein kinase (58).

To date, it is also not clear whether melanocytic nonmalignant cells can enter the bloodstream. There is a single case report on the detection of circulating melanocytic cells following the surgical excision of a benign, congenital cutaneous nevus. Since no cells were found before surgery, it was assumed that the release of these cells was caused by surgery (59).

CONCLUSION

The detection and genotypic characterization of CMC has a substantial potential to enable a better understanding of UM. Isolated CMC can be used in experimental studies to elucidate how these cells gain access into the bloodstream, acquire the ability to settle, become dormant, and eventually proliferate to form clinically evident metastases. These findings would in turn contribute to the development of targeted and possibly preventive therapies to improve the survival rate of patients with this devastating disease.

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