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TOPIC HIGHLIGHT

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Prognostic relevance of minimal residual disease in colorectal cancer

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Abstract

Presence of occult minimal residual disease in patients with colorectal cancer (CRC) has a strong prognostic impact on survival. Minimal residual disease plays a major role in disease relapse and formation of metastases in CRC. Analysis of circulating tumor cells (CTC) in the blood is increasingly used in clinical practice for disease monitoring of CRC patients. In this review article the role of CTC, disseminated tumor cells (DTC) in the bone marrow and micrometastases and isolated tumor cells (ITC) in the lymph nodes will be discussed, including literature published until September 2013. Occult disease is a strong prognostic marker for patient survival in CRC and defined by the presence of CTC in the blood, DTC in the bone marrow and/or micrometastases and ITC in the lymph nodes. Minimal residual disease could be used in the future to identify patient groups at risk, who might benefit from individualized treatment options.

Key words: Colorectal cancer; Circulating tumor cells; Disseminated tumor cells; Isolated tumor cells; Micrometastases; Occult disease; Minimal residual disease

Core tip: Occult disease or minimal residual disease is defined by the presence of tumor cells in the blood, bone marrow or lymph nodes not found in conventional staging procedures. Occult disease in form of circulating tumor cells in the blood, disseminated tumor cells in the bone marrow and micrometastases and isolated tumor cells in the lymph nodes is a prognostic marker for survival in colorectal cancer. Future research should be directed to test the predictive value of occult disease as an additional staging tool to identify high risk patients. The patient group at risk might benefit from additional individualized treatment options and this should be investigated in future clinical trials.

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INTRODUCTION

Although tremendous progress has been made in surgical technique and medical treatment, colorectal cancer remains the third most common malignancy among both genders with estimated incidence rates of approximately one million new cases each year[1,2]. Five year survival rates exceed 80% for Union for International Cancer Control (UICC) stage I disease and are below 10% for patients in stage IV[1,2]. However, colorectal cancer (CRC)

is still the third most common cause of cancer related death worldwide[1]. Patients with UICC stage I and II disease are regarded as patients with non-systemic, localized disease and are in general treated with surgery alone and no further adjuvant medical treatment[3,4]. However, up to 25% of the patients with localized disease will die of disease relapse and metastases, potentially due to occult disease not recognized during conventional staging[2].

Research about minimal residual disease in solid cancers and our knowledge about the role of circulating tumor cells (CTC), disseminated tumor cells (DTC) and micrometastases in colorectal cancer has rapidly progressed during the last two decades. Understanding the role of CTC in the blood, DTC in the bone marrow and disseminated (isolated) tumor cells or micrometastases in lymph nodes has helped us deciphering the pathways of disease relapse and metastases formation in colorectal cancer and will be focused in this review article.

DEFINITION OF OCCULT DISEASE: MINIMAL RESIDUAL DISEASE IN CRC

The first description of a circulating tumor cell in the blood-stream dates back to Ashworth in 1869[5]. However, research on CTC only began to progress during the last two decades after the introduction of modern molecular biology techniques, which made it possible to identify and isolate CTC from cancer patients[5,6].

CTC are defined as circulating cells in the blood-stream originating either from the primary tumor or distant metastases. They can be found in the central venous blood compartment, the peripheral blood compartment, the tumor draining veins, the portal venous system or within the arterial blood system. It is estimated that approximately 10^6 cells per gram of primary tumor are released into the systemic circulation on a daily basis[7]. However, most of these cells will not have the capacity to survive in the bloodstream or to form distant metastases as they will ultimately undergo cell death by apoptosis or will die due to shearing forces within the blood-stream. Data from animal models show that less than 0.1% of tumor cells released into the circulation have the ability to form distant metastases[8]. It is now well understood, that a key prerequisite for metastases formation is the dissemination of CTC from epithelial malignant cells into the blood-stream by the process of epithelial mesenchymal transition (EMT). EMT and the reverse process, which is called mesenchymal epithelial transition (MET) are key processes in early embryogenic development and differentiation of cells and tissues[9,10]. During early embryogenic development EMT is necessary for the differentiation of the mesoderm into different tissues, whereas later during development MET is important for the differentiation of mesodermal cells into epithelial organs, such as kidney or colon[11]. Various key transcription factors for the embryological induction of EMT and MET have been linked to malignant capacities, such as evasion of apoptosis, invasive growth patterns and motility of neoplastic cells[12-17]. Metastasis requires disseminated cells having self-renewing capacities, similar to the process of organ formation and is at least theoretically similar to processes occurring during embryological development. Therefore, it is likely that EMT and MET play important roles in generating cells within tumors, which may enter the blood-stream and have the capabilities to survive in the circulation and to form distant metastases in secondary tissues. These CTC can also enter the bone marrow as DTC as an exit reservoir and remain there in dormant states until reactivation before entering the blood-stream again[10,18]. CTC can also be released by manipulation of the tumor, *e.g.*, by mechanical forces during surgery or colonoscopy[19-22]. They may also be found as cell clusters or tumor microemboli[23].

Figure 1 summarizes the metastatic cascade with the key elements in CRC: Tumor cells are disseminated from the primary CRC and may enter either the circulation or the lymphatic vessels and lymph nodes. Once tumor cells have entered the circulation or lymphatic system most of them will undergo apoptotic cell death, while some will have the capacity to form distant metastases; for CRC mostly in the lung and liver. It is assumed that CTC in the blood will also have the capacity to enter the lymphatic system and/or that ITC in the lymphatic system have the capacities to enter the blood vessels as CTC and/or to form distant metastases. It is likely, but has not been proven, that metastases themselves have the ability to release tumor cells into the circulation or lymphatic system as secondary DTC. At any point tumor cells can also enter the bone marrow as DTC and remain there in a dormant state or evade the vascular structures again by means of MET. The whole process must be regarded as a dynamic state with various interactions and structural changes occurring at all times[24-26].

DTC are tumor cells, which can be found in the bone marrow or lymph nodes. Most research about DTC originates from patients with breast cancer and is usually undertaken on biopsies from the bone marrow of the iliac crest. Tumor cells in the bone marrow often enter a dormant state and can eventually be reactivated after several years of dormancy. DTC are thought to be one of the key elements in late disease recurrence[24,27]. DTC in lymph nodes are called isolated tumor cells (ITC). Micrometastases and ITC in lymph nodes are tumor cells in lymph nodes not detected by conventional hematoxylin-eosin (HE) staining done by the pathologist for routine staging. They will only be found by using molecular biology techniques, such as staining with tumor specific antibodies, reverse-transcriptase polymerase chain reaction (RT-PCR) or FACS analysis of lymph node tissue[28].

CTC, DTC, ITC and micrometastases are all regarded as occult tumor burden and are termed as minimal residual disease. Minimal residual disease is a sign for a systemic disease progression which may have significant impact on patient survival and disease progression in many solid cancers. Most research about minimal residual disease was performed in patients with breast-, prostate

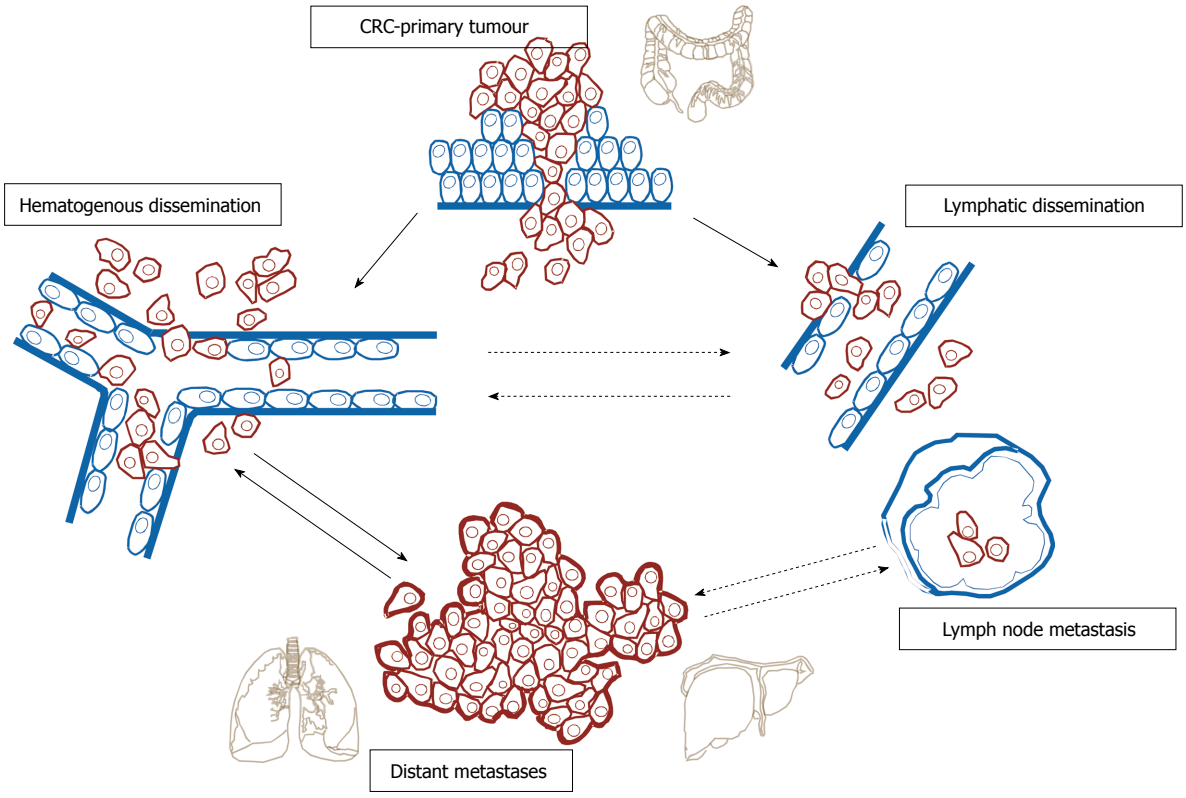

Figure 1 Model of the metastatic cascade in colorectal cancer. Cancer cells (red cells in Figure 1) are released from the primary tumor and enter the circulation and/or the lymphatic system (lymphatic vessels and lymph nodes) by various mechanisms, such as epithelial mesenchymal transition. The circulating tumor cells in the circulation are called circulating tumor cells (CTC) and may form distant metastases, for colorectal cancer (CRC) commonly in the liver and lung. However, more than 90% of the CTC in the vascular system will undergo cell death and will not have the malignant traits necessary to form distant metastases. Disseminated tumor cells (DTC) in the lymph nodes can either form micrometastases or remain in the lymph nodes as isolated tumor cells which are not detected by conventional HE staining. Cross talk between the vascular and lymphatic system likely exists and it is hypothesized that disseminated tumor cells in the lymphatic system may also form distant metastases. It is also assumed that metastases themselves have the capacity to release tumor cells back into the systemic or vascular circulation. Cross talk also exists with DTC (not shown in the figure) in the bone marrow, where cells enter from the blood-stream and remain in a silent state for several years before they may reenter the circulation to form distant metastases. Dotted lines indicate hypothesized pathways and straight lines show established and accepted mechanisms of tumor cell dissemination.

and colorectal cancer.

DETECTION METHODS OF OCCULT DISEASE

To date there are numerous detection methods for minimal residual disease in colorectal cancer patients, which will be discussed briefly below. Most of the detection methods are not standardized and protocols vary widely between research groups, which makes comparison of results difficult. Endpoints of clinical trials and the used markers and detection methods are not standardized and many trials have low sample sizes. In addition, CTC and micrometastases represent an extremely heterogeneous group of cells and, depending on the detection method, different subpopulations of CTC or micrometastases will be detected while other subgroups will not be identified.

Usually, for identification and enumeration of CTC blood will be taken from a central venous catheter (central venous blood compartment) or from a peripheral vein (peripheral venous blood compartment, PVBC). For patients undergoing surgery various sites of blood sampling exist: Once the abdominal cavity is opened, blood can also be taken from the tumor draining veins (mesenteric venous blood compartment), the portal vein (PVBC) or the liver veins (liver venous blood compartment). Several groups have compared cell count of CTC in CRC patients in these different blood compartments[29,30].

A CTC is described as an intact cell, originating from either a primary tumor or a metastasis, which has an intact nucleus and positive staining for cytokeratins (in case of cancers originating from epithelia). CTC can be discriminated from leucocytes *via* staining for CD45, a panleucocyte marker. Tests for CTC need to be very specific and sensitive, as CTC are relatively rare compared to the high number of hematopoetic cells in the blood: Depending on the tumor load and CTC load of the patient one CTC needs to be identified within 5^6-10^6 leucocytes and 5×10^9 red blood cells per mL of blood[31]. Almost all tests for CTC in the blood use an enrichment procedure, usually *via* density gradient centrifugation and/or lysis of red blood cells. This step is in general followed by isolation from contaminating leucocytes prior to the CTC isolation step. For CRC patients the enrichment step can be done *via* an epithelial cell adhesion molecule (EPCAM) dependent step, using the cell surface protein

EPCAM for detection of CTC, which is expressed on epithelial cells and many carcinoma cells. Non-EPCAM dependent enrichment techniques for CTC use other tumor cell traits, such as cell size or cellular morphology. The only completely standardized and United States food and drug agency (FDA) approved detection method is the Cell Search technique, which uses an EPCAM dependent enrichment step and is described elsewhere in detail[30,32]. This system has been FDA approved only for the detection and enumeration of CTC in patients with metastasized colorectal-, prostate- and breast cancer. New technologies are usually compared to the Cell Search system. In brief, the system uses 7.5 mL of patient blood, which are initially processed during an enrichment step *via* density gradient centrifugation. Blood samples are then processed and the CTC immunomagnetically separated from contaminating cells by EPCAM labeled ferrofluids. The nucleus and cytokeratins 8, 18 and 19 (for positive CTC detection) are stained with immunofluorescent antibodies. CD45 is stained with a labeled antibody for negative leucocyte exclusion. Cells are than analyzed and photographed by a semiautomatic microscope and trained operators identify CTC according to certain specified criteria (cell and nuclear morphology, size and staining behavior)[30]. However, the Cell Search system will only detect the EPCAM positive CTC subpopulation, which makes detection of CTC in pancreatic cancer and other cancer entities with low EPCAM expression difficult. It is also not possible to detect the subpopulation of CTC in CRC patients which may have significantly downregulated EPCAM expression (for example due to EMT). Other detection techniques are described elsewhere in more detail[33]. In brief, many mRNA-based strategies were used in the early years of CTC detection, targeting specific genes, such as CK18, CK19, CK20, MUC1, CEA and others. However, these markers are also present at low levels in normal blood, the tests are not standardized between laboratories and quantitative RT-PCR is necessary with validated cut-off values for each test[34,35]. Other techniques, such as the non EPCAM dependent EPISPOT assay (Epithelial ImmunoSPOT assay) do exist and are currently validated[35]. Various microfluid devices have been developed during the recent years, isolating CTC *via* morphology and cell size or by surfaces coated with antibodies, such as EPCAM[36-38]. As CTC are very rare cells the introduction of *in vivo* tests with the capability of analyzing higher volumes of blood are promising. One such technique is a nanodetector device inserted into a peripheral arm vein for 30 min, coated with anti EPCAM antibiodies and capturing CTC from a bypassing blood volume of approximately 1.5 L during 30 min[39]. Live CTC and DTC can also be manually picked under the microscope with a micromanipulator and be further processed or used for single-cell analyses.

DTC are identified in the bone marrow, which can be taken from the iliac crest in patients undergoing surgery for CRC or CRC liver metastases resection. The procedure is invasive, compared to a simple blood draw, but safe in experienced hands and from major research groups doing routine sampling of bone marrow for research no serious adverse events have been reported to date. Detection and isolation of DTC is similar as for CTC while various enrichment and detection techniques can be used. Detection methods for DTC are not standardized and vary widely between research groups. The Cell Search system cannot be used for DTC detection.

Micrometastases and ITC in lymph nodes are identified from the lymph nodes, which are routinely sent to pathology. Occult disease in lymph nodes can be identified using either RT-PCR detection methods and/or immunohistochemistry. The majority of published studies uses cytokeratins as a marker for molecular tumor cell detection, while some studies use other markers, such as CEA or GUCYC[28]. In general, detection methods are not standardized and vary between laboratories. Additionally, no technology exists so far which would make it feasible to routinely analyze all harvested lymph nodes for occult disease outside of clinical studies. Harvesting of sentinel lymph nodes in CRC surgery has been investigated by several groups, however with frustrating results, due to the complex abdominal lymphatic drainage, which makes routine use of sentinel node techniques in CRC difficult[28].

CTC AS PROGNOSTIC MARKERS

The prognostic relevance of CTC in CRC has been shown in large trials and was confirmed in a meta-analysis[29,32,40-44]. Recently, the notion of CTC as the primary driving force of metastases formation has been widely accepted. In the past, many studies about the role of CTC in CRC had controversial results, lacked statistical power and used different study protocols or non-standardized detection methods of CTC detection. The introduction of the Cell Search system for detection and enumeration of CTC has brought further insights to CTC research and large trials have been published about the prognostic role of CTC in patients with UICC stage Ⅳ CRC[32].

In a recent meta-analysis about the role of CTC in CRC 3094 patients from 36 studies were included and showed that detection of CTC in the peripheral blood of patients with CRC is a strong prognostic marker for overall survival. Pooled analysis from all sampling sites showed an association of CTC detection with poor recurrence free survival [HR = 3.24 (95%CI: 2.06-5.1)] and overall survival [HR = 2.28 (95%CI: 1.55-3.38)]. Interestingly, data for CTC detection in the mesenteric venous blood and portal venous blood were inconsistent; mainly due to the low number of studies performed on CTC in CRC in different blood compartments these results were not significant prognostic markers in the meta-analytic approach[40].

In the era of personalized medicine the idea of CTC as a liquid biopsy, easily taken at different time points *via* a simple blood draw, provides a promising tool for individualized therapeutic and treatment decisions and

prognostic outcome calculations. Until now routine measurements of CTC in CRC patients has not become the standard of care and it is not clear which detection method will provide the most valuable information: Using the Cell Search System only EPCAM positive CTC will be identified, possibly missing a high number of CTC within this heterogeneous cell group with low EPCAM expression (for example due to low EPCAM because of EMT). So far the Cell Search system has only been approved for the purpose as a prognostic marker in patients with metastasized CRC in UICC stage Ⅳ[45]. It is likely that CTC detection in patients with CRC UICC stage Ⅰ-Ⅲ may also have a prognostic relevance, which has been already proven for CTC detection in breast cancer[46].

Recently, in a trial including 200 patients with CRC, it was shown that the detection rate and number of CTC in the mesenteric venous blood compartment is significantly higher than in the central venous blood compartment[30]. There was also a significant correlation between the number of detected CTC in the mesenteric venous blood compartment and the central venous blood compartment, supporting the theory of the liver acting as an incomplete CTC filter[34,47]. It was also shown that the count of tumor cells in the central venous blood compartment is higher for patients with low rectal tumors compared to patients with high rectal tumors. This is possibly due to the filtering function of the liver as the blood from the lower rectum drains *via* the iliac veins into the inferior vena cava, therefore bypassing the liver[30,48].

A recent analysis of more than 20000 Medicare CRC patients in the United States showed no benefits of adjuvant chemotherapy in patients with stage Ⅱ disease with or without the routinely used prognostic criteria[49]. CTC could provide a potential decision tool for this patient population regarding further adjuvant therapy regimes. More randomized controlled trials are needed in the future to implement the use of routine pre-, intra- and postoperative CTC detection for therapeutic treatment guidance.

DTC AS PROGNOSTIC MARKERS

DTC have been widely accepted as prognostic markers for patients with breast cancer, as most research about DTC and metastases formation has been done in these patients[24,25,27]. For CRC the role of DTC remains controversial. A recent meta-analysis included six studies investigating the prognostic role of DTC detection in the bone marrow in patients with CRC. However, the analysis failed to show a prognostic effect of DTC in CRC patients[40]. This was mostly due to the lack of studies done in this field and the high inter-study heterogeneity, as to date there are only few trials investigating the role of DTC in CRC patients and most of them lack statistical power. It is highly likely, that just as in breast cancer patients DTC play a special role in tumor cell dormancy and late relapse after curative resection. It has been shown that in patients with solid carcinomas DTC in the bone marrow will be found in 15%-40% of the patients, independent of nodal or metastatic stage of disease[50]. The exact role of DTC and contribution in the metastatic cascade has not been elucidated. It is estimated that depending on the primary cancer DTC possess varying degrees of capability to undergo dormancy, migrate into the blood and/or form distant metastases or bone metastases[19,40,51]. A recent study has shown that DTC in the bone marrow negatively influence survival after resection of colorectal liver metastases[52]. Further studies about the role of DTC in tumor cell dormancy, prognosis, disease progression and late relapse of disease are necessary.

MICROMETASTASES AND ISOLATED TUMOR CELLS IN LYMPH NODES AS PROGNOSTIC MARKERS

Tumor infiltration of loco-regional lymph nodes, represents one of the strongest prognostic factors in CRC[2]. Resected lymph nodes are routinely isolated from the pathological specimen and several representative sections of each lymph node will be analyzed by the pathologist using routine hematoxylin and eosin (HE) staining procedures. However, using conventional staining techniques only major tumor infiltration of lymph nodes will be observed, and ITC and micrometastases cannot be detected with this method. Various groups have observed ITC and micrometastases in lymph nodes of up to 50% of the patients staged lymph node negative (UICC stage Ⅰ and Ⅱ) in conventional HE staining[53-55]. The prognostic role of micrometastases and ITC in lymph nodes was confirmed in a meta-analysis including a cumulative sample size of more than 4000 patients from 39 trials[28]. Independent of the used detection methods (RT-PCR, immunohistochemistry, FACS analysis) and the used markers (mostly cytokeratins) the detection of micrometastases and/or ITC in lymph nodes had a strong prognostic effect on OS and PFS in CRC patients[28]. Molecular detection of minimal residual disease in regional lymph nodes was significantly associated with poor overall survival [HR = 2.20 (95%CI: 1.43-3.4)], disease specific survival [HR = 3.37 (95%CI: 2.31-4.93)] and disease free survival [HR = 2.24 (95%CI: 1.57-3.20)]. Similar to CTC and DTC detection the analysis of micrometastases and ITC in lymph nodes could help to build additional criteria to identify patients at risk for disease recurrence in early CRC. Current criteria to identify the 25% subgroup of stage Ⅱ disease CRC patients who will develop disease recurrence are insufficient[49]. However, limitations in human-, infrastructural- and financial resources would currently make it unfeasible to screen all retrieved lymph nodes for ITC and micrometastases. Using techniques to identify and thoroughly analyze the sentinel lymph node (as in breast cancer treatment) have mainly failed and provided frustrating results for CRC[28]. Until the development of high throughput standardized screening techniques, based on techniques such as FACS analysis and/or RT PCR routine screening

of all lymph nodes for minimal residual occult disease will likely remain unfeasible outside of clinical research use. Future research is needed in the development and evaluation of standardized screening methods for ITC and micrometastases in lymph nodes and their implementation into routine use in clinical practice[28].

LESSONS FROM *IN VITRO* AND *IN VIVO* EXPERIMENTS

Translational research

CTC and DTC are the biological correlate of tumor cell dissemination and their occurrence significantly influences the patients' fate[40,42]; therefore, numerous groups have focused on the in-depth characterization of these cells to find novel therapeutic targets in these cells. Unfortunately, translational CTC/DTC research in CRC faces two major problems: (1) CTC and, even more so, DTC are extremely rare cells in CRC[30,34]. Especially in early stage patients, CTC and DTC can be found in only few patients. To overcome this problem, researchers have focused on late-stage patients[32], obtained blood samples directly from tumor-draining veins[30], or analyzed significant amounts of peripheral blood. The first two approaches may bias the results of the analyses; the latter may produce ethical problems, especially in serial blood draws of significant volumes. Despite all efforts, insufficient sample size has limited research on CTC/DTC until recently, when better amplification protocols and highly sensitive analytic tools such as next-generation sequencing became available; and (2) The true origin of CTC/DTC is not always clear and their phenotype may change over the course of disease or even during the process of dissemination. The currently most widely used definition of CRC-derived CTC is set by the Cell Search System as intact $EPCAM^+$, cytokeratin 8, 18 or 19^+ and $CD45^-$ cells[31]. As the Cell Search System is currently the only FDA approved CTC detection method, almost all clinical trials and many preclinical trials have used this system to isolate and quantify CTC. However, it is unclear whether CTC retain their epithelial phenotype, especially during EMT. A significant downregulation of epithelial genes such as EPCAM or cytokeratins in CTC seems to be possible. This would mean that the $EPCAM^+$ cell fraction does not contain all CTC; in fact, the metastasis-inducing CTC may be contained in the EPCAM- fraction as these cells have a more mesenchymal and thus more invasive phenotype. Different detection methods (*e.g.*, cell size, elasticity, novel surface markers) are currently being developed and tested.

Despite these obstacles, very interesting findings have been published during the last year. Genomic analyses of single CTC have revealed significant genetic heterogeneity between CTC and the tumor as well as between CTC themselves[56,57]. In fact, mutations detected in CTC that were previously unknown in the tumor could be confirmed by deep sequencing of the primary tumor, indicating the possibility of CTC-based "liquid biopsies" enabling the clinician to follow non-invasively the genetic evolution of the disease.

Mouse models

The metastatic cascade is a complex, multi-step process that cannot be reproduced by current *in vitro* methods. Owing to the above mentioned obstacles in translational CTC research, significant efforts have been made to reproduce tumor cell dissemination in animal models. Several mouse models of metastatic CRC have been published. They can be categorized into chemically induced mouse models[58-60], models based on tumor cell injection[61-65] and genetically engineered mouse models (GEMM)[66,67]. Chemically induced mouse models develop genuine mouse tumors and therefore realistically mimic the disease; however, their induction is bothersome and the incidence of tumors varies both temporally and spatially[58-60]. Also, the detection of CTC is complicated by the same problems (rarity, detection method) as in humans.

Depending on the cell line, tumor cell injection-based tumor models yield highly aggressive and widely metastatic tumors[64]. The detection of CTC is easy as the cells can be labeled prior to injection. However, as most CRC cell lines have been in culture for decades and are monoclonal and highly anaplastic, the tumors as well as the resulting CTC may not be representative of human disease. Genetically engineered mouse models, in which specific CRC-related driver mutations (*e.g.*, APC, KRAS, TP53) are induced in the colonic epithelium, develop genetically well-defined, invasive tumors which also metastasize and mimic well the clinical situation[66]. Analyses of GEMM-derived CTC are currently underway in several laboratories. The results may add significant information to the field of CTC research.

CONCLUSION

Occult disease as guidance for treatment decisions and monitoring disease progression

According to current treatment guidelines patients with non-systemic CRC disease (*i.e.*, UICC stage I and II) are cured by surgery alone[4]. However, up to 25% of these patients will ultimately die of tumor relapse and/or distant metastases[2]. Current staging procedures are insufficient to identify the patient cohort at high risk, who might benefit from additional adjuvant medical treatment and/or close follow up surveillance. Minimal residual disease has been shown to act as a strong prognostic parameter in these patients and could ultimately be used to identify patients with occult systemic disease who will need additional treatment besides surgical therapy. Therefore, the idea to use occult disease as guidance for treatment decisions and monitoring disease progression seems very promising. Occult disease such as CTC in the blood, DTC in the bone marrow and micrometastases and ITC in the lymph nodes of patients with CRC is a strong prognostic marker for patient survival. Future re-

search should be directed to identify patients with UICC stage I and II who have already systemic disease despite negative conventional staging. These patients at risk might benefit from additional adjuvant treatment and this should be investigated in future randomized controlled trials.

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