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Cytokeratin 18 (CK18) and CK18 Fragments for Detection of Minimal Residual Disease in Colon Cancer Patients

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1. Introduction

Despite advances in therapeutic approaches for patients with colorectal cancer (CRC), approximately 20-45% of those who undergo apparently curative surgery subsequently develop local or distant tumor recurrence (Harrison & Benziger, 2011). The liver constitutes the preferred metastatic site in approximately 30% of cases. According to the TNM staging system, a stage-specific 5-year survival rate of approximately 93% has to be expected for stage I, 72-84% for stage II, 44-83% for stage III and 8% for stage IV colon cancer (O'Connell et al., 2004). An early identification of patients at risk of developing metastatic disease after surgery would be of great importance for improving the clinical outcome. Currently, there are no reliable methods to identify patients who are at increased risk of relapse and in need of adjuvant chemotherapy. Aggressive postoperative treatment with cytotoxic drugs without certain indication leads to overtreatment of patients and severe side effects. Appropriate biomarkers for the identification of CRC patients at high risk for recurrence and/or poor prognosis are required to facilitate individually tailored therapy (Gangadhar et al., 2010).

1.1 Biomarkers and CRC

In general, an eligible biomarker in reference to chemotherapy is a characteristic that is objectively measured and evaluated as an indicator of response. A range of assays, including immunohistochemistry, gene analysis, gene and protein expression assessments and detection of single nucleotide polymorphisms (SNPs) have served to identify biomarkers in recent years (Saijo, 2011). Response rates to anticancer drugs applied in CRC may be as low as approximately 20% and, therefore, it is of great importance to identify patients most likely to benefit from a specific agent. For example, biomarkers such as expression of HER2 for breast cancer or EGFR mutation for lung cancer and KRAS mutation in CRC aid in selection of drug-sensitive patients. Personalized CRC care has improved patient outcome significantly over the last decades in both the adjuvant and metastatic

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settings (Catenacci et al., 2011). Microarray-based gene expression profiling has been frequently used to formulate prognostic signatures and, to a lesser degree, predictive signatures in CRC; however, common problems associated with these markers are clinical study design, reproducibility and interpretation of the results (Van Schaeybroeck et al., 2011).

1.2 CRC and disseminated tumor cells (DTCs)

Approximately 50% of CRC patients undergoing curative resection die from metastatic disease within 5 years and the relapse rate is 30% even in lymph node-negative patients (Iddings & Bilchik, 2007). Appearance of disseminated tumor cells (DTCs) in cancer patients may precede the occurrence of detectable metastases and, thus, may be used to adapt the aggressiveness of therapeutic regimens (Lugo et al., 2003; Pantel & Alix-Panabières, 2010). First evidence for such a role of bone marrow DTCs (BM-DTCs) or circulating tumor cells (CTCs), respectively, was obtained for breast cancer, where BM-positive patients had an approximately twofold increased risk of relapse within ten years (Braun et al., 2000). However, the situation is less clear for other solid tumors like CRC (Thorsteinsson & Jess, 2011). Both solitary cells and micrometastases may remain in "dormancy" for years, being cell cycle arrested and not undergoing apoptosis (Luzzi et al., 1998). Cancer cells that left the primary tumor can seed metastases in distant organs, which is thought to be a unidirectional process. However, in a process that is called "tumor self-seeding" CTCs can also colonize their tumor of origin (Kim et al., 2009).

As further discussed below, analysis of cytokeratins (CKs), either by RT-PCR, immunohistochemistry or quantification of soluble CK protein fragments released by tumor cells, is the mainstay of the detection of DTCs. Studies indicate that BM is a common homing organ and serves as reservoir for DTCs derived from various primary sites including tumors of the breast, prostate, lung and colon (Pantel & Alix-Panabières, 2010). However, peripheral blood analyses are obviously more convenient for patients than invasive BM sampling.

1.3 CKs as marker of epithelial tumor cells

Antibodies directed to CKs, that are members of the intermediate filament (IF) proteins, are useful tools particularly in the diagnostics of carcinomas (Barak et al., 2004; Karantza, 2011). These proteins protect epithelial cells from mechanical and non-mechanical stressors (Coulombe & Omary, 2002). At present, more than 20 different CKs have been identified, of which CKs 8, 18, and 19 are the most abundant in simple epithelial cells and carcinomas of the breast, prostate, lung, colon, ovary, among others (Moll et al., 1982; Bragulla & Homberger, 2009). Low molecular weight acidic type I CKs, such as CK18, are normally complexed with high molecular weight basic or neutral type II CKs, such as CK8. The bulk of cellular CKs are part of the IF system and mostly insoluble at physiological salt concentrations until they are cleaved to yield soluble fragments. CKs are in use for the detection of epithelial cancer cells in BM with the help of pan-CK antibodies directed to the CKs 8, 18, 19 and, furthermore, these proteins seem to be of functional importance for BM-DTCs. Alix-Panabieres et al. demonstrated that full-length CK19 was shed by viable epithelial breast tumor cells and that such cells might constitute a biologically active subset of breast cancer cells with highly metastatic properties (Alix-Panabieres et al., 2009).

The production of soluble CK fragments may be triggered by hypoxia that has resulted in network disassembly and CK8/CK18 degradation (Na et al., 2010). Similarly, the keratin cytoskeleton in mammary epithelial cells disintegrates under metabolic stress of glucose and oxygen deprivation, which mimicks the tumor microenvironment (Nelson et al., 2004) Phosphorylation of CKs regulates their distribution into an insoluble filamentous cytoskeletal fraction and a soluble cytosolic hyperphosphorylated pool (Omary et al., 2006) and plays a role in CK ubiquitination and turnover by the proteasome (Ku & Omary, 2000; Jaitovich et al., 2008) and, likely, by autophagy (Kongara et al., 2010). CKs released from proliferating or necrotic/apoptotic cells are useful markers for prediction of tumor progression/recurrence or response to therapy (Linder, 2007). The three most applied CK markers for the assessment of cell activity used in the clinic are tissue polypeptide antigen (TPA), tissue polypeptide-specific antigen (TPS) and CYFRA 21-1. The broad spectrum TPA test measures CKs 8, 18, and 19 concentrations, while TPS and CYFRA 21-1 assays are more specific and determine CK18 and CK 19 levels, respectively (Linder, 2007). More recently, the ratio of caspase-cleaved (M30) to total CK18 (M65), which can be assessed in the serum or plasma using commercially available enzyme-linked immunosorbent assay (ELISA) kits, has been evaluated as a biomarker for monitoring therapy efficacy in carcinoma patients (Linder et al., 2010).

2. Assessment of DTCs in CRC

The prognosis of colon cancer patients is largely determined by the occurrence of systemic disease (Negin & Cohen, 2010). In patients with primary tumors relapse is mainly due to clinically occult micrometastasis that exists in secondary organs already at first presentation, but is not detectable with imaging procedures currently used (Riethdorf et al., 2008). Sensitive and specific immunocytochemical and molecular tests enable the assessment and characterization of DTCs at the single cell level in BM, which constitutes the common homing site of DTCs. Although many assays were developed to prove CTCs, two main approaches that are used involve either cytology based on immunocytochemical staining or polymerase chain reaction (PCR) analysis (Cristofanilli et al., 2007). Employing the US Food and Drug Administration (FDA)-approved Cellsearch® system, the value of CTCs as a predictive marker for survival was substantiated in metastatic breast cancer and CRC (Paterlini-Brechot et al., 2007). Still, there is an urgent need for standardized methods because of the high variability of results in the detection of DTCs and CTCs, respectively.

2.1 Detection of DTCs by RT-PCR

The detection of CTCs can be very difficult with as few as one CTC in 100 million leukocytes in peripheral blood and, thus, highly sensitive methods like PCR are employed to detect tumor-specific DNA or RNA. Since RNA is unstable and therefore disappears quickly from the blood after cell death, presence of RNA must be due to the occurence of viable tumor cells (Mostert et al., 2009). The RNA markers commonly used in CRC are carcinoembryonic antigen (CEA), CK19 and CK20 (Sergeant et al., 2008). The advantage of RT-PCR is a higher sensitivity compared to immunocytochemical techniques. Unfortunately, the specificity of RT-PCR is hampered by high numbers of false-positive results either due to contamination or target genes expressed in other nonmalignant cells. Novaes et al. demonstrated that mononuclear cells from peripheral blood of healthy donors express CK19 in RT-PCR assays (Novaes et al., 1997). Furthermore, both CEA and CK20 transcripts are elevated in patients

with inflammatory diseases (Dandachi et al., 2005). Another important limitation of PCR-based methods is that CTCs cannot be isolated for further analysis. Nevertheless, Allen-Mersh et al. demonstrated that poor disease-free survival (DFS) was associated with the occurrence of CEA or CK20 24 hrs postoperatively using RT-PCR on blood samples from 147 CRC patients with TNM-stage I-III tumors (Allen-Mersh et al., 2007). In another study, four RNA markers including human telomerase reverse transcriptase (hTERT), CK19, CK20 and CEA mRNA were used to detect CTCs in stages II and III CRC patients who underwent curative resection to determine the significance of CTCs in prediction of early relapse (Lu et al., 2011). The presence of persistent postoperative CTCs was proved as independent predictor for early recurrence (hazard ratio: 11.04) and correlated with a poorer DFS and overall survival (OS). CK20 is a commonly used RT-PCR marker in analysis of BM from CRC patients; however, the number of investigated patients was larger than 100 with DTC detection rates of 11-35% only in 3 out of 10 studies. Two groups reported no association to survival in metastatic patients, whereas four groups found a correlation between the presence of CK20 transcripts and worse OS (Koch et al., 2006).

Rahbari et al. also found postoperative tumor cell detection can predict poor recurrence-free survival in curatively resected CRC patients in a metaanalysis (Rahbari et al., 2010). The included studies reported postoperative detection rates of 22-57% DTCs in CRC with RT-PCR. However, application of the RT-PCR approach is hampered by an absence of an international standard on choice of markers, enrichment procedures and laboratory techniques. Nevertheless, combining the TNM staging system and proof of CTCs could aid in decision-making as to which patients should be offered adjuvant chemotherapy.

2.2 Detection of DTCs by immunohistochemical methods

Immunohistochemistry utilizes labeled monoclonal antibodies directed against epithelial or tumor-associated antigens and automated digital microscopy or flow cytometry to isolate and count CTCs (Alix-Panabieres et al., 2008; Pantel et al., 2009; Allen & Keeney, 2010). This method allows identification of intact tumor cells occurring in the periphery for further characterization (Smirnov et al., 2005; Cohen et al., 2006). Most studies describing immunocytochemistry for the detection of DTCs in CRC patients either used the monoclonal antibody CK2 against CK18 or the pan-cytokeratin antibody A45-B/B3, while few studies employed a cocktail of several antibodies against different epithelial antigens including CKs. The DTC detection rate in studies with CK2 was 16-32%, whereas it was clearly higher in investigations using the A45-B/B3 antibody (24-55%). This difference might probably be due to a potential downregulation or loss of CK18 expression on DTCs (Pantel et al., 1994). Both antibodies rarely detect CK-positive cells in BM of noncancer control patients (0-5.5%). The largest study so far conducted by Flatmark et al. included 275 CRC patients and 206 noncancer control patients (Flatmark et al., 2011). An immunomagnetic bead enrichment system capturing BM-DTCs by the antibody MOC31 directed against epithelial cell adhesion molecule (EpCAM) was applied and 17% of the patients showed presence of DTCs, whereas only 1.5% of control samples were positive. However, the BM status did not correlate significantly with disease stage or other clinical parameters, a finding we confirmed in a small series of patients (Buxhofer-Ausch et al., 2009). In conclusion, several studies were carried out to elucidate the clinical relevance of DTCs in CRC. These studies, however, present a very heterogeneous picture differing in patient groups, sample sizes, follow-up

times, staining methods and target antigens, all of which probably contributed to the observed variation in DTC detection rates and association to clinical parameters (Riethdorf et al., 2008).

For the analysis of CTCs an automated microscopic system including an immunomagnetic tumor cell enrichment step was developed (Miller et al., 2010). This CellSearch System (Veridex LLC, Raritan, NJ) gained approval for metastatic breast cancer from the FDA in 2004 and is now also accepted for metastatic prostate and colorectal cancer. In this detection method CTCs must feature: a round to oval shape by light scatter, an evident nucleus by DAPI staining, EpCAM positivity and $CK8^+$, $CK18^+$, $CK19^+$ and $CD45^-$ by immunofluorescence (Allard et al., 2004). The sensitivity of approaches using EpCAM might be limited by the fact that EpCAM-negative CTCs would not be detected, which would lead to false-negative results. Rao et al. demonstrated that the expression of EpCAM on CTCs was approximately tenfold lower than on primary and metastatic tissues (Rao et al., 2005). The CellSearch CTC detection system was combined with a monoclonal antibody (M30) targeting a neoepitope disclosed by caspase cleavage at CK18 in early apoptosis (Rossi et al., 2010). M30-positive CTC could be detected in >70% of CTC-positive carcinoma patients, which were free from both chemotherapy and radiologic treatments. The fraction of M30-positive CTC varied from 50% to 80%, depending on the histotype.

In a study by Sastre et al., CTCs were detected in 34 of 94 patients (Sastre et al., 2008). Only tumor stage correlated with positive CTCs (20.7% in stage II, 24.1% in stage III and 60.7% in stage IV). Cohen et al. demonstrated that the CTC level at baseline and follow-up is an independent prognostic factor in metastatic CRC. Patients were divided into unfavorable and favorable prognostic groups by their CTC levels of ≥3 or <3 CTCs/7.5 ml blood, respectively. PFS as well as OS was shorter for unfavorable compared with favorable baseline CTC patients (Cohen et al., 2009). Approximately one quarter of patients with metastatic disease thereby categorized in this poor prognosis group (Negin & Cohen, 2010). In a similar study by Tol et al. the CTC count before and during treatment with chemotherapy plus targeted agents independently predicted PFS and OS in advanced CRC patients (Tol et al., 2010). A metaanalysis of available studies to assess whether the detection of tumor cells in the blood and BM of patients diagnosed with primary CRC can be used as a prognostic factor included a total of 36 reports, comprising 3094 patients (Rahbari et al., 2010). Pooled analyses that combined all sampling sites showed an association of the detection of tumor cells with poor DFS (hazard ration: 3.24) and OS (hazard ratio: 2.28). Now there is compelling evidence that CTCs predict clinical response in metastatic CRC (Allen & El-Deiry, 2010).

Although the presence of CTCs can be a strong marker of poor prognosis in patients with metastatic disease, the prognostic role of CTCs in nonmetastatic CRC (TNM stages I-III) is less clear (Thorsteinsson & Jess, 2011a). By using the CellSearch method to detect CTCs from blood samples taken 4-12 weeks after apparently curative surgery, Maestro et al. found >2 CTCs/7.5 ml blood in 25 of 164 patients with localized CRC (Maestro et al., 2009). CTCs were detected with the CellSearch system preoperatively in one out of 20 patients with TNM stages I-III, and none of the four different postoperative blood samples had CTC levels above the cut-off value of ≥2 CTCs/7.5 ml blood (Thorsteinsson et al., 2011b). The presence of CTCs at least 24 hrs after CRC resection was suggested as independent prognostic marker of recurrence (Peach et al., 2010). It was concluded that the presence of CTCs in

nonmetastatic colon cancer is rare and barely detectable with the only commercially available assay for determination of CTCs, the CellSearch system. Further studies are needed to clarify the optimal time point for blood sampling and the benefit of chemotherapy in CTC-positive patients with stage II disease. The low incidence of CTCs in nonmetastatic CRC requires highly sensitive and specific detection methods. A gastrointestinal-specific anti-CK20 antibody was developed by Wong et al. and demonstrated to detect CTCs in 58 of 101 patients with stage I-III CRC preoperatively and, furthermore, a decrease in CTCs in 51 of these 58 patients after surgery (Wong et al., 2009). Another study by this group found CK20 expression in lymph nodes and blood of CRC patients and a follow-up study reported that these cells predicted metastasis (Wong et al., 2009).

Recently, it was observed that CTCs are often undetected in metastatic breast cancer patients treated with bevacizumab (Gazzaniga et al., 2011). Due to the frequent use of bevacizumab as first-line medication for metastatic CRC, the predictive value of the CTC count in patients treated with first-line chemotherapy plus bevacizumab compared with those treated with chemotherapy plus cetuximab was investigated. In the bevacizumab-treated patient group the median number of baseline CTCs was 2.7/7.5 ml blood and dropped to zero in most patients. However, half of the apparently CTC-negative patients proved to have progressive disease. Thus, bevacizumab-induced hypoxia in the primary tumor may generate a selected population of cells undergoing epithelial-mesenchymal transformation (EMT) with downregulation of epithelial markers such as EpCAM and CKs making these cells undetectable by the CellSearch system. Mego et al. for the first time used the term 'undetectable CTCs' due to the obvious underestimation of CTCs that underwent EMT by the CellSearch system (Mego et al., 2011).

2.3 Surgery and tumor cell dissemination

Disturbing tumors mechanically, which may lead to shedding of cancer cells during surgery, constitutes a possible mechanism of tumor dissemination. The quantification of CTCs can be applied to assess the extent of this phenomenon. Lu et al. published a large-scale study involving stage III and IV CRC patients who underwent curative resections. This study found that postoperative relapse was strongly correlated with laparotomy versus laparoscopic surgery, lymph node metastases, as well as CTC levels if elevated at both pre- and postoperative time points (Lu et al., 2011). In contrast, another study reported no statistically significant difference of the $CD45^-/CK^+$ tumor cell count in the blood at time of surgical incision, after tumor resection and at the end of operation (Tralhão et al., 2010). A similar result was published by Wind et al. for the respective type of operative procedures, revealing that the cumulative percentage of samples containing CTCs was significantly higher during open surgery as compared to the laparoscopic approach (Wind et al., 2009). However, dissemination of CK^+ cells during surgery of hepatic metastases, a frequent event in colon cancer patients, did not predict extrahepatic recurrence (Schoppmeyer et al., 2006, Koch et al., 2007). In conclusion, CTCs seem to be generated during surgery; however, their significance for the occurrence of relapses is not clear.

2.4 Epithelial-mesenchymal transition (EMT) and CKs

EMT is considered an essential process in the metastatic cascade enabling the cells to aquire a mesenchymal cell phenotype characterized by increased motility and altered morphology

(Kalluri and Weinberg, 2009). The appearance of dot-like α-smooth muscle actin (α-SMA)-staining in CK^+ cells during tumor progression may indicate the initial phase of EMT in CRC (Valc et al., 2011). Increasing intraepithelial α-SMA concomitant with decreasing E-cadherin expression points to a loss of epithelial cell contact in the beginning of EMT. However, downregulation of epithelial markers like EpCAM and CKs in EMT-transformed tumor cells implicates the infeasibility to detect the most aggressive CTCs by methods relying on these markers (Mego et al., 2011). Epithelial cancer cells are likely to undergo EMT before they enter the peripheral circulation. Both EpCAM and CKs are downregulated as part of an oncogenic pathway promoting increased invasiveness and metastatic potential (Woelfle et al., 2004; Willipinski-Stapelfeldt et al., 2005). EpCAM is expressed in most but not all tumors and there is evidence for its modulation with cancer progression and metastasis (Mikolajczyk et al., 2011). Again, EpCAM expression may be suppressed to allow dissociation of epithelial cancer cells from the tumor and structural cytoplasmic CKs are downregulated to facilitate cell plasticity and migration. It is not always clear whether the loss of CKs is a result of independent oncogenic processes or whether it is always related to EMT. Aberrant occurrence of CKs in BM appears to be more common than in peripheral blood, and CK expression caused by inflammation can also contribute to false-positive observations (Dandachi et al., 2005). Although reduced expression of CK8 and CK20 is associated with EMT in CRC, which is generally indicative of higher tumor aggressiveness and decreased patient survival, several studies provided evidence supporting an active role of CKs in cancer cell invasion and metastasis (Knosel et al., 2006). Transfection of CK8 and CK18 into vimentin-positive mouse L fibroblasts resulted in higher migratory and invasive ability, indicating that CKs may influence cell shape and migration through interaction with the extracellular environment (Chu et al., 1993). Similarly, experimental coexpression of vimentin with CK8 and CK18 increases invasion and migration of human melanoma and breast cancer cells in vitro (Hendrix et al., 1997)

EMT in breast tumor cells is characterized by upregulation of expression of vimentin, Twist, Snail, Slug and Sip1, among others (Kalluri and Weinberg, 2009). A recent study in early and metastatic breast cancer patients found that immunomagnetic separation of CTCs and triple-immunofluorescence obtained with anti-CK/anti-Twist/anti-vimentin antibodies indicated that the mesenchymal marker could be coexpressed in the same CK^+ cell, since 64% of the total identified CTCs were triple-stained (Kallergi et al., 2011). Among patients with early disease, approximately half of the CK^+ CTCs were double-stained with anti-vimentin and anti-Twist antibodies, while the corresponding values for metastatic patients were 74% and 97%, respectively. The median expression of CK^+ $vimentin^+$ and CK^+ $Twist^+$ cells per patient in metastatic patients was nearly 100% and in an adjuvant chemotherapy setting approximately 50%, respectively. The high incidence of coexpressing cells in metastatic breast patients compared to early stage tumors point to a high metastatic potential of CTCs with EMT phenotype. Similar results were found for non-small cell lung cancer (NSCLC) and prostate tumor patients, where hybrid CTCs with an EMT phenotype coexpressing vimentin and CKs were detected (Lecharpentier et al., 2011; Armstrong et al., 2011). Furthermore, DTCs in BM may reverse EMT in a process termed mesenchymal-epithelial transition (MET) and regain an epithelial phenotype with loss of mesenchymal migratory properties (van der Pluijm, 2011). Therefore, antibodies to CKs are still expected to help in the identification of tumor cells that underwent EMT, dependent on the respective histological origin.

2.5 CK-positive CTCs and cancer stem cells (CSCs)

The CSC concept hypothesizes that tumors arise from a small population of stem cells, which may disseminate from the primary tumor to a stem cell niche until relapse. The relationship of cancer stem cells (CSCs) with CTCs is entirely unclear at this point. Thus, CTCs have additional intravasation and extravasation properties and, possibly, stem cell characteristics. CD133 (prominin) is one of the key markers of CSCs in CRC and CD133-positive cells have high tumorigenic ability in nude mice (Smirnov et al., 2005). Furthermore, it was reported that CSCs are often characterized by downregulation of epithelial markers such EpCAM and CKs. Stem cell markers are frequently overexpressed in CTCs of patients with metastatic breast cancer. This stem cell-like subpopulation of CTCs is characterized by a nonproliferative and chemoresistant phenotype (Lianidou & Markou, 2011). These facts would suggest that a new marker is necessary for the detection of CK-downregulated, aggressive CTCs, which may comprise CSCs as well. The surface markers of colon CSCs, namely CD133, CD44, CD166, Musashi-1, CD29, CD24, leucine-rich repeat-containing G-protein-coupled receptor 5 and aldehyde dehydrogenase 1 were reported (Dhawan et al., 2011). The clinical significance of CSC-like CTCs as a prognostic factor for OS and DFS in the peripheral blood of CRC patients was published (Iinuma, et al., 2011). CTCs of CRC patients who had undergone curative surgery expressed CEA, CK19, CK20 and/or CD133 mRNA in peripheral blood. In particular, these $CEA^+/CK^+/CD133^+$ CTCs demonstrated significant prognostic value in patients with Dukes' stage B and C cancer, but not Duke's A. Again, this finding demonstrates that CTCs with CSC characteristics can still express CKs and may be detected with respective pan-CK antibodies.

2.6 Soluble CK18 fragments as markers of residual tumor load

There are several reports dealing with determinations of CK fragments in BM samples of cancer patients. An investigation using BM aspirates of breast cancer patients reported a positive relationship between the detection of micrometastic tumor cells in immunocytochemistry with the pan-CK antibody A45-B/B3 and measurements of CK fragment CYFRA 21-1 expression (Pierga et al., 2004). CYFRA 21-1 was significantly elevated in patients with disseminated tumors and both markers were associated with a poorer survival for patients with stage I to III breast cancer. The CK-positive cells in BM aspirates were reported to lack expression of urokinase plasminogen activator which is associated with metastasis (Werther et al., 2002). In order to avoid tedious immunohistochemical methods, a new enzyme immunoassay for detection of occult tumor cells in BM was developed that was designed to detect intracellular CK19 released from epithelial tumor cells after they had been lysed by freezing/thawing cycles (Riethmüller et al., 1997). Comparison of immunohistochemistry with this new assays revealed higher incidence of epithelial cells in advanced T-stage of CRC patients by the ELISA test. In comparison with controls, BM samples of cancer patients were found to have significantly elevated levels of CK19 and in the analysis of almost 400 BM aspirates of cancer patients, a significant correlation of ELISA and immunohistochemistry to detect CK^+ cells was observed. However, most discordant samples were ELISA-positive and the CK status detected by this method did not correlate with the TNM stage and the histological grading. This immunoassay was reported to allow for sensitive and specific detection of DTCs in a faster, less laborious and more objective manner compared to immunohistochemistry.

Quantification of soluble CK fragments that are shed by epithelial tumor cells by newer methods may serve as an alternative way for detection of occult residual tumor load by DTC counting. In particular, CK18, an intracellular, mainly insoluble protein highly expressed by various types of epithelial cells, is released in form of a caspase-cleaved 30 kD (ccCK18) and a 65 kD fragment into the extracellular compartment during apoptosis and necrosis, respectively, though active export of intact CK19 has also been reported (Linder, 2011; Alix-Panabieres et al, 2009). The 30 and 65 kD fragments can be quantified by the M30-Apoptosense® ELISA and the M65® ELISA assay (Peviva, Bromma, Sweden), respectively. So far, a single study has been conducted in small cell lung cancer (SCLC) patients undergoing standard chemotherapy to evaluate cell death assays detecting CK18 fragments (ccCK18 and M65) and CTC profiles using the CellSearch system (Hou et al., 2009). In this study both ccCK18 and M65 correlated with known clinical and biochemical prognostic factors including tumor stage and expression of lactate dehydrogenase. The number of CTCs had prognostic significance and correlated with pretreatment M65 levels. The accordance between levels of the circulating apoptotic-specific protein ccCK18 and the proportion of morphologically apoptotic CTCs corroborate the role of ccCK18 to indicate tumor cell apoptosis.

Although the levels of CKs in serum of cancer patients have been widely used for monitoring progression of tumor growth and effectiveness of treatment, the mechanisms of the release of CK fragments from cells is not clear (Linder et al., 2010). Studies in patients have shown that the release of CKs by tumors is a complex process, which seems to be not simply correlated to the number of proliferating cells or to the tumor mass but may also be dependent on the rate of cell damage (Oehr et al., 1997). During the development of CK18 as tumor marker the focus has switched from the perspective as proliferation marker to its significance as parameter of tumor cell death (Ausch et al., 2010). The demonstration of elevated levels of both ccCK18 and M65 in venous blood from tumors of endometrial cancer patients proved that these proteins were derived from the malignant tissue (Kramer et al., 2004). Additionally, and in contrast to the assumption that CKs are only released in fragmented form by epithelial cells as a result of necrosis/apoptosis, evidence for the active release of full-length CK19 by viable epithelial tumor cells was published (Alix-Panabieres et al., 2009). According to this study CK19-releasing cells were detected in BM of 44-70% of breast cancer patients and correlated to the presence of manifest metastases.

2.6.1 Effect of radical tumor surgery on circulating CK18 fragments in CRC patients

We quantified the CK18 fragments M65 and ccCK18 in serum samples of CRC patients pre- and postoperatively using ELISA assays and demonstrated higher levels of circulating M65 and ccCK18 in patients with either low grade tumors or disseminated metastatic CRCs in comparison to nontumor control individuals. Due to tumor surgery approximately 60% of the CRC patients responded with a mean drop of soluble M65 blood levels of 30%, while the remaining 40% of patients showed a mean increase of 40%, respectively (Ausch et al., 2009a). The group with increased postoperative levels of M65 was characterized by a higher incidence of BM-DTCs in comparison to patients with postoperatively normalizing M65. In the case of circulating ccCK18 almost 80% of patients exhibited a significant decrease of approximately 75% in response to tumor removal; however, the remaining 20% revealed a

mean increase of 40% of these CK fragment levels postoperatively (Ausch, et al., 2009b). The frequency of BM-DTCs, as detected before surgery with help of the pan-CK antibody A45-B/B3, was not significantly different for these two groups of patients with normalizing and increased ccCK18 levels. However, the course of ccCK18 correlated well with an increased number of recurrences in the group with persisting serum fragment levels within a follow-up of three years (hazard ratio: 8.3). In conclusion, radical removal of the tumor, which is supposed to be the main source of the circulating CK18 fragments, failed to result in a decline of M65 levels to normal values in this subgroup of patients characterized by occurrence of BM-DTCs and increased risk of early relapses. In the same group of CRC patients the overall rate of BM-DTCs detected was 23% independent of the respective tumor stage. No difference was found in relapse and OS between patients with or without BM-DTCs preoperatively after a median follow-up of 35.4 months (Buxhofer-Ausch et al., 2009). The BM-DTC status was found to be changed for a second BM aspiration after twelve months in a quarter of the patients. Thus, failure to yield postoperative reduction of circulating CK18 fragments in individual CRC patients seems to be indicative of remaining tumor tissue.

CTCs released during surgery may undergo apoptotic cell death due to a failure of homing of the majority of cells to a suitable environment, while a minor fraction eventually manages to settle at distant sites and causes tumor recurrence (Rossi et al., 2010). Retsky et al. suggested that surgery to remove the primary tumor often terminates the dormancy DTCs resulting in accelerated relapses, as demonstrated in over half of the metastatic cases (Retsky et al., 2008). Another explanation for the CK release may be intracellular degradation of CK18 by caspases and shedding into the circulation as part of EMT without cell death or, alternatively, secretion of CK18 in intact form, as exemplified for CK19, followed by cleavage by caspases in the BM or serum (Alix-Panabieres et al, 2009).

2.6.2 Course of M65 concentrations in BM aspirates of CRC patients

In the present study we extended the measurement of M65 to BM aspirates of CRC patients obtained preoperatively and one year after surgery using the methods described by Ausch et al. (Ausch et al., 2009a).

2.6.2.1 Patients and methods

A total of 56 patients with colorectal cancer who were treated between January 2002 and December 2004 at the Donauspital, Vienna and had a follow-up period of more than 3 years. 30 patients underwent surgery for primary colorectal carcinoma and none of the patients had received chemotherapy and/or radiotherapy prior to surgery. All patients were checked for infections by viral tests, blood count and chemistry, including determination of C-reactive protein. Twenty-three nontumor patients admitted to the outpatient department for minor complaints served as controls. Collected blood was centrifuged 2000 rpm for 10 minutes and stored at -20 °C. Written informed consent was obtained from all patients. The study was approved by the local ethics committee and the institutional review board. BM aspirates were obtained from both upper iliac crests (5ml each) by needle aspiration immediately prior to the operation under general anesthesia. Mononuclear cells of bone marrow aspirates were separated by Ficoll-Hypaque density gradient centrifugation (GE Healthcare, Little Chalfont, UK). Cytospins containing $1x10^6$ cells/slide were fixed in acetone and stained using pancytokeratin pan-CK antibody A45-B/B3 (Micromet, Munich,

Germany; final concentration 5 µg/ml; 20 min). All staining steps, including blocking and washes, were performed using the Idetect-Super- Stain-(alkaline phosphatase)-Fast-Red kit according to the manufacturer's instruction (ID Labs, London, ON, Canada) and mouse monoclonal isotype controls were included. For assessment of BM-DTCs at least $2x10^6$ cells per specimen were screened blinded by two pathologists and a minimum of one tumor cell per $2x10^6$ mononuclear cells was regarded as a positive result for A45-B/B3. From all BM aspirates the concentrations of ccCK18 and and total CK18 (M65) were determined using the M30-Apoptosense® and the M65-ELISA assay® according to the manufacturer's instruction (Peviva, Bromma, Sweden), respectively. The coefficient of variance for the duplicate measurements of M30/M65 was < 7.5%.

2.6.2.2 Result of determinations of M65 in BM aspirates of CRC patients

The comparison of M65 concentrations determined from 30 CRC patients is shown in Figure 1. In the group of 16 patients with significant reduction (>10 %) of BM M65 levels, four cases of local relapses (Pat. # 2, 14, 52 and 68) were observed, whereas all four systemic relapses (Pat. # 3, 11, 43 and 45) occured in the second group who exhibited increasing M65 levels or minor reduction of <10%. Thus, the difference in respect to systemic relapses was highly significant (0/16 versus 4/14; 0 versus 28.6%; $p < 0.001$; Fisher's exact test). Mean differences of M65 for the two groups and the two time points were -42.5 ± 17.2% and +12.3 ± 19.2%, respectively. Obviously, the determination of BM M65 levels is not adequate to predict local relapses in CRC patients, but systemic relapses in this small group of CRC patients correlated with perioperative changes in this CK fragment. The course of BM M65 levels during the first postoperative year was studied for the BM DTC-positive and -negative groups. Assessment of the BM for pan-CK-positive tumor cells by inspection of two million mononuclear cells showed no correlation with the course of M65 for the first year after surgery: M65 decreased by -24.0 ± 36.8% for the BM-DTC-positive (n = 15) and by -9.8 ± 28.0% for the BM-DTC-negative patient group (n = 15), respectively. The former group comprised two systemic relapses while the latter exhibited two systemic and one local relapses (difference not significant). Although BM-DTCs were detected in 50% of these CRC patients, the status of BM did not correlate with the frequency of relapses during the follow-up of five years. However, analysis of BM-DTCs was performed without any enrichment of tumor cells and the analysis of CTCs actually seems to be more suitable to obtain prognostic results (Allen & El-Deiry, 2010). In most cases, concentrations of M65 in BM and peripheral blood showed good correlation (Olszewski-Hamilton et al., 2011). In conclusion, a reduction of BM M65 concentrations during the first postoperative year points to PFS in respect to systemic disease, whereas an increase or a minor reduction seems to be associated with residual tumor load and systemic recurrence. Increased preoperative or pretreatment blood concentration of M65 was described as independent prognostic parameter, possible reflecting tumor burden of the individual patients. However, the individual time courses of CK fragments may more accurate mirror the effects of tumor treatment. Although determination of the individual differences in circulating CK18 levels before and after tumor surgery is prone to interference by inflammation and benign disease, it represents a cost-effective alternative to the expensive automated counting of CTCs in nonmetastatic CRC. However, these results were obtained in a small group of CRC patients and need to be confirmed in a larger study.

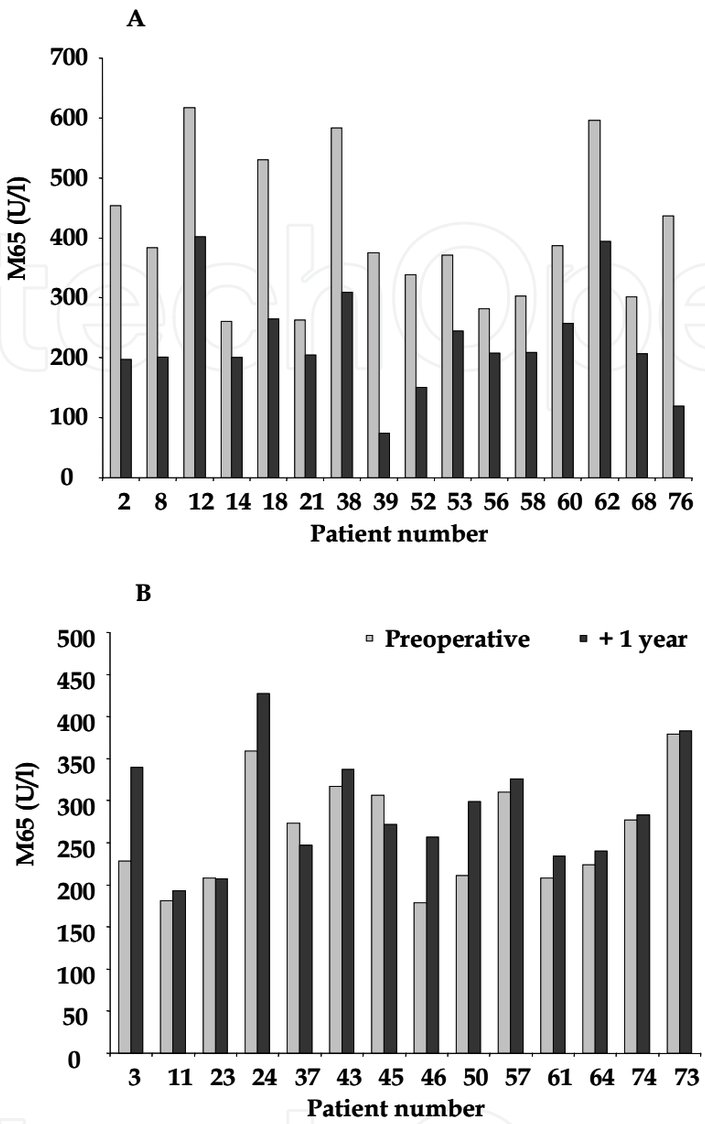

Fig. 1. Comparison of the differences of BM M65 concentrations in two CRC patient groups between preoperative and follow-up samples taken one year later. 30 patients were classified into a group of 16 showing significant reduction (>10%) of the BM M65 concentrations during the first year after surgery (A) and a group of 14 patients exhibiting <10% reduction of M65 (B). Data are shown as mean values (SD <10%).

3. Conclusions

Dissemination of tumor cells from primary tumors in the circulation seems to be an early event in tumor development for specific histological types. The presence of these DTCs in peripheral blood, bone marrow and distant organs is tested for providing the rationale for adjuvant systemic treatment (Bidard et al., 2011; Lin et al., 2011). Detection of DTC in bone marrow aspirates from breast cancer patients and other solid tumors at the primary diagnosis impacts the prognosis of disease. Technological advances in immunological and

quantitative real-time PCR-based analysis allow for detection, enumeration and characterization of disseminated tumor cells in cancer patients. Proof of the expression of CKs by use of antibodies is the mainstay of most methods to detect DTCs and gain prognostic data of patients with carcinomas. Despite assumed downregulation of epithelial markers like CKs, during EMT they are still expressed in vimentin-positive hybrid CTCs and allow for the assessment of tumor dissemination. Determinations of individual courses of the levels of circulating CK fragments before and after tumor surgery in blood or BM of CRC patients seem to constitute a valuable technique to prove residual tumor load, provided that these CK fragments are not released due to nontumor causes. The advantage of measuring CK fragments in the circulation or BM aspirates may be the sensitive detection of DTCs independent of their localization. For reliable results in regard to systemic relapses careful monitoring of individual time courses of the levels of the CK fragments in tumor patients seem to be necessary.

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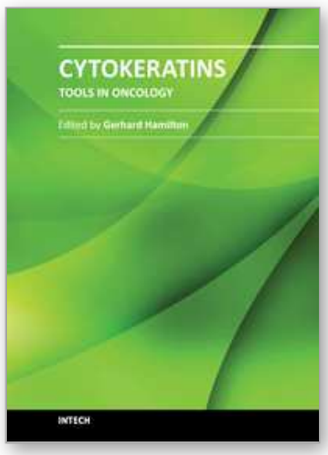

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The first chapters of the volume "Cytokeratins - Tools in Oncology" discuss multiple functions of cytokeratins in organization of the intermediary filaments in normal intestine and liver as well as microfold L cells and the usability of cytokeratins 7, 8 and 20 in tumor diagnosis in detail. Epithelial to mesenchymal transition as a mechanism important in pathogenesis is touched in another chapter, followed by several articles dealing with the role of cytokeratins for detection of disseminated tumor cells and as response markers during chemotherapy. This book is therefore destined to all cancer researchers and therapists who want to understand the diagnostic application of cytokeratins in histology and, especially, the use of anti-cytokeratin antibodies to identify viable residual tumor cells accounting for a higher risk of tumor recurrence or cancer cells responding to chemotherapy, respectively.

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