Molecular Detection of Circulating Tumor Cells in peripheral blood of colon cancer patients

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AIM: The aim of our pilot study was to evaluate the use of a commercial kit (AdnaTest) for the detection of circulating tumor cells of patients with colon cancer.

METHODS: After immunomagnetic enrichment and mRNA extraction, multiplex RT-PCR was performed in peripheral blood specimens from 50 patients and 40 healthy donors (control group) for beta-actin gene (as an internal control) and three tumor genes.

RESULTS: The internal PCR-control band of beta-actin was present in all samples. In the peripheral blood of four patients, aberrant expression of the CEA and GA 733-2 tumor genes was observed. Three of these patients were stage IV (Dukes D), with liver metastasis according to the histopathological examination. The other one was stage III (Dukes C) with no confirmed histopathological metastasis.

CONCLUSIONS: Further studies are needed to validate both technically and clinically the use of this kit in the peripheral blood of patients with colon cancer.

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Key words: Circulating Tumor Cells (CTCs); Colorectal cancer (CRC); mRNA; Multiplex RT-PCR
in the peripheral blood could become not only an excellent non-invasive prognostic tumor marker but also an assistant in the classification of the disease and choice of therapeutic intervention[18,19].

There are several laboratory methods that are investigated for metastatic diagnosis, besides gold standard radiometric examinations (CT, MRI), such as immunoassays, flow cytometry and molecular techniques (Real-time PCR, multiplex PCR, qRT-PCR, EPISPOT, etc)[20]. Besides the mere detection of CTC/DCs, can provide important information on the profile of secreted proteins potentially relevant for metastasis formation.[21,22,23,24]

Significant technical challenges exist in identification of these rare cells in the circulation and in distinguishing them from more prevalent hematologic and normal epithelial cells. Sensitive enrichment methods for the tumor cell population can significantly increase the yield. Capture and enrichment are performed using specific morphologic and phenotypic characteristics of tumor cells such as size, density, and specific protein expression. These methods should be specific to cancer patients, with zero or only rare cells found in healthy individuals.[8,9]. Because tumor cells are thought to be of lower density than the other cells found in the circulation, some in-house (Ficoll-Hypaque), or commercially available assays (OncoQuick Greiner Bio-One, Monroe, NC) use a density gradient for cell enrichment[16]. These methods still lack the required sensitivity and result in low purity samples secondary to loss of tumor cells during the isolation process[25]. The most commonly used methods for enrichment are based on immunomagnetic techniques—MACS Systems (Miltenyi Biotech, Germany), RARE™ (StemCell Technologies, Canada), AdnaTest (AdnaGen AG, Germany), macro-iron beads, magnetic beads, (CellSearch, Veridex, Raritan, NJ)[11,12,19]. These are able to separate tumor cells from other cells in the circulation based on specific surface markers. Some of these methods use negative selection by removing mononuclear cells with an anti-CD45 antibody, a panleukocyte antibody. Others use positive selection by the use of monoclonal antibodies targeting epithelial markers located on tumor cells[23]. These systems have been found to be more sensitive than the density gradient assays[20]. Yet because of lack of highly specific tumor antigens, some CTCs may be lost during the process. In addition, false-negative results are possible secondary to loss of tumor-specific antigens by the CTCs[27]. The separation of CTCs according to Richard Cote[20,29] is the newest addition and is based on a novel membrane microfilter device to isolate CTCs from blood with recovery rates of spiked tumor cells greater than 90% by exploiting size differences between tumor and normal blood cells. His group developed a quadruplex multimarker immunofluorescent assay that can be performed and evaluated directly on the microfilter device simultaneously, assessing CD44, CD24, ALD1 and cytokeratin using quantum dots as labels. The ability to efficiently capture CTCs on a small filter area and then analyze these cells on the same platform can substantially facilitate further detailed analysis of early and late events in metastasis. The size by size of epithelial tumor cells (ISET) assay is an enrichment method using CTC size to separate cells that are 2-8 μm[30]. To date, studies have not validated the assumption that all CTCs are 2-8 μm[19]. Finally, another novel technology for simultaneous isolation, detection and characterization of CTCs is based on a combination of hyper-spectral microscopic imaging and a microchip instrument that precisely quantifies 17 different tumor markers in individual breast cancer cells from touch preps of the primary tumor or CTCs in patients with tumor progression (Jonathan Uhr, personal communication)[19].

Whichever the separation/enrichment method, the detection of circulating tumor cells in peripheral blood of patients with cancer is most frequently done worldwide with RT-PCR gene expression indicators that contribute significantly to the RNA which is extracted from the CTCs enriched sample[19]. The expression of genes like CEA, EGFR, GA 733-2 in colon cancer is consistent with disease progression, staging and also response to treatment as shown previously[31]. This is the detection method that was used in our pilot study.

**MATERIALS AND METHODS**

**Patient characteristics and specimen collection**

In our study, 50 patients were included after signing informed consent, 20 preoperatively (from the 4th Surgical Department), whose characteristics were described elsewhere[20] and 30 before and/or after chemotherapy (from the Oncology Sector) (see Table 1a, 1b, Figures 1-4). Patients were followed up for a median of 36 months (3 years) up to now. As a control group, we used 40 healthy blood bank donors, 3:1 male to female, between 40-60 years old. Before surgery, venous blood was obtained from each of surgical patients. Also, for oncology patients blood samples were drawn before the application of therapeutic substances, and not earlier than 5 days after the last chemotherapeutic intervention. In order to prevent epithelial cells from the vein puncture to contaminate the sample, the first 5 ml were discarded. The samples were collected in special blood collection tubes (AdnaCollect tubes) that contain EDTA and an agent preventing illegitimate mRNA expression (150 μl liquid). AdnaCollect tubes have a non-return valve to prevent a possible backflow of blood. The samples were analyzed within 24 hours for the presence of circulating tumor cells using the AdnaTest AdnaTest ColonCancerSelect & Detect. The blood collection tubes have to be stored at 2-4°C in a dark place until use, but a limitation is that results cannot be guaranteed after 24 hours. The study was approved by the local Ethics Committee and conformed to the ethical standards of the Helsinki Declaration.

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<th>Table 1 Patients’ demographic characteristics.</th>
<th>Man Sex</th>
<th>Mean age (years) ± SD</th>
<th>Oncology department</th>
<th>4th Surgery clinic</th>
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<tbody>
<tr>
<td>Woman</td>
<td>30</td>
<td>68 ± 10.76</td>
<td>20</td>
<td>30</td>
<td>50</td>
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<tr>
<td>Men</td>
<td>20</td>
<td>68 ± 10.76</td>
<td>20</td>
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<td>Mean age(years) ± Std Deviation in sex groups</td>
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**Circulating tumor cells (CTCs) selection and molecular detection**

Antibodies against epithelial and tumor associated antigens are conjugated to magnetic beads (Dynabeads) for the enrichment of tumor cells from peripheral blood.

The immunomagnetic enrichment of cancer cells in peripheral
blood of patients with colorectal cancer performed by Adnatest is based on the following antibodies: MOC-31 (monoclonal antibody that recognizes the transmembrane surface glycoprotein EGP-2), BerEP4 (monoclonal antibody that recognizes cell surface glycoprotein attachment of EpCAM) and monoclonal antibody clone 8B6.

The labeled cells are extracted by a magnetic particle concentrator and are subsequently lysed. From the cell lysate, mRNA extraction takes place with the use of the oligo(dT)25-coated beads.

mRNA was extracted from the isolated tumor cell lysates and used in a subsequent reaction with reverse transcriptase (Sensiscript, QIAGEN, Germany). The produced cDNA was subjected to a multiplex PCR with HotStar Taq polymerase (QIAGEN) for the beta-actin (as an internal control), and for 3 tumor genes: CEA, EGFR (ErbB-1) and GA 733-2 (EpCAM) (primers from AdnaTest Colon Cancer Detect, kit AdnaGen, Germany) selected as previously described[31]. The primers generate fragments in positive samples for the GA733-2: 383bp, CEA: 226bp, EGFR: 161bp, beta-actin: 114bp (internal PCR control) sizes.

PCR products were then run in a 3% agarose electrophoresis gel 3:1 (Nusieve, Lonza), ethidium bromide-stained and photographed under a UV transiluminator (Vilbert-Lourmat, France), whereas according to the manufacturer’s instruction the data could also be generated on a Agilent Bioanalyser 2100 by a DNA 1000 Labchip.

For the interpretation of the results, the following criteria must be evaluated: a) the fragment of beta-actin must be present in all samples providing that cell separation was successful, b) multiplex PCR must be successful (as indicated by the presence of all 4 bands in a provided control), c) the C- and the RT control mustn’t show any band of more than 80bp, and finally d) a band>1kb indicates a contamination with genomic DNA, meaning a not successful extraction procedure.

### RESULTS

The method was performed according to the manufacturer’s instructions and the internal control band of beta-actin was present in all samples at the correct size.

In the peripheral blood of four patients the presence of transcripts of tumor genes was observed (aberrant expression of the CEA and GA 733-2 in two and three cases respectively), (characteristic result from a gel photo Figure 5).

In more detail, the results are as follows: One case from the surgical clinic, Dukes C stage III was positive for GA733-2 gene (395 bp), but with no confirmed histopathological metastasis. Second case was from the surgical clinic, Dukes D stage IV positive for GA733-2 gene (395 bp), with liver metastasis. No frozen tissue specimens were available from these surgical patients to corroborate the aforementioned results in the corresponding tissue as well. Third case was from the oncology department positive for CEA gene (226 bp). The sample belonged to patient with liver metastasis Dukes D stage IV, drawn before chemotherapy. The fourth case was from the oncology department; Dukes D stage IV and was positive for both CEA and GA 733-2 genes. A second sampling from this patient after treatment rendered the molecular result negative.

The patients with negative molecular investigations have not confirmed metastasis so far, after a median of 36 months (3 years) follow up. The results from the control group were negative for all tumor genes investigated.

### DISCUSSION

The advantage of detecting cancer cells in the bloodstream (CTCs) is the detection of relapse several months before clinical signs of the metastatic process become evident[31] e.g. before a significant increase in serum tumor markers above the reference values. Besides providing independent prognostic information, it could also be an independent biomarker to monitor the patient during treatment, but also a tool for selecting appropriate personalized treatment[33] by a non-invasive way.

The concentrations of serum tumor markers correlate with tumor size[31,34] but still they do not reflect the viability of dispersed tumor cells[31].

So far, the most established method for CTC detection is the image cytometric FDA-approved Cell-search method by Veridex: it is a semi-
automated system combining positive selection with the antiepithelial antibody (EpCAM) in the enrichment stage and enumeration of CD45(−), cytokeratin 8/18/19 positive cells after labeling in a microscope slide. The advantages of Cell-search are that multiple large validation studies have been performed and it is adequately standardized while additional studies can be executed on the same cells. Among the disadvantages are the false positives results from identification of normal cells aberrantly expressing the detection antigens (artifacts). Also, false negatives may arise from CTCs being EpCAM negative (−). Finally, this semi-automated system is expensive and that it requires specialized training.

Our goal was to evaluate the use of another-simpler to perform-commercial kit for the molecular detection of circulating tumor cells of patients with colon cancer. The AdnaTest's combines immunomagnetic enrichment selection method with 3 monoclonal antibodies targeting different antigens with RT-PCR analysis of expression of 3 tumor genes. An advantage of the method is that also maintains cell structure for additional evaluation studies after separating CTCs from non-needed cells. Disadvantages are the false positives results from the isolated normal cells with illegitimate expression of the detection genes, but also false negative results from CTCs depleted those 3 specific selection antigens. Finally, it is not automated.

This study attempted to detect circulating tumor cells in patients with colon cancer, sampled either a) preoperatively and before any treatment or b) after surgery and before any other treatment. Our aim was to predict any possible metastases at different time points (e.g. TNM staging). All 50 patients were studied for mRNA expression of GA733-2, CEA and EGFR tumor genes. In a separate study, we analyzed the usefulness of measuring the protein concentrations of the same genes in the patients' sera since only one of them is an established tumor marker (CEA). Tumor cells were detected in only 4 patients (8%).

Regarding CEA mRNA detection, it was detected in two patients stage IV (Dukes D). One of them had a second sample after chemotherapy that turned out negative. The gene for carinoembryonic antigen (CEA/CEACAM5) is up-regulated in patients with metastatic colorectal cancer[21,34,36] and is associated with the stage and the progression[31]. After treatment, the expression of CEA in colon cancer metastasis has been shown to be as high as 66%[31]. CEA probably has prognostic value in patients with colorectal cancer and seems to help in early detection of recurrence[31].

The GA733-2 (EpCAM) gene was detected in three patients (6%)[31]. One case, Dukes C stage III with no confirmed histopathological metastasis and the other case was Dukes D stage IV with liver metastasis. It is known that the mRNA expression of GA 733-2 is associated with tumor size, stage and lymph node infiltration[31,37]. Overexpression serves either as a marker in the diagnosis and treatment of cancer, or as an indicator for poor outcome of the patient. Moreover it has also negative correlation with the progression of staging from Dukes M0 to Dukes M1[31]. EpCAM is a major goal in immunotherapy with monoclonal antibodies in colorectal cancer because of its excellent specificity for tumor cells (e.g. surgery after administration of EpCAM-specific murin monoclonal antibody 17-1A in patients with colon cancer stage Dukes C)[30,37]. The use of two EpCAM antibodies (mAb BerEP4 and mAb KS1/4) in another study for immunomagnetic enrichment in blood proves that isolation and detection of CTC is critically dependent on the used EpCAM clone and suggested that further analysis regarding the clinical importance of heterogeneous expression of the EpCAM molecule in CTC of CRC patients is urgently needed[31]. Further studies with a larger number of patients should clarify if the enrichment protocol influences the prognostic value of the tumor cell detection protocol.

In contrast, expression of EGFR was not detected in any sample. Preoperatively this could be justified by the administration to the patients of Erbitux, which targets the receptor for epidermal growth factor[38]. It is mentioned in the literature that in patients diagnosed with metastasis, EGFR expression could be increased in up to 15% of cases[8,23,31,39]. The EGFR is expressed in metastatic and early-stage disease (TNM stage I)[31,41,42,44,44]. It is suggested in the literature that further investigations are needed for EGFR mRNA usefulness as an indicator of circulating tumor cells in peripheral blood of patients with solid tumors[46], because of the recent discovery of two new splicing isoforms (deletion variations: cEX12_15del, cEX12_14del) affecting the extracellular region of the molecule (cysteine rich extracellular domain-ligand binding site). These new findings could explain the absence of mRNA expression of EGFR in the used kit.

The small number of positive samples detected in our study (a severe limitation) doesn’t permit the statistical correlation between CTCs in peripheral blood and clinical characteristics of colon cancer patients. 38% of metastatic patients (3 out of 8) were tested positive. From stage III only 1 patient out of 16 tested positive (6%). We can hypothesize that the small number of positive samples detected in our study could be probably due to the “liver filter” which prevents the passage of tumor cells in circulation[47-49]. In fact, the latest studies recommend the collection be made either by the mesenteric vein (drainage blood samples)[47,48], or the central venous blood, or even portal blood, considering that peripheral blood has low sensitivity in the detection of CTCs in colon cancer patients.

However, regarding the majority of the samples that were tested negative in our study, it is of interest that no one has metastasized so far after 3 years (neither though our first case that was tested positive). The detection of circulating tumor cells in colon cancer patients especially in Dukes C stage III, would enhance the usefulness of the kit as an early indicator of distant metastases who are not yet overt.

It seems essential for the technical validity of the kit to include stabilized cellular controls with low levels of cells expressing the selection antigens in order to assess the efficiency of selection process and consequent mRNA extraction[46]. This will help in the achievement of a standardized level of quality and will permit assessment of the reproducibility and the analytical sensitivity of the kit. Others claim that linearized plasmids would improve the method’s accuracy[49]. In the tested kit, cDNA synthesis is controlled adequately by beta-actin amplification and multiplex PCR is controlled by the positive control that is provided along with the kit. Availability of suitable external quality assessment (EQA) schemes for the CTC parameter is also of utmost importance for the laboratory performing this kind of testing.

Due to the fact that transcripts of the so-called “tumor-specific” biomarkers such as: EpCAM, EGFR, CEA, can also be identified in normal bone marrow, blood or lymph node tissue, clear cut off values need to be defined and validated in multicenter trials[22,25]. Weak positive samples might not be of clinical significance. Therefore, larger and long-term clinical studies are needed to confirm the prognostic value of this kit regarding the detection of tumor cells in peripheral blood of patients with colorectal cancer at different stages of the disease, before and after surgery, before and after chemotherapy[21,22,45,46,50,53,52].

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