

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

Antonia Mourtzikou, Christos Kroupis, Nikoleta Poumpouridou, Spyridon Christodoulou, Maria Skondra, Anastasia N Kastania, Georgios Athanasas, Demetrios Pectasides, Kleanthi Dimas

Antonia Mourtzikou, Christos Kroupis, Nikoleta Poumpouridou, Kleanthi Dimas, Department of Clinical Biochemistry, Attikon University Hospital, Athens 12462, Greece.

Spyridon Christodoulou, Georgios Athanasas, Fourth University Surgical Clinic, Attikon University Hospital, Athens 12462, Greece.

Maria Skondra, Demetrios Pectasides, Oncology Sector, Second Department of Pathology, Attikon University Hospital, Athens 12462, Greece.

Anastasia N Kastania, Bioinformatics and Medical Informatics Team, Biomedical Research Foundation of the Academy of Athens, Greece.

Correspondence to: Antonia Mourtzikou, MSc, PhD candidate, EurClinChem, Department of Clin. Biochemistry, Attikon University Hospital, University of Athens Medical School, Athens 12462, Greece, [antoniamour@yahoo.com](mailto:antoniamour@yahoo.com)

Telephone: +30-210-5831915 Fax: +30-210-5831942

Received: January 10, 2012 Revised: March 18, 2012

Accepted: May 23, 2012

Published online: June 21, 2012

### ABSTRACT

**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.

© 2012 Thomson research. All rights reserved.

**Key words:** Circulating Tumor Cells (CTCs); Colorectal cancer (CRC); MRNA; Multiplex RT-PCR

Mourtzikou A, Kroupis C, Poumpouridou N, Christodoulou S, Skondra M, Kastania A.N, Athanasas G, Pectasides D, Dimas K. Molecular Detection of Circulating Tumor Cells in peripheral blood of colon cancer patients. *Journal of Gastroenterology and Hepatology Research* 2012; 1(5): 74-79 Available from: URL: <http://www.ghrnet.org/index./joghr/>

### INTRODUCTION

Epidemiologically, worldwide colon cancer is the third most frequent cancer, with equal rate for males and females and the second cause of all-cancer deaths<sup>[1,2]</sup>. Every year, half million new incidents are diagnosed with colon cancer. In Europe a higher percentage of deaths caused by colon cancer is observed among males and females from East and Northwest Europe, compared to those from South Europe<sup>[1,3]</sup>.

Metastasis is the main cause of death in patients with solid epithelial malignancies that are resistant to the conventional therapeutic schemes<sup>[4]</sup>. The blood dispersion of tumor cells from the primary tumor is the decisive step towards the development of metastases<sup>[5,6]</sup> and plays a significant effect on patients' prognosis<sup>[7,8]</sup>.

Early detection of the metastatic process is very important in clinical practice because it allows the prediction, the evaluation of the therapeutic scheme, the monitoring of the effectiveness of treatment and the possibility of individualization of drug therapy for each patient and each different situation<sup>[8,9,10]</sup>. Circulating Tumor Cells (CTCs) are defined as epithelial cells detected in blood or bone marrow of patients with cancer and have specific phenotypic characteristics reminiscent of the primary tumor, such as: expression of Growth Factor Receptors e.g. (EGFR), Mucin receptor (MUC-1), cytokeratins (CK19, CK20) etc<sup>[11,12,13,14]</sup>. Their numbers in circulation are low and their detection requires adequate enrichment<sup>[15]</sup>. The limited life span of cancer cells in the systemic circulation—often less than 24 hours—ensures that the detection in blood of patients diagnosed with cancer is evidence of active metastatic process<sup>[16,17]</sup>. Furthermore, they can be used as an additional predictor for disease progression in newly diagnosed patients with malignant cancers and therefore are characterized as “real-time liquid biopsy”<sup>[18]</sup>. The molecular profile of CTCs could also identify therapeutic targets such as the HER2 or EGFR for individualized therapy through the action of biological therapies with monoclonal antibodies such as Herceptin or Erbitux<sup>[8]</sup>. Therefore, detection of CTCs

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<sup>[19,20,21]</sup>.

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)<sup>[22]</sup>. Besides the mere detection of CTC/DTCs, can provide important information on the profile of secreted proteins potentially relevant for metastasis formation<sup>[21,23,24,25,26]</sup>.

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<sup>[8,9]</sup>. 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<sup>[4]</sup>. These methods still lack the required sensitivity and result in low purity samples secondary to loss of tumor cells during the isolation process<sup>[20]</sup>. 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)<sup>[4,11,12,19]</sup>. 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<sup>[13]</sup>. These systems have been found to be more sensitive than the density gradient assays<sup>[20]</sup>. 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<sup>[27]</sup>. The separation of CTCs according to Richard Cote<sup>[28,29]</sup> 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 isolation by size of epithelial tumor cells (ISET) assay is an enrichment method using CTC size to separate cells that are  $>8 \mu\text{m}$ <sup>[4,10]</sup>. To date, studies have not validated the assumption that all CTCs are  $>8 \mu\text{m}$ <sup>[19]</sup>. 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)<sup>[18]</sup>.

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<sup>[30]</sup>. 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<sup>[31]</sup>. This is the detection method that was used in our pilot study.

## MATERIALS AND METHODS

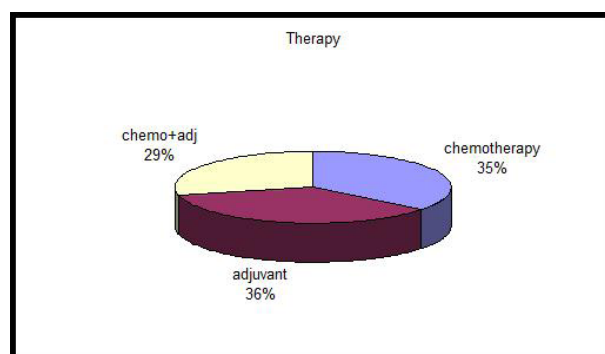
### Patient characteristics and specimen collection

In our study 50 patients were included after signing informed consent, 20 preoperatively (from the 4th Surgical Department), whose characteristics were described elsewhere<sup>[32]</sup> 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  $\mu\text{l}$  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.

**Table 1** Patients' demographic characteristics.

| Man<br>♂   | Woman<br>♀ | Age-years<br>(mean)                        | Oncology<br>department | 4 <sup>th</sup> Surgery<br>clinic | N patients |
|------------|------------|--|------------------------|-----------------------------------|------------|
| 30         | 20         | 69   | 20                     | 30                                | 50         |
| <b>Sex</b> |            | <b>Mean age (years) <math>\pm</math>SD</b> |                        | <b>Number of cases</b>            |            |
| Men        |            | 68.70 $\pm$ 10.76                          |                        | 30                                |            |
| Women      |            | 68.15 $\pm$ 9.91                           |                        | 20                                |            |

Mean age(years)  $\pm$  Std Deviation in sex groups



**Figure 1** Percent of cases per therapy group.

### CTC 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.

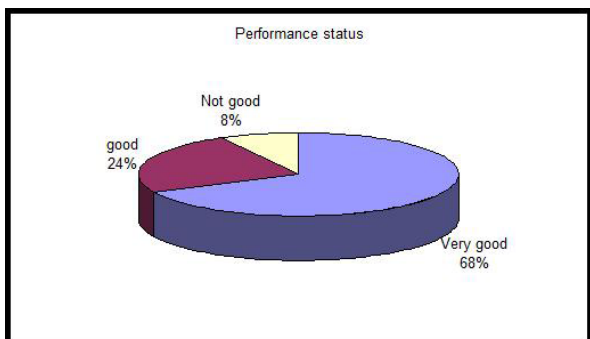


Figure 2 Percent of cases per Performance status group.

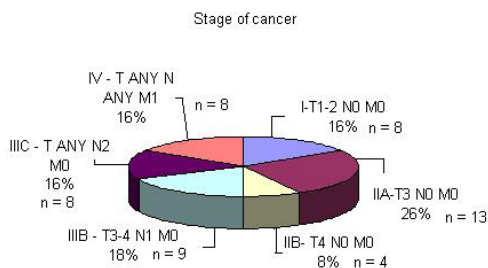


Figure 3 Percent of cases per stage of cancer.

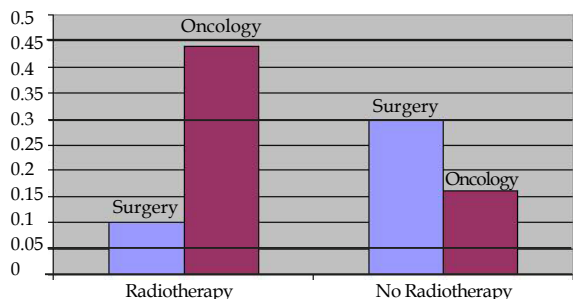


Figure 4 Percent of cases per type of clinic for patients treated with Radiotherapy (or not).

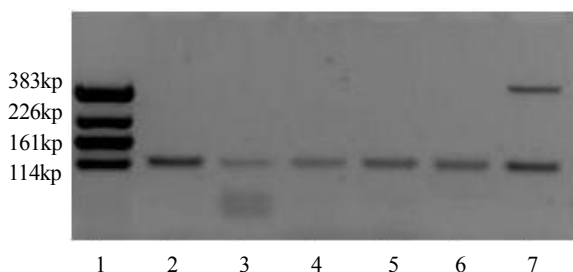


Figure 5 Agarose gel photo with multiplex PCR results: lane 1, positive control C, lanes 2-6, negative samples, lane 7, positive sample for GA733-2 expression

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<sup>[31]</sup>. 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 transilluminator (Vilbert-Lourmat, France), whereas according to the manufacture’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<sup>[31]</sup> 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<sup>[33]</sup> by a non-invasive way. The concentrations of serum tumor markers correlate with tumor size<sup>[31,34]</sup> but still they do not reflect the viability of dispersed tumor cells<sup>[31]</sup>.

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<sup>[35]</sup> 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 carcinoembryonic antigen (CEA/CEACAM5) is up-regulated in patients with metastatic colorectal cancer<sup>[31,34,36]</sup> and is associated with the stage and the progression<sup>[21]</sup>. After treatment, the expression of CEA in colon cancer metastasis has been shown to be as high as 66%<sup>[31]</sup>. CEA probably has prognostic value in patients with colorectal cancer and seems to help in early detection of recurrence<sup>[31]</sup>.

The GA733-2 (EpCAM) gene was detected in three patients (6%)<sup>[31]</sup>. 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<sup>[31,37]</sup>. 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<sup>[23]</sup>. 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)<sup>[16,17]</sup>. The use of two EpCAM antibodies (mAb BerEP4 and mAb KSI/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<sup>[38]</sup>. 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<sup>[8]</sup>. It is mentioned in the literature that in patients diagnosed with metastasis, EGFR expression could be increased in up to 15% of cases<sup>[6,8,23,31,39]</sup>. The EGFR is expressed in metastatic and early-stage disease (TNM stage I)<sup>[40,41,42,43,44,45]</sup>. 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<sup>[46]</sup>, 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<sup>[47,48]</sup>. In fact, the latest studies recommend the collection be made either by the mesenteric vein (drainage blood samples)<sup>[47,48]</sup>, 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<sup>[40]</sup>. 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<sup>[49]</sup>. 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<sup>[22]</sup>. 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<sup>[21,22,45,48,50,51,52]</sup>.

## REFERENCES



- 1 Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. *CA Cancer J Clin* 2009; **59**: 225-249
- 2 Allen JE, El-Deiry WS. Circulating Tumor Cells and Colorectal Cancer. *Curr Colorectal Cancer Rep* 2010; **6**: 212-220
- 3 Huerta S. Recent advances in the molecular diagnosis and prognosis of colorectal cancer. *Expert Rev MolDiagn* 2008; **8**: 277-288
- 4 Pantel K, Brakenhoff RH. Dissecting the metastatic cascade. *Nat Rev Cancer* 2004; **4**: 448-456
- 5 Jiang WG, Martin TA, Mansel RE. Molecular detection of micrometastasis in breast cancer. *Crit Rev OncolHematol* 2002; **43**(1): 13-31
- 6 Paterlini-Bréchet P. Organ-specific markers in circulating tumor cell screening: an early indicator of metastasis-capable malignancy. *Future Oncol* 2011; **7**: 849-871
- 7 Tímár J, Csuka O, Orosz Z, Jeney A, Kopper L. Molecular pathology of tumor metastasis. II. Molecular staging and differential diagnosis. *PatholOncol Res* 2002; **8**: 204-219
- 8 Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 2001; **344**: 783-792
- 9 Hauch S, Zimmermann S, Lankiewicz S, Zieglschmid V, Böcher O, Albert WH. The clinical significance of circulating tumour cells in breast cancer and colorectal cancer patients. *Anticancer Res* 2007; **27**: 1337-1341
- 10 Hardingham JE, Hewett PJ, Sage RE, Finch JL, Nuttall JD, Kotasek D, Dobrovic A. Molecular detection of blood-borne epithelial cells in colorectal cancer patients and in patients with benign bowel disease. *Int J Cancer*. 2000 Jan 20; **89**(1): 8-13
- 11 Elshimali YI, Grody WW. The clinical significance of circulating tumor cells in the peripheral blood. *DiagnMolPathol* 2006; **15**: 187-194
- 12 Stathopoulou A, Gizi A, Perraki M, Apostolaki S, Malamos N, Mavroudis D, Georgoulas V, Lianidou ES. Real-time quantification of CK-19 mRNA-positive cells in peripheral blood of breast cancer patients using the lightcycler system. *Clin Cancer Res* 2003; **9**: 5145-5151
- 13 Vlems FA, Diepstra JH, Cornelissen IM, Ruers TJ, Ligtenberg MJ, Punt CJ, van Krieken JH, Wobbes T, van Muijen GN. Limitations of cytokeratin 20 RT-PCR to detect disseminated tumour cells in blood and bone marrow of patients with colorectal cancer: expression in controls and down-regulation in tumour tissue. *MolPathol* 2002; **55**: 156-163
- 14 Wong IH, Yeo W, Chan AT, Johnson PJ. Quantitative relationship of the circulating tumor burden assessed by reverse transcription-polymerase chain reaction for cytokeratin 19 mRNA in peripheral blood of colorectal cancer patients with Duke's stage, serum carcinoembryonic antigen level and tumor progression. *Cancer Lett*. 2001; **162**(1): 65-73.
- 15 Deng G, Herrler M, Burgess D, Manna E, Krag D, Burke JF. Enrichment with anti-cytokeratin alone or combined with anti-EpCAM antibodies significantly increases the sensitivity for circulating tumor cell detection in metastatic breast cancer patients. *Breast Cancer Res*. 2008; **10**(4): R69
- 16 Patel H, Le Marer N, Wharton RQ, Khan ZA, Araia R, Glover C, Henry MM, Allen-Mersh TG. Clearance of circulating tumor cells after excision of primary colorectal cancer. *Ann Surg* 2002; **235**: 226-231
- 17 Allen-Mersh TG, McCullough TK, Patel H, Wharton RQ, Glover C, Jonas SK. Role of circulating tumour cells in predicting recurrence after excision of primary colorectal carcinoma. *Br J Surg* 2007; **94**: 96-105
- 18 Lianidou ES, Mavroudis D, Sotiropoulou G, Agelaki S, Pantel K. What's new on circulating tumor cells? A meeting report. *Breast Cancer Res* 2010; **12**: 307
- 19 Hayes DF, Smerage JB. Circulating tumor cells. *ProgMolBiol-TranslSci* 2010; **95**: 95-112
- 20 Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer* 2003; **3**: 453-458
- 21 Zhang L, Wu G, Pantel K. Detection of circulating tumor cells by RT-PCR significantly associated with poor prognosis in breast cancer. *Breast Cancer Res Treat* 2011; **130**: 359-364
- 22 Alix-Panabières C, Riethdorf S, Pantel K. Circulating tumor cells and bone marrow micrometastasis. *Clin Cancer Res* 2008; **14**: 5013-5021
- 23 Pantel K, Alix-Panabières C. The clinical significance of circulating tumor cells. *Nat ClinPractOncol* 2007; **4**: 62-63
- 24 Alix-Panabières C, Brouillet JP, Fabbro M, Yssel H, Rousset T, Maudelonde T, Choquet-Kastylevsky G, Vendrell JP. Characterization and enumeration of cells secreting tumor markers in the peripheral blood of breast cancer patients. *J Immunol Methods* 2005; **299**: 177-188
- 25 Czerkinsky CC, Nilsson LA, Nygren H, Ouchterlony O, Tarkowski A. A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. *J Immunol Methods*. 1983 Dec 16; **65**(1-2):109-121
- 26 Alix-Panabières C, Vendrell JP, Pellé O, Rebillard X, Riethdorf S, Müller V, Fabbro M, Pantel K. Detection and characterization of putative metastatic precursor cells in cancer patients. *ClinChem* 2007; **53**: 537-539
- 27 Dotan E, Cohen SJ, Alpaugh KR, Meropol NJ. Circulating tumor cells: evolving evidence and future challenges. *Oncologist* 2009; **14**: 1070-1082
- 28 Zheng S, Lin HK, Lu B, Williams A, Datar R, Cote RJ, Tai YC. 3D microfilter device for viable circulating tumor cell (CTC) enrichment from blood. *Biomed Microdevices* 2011; **13**: 203-213
- 29 Lin HK, Zheng S, Williams AJ, Balic M, Groshen S, Scher HI, Fleisher M, Stadler W, Datar RH, Tai YC, Cote RJ. Portable filter-based microdevice for detection and characterization of circulating tumor cells. *Clin Cancer Res* 2010; **16**: 5011-5018
- 30 Smirnov DA, Zweitzig DR, Foulk BW, Miller MC, Doyle GV, Pienta KJ, Meropol NJ, Weiner LM, Cohen SJ, Moreno JG, Connelly MC, Terstappen LW, O'Hara SM. Global gene expression profiling of circulating tumor cells. *Cancer Res* 2005; **65**: 4993-4997
- 31 Zieglschmid V, Hollmann C, Mannel J, Albert W, Jaeschke-Melli S, Eckstein B, Hillemann T, Greten TF, Gross E, Böcher O. Tumor-associated gene expression in disseminated tumor cells correlates with disease progression and tumor stage in colorectal cancer. *Anticancer Res* 2007; **27**: 1823-1832
- 32 Pavlidou A, Dalamaga M, Kroupis C, Konstantoudakis G, Belimezi M, Athanasas G, Dimas K. Survivin isoforms and clinicopathological characteristics in colorectal adenocarcinomas using real-time qPCR. *World J Gastroenterol* 2011; **17**: 1614-1621
- 33 Pantel K, Brakenhoff RH, Brandt B. Detection, clinical relevance and specific biological properties of disseminating

- tumour cells. *Nat Rev Cancer* 2008; **8**: 329-340
- 34 Hampton R, Walker M, Marshall J, Juhl H. Differential expression of carcinoembryonic antigen (CEA) splice variants in whole blood of colon cancer patients and healthy volunteers: implication for the detection of circulating colon cancer cells. *Oncogene* 2002; **21**: 7817-7823
- 35 Mourtzikou A, Stamouli M, Kroupis C, Christodoulou S, Skondra M, Kastania A, Pectasides D, Athanasas G, Dimas C. Evaluation of CEA, EGFR, EpCAM (GA733-2) and CA19-9 levels in colorectal cancer patients and correlation with clinicopathological characteristics. *Clinical Laboratory* 2012; **58** (5-6): 441-448
- 36 Park S, Lee B, Kim I, Choi I, Hong K, Ryu Y, Rhim J, Shin J, Park SC, Chung H, Chung J. Immunobead RT-PCR versus regular RT-PCR amplification of CEA mRNA in peripheral blood. *J Cancer Res Clin Oncol* 2001; **127**: 489-494
- 37 Baeuerle PA, Gires O. EpCAM (CD326) finding its role in cancer. *Br J Cancer* 2007; **96**: 417-423
- 38 Antolovic D, Galindo L, Carstens A, Rahbari N, Büchler MW, Weitz J, Koch M. Heterogeneous detection of circulating tumor cells in patients with colorectal cancer by immunomagnetic enrichment using different EpCAM-specific antibodies. *BMC Biotechnol* 2010; **10**: 35
- 39 Braun S, Kentenich C, Janni W, Hepp F, de Waal J, Willgeroth F, Sommer H, Pantel K. Lack of effect of adjuvant chemotherapy on the elimination of single dormant tumor cells in bone marrow of high-risk breast cancer patients. *J Clin Oncol* 2000; **18**: 80-86
- 40 Lankiewicz S, Rother E, Zimmermann S, Hollmann C, Korangy F, Greten TF. Tumour-associated transcripts and EGFR deletion variants in colorectal cancer in primary tumour, metastases and circulating tumour cells. *Cell Oncol* 2008; **30**: 463-471
- 41 Mitsuhashi A, Tanaka N, Suzuka K, Matsui H, Seki K, Sekiya S. Detection of epidermal growth factor receptor mRNA in peripheral blood of cervical cancer patients. *Gynecol Oncol*. 2003 Jun; **89**(3): 480-5.
- 42 Leitzel K, Lieu B, Curley E, Smith J, Chinchilli V, Rychlik W, Lipton A. Detection of cancer cells in peripheral blood of breast cancer patients using reverse transcription-polymerase chain reaction for epidermal growth factor receptor. *Clin Cancer Res* 1998; **4**: 3037-3043
- 43 De Luca A, Pignata S, Casamassimi A, D'Antonio A, Gridelli C, Rossi A, Cremona F, Parisi V, De Matteis A, Normanno N. Detection of circulating tumor cells in carcinoma patients by a novel epidermal growth factor receptor reverse transcription-PCR assay. *Clin Cancer Res* 2000; **6**: 1439-1444
- 44 Gazzaniga P, Gandini O, Giuliani L, Magnanti M, Gradilone A, Silvestri I, Gianni W, Gallucci M, Frati L, Aglianò AM. Detection of epidermal growth factor receptor mRNA in peripheral blood: a new marker of circulating neoplastic cells in bladder cancer patients. *Clin Cancer Res* 2001; **7**: 577-583
- 45 Tsouma A, Aggeli C, Lembessis P, Zografos GN, Korkolis DP, Pectasides D, Skondra M, Pissimissis N, Tzonou A, Koutsilieris M. Multiplex RT-PCR-based detections of CEA, CK20 and EGFR in colorectal cancer patients. *World J Gastroenterol* 2010; **16**: 5965-5974
- 46 Clarke LE, Leitzel K, Smith J, Ali SM, Lipton A. Epidermal growth factor receptor mRNA in peripheral blood of patients with pancreatic, lung, and colon carcinomas detected by RT-PCR. *Int J Oncol* 2003; **22**: 425-430
- 47 Iinuma H, Okinaga K, Egami H, Mimori K, Hayashi N, Nishida K, Adachi M, Mori M, Sasako M. Usefulness and clinical significance of quantitative real-time RT-PCR to detect isolated tumor cells in the peripheral blood and tumor drainage blood of patients with colorectal cancer. *Int J Oncol* 2006; **28**: 297-306
- 48 Wind J, Tuynman JB, Tibbe AG, Swennenhuis JF, Richel DJ, van Berge Henegouwen MI, Bemelman WA. Circulating tumour cells during laparoscopic and open surgery for primary colonic cancer in portal and peripheral blood. *Eur J Surg Oncol* 2009; **35**: 942-950
- 49 Prost à la Denise J, Dekairelle AF, Desbene C, Moutereau S, Douard R, Devanlay M, Eschwege P, Gala JL, Loric S. A bias in quantitative RT-PCR limit of detection is induced by the use of cancer cell lines in the molecular detection of circulating tumor cells. *Clin Chem Lab Med* 2011; **49**: 1073-1075
- 50 Personeni N, Fieuws S, Piessevaux H, De Hertogh G, De Schutter J, Biesmans B, De Roock W, Capoen A, Debiec-Rychter M, Van Laethem JL, Peeters M, Humblet Y, Van Cutsem E, Tejpar S. Clinical usefulness of EGFR gene copy number as a predictive marker in colorectal cancer patients treated with cetuximab: a fluorescent in situ hybridization study. *Clin Cancer Res* 2008; **14**: 5869-5876
- 51 Moroni M, Sartore-Bianchi A, Veronese S, Siena S. EGFR FISH in colorectal cancer: what is the current reality? *Lancet Oncol*. 2008 May; **9**(5): 402-403
- 52 Lianidou ES, Markou A. Circulating tumor cells as emerging tumor biomarkers in breast cancer. *Clin Chem Lab Med* 2011; **49**: 1579-1590

**Prereviewer:** Mitsunori Yamakawa, Professor, Department of Pathological Diagnostics, Yamagata University Faculty of Medicine, 2-2-2 Iida-Nishi, Yamagata 990-9585, Japan; Hiroto Kita, Professor and Chair, Department of Gastroenterology, Director of Endoscopy, Saitama Medical University International Medical Center, 1397-1, Yamane, Hidaka, Saitama, Japan; Maha Maher Shehata, Professor, Internal Medicine Department, Gastroenterology and Hepatology Unit, Mansoura University, Specialized Medical hospital, 35516, Mansoura, Egypt.