Trakia Journal of Sciences, No 3, pp 309-324, 2014

Available online at:
http://www.uni-sz.bg
ISSN 1313-7050 (print) **doi:10.15547/tjs.2014.03.014**
ISSN 1313-3551 (online)

Review

MINIMAL RESIDUAL DISEASE IN EARLY COLORECTAL CARCINOMA

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ABSTRACT

Introduction: The detection and importance of cancer cells circulating in blood is a subject to discussion for more than a century. Their prognostic value for patients with colorectal cancer is still disputable.

Aim: The present study provides information about the specificity and sensitivity of molecular biology, oncogenetics and histopathological methods for early diagnostics of minimal residual disease in the blood of colorectal cancer patients.

Scope: PUBMED query results from 1975 to 2011 were analyzed. The information provides evidence that the presence of such cells is essential for monitoring the progression and therapeutic response of this oncological disease.

Conclusions: During the last decade, new technologies have allowed to use methods with substantial sensitivity and specificity for detection of minimal residual disease in oncological patients, including those with colorectal carcinoma. Data have shown that these cells were with considerable metastatic potential and that their eradication had a direct effect on patients' survival. Therefore, the improvement of methods for early diagnostics and the subsequent specific characterization of initial tumour levels have caused a revision of concepts for operative and conservative treatment of oncological patients. Their further research would give new possibilities for individual assessment of the risk for metastasis, different from the standardized and commonly accepted TNM staging.

Key words: colorectal cancer, circulating tumour cells, residual disease, survival, prognosis.

INTRODUCTION

The spread of malignant tumours occurs mainly via lymph and blood vessels, resulting in metastases in regional lymph nodes and internal organs. Lymphatic spread is characteristic for most neoplasms in humans, but carcinoma metastases in lymph nodes are not always fatal. In some cancer types however, the existing lymphatic spread is a prognostic factor that influences the therapy and the prognosis of disease. On the other hand, haematogenous spread of solid carcinomas is the greatest clinical challenge in oncology and is essential for the outcome of the disease.

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On a global scale, the treatment of colorectal cancer patients remains a challenge from a diagnostic and therapeutic point of view. Among neoplasms, it is the third commonest cause of death in males after prostate and lung cancer (10.0%), while second in females after breast cancer (9.4%). According to Parkin et al. colorectal cancer is 2.4 times more frequent than rectal cancer (1).

In 2008, newly registered patients with this disease are 1,235,108 (663,904 men and 571,204 women). The death rate for the same year attains 609,051 (men – 320,397; women – 288,654). In Bulgaria, new cases for 2008 are 4,625 or 0.38%) (men – 2,591 or 0.39%; women – 2,034 or 0.35%, and fatal outcomes are 2,541 or 0.42% (2).

Despite that, the total mortality rate related to this disease has considerably decreased during the last decades, the five-year survival rate continues to range between 80-90% for patients in clinical stage I, decreased to 70-75% in those in stage II. Survivors in stage III are only 50-35%. Data about stage IV are even more discouraging, as only 7% of patients remain alive beyond the fifth year (3, 4, 5).

According to data reported by August DA et al. 1984, Ghossein RA et al. 2000, and Tebbutt NC et al. 2002, 80% of patients undergo a radical treatment during the first surgery but subsequently, 30 to 50% experience remote metastatic disease. The reason is the presence of residual cancer cells in blood and other organs, which at a later stage cause further progression of the disease (6, 7, 8).

The precise diagnosis of malignancy is essential for scheduling a proper therapy. The modern development of biomedical technologies allows detecting tumour cells at a molecular level. An important and unsolved issue is whether molecular staging is accurate enough for prognostication of a given oncology patient.

Metastases are responsible for the major part of oncological death rate and it could be therefore assumed that the detection of metastatic cells should entail a decision for aggressive therapy. The prognostic value of the different tumour cells in blood or lymph nodes (circulating tumour cells, CTC) is recently questioned. Decisions about the type of treatment in metastatic colorectal cancer are more difficult to make due to the increased likelihood to detect such cells in peripheral blood or bone marrow at the background of conflicting data about their prognostic value for patient's outcome (9).

According to Keene SA et al. 2001, the prognostic value of detected circulating cells is yet uncertain, necessitating a more extensive investigation in the problem (10).

Braun S et al. 2005 reviewed a number of strategies after the discovery of single metastatic cells in bone marrow or blood. They discussed the techniques and the probability for detection of micrometastases as precursors of a future recurrence. As an important contribution, they reviewed the attempts for geno- and phenotyping of these cells, important for monitoring of processes in adjuvant and metastatic conditions (11).

The fast development of cytology, immunohistochemistry and molecular biology has largely provided an insight to oncological disease progression. The terms minimal residual disease and minimal tumour load were introduced in clinical practice.

There is not a clear definition or standard terminology with respect to micrometastasis. Synonyms used are isolated tumour cells, disseminated tumour cells, occult metastatic cells and micrometastatic cells. In a general sense, micrometastasis is regarded a persisting malignant cell population that is not eradicated with therapy and that has remained under the sensitivity threshold of existing conventional diagnostic techniques.

Initially, micrometastases were defined as tumour cell depots that could be detected only by light microscopy. In the view of Haermanek P et al. 1999, isolated tumour cells should be distinguished from micrometastases on the basis of contact with vascular or lymph sinus wall, extravasation, extravascular stromal reaction and tumour cell proliferation (12).

At present, minimal residual disease is considered as precursor of micrometastasis and is regarded as the clinical condition, in which tumour cells are detected in patient's blood or bone marrow by contemporary diagnostic techniques, but without clinical signs of disease (13). Its importance for cancer further progression will be investigated from now on.
While the importance of minimal residual disease for breast cancer patients was partly established by Sharp JG /1992/ (14) and Stadmauer EA /1999/ (15), the issue with tumour cells detected in the blood of colorectal and rectal cancer patients is different (16, 17).

The detection of tumour cells in peripheral blood predetermines the real behaviour of the tumour (18), and their detection prior to the clinical disease makes them a factor for consequent recurrence or metastases (19, 20), related to change in the cytostatic drug treatment protocols and duration (6, 7, 8).

Clinically, numerous tumour cells could be detected in the blood of cancer patient and at the

same time, a very small proportion of them could give rise to metastases (21).

In the view of Kerr et al., 20% of patients with early colorectal cancer exhibit lymph node metastases (22), which predetermine the fatal outcome in 90% of cases (23). In an earlier report of Liefers GJ et al. (1998), the failure of some of used cytostatic treatment protocols was shown to be due to detected lymph node micrometastases (24).

Thus, factors linked to local spread, histological features and lymph nodes metastases remained clinically important for survival (25, 26, 27).

The authors concluded that the presence of these cells in peripheral blood did not correlate to lower survival rates in patients with early breast cancer, while Karina Kl, /1998/ showed that after experimental implantation of tumour cells in blood, despite the substantial initial concentration, the final result was also lack of metastases (28, 29, 30).

Of particular importance is whether the metastatic potential of detected circulating tumour cells is high or low. To confirm these hypotheses, the following techniques were developed and implemented during the recent years: *in vivo* videomicroscopy, ELISA, immunological methods, PCR or RT-PCR and CellSearch system (31).

Further investigations have shown that treatment failures were partly due to CTC in blood or in peritoneal fluid and bone marrow (24, 32, 33). Parallelly, it was established that these cells reduced the five-year survival rate of colorectal cancer patients in the second clinical stage from 75% in the group of patients negative for minimal residual disease to 36% in the CTC-positive group (24).

Therefore, the development of new methods allowing for early diagnostics and eradication of detected tumour cells in blood would substantially slow down the relapse and would increase the five-year survival rate (34).

To support these hypotheses, numerous publications have been devoted to the problem during the last years, each of them reporting a method for diagnostics of blood tumour cells in oncology patients (35).

METHODS FOR DIAGNOSTICS OF TUMOUR CELLS IN THE BLOOD OF ONCOLOGY PATIENTS

Today, these diagnostic methods could be conditionally assigned to one of the following groups (8):

1. Conventional cytology (cytological and histopathological);
2. In vivo videomicroscopy;
3. Immunological assays – ELISA, flow cytometry etc.
4. Molecular biology techniques: polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR).
5. The CellSearch system.

Conventional cytology

During the past century, the detection of tumour cells in blood depended upon the cytological examination of routinely stained blood smears. Tumour cells' detection rate by routine cytological and histopathological methods remains with a low diagnostic value up to 4% of cases (36, 37).

It was then established that the incidence of false positive results attained 67% due to the non-specific staining of mononuclear lymphocytes (38, 39, 40). During the last two decades however, a number of new techniques were developed and numerous studies have been carried out permitting the specific detection and characterization of circulating tumour cells in breast and colorectal cancer patients. In general, patients with tumour cells in the blood have a poorer prognosis compared to those without such cells (11, 41, 42, 43).

Nevertheless, the identification of circulating tumour cells could be problematic due to the different sensitivity and specificity of these methods. For example, single necrotic cells shed from the primary tumour during the surgery, could be detected in peripheral blood, but they are not viable and therefore, their presence is not indicative for the further disease progression. On the contrary, the detection of multiple cells could be more relevant to the outcome as these cells are probably indicating an earlier stage of haematogenous spread. The issues with the blood cell counts needed for analyses are still not clear (44).

In vivo videomicroscopy

In vivo videomicroscopy method is based on a relatively simple and fast procedure for clear CTC identification. It includes intravenous infection of a tumour-specific fluorescent ligand, followed by imaging of superficial blood vessels by multiphoton fluorescence. By this technique, about 2 CTC/ml blood could be identified. The non-invasive imaging of peripheral blood tumour cells could improve the sensitivity of detection permitting analysis of a relatively larger blood volume (virtually the entire patient's blood volume) but so far, these analyses have been proved efficient only when tumour cells were marked ex vivo, prior to intravenous injection (45).

The method is promising for screening, early detection of disease progression and evaluation of therapeutic response for numerous cancers. It could easily detect less than one circulating epithelial cells per ml blood (46). The in vivo microscopy reveals the role of tumour cells during both primary and metastatic tumour growth (45, 47).

A disadvantage of the method is the need for a specific marker to visualize circulating tumour cells.

Immunological methods

ELISA (enzyme-linked immunosorbent assay)

ELISA was firstly used in 1971. Later, telomerase was used as a more precise marker for early diagnostics of tumour cells in the blood of colorectal cancer patients **(Table 1)** (48). The implementation of immunomagnetic separation increased the sensitivity of the method. Nanoparticles coated with BerEP4 **(Figure 1)** are used, and the sensitivity attained 1 tumour cells per 1 million epithelial cells (49). Nevertheless, the issues with finding and applying serum markers to detect such cells in blood are not solved, thus making the technique less appropriate.

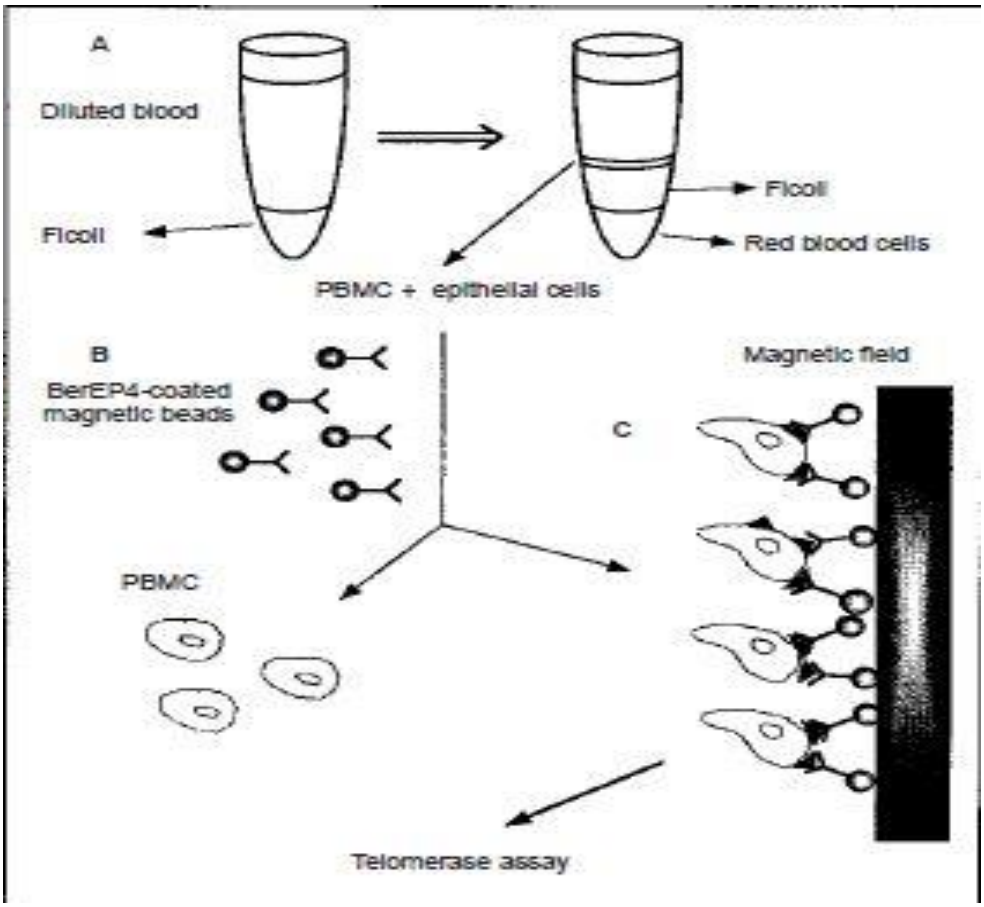

Figure 1. Schematic representation of the method for detection of telomerase activity of CTC, using immunomagnetic separation with BerEP4 (Koelink et al, BMC Cancer 2009; 9:88) (49)

Table 1. *Sensitivity and specificity of ELISA*

Year	Author	Method	Number of patients	Sensitivity	Specificity
2001г.	Gauthier LR	Telomerase activity	11	72%	P > 0.0001
2009	Koelink PJ	CK18-Asp396	49	95%	P ≤ 0.05
		total CK18			

Immunocytological and immunohistochemical methods

By the end of 1980, the analysis of circulating tumour cells by immunohistochemistry was introduced. Specific antibodies are used for detection and selection. Immunological methods are used for high-sensitivity detection of micrometastases in bone marrow, lymph nodes and peripheral blood (32, 50, 51, 52).

The concentration of tumour cells in bone marrow aspirate or blood is done by centrifugation of the aspirate in separation medium resulting in CTC migration in the mononuclear cell layer. They are used for cytological smears or slides that are further processed with monoclonal or polyclonal antibodies recognizing a specific epithelial antigen, which are then identified by immunocytochemical or immunofluorescence methods **(Figures 2, 3 and 4).**

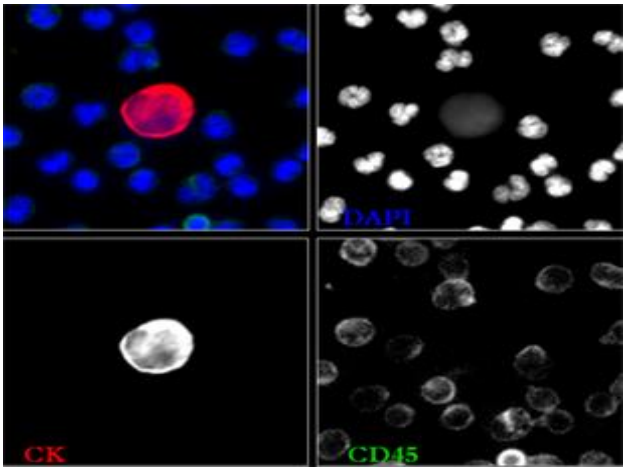

Figure 2. Identification of tumour cells by fluorescence microscopy (Fehm et al, Clin Cancer Res. 2002; 8(7):2073-84)

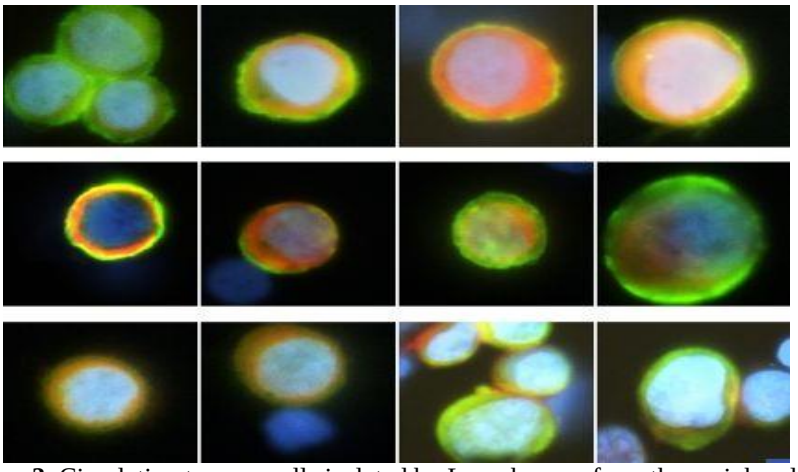

Figure 3. Circulating tumour cells isolated by Lymphoprep from the peripheral blood of colorectal cancer patients (Ntouroupi et al., British Journal of Cancer 2008; 99: 789–795)

The disadvantages of the method are related to the risk of false positive results due to non-specific binding to normal non-epithelial cells typical for healthy subjects with inflammation, trauma, surgical intervention or benign epithelial disease (53, 54).

These results are particularly dangerous in evaluating the tumour response after therapy. Yet, in general, immunocytological techniques are a method of choice for detection of micrometastases in bone marrow (52, 55, 56).

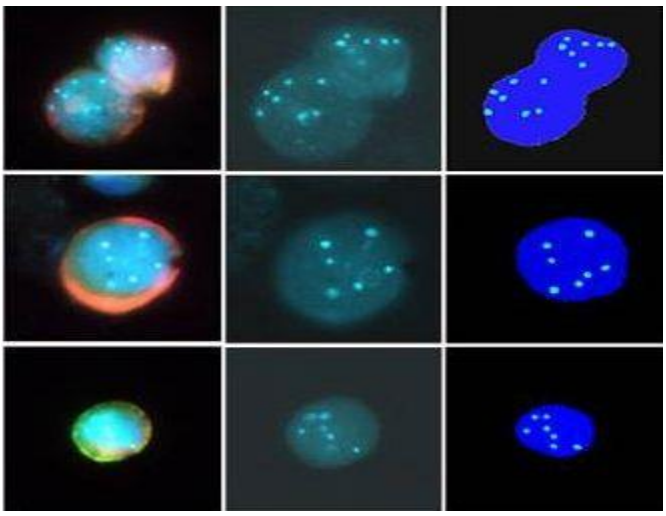

Figure 4. Circulating tumour cells isolated by peripheral blood filtration in colorectal cancer patients /Jian-Mei Hou et al, *American Journal of Pathology.* 2009;175:808-816)

Despite the flaws, immunocytochemistry has the advantage to preserved cell morphology and permits the identification of different groups of cells. It permits a simultaneous assessment of other cell traits as proliferative activity and oncogene expression (57, 58).

Molecular biology techniques

Polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR)

PCR is introduced in 1990. At present, it is the most commonly used technique for CTC detectin (59, 60).

The specificity of the method is based on the possibility to find out specific DNA or mRNA regions by their amplification with specific oligonucleotide primers flanking these sequences.

DNA is more stable than RNA and thus, more suitable for investigation. Freely circulating DNA fragments could be detected in blood, for example consequently to release from nonviable tumour cells, hence the risk of false positive results. It was found out that only a few chromosome abberations are frequent and sensitive enough for identification of CTC.

That is why, modern techniques are focused on CTC detection by proving and determining the mRNA of specific tumour or tissue-specific genes by RT–PCR. After isolation of total RNA from blood, a complementary DNA is first synthesized by reverse transcriptase, also called copy DNA. It is further used as a matrix for amplification of a pair of specific primers, limiting gene sequence that is to be determined, evidencing an expression of this gene **(Figure 5).** The number of mRNA copies for a given gene could vary during the cell life cycle and yield a false positive result. This limitation is avoided by using two approaches for gene selection in RT-PCR reaction (61).

The first is based on detection of tumour-specific anomalies occurring in s given gene region by either PCR or RT-PCR. The second approach uses detection of the expression of tissue-specific markers on the surface of tumour cells by RT-PCR. Now, this method is considered as the most sensitive for detection of CTC (1 tumour cell per 1-10 million normal cells, approximately 1 tumour cell per 1-10 mL blood) (20, 61, 62, 63). The advantage of the method is its high specificity **(Table 2).** Its high efficiency is due to the possibility to analyze the entire genomic DNA or RNA in one run. There are

however some limitations. A disadvantage of the method is the impossibility to perform a further analysis of tumour cells. As a paradox, its high sensitivity is also a drawback. An erroneous transcription (low level of gene transcription in all cells), contamination of the sample (64) or the presence of pseudogenes (genes without intron sequences) could yield false positive results after the PCR amplification. Other causes for erroneous results are markers with low specificity (65) or potential spread of normal/benign cells after invasive procedure (64). On the contrary, variability in PCR sensitivity could result in false negative results. Possible causes for such variability could be: a) circulating tumour cells do not express equally the studied marker; b) presence of PCR inhibitors in tissues and body fluids reducing the sensitivity of the reaction; в) damage of the target gene by performed therapy (8, 42, 66, 67, 68).

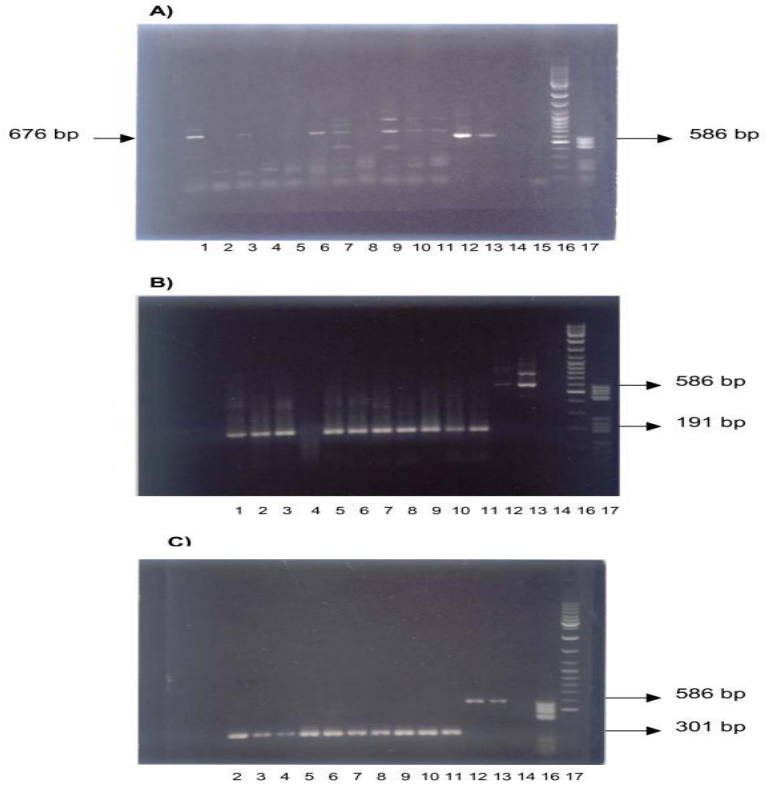

Figure 5. RT-PCR amplification products: A), PRSS22, B) RANBP3 and C) TMEM69 gene expression. Gene's lanes are marked as followed: 1-4 – blood RNA from healthy subjects, 5 – RNA from normal leukocytes, 6 – blood from colorectal cancer patients, 7- RNA from normal colonic mucosa, 8- RNA from colon adenocarcinoma patient, 9 –normal mucosa from control subjects , 10-11 – disease-free mucosa and colon cancer from the same patient, 12-13 – PCR-positive controls, 14-15 – negative controls, 16-17 - 1 μL molecular markers: MBI V and GeneRuler (Genenco), respectively (Bertolini et al., Nat. Rev. Cancer 2006; 6(11): 835–845)

The analysis of causes for false positive and false negative results in RT-PCR method shows that it is not easy to be automated. Although, it permits to distinguish tumour cells from normal cells, an exact quantitation of CTC in the sample is not possible. The aforementioned disadvantages of the methods could be overcome by a correct and consecutive combination between them (68, 69, 70, and 71).

The most commonly used markers for CTC detection in colorectal cancer by RT–PCR are CK-19, CK-20, K-PAH, MUC and guanylin cyclase **(Figure 6)** (72, 73, 74, 75, 76).

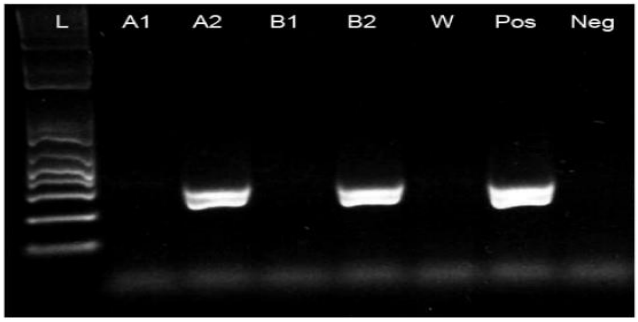

Figure 6. RT-PCR amplification products for mRNA of cytokeratin-20 after immunomagnetic enrichment with Ep-Cam antibodies (Antolovic et al., *BMC Biotechnology* 2010; 10:35)

Table 2. *Sensitivity and specificity of PCR and RT-PCR.*

Year	Author	Method	Number of patients	Sensitivity	Specificity
2004	R. SCHUSTER	proTM	129	17%	P=0.12
		CEA		86%	P=0.055
		CK20		88.4%	P=0.018
2005г.	Zhang XW	CK20	58	69,0%	P<0.01
2006	Wang JY	CK-19	72	66.7%	P=0.006
		CK-20		52.8%	
		CEA		72.2%	
2008	Yie SM	RT-PCR ELISA	86	44%	P < 0.001
2010г.	Tsouma A	CEA	88	84/95.5%,	P < 0.001
		CK20		69/78.4%	
		EGFR		17/19.3%	
2010	Vogelaar FJ	CK-ICC	46	33%	p = 0.002

CellSearch system

Recently, the FDA approved a system for detection of circulating tumour cells in peripheral blood. It is an automated immunomagnetic method for tumour cells detection **(Figures 7 and 8)** (77, 78).

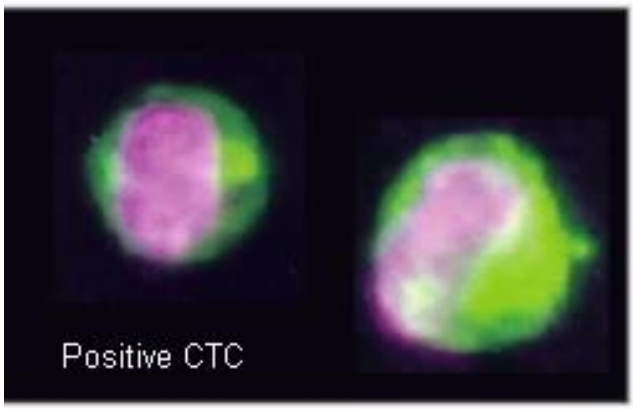

Figure 7. Visualization of positive CTC by the CellSearch system (CD45-, EpCAM+, and cytokeratins 8, 18+, and /or 19+) in blood (CTC (Circulating Tumor Cell Detection) Assay is available at the UCLA Biomarker Innovations Laboratory)

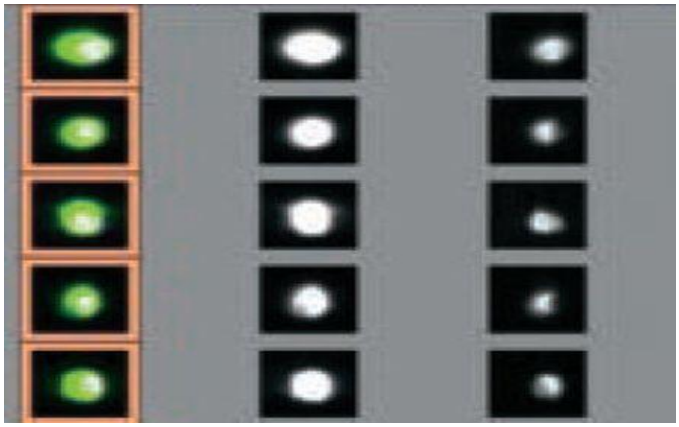

Figure 8. Visualization of circulating tumour cells (CK-PE +, DAPI +, CD45-APC-), as seen on a CellSearch analyzer (The CellSearch Assay for Circulating Tumor Cells: An Advance in Cancer Prognosis and Treatment, Copyright © 1995– 2011 Mayo Foundation for Medical Education and Research)

This system was utilized by Miller et al. 2010, which found out that the amount of tumour cells in the blood of colorectal cancer patients correlated with disease stage, but not with other morphological parameters P= 0.005 **(Table 3)** (79, 80, 81).

Table 3 *Sensitivity and specificity of CellSearch system.*

Year	Author	Number of patients	Sensitivity	Specificity
2008	J. Sastre	94	36,2%	P= 0.005
2010	Miller MC	413	38%	P < 0.001
2010	**Yalcin S**	130	32%	p = 0.001

DISCUSSION

The detection of circulating tumour cells is essential from both theoretical and practical points of view. It allows obtaining in vivo information about the metastatic process, and in the future, additional information about the interaction between the tumour and immune system could be anticipated. This would add to our knowledge on the mechanism of tumour growth and could help the development of new drugs and therapeutic protocols (82, 83, 84).

Metastases occur secondary to the interaction between tumour cells and patient's cells. During their movement in peripheral blood, many tumour cells are killed by immune response, but the metastatic potential of remaining ones could not be underestimated or excluded. The occurrence of tumour cells in systemic circulation entails tumour relapse. That is why, the early detection of these cells followed by their immediate eradication by cytostatic drugs or perhaps following individual cytostatic treatment protocol could prevent the development of metastases (58, 85).

The ability of tumour cells to migrate is a part of their metastatic potential (86), and methods developed for their detection in blood circulation resulted in reassessment of the risk for relapse and provided new therapeutic possibilities (87). It was proved that minimal residual disease in bone marrow, peritoneal punctate or in lymph nodes correlates to poorer prognosis and shorter survival period (88, 89, 90).

Papavasiliou et al. established a significant correlation between post operative presence of CTC, the progression of disease and the overall survival rate. In their belief, from a clinical point of view, the post operative detection of tumour cells in peripheral blood could be the most suitable indicator for occult clinical disease (91). Vardakis et al. provided evidence for considerable lower disease-free survival (DFS)

(P<0,001), and overall survival (P=0.017) in patients with circulating tumour cells detected by RT-PCR. These data show that CTC are an independent prognostic factor for reduction of DFS (92).

Rahbari et al. performed a meta-analysis showing a bad prognosis on the basis of blood CTC detection in colorectal cancer patients (93).
In cancer treatment, the search for and the detection of minimal residual disease has the following important functions: to determined the efficacy of performed therapy, to compare the different therapeutic protocols, to monitor patients at risk for progression of the disease and last but not least, to identify patients needing more aggressive therapy (77, 94, 95).

At present, the treatment of colorectal cancer patients (Dukes A and B, TNM stages I and II) is operative (96). After diagnosing a progression (Dukes C and D, TNM stages III and IV) adjuvant chemotherapy is indicated apart surgery (97, 98, 99).

Nevertheless, the result from the treatment is rather variable and therefore, the prognostication of therapeutic response on the basis of conventional criteria is not consistent (96, 99, 100).

The detection of the amount of peripheral blood CTC could be useful for the consequent monitoring of cytostatic drug efficacy and the related therapeutic effect (101, 102).

According to Hampton et al., detected tumour cells are markers of therapy's efficiency, including for patients treated surgically in an early stage of disease (103).

The detection and quantitation of tumour cells in peripheral blood could be a unique method for diagnostics, prognostication and therapeutic efficacy evaluation.

That is why, a considerable interest is raised for development of more stable and more predictable disease markers (104, 105).

Markers based on circulating CEA and various levels of tumour-associated gene mutations, including microsatellite instability, loss of heterozygocity of the 18p and DCC genes, and mutations in K-RAS, B-RAF and PIK3CA genes are with proved prognostic value (44, 79, 104, 106).

In particular, mutations in K-RAS, B-RAF and PIK3CA genes were recently advanced as reliable marker to predict the response to EGFR targeted immunotherapy (107,108).

CONCLUSION

The appearance of tumour cells in peripheral blood is an early sign of metastasis. Despite the systemic mechanisms of defense, single cancer cells could be detected in remote areas under the form of colonies as an initial stage for formation of a distal metastasis (109)

As forerunner of a secondary relapse, circulating tumour cells detected after radical surgery could be used for prediction and monitoring of disease, being independent prognostic factors of the further progression of the neoplasm (75, 81, 110).

They could broaden the therapy options both with approved drugs and by using epithelially targeted monoclonal antibodies. A highly sensitive method for prognosis of the metastatic potential and clinical results is needed for undertaking a proper treatment. These markers could provide prognostic information independently from and in addition to traditional tumour markers (111, 112).

In conclusion, circulating tumour cells possess a considerable metastatic potential and their eradication influences directly the survival of patients. This way, the improvement of methods for early diagnostics and the subsequent specific characterization of initial tumour levels have caused a revision of concepts for operative and conservative treatment of cancer patients. Future research would provide new alternatives for individual assessment of risk for metastasis, different from the standard and commonly accepted TNM staging.

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