

# Colorectal carcinomas in 2014: The search for powerful prognostic markers is still on the go!

Julien Coget<sup>a</sup>, Francesco Borrini<sup>b</sup>, Sergiu Susman<sup>c</sup> and Jean-Christophe Sabourin<sup>a,d,\*</sup>

<sup>a</sup>Laboratory of Cancer Genetics, Department of Pathology, Rouen University Hospital, Rouen, France

<sup>b</sup>Department of Surgery- Pietro Valdoni, Rome University Hospital-La Sapienza, Rome, Italy

<sup>c</sup>Department of Morphological Sciences, University of Medicine and Pharmacy, Cluj-Napoca, Romania

<sup>d</sup>INSERM Unit 1079: Genetics of Cancer and Neurogenetics, Institute for Research and Innovation in Biomedicine, University of Rouen, Rouen, France

**Abstract.** Colorectal cancer (CRC) is the third cause of cancer worldwide after prostate cancer and breast cancer. Patients have a survival rate of 5 years, which varies between 10 and 95% depending on CRC stage. Today, the management of patients with CRC is based on parameters such as TNM and classic histologic parameters, but new molecular and cell markers have been created to improve treatment and survival. Determining the expression of a characteristic set of genes either from formalin-fixed paraffin-embedded tissue (Oncotype DX test<sup>TM</sup>) or from fresh tissue (AGENDIA© ColoPrint<sup>®</sup>) has led to encouraging results, but there is a need for clinical validation on a large number of patients. Also, next-generation sequencing (NGS) technologies may be the next step in the molecular approach of CRC tumor samples, allowing tumor characterization by gene signature arrays. In addition to molecular markers, evaluation of the presence of cellular markers such as circulating tumor cells (CTC) in the blood of patients with CRC can optimize prognostic evaluation and response to treatment. CTC isolation methods used today have different sensitivities and specificities, due not only to the very small number of these cells but also to the epithelial-mesenchymal transitional process (EMT). This paper presents the preliminary results of our study conducted on CTC isolation in patients with CRC by filtration method (ScreenCells Cyto<sup>®</sup>). This fast and efficient method identifies CTCs and also isolates cells in EMT, which explains its high efficiency compared to technologies based on immunomagnetic and microfluidic separation reliant on EpCAM presence on the cell surface.

Keywords: Colorectal carcinoma CRC, circulating tumor cells CTC, epithelial-mesenchymal transitional process EMT, biomarkers

## 1. Introduction

Cancer is the result of the accumulation of genetic abnormalities in tumor cells. In solid tumors, we now know that an average of 100 alterations is present in tumor cells. Only a dozen key alterations are needed for the survival, growth and progression of a malignancy (i.e., driver mutations) while other anomalies are related to the genetic instability present in tumor cells (i.e., passenger mutations).

Although advances have been made in colorectal cancer biology, the complexity of the dysfunctions in-

duced by these genetic alterations explains why, in 2013, management of these cancers is still based on morphological analysis (reflecting genetic abnormalities) and clinical staging (considered to be the gold standard of prognostic markers). However, with the emergence of new treatments, the need for powerful prognostic factors is becoming increasingly urgent.

In this presentation, we initially review the major prognostic factors used in digestive oncology and then present a new biomarker that could be useful in the management of these patients.

## 2. Epidemiology of colorectal cancer

Colorectal carcinoma (CRC) is a major cause of death in western countries. With an annual incidence

---

\*Corresponding author: Jean-Christophe Sabourin, Laboratory of Cancer Genetics, Department of Pathology, Rouen University Hospital, Rouen, France. E-mail: Jean-Christophe.Sabourin@chu-rouen.fr.

of 37500 new cases in France, CRC is the third most common cancer after prostate cancer and breast cancer [5,9]. CRC is the second leading cause of cancer death after lung cancer, with nearly 17000 deaths annually [5,19]. For all stages combined, the 5-year survival rate varies from 10 to 95% [17].

### 3. The TNM grading system

Prognosis is mainly related to the anatomic extent of the tumor (locally through the digestive wall and then at distance from the colon-rectum). This extension is currently determined by the TNM (tumor-node-metastasis) staging system of the International Union Against Cancer (UICC) and the American Joint Committee on Cancer (AJCC).

This staging allows for the differentiation of localized forms from metastatic cases. For localized tumors, the risk of local recurrence and/or metastasis varies from 20 to 30% depending on the presence or absence of lymph node involvement and the performance of adjuvant chemotherapy [3,16]. For metastatic cases, different therapeutic strategies are available depending on the resectable or unresectable character of the metastatic lesions. Thus, 5-year survival for patients with resectable metastatic lesions is 35%, while median survival is 15 to 25 months for patients with unresectable metastatic lesions [1].

If the regularly updated TNM classification is the current reference for prognosis (our gold standard), it is imperfect, with different evolutionary profiles within the same stage. Therefore, other clinical, histologic and molecular factors are being sought to complete this classification and assist clinicians in treatment decisions.

At diagnosis, the distribution of colorectal cancers involves 20% at early stage (stage I), 25% at major parietal invasion stage (stage II) and 30% at nodal involvement stage (stage III) [4]. Stage I CRCs are curable after surgical resection [12] with a recurrence risk of approximately 10% after treatment. Adjuvant chemotherapy after curative surgery is no longer recommended for stage II tumors (T3-T4N0) [12]. Stage II cases are generally associated with a favorable prognosis [30] demonstrating no benefit for adjuvant chemotherapy. However, these tumors have a relatively heterogeneous prognosis, with 5-year survival ranging from 60 to 90% in stage II CRC.

Poor prognostic factors have been identified within Stage II CRCs, including tumors with involvement of

the visceral peritoneum or adjacent organs (T4); tumors with perivascular or peri-nervous invasion; undifferentiated tumors; fewer than 12 nodes examined; or the existence of perforation/occlusion at diagnosis. Therefore, the 5-year survival rate of patients with stage II CRCs with one or more of these factors is similar to that of stage III tumors [16].

### 4. Classic prognostic factors

The *R staging system*: “R” stage corresponds to tumor status after surgery (residual tumor RT): no microscopic RT = R0; microscopic RT = R1; macroscopic RT = R2. If R has a strong prognostic value (R1 or R2), it refers to advanced tumors for which therapeutic management is radically different to localized R0 tumors.

High rates of pre-surgery carcino-embryonic antigen (*CEA serum*) (> 5.0 ng/mL) have been known for many years to be a poor prognostic factor, independent of TNM stage [18,23].

*Tumor grade* represents the degree of gland formation and allows classification of CRC according to degree of differentiation: well differentiated (grade 1) to undifferentiated (grade 3). Tumor grade has been shown to be a poor prognostic factor in univariate and multivariate analysis [8,16]. Nevertheless, studies have failed to prove this prognostic association in multivariate analysis [25]. Moreover, histologic classifications are not homogeneous across studies: classification is either basic (two-tier system) with “low grade” and “high grade” or with three to four levels of differentiation. This mainly explains why tumor differentiation grade is not recommended for therapeutic purposes.

*Tumor “budding”* (TB) is defined as a budding of clusters of less than 5 tumor cells or as independent cells, especially at the tumor invasion front. TB is a criterion for tumor aggressiveness: it is associated with lymph node metastasis in univariate and multivariate analysis (OR = 3.7) [38]. It is also considered to be a useful and simple histologic criterion predicting metastasis occurrence after curative surgery; however, inter-observer variability in its assessment limits its use [24]. Further studies are needed to standardize TB evaluation criteria.

*Lymphatic and venous tumoral invasion* is a key step in the development of micrometastases. Venous invasion is a tumor aggressiveness factor but is not systematically mentioned in the pathology report: its detection is sometimes difficult to perform due to fibrosis

and/or fixation artifacts. Observation variability is often reported and currently there is no reference classification to harmonize its evaluation [11].

*Peri-nervous tumoral infiltration* is a poor prognostic factor in multivariate analysis (independent factor) [11].

The initially obstructive or perforated state of the tumor also has a potentially prognostic value.

Other factors still under investigation are known for their potential prognostic impact on CRC, including *intra-tumoral lymphocyte reaction*, which could be the most promising [28,31]. Lymphocyte reaction in contact with infiltrating CRC tumor cells appears to have a prognostic value greater than TNM [6]. However, this lymphocyte reaction is frequently associated with dMMR tumors, as microsatellite instability phenotype produces abnormal proteins (i.e., neoantigens) that are likely to be recognized as “no self” proteins by the immune system. This data was missing from the Galon team’s work [28]. Thus, a French clinical study is underway to validate this new marker (lymphoid grade) while specifying tumoral MMR status.

## 5. New prognostic markers

### 5.1. Molecular markers

Recently, molecular markers have been introduced in the U.S. for managing patients with CRC. The *Oncotype DX test*<sup>TM</sup>(GENOMIC HEALTH©) is based on the molecular signature of 12 genes detected by RT-PCR on formalin-fixed paraffin-embedded tumoral tissues. AGENDIA© *ColoPrint*<sup>®</sup> test is based on the RNA profile obtained from fresh/snap-frozen specimen or preserved in a special preservative solution such as *RNAlater*<sup>®</sup>. These molecular tests (which do not share the same signature genes) need further validation in studies covering a large patient series.

Next-generation sequencing NGS technologies may be the next step in a molecular approach for CRC tumor samples. NGS will allow tumoral characterization with prognostic and predictive values: gene signature arrays (involving the main targeted therapy pathways such as EGFR pathway) are under development.

### 5.2. Circulating tumor cells

Metastases generally result from the hematologic spread of disease [32]. Therefore, detection of tumor cells (i.e., circulating tumor cells CTC) in peripheral

blood seems interesting in terms of prognosis. Several studies of different cancers have shown that the presence of CTC is associated with worse prognosis [13–15,29].

A large U.S. study of 430 patients demonstrated the value of CTC detection in metastatic colorectal cancer (mCRC) by showing a link between the presence of CTC and reduced disease-free survival [10]. In multivariate analysis, including lymph node involvement and vascular invasion, the presence of perioperative CTC appears as an independent predictor of recurrence with a hazard ratio of 29.5 (95% CI, range 10.3 to 87.8) [36,37,40,41]. Finally, a recent (2010) meta-analysis of 36 studies demonstrated that CTC detection had a negative influence on overall survival and disease-free survival in a series of over 3000 patients with stages I to IV CRC [34].

This new biomarker is potentially interesting, not only in terms of prognosis but also for early detection of recurrences and assessment of the sensitivity of CRC to chemotherapy.

CTCs reflect the hematologic spread of disease. These CTCs arise from the primary tumor [32] at the earliest stages of vascular invasion and spread daily in the blood as “tumoral seeding” [7]. CTCs have acquired a particular phenotypic profile (by undergoing EMT), which allows them to migrate and circulate in the peripheral blood. While they mostly disappear (by apoptosis or by action of the immune system), a small proportion survives in the blood circulation and then locates in the bone marrow, liver, lungs, etc. Within these target organs [35], CTCs may:

- Stay quiescent (G0 phase, dormancy) [2],
- Turn to micro-metastases,
- Spread again into the blood stream,
- Be destroyed.

On a technical level, the challenge is to identify rare tumor cells among 10 million leukocytes and 5 billion erythrocytes in 1 ml of blood [32]. The problem is complex because not all CTCs have the ability to become metastatic and the sensitivity and specificity of the techniques available vary. These techniques are used to define CTCs based on their morphological appearance associated with immunostaining (EpCAM antibody) or by molecular analysis (RT-PCR) detecting tumoral mRNA (CEA, cytokeratins) [27,32].

ScreenCell<sup>®</sup> laboratory (Paris – France) has developed an isolation-by-size technique to detect CTCs [14]. Circulating blood cells are among the smallest cells in the body (< 8  $\mu$ m). Filtering the blood through a membrane whose pores are calibrated to 8  $\mu$ m can elimi-

nate them. After filtration, the remaining cells present on the membrane are then morphologically analyzed to assert their tumoral nature. This simple and rapid technique does not require expensive equipment (a single-use device) and can be used easily in routine practice.

The feasibility of this detection method was evaluated in various cancers, including breast carcinomas [33], hepatocellular carcinomas [39] and non-small cell lung carcinomas [20–22], by filtration using a similar process (ISET<sup>®</sup> system – Metagenex – Paris), and the same filters with 8  $\mu\text{m}$  pores but associated with a filtration device.

However, to our knowledge, no study has evaluated this method in mCRC. The primary aim of this study was to demonstrate the feasibility of CTC filtration in a clinical situation.

## 6. Material and methods

A sampling of peripheral venous blood was performed on 39 patients with mCRC before starting chemotherapy regimen. For cytology, 4 ml of blood were filtered using 4 Screencells Cyto<sup>®</sup> devices. After staining with hematoxylin, each filter was analyzed by a cytopathologist. Cells visualized on the filter were considered tumoral if they met the following morphological criteria: nuclear diameter  $> 7 \mu\text{m}$ , anisocytosis, membrane irregularities, and the presence of large nucleolus. Immunostaining with pancytokeratin, CDX2 and EpCam antibodies was also performed on the filters.

## 7. Results

All samples were filtered within 4 hours of collection. Three samples were not analyzed due to blood coagulation. The various stages of cytology identification were performed in less than 60 minutes. Presence of CTCs was identified in 23/36 patients with mCRC (64%). Cytokeratin antigen was mainly lost in CTC probably due to EMT. EpCam and moreover CDX2 were detected on CTC, confirming their digestive origin.

## 8. Conclusion

Screencells<sup>®</sup> Technology allows cytological testing that appears to be fast and efficient for the detection of

CTC in mCRC patients. CTCs undergo EMT; thus, it is important to choose the right antibody for their characterization. This finding could also explain why microfiltrating approaches seem to be more efficient than technologies based on immunomagnetic or microfluidic separation, which rely on the presence of EpCam antigen at the surface of the CTC.

## 9. General conclusion

In 2014, patients with colorectal cancer are still easily managed using tumor-node-metastasis staging and histopathological criteria (i.e., “gold standards”). Nevertheless, in an age of personal medicine with targeted therapies, the search for new strong prognostic and predictive markers is ongoing. For metastatic colorectal cancer patients, circulating tumor cells, which are easily detected and are characterized using simple and efficient microfiltrating technology, could be one of these markers, offering both morphological and molecular characterizations.

## Acknowledgments

The authors thank Nikki Sabourin-Gibbs, Rouen University Hospital, for her help in editing the manuscript.

## References

- [1] R. Adam, V. Delvart, G. Pascal, A. Valeanu, D. Castaing, D. Azoulay, S. Giacchetti, B. Paule, F. Kunstlinger, O. Ghéard, F. Levi and H. Bismuth, Rescue surgery for unresectable colorectal liver metastases downstaged by chemotherapy: a model to predict long-term survival, *Ann Surg* **4** (2004), 644–57.
- [2] J.A. Aguirre-Ghiso, Models, mechanisms and clinical evidence for cancer dormancy, *Nat Rev Cancer* **11** (2007), 834–46.
- [3] T. André, C. Boni, M. Navarro, J. Tabernero, T. Hickish, C. Topham, A. Bonetti, P. Clingan, J. Bridgewater, F. Rivera and A. de Gramont, Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial, *J Clin Oncol* **19** (2009), 3109–16.
- [4] A.D. Benson, D. Schrag, M.R. Somerfield, A.M. Cohen, A.T. Figueredo, P.J. Flynn, M.K. Krzyzanowska, J. Maroun, P. McAllister, E. Van Cutsem, M. Brouwers, M. Charette and D.G. Haller, American Society of Clinical Oncology recommendations on adjuvant chemotherapy for stage II colon cancer, *J Clin Oncol* **16** (2004), 3408–19.
- [5] A.M. Bouvier, Épidémiologie descriptive du cancer colorectal en France, *Bull Epidem Hebdo* **2-3** (2009), 14–16.

- [6] E.K. Broussard and M.L. Disis, TNM Staging in Colorectal Cancer: T Is for T Cell and M Is for Memory, *J Clin Oncol* **29** (2011), 601-3.
- [7] Y.S. Chang, E. di Tomaso, D.M. McDonald, R. Jones, R.K. Jain and L.L. Munn, Mosaic blood vessels in tumors: frequency of cancer cells in contact with flowing blood, *Proc Natl Acad* (2000), 14608-14613.
- [8] P.H. Chapuis, O.F. Dent, R. Fisher, R.C. Newland, M.T. Pheils and E. Smyth, A multivariate analysis of clinical and pathological variables in prognosis after resection of large bowel cancer, *Br J Surg* **9** (1985), 698-702.
- [9] K. Chevreul, Colorectal cancer in France, *Eur J Health Econ* **10** (2010), S15-S20.
- [10] S.J. Cohen, C.J. Punt, N. Iannotti, B.H. Saidman, K.D. Sabbath, N.Y. Gabrail, J. Picus, M.A. Morse, E. Mitchell, M.C. Miller, G.V. Doyle, H. Tissing, L.W. Terstappen and N.J. Meropol, Prognostic significance of circulating tumor cells in patients with metastatic colorectal cancer, *Ann Oncol* **7** (2009), 1223-1229.
- [11] C.C. Compton, Colorectal carcinoma: diagnostic, prognostic, and molecular features, *Mod Pathol* **4** (2003), 376-388.
- [12] Consensus conference. Prevention, diagnosis and treatment of colon cancer, *Gastroenterol Clin Biol* **2** (1998), 205-26.
- [13] M. Cristofanilli, G.T. Budd, M.J. Ellis, A. Stopeck, J. Matera, M.C. Miller, J.M. Reuben, G.V. Doyle, W.J. Allard, L.W. Terstappen and D.F. Hayes, Circulating tumor cells, disease progression, and survival in metastatic breast cancer, *N Engl J Med* **351** (2004), 781-91.
- [14] I. Desitter, B.S. Guerrouahen, N. Benali-Furet, J. Wechsler, P.A. Jänne, Y. Kuang, M. Yanagita, L. Wang, J.A. Berkowitz, R.J. Distel and Y.E. Cayre, A new device for rapid isolation by size and characterization of rare circulating tumor cells, *Anticancer Res* **2** (2011), 427-41.
- [15] Y.I. Elshimali and W.W. Grody, The clinical significance of circulating tumor cells in the peripheral blood, *Diagnostic Molecular Pathology* **4** (2006), 187-194.
- [16] S. Gill, C.L. Loprinzi, D.J. Sargent, S.D. Thomé, S.R. Alberts, D.G. Haller, J. Benedetti, G. Francini, L.E. Shepherd, J. Francois Seitz, R. Labianca, W. Chen, S.S. Cha, M.P. Heldebrandt and R.M. Goldberg, Pooled analysis of fluorouracil-based adjuvant therapy for stage II and III colon cancer: Who benefits and by how much? *J Clin Oncol* **10** (2004), 1797-806.
- [17] L.L. Gunderson, J.M. Jessup, D.J. Sargent, F.L. Greene and A.K. Stewart, Revised TN categorization for colon cancer based on national survival outcomes data, *J Clin Oncol* **2** (2010), 264-71.
- [18] L.E. Harrison, J.G. Guillem, P. Paty and A.M. Cohen, Preoperative carcinoembryonic antigen predicts outcome in node negative colon cancer patients: a multivariate analysis of 572 patients, *J Am Coll Surg* **1** (1997), 55-9.
- [19] C. Hill and F. Doyon, La fréquence des cancers en France: mortalité en 2003, évolution depuis 1968 et cancers de l'enfant, *Bull Cancer* **1** (2007), 7-13.
- [20] V. Hofman, C. Bonnetaud, M.I. Ilie, P. Vielh, J.M. Vignaud, J.F. Fléjou, S. Lantuejoul, E. Piaton, N. Mourad, C. Butori, E. Selva, M. Poudenx, S. Sibon, S. Kelhef, N. Vénissac, J.P. Jais, J. Mouroux, T.J. Molina and P. Hofman. Preoperative circulating tumor cell detection using the isolation by size of epithelial tumor cell method for patients with lung cancer is a new prognostic biomarker, *Clin Cancer Res* **4** (2011), 827-35.
- [21] V. Hofman, M.I. Ilie, E. Long, E. Selva, C. Bonnetaud, T. Molina, N. Vénissac, J. Mouroux, P. Vielh and P. Hofman, Detection of circulating tumor cells as a prognostic factor in patients undergoing radical surgery for non-small-cell lung carcinoma: comparison of the efficacy of the CellSearch Assay<sup>TM</sup> and the isolation by size of epithelial tumor cell method, *Int J Cancer* **129** (2011), 1651-60.
- [22] V.J. Hofman, M.I. Ilie, C. Bonnetaud, E. Selva, E. Long, T. Molina, J.M. Vignaud, J.F. Fléjou, S. Lantuejoul, E. Piaton, C. Butori, N. Mourad, M. Poudenx, P. Bahadoran, S. Sibon, N. Guevara, J. Santini, N. Vénissac, J. Mouroux, P. Vielh and P.M. Hofman, Cytopathologic detection of circulating tumor cells using the isolation by size of epithelial tumor cell method: promises and pitfalls, *Am J Clin Pathol* **1** (2011), 146-56.
- [23] J.W. Huh, B.R. Oh, H.R. Kim and Y.J. Kim, Preoperative carcinoembryonic antigen level as an independent prognostic factor in potentially curative colon cancer, *J Surg Oncol* **5** (2010), 396-400.
- [24] J.R. Jass, Y. Ajioka, J.P. Allen, Y.F. Chan, R.J. Cohen, J.M. Nixon, M. Radojkovic, A.P. Restall, S.R. Stables and L.J. Zwi, Assessment of invasive growth pattern and lymphocytic infiltration in colorectal cancer, *Histopathology* **6** (1996), 543-8.
- [25] C. Liebig, G. Ayala, J. Wilks, G. Verstovsek, H. Liu, N. Agarwal, D.H. Berger and D. Albo, Perineural invasion is an independent predictor of outcome in colorectal cancer, *J Clin Oncol* **31** (2009), 5131-7.
- [26] C. Liebig, G. Ayala, J. Wilks, G. Verstovsek, H. Liu, N. Agarwal, D.H. Berger, D. Albo, J.M. Lloyd, C.M. McIver, S.A. Stephenson, P.J. Hewett, N. Rieger and J.E. Hardingham, Identification of early stage colorectal cancer patients at risk of relapse post-resection by immunobead transcription-PCR analysis of peritoneal lavage fluid for malignant cells, *Clin Canc Res* **12** (2006), 4217-24.
- [27] G. Lurje, M. Schiesser, A. Claudius and P. M. Schneider, Circulating tumor cells in gastrointestinal malignancies: current techniques and clinical implications, *J Oncol* 2010, ID 392652.
- [28] B. Mlecnik, M. Tosolini, A. Kirilovsky, A. Berger, G. Bindea, T. Meatchi, P. Bruneval, Z. Trajanoski, W.H. Fridman, F. Pages and J. Galon, Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction, *J Clin Oncol* **29** (2011), 610-618.
- [29] F. Nolé, E. Munzone, L. Zorzino, I. Minchella, M. Salvatici, E. Botteri, M. Medici, E. Verri, L. Adamoli, N. Rotmensz, A. Goldhirsch and M.T. Sandri, Variation of circulating tumor cell levels during treatment of metastatic breast cancer: prognostic and therapeutic implications, *Ann Oncol* **19** (2008), 891-7.
- [30] J. O'Connell, M.A. Maggard and C.Y. Ko, Colon cancer survival rates with the new American Joint Committee on Cancer sixth edition staging, *J Nat Cancer Inst* **19** (2004), 1420-5.
- [31] S. Ogino, K. Nosho, N. Irahara, J.A. Meyerhardt, Y. Baba, K. Shima, J.N. Glickman, C.R. Ferrone, M. Mino-Kenudson, N. Tanaka, G. Dranoff, E.L. Giovannucci and C.S. Fuchs, Lymphocytic reaction to colorectal cancer is associated with longer survival, independent of lymph node count, microsatellite instability, and CpG island methylator phenotype, *Clin Cancer Res* **2** (2009), 6412-20.
- [32] P. Paterlini-Brechot and N.L. Benali, Circulating tumor cells detection: clinical impact and future directions, *Cancers Letters* **253** (2007), 180-204.
- [33] P. Pinzani, B. Salvadori, L. Simi, S. Bianchi, V. Distante, L. Cataliotti, M. Pazzagli and C. Orlando, Isolation by size of epithelial tumor cells in peripheral blood of patients with breast cancer: correlation with real-time reverse transcriptase-polymerase chain reaction results and feasibility of molecu-

- lar analysis by laser microdissection, *Hum. Pathol* **37** (2006), 711-718.
- [34] N.N. Rahbari, M. Aigner, K. Thorlund, N. Mollberg, E. Motschall, K. Jensen, M.K. Diener, M.W. Büchler, M. Koch and J. Weitz, Meta-analysis shows that detection of circulating tumor cells indicates poor prognosis in patients with colorectal cancer, *Gastroenterology* **138** (2010), 1714-1726.
- [35] J.P. Thiery and J.P. Sleeman. Complex networks orchestrate epithelial-mesenchymal transitions, *Nat Rev Mol Cell Biol* **7** (2006), 131-142.
- [36] Y.H. Uen, S.R. Lin, D.C. Wu, Y.C. Su, J.Y. Wu, T.L. Cheng, C.W. Chi and J.Y. Wang, Prognosis significance of multiple molecular markers for patients with stage II colorectal cancer undergoing curative resection, *Ann Surg* **246** (2007), 1040-6.
- [37] Y.H. Uen, C.Y. Lu, H.L. Tsai, F.J. Yu, M.Y. Huang, T.L. Cheng, S.R. Lin and J.Y. Wang, Persistent Presence of Post-operative Circulating Tumor Cells is a Poor Prognostic Factor for Patients with Stage I-III Colorectal Cancer after Curative Resection, *Ann Surg Oncol* **15** (2008), 2120-8.
- [38] H. Ueno, H. Mochizuki, Y. Hashiguchi, H. Shimazaki, S. Aida, K. Hase, S. Matsukuma, T. Kanai, H. Kurihara, K. Ozawa, K. Yoshimura and S. Bekku, Risk factors for an adverse outcome in early invasive colorectal carcinoma, *Gastroenterol* **2** (2004), 385-94.
- [39] G. Vona, L. Estepa, C. Bérout, D. Damotte, F. Capron, B. Nalpas, A. Mineur, D. Franco, B. Lacour, S. Pol, C. Bréchet and P. Paterlini-Bréchet, Impact of cytomorphological detection of circulating tumor cells in patients with liver cancer, *Hepatology* **39** (2004), 792-797.
- [40] J.Y. Wang, S.R. Lin, D.C. Wu, C.Y. Lu, F.J. Yu, J.S. Hsieh, T.L. Cheng, L.B. Koay and Y.H. Uen, Multiple molecular markers as predictors of colorectal cancer in patients with normal perioperative serum carcinoembryonic antigen levels, *Clin Cancer Res* **13** (2007), 2406-13.
- [41] J.Y. Wang, C.H. Wu, C.Y. Lu, J.S. Hsieh, D.C. Wu, S.Y. Huang and S.R. Lin, Molecular detection of circulating tumor cells in the peripheral blood of patients with colorectal cancer using RT-PCR: significance of the prediction of postoperative metastasis, *World J Surg* **30** (2006), 1007-13.