Novel Cellular and Molecular Approaches to Stratification and Treatment of Colorectal Cancer

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Abstract
Colorectal cancer is an important disease of the modern world, generating significant mortality and morbidity rates. Its therapy, although considerably improved, continues to be unhelpful for a large percentage of patients, especially for those in advanced stages. This justifies the efforts toward producing new therapies, as well as for stratifying patients according to risk factors and prediction of therapeutic response. In this respect, the contributions of modern science are essential for defining the most intimate mechanisms and players of tumorigenesis and for proposing new biomarkers. The study of antitumor immune responses has revealed new insights into the tumor microenvironment, leading to the development of vaccines and adoptive transfer of immunocompetent cells. Circulating tumor cells are a real opportunity to detect early relapses and to define risk categories, while miRNAs, a family of small non-coding RNA implicated in regulation of gene expression, evolved as a new class of biomarkers with high potential for diagnosis, prognosis and prediction of colorectal cancers.

Key words

Abbreviations

Introduction
Colorectal cancer (CRC) represents the fourth death cause in the world, with over 600,000 deaths per year, of which almost 70% in poorer countries [1]. According to GLOBOCAN statistics for Romania 2008, age-standardized rate for CRC ranked 4th in incidence and 3rd in mortality among all tumor types [2]. Its incidence is still rising, except countries such as the USA, where CRC showed a slight decline in incidence and mortality during the last 20 years [3]. Although the treatment of CRC definitely improved during the past decades, there are patients who do not benefit from the current therapeutic schemes, especially in advanced stages. This justifies the intensive research conducted to
establish the prognostic and predictive role of biological markers. This review summarizes the latest contributions of antitumor immunology, morphopathology of circulating tumor cells, and microRNA assessment in identifying novel biomarkers for CRC. Nonetheless, we present several new therapies which have emerged as alternatives to conventional approaches. Parts of these evaluations are intended to be developed in our research study financed by PN-II-ID-PCE-2011-3-0753 grant.

**Immunological markers and therapies for colorectal cancer**

An important number of cells and molecules of the immune system are now correlated with survival and response to different therapies, due to a large body of data provided by numerous studies performed during the past decades. First of all, the presence of tumor-infiltrating lymphocytes (TILs) was recognized as an independent prognostic factor in CRC since 1987 [4]. Jass et al observed a direct correlation between the number of TILs and survival, and an inverse relation between the presence of TILs and tumor stage. In another study done by Phil et al [5], the density and type of local lymph node-infiltrating lymphocytes were proved to exert an influence on survival, establishing a direct link between the antineoplastic immune response mounted by the organism and the tumor evolution. More recently, the researchers are not focused on TILs as a whole, but on their subtypes, knowing that CD4+ T helper cells, CD8+ T cytotoxic cells, and regulatory T cells (Tregs) play different roles in oncologic surveillance [6].

**CD8+ cytotoxic T lymphocytes** can kill tumoral cells by recognizing the tumor specific antigens presented in the context of MHC class I molecules. In a study conducted on 602 early-stage (TNM I and II) CRC, the density of intratumoral CD8+ T cells with memory phenotype (CD45RO+) proved to be a very good prognostic marker, since recurrence rates were 4.8% versus 75% (p<0.0001) in high and, respectively, low density CD8+CD45RO+ T cells CRC [7]. Similar studies have shown that CRC free of early metastatic invasion had important infiltrates of CD8+ T cells [8]. In 2011, a French group of researchers demonstrated that the density of CD8+CD45RO+ T intratumoral cells is a far better prognostic tool in CRC than TNM classification [9, 10].

**Regulatory T cells** are characterized by their ability to inhibit the auto-reactive and unwanted immune responses. Their role was better understood in the last decade, when it became clear that an immune response is the result of a complex balance between action and suppression. As would be expected, Tregs are detrimental in oncologic surveillance, inducing poor clinical outcomes if accumulated in large quantities in solid tumors [11, 12]. Regarding the impact of Tregs in CRC, there has been a large debate over the latter years, whether these types of cells, if considerably accumulated in tumors, are connected, or not, with a poor clinical outcome. The initial assumption, that Tregs are detrimental in CRC, was questioned by several reports published beginning with 2005 [7]. In 2009, a study conducted on 967 patients diagnosed with stage II and III CRC, evaluated by immunohistochemistry the presence of intratumoral FoxP3+ Tregs, concluding – by multivariate analysis – that a high density of these cells is positively correlated with survival. Treg cells would thus be granted the value of a positive prognostic marker in CRC patients. A possible explanation for this apparent paradox would be the fact that CRC develops in a septic environment, and bacterial products from the intestinal lumen would act as stimulators of inflammation, chronic inflammation being incriminated as a risk factor developing CRC [13]. In 2010, Frey et al [14] demonstrated, on a cohort of 1420 patients with non-metastatic CRC, that the presence of Tregs, characterized by the CD4+FoxP3+ combination, is an independent prognostic marker in MMR (Mismatch Repair) genes-proficient CRC, both in univariate and multivariate analysis; according to this study, CD4+FoxP3+ Tregs correlate with better survival rates. In MMR-deficient CRC, this correlation was kept only in univariate statistical analysis, which has triggered live debates, especially after several researchers failed to identify the above mentioned correlations in their studies [15, 16]. Stratification of CRC patients according to the status of MMR genes in this type of studies is performed due to the fact that a deficiency of DNA mismatch repair genes generates an advanced genomic instability, leading to the appearance of numerous mutated proteins, recognized as tumor associated antigens (TAA), capable of inducing livelier immune responses. MMR-deficient patients have better survival, and this is why the confirmation of novel prognostic markers in CRC takes into account the MMR state [17]. In conclusion, involvement of Tregs in the development of CRC is still under debate, and their function as prognostic markers is yet to be ascertained.

**Myeloid-derived suppressor cells** (MDSC) are classically identified based on the CD11b+CD14-CD33+ phenotype. MDSC inhibit anti-tumor immune responses; they can reach 10 times higher numbers than normal in the peripheral blood of oncologic patients [18, 19]. Recently, a new molecular marker was proposed for identification of these cells: S100A9 [20]. They can have double origin – myeloid or monocyte – and are capable of inhibiting cytotoxic T lymphocytes [21]. Their number increases progressively, from stage I to stage IV of a tumor, metastatic patients presenting with the highest numbers of MDSC in peripheral blood [22]. The role of MDSC as prognostic markers in CRC has not yet been fully established.

**β2-microglobulin** stabilizes the MHC-I complex, when the tumor associated antigens are presented to CD8+ T lymphocytes by tumor cells. Downregulation of β2-microglobulin expression in neoplasias is one of the many evasion strategies observed in CRC [23], as well as in other malignancies or chronic viral infections. Yet tumors which completely suppress β2-microglobulin synthesis and, implicitly, MHC class I assembly, become the target of NK cells. Therefore, it is not surprising to observe that, in CRC
patients, an intermediate expression of MHC-I is associated with reduced survival, indicating that tumors which down-regulate the expression of HLA class I molecules without fully suppressing it, can avoid cytotoxicity caused by T cells and NK cells [24].

**Immunotherapy in CRC** has a history of over one hundred years; at its beginnings, non-specific immune stimulators such as the Coley extract (heat-inactivated extract of Streptococcus and Serratia) were used to boost the anti-oncogenic immune response. During the past decades, clinical trials have tested numerous immunotherapies, ranging from nonspecific to specific – vaccines and monoclonal antibodies. Among the first agents used in a clinical setting, we must mention interleukin 2 (IL-2), a stimulator of T cells. A meta-analysis targeting publications between 1990 and 2004 [25] has shown that IL-2 is efficient only if associated with standard therapy, in early stages of metastatic colorectal cancer (mCRC).

**Antitumor vaccination**

CRC was not initially described as an immunogenic tumor, but recent studies have demonstrated the existence of an antitumor immune response in this case, too: the presence and number of intratumoral T cells is inversely correlated with the tumor stage, and directly correlated with overall survival (OS). Clinical and immunological responses to specific active immunotherapy in CRC were assessed by a meta-analysis including 32 clinical trials and 527 patients [26]. The clinical benefit rate was 11.2%. Antitumor responses were detected in 59% of treated patients. Cell-mediated antitumor responses were present in 44% of patients.

In a more recent study [27], a βHCG-based (beta-Human Chorionic Gonadotropin) vaccine which was administered to 77 patients with mCRC, in a phase II clinical trial: 73% of patients developed anti-βHCG antibodies post-vaccination, 11 patients developed circulating tumor cells (CTC) by reproducible manner, thus allowing the assessment of their presence and number of intratumoral T cells. Vaccination with peptides resulted from IDO fragmentation, induces a cell-mediated immune response based on specific anti-tumor cytotoxic T cells or tolerogenic DC [28]. In a similar fashion, it can be speculated the induction of active immunity toward other immunosuppressive molecules such as TGF-β (Transforming Growth Factor Beta). In a mini-study enrolling 19 CRC patients, vaccination with a synthetic peptide TGFβRII-like, in association with GM-CSF (Granulocyte-Macrophage Colony Stimulating Factor) as a booster of antigen presenting cells, managed to induce an antitumor immune response in all 19 patients [29].

Another antigenic molecule for CRC, the glycoprotein MUC1, was speculated as tumor antigen in CRC patients [30]. L-Blp25, a molecule inducing anti-MUC1 responses, is currently being tested in association with chemotherapy in metastatic CRC. After complete resection of primary tumor and all liver metastasis, the patients are randomized 2:1 to receive either L-Blp25 or placebo. The study will end in 2017 [31].

**Adoptive cell therapy**

In adoptive immunotherapy, T lymphocytes are harvested from the host, conditioned *ex vivo* by stimulation with tumor extracts, expanded and re injected. T cells obtained in this manner are generally CD8+ memory T cells. There are many attempts of adoptive immunotherapy in CRC, of which we are presenting two studies which appear to be more promising. Karlsson and collaborators have harvested T cells from sentinel lymph nodes in 16 patients with stage II-IV CRC; after conditioning and reinjection, 8 patients from the study group were also subjected to chemotherapy. Among patients with disease in stage IV, the outcome was: 4 stable disease, 1 partial response, and 4 complete remissions. There was also a significant increase of OS (2.6 versus 0.8 years). Patients in stages II-III were recurrence-free at 3 years, with the exception of one patient who presented with liver metastases at 6 months [32].

A second clinical trial has combined immunotherapy with chemotherapy to increase the chance of a successful intervention. Correale et al conducted a phase II clinical trial on 47 patients with pretreated mCRC. The patients were administered the GOLFIG combination (FOLFOX + gemcitabine + IL-2 + GM-CSF), with promising results: 10 complete remissions, 16 partial remissions and 16 patients with stable disease. The survival indices were as follows: overall response rate - 43%, time to progression - 12.26 months, OS - 18.76 months. These encouraging results have prompted the Italian group to start a phase III trial entitled GOLFIG-2, which is ongoing, comparing GOLFIG with FOLFOX-4 (oxaliplatin, 5-fluorouracil, leucovorin) in the first line of treatment of mCRC patients. High CD8+ T cells CCR7+ tumor infiltration score has already emerged as a favorable prognostic factor for mCRC in this new trial [33].

**Circulating tumor cells**

An essential element in CRC progress is the process of metastasis [34, 35]. This implies malignant cells leaving the primary tumor and migrating to distant organs, using the vascular or lymphatic system [36]. The presence, in the blood, of cells with morphologic similarities to primary tumor cells, was first spotted by the Australian pathologist T. Ashworth more than 100 years ago [37].

Only recently have the cellular detection methods enabled the isolation of circulating tumor cells (CTC) by reproducible manner, thus allowing the assessment of their role in the clinical evolution of patients [38, 39]. The studies performed at present for CTC identification have proven that these cells have an important role in establishing the
diagnosis, prognosis, or in monitoring of recurrences and therapeutic response for patients with CRC, breast and prostate cancer [40-42]. Existing methods are limited in terms of predicting the clinical evolution of CRC patients or response to therapy, which makes CTC determination a potentially valuable tool for providing new information in this respect [43, 44].

**CTC isolation methods**

Few CTC isolation methods are used today for CRC patients, each of them presenting advantages and disadvantages; they are mainly based on the different morphological and molecular characteristics of epithelial-derived CTC, as opposed to blood cells. At present, most studies are performed using the immunomagnetic separation (IMS) method. This can be achieved by using a classic separation system (MACS, Miltenyi Biotec), or an automatic one (CellSearch, Veridex™), the latter being approved by the FDA [45]. The automatic method uses microbeads covered by anti-EpCAM (Epithelial Cell Adhesion Molecule) antibodies for the isolation of epithelial cells from peripheral blood. Afterwards, the isolated cells are identified by positivity for CKs (cytokeratins) 8, 18, 19 and DAPI (4',6-diamidino-2-phenylindole), and negativity for CD45 [46]. This technique presents the advantages of a high sensitivity and specificity, as well as reproducibility. Being commercially available and approved by the FDA, it is currently used in most of the clinical studies with large numbers of patients. Nevertheless, it also presents a few disadvantages such as selection of a small number of EpCAM-expressing cells, and the impossibility of their subsequent molecular analysis [47, 48]. Another method, based on the size difference between epithelial cells and blood cells, is ISET (Isolation by Size of Epithelial Tumor cells) [49]. In this case, filters with pores of about 8 micrometers are used to obtain a mechanical separation of CTC. It is a quick and easy method; it does not destroy the cells, enabling their further molecular analysis. In terms of disadvantages it has a low specificity, but it also retains cells in epithelial–mesenchymal transition, which do not express EpCAM [50].

Apart from the gene amplification technique, other methods used to confirm isolated cells as CTC rely on identifying certain morphological characteristics of the cell, which requires the experience of a trained pathologist. From this point of view, it was agreed upon several criteria for CTC identification: presence of cytoplasm, irregular nuclear membrane, size of nucleus (>24 micrometers), anisokaryosis (ratio>0.5), an increased nuclear-cytoplasmic ratio and the presence of grouped cells. Depending on such criteria, the cells are categorized in: malignant – 4 criteria, non-determined malignant potential – 2 criteria, and benignant – absence of all criteria. For an accurate identification, several molecular criteria were added (e.g. positivity for CK 8, 18, 19) [51].

Following isolation, PCR (Polymerase Chain Reaction) assays are efficient in vitro assays for amplification of RNA sequences specific for the epithelial phenotype or for stem cells, in order to obtain a molecular characterization of CTC, but do not distinguish between viable and nonviable cells and do not allow further molecular analysis of the isolated cells [52, 53].

**Clinical studies**

The first study showing a correlation between CTC disseminated in the bone marrow and the prognosis of CRC patients was published more than two decades ago [54]. The following studies confirmed the initial results, but they were heterogeneous in respect of detection methods; therefore a standardization of CTC detection methodologies is mandatory for achieving comparability and validation of large scale studies [55, 56].

Regarding the clinical studies which have been so far conducted, there is a difference between those enrolling patients with CRC stage I-III, on one hand, and metastatic stage IV, on the other hand. For stages I-III CRC, the number of isolated CTC is lower, which makes the methods used more sensitive, most of them being based on the PCR technique. Even so, the CTC presence was correlated with poor disease-free survival and overall survival [57, 58].

Metastatic colon cancer has been the subject of even more studies, those including the highest number of patients being presented in Table I. Our presentation is focused on two of the most important, conducted by Cohen et al (430 patients) and Tol et al (467 patients). Presence of CTC in the patients’ blood (3 CTC/7.5 ml) was linked in both studies with lower PFS (progression-free survival) and OS, confirming the prognostic value of CTC in CRC. Concerning the response to treatment, the patients who became negative for CTC after 3-5 weeks (Cohen study) or 1-2 weeks (Tol study, Cairo-2) had a better PFS than those who remained CTC-positive during these intervals [59, 60], stating the CTC as a predictive marker, as well. Unlike other tumors, such as, for example, lung, breast or prostate, for which the threshold of CTC positivity is considered to be at least 5 CTC/7.5 ml blood, in CRC this was established at only 3 CTC/7.5 ml. This is due to two facts: 1) the liver acts as a “filter” for CTC from colorectal tumors, leaving fewer to escape in the systemic circulation; 2) CTC-derived CTC are present more as groups, rather than isolated cells.

Other studies, with smaller number of patients, have produced similar results, indicating the value of CTC assessment in the management of patients suffering from metastatic CRC [61]. An interesting study has attempted to evaluate the relationship between the type of hepatic metastases ablation and the number of CTC, in patients with stage IV CRC. The data collected by this group showed that the CTC number drops when the ablation is made surgically, unlike radiofrequency ablation, when the CTC number increases [62]. If the presence and especially the CTC number were proven to have an impact upon OS and PFS, it remains yet to establish the CTC role in indicating and monitoring systemic chemotherapy [63]. This would be clinically significant, because from stage II CRC patients 25% will progress, and chemotherapy would be useful in their case.
Stratification and treatment of CRC

Whereas at present there are no means of identifying these patients, highlighting CTC could serve as a guide for the therapeutic decision. Additionally, in those patients who already have an indication for systemic treatment, choosing the type and dosage of chemotherapy could be assisted by CTC determination at different time points [64, 65].

**MicroRNAs as new molecular tools for clinical applications**

MicroRNAs (miRNAs) represent small non-coding RNAs of about 22-nucleotides (nt), transcribed from individual genes, mostly located in intergenic regions, in introns of protein coding genes or in introns and exons of non-coding genes [66]. miRNAs are processed from stem-loop precursors into miRNA/miRNA* duplexes. One strand of these duplexes is usually incorporated into the RNA-induced silencing complex (RISC) which interacts with the 3’untranslated region (3’-UTR) of the mRNA targets, inducing translational repression or mRNA (messenger RNA) cleavage [67]. Generally, miRNAs are involved in negative regulation of gene expression at the post-transcriptional level, but there have been reports about positive regulation of gene expression mediated by miRNAs [68]. Their activities are involved in many crucial biological processes, including metabolism, survival, differentiation, apoptosis and proliferation [69, 70].

A considerable advantage of implementing miRNAs as novel molecular tools derives from the fact that a single miRNA can target and regulate the expression of hundreds of miRNAs. Therefore, it is easier to work with a small number of miRNAs than with miRNAs to discover biomarkers of interest with higher sensitivity and specificity. The data obtained by genomic high-throughput technologies provide specific profiles of up-regulated and down-regulated miRNAs for many cancer types [71, 72]. Another advantage of miRNAs is their high stability and resistance to storage and handling, including archived formalin fixed paraffin embedded (FFPE) tissues and body fluids [73, 74].

Several methods including quantitative real-time PCR (qRT-PCR), miRNA-microarray and deep sequencing (next-generation sequencing) are currently available for miRNA detection. Generally, qRT-PCR is used to evaluate a small number of miRNAs, while miRNA-microarray and next-generation sequencing are used for assessing comprehensively screening panels of miRNA. Even if qRT-PCR is considered the most sensitive and reproducible method for miRNA quantification, there are some limitations regarding the consensus about housekeeper miRNAs or endogenous controls used for normalization. The common controls used for miRNAs quantification are: U6 small nuclear RNA (RNU6B), 5SrRNA, let-7a, mir-16, mir-345, mir-16, mir-223 [75-77].

**MiRNAs in colorectal pathology**

**MiRNAs in CRC diagnosis**

Nowadays, early diagnosis of CRC is based on colonoscopy and fecal occult blood test (FOBT). Colonoscopy represents the most reliable method for CRC detection, but has a major drawback due to its invasive nature. Instead, FOBT represents a widespread CRC noninvasive test, but has low sensitivity especially for pre-neoplastic lesions [78]. Thus, it is necessary to identify a new class of noninvasive biomarkers for early detection of CRC, miRNAs being regarded as valuable candidates to this challenge. miRNAs could influence colorectal carcinogenesis by direct regulation of their mRNA targets, represented by oncogenes or tumor suppressor genes. Wnt/β-catenin, PI3K, KRAS, p53 or regulators of extracellular signals are the most important pathways mediated by miRNAs [79, 80].

Identification of miRNAs in the body fluids has followed the discovery of tissue miRNAs. The studies on serum or plasma from patients suffering from CRC are relatively recent and suggest the possible use of miRNAs as novel noninvasive biomarkers for cancer detection [81-84]. In one of these studies, Pu et al have analyzed plasma miR-21, miR-221 and miR-222 as possible noninvasive biomarkers for cancer detection [81-84]. In another study, Huang et al identified miR-29a and miR-92a as...
noninvasive biomarkers for early detection of CRC versus advanced adenoma. Ng et al reported increased levels of miR-135b, miR-95, miR-222, miR-17-3p, and miR-92 in the plasma of CRC patients when compared with plasma of healthy subjects. They showed that miR-92 plasma levels can distinguish patients with CRC from gastric cancer, inflammatory bowel disease (IBD) and healthy subjects, with 89% sensitivity and 70% specificity. Cheng et al tried to correlate CRC stage of evolution with plasma levels of three miRNA types: miR-21, miR-92, and miR-141. They found that only miR-141 was significantly associated with stage IV CRC versus other stages, with 77.1% sensitivity and 89.7% specificity. The authors proposed a more accurate screening program for CRC patients, combining the miR-141 level with the CEA test.

Link et al [85] identified an alternative miRNA test using fecal samples. They found that miR-21 and miR-106a are up-regulated in CRC fecal samples compared with healthy subjects and the expression of these two miRNAs decreases with advanced stages.

The role of miRNAs in CRC prognosis

Xi et al [86] reported for the first time the utility of miRNAs in CRC prognosis. They found that a high level of miR-200c in CRC is significantly correlated with patient survival. In a miRNA-microarray study, the group of Motoyama identified miR-18 as being involved in CRC prognosis. They reported that CRC patients with high levels of miR-18 have a poorer clinical prognosis compared to the low expression group [87]. Moreover, high levels of miR-21, which acts as a putative oncogene, were correlated with poor survival in CRC patients [88]. Schepeler et al [89] reported a molecular signature based on four miRNAs (miR-142-3p, miR-212, miR-151, miR-144), used for predicting tumor recurrence in stage II colon cancer. Even if these data reveal a significant potential for miRNAs as prognostic markers in CRC, larger studies are required to strengthen these facts.

Predicting treatment response in CRC based on miRNAs

An important step in CRC treatment involves modulating the response of CRC cells to chemotherapeutic drugs. Song et al [90-92] reported miR-192, miR-215 and miR-140 as being involved in modulation of chemoresistance of CRC cells. Overexpression of miR-192 led to inhibition of dihydrofolate reductase (DHFR) and an increased chemosensitivity of CRC to methotrexate (MTX). The authors also found that overexpression of miR-192 could lead to an increased chemoresistance to MTX and tomudex (TDX), and overexpression of miR-140 is linked with increased chemoresistance to MTX and 5-fluorouracil (5-FU). In another study, Wang et al [93] reported that the inhibition of miR-31 in CRC could increase the sensitivity to 5-FU at an early stage.

Other strategies for boosting chemo-responsiveness in CRC cells are based on increasing the tumor suppressor-like activity and/or on repressing the oncogenic-like activity of miRNAs. In this respect, the overexpression of miR-143 [94] and miR-34a [95] in CRC cell lines have led to increased chemosensitivity to 5-FU. Li et al [96] have shown that overexpression of miR-22 in p53-mutated CRC cell lines has increased the chemosensitivity to paclitaxel. The inhibition of miR-21, which acts as a potential oncogene, may improve the response to CRC therapy [97].

Conclusions

Although none of the cells or molecules described above has been included in clinical guidelines for the management of CRC, there is hope that the oncology practitioner will soon benefit from this extensive biomedical research. The immunologic approach shifts the focus from the tumor toward the other major protagonist involved in oncogenesis, namely the immune system of the host. Antitumor immunity can offer clinically significant biomarkers, and opportunities for improving existing therapies. CTC isolation and molecular analysis could open new perspectives in understanding the metastatic process, leading to the development of new treatments or to the customization of those already existing. The high stability of miRNAs renders them important candidates for a new class of biomarkers, potentially useful for improving diagnosis, prognosis and prediction in CRC. Larger studies are required to validate the clinical usefulness of these biomarkers in colorectal carcinoma, their role as prognostic or predictive factors, either alone or in combination with other known markers.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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