

Improving diagnosis, prognosis and prediction by using biomarkers in CRC patients (Review)

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Abstract. Colorectal cancer (CRC) is among the most common cancers. In fact, it is placed in the third place among the most diagnosed cancer in men, after lung and prostate cancer, and in the second one for the most diagnosed cancer in women, following breast cancer. Moreover, its high mortality rates classifies it among the leading causes of cancer-related death worldwide. Thus, in order to help clinicians to optimize their practice, it is crucial to introduce more effective tools that will improve not only early diagnosis, but also prediction of the most likely progression of the disease and response to chemotherapy. In that way, they will be able to decrease both morbidity and mortality of their patients. In accordance with that, colon cancer research has described numerous biomarkers for diagnostic, prognostic and predictive purposes that either alone or as part of a panel would help improve patient's clinical

management. This review aims to describe the most accepted biomarkers among those proposed for use in CRC divided based on the clinical specimen that is examined (tissue, faeces or blood) along with their restrictions. Lastly, new insight in CRC monitoring will be discussed presenting promising emerging biomarkers (telomerase activity, telomere length and micronuclei frequency).

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Abbreviations: CRC, colorectal cancer; CIN, chromosomal instability; MSI, microsatellite instability; CIMP, CpG island methylation phenotype; CEA, carcinoembryonic antigen; ctDNA, circulating tumor DNA; CA 19-9, cancer antigen 19-9; CTCs, circulating tumor cells; PI3K, phosphoinositide 3-kinase; CKs, cytokeratins; APC, adenomatous polyposis coli; CDH17, cadherin 17; PFS, progression-free survival; OS, overall survival; hTERT, human telomerase reverse transcriptase; MN, micronuclei; TERC, telomerase RNA component

Key words: colorectal cancer, diagnosis, prognosis, prediction, biomarkers, DNA markers, non-DNA markers

1. Introduction

Colorectal cancer (CRC) is ranked as one of the most common types of cancer. In fact, according to data from the USA, for the male population it is ranked third of the most diagnosed, after lung and prostate cancer, and for the female population, it is ranked second, following breast cancer (1). Moreover, it is considered as one of the leading causes of cancer-related death worldwide (2,3) In the USA alone, CRC is responsible for the second greatest number of cancer-related deaths. As a matter of fact, the American Cancer Society estimated that for 2013 alone, the number of first diagnosed CRC cases and that of deaths due to CRC was as high as 142,820 and about 50,830, respectively (4). However, regardless of the fact that both public and medical awareness concerning CRC has risen during the last decade, approximately 50% of the patients referred with CRC, at the time of diagnosis present distant metastases. It is clear that CRC is a rather heterogeneous disease by means of its various clinical manifestations, biological behavior and

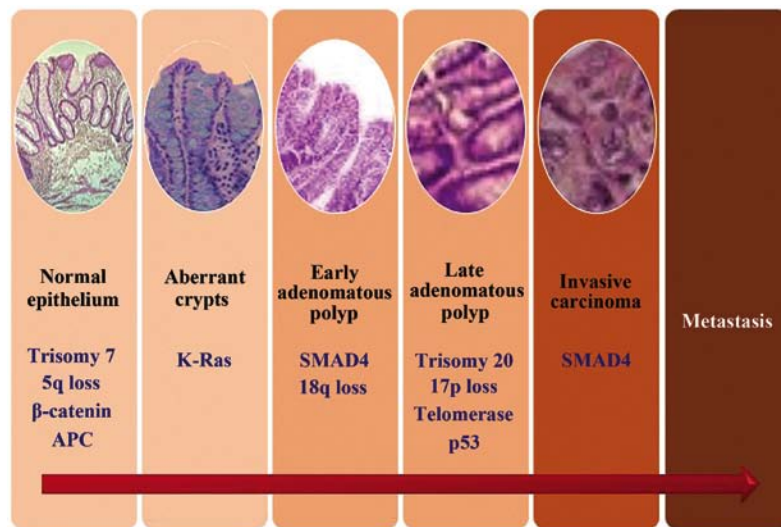


Figure 1. Steps of CRC progression and a summarized rendering of the pathogenetic model. CRC, colorectal cancer; APC, adenomatous polyposis coli.

in-tumor variety of mutations (5,6) making it a true challenge, not only to detect in an early stage, but also to treat or even manage in the long term. Nowadays, it is evident that CRC is a multifactorial/polygenic disease arising both due to epigenetic as well as genetic manifestations occurring in a number of genes with an unparalleled role for the maintenance of normal cellular homeostasis. Such genes may be tumor suppressor genes, oncogenes, mismatch repair genes and cell cycle regulating genes in colon mucosal cells (7). Intense research has highlighted the importance of three major molecular pathways as the main cause of these genetic alterations that in turn result in carcinogenesis: Chromosomal instability (CIN), microsatellite instability (MSI), and the CpG island methylation phenotype (CIMP) (5,8). All these pathways attribute to the transformation of an adenoma to carcinoma, a multistep carcinogenic process known as the adenoma-carcinoma sequence (9), which is thought to be a common site for all CRCs. An illustrated form of this process is presented in Fig. 1. However, the period between those two hallmarks provides a diagnostic window for the early detection of CRC. As a result, the current research trend aims to describe new markers for diagnosis (these markers are meant to be used for risk stratification and early detection of colorectal polyps), prognosis (these markers will give an indication of the likely progression of the disease) and prediction of the biological behavior of a certain therapeutic regimen, making the clinician able to transform the knowledge of the tumor biology into a personalized decision-making process for each patient (8,10,11). Therefore, the information coming from CRC biology combined with the assessment of serum and tissue markers with prognostic and predictive value, currently constitutes, the pillars in the treatment of early-stage cases as well in the clinical management of advanced disease offering new methods to estimate the therapeutic efficacy and the overall outcome. An overview of the current and potential biomarkers used in CRC is shown in Fig. 2. In addition to CRC biomarkers, novel molecular imaging techniques using hybrid positron emission tomography (PET)/computed tomography (CT) systems enable accurate initial staging, efficient assessment of treatment response, and follow-up, facilitating

individualized treatment strategies in CRC patients (12). Furthermore, novel positron emitting radiopharmaceuticals together with hybrid PET/magnetic resonance (MR) systems, which provide enhanced soft tissue resolution and incremental diagnostic information from functional MR methods such as diffusion-weighted imaging, hold promise for increased accuracy in CRC staging, restaging, early detection of recurrence, and accurate treatment planning for radiotherapy (13).

2. Diagnostic markers

General. Concerning the detection of CRC in the general population, the screening methods most commonly offered until now have included faecal occult blood testing (FOBT), flexible sigmoidoscopy and colonoscopy while CT colonography is a more recent addition to the CRC screening modalities (14). Even though screening has clearly proved to decrease the risk of CRC-associated mortality, screening effectiveness is restricted by limitations of test performance, inadequate access to CRC screening tests and loose screening compliance. Consequently, a great number of patients at the time of diagnosis present with locally advanced or metastatic disease, a phenomenon that is observed even among prosperous nations, including the United States (15). In accordance to that, CRC researchers focus their research on innovative ideas to identify molecular markers for the development of highly accurate, non-invasive screening tests for CRC in the hope of increasing the compliance of the population and to decrease potential unwanted side-effects which accompany the more invasive techniques. Several molecular classes have been tested for their potential use in CRC screening: DNA (16-18), proteins (19), messenger RNA (mRNA) (20) and microRNA (miRNA) (21-24), and have all proven to be quite promising in early phase biomarker studies (25). However, until now, only two tests (faecal haemoglobin and DNA-based markers) meet the pre-clinical and clinical criteria required for their efficient transduction from the laboratory to the clinical setting. In fact, recently, a multi-target stool DNA (MT-sDNA) test has proven better sensitivity, although with lower specificity, to faecal haemoglobin by immunochemical testing for

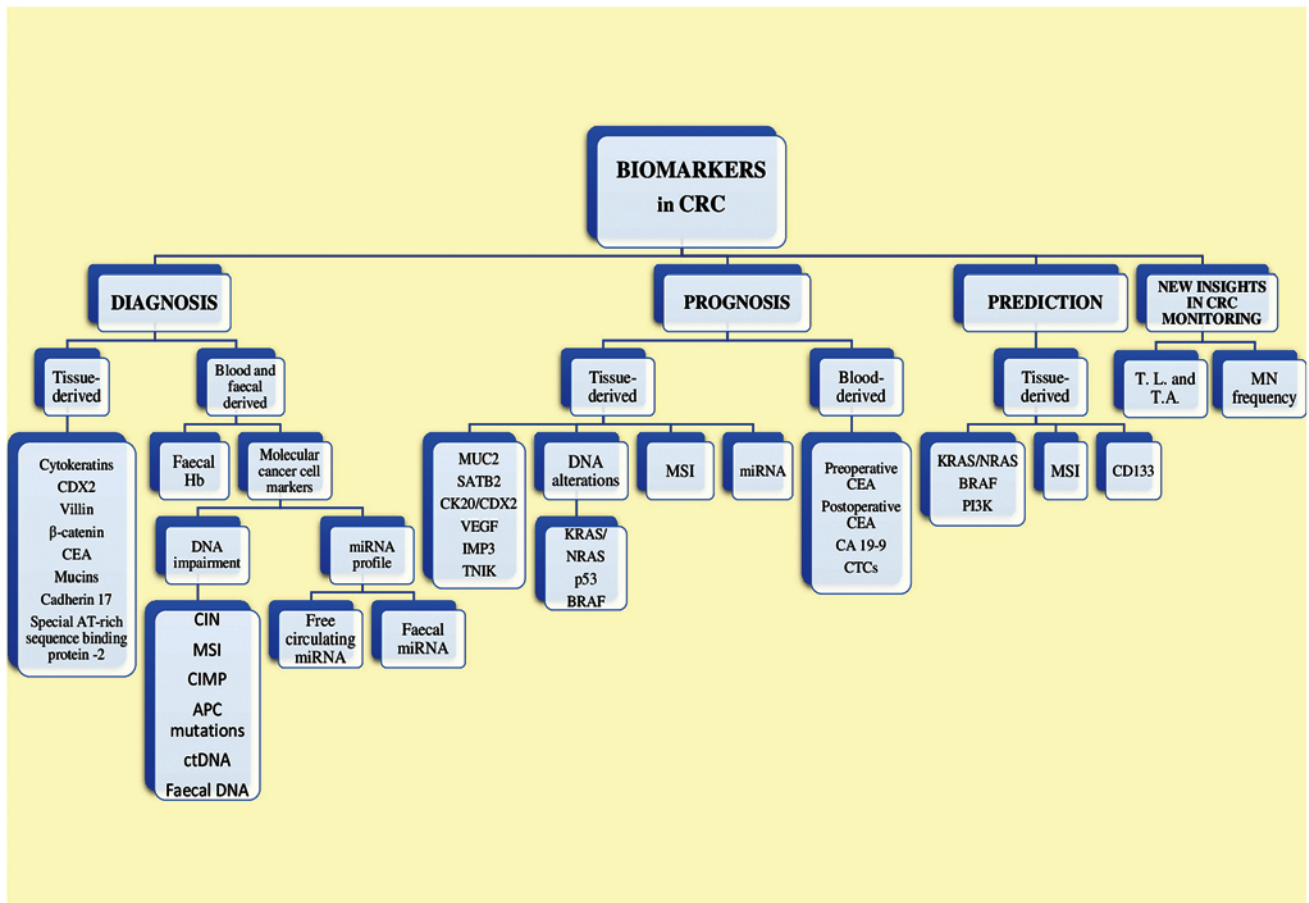


Figure 2. A graphic overview of the current and potential biomarkers used in CRC. CRC, colorectal cancer; CDX2, caudal type homeobox 2; CEA, carcinoembryonic antigen; CIN, chromosomal instability; MSI, microsatellite instability; CIMP, CpG island methylation phenotype; APC, adenomatous polyposis coli; ctDNA, circulating tumor DNA; miRNA, microRNA; SATB2, special AT-rich sequence binding protein 2; CK, cytokeratin; VEGF, vascular endothelial growth factor; IMP3, insulin-like growth factor-II mRNA-binding protein 3; TNK, Traf2- and Nck-interacting kinase; BRAF, B-rapidly accelerated fibro-sarcoma (proto-oncogene); MSI, microsatellite instability; CA 19-9, cancer antigen 19-9; CTCs, circulating tumor cells; PI3K, phosphoinositide 3-kinase; MN, micronuclei.

the detection of curable-stage CRC and advanced adenomas while exhibiting overall cancer detection similar to colonoscopy (26). As a result, stool DNA testing was approved in the United States by FDA for general population screening of average risk, asymptomatic individuals in 2014.

Invasive techniques and diagnostic tissue markers

The most commonly invasive technique used, colonoscopy, provides the clinician a great advantage, to evaluate in real time the presence/absence of a polyp and to resect it at the same time, if possible, thus representing the standard tool of practice for CRC evaluation (10). Resecting a polyp gives the clinician an opportunity to use immunohistochemical (IHC) staining, typically used in the diagnosis of gastrointestinal neoplasms, in order to facilitate accurate tumor classification. By doing that, two main goals are set: The first one is to exclude morphologic mimickers or to identify the closest morphologically tissue or organ of origin in cases of metastatic carcinoma of unknown origin and thus confirm the diagnosis. The second one is to help estimate the most likely prognosis and even predict response to a given chemotherapy or novel molecular-targeted therapy (15,26). Diagnostic tissue biomarkers therefore provide additional and fundamental information that complement clinical colonoscopy findings.

Cytokeratins (CKs). CKs, proteins expressed by epithelial cells, are members of the intermediate filaments family along with vimentin, desmin, neuro-filament, and glial-filamen. Numerous studies have attempted to identify a possible expression pattern of CKs and connect it with either the organ of origin (in order to determine whether it is a primary CRC) or with tumor progression. However, as more and more studies are conducted it is becoming clear that such a connection is not likely to be identified in the near future. To begin with, CK7 and CK20 are helpful when the clinician needs to differentiate metastases from CRC, which are usually CK7/CK20⁺, from other tumors (27). CK20 almost selectively stains the normal gland cells of the colonic mucosa and Merkel cells while its expression is rarely may be seen in the urothelium or other mucosas (28,29). By contrast, CK7 is usually expressed in urinary bladder and female genital tract epithelia, mesothelium, normal lung, and, rarely, it may be observed in gastric and intestinal normal glands. However, the majority of researchers agree that it is not found in normal colonic mucosa (28,30). Based on these findings, the immunophenotype CK7/CK20 is used as a routine in order to differentiate CK20-expressing metastasis of colorectal adenocarcinomas from lung, ovarian or bladder carcinomas, which are usually stained with CK7 (31). However, it is reported that

non-neoplastic colonic mucosa proximal to the rectum exhibits a CK7/CK20⁺ phenotype, as is the case for 90% of CRCs (32). When CK17 is included in the diagnostic panel, the efficacy of the test is improved as less than 10% of CRCs express CK17 in contrast to other carcinomas that are more often positive for CK17 (including stomach, endometrium and urine bladder). In addition, pancreatic ductal carcinomas are consistently positive and a number of carcinomas from other sites, may exhibit CK17 expression (33). Furthermore, when CRC progression is studied, CK20 and CK7 can be useful. Results indicate that advanced CRCs were more often CK20⁺/CK7⁺ compared to early-stage cancers, which were predominantly CK20⁺/CK7⁻. Thus, CK7 expression may be a differentiating marker for the progression of CRC (34).

Caudal type homeobox 2 (CDX2). CDX2 is a transcription factor encoded by *CDX2* gene, a member of the caudal subgroup of homeobox genes. Its main role is to ensure maintenance of a cellular intestinal phenotype during the *in utero* and *ex utero* life (35). CDX2 presents strong expression patterns in epithelia of the normal small intestine, appendix, colon, and rectum as well as in the pancreatic centroacinar and interacinar ductal cells (36). It is revealed that loss of CDX2 may give birth to human CRC. CRCs, beside those exhibiting MSI, are consistently CDX2-positive (37). In fact, a quite interesting research recently investigated the effect of restoration of CDX2 expression on colon cancer cell viability, colony formation, cell cycle distribution, apoptosis, invasion ability and xenograft tumor growth in nude mice (38). According to the researchers, CDX2 upregulation significantly reduced the life span and inhibited colony formation, and the invasion and migration ability of LoVo cells. Moreover, it was able to induce cell cycle arrest and apoptosis *in vitro*, especially under hypoxic culture conditions (38). According to data from histological studies, expression patterns of CDX2 are found in a variety of neoplastic tissues such as adenocarcinomas that exhibit intestinal-type differentiation, including adenocarcinomas of the gastroesophageal junction, bladder, urachus, small bowel, pancreas, appendix, and ovary (37).

Villin. Villin is a cytoskeletal actin-binding protein that is associated with the microfilament family. It is normally found in cells that exhibit highly specialized, brush border-type microvilli, similar to enterocytes (39). Villin is associated with elimination of polarity that the epithelium exhibits, thus altering tissue architecture (40). Thus, in CRCs containing a micropapillary pattern, villin IHC has been effective for tracking the polarity in this type of CRC (41). The specificity of villin as a marker of intestinal origin is limited, similar to CDX2, as positivity may also be marked in adenocarcinomas with intestinal differentiation arising from a wide variety of organs including stomach, lung, and ovary as well as in malignancies of other sites such as the endocervix and liver (the latter more rarely, though) (42).

β-catenin. β-catenin is a multifunctional protein that is involved both in cell adhesion and intracellular signaling, with the latter being accomplished through β-catenin's actions through the Wnt signaling pathway (43). Activation of the Wnt signaling pathway (Fig. 3) increases the cytoplasmic pool of free β-catenin and, to a smaller extent, the nuclear pool where it induces proliferation. The Wnt signaling pathway in its normal form is highly activated in the majority of CRCs

as a result of mutant adenomatous polyposis coli (*APC*) or β-catenin. Moreover, upregulated Wnt signaling has a key role in the pathogenesis of CRC (44). In the absence of functional *APC*, as it often happens in CRC, nuclear β-catenin can be identified immunohistochemically (44,45). Although nuclear expression of β-catenin is not unique to CRC, it can be proven useful as part of a diagnostic panel.

Carcinoembryonic antigen (CEA). CEA are membrane-associated glycoproteins playing a number of roles in cell adhesion or signal transduction (46). Monoclonal CEA (mCEA) may be expressed in a wide variety of adenocarcinomas, including those arising from the colon (47). Although this lack of specificity limits its value as a diagnostic marker of CRC, it remains a useful component of a broad diagnostic panel. In a meta-analysis study by Tan *et al* (48) based on 20 different studies, serum CEA has been demonstrated to comprise an exam of elevated specificity, although sensitivity was inadequate when tracing CRC recurrent conditions. The cut-off range varied among these studies from 3 to 15 ng/ml, suggesting that a measure of 2.2 ng/ml would be the optimal regarding sensitivity and specificity (48). Evidently, at this point circulating CEA may constitute a primary means of recording progress in patients' surgical follow-up, in accordance with the complementary tools including clinical picture, radiological response and histological results.

Mucins. Mucins are high molecular-weighted, heavily glycosylated proteins (49). Within the polypeptide of these glycoprotein molecules tandemly repeated sequences of amino acids rich in threonine and serine moieties lead to various combinations of these repeats making each mucin type unique. In colon, a mixture of neutral, sialomucin, and sulphomucin are normally met, with MUC2 being the most prominent, primarily in goblet cells. MUC4 is also abundant in the colon and its expression is found both in goblet and columnar cells, whereas MUC3 appears to be expressed primarily within enterocytes (50). MUC1, MUC5AC, and MUC6 under normal conditions are not expressed in the colonic mucosa (50). MUC2 is frequently expressed in mucinous CRC as well as mucinous carcinomas of the ovary, breast, and pancreas (50). Gastric mucins may also be expressed in CRC (51). MUC5AC expression is associated with mucinous differentiation and MSI, while most mucinous carcinomas exhibit a MUC2⁺/MUC5AC⁺ phenotype (52). Additionally, MUC21 has recently proven to be expressed in CRCs (53). Notably, although a correlation between mucin expression and clinical characteristics was observed by Wang *et al* (54) an increased expression of MUC5 was found to be associated with poor cellular differentiation, lymph node metastasis, advanced tumor stage and a poor overall prognosis in CRC. By contrast, a decreased expression of MUC2 was reported in cases with lymph node metastasis, poor cellular differentiation and an advanced tumor stage in CRC. These results suggest that MUC2 and MUC5 levels could be associated with tumor progression and even be used in order to facilitate the early diagnosis and clinical characterization of CRC (54).

Cadherin 17 (CDH17). CDH17 was first described in the liver and intestine of rats (55). Later on, the research in humans revealed that its expression is limited to the intestine (both small and large) and in a part of the pancreatic duct. As for its function, it serves as an intestinal peptide transporter (56). However, its

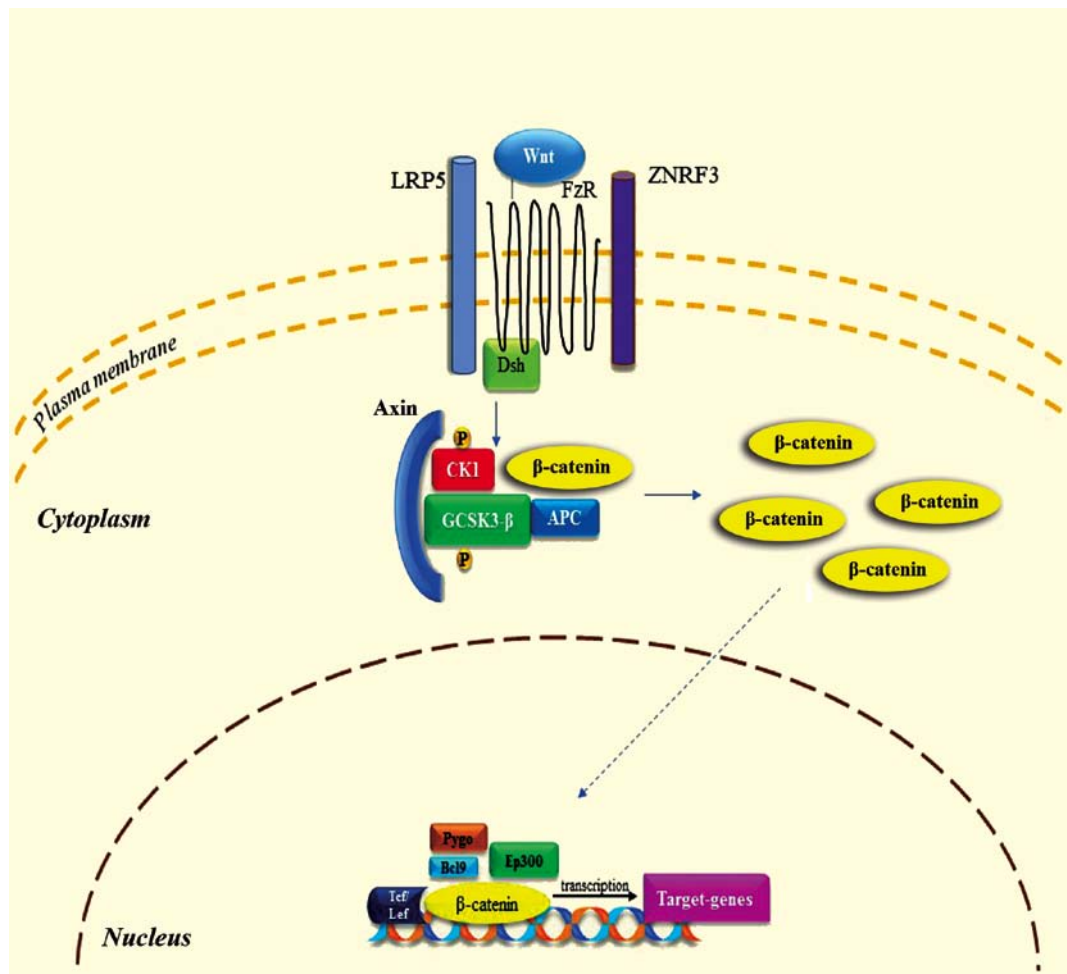


Figure 3. Wnt/ β -catenin signaling pathway. When Wnt is present, it binds to FzR and LRP5 receptor. Dsh enables the phosphorylation of GSK-3 β and CK1, resulting in the binding of axin. Following to accumulation of β -catenin and translocation to the nucleus, it binds to various factors in order to modulate the transcription of target-genes. These processes lead to antiapoptotic results and promote cellular proliferation. FzR, frizzled receptor; LRP5, low-density lipoprotein receptor-related protein 5; GSK3- β , glycogen-synthase kinase 3 β ; CK1, casein kinase 1; ZNRF3, zinc and ring finger 3; APC, adenomatosis polyposis coli; Pygo, pygopus; Bcl9, B-cell CLL/lymphoma 9; Ep300, E1A binding protein p300; Tef, T-cell factor; Lef, lymphoid enhancer-binding factor 1.

clinical applicability in GI tumors diagnosis was only recently recognized (57). Recent data indicate that positive CDH17 immuno-reactivity is frequently seen in colorectal adenocarcinomas up to 96% and to a significant portion of gastric (58), pancreatic, and biliary adenocarcinomas (25-50%) (59). By contrast, it is rarely found in adenocarcinomas arising from extra-GI tract (1-10%). Notably, even though CDH17 is transcriptionally regulated by CDX2, it is more sensitive and specific than CDX2 for the identification of primary and metastatic colorectal adenocarcinoma (57-59). CDH17 also seems to be useful in diagnosing CRC variant with poorly differentiated or even undifferentiated morphology, such as medullary carcinoma, which characteristically lacks the expression of conventional intestinal differential markers, such as CK20 and CDX2 (57). In a study where the effect of the downregulation of CDH17 upon human colon cancer cells was examined, the researchers found that the downregulation of extrinsic *CDH17* gene can conspicuously promote apoptosis-inducing effects of nospapine on specific human colon cancer cell lines, which may indicate a novel strategy to improve the chemotherapeutic effects on colon cancer (60).

Special AT-rich sequence binding protein 2 (SATB2). SATB2 is a member of the nuclear matrix-associated

transcription factors family whose role is to serve as epigenetic regulators of gene expression with high tissue selectivity (61). Research has shown that SATB2 has a variety of biologic functions. However, the exact role of SATB2 in the GI tract is still unknown. Recently, Magnusson *et al* (61) suggested that SATB2 immunoreactivity was mainly encountered in the glandular lining cells of the lower GI tract, including appendix, colon, and rectum. In fact, SATB2 is a highly sensitive and specific marker for adenocarcinomas of the colon and rectum, with a diagnostic sensitivity of 97% (121 of 125 cases) in CRCs and of 81% in CRC metastases (61). Moreover, Bian *et al* (59) found that the combination of CDH17 and SATB2 serves as potential optimal markers for the differential diagnosis of pulmonary enteric adenocarcinoma, a rare type of non-small cell lung cancer exhibiting similar histological and immunohistochemical morphology to colorectal adenocarcinoma, and metastatic colorectal adenocarcinoma (59).

Non-invasive techniques and diagnostic blood-derived and faecal markers

Faecal haemoglobin detection tests. Until recently, non-invasive techniques used for diagnostic purposes were represented mainly by the guaiac faecal occult blood test (gFOBT) and

faecal immunochemical test (FIT). The gFOBT detects blood loss from peptic ulcer and gastrointestinal cancer. However, its validity in terms of CRC diagnosis, is rather restricted as gFOBT is, not only unable to distinguish bleeding between the upper and the lower GI tract, but also lacks the ability to specifically distinguish human from non-human haeme (8,10). Moreover, gFOBTs are not sensitive to small bleedings, while specificity can be affected by diet or drugs and they have a fixed hemoglobin concentration cut-off determining positivity. As a result, gFOBT exhibits low sensitivity in terms of the detection of cancerous and preneoplastic lesions (30-40%) (62), whereas the sensitivity to detect an advanced adenoma (polyp, >9 mm; villous features or high grade dysplasia) is only 18% (63). On the other hand, FIT is able to detect human globin by means of an antibody-based assay providing a qualitative or quantitative result (depending on the kit used) expressed as faecal haemoglobin concentration per gram of faeces. FITs are analytically more specific, capable of quantitation and hence provide flexibility to adjust cut-off concentration for positivity and the balance between sensitivity and specificity. FITs are clinically more sensitive for cancers and advanced adenomas, and because they are easier to use, acceptance rates are higher (64). However, FIT can only detect bleeding from colonic pre-neoplastic lesions. Thus, when used for CRC screening, the sensitivity FITs demonstrates in detecting adenomas >1 cm in diameter is only 20-30%, while for advanced neoplasia or large adenomas sensitivity ranges between 20 and 67%, which is comparable or superior to the sensitivity of gFBOT (64,65). Another very important aspect that limits occult blood testing is the fact that it detects significantly more in the left- than right-sided lesions in the colon (66), which is a significant issue given the increased incidence of right-sided CRCs that has developed over the last two decades (67). In other words, it is evident that those two non-invasive diagnostic techniques are limited by their intrinsic lack of sensitivity and specificity (with the latter being the case for gFBOT) making them unable to stand on their own as diagnostic tools. Table I presents a summarization of bibliographical references regarding FOBT sensitivity and specificity.

Blood-derived and faecal molecular cancer cell markers. Our improved knowledge of the molecular pathogenesis that conditions the polyp→adenocarcinoma progression sequence has made clear that the molecular changes found in polyps and adenocarcinomas have the potential to serve as neoplasm-specific molecular markers for these lesions. The concept of using these molecular markers for CRC screening is indeed the next step for a well-established non-invasive detection method for CRC. In contrast to heme detecting tests, tests focusing on molecular markers derived from neoplastic cells of the colon can prove to be more accurate. CRCs are known to bear distinguishable genetic and epigenetic changes as they develop and progress, which forms the rational of stool-based DNA and RNA testing.

DNA impairment. Approximately 90% of CRCs develop sporadically, and only a few cases (<10%) are hereditary, with familial adenomatous polyposis, hereditary non-polyposis colorectal cancer (HNPCC), MUTYH-associated polyposis, Peutz-Jeghers syndrome (PJS) and serrated polyposis syndrome being the main representatives of hereditary causes of CRC. Currently, three major paths for CRC development have been

described, with CIN being the most common accounting for 70-80% of CRCs, the MSI pathway positioned in the second place accounting for 5-20% of tumors and in the third place the CIMP, which represents approximately 15% of CRCs (68). These pathogenetic pathways can be examined either in serum samples (CIN, MSI, CIMP, APC) using immunohistochemical techniques or in faeces (CIMP, APC).

a) CIN. CIN is the hallmark characteristic of most CRC cases (80-85%), and its main characteristic is the extensive abnormality in chromosome number (aneuploidy) and loss of heterozygosity. CIN can be observed in several forms, including chromosomal numerical abnormalities, small sequence modifications such as base deletions or insertions, chromosomal rearrangements and gene amplification (5).

b) MSI. Microsatellites (MSs) are short tandem-repeated base pairs of 1-6 scattered all over the genome. For the normal human genome the number of MSs is approximately half a million. Genome studies revealed that MSs are prone to duplication errors. However these errors are usually corrected by the MMR system (5,69,70). Consequently it is logical to assume that a defective MMR system would result in the accumulation of DNA mistakes and thus MSI. Indeed, MSI arises by the inhibition of MMR system either via defective methylation of *MLH1* in CpG island or point mutation of any MMR genes (*hMLH1*, *hMSH2*, *hMSH6*, *PMS1* and *PMS2*) especially hypermethylation of *hMLH1* promoter (5). It is estimated that about 15-20% of CRC patients present MSI with a small fraction of which 2-4% are related to HNPCC (71). In order to estimate MS status, Bethesda panel was agreed in which five MS loci were included (*BAT25*, *BAT26*, *D5S346*, *D2S123*, and *D17S250*) (72). However, some researchers suggested an expanded Bethesda panel include 10 loci. Based on this panel, MSI can be divided into three groups: MSI-high (MSI-H), defined as having ≥30% unstable loci using mononucleotide or dinucleotide markers (8,73); MSI-low (MSI-L), with 10-30% unstable loci; and microsatellite stable (MSS), with <10% unstable loci (73). MSI status varies according to a given CRC stage: Stage II CRC exhibits high prevalence of MSI (20%) while in stage IV CRC MSI is approximately 4% (74,75). Moreover, differences based on the MSI status are found when prognosis is examined. For example, cases with MSI-H CRC share a better prognosis than that with MSS CRC (5,76).

c) CIMP. It is well accepted that DNA methylation is a key process for the normal growth of eukaryotes. If occurs in cytosine number 5 within CpG island which is present in 50% of tumor suppressor gene promoters (77). CpG island accounts for >70% of CG sequences that extend to 0.4 kB on the genome (78). Even though hypermethylation of CpG island cytosine represents a hallmark for cancer progression, both hypomethylation and hypermethylation may lead to the transformation of normal mucosa to adenoma and subsequently to the development of CRC (79). Disturbance of epigenetic programming (epigenetic modification including DNA methylation, histone modification and post-transcriptional gene regulation) is closely related to the development of CRC (80). It is reported that a wide spectrum of aberrant methylated genes in CRC, regulates crucial functions in the normal cell regarding proliferation and maintenance of genome stability (77). In the clinical setting, abnormal DNA methylation patterns can be detected in patient's blood or faeces samples from which CRC

Table I. A summarization of bibliographical references to FOBT sensitivity and specificity.

Author/(Refs.)	Faecal occult blood testing
Kronborg <i>et al</i> (185), Scholefield <i>et al</i> (186)	Reduction in CRC mortality with gFOBT biannually [relative risk reductions of 13% (UK trial) and 16% (Danish trial)] No significant reduction in overall mortality gFOBT: Low sensitivity for CRC detection (UK trial, 45%; Danish trial, 54%) True-positive rate: 50% (UK and Danish trials) False-positive rate: 5-10% (UK and Danish trials) True-negative rate: 90-95% (UK and Danish trials) False-negative rate 50% (UK and Danish trials)
Medical Advisory Secretariat (187), Dancourt <i>et al</i> (188), Faivre <i>et al</i> (189)	iFOBT sensitivity superior to those of gFOBT for CRC detection: Two studies showed sensitivity for gFOBT, 13 and 25%, respectively; pooled iFOBT sensitivity, 81% iFOBT and gFOBT: Lower sensitivities for adenoma detection than for CRC detection: Rehydrated gFOBT, 22%; pooled iFOBT, 28%
Lin <i>et al</i> (190)	FIT sensitivity, 73.8% (95% CI, 62.3 to 83.3) for quantitative (n=9,989) test categories; 78.6% (95% CI, 61.0 to 90.5) for qualitative (n=18,296) test categories
Koo <i>et al</i> (191), Moss <i>et al</i> (192)	Positive predictive value of FIT > positive predictive value gFOBT for advanced adenoma (1.73 vs. 0.35%) and all neoplasias (3.74 vs. 0.76%) FIT detects twice more CRCs and advanced adenomas
Gonzalez-Pons and Cruz-Correa (8)	gFOBT: ↓ ability to define bleeding between upper/lower GI tract
Kuipers <i>et al</i> (10)	gFOBT: ↓ ability to distinguish human haeme
Valori <i>et al</i> (62)	gFOBT: Not sensitive in small bleedings gFOBT ↓ sensitivity in detecting cancerous/preneoplastic lesions gFOBT: Specificity affected by diet/drugs
Lieberman <i>et al</i> (63)	gFOBT: 18% sensitivity in detecting advanced adenomas
Whitlock <i>et al</i> (65), Young <i>et al</i> (64)	FITs sensitivity for advanced adenomas: ~20-67% (↑ than FOBT)
Dancourt <i>et al</i> (188) Imperiale <i>et al</i> (25)	FIT detects more CRC and advanced neoplasia than gFBOT (similar positive predictive value)
Rozen <i>et al</i> (193)	Comparative performance of gFOBT and FIT depends on the number of samples and threshold chosen for the quantitative FIT
Hoffman <i>et al</i> (194)	Screening adherence with FIT was higher than with gFOBT (61.4 vs. 50.5%)
Brenner and Tao (195)	Sensitivity of FITs for detecting CRC/any advanced neoplasm/any neoplasm: 2-3 times higher than gFBOT Increased levels of FITs specificity vs. gFOBT
Fitzpatrick-Lewis <i>et al</i> (196)	iFOBT vs. gFOBT on mortality outcomes: iFOBT has higher sensitivity and comparable specificity (insufficient evidence from RCTs)
Murphy <i>et al</i> (197)	Total financial burden: Lower for FIT at any threshold (expressed in µg Hb/g faeces) than for gFOBT, and this difference increases as the FIT threshold is decreased (Cohort-based Markov state transition model)
Lee <i>et al</i> (198)	FIT sensitivity, 79%; FIT specificity, 94%
Morikawa <i>et al</i> (66)	gFOBT detect notably more lesions in the left (compared to the right colon)

FOBT, faecal occult blood test; CRC, colorectal cancer; gFOBT, guaiac faecal occult blood test; FIT, faecal immunochemical test; RCT, randomized controlled trials.

cells are examined. A number of abnormal methylated genes with variable level of specificity and sensitivity can serve as diagnostic biomarkers in CRC patients. These genes include *WIF-1*, *AIX4*, *PGR*, *FBNI*, *P53*, *TIMP3*, *SEPT9*, *MGMT*, *Vimentin* and *GATA4* (77,80). A number of studies have

indicated that instead of looking for a single gene methylation pattern, using a panel of several methylated genes is better in terms of specificity and sensitivity. Lind *et al* (81) tested a panel of epigenetic biomarkers for use as CRC and adenoma screening. In this study, approximately 523 human samples

were examined using a gene panel consisting of: *CNRIP1*, *FBNI*, *INA*, *MAL*, *SNCA* and *SPG20*. According to their study, high level of sensitivity and specificity was achieved. Hence, those authors suggested that a combination of the six genes may serve as a non-invasive biomarker with high specificity and sensitivity for early diagnosis of CRC (81).

d) APC mutations. *APC* gene regulates the Wnt signaling pathway via encoding a multifunctional protein. Specifically, *APC* regulates Wnt pathway through the destruction of the transcription factor β -catenin, which enhances the activity of Wnt pathway. Hence, *APC* conversely organizes Wnt signaling (82). In addition, *APC* gene is involved in cell cycle regulation, cytoskeleton stabilization, intracellular adhesion, as well as apoptosis. In an attempt to determine the exact role of *APC* gene in CRC, Dow *et al* (83) investigated whether *APC* mutation is essential for CRC protection. For their study a CRC mouse model with inhibited *APC* was used. According to their findings, inhibiting *APC* gives rise to adenomas in colon and small intestine (83). In general, about 90% of CRC patients demonstrate *APC* gene mutation, which highlights its applicability as a molecular biomarker for CRC diagnosis (8). Liang *et al* (84) conducted a meta-analysis study to correlate *APC* polymorphism (*D1822V*, *E1317Q*, *I1307K*) and CRC risk. They concluded that *E1317Q* significantly increased adenoma risk. However, *I1307K* is associated with high risk of CRC (84). CRC-related tumor suppressor genes are thought to be altered in the early phase of cancer development, and *APC* mutation is the first step in the translation of normal mucosa to neoplastic tissue, leading to the activation of the WNT pathway. Subsequent mutations that occur in genes, such as *KRAS*, *TP53*, *SMAD4* and type II TGF- β receptor (*TGFBR2*), lead to the progression from polyp to cancer similar to the process that takes place in other gastrointestinal carcinomas (85).

e) Circulating tumor DNA (ctDNA). Circulating free DNA (cfDNA) is thought to be a natural phenomenon, existing as a result of cellular apoptosis and necrosis of both normal and cancer cells, with secretion from cancer cells being an additional source that remains to be elucidated (86). ctDNA, a subclass of a patient's cfDNA, is the material used for testing in order to identify potential DNA impairments that have been associated with CRC. Owing to the circulating DNAases, the half life ctDNA is restricted to a few hours allowing clinicians to have a more accurate look at a patient's cfDNA profile. Findings have shown that the cfDNA quantity is quite higher in patients with cancer than that in healthy individuals. Moreover, cfDNA quantity seems to be positively correlated with the cancer stage (87). Of note, benign tumors or non-neoplastic lesions in general do not lead to ctDNA avoiding, therefore, a potential pitfall of false positives (88). To date, the main DNA defect that is studied is the hypermethylated *SEPT9* gene in the plasma. However, following the initial attempts to set a diagnostic tool based solely on *SEPT9*, researchers realized that combining multiple gene loci in a single panel in order to achieve better sensitivity and specificity is optimal. For example, T nzer *et al* (89) showed that the combination of *SEPT9* with *ALX4* achieved a sensitivity of 71% and a specificity of 95% for advanced adenomas, thus supporting *SEPT9/ALX4* as a biomarker for precancerous lesions (89). In parallel to that, apart from *SEPT9*, a number of genes have been investigated including *HJC1*, *CYCD2*, *PAX5*, *RBI*, *SRBC*, *NPY*, *PENK*,

WIF1, *ALX4*, *HLFT*, *HPPI*, *MLH1*, *APC*, *CDKN2A/P16h*, *TMEFF2*, *NGFR*, *FRP2*, *NEUROG1* and *RUNX3* (90).

f) Faecal DNA. As with circulating DNA, stool DNA is examined for the presence of abnormalities in specific genes. Stool-based assays have proven to be the most efficient assay type for a number of reasons. Direct histologic observations show that CRCs and polyps exfoliate neoplastic cells and their debris into the mucocellular layer of the colonic lumen at a steady continuous rate (91). However, at first sight, one would wonder how exfoliated cells arising from a right-sided polyp or CRC could survive in the intraluminal environment in order to be detected. Indeed, most of these cells go through lysis, preserving however to a certain extent their DNA content thus allowing a DNA analysis to be conducted. To date, along with mutant *KRAS* a variety of hypermethylated genes including *APC*, *ATM*, *BMP3*, *CDKN2A*, *SFRP2*, *GATA4*, *GSTP1*, *HLTF*, *MLH1*, *MGMT*, *NDRG4*, *RASSF2A*, *SFRP2*, *TFPI2*, *VIM* and *WIF1* have been analyzed in faecal DNA for the early detection of CRC, with *SFRP2* and *VIM* proving to be the most promising ones (92,93). When these tests for both mutant and methylated DNA markers and FIT, collectively known as MT-sDNA, are combined, it is proven that they demonstrate the best clinical performance of CRC molecular marker screening assays to date.

Blood and faecal miRNA profile. miRNAs are single-stranded small non-coding RNAs that are 18-25 nucleotides in length. Their role remained unknown until 1993 when it was recognized that they act as negative post-transcriptional regulators in *Caenorhabditis elegans* (94,95). Until 1993, miRNA (as any other member of the non-coding RNA family) was considered to be a useless RNA product of 'junk DNA'. However, it was shown that this is not the case as it was proven that it is able to regulate gene expression at a post-transcriptional level, either by blocking mRNA translation or by inducing their degradation. By binding miRNA to its target mRNA, miRNA can trigger the degradation of its target or otherwise inhibit its translation into protein, with the degree of sequence complementarity between the miRNA and mRNA determining which mechanism is employed (96-98). Over the last few years, research in human cancer focusing on the potential of miRNA to serve as a biomarker has increased dramatically mainly due to their unique properties. First of all, miRNAs are very stable under a variety of conditions both in the experimental and laboratory setting. Moreover, RNases cannot degrade them owing to their small size and the hairpin-loop structure (99). In addition to that, cell-free miRNAs occur in large numbers as they are packed in high density lipoprotein particles, apoptotic bodies, microvesicles, exosomes or by their binding to argonaute-2, properties that synergistically result in an increased stability (100,101). Thus, it is easy to isolate them from different forms of clinical specimens (99). Lastly, miRNAs are actively secreted by cancer cells into the circulatory system and digestive tract (102). Hence, in the clinical setting, circulating cell-free miRNAs and faecal miRNA are the main forms of RNA used as diagnostic biomarkers.

a) Blood-derived (circulating) cell-free miRNA. Various studies have shown that a series of miRNAs are pathologically excreted in CRC plasma or serum samples. However, due to the fact that standardized techniques for miRNA extraction, normalization and quantification are yet to be found, one needs

to bear in mind that most of the available results in literature are not easily reproducible thus making sometimes controversial. Circulating cell-free miRNA was first evaluated in a more comprehensive and systematic way in patients with CRC by Ng *et al* (103) in 2009 who found an altered miRNA expression profile in tissue and plasma samples from CRC patients and healthy subjects. However, a very important observation was that two miRNAs, miR-92a and miR-17-3p, were able to indicate patients with CRC, differentiating from healthy subjects, based on their high expression profile (sensitivity: 64 89%, specificity: 70, 70% for each miRNA, respectively) (103). Since then, numerous miRNAs have been identified as indicators of CRC. However, even though a few single miRNAs have proven to be enough to make the distinction with increased sensitivity and specificity, the addition of even more dysregulated miRNAs into a single panel usually achieved better diagnostic results. Indicatively, some of the dysregulated miRNAs and their testing samples are described: miR-17-3p plasma, miR-18 plasma, miR-21 plasma, miR-21 serum, miR-21 serum (exosome), miR-29a plasma, miR-92a plasma, miR-92a serum, miR-155 serum, miR-200c plasma, miR-221 plasma, miR-21, miR-31, miR-92a, miR-181b, miR-203, let-7g (panel), miR-7, miR-93 and miR-409-3p (panel) (104). However, only three upregulated miRNAs, miR-19a-3p, miR-21 and miR-92, were identified as promising diagnostic biomarkers by more than one study (105,106).

b) Faecal miRNA. Identification of CRC-specific miRNAs is also feasible in stools. Link *et al* (107), using RT-PCR and microarray analysis proved that faeces from patients with CRC and colorectal adenoma contained higher levels of miR-21 and miR-106a as opposed to that from healthy controls (107). A subsequent study that assessed faecal miR-21 and miR-92a levels revealed that faecal miR-92a expression was able to differentiate patients with CRC or adenoma from healthy subjects or even those with lower-risk polyps (22). More interesting data came from the study of Zhu *et al* (108) as they found that stool miR-29 was significantly present in patients with rectal cancer than in those with cancer in the rest of the large intestine. Thus, the use of a panel of miRNA expression patterns can create a form of a cancer fingerprint (108). A sum of the useful stool-derived dysregulated miRNAs for diagnosis is miR-17-92 cluster, miR-20a, miR-21 up miR-135 miR-144 miR-29a, miR-223, miR-221, miR-92a and miR-224 (109).

3. Prognostic markers

General. Once CRC is diagnosed, the clinician has to take the next step in the clinical management, that is to evaluate the prognosis for this patient or in other words to estimate the likely progression of the cancer and the aggressiveness that it may exhibit (recurrence likelihood, progression and/or chance for metastasis despite adjuvant therapy). The current practice for prognosis assessment is based on radiological (CT, MRI) and pathological (TNM, lymphovascular, perineural and venous invasion) criteria. In fact, TNM staging remains the strongest prognostic tool (110). However, not only do none of the prognostic tools mentioned above provide clear evidence on which of these CRC cases is more prone to relapse, give metastases or are proven to be resistant to chemotherapy, but they also are not suitable for the personalized treatment of

each patient. Thus, much effort has been made for the evaluation of the potential of several molecules and gene alterations to serve as prognostic biomarkers, taking us a step closer to a true personalized treatment.

Tissue-derived prognostic biomarkers

Molecular prognostic biomarkers. Some of the tissue-derived molecular biomarkers meant for diagnostic purposes, have demonstrated a promising potential for also serving as prognostic markers. Such markers are mucins (MUC2), SATB2 protein, CK20/CDX2, VEGF, insulin-like growth factor-II mRNA-binding protein 3 (IMP3) and Traf2- and Nck-interacting kinase (TNIK) expression. Even though some MUC2 expression profile studies have yielded mixed results, it is evident that MUC2 loss of expression may be an indicator of poor prognosis in both mismatch repair protein (MMR)-proficient and *MLH1*-negative CRC (111). On the other hand, studies on the SATB2 expression profile showed that an upregulated SATB2 was connected with good prognosis in CRC and could even increase sensitivity to chemotherapy and radiation, whereas a downregulated SATB2 in cases of colorectal adenocarcinomas was associated with poor prognosis, as tumor invasion, infiltrated lymph nodes and distant metastases were observed (112,113). Studies on CDX2 revealed that loss of CDX2 expression is associated with proximal origin, infiltrative characteristics and advanced TNM stage. Moreover, it was found to be an independent poor prognostic factor of progression-free survival (PFS) and overall survival (OS) (114). Vascular endothelial growth factor or VEGF is one of the main angiogenic factors in CRC as it is expressed in approximately 50% of the cases. Thus, VEGF-1 expression is correlated with a worse prognosis (115). Fig. 4 shows the connection between chronic inflammation and the development of CRC. Moreover, studies on IMP3 found an increased expression in colon cancer compared to normal colonic mucosa. Of note is the fact that its expression was found to be higher in cases with lymph node infiltration with cancer cells (93%) than in primary colon cancer (65%) or normal mucosa (3.9%) (116). Lastly, research on TNIK reported that high levels of TNIK protein in primary tumors could indicate distant metastasis after surgery of stage II and III CRC patients as well as invasive characteristics of CRC (117).

DNA alterations with prognostic value

KRAS and NRAS. The family of RAS proteins (H-, K-, and N-RAS) is located in the intracellular side of the cell membrane involved in G-protein mediated signal transduction. Activation of the epidermal growth factor receptor (EGFR) from its ligand (e.g., EGF, TGF- α , amphiregulin) results in a change from GDP- to GTP-form of the KRAS, leading to increased concentrations of B-rapidly accelerated fibrosarcoma (proto-oncogene) (BRAF) to the plasma membrane (Fig. 5). BRAF activates the mitogen-activated protein kinases (MAPK) signaling pathway that results in the expression of proteins involved in several pathways with a crucial role including cell proliferation, differentiation, survival, angiogenesis, and cell motility. Mutant KRAS proteins present as locked in the active form as a result of an impaired GTPase activity, leading to an increased proliferating rate that is unregulated and thus resulting in an overall malignant transformation of the cells. Therefore, it is logical to assume that mutation of these

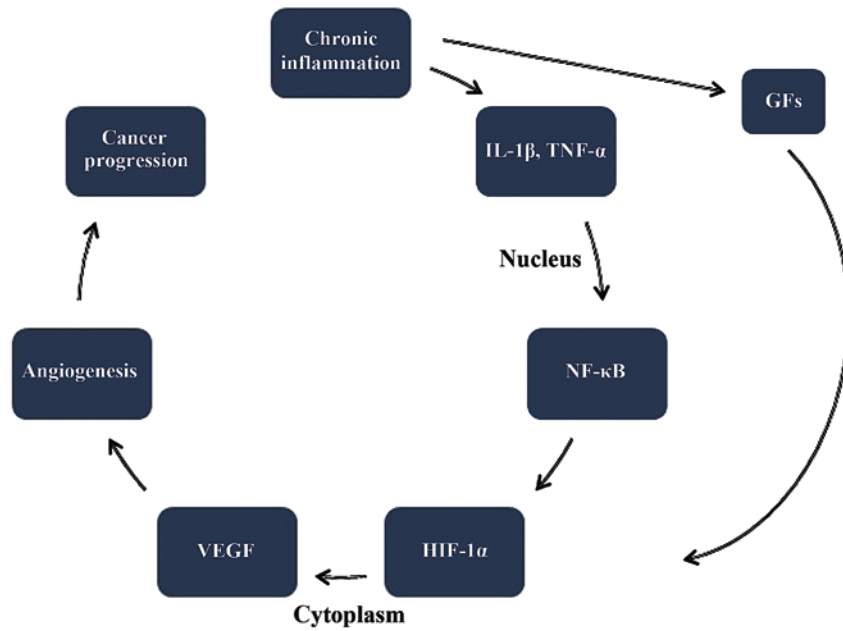


Figure 4. The connection between chronic inflammation and development of CRC. CRC, colorectal cancer; GFs, growth factors; IL-1β, interleukin 1β; NF-κB, nuclear factor κB; HIF-1α, hypoxia inducible factor-1α; VEGF, vascular endothelial growth factor.

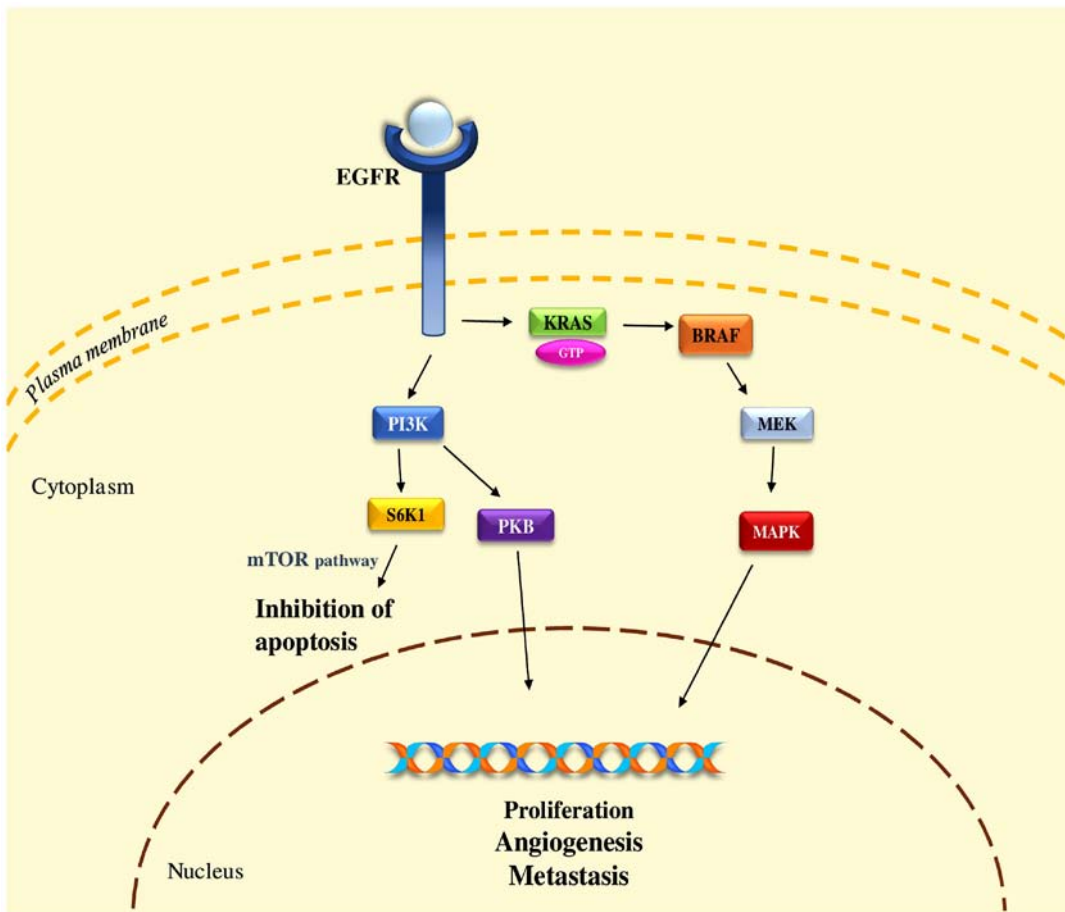


Figure 5. Intracellular signals for CRC manifestation via EGFR. CRC, colorectal cancer; EGFR, epidermal growth factor receptor; BRAF, B-rapidly accelerated fibrosarcoma (proto-oncogene); MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; S6K1, ribosomal protein S6 kinase β-1; PKB, protein kinase B; mTOR, mechanistic target of rapamycin.

oncogenes poses a great threat for carcinogenesis (118). Indeed, it is well documented that alterations of *KRAS* gene can act

as the first step towards carcinogenesis in approximately 50% of the CRC cases (119). However, it is not that clear whether

it could serve as a prognostic marker in the clinical setting. Recently, a meta-analysis of seven studies failed to associate *KRAS* mutation status with prognosis (however, a common limitation of such studies is the small pool of patients) (120). On the other hand, two large multicenter studies, demonstrated that only one mutation of *KRAS*, of codon 12, could be linked with a more aggressive progression of cancer cells. However, according to their data, *KRAS* mutations failed to be associated with tumor location or stage and recurrence of disease (121,122). On the other hand, the possible prognostic value of *NRAS* mutations is less examined, even though they appear to act in a similar way with *KRAS* as for the degree of negative prognostic significance (123).

p53. p53 is a transcription factor that participates in a variety of cellular reactions to several stress situations such as mutagenic DNA damage, oncogene activation, hypoxia and telomere shortening. In contrast to other mutations, p53 mutation seems to occur late in the development of CRC since few or even no cases of mutations are described in precancerous lesions from sporadic adenomas and polyps. Interestingly, data from one study showed that mutations of p53 in CRC exhibits a dependence on the primary tumor site thus suggesting a prognostic value of this marker. In more detail, patients with a primary tumor site in the proximal colon (caecum, ascending colon) and mutant p53 gene exhibited better survival when treated with a combination of chemotherapy and surgical removal compared to those treated by surgery alone (124).

BRAF. BRAF is serine-threonine protein kinase that is recognized downstream in the *KRAS* signaling cascade. *BRAF* mutations are linked with a poor outcome thus proving their clinical applicability as a prognostic marker in the adjuvant setting. Another indication of a poor outcome is found in stage II and III CRC cases where *BRAF* mutations were associated with worse OS (125). A rather interesting finding is that *BRAF V600E* mutation is able to predict a poor prognosis in right-sided MSS CRC (126,127). Further examination of the *BRAF* prognostic value was carried with a study investigating the correlation of *BRAF* mutation with MSI. The existence of *BRAF* wild-type and MSI-H exhibited favorable outcomes. Furthermore, *BRAF*-wild/MSS and *BRAF*-mutated/MSI-H exhibited intermediate outcomes (128).

MSI. MSI status is a well-studied diagnostic marker for CRC as mentioned above. However, its clinical relevance does not stop in the diagnostic setting but continues in the prognostic as well. MSI-H is associated with better survival rates than both MSI-L and MSS, not only in HNPCC, but also in sporadic cases (129,130).

miRNA with prognostic value. During the last few years, a wide variety of miRNAs has been evaluated in order to identify their prognostic value in CRC. Indeed, numerous miRNAs, among which are miR-101, the let7 family, miR-133b, miR-126, miR-337, miR-944, miR-646, miR-497 and miR-142-3p have been identified to behave as tumor suppressors (tumor-suppressive miRNAs) (131-137) while others, including miR-7, miR-20a, miR-21, miR-29a, miR-92a, miR-130b, miR-155 and miR-552, were characterized as key players in creating a favorable microenvironment for cancer cells. These miRNAs are known as oncomirs (oncogenic miRNAs) (138-141). miR-20a-5p was found to promote tumor invasion and metastasis via downregulation of SMAD4 (142). Moreover, elevated

miR-21 levels correlate with CRC cell proliferation, invasion, lymph node metastases, advanced clinical stage, poor overall and disease-free survival in the different Duke stages (143,144). Increased expression of miR-29a is found to strongly correlate with metastases and especially liver metastases as, if present, it can serve as an early detector of liver metastases (145,146). High preoperative miR-155 closely correlates with advanced stage and metastasis while persistent postoperative expression correlates with recurrence and metastasis. Thus, miR-155 could be considered a prognostic marker for overall and disease-free survival (147). In a recent study, Zhao *et al* (148) presented that miR-411 functions as a tumor-suppressive miRNA directly targeting PIK3R3 and indirectly regulating the AKT/mTOR signaling pathway. In other words, an increase of miR-411 would result in the downregulation of PIK3R3 directly and AKT/mTOR indirectly. Additionally, they observed that a decreased expression of miR-411 was correlated with worse findings such as lymph node metastasis, distant metastasis and worse TNM stage (148).

Blood-derived prognostic biomarkers

Preoperative CEA levels. CEA may be tested in the preoperative setting in patients with CRC in order to assist staging and surgical treatment planning. Previous findings showed that increased preoperative CEA (>5 ng/ml) correlates with poorer prognosis. In fact, a study with 2,230 patients proved that pre-operative CEA levels was an important independent prognostic factor when outcome prediction was encountered (149). Similarly, another study with 1,146 patients with CRC found that, following use of a multivariate analysis, preoperative CEA levels proved a highly significant prognostic factor even when stage and grade were introduced in the model (150). In addition, elevated preoperative CEA in stage III and IV CRC are considered to be a potent independent risk factor as far as local relapse, short disease-free survival and OS are concerned (151).

Postoperative CEA levels. After surgical removal of the tumor, CEA levels should be checked as it is shown that persistent by elevated CEA levels suggest further evaluation for metastatic disease. In addition, elevated CEA is very efficient for revealing recurrence in asymptomatic patients and is the most sensitive detector for liver metastases (152).

Cancer antigen 19-9 (CA 19-9) levels. CA 19-9, also termed sialyl Lewis a, is a documented marker with prognostic value for CRC. It is shown that cases with increased CA 19-9 present more frequently metastases thus making it a marker of poor prognosis (152). Similarly, a recent study with stage IV CRC proved that the preoperative serum CA 19-9 level can be a promising marker of tumor recurrence and prognosis in cases submitted to curative resection. In detail, high levels of CA 19-9 were connected with worse 3-year relapse-free survival and 3-year overall than that in the normal CA 19-9 group (153).

Circulating tumor cells (CTCs). Detection of CTC has demonstrated prognostic significance in predicting patient outcome in cases of metastatic CRC. Recently, two groups investigated the prognostic significance of CTCs in CRC (154,155). Both of them found that patients with high CTC count were more likely to experience worse PFS and OS in contrast to those patients with low CTC count.

4. Predictive markers

General. The final step in the clinical management of a patient with cancer, is the determination of the most suitable therapeutic regimen. It is well known that patients with cancer, exhibit different responses to a certain therapy and some patients can benefit the most from the use of a biologic factor. Thus, it is of paramount importance for us to be able to know beforehand which patient will receive each therapy or in other words to predict the most likely response to a given therapy. For this reason, predictive markers are used.

Tissue-derived predictive markers

DNA alterations

KRAS and NRAS. Mutations of *KRAS* gene have proven their clinical use as a predictive biomarker in response to the EGFR inhibitors clinically used, as various mutations of *KRAS* present resistance to therapy with EGFR receptor monoclonal antibody blockers such as cetuximab (156). In detail, anti-EGFR therapy on cases of metastatic CRC who display *KRAS* mutations either of the codon 12 or 13, present no benefit (157). However, a mutant *KRAS* does not always mean that it is a sign of bad response. For example, De Roock *et al* (158) have found that patients with metastatic CRC with *P.G13D KRAS* mutation treated with cetuximab exhibited better results than other *KRAS* mutations (158). Under the light of these findings, evaluation of an extended panel of *RAS* mutations including mutations in *KRAS* exon 2, 3 and 4 and *NRAS* exons 2, 3, and 4 can better discriminate which patients are not good candidates for treatment with anti-EGFR therapy (159).

BRAF. *BRAF* mutations and especially *BRAF V600E* is among the most common mutations found in CRC cases as it is present in approximately 8-10% of all cases (160). *BRAF* mutation is often used as a discrimination factor between familial and sporadic CRC as the existence of *BRAF V600E* mutation in MSI CRCs can virtually exclude Lynch syndrome (161). However, numerous studies have shown that tumors that exhibit *BRAF* mutation are resistant to anti-EGFR therapy (162).

Phosphoinositide 3-kinase (PI3K). PI3K is a downstream mediator of the EGFR signaling cascade along with AKT and PTEN. An interesting finding regarding *PI3K* mutations lies on the use of cetuximab in patients with CRCs who display mutations of *PI3K* and especially at the *PIK3CA* exon 20 (which is the kinase domain) in contrast to patients with *PIK3CA*-wild-type CRCs. Patients with mutant *PI3K* exhibit much worse results (163). However, the mutation of a different exon, *PIK3CA* exon 9 (which is the helical domain), cannot serve as a predictive marker for anti-EGFR therapy, a fact that reflects the high complexity of the effects of specific mutations on different functions of the mutant kinases (163).

MSI status. Recent studies have investigated the application of level of MSI status as a potential predictive marker of adjuvant therapy. While there is enough evidence supporting that MSI-H may predict response to 5-fluorouracil (5-FU)-based adjuvant therapy in stage III colon cancer, numerous recent studies demonstrated that there is no significant difference between patients with MSI-H and MSS tumors when 5-FU-based adjuvant therapy is used (164-166). This finding is very important for the group with stage II disease, in which

adjuvant chemotherapy (5-FU alone) is reported to improve survival by approximately 3%, allowing some investigators to suggest that stage II colon tumors should be analyzed for MSI status as well in order to assist guide decisions on the use of adjuvant therapy (165).

CD133. CD133 is a surface protein that has been associated with tumor angiogenesis and recurrence and especially with VEGF cascade (167). Previous findings have shown that the expression of CD133 was associated with worse survival rates in patients treated with surgery as a monotherapy and in patients treated with 5-FU-based chemotherapy. By contrast, CD133 expression in patients with stage III tumors, was able to determine that the absence of CD133 could benefit more from 5-FU treatment as expressed by their survival rates but not those with present CD133 expression. Positive expression of CD133 was also associated with worse clinical response to chemotherapy in stage IV patients (168).

5. New insights in CRC monitoring

General. Going through the list of the markers used for diagnostic, prognostic and predictive purposes, it is evident that even though a plethora of markers are available, the clinician still cannot achieve the best possible monitoring of his patient's disease, even when a wide panel of markers is used. For this reason, great effort is made by researchers worldwide in order to identify new markers that will be able to lead to a better, if not the best, disease monitoring. A number of these markers seem to be quite promising. We will discuss three of these markers [telomere length (TL), telomerase activity and micronuclei (MN) frequency].

TL and telomerase activity. Telomeres are DNA-protein complexes that are enrolled to guard the ends of eukaryotic chromosomes and composed of a repetitive nucleotide sequence (5'-TTAGGG-3') which is added on by the telomerase. Telomerase is an enzyme complex that consists of two subunits, the reverse transcriptase protein human telomerase reverse transcriptase (hTERT), which is the catalytic subunit, and the telomerase RNA component (TERC), an RNA template-hTR (human telomere RNA), which serves as a template for directing the appropriate telomeric sequences onto the 3' end of a telomeric primer (169). When telomeres become too short, cells may be unable proliferate, a situation that has been linked to the development of a variety of age-related diseases, such as cancer, cardiovascular diseases, diabetes and psychiatric disorders (170-172). A number of studies have examined the role of telomeres and telomerase in CRC in generating CIN. In CRC telomere, shortening is an initial event that directly reflects pathologic cell proliferation because of telomere's shortening in CRCs and in well-differentiated tumors (173,174). Telomeres and telomerase have been proposed as potential prognostic and diagnostic biomarkers in CRC. Telomeres and telomerase activity have universal changes along the CRC process (175). Fernández-Marcelo *et al* (176) examined the ratio of TL in cancer to non-cancer tissue, telomerase activity and TERT levels and their role as prognostic markers. In tumors, TL was shorter than that in non-tumor tissues and more than 80% of CRCs displayed telomerase activity and thus the use of

telomere status as a prognostic factor has been suggested (176). Gertler *et al* (177) analyzed TL and hTERT expression in matched cancer and adjacent non-cancer mucosa samples and found that telomeres in CRC tissues were significantly shorter compared with adjacent normal mucosa samples. Another study comparing peripheral blood cell TL in CRC patients and healthy subjects, TL has an anomalous behavior. We can conclude that telomeres and telomerase emerge as useful diagnostic and prognostic markers in the clinical management of CRC (5).

MN frequency. MN are extra-nuclear bodies recognized in dividing cells, that contain chromosome fragments and/or whole chromosomes that failed to incorporate into the nucleus after cell division. It is well established that MN formation can be induced by defects in the DNA repairing system, leading to the accumulation of DNA damages and chromosomal aberrations (178). As MN formation is a result of CIN and indicative of a malfunctioning cell, it is reasonable to assume that MN could, under certain circumstances, be linked with cancer development (179). Indeed, high MN frequency is recognized in a number of cancer types. Specifically, in lung cancer MN frequency has become a useful marker for the identification of small and non-small cell lung cancer using peripheral blood samples (180). As far as CRC is concerned, data from various studies using peripheral blood samples indicate that MN frequency is a promising biomarker for the early detection of CRC while it could also be used as a prognostic biomarker (181-184). However, more studies are needed in order to describe with certainty the true potential of this biomarker.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JT conceived and designed the study. TKN and LV researched the literature, performed analysis of data and drafted the manuscript. PF and GP drafted the manuscript. JT, TMS, DAS and AMT critically revised the article for important intellectual content.

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

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