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Review

Prognostic and Predictive Molecular Markers in Colorectal Carcinoma

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Abstract. *Tumour markers are molecules occurring in blood or tissue that are associated with cancer and whose measurement or identification is useful in patient diagnosis or clinical management. The ideal marker would occur only in patients with malignancy, and would correlate with stage and response to treatment, however, to date there are few reliable prognostic markers in colorectal cancer (CRC), consequently much research is focused on identifying such markers. This review aims to summarise the most important currently available markers in CRC that provide prognostic or predictive information. Amongst others, it covers serum markers such as CEA and CA19-9, markers expressed in tumour tissue such as thymidylate synthase and also the expression/loss of expression of certain oncogenes and tumour suppressor genes such as K-ras and p53. The prognostic value of genomic instability, angiogenesis and proliferative indices such as apoptotic index is discussed.*

Colorectal cancer (CRC) is an important public health problem; it is a leading cause of cancer mortality in the industrialized world, second to lung cancer: there are nearly one million new cases of CRC diagnosed worldwide each year and half a million deaths (1). Globally, the incidence of CRC varies 10-fold, with the highest incidence rates in North America, Australia and northern and western Europe; developing countries have lower rates, particularly Africa and Asia (1). These geographic differences appear to be attributable to differences in dietary and environmental exposures that are imposed upon a background of genetically determined susceptibility. The overall mortality from CRC is 60%, which represents the second leading cause of cancer death in western societies (2). However, there have not been great improvements in patient survival despite advances in the understanding of the disease and in chemotherapy practice (3). Postoperative adjuvant chemotherapy improves the outcome in stage III (Dukes' stage C) colon cancer and is widely accepted as a standard therapy although no reliable prognostic predictors have been identified in such cases. Nevertheless, the most important aspect of treatment is curative surgery. The majority (up to 90%) of CRC patients undergo surgery. However, approximately 50% of those patients initially believed to be cured by surgery subsequently relapse and die of their disease (4). Once metastasis has occurred treatment is mostly palliative. Consequently, much research is focused on identifying molecular markers to predict the biological behaviour and response to therapy of CRCs.

In most cases CRC develops from a pre-existing adenoma: the adenoma-carcinoma sequence (5). This sequence is characterised by an accumulation of molecular genetic alterations causing disorders in cell growth, differentiation and apoptosis (6). It is generally believed that the balance between the rates of cell growth and apoptosis maintains intestinal epithelial cell homeostasis (7) and that during cancer development this balance becomes progressively disturbed (8). Changes in cell proliferation have been well studied in the adenoma-carcinoma sequence with the number of proliferating cells increasing in proportion to the severity of dysplasia (9). When detected early, CRC is highly treatable and curable. Most colon cancers arise from adenomatous polyps. About 5% of adenomatous polyps are estimated to become malignant and this process takes approximately 10 years (10). Cancer can grow inward toward the lumen of the colon or rectum and/or outward through the walls of these organs. Advanced disease can cause perforation of the bowel, leading to

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infection. Metastasis of the disease may occur to the lymph nodes, liver, lung, peritoneum, ovaries and brain.

Age is a major risk factor for sporadic CRC. It is a rare diagnosis before the age of 40, the incidence begins to increase significantly between the ages of 40 and 50 and age-specific incidence rates increase in each succeeding decade thereafter (11). The lifetime incidence of CRC in patients at average risk is about 5 %, with 90 % of cases occurring after age 50 (12). The incidence is obviously higher in patients with specific inherited conditions that predispose them to the development of CRC.

The most important etiological factor to date related to colon cancer is genetic predisposition. Genetic alteration such as mutation of the APC (adenomatous polyposis coli) tumour suppressor gene, K-ras proto-oncogene and p53 has been demonstrated to lead to polyps and cancer formation in the large intestine (13, 14).

Understanding of the molecular pathogenesis of CRC (both sporadic and inherited) is evolving rapidly. Several specific genetic disorders have been identified, all of which are inherited in an autosomal dominant fashion and are associated with a very high risk of developing colon cancer. Sporadic CRC is estimated to account for 80% of all CRCs with hereditary forms account for the remaining 20% (15). The hereditary syndromes include familial adenomatous polyposis (FAP) which accounts for 1% of all CRC (16), hereditary non-polyposis colon cancer (HNPCC) which accounts for 5-10% of all CRC (17) and familial colon cancer (FCC) which accounts for the remaining 10-15%. FCC is most likely to be of multifactorial origin and remains largely unexplained at this time. Epidemiological studies suggest that several exogenous agents, for example meat and tobacco smoke, may increase the risk of developing CRC. Others, such as NSAID's (non-steroidal anti-inflammatory drugs), vegetables and hormone replacement therapy, may reduce the risk (18). Our knowledge of agents responsible for the development of CRC is still limited. High energy intake, especially from saturated fat, seems to be a definite risk factor and high consumption of dietary fibre and vegetables seems to be protective, especially when combined with physical exercise (19). However there is still much controversy concerning this "fat –fibre"-hypothesis, which proposes that the epithelium in the colon and rectum will be exposed to mutagens for longer times due to prolonged transit time in the gut caused by fat rich and low fibre diets combined with low physical activity (20).

Prognostic and Predictive Markers

Proliferation indices

Apoptotic index. The term "apoptosis" describes a process of programmed cell death clearly different from the other type of cell death, necrosis. Some of the main morphological characteristics of apoptosis are cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation and finally formation of apoptotic bodies (7, 21). These morphological changes result from a vast series of cellular and biochemical processes triggered by physiological stimuli or activated in response to various forms of cell injury and stress (7, 21). Apoptosis, or programmed cell death, plays an important role in many physiological and pathological processes (22). Among others, an important function of apoptosis lies in the elimination of damaged cells. For example, cells with genetic damage caused by exposure to carcinogens may be removal by undergoing apoptosis, thereby preventing their replication and the accumulation of clones of abnormal cells. There is increasing evidence to support the hypothesis that failure of apoptosis may be an important factor in the evolution of CRC and its poor response to chemotherapy and radiation (23). Inhibition of apoptosis causes an imbalance in normal tissue homeostasis promoting cell growth and it also allows the survival of genetically damaged cells, both contributing to tumour development and progression (24). However, considerable controversy exists as to whether the frequency of apoptosis increases or decreases during the adenoma-carcinoma sequence (25). A large number of studies have assessed the proportion of cells undergoing apoptosis, both *in vitro* and in human colorectal tissue sections.

The balance between cell production through proliferation and cell loss through apoptosis determines how fast a tumour grows and is an important determinant of tumour behaviour. Most colorectal adenomas are stable for a long time before, if ever, transforming to malignancies. For example, 3-5 years after the initial diagnosis of adenomas, 70% showed no change in size (26). As increased cell death may be an attempt to limit the expansion of the tumour cell population, several studies have linked the rate of apoptosis with the proliferative rate of adenomas and carcinomas, yielding conflicting results. Levels of proliferation were associated with those of apoptotic cell death in adenomas and carcinomas in a number of studies (27-29) whereas others found no correlation (30, 31). The finding that apoptosis and proliferation are not correlated is often used as an argument to support the assumption that an imbalance between these processes emerges in the course of the adenoma-carcinoma sequence. Supporting this assumption are results from a study in which proliferative activity was shown to be correlated with apoptotic index in adenomas with low-grade dysplasia whereas this correlation was lost in adenomas with high-grade dysplasia and carcinomas (32). There is general agreement that the deregulation of apoptosis contributes to malignant transformation; the potential predictive or prognostic value of the degree of apoptosis in CRC is controversial. Several

studies have examined the prognostic value of the apoptotic index in CRC, producing conflicting results (24, 29, 33, 34). Schwandner *et al.* showed that the apoptotic index was not predictive of prognosis in a series of 160 cases of rectal cancer (34). In three other studies, no prognostic significance was found in large groups of carcinomas (29, 35, 36). However, stratification by tumour site revealed that the apoptotic index was an independent predictor of survival in a series of 82 distal tumours (distal to splenic flexure) (29). In two studies, it was shown that a low apoptotic index in the tumour was associated with poor survival (37, 38). Two reports showed that apoptotic indices were higher in tumours that were more highly differentiated and had not invaded or metastasised than in those that were poorly-differentiated and invasive or metastasising (39, 40). Tanako *et al.* also found higher apoptotic indices in tumours without lymph node or distant metastases in comparison to tumours that had metastasised, but they found no correlation with the degree of tumour differentiation (41). On the other hand, Hawkins *et al.* demonstrated that Dukes' A carcinomas had lower apoptotic indices than Dukes' B-D carcinomas (24).

MIB-1 (KI-67). The Ki-67 protein is a proliferation antigen, which is present in G1-, S-, G2- and M- phases of the cell cycle. Quiescent or resting cells in the G0- phase of the cell cycle do not express the Ki-67 antigen. Thus, determination of Ki-67 is an excellent correlate of the "growth fraction" of a population of cells, neoplastic or otherwise (42). Ki-67 is virtually restricted in its role as a proliferation antigen, making it a more specific determinant of growth fraction. Studies over the last decade have convincingly established the validity of assessing the Ki-67 antigen-expressing fraction ("MIB-1 labelling index") in tumours to indicate growth fraction, and correlated this value with a variety of clinicopathological parameters (43, 44). The prognostic value of the MIB-1 labelling index (MLI) is of greatest value in those tumours where the clinical course is difficult to predict by histological parameters alone (45). A few tumours appear to show little correlation between Ki-67 expression and patient survival, *e.g.* large bowel cancer. Indeed, the two studies of CRC which did show a correlation were contradictory; Palmqvist *et al.* (46) concluded that CRC with low pKi-67 expression at the invasive margin had a poor prognosis, whilst Kimura *et al.*, found that those cases showing a high pKi-67 expression at the site of deepest invasion had a worse prognosis (47).

Proliferating cell nuclear antigen (PCNA). One of the first steps in multistage colonic carcinogenesis is increased cell proliferation. PCNA is known as a cyclin and an auxiliary factor in DNA polymerase. PCNA is the 36 KD polypeptide which is synthesized and expressed just in proliferating cells. It has been proved that PCNA expression is related to the cell generation cycle (48). Expression of PCNA increases in G1-phase gradually, peaks in S-phase and decreases in G2/M-phase. Determining PCNA indices can play an important role in understanding the cell generation state. The higher the PCNA expression, the higher the cell malignancy trend (49, 50). PCNA plays a very important role in DNA replication (51). Because of this direct relationship with cell proliferation, PCNA is considered to be an important factor in prognosis. In fact, it has been described as a significant factor in the prognosis of CRC in several studies (52, 53).

Oncogenes/Tumour suppressor genes

P53. P53 is a tumour suppressor gene on the short arm of chromosome 17 encoding a protein that is important in the regulation of cell division. It is normally expressed when a cell senses DNA damage, producing a protein product that causes growth arrest and apoptosis (programmed cell death) in rapidly-dividing cells. In this way, p53 acts as a tumour-suppressor gene by aborting the growth of potentially malignant cells and/or established malignancies.

Mutations of the p53 gene occur in various human tumours, including CRC (54). Deletions and mutations of the p53 gene can be detected in up to 85% of colorectal tumours and usually occur during the transition from adenoma to adenocarcinoma (5). Several functions have been ascribed to the p53 tumour suppressor gene, reviewed by Levine (54) and Sigal (55). Its product, the p53 protein, may respond to DNA damage by triggering either growth arrest during the G1- or G2- phase of the cell cycle or programmed cell death. In this manner, p53 may protect the normal cell from proceeding to replicate damaged DNA. A study by Valentini *et al.* demonstrated higher p53 expression in colorectal tumours with microsatellite instability (56). The wild-type p53 protein, but not the mutant, can initiate apoptosis. The mutated p53 protein may block the function of the wild-type p53 protein and thereby inhibit the induction of apoptosis. It has also been found that p53 has profound effects on responses to chemotherapeutic drugs used in CRC, and these effects vary considerably depending on the drug (57). Several studies have assessed the correlation between p53 protein expression and apoptosis in colorectal neoplasms. Some studies have indicated that adenomas and/or carcinomas with a high percentage of cells expressing the p53 protein were more likely to have a low apoptotic index (6, 31, 34, 58), whereas most studies did not show such a relationship (24, 32, 35, 36, 59-61). In summary, the fact that neither the immunohistochemical overexpression of p53 nor p53 mutations correlated to the frequency of apoptotic cell death in the majority of studies does not support a major role for the mutant p53 protein as an inhibitor of apoptosis in the development of CRC.

Bcl-2. Bcl-2 is an intracellular membrane protein capable of inhibiting programmed cell death (62). The bcl-2 gene is overexpressed in follicular B-cell non-Hodgkin's lymphomas resulting from a t(14;18) translocation; however, overexpression of bcl-2 has also been detected in human epithelial tumours without translocation (63).

At least 15 bcl-2 family member proteins have been identified in mammalian cells, including proteins that promote apoptosis and those that prevent it (64). The bcl-2 protein is normally expressed only in the lower half of the crypts of the colon, corresponding to the stem cell compartment, where bcl-2 is believed to protect stem cells from apoptosis (65). Most colonic adenomas express bcl-2 protein at high levels throughout the neoplastic epithelium, (6, 66, 67) while non-neoplastic polyps have a normal pattern of bcl-2 expression (68, 69). Overexpression of bcl-2 may therefore contribute to the transition between hyperplastic epithelium and adenomas. The bcl-2 protein expression in CRCs is higher than in normal mucosa, but lower than in adenomas (24, 66-70). An inverse correlation has been reported between bcl-2 expression and the apoptotic index of colonic tissues (6, 24, 32, 37, 71), whereas others have found no such correlation (29-31, 34, 35, 59, 60). With respect to the correlation between bcl-2 expression and prognosis in CRC, reports are conflicting (35, 72-74). Considering the relationship between bcl-2 and p53 overexpression, some have found an inverse correlation in adenomas and carcinomas (67, 71). Bcl-2 expression in poorly-differentiated clusters of cancer cells in the tumour growth zone has been found to be a probable factor determining the biological malignancy of CRC (75).

Bcl-2 expression seems to be gradually reduced in the course of the adenoma-carcinoma sequence and inversely related to p53 overexpression. As most studies show a gradual increase in frequency of apoptotic cell death, a possible relationship with the down-regulation of bcl-2 can be hypothesised. However, bcl-2 is probably only one of the genes that determine the incidence of apoptotic cell death in colorectal neoplasms. Indeed, changes in the expression of other members of the bcl-2 family have been shown during the progression of colorectal tumours, such as the anti-apoptotic proteins bcl-XL, mcl-1 and the pro-apoptotic protein bak, which may be more important than bcl-2. Krajewska *et al.* showed that the expression of bcl-XL is increased in undifferentiated primary CRCs, often with accompanying reciprocal decreases in the anti-apoptotic proteins bcl-2 and mcl-1 and the pro-apoptotic protein Bak, whereas Bax expression is relatively constant. Thus, a shift from expression of the anti-apoptotic proteins bcl-2 and mcl-1 to the bcl-XL protein may occur during progression of colorectal tumours (76).

K-ras. The ras family of oncogenes comprises three principal members - K-ras, H-ras and N-ras - all of which have been implicated in the development of human malignancies (77). The K-ras oncogene located on chromosome 12p12 encodes a 21-kD protein (p21ras) involved in the G-protein signal transduction pathway, modulating cellular proliferation and differentiation (78). Mutations of the K-ras oncogene result in constitutive activation of this signal transduction pathway and, consequently, unregulated proliferation and impaired differentiation. Activating K-ras mutations are present in greater than 50% of colorectal adenomas and carcinomas and the vast majority occur at codon 12 of the oncogene (5). K-ras abnormalities are one of the earliest events in the stepwise progression of colorectal neoplasms, being detectable even in histologically unremarkable epithelium and aberrant crypt foci adjacent to cancers (5). The clinical significance of K-ras mutations is controversial, although some studies have shown lower median survival times in patients with mutation-positive tumours. Amongst other gastrointestinal malignancies, K-ras mutations are one of the most common genetic abnormalities in pancreatic and bile duct carcinomas, detectable in more than 75% of tumours (79). Measurement of ras oncogene abnormalities has not been demonstrated to be an independent predictor for either survival, quality of life, or disease-free survival in patients with large bowel carcinoma (80). Abnormalities of ras in colorectal tissue may correlate with increased relapse rate and decreased survival (79). However, currently available data do not demonstrate that ras oncogene analysis provides independent prognostic information in CRC.

Deleted in colorectal cancer (DCC). The development of colonic carcinoma is associated with the mutation of a specific set of genes. One of these, named DCC (deleted in colon cancer), is a candidate tumour-suppressor gene (81). The contribution of molecular genetics to CRC has been largely restricted to relatively rare inherited tumours and to the detection of germ line mutations predisposing to these cancers. However, much is now known about the somatic events leading to CRC in general. Several studies have examined the relationship between genetic features and prognosis.

Inactivation of the gene DCC located on chromosome 18 is known to be associated with the tumorigenesis and metastasis of CRC (82). Loss of heterozygosity (LOH) at the DCC gene locus was detected in colorectal tumours and this LOH is possibly related to metastasis. Saito *et al.* suggested that a decrease in DCC expression may play an important role in the progression of CRCs and may be a biological marker of prognostic significance (83). Reymond *et al.* confirmed that DCC protein is a useful prognostic marker in patients with rectal carcinomas (84). The deletion of the DCC gene predicted a poor outcome in patients with

diploid or low S-phase fraction tumours (85). Therefore, immunohistochemistry to detect loss of protein expression may be considered as a practical test to assess prognosis in this subgroup of patients (85).

Genomic instability

Most cancers of the colon and rectum display a phenomenon termed genomic instability. There are apparently two distinct forms of genomic instability which reflect different genetic pathways of tumorigenesis. One form is observed at the nucleotide level, frequently resulting in deletions or insertions of a few nucleotides, and is termed microsatellite instability (MSI). Microsatellites are polymorphic tandem repeats of short nucleotide sequences distributed through the genome. The inherent instability of microsatellite loci is primarily due to changes in the number of repeats during DNA replication as a result of inefficiencies in a proof-reading enzyme such as the mismatch-repair enzymes MLH1 and MSH2. Germline mutations in the genes that encode these enzymes are known to result in hereditary non-polyposis colorectal cancer (HNPCC), but in addition somatic alterations in this group of genes can result in the observed high-level MSI that is found in 10-15% of colorectal tumours (86).

Colorectal tumours that exhibit MSI surprisingly have a normal complement of chromosomes *i.e.* they retain a diploid karyotype (87). In contrast, however, about 80% of colorectal tumours are microsatellite *stable*, exhibiting a wide variation in chromosome number. This type of genomic instability has been termed chromosomal instability and, at the molecular level, is characterised by frequent cytogenetic abnormalities and allelic imbalance (AI), which represents losses or gains of defined chromosomal regions. This pathway of genetic instability is selected very early in tumorigenesis (88) and is possibly caused by the inappropriate chromosome segregation at mitosis that occurs when the APC gene is mutated, an event that occurs very early in most sporadic and hereditary colorectal tumours (14).

MSI-positive tumours occur more frequently in the right side of the colon with approximately 75% of HNPCC and up to 90% of sporadically occurring MSI-positive cancers detected proximal to the splenic flexure (89, 90). Survival data from several studies suggest that patients with HNPCC have a better prognosis than those with sporadic disease (91, 92). A Finnish study described an improved 5-year survival for both localised (85 *vs* 68%) and advanced (40 *vs* 18%) HNPCC compared to the population (93). Another study found a significantly improved 5-year survival in Dukes' stage C cancers (61 *vs* 21%, $p=0.01$) in HNPCC compared to the population, but no difference in survival in Dukes' stage A and B cancers (94).

The impact of 5-FU chemotherapy in 656 patients with Dukes' C CRC (median follow-up of 54 months) demonstrated a clear survival advantage (90 *vs* 35%) for MSI-positive disease (95). However, contrary to this, Halling *et al.* studied tumours from 508 patients and did not detect a difference in survival between MSI-positive and negative CRCs in response to chemotherapy (96). Similarly, a recent study by Ribic *et al.* found, among 287 patients who did not receive adjuvant therapy, those with tumours displaying high-frequency microsatellite instability had a better five-year rate of overall survival than patients with tumours exhibiting microsatellite stability. However, among patients who did receive fluorouracil-based adjuvant chemotherapy, high-frequency microsatellite instability was not correlated with increased overall survival (97).

Little is known about how these two types of genetic instabilities might interact with specific therapies. However, even a small variation in the response of MSI-positive or -negative cancers to chemotherapy may have an important effect on clinical outcome. Clearly, more studies are required to assist in the choice of suitable chemotherapeutic agents.

Angiogenesis

Vascular endothelial growth factor (VEGF). The realization that the growth and spread of tumours is dependent on angiogenesis has created new avenues of research designed to help us to better understand cancer biology and to facilitate the development of new therapeutic strategies. However, the process of angiogenesis consists of multiple sequential and interdependent steps with many positive and negative regulators of angiogenesis being involved. The expansion of tumours and their metastases are dependent on the balance of endogenous angiogenic and antiangiogenic factors such that the outcome favours increased angiogenesis (98). Recently, angiogenesis has gained increasing interest as a prognostic factor in a variety of solid tumours. The immunohistochemical analysis of the newly-formed vessels is the most widely used index of angiogenesis in solid tumours (99). However, the levels of angiogenic factors have also been studied as a marker of angiogenesis and prognostic factor. Vascular endothelial growth factor (VEGF) is a glycoprotein similar to platelet-derived growth factor, and it is a potent angiogenic factor and one of the more widely studied as a prognostic factor in cancer patients (100-103), since it is considered to be the main angiogenic stimulator (104). Higher levels of VEGF are reported to correlate with the tumour burden and poor prognosis in patients with solid tumours (100, 101, 105). VEGF expression may also be induced by mutation of the p53 gene and activation of the ras/MAPK pathway in human CRC (106).

Several growth factors have been identified that regulate angiogenesis in colon cancer; the most important of these is thought to be VEGF (98). VEGF is the major growth factor promoting angiogenesis in CRC. Overexpression can be identified in about 50% of cases. Most of the reported studies have suggested that VEGF expression is associated with an adverse prognosis (105, 107). Evidence for the predictive role of VEGF with current available agents is lacking. However, in the future, tumours with high VEGF expression may be treated with targeted antibody therapy (108). The natural direct antagonist of VEGF is FLT-1, which may have a role in anti-angiogenesis therapy. It is detectable in the sera of cancer patients, however, its prognostic significance remains to be determined (109).

Markers of invasion/metastasis

Matrix metalloproteinases (MMPs). To invade the surrounding tissue and metastasise, tumour cells need to secrete enzymes that will break down the components of the surrounding extracellular matrix (ECM). Such enzymes include the matrix metalloproteinases (MMPs), a family of neutral metalloenzymes that together are able to degrade all the components of the ECM. The MMPs are secreted as latent proenzymes. They require activation through proteolytic cleavage of the amino-terminal domain and their activity depends on the presence of Zn^{2+} and Ca^{2+} (110, 111). Five MMP subclasses have been defined, grouped according to substrate specificity; interstitial collagenases (112), gelatinases, stromelysins, (113), metalloelastase (114) and membrane type-MMPs (MT-MMPs).

MMPs are now known to contribute to multiple steps of tumour progression in addition to invasion, including tumour promotion, angiogenesis and the establishment and growth of metastatic lesions in distant organ sites. In addition, it is recognized that MMPs not only can be synthesized by tumour cells but are frequently produced by surrounding stromal cells, including fibroblasts and infiltrating inflammatory cells. Finally, although creating gaps in matrix barriers remains a role for MMP activity, MMPs are also known to solubilize cell surface and matrix-bound factors that can then act in an autocrine or paracrine manner to influence cellular properties such as growth, death and migration (115). It has also been demonstrated that MMP and CD44 interactions are important in controlling tumour cell invasion and migration (116, 117).

In numerous work with different types of cancer, the expression levels of particular MMPs and their correlation with the clinicopathological characteristics of the patients have been studied. In general, there are 2 important aspects related to cancer progression in several studies: (i) the association between MMP expression and tumour grade or aggressiveness and (ii) the correlation of MMP expression and activity with recurrence or metastasis risk. Increased MMP activity and overexpression has been shown to correlate with tumour aggressiveness and metastatic potential in a wide range of cancers (110, 113, 118-120).

Inhibition of MMP activity in the extracellular space has been extensively studied as an approach to inhibit growth and invasion of neoplastic cells. MMP inhibitors (MMPIs) have shown efficiency against malignant tumours in preclinical studies (121). MMPIs are currently being assessed in several phase II and III trials in the treatment of different cancers.

Biochemical markers

Thymidylate synthase (TS). Thymidylate synthase plays an essential role in catalyzing the reductive methylation of deoxyuridylate (dUMP) to thymidylate (dTMP), which provides the sole intracellular *de novo* source of dTMP (122). Once synthesized, dTMP is then metabolized intracellularly to dTTP (the triphosphate form), an essential precursor for DNA biosynthesis. This reaction is critical as it maintains the essential metabolic requirements for cellular proliferation and growth.

Because of its essential role in DNA replication, human TS is an anticancer drug target. TS is the target for the widely used anticancer agent 5-FU, which is active against solid tumours like breast, head and neck and colon cancers. TS has been suggested as a prognostic factor of survival in CRC (123, 124) and of the response of tumour cells to 5-FU therapy (125, 126) where high TS levels correlated with a poorer prognosis. Increased TS levels in tumours are associated with resistance to chemotherapy with 5-FU. TS expression has been shown to be an independent prognostic factor in several cancers. Higher TS levels in hepatic metastases and resection margin are independent predictors of survival and progression in patients with metastatic CRC (127). Comparable results have been found in other tumour types such as gastric, (128) and cervical (129) cancers, with TS (+) tumours having significantly worse outcome compared to TS (-) cases.

Serum markers

Carcino embryonic antigen (CEA). CEA is a serum glycoprotein with a molecular weight of 180 kd that is one of at least 19 related molecules that are members of the immunoglobulin gene superfamily. As such, CEA functions as a homotypic intercellular adhesion molecule that promotes the aggregation of human colorectal carcinoma cells (130). CEA may facilitate metastasis of CRC cells to the liver and lung.

CEA is a normal cell product that is overexpressed by adenocarcinomas, primarily of the colon, rectum, breast and

lung (131, 132). Smokers have a higher circulating CEA concentration than non-smokers, but there are no significant effects of age, sex, or ethnic group on the normal range (133). The liver is the major site for clearance of CEA. Moderate to significant elevations of serum CEA can be observed in a variety of chronic and acute inflammatory diseases, including alcoholic cirrhosis, cholelithiasis, obstructive jaundice, cholangitis, liver abscess, emphysema, bronchitis, gastric ulcer, gastritis, diverticulitis, diabetes and collagen vascular diseases (134). CEA level elevations are not unique to CRC, but are observed in several carcinomas.

Elevated preoperative CEA is a prognostic factor independent of Dukes' stage in patients with CRC and is associated with poor prognosis (135, 136). The majority of the preoperative CEA studies showed that it was a useful prognostic indicator that remained significant even when Dukes' stage and histological grade were factored into a multivariate analysis (135-137). Some of the results were conflicting, especially on the issue of the prognostic significance of an elevated preoperative CEA for Dukes' B *versus* C, or colon *versus* rectal cancer (135, 136).

As a postoperative prognostic indicator following complete surgical resection of colon carcinoma, elevated plasma CEA levels should return to normal within 4 to 6 weeks (138). An elevated postoperative CEA is an adverse prognostic indicator. Recently, CEA mRNA levels have also been found to be useful for the evaluation of CRC progression, with elevated post-operative CEA mRNA predicting the presence of micrometastasis (139).

CA 19-9. The CA 19-9 assay measures a tumour-related mucin that contains the sialylated Lewis-a pentasaccharide epitope, lacto-N-fucopentaose II (140). CA 19-9 is produced by adenocarcinomas of the pancreas, stomach, gall bladder, colon, ovary and lung, and it is shed into the circulation. The upper limit of normal for healthy subjects has been defined by the cut-off value of 37.0 U/mL (141). CA 19-9 has become an established marker for pancreatic cancer (142, 143), but it is still regarded as a research test for CRC.

Numerous studies have addressed the potential utility of CA 19-9 in adenocarcinoma of the colon and rectum. The reported incidence of elevated serum CA 19-9 in CRC ranges from 20% to 40% (144, 145). The incidence of elevated CA 19-9 is stage-related, with the highest sensitivity occurring in patients with metastases (146, 147). However, the sensitivity of CA 19-9 was always less than that of the CEA test for all stages of disease (136, 144-146). The false-positive rate (> 37.0 U/mL) is 15% to 30% in patients with non-neoplastic diseases of the pancreas, liver and biliary tract (147, 148). Consequently, CA 19-9 cannot be used for screening asymptomatic populations.

Significant post-surgical decreases are observed for CA 19-9, but these decreases have not been correlated with survival or disease-free interval (149). Filella *et al.* (150) reported that, in 370 patients with CRC, progressive increases of serum CA 19-9 were observed in 48% of 96 patients with relapse. However, the CA 19-9 abnormality preceded clinical manifestation of the disease in only 25% of the cases and provided a median lead time of only 3 months (150). Kouri *et al.* reported that serum CA 19-9 is one of the most significant prognostic factors in chemotherapy-treated patients with advanced CRC and elevated significantly more often in patients with liver metastases (151).

Serum CA 19-9 elevations may be observed in as many as 20% to 40% of patients with late-stage CRC, but cannot be regarded as a diagnostic aid nor can it be used to detect early stage disease. Progressive increases of the marker may signal disease progression in 25% of the patients who express the CA 19-9 marker, but this monitoring provides only a minimal lead time of 1 to 3 months. Monitoring with CA 19-9 has not been shown to improve the management of patients with CRC.

Circulating tumour cells

Circulating tumour cells of CRC have been of great interest in cancer research. The detection of circulating cancer cells in peripheral blood has become the primary goal of this research, whereas histological sections, lymph nodes and bone marrow were previously studied. New methods of detection include rare cell enrichment and detection techniques like fluorescence microscopy, flow cytometry, RT-PCR and methylated DNA PCR, of which RT-PCR is the most widely used molecular method for the detection of circulating tumour cells (152) Among the target genes for RT-PCR, CEA mRNA seems to be the most frequently screened for in the blood of patients with gastrointestinal carcinomas (153, 154). A lot of different epithelial markers are targeted with these techniques *e.g.* cytokeratins, EGFR, CEA and EMA (152, 155). Clinically, circulating tumour cells have been found to be independent prognostic factors in lymph nodes and bone marrow. In blood, their presence appears to be an early marker for recurrence and relapse (156, 157).

Conclusion

A number of prognostic and predictive molecular markers for CRC can be measured and have been outlined in this review, but it is not yet clear whether they have prognostic value or therapeutic implications. They may well be useful in making decisions regarding the prognosis of CRC patients but further prospective trials are clearly required. By identifying and understanding molecular markers the effectiveness of treatment can be improved in several ways, for example it can lead to the development of marker-specific therapies. Prognostic markers may also improve the selection of patients for adjuvant therapy by identifying

those who will benefit most and therefore avoid toxic side-effects of treatment in patients with the least risk for recurrence. As yet predictive markers remain an open question but clearly they will have an important role to play in the future. For example they may help to select the patients who will respond the best to specific treatments.

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