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REVIEW

Novel biomarkers for patient stratification in colorectal cancer: A review of definitions, emerging concepts, and data

Manish Chand, Deborah S Keller, Reza Mirnezami, Marc Bullock, Aneel Bhangu, Brendan Moran, Paris P Tekkis, Gina Brown, Alex Mirnezami, Mariana Berho

Manish Chand, GENIE Centre, University College London, London W1W 7TS, United Kingdom

Deborah S Keller, Department of Surgery, Columbia University Medical Centre, New York, NY 10032, United States

Reza Mirnezami, Department of Surgery, Imperial College London, London SW7 2AZ, United Kingdom

Marc Bullock, Department of Surgery, University of Southampton, Southampton SO17 1BJ, United Kingdom

Aneel Bhangu, Department of Surgery, University of Birmingham, Birmingham B15 2QU, United Kingdom

Brendan Moran, Department of Colorectal Surgery, North Hampshire Hospital, Basingstoke RG24 7AL, United Kingdom

Paris P Tekkis, Department of Colorectal Surgery, Royal Marsden Hospital and Imperial College London, London SW3 6JJ, United Kingdom

Gina Brown, Department of Radiology, Royal Marsden Hospital and Imperial College London, London SW3 6JJ, United Kingdom

Alexander Mirnezami, Department of Surgical Oncology, University of Southampton and NIHR, Southampton SO17 1BJ, United Kingdom

Mariana Berho, Department of Pathology, Cleveland Clinic Florida, Weston, FL 33331, United States

ORCID number: Manish Chand (0000-0001-9086-8724); Deborah S Keller (0000-0002-8645-6206); Reza Mirnezami (0000-0003-4572-5286); Marc Bullock (0000-0002-2355-9494); Aneel Bhangu (0000-0001-5999-4618); Brendan Moran (0000-0002-9862-6241); Paris P Tekkis (0000-0002-0730-3907); Gina Brown (0000-0002-2336-622X); Alexander Mirnezami (000-0002-6199-8332); Mariana Berho (0000-0000-1111-1111).

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Correspondence to: Manish Chand, FRCS (Gen Surg), PhD, Associate Professor, Surgeon, Consultant Colorectal Surgeon and Senior Lecturer, GENIE Centre, University College London, Charles Bell House, 43 Foley Street, London W1W 7TS, United Kingdom. m.chand@ucl.ac.uk
Telephone: +44-20-34475879
Fax: +44-20-34479218

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Abstract

Colorectal cancer (CRC) treatment has become more personalised, incorporating a combination of the individual patient risk assessment, gene testing, and chemother-

apy with surgery for optimal care. The improvement of staging with high-resolution imaging has allowed more selective treatments, optimising survival outcomes. The next step is to identify biomarkers that can inform clinicians of expected prognosis and offer the most beneficial treatment, while reducing unnecessary morbidity for the patient. The search for biomarkers in CRC has been of significant interest, with questions remaining on their impact and applicability. The study of biomarkers can be broadly divided into metabolic, molecular, microRNA, epithelial-to-mesenchymal-transition (EMT), and imaging classes. Although numerous molecules have claimed to impact prognosis and treatment, their clinical application has been limited. Furthermore, routine testing of prognostic markers with no demonstrable influence on response to treatment is a questionable practice, as it increases cost and can adversely affect expectations of treatment. In this review we focus on recent developments and emerging biomarkers with potential utility for clinical translation in CRC. We examine and critically appraise novel imaging and molecular-based approaches; evaluate the promising array of microRNAs, analyze metabolic profiles, and highlight key findings for biomarker potential in the EMT pathway.

Key words: Biomarker; Colorectal cancer; Epithelial-to-mesenchymal-transition pathway; Molecular biomarker; MicroRNA; Metabolic biomarker; Imaging biomarker; Tumour regression grade

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Core tip: Biomarkers are an emerging field that can potentially guide the diagnosis, prognosis, and treatment course in rectal cancer. Here, the current definitions, classifications, recent developments and emerging biomarkers with potential utility for clinical translation in colorectal cancer are reviewed by international experts for a better understanding in surgery.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common types of cancer and cancer related deaths worldwide, with more than a third of the incidence involving the rectum[1,2]. Historically, rectal cancer was associated with the worst oncological outcomes[3]. The choice of treatment for rectal cancer was traditionally based upon the histologic type of malignancy, stage of the disease, the tumour-node-metastasis (TNM) staging system, and circumferential resection margin (CRM) status[2,4]. These variables provide clinical utility, help determine the need for neoadjuvant chemoradiotherapy (CRT) in patients with a threatened or involved CRM, post-operative adjuvant treatment in stage Ⅲ disease, and are prognostic of oncological outcome. Nevertheless, they provide an incomplete picture, as many patients with predicted early-stage disease harbour lymph node and systemic micrometastases, which can ultimately result in local and/or distant disease recurrence. Administration of neoadjuvant CRT is also sub-optimal as this treatment modality has many side effects, some of which are fatal, while others impair quality of life (QOL). Response to CRT is also unpredictable; up to 30% of patients will have a complete pathological response (pCR = tumour regression grade 1, TRG1), and could have omitted surgery altogether[5,6]. In 10% of cases however, no reduction in tumour volume is achieved, (tumour regression grade 5, TRG5); patients get no benefit from CRT, but are exposed to its side effects and may also experience cancer progression from delay to surgery[7]. These observations underscore the limitations of current methods for accurate stratification of patients with rectal cancer, and highlight the pressing need to identify biomarkers indicative of aggressive disease and/or response to CRT, in order to avoid patient under- or over-treatment.

With the advent of the "holy plane", standards for utilising chemoradiation, the application of minimally invasive surgery, and multidisciplinary tumour boards to guide care, the diagnosis, staging and management of rectal cancer has improved significantly in the past 25 years[8-18]. However, considerable variation still exists in management and outcomes, and recurrence continues to be a problem, with 5-year survival rates stubbornly below 60% in most European countries[19]. To further improve outcomes, there is a paradigm shift in the methods of diagnosis, staging, determining the patient's prognosis, and developing a personalized therapeutic course using advances in molecular biology, genetics, biochemistry, imaging, and the individual patient's personal risk assessment, neoadjuvant chemoradiotherapy, and adjuvant chemotherapy with surgery to optimise care[20].

The routine evaluation of microsatellite instability (MSI) and KRAS/NRAS/BRAF mutational status in clinical practice, for risk stratification in stage Ⅱ CRC and to determine the utility of monoclonal antibody-based adjuvant therapy, such as panitumumab or cetuximab, in metastatic disease, provides a clear proof-of-concept that more tailored therapeutic strategies can be translated to improve patient care through identification of biomarkers with functional activity. In this review, we explore the recent developments and emerging biomarkers with potential utility for clinical translation in CRC. We examine and critically appraise both novel imaging and molecular pathology based approaches; evaluating the promising array of microRNAs with biomarker potential; examining the developing techniques

and studies analysing metabolic profiles, and highlight key findings in the biomarker potential in the epithelial-to-mesenchymal-transition (EMT) pathway.

BIOMARKERS: TERMS OF REFERENCE, CONCEPTS, AND CLASSIFICATION

From the Biomarkers Definitions Working Group, the formal definition of a biomarker is a tumour characteristic that can be objectively measured and evaluated as an indicator(s) of normal biological or pathogenic processes, or pharmacologic responses to a therapeutic intervention that identify increased or decreased risk of patient benefit or harm[21,22]. Biomarkers can take multiple forms when used to detect or confirm presence of disease or to identify affected individuals[23]. Table 1 shows the categorisation of biomarkers. Most biomarkers applicable in CRC are prognostic - providing information about the likelihood of a condition, disease recurrence or progression; or predictive - providing information about the likelihood to respond to specific treatments. A cause of confusion around biomarkers has been the loose application of their definition and application. Distinguishing between predictive and prognostic biomarkers- which may not be mutually exclusive- has been another source of confusion in patient stratification and developing treatment strategies[23]. Another source of confusion is the inconsistent terminology previously used, restricting the scope of biomarkers to describing biological molecules or monitoring the treatment response. The current definition laid out by Cancer Research United Kingdom provides a standardised vocabulary for investigators, explicitly stating, "molecular, histologic, radiographic or physiologic characteristics are examples of biomarkers"[24]. With this progression, biomarkers may be used in a variety of situations and serve a number of purposes - as a diagnostic tool; for risk-stratification and staging of disease; as an estimator of prognosis; and, for prediction of disease response. The study of such biomarkers can be broadly divided into metabolic; miRNA; EMT; and imaging biomarkers. This review describes the current status of biomarkers in CRC within this framework.

MOLECULAR MARKERS ASSOCIATED WITH CARCINOGENESIS PATHWAYS

The search for molecular markers in CRC has been of significant recent interest. Extensive research has revealed that CRC develops through three major pathways: (1) chromosomal abnormalities that lead to mutations of oncogenes and tumour suppressor genes (classic pathway), characterised by the adenoma-carcinoma progression; (2) the microsatellite instability pathway that results from defects in the DNA repair system; and (3) the methylation pathway characterized by the epigenetic (post cellular division) methylation of numerous genes (methylator pathway). Hundreds of molecules involved in the chromosomal instability pathway have been associated with prognosis, however, only 1 single marker- the epidermal growth factor receptor (EGFR) pathway-has successfully proven clinical utility to date, largely due to the complexity and redundancy of cellular pathways, as well as the lack of therapies that can target the different biomarkers.

The EGFR pathway is the most clinically relevant molecule involved in the chromosomal instability pathway, and the EGFR serves as the main target for treatment in locally advanced CRC. However, this treatment is only useful for patients with wild-type KRAS (wtKRAS)[25]. Abnormal activation of the EGFR signalling pathways in CRC is mainly associated with three mutations in the mitogen-activated protein kinase and phosphatidylinositol-3-kinase (PI3K) pathways - KRAS, NRAS, and BRAF; these three mutations are reported to occur in more than half of all CRC cases[26]. Mutation of some of the components of the EGFR pathway, specifically BRAF V600E, KRAS (exon 2, 3, 4), and NRAS mutation (exon 2, 3, 4) cause the malignant cells to become resistant to anti-EGFR therapy; thus, patients should not be treated with either cetuximab or panitumumab. As a result, all patients with metastatic CRC should have investigation of KRAS/NRAS and BRAF mutation status prior to the start of treatment. KRAS/NRAS and BRAF mutational status may be performed by a variety of techniques, detailed discussion of the different methodologies is out of the scope of this review, however it is essential to emphasize that several technical factors including tissue fixation and tumour volume amongst others may affect the accuracy of the test results leading to erroneous information with the consequent impact on the decision making process. Furthermore, any tumour molecular analysis should be performed only by a certified laboratory that can prove competency and proficiency to perform testing.

Microsatellite instability status (MSI) (high or low) is the primary molecular marker for stratification of stage Ⅱ CRC. In node negative CRC, patients that are MSI-high have better outcomes than MSI-low tumours; therefore, adjuvant chemotherapy is usually not indicated in MSI-high tumours. MSI-high tumours arise in the setting of a defective DNA repair machinery, although several proteins have been implicated in DNA repair, abnormalities in MSH2, MSH6, PMS2 and MLH1 are the most commonly described. MSI-high tumours may be the result of an inherited mutation of the DNA repair genes (Lynch syndrome) or, more commonly, the abnormal epigenetic methylation of the *MLH1* promoter gene (sporadic MSI-high CRC). Analysis of the DNA repair system may be directly investigated by the tissue expression of MSH2, MSH6, PMS2 and MLH1 by immunohistochemistry, or alternatively by determination of microsatellite status by PCR.

The CpG Island Methylator Phenotype (methylator) pathway has been associated with a constellation of clinical (elderly patients, female, right-sided colon tumours) and histological features (poorly differentiated

Table 1 Biomarker types and definitions

Biomarker type	Objective
Diagnostic biomarker	These aim to identify the type of cancer, *e.g.*, PSA, CEA. They may also be used to monitor or detect disease recurrence
Pharmacological biomarker	These are used to measure response to a specific drug treatment. They are based on accurate pharmokinetic data and measure treatment response in early drug trials, *e.g.*, drug therapy to angiogenesis
Predictive biomarker	These are used to identify individuals who will most likely show a survival benefit to a specific targeted treatment, *e.g.*, improvement in local recurrence risk following treatment for circumferential resection margin involvement
Prognostic biomarker	These indicate the progress of disease and to estimate the risk of disease recurrence for example. They are used to estimate survival outcome and are independent of treatment strategy, *e.g.*, nodal disease
Risk/predisposition biomarker	These aim to identify individuals who are at significant risk of developing tumours, *e.g.*, *MLH1* gene
Screening biomarker	These are used to identify disease at an early stage, *e.g.*, PSA
Surrogate response biomarker	These can be used as an alternative to a clinically meaningful endpoint. Therefore there must be correlation with a clinical endpoint, *e.g.*, CEA

tumours and advanced stage disease). This pattern seen in approximately 15%-20% of CRCs, and involves atypical methylation of the mismatch repair gene *MLH1*. The precursor lesions in CIMP cancers are serrated polyps, not adenomatous lesions, with the initial mutation occurring most often in the *BRAF* oncogene[27]. BRAF mutations transform normal mucosa to aberrant crypt foci, hyperplastic, or sessile serrated polyps (SSP). With promoter methylation, loss of p16 occurs, allowing cells to progress to advanced polyps[28]. Increasing activity leads to methylation of MLH1, silencing transcription. Loss of MLH1 results in MMR deficiency and the MSI-H CRC phenotype. This is clinically important for diagnosis and therapeutic planning. An estimated 85% of MMR deficiency CRC is due to methylation of the MLH1 promoter region. BRAF can be used to distinguish between MLH1 promoter methylation and Lynch syndrome as the cause of CRC. A positive BRAF mutation is associated with the methylator pathway, and indicates MLH1 down-regulation through somatic methylation of the gene's promoter region, not through a germline mutation. BRAF mutations are rare in Lynch Syndrome-related CRC. On the converse, MLH1 promotor methylation in the absence of a BRAF mutation is consistent with Lynch Syndrome. Figure 1 shows a clinical algorithm for testing MMR deficiency. Several promising new therapies aimed at demethylation of genes are being developed.

METABOLIC PROFILING APPROACHES

In recent years the majority of molecular profiling approaches applied to the study of rectal cancer have focused on macromolecules (DNA, RNA, protein). While these avenues of research continue to offer significant insights into rectal cancer development and progression[29,30], it is widely accepted that a macromolecular, "bottom up" view of system activity cannot provide all the answers to facilitate precision approaches for rectal cancer diagnosis, prognosis and therapeutic personalisation[31]. Metabonomics (metabolomics/metabolic profiling) offers a dynamic "top down" view of system activity and is defined as the systematic, time-dependent measurement of metabolic shifts occurring in response to drugs, environmental stimuli or disease[32-34]. This approach provides rich *micro*molecular data downstream of the genome and proteome, offering a genuine functional "snapshot" of system activity[33].

The basic concept of altered cancer metabolism is well described across a variety of cancer subtypes[35-38]; the Warburg effect[39] is central to our understanding of cancer metabolism and glycolytic flux forms the basis for [^{18}F]-fluorodeoxyglucose enhanced positron emission tomography (FDG-PET) solid tumour imaging[40]. Current and next-generation nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS)-based profiling platforms offer a means of interrogating the cancer metabolome in unprecedented detail and moving beyond the Warburg phenomenon to identify an entirely new pool of disease-relevant biomolecular data. These profiling approaches are likely to have three main areas of application in rectal cancer phenotyping: (1) to identify novel metabolic fingerprints for accurate and ultra-fast tumour tissue diagnosis, staging and grading; (2) to develop metabolite-based models for prediction of response to chemo and/or radiotherapy; and (3) to devise novel next-generation targeted therapies designed to disrupt specific metabolic pathways implicated in rectal cancer.

NMR spectroscopy techniques are highly versatile and have been developed and applied for metabolic profiling of liquid-state and solid-state systems[41,42]. The technique of HR-MAS NMR has been introduced more recently to overcome spectral line-broadening effects seen with conventional NMR analysis of solids[41]. This approach allows acquisition of tissue-specific high-resolution spectra, which in combination with chemometric data treatment methods have the capacity to identify novel molecular signatures within rectal cancer tissue[43]. Recent work in this area has demonstrated increased abundance of taurine, glycine, lactate and scyllo-inositol in cancerous relative to healthy rectal mucosa, with a relative reduction in abundance observed for lipids and glucose[44] (Figure 2). These findings can be used to determine tissue status (cancerous or healthy) by entirely biochemical means, and have also revealed strong differences in metabolite profiles according to tumour stage[44]. From a pharmaco-

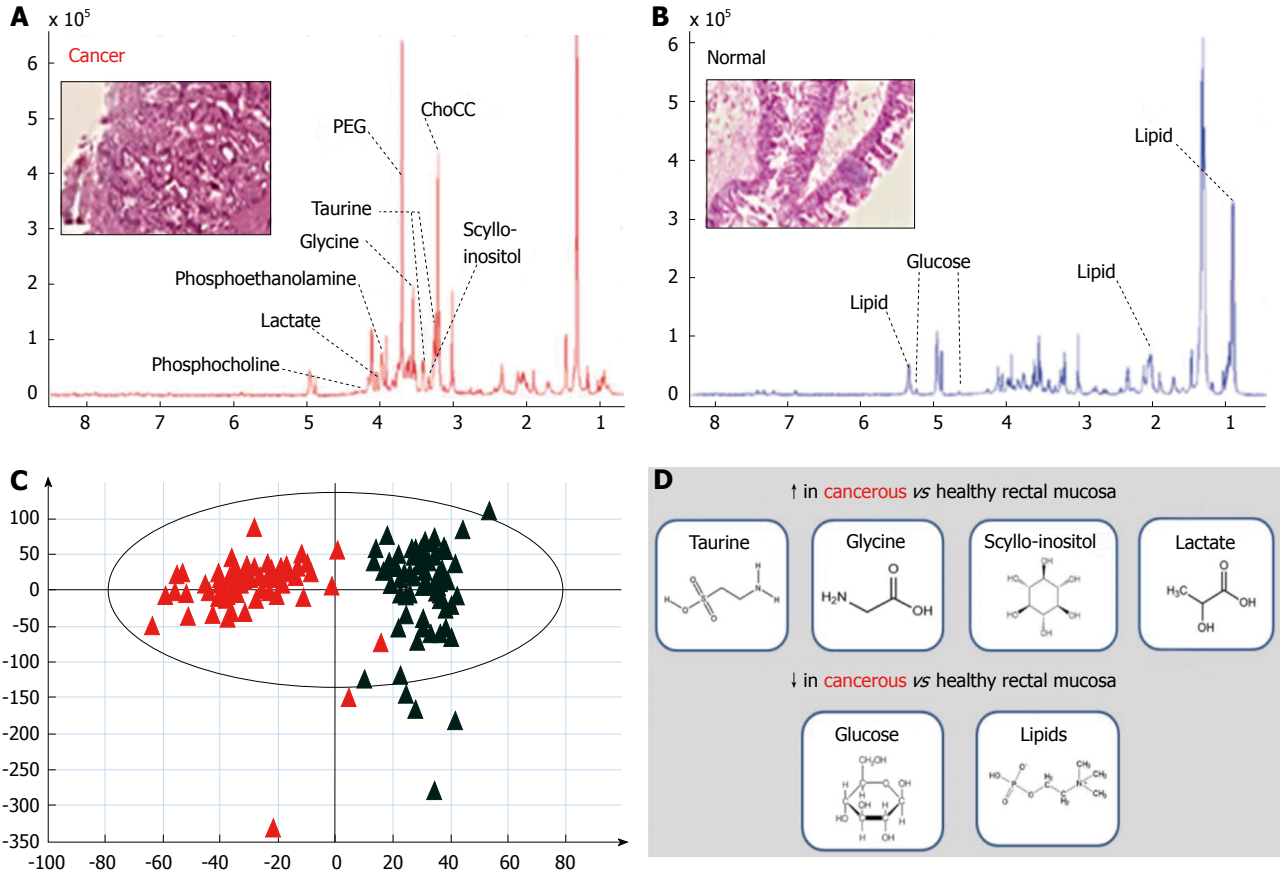


Figure 1 High-resolution magic angle spinning nuclear magnetic resonance spectroscopy of intact rectal cancer tissue biopsies. A and B: Annotated representative HR-MAS NMR spectral metabolite pattern for rectal cancer (A) and healthy rectal mucosa (B); C and D: Acquired data can then be subjected to supervised and un-supervised multivariate analysis using PCA and PLS-DA (C) to determine metabolic processes up- and down-regulated in cancerous tissue (D) (original data). NMR: Nuclear magnetic resonance; PCA: Principal component analysis; PLS-DA: Partial least squares discriminant analysis.

therapeutic perspective these discoveries offer the chance to develop novel anti-cancer agents; for example, taurine (2-aminoethane sulphonic acid), a common beta-amino acid has a known role in a number of fundamental physiological functions including cellular osmoregulation, cell-membrane stabilization and protein assembly[45]. Exploiting this finding by disrupting taurine handling within the rectal cancer microenvironment may offer a means of developing next-generation targeted agents for rectal cancer down-staging[46].

Mass spectrometry approaches have shown recent promise in the development of metabolite-based biomarker discovery for prediction of response to chemoradiotherapy. Crotti *et al*[47] described novel peptidomic methodology in an analysis of samples of serum collected pre- and post-CRT subjected to matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry. A comparison of pre-treatment serum fingerprints from responders [Mandard tumour regression grade (TRG) 1 and 2] and non-responders (Mandard TRG 3-5) identified three peptides (m/z 1082.552, m/z 1098.537 and 1104.538) that were capable of robust class separation. Kim and colleagues also used a MALDI-based approach, but specifically sought to evaluate the abundance of low-mass ions (< m/z 1000) in serum samples acquired from 73 patients with locally advanced rectal cancer, prior to CRT[48]. A panel of nine low-mass ions were found to have discriminatory capacity, with hypoxanthine (HX; m/z 137.08) and phosphoenolpyruvic acid (PEP; m/z 169.04) highlighted as the most significant. Lower levels of HX and higher levels of PEP were shown to strongly correlate with improved response to CRT (TRG 1, 2). These studies indicate the exciting potential for the development of a circulating biomarker panel to predict chemoradiosensitivity prior to commencing therapy.

MiRNA AND RESPONSE TO TREATMENT

MicroRNAs (miRNA) are highly conserved, short, non-coding nucleotide segments that regulate gene expression post-transcriptionally through repressing translation or targeting mRNAs for degradation[49]. miRNA genes account for between 2%-5% of the human genome and are commonly clustered within introns[50]. Each miRNA is estimated to interact with multiple mRNA targets and, as a consequence, thus, these sequences may regulate more than 30% of all human genes[51,52]. Oncogenes and tumour-suppressor genes are being discovered under miRNA control, with the majority of miRNA genes found within cancer-associated genomic regions[53,54]. In CRC,

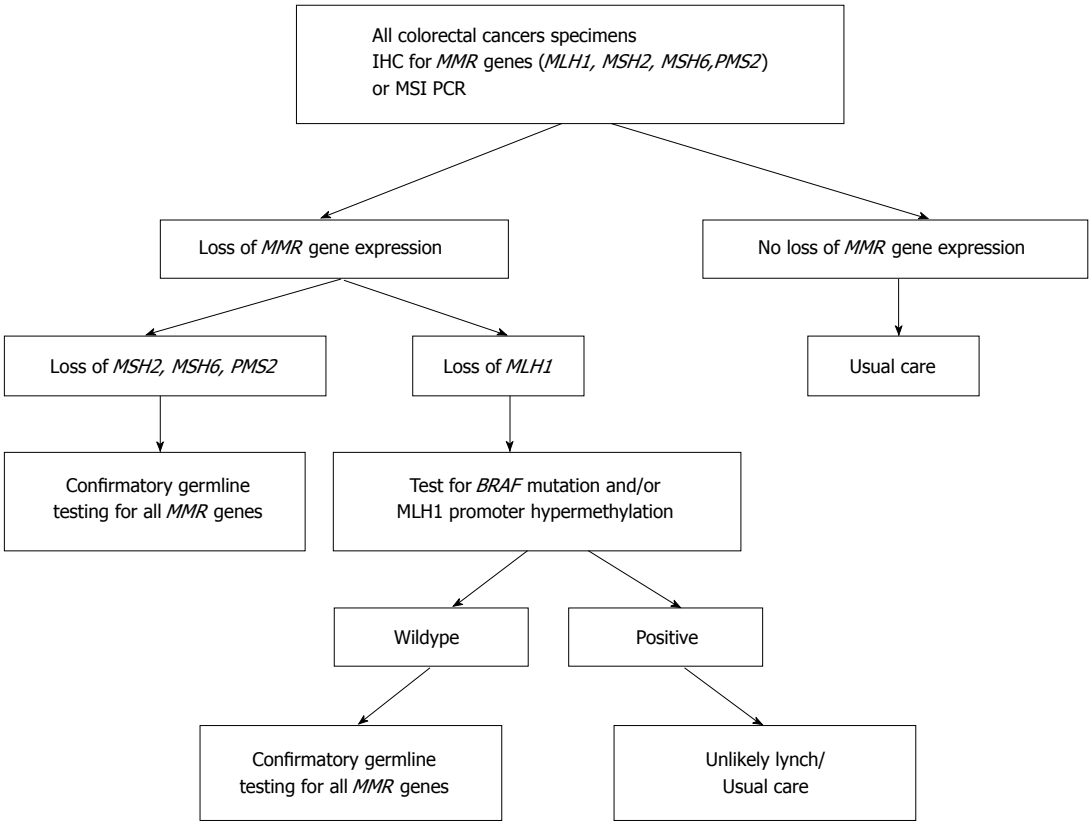


Figure 2 Algorithm for testing of mismatch repair genes in colorectal cancer for Lynch syndrome. MMR: Mismatch repair; MSI: Microsatellite instability.

abnormally expressed miRNAs disrupt cellular signal transduction and cell survival pathways, such as Wnt/β-catenin, EGFR, and p53, linking miRNA to known events in the pathway of malignant transformation[55].

Accumulating evidence suggests that miRNAs may also have powerful clinical applications. miRNA expression profiles are capable of discriminating tumours of different developmental origin[56]. Furthermore, the expression of individual miRNAs may be used to predict patient survival, tumour stage, the presence of lymph node metastases and the response to therapy in CRC[55,57,58].

Three studies have specifically examined the utility of miRNA expression signatures in predicting chemoradiotherapy response in rectal cancer[59-61]. Della Vittoria Scarpati *et al*[59] examined miRNA expression in fresh-frozen pre-treatment tumour specimens from 38 patients with locally advanced (T3/T4 Node +ve) rectal cancer and compared miRNA profiles in patients with complete (Mandard TRG 1; n = 9) and incomplete (Mandard TRG > 1; n = 29) pathological responses to a standardised neoadjuvant chemoradiotherapy regime consisting of capecitabine, oxaliplatin and 45 Gy of pelvic conformal radiotherapy. Thirteen significantly differentially expressed miRNAs were subsequently validated using high sensitivity TaqMan® qRT-PCR, of which 2; miR-622 and miR-630, were found to predict chemoradiotherapy response with 100% sensitivity and specificity[59].

A similar analysis of 20 patients undergoing combined radiotherapy and capecitabine/5-FU chemotherapy compared "responders", namely those displaying a positive response to treatment (Mandard TRG 1 and 2) with "non-responders" (Mandard TRG 3-5). TaqMan Low Density Arrays identified a miRNA signature consisting of 8 miRNAs capable of correctly classifying 90% (9/10) of responders and 90% (9/10) of non-responders[60].

A third study, which used formalin fixed rather than fresh rectal cancer specimens identified a miRNA signature consisting of just 3 miRNAs (miR-153, miR-16 and miR-590-5p), capable of distinguishing patients with complete and incomplete responses to therapy, however the value of this data is unclear as patient demographics, tumour characteristics, study end-points and the neoadjuvant treatment strategy were not clearly described[61].

As profiling methodology and the definition of tumour regression vary between these 3 studies, inter-study comparisons are of limited value; however it is important to note that no overlap is observed between the miRNA signatures described. This suggests that an miRNA based "therapy-response" prediction tool is some way from becoming a reality however; other studies have clearly established that miRNAs do play a role in regulating the tissue response to neoadjuvant therapy in CRC[62-64]. Perhaps by focusing on the contribution of miRNAs within the biological pathways that govern resistance and/or sensitivity to neo-adjuvant therapy in rectal cancer, more clinically pertinent data will emerge on the role of miRNA as a potential biomarker in cancer treatment strategies[65].

EMERGING TECHNOLOGY, LIQUID BIOPSIES

The term "liquid biopsy" in cancer arose when circulating tumor cells (CTC) were proposed as alternatives to conventional tissue biopsy in breast cancer for prognosis and evaluation of treatment responses[66]. The theory has continued to grow experimentally and has gained particular traction in CRC. The clinical applications of liquid biopsy in CRC continue to grow, including detecting premalignant and early-stage cancers, identification of aggressive phenotypes and high-risk patients, assessing tumor heterogeneity, residual, and recurrent disease, and monitoring treatment response[67]. In colon cancers, liquid biopsies may hold prognostic information beyond the nodal status for determining whether to administer adjuvant chemotherapy, while in rectal cancer, liquid biopsy may have roles for both primary disease evaluation and monitoring treatment response[68]. Possible sources of liquid biopsies include blood, urine, saliva, and stool, which contain cancer-derived subcellular components, such as circulating tumor DNA (ctDNA) and circulating miRNAs.

Tumour-tissue remains the "gold standard", but the advent of ctDNA analysis from blood samples has promise as a non-invasive biomarkers. Studies have reported a direct relationship between ctDNA levels and tumor burden, stage, vascularity, cellular turnover, and response to therapy[69-71]. It can enable efficient temporal assessment of disease status, response to intervention, and early detection of recurrence superior to current strategies, such as CEA[72]. ctDNA can monitor and recognize high-risk individuals, as the plasma tumour DNA levels are significantly higher in patients with increased advanced/stage Ⅳ disease, recurrence, or metastasis[73,74]. ctDNA may be sensitive to detect with early, presumably curable CRC from common mutations, which could have implication for diagnostic testing[75]. Meta-analysis has demonstrated high overall sensitivity and specificity for detecting the *KRAS* oncogene mutation in CRC, showing it may be a viable alternative to tissue analysis for the detection of *KRAS* mutations and subsequent therapeutic planning[75]. Further, comparative analysis between CTCs and ctDNA in metastatic CRC has shown strong concordance between ctDNA and tissue for RAS, BRAF, and ERBB2 mutations (84.6%) and greater detectability than CTCs with a smaller amount of blood sampling[76]. ctDNA may hold specific promise as a biomarker to guide therapy in post-operative locally advanced rectal cancer, but further studies are needed for validation[77]. There are limitations to ctDNA as a biomarker. Although ctDNA targets offer a high specificity, it is scarce in circulating biofluids- representing less than 1% of the total circulating free DNA and may be inadequate as clinically applicable diagnostic biomarkers. The best source of ctDNA is still uncertain and the size of the DNA released from dead cancer cells is longer than that of non-neoplastic DNA[70,78]. Large scale controlled trials are needed for validation.

miRNA is an alternate for liquid biopsy. miRNAs have features making them ideal candidates for development as disease-specific biomarkers, and may offer superior sensitivity and specificity compared with ctDNA for diagnosing CRC[79]. miRNAs are generally stable in blood and other body fluids due to their small size and their ability to escape from RNase-mediated degradation. miRNA expression levels are different in tumour compared to normal colon tissues[80]. miRNA are actively secreted from living cells, while most ctDNA is dependent on release from apoptotic or necrotic cells[81,82]. miRNA-based diagnostic markers and panels have been identified for early detection, risk of recurrence at the time of diagnosis, complement to CEA for identification of distant metastasis, and stratification of patients with poor prognosis and greater likelihood of metastasis to the lymph nodes, liver, and peritoneum[80,83-88]. These miRNAs are detailed in Table 2. While a promising tool for "precision medicine", there are limitations of circulating miRNAs as biomarkers in CRC. The existing studies use relatively small sample sizes, are retrospective in design, and utilized non-standardized sampling procedures. Larger, controlled studies are needed in order to validate the best purification method and clinical use of circulating miRNAs in CRC.

An example of a blood sample-based diagnostic biomarker that could make a clinical impact is methylated *Septin 9* (m*SEPT9*), which is validated to distinguish CRC from normal blood using real-time PCR[89]. This non-invasive, blood-based tool for CRC could improve screening and surveillance compliance over colonoscopy and other screening methods[90]. While monitoring of mSEPT9 may hold promise for CRC screening, a larger study population and more prospective studies are needed to validate mSEPT9 as a diagnostic biomarker in CRC.

ROLE OF EPITHELIAL MESENCHYMAL TRANSITION IN PRODUCING RECTAL CANCER CELLS WITH A RADIORESISTANCE PHENOTYPE

EMT is a physiological process resulting in transformation of stable epithelial cells into mobile mesenchymal cells[91]. While EMT is a normal process during human development, it has also been shown to occur in carcinogenesis[92]. In this situation, the resulting abnormal mesenchymal cells, which evade the influence of normal cellular control mechanisms, display an aggressive and invasive phenotype. These cells are increasingly linked to formation of micro-metastases, and causation of resistance to the effects of radiotherapy.

EMT cellular biology

Down-regulation of membranous E-cadherin is the classical finding of EMT. This results in loss of intercellular epithelial junctional complexes, promoting migration of

Table 2 Candidate liquid biopsy/circulating miRNA biomarkers[145]

Expression level	Diagnostic biomarker	Prognostic biomarker (malignant potential, tumor recurrence)	Predictive biomarker (chemosensitivity)
High	miR-92a, miR-141, let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, miR-23a, miR-378	miR-141, miR-320, miR-596, miR-203	miR-106a, miR-484, miR-130b
Low		miR-15a, miR-103, miR-148a, miR451	

Adapted from Tsutomu Kawaguchi *et al*. Circulating MicroRNAs: A Next-Generation Clinical Biomarker for Digestive System Cancers. *Int J Mol Sci* 2016; 17: 1459.

cells[93-95]. The microRNA-200 family has been identified as a key post-transcriptional regulator of this process, through its targeting of E-cadherin transcriptional receptors[96]. Subsequent escape from growth factor control, with uncontrolled proliferation, results from the EMT process[94,95]. An end consequence of this pathway is tumour budding, defined as the presence of single cells or small cell clusters at the invasive front of tumour growth[97]. Tumour budding is highly likely to be associated to EMT at the poorly differentiated invasive front[97-100].

Current evidence

There is increasing evidence linking EMT to chemoresistance in ovarian, pancreatic and breast cancer cell lines[101-104], and in human lung cancer specimens[105]. Emerging evidence is also relating EMT to response to chemoradiotherapy in CRC. This initially arose from testing chemoresistance in colorectal cell lines[106-108]. However newer human evidence is relating EMT as an independent biomarker of tumour budding, lymph node metastases, and radioresistance[109]. The largest of these demonstrated that, in 103 patients with advanced rectal cancer, an EMT phenotype was associated with non-response to neoadjuvant therapy and reduced cancer specific survival[110]. More evidence from human rectal cancer tissue is urgently needed to assess its potential as a biomarker.

Windows for intervention

A genetic predisposition to loss of E-cadherin and subsequent EMT may be causative, meaning that pre-treatment biopsy analysis presents a window for intervention. Radiotherapy may also be a traumatic triggering stimulus which forces some cells into an EMT phenotype, meaning other methods for patient selection may be required; overlap in causation is likely.

EMT as a prognostic and therapeutic biomarker

The biological action of metformin down-regulates the EMT transcription factors and up regulations E-cadherin[110]. Its low toxicity profile makes it a feasible option in EMT prevention attempts, with subsequent improvements in response to neoadjuvant therapies[111,112]. Additionally, cyclo-oxygenase (COX) inhibitors have shown potential to prevent EMT by reducing vimentin expression and increasing cell surface E-cadherin expression in cell line models[113]. However, due to their serious associated cardiovascular side-effects, the particular COX agent and dose require optimisation before widescale use[113,114]. The potential role of post-transcriptional microRNA-200 regulation presents a further potential therapeutic target[96].

ROLE OF IMAGING BIOMARKERS IN DETECTION AND MONITORING DISEASE

The concept of an imaging biomarker is relatively new, but one which is becoming an increasingly important component of many phase Ⅱ/Ⅲ clinical trials as a surrogate endpoint. Imaging biomarkers may allow objective assessment of the tumour response to therapy and/or non-invasively detect early disease. Currently, the imaging techniques that seek to quantify treatment response in CRC can be broadly divided into those which measure tumour size and those which measure tumour activity. Whilst size criteria are the more commonly used biomarkers to assess radiological response in clinical trials because of their association with survival outcomes, it is the functional imaging techniques which are feted as having the greatest potential in uncovering the underlying biological processes which lead to cancer.

Measuring changes in tumour size

Reduction in tumour size has been shown to be a useful biomarker[115]. This can be measured in one-, two- or three-dimensions by various routine imaging techniques such as CT and MRI[116]. However, the two commonly used criteria - WHO[117] and RECIST[118] (Table 1); have contrasting characteristics, in particular in the technique used to measure tumour size - only one dimension using RECIST criteria. Further limitations to using size measurements have been deciding on what degree of tumour bulk reduction constitutes a significant clinical response. An example of this is has been shown by Morgan *et al*[119], who investigated the effect of a VEGF receptor inhibitor on colorectal metastases, whereby significant size reduction was not met with an equally significant overall response (< 10%). However the novel MRI-based tumour regression grade (mrTRG), which stratifies response on the degree of fibrosis visualised in the tumour following chemoradiotherapy, has been shown to be a useful clinical tool[120]. The degree of fibrosis seen on MRI following CRT on a scale analogous to histopathological tumour regression grade (TRG)[121] - tumour signal that has been completely replaced by radiological evidence of fibrosis is defined as radiological

complete response (mrTRG1-2)[122]. These findings have been validated in a prospectively enrolled, multicentre study[123] and used to influence treatment decisions in particular "deferral of surgery" programs. In the above study, multivariate analysis showed mrTRG hazard ratios (HR) were independently significant for overall and disease-free survival. Using fibrosis as a radiological feature is not limited to measuring tumour size but can be used to quantify other prognostic factors such as extramural venous invasion (EMVI), for example[120]. A further study using prospectively collected data on EMVI response to neo-adjuvant chemoradiotherapy showed hazard ratio of 2.37 for DFS in tumours which had undergone more than 50% fibrosis of tumour signal in extramural vasculature[124].

Measuring tumour activity

These techniques involve analysis of images to quantify the functional activity of tumours. The most common example of this is positron emission tomograhy (PET) with Fluorodeoxyglucose (18-FDG), which relies on the principle of a differential glycolytic rate seen in tumour cells. Using the glucose analogue 18-FDG gives an assessment of tumour metabolism[125,126] by quantification of standard uptake values (SUV). However as timing of the scans from administration of the 18-FDG and subsequent clearance rates may vary between centres and patients, comparisons and standardisation of technique has been difficult. It is also important to note that until now, there has been no validation of response.

Dynamic contrast-enhanced (DCE) CT/MRI provides a detailed assessment of tumour bloodflow through acquisition of data as specific contrast material passes through the vasculature. DCE-CT has the potential to identify angiogenesis and has been shown to be able to distinguish from diverticular disease as well as detect early liver metastases[127,128]. Although reports have identified a correlation between tumour blood flow, the development of metastases, and decreased survival outcomes[129,130], this has not been translated to widespread clinical application. Vascular endothelial growth factor (VEGF) is upregulated in up to 78% of CRCs[131,132] and is a potential target for functional imaging techniques. Bevacizmab is an anti-VEGF-A monoclonal antibody and DCE-MRI has been used in rectal cancer to evaluate treatment response using conjugation with a radioclueotide[133-135]. The analysis in DCE-MRI uses two compartments of plasma and extravascular-extracellular space to compare contrast agent - K^{trans} is the constant which is used to depict the bloodflow. Several studies have validated K^{trans} with expression of growth factors, such as VEGF and immunohistochemical confirmation of vessel architecture[136-139]. Reduction in K^{trans} using Vatalanib (tyrosine kinase inhibitor which target VEGF receptor-2) for metastatic CRC with liver disease have shown promising results in the phase Ⅰ/Ⅱ setting[119,140] but not been translated to survival benefit in phase Ⅲ trials.

Diffusion weighted imaging (DWI) assesses the movement of water molecules within cells using diffusion-weighted gradients to T2 sequences. Quantitative analysis is possible by calculation of the apparent diffusion coefficients (ADC), which are inversely correlated with tumour cellularity. DWI has been effective in detecting small liver metastases and differentiation from inflammatory lesion[141-143], as well as detecting lymph node metastases[144], but application has been limited to mainly experimental work.

CONCLUSION

The interest in biomarkers relating to rectal cancer is clearly increasing. They form a new aspect of clinical and laboratory research which help translate these concepts to more meaningful applications in patient management. Much of the current literature is still in its embryonic stage, but as more results from clinical trials using biomarker endpoints and outcome measures become available, there will be a better understanding by clinicians of their potential, with possible future application to improve the predictive and prognosis of rectal cancer.

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