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Predictive and prognostic biomarkers for neoadjuvant chemoradiotherapy in locally advanced rectal cancer

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Abstract

Locally advanced rectal cancer is regularly treated with trimodality therapy consisting of neoadjuvant chemoradiation, surgery and adjuvant chemotherapy. There is a need for biomarkers to assess treatment response, and aid in stratification of patient risk to adapt and personalise components of the therapy. Currently, pathological stage and tumour regression grade are used to assess response. Experimental markers include proteins involved in cell proliferation, apoptosis, angiogenesis, the epithelial to mesenchymal transition and microsatellite instability. As yet, no single marker is sufficiently robust to have clinical utility. Microarrays that screen a tumour for multiple promising candidate markers, gene expression and microRNA profiling will likely have higher yield and it is expected that a combination or panel of markers would prove most useful. Moving forward, utilising serial samples of circulating tumour cells or circulating nucleic acids can potentially allow us to demonstrate tumour heterogeneity, document mutational changes and subsequently measure treatment response.

Keywords: Rectal cancer; Neoadjuvant; Biomarker; Chemoradiation

1. Introduction

Colorectal cancer is one of the leading causes of mortality in the developed world, with rectal cancers comprising a third of cases. Rectal cancers carry a worse prognosis and in the locally advanced setting, are treated differently to colon cancers, with trimodality therapy consisting of neoadjuvant chemoradiation, surgery and adjuvant chemotherapy. Neoadjuvant therapy consists of either long-course chemoradiation or short-course radiation. Long-course chemoradiation is given as 45–50.4 Gy radiation in 25–28 fractions, typically over 5–6 weeks, and short-course as 25 Gy in 5 fractions over a week. Long-course therapy is recommended for patients with mid to lower tumours as it provides a higher rate of sphincter preservation due to tumour downstaging [1]. Higher tumours may be treated with short-course radiotherapy. Short-course radiation has been shown to have effective local control and no difference in overall survival compared to long-course chemoradiotherapy [2,3]. Surgery follows neoadjuvant therapy, consisting of proctectomy with total mesorectal excision. Despite trimodality therapy, around 40% of patients still recur [1].

1.1. Current benchmark – tumour regression and downstaging

The clinicopathological variables that are currently used to guide therapy and prognosticate include post-treatment pathological stage (ypT and ypN) and tumour regression grade (TRG) that are assessed microscopically [4,5]. TRG and tumour downstaging have become universally accepted for assessing tumour response to neoadjuvant therapy in locally advanced rectal cancer [4–6]. Hence it is used in the majority of studies as the benchmark for comparison with newer biomarker candidates. TRG and downstaging after neoadjuvant therapy have resulted in an increased rate of sphincter preservation with curative resection, 9–30% with complete tumour regression and 46–60% with tumour downstaging [4,7]. A survival benefit has also been demonstrated, with pathological complete response associated with superior survival. A recent study reported both complete and intermediate tumour regression to be associated with improved long-term outcomes with a follow-up period of 132 months, based on the Dworak classification [8]. It is important to note that there is no standardised method of reporting regression, with various different grading classifications, based on degree of fibrosis and percentage of viable tumour cells [9–11]. This lack of standardisation perhaps explains the lack of association between tumour regression and survival in a study of 297 patients that looked at both cellular response/regression (based on Mandard and Rodel classifications) and downstaging [12]. Nevertheless, tumour regression is included as a prognostic factor in the 7th edition cancer staging manual of the American Joint Committee on Cancer (AJCC) [13].

Studies have also demonstrated the importance of pathological stage [8,14–16]. A retrospective study of 77 patients correlated the residual pathological stage and survival outcomes, both local and distant [5]. Survival by pathologic stage was reported to be 100% for ypT0–2, N0 cancers, 80% for ypT3–4, N0 and 73% for ypTx, N1–2. Local recurrence of disease was observed in no patients with ypT0–2, N0 as compared with 13% (3/24) in ypT3–4, N0 and 16% (5/31) in ypT0–4, N1–2 patients. Downstaging was shown in multivariate analyses to be significant for survival in the aforementioned study [12].

Other histological factors have also been investigated along with tumour response in a multivariate analysis in 297 patients [7]. This demonstrated that a pathological response of more than 95%, positive lymph nodes, lymphovascular invasion and perineural invasion were significantly

associated with recurrence-free and overall survival. The treatment consisted of long-course radiotherapy with concurrent chemotherapy (93% treated with 5-fluorouracil (5-FU) and the other 7% treated with irinotecan). Interestingly, they showed that there may not be a difference between a pathological response of greater than 95% and pCR. Correlation of clinicopathological factors and tumour characteristics with TRG was explored in another study of 98 patients with rectal cancer treated with chemoradiotherapy [17]. Non-fixed tumours and tumours with circumferential extent less than or equal to 50% were associated with good response. Tumour circumferential extent of greater than 60% was associated with lower pCR in a study of 562 patients treated with concurrent radiotherapy +/− chemotherapy (5-FU in 77% and uracil with tegafur in 2%) [18]. This remained significant in multivariate analysis ($p = 0.033$). Greater tumour circumferential extent ($p = 0.020$) as well as greater distance from anal verge ($p = 0.010$) predicted lower tumour downstaging.

Time to surgery is also becoming a focus of interest, based on the observation that a longer duration from radiation to surgery results in greater tumour regression [19]. One hundred and sixty-seven patients treated with neoadjuvant radiation were assessed for predictive and prognostic markers of tumour downstaging and tumour response [16]. Time before surgery was found to be a predictor of tumour downstaging.

These current factors that determine prognosis are measured on the surgical specimen, and as yet there are no robust biomarkers that can aid in stratification of patient risk in order to adapt and personalise therapy. In studies that have adopted a 'watch-and-wait' approach, a combination of radiologic, clinical and endoscopic criteria is used after completion of neoadjuvant treatment, and intense surveillance is adopted in cases of complete clinical response that did not proceed to surgery [20]. There is a great need to find markers that can predict tumour radiosensitivity and response, particularly upfront or early on in the treatment course. Hence this is particularly relevant in the neoadjuvant phase, given that patients who experience a complete response may not need to proceed with surgery or additional therapy.

Many biomarkers have been investigated and compared against the current standard of TRG. These include tumour tissue biomarkers of either biopsy or surgical specimens, and blood-based biomarkers as well as tumour related nucleic acids and circulating tumour cells. Our review aims at providing a summary of standard and potentially novel clinical and pathological biomarkers after treatment with neoadjuvant chemoradiation. Selected studies on short-course radiation are included to illustrate certain points. For the most part, interesting correlations exist. However, some data are conflicting and there is as yet no single marker that is robust

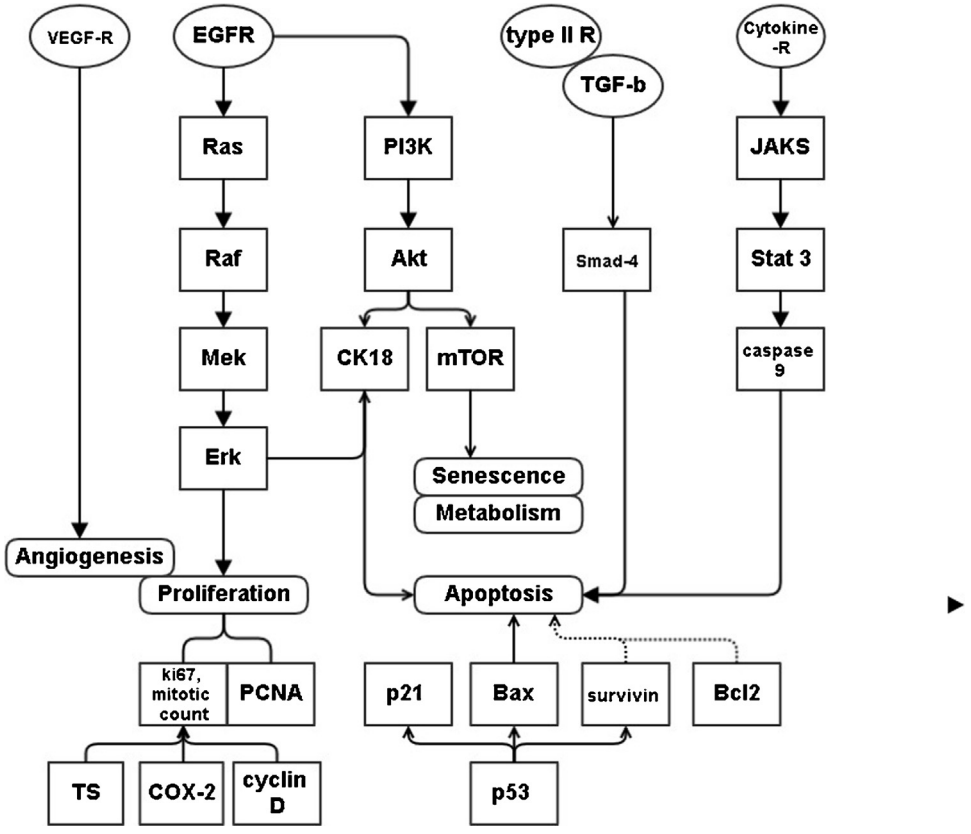

Fig. 1. Pathway modulators that have been investigated as potential biomarkers of response in rectal cancer (dotted lines indicate an opposing effect).

enough to be clinically useful on its own. It is expected that a combination of these markers may be required to have the most clinical utility.

2. Solid tissue-based biomarkers

2.1. Biomarkers under investigation

Potential biomarker candidates have included markers of cell proliferation, apoptosis, and angiogenesis (Fig. 1). Aberrations in these pathways have been implicated in radioresistance. Specific examples include EGFR Sp1-216 G/T polymorphisms, DNA double-strand break repair pathways, and PI3K-Akt hypoxia-related resistance [21–24]. Many studies have investigated a single biomarker, while others have classified biomarkers based on mechanism of action, or included multiple candidates that have shown promise in earlier studies (Table 1). Many of these studies have relatively small patient number, are retrospective in nature and differ in methodologies, making it difficult to draw firm conclusions or carry out cross-study comparisons. Moreover, there is a lag time bias in these studies, as the effect of chemoradiotherapy is continual over time [25]. Some pertinent studies are discussed herein to illustrate certain concepts in an attempt to draw meaningful conclusions.

Biomarker candidates include p53, epidermal growth factor receptor (EGFR), thymidylate synthase, Ki67, p21, bax/bcl-2, COX-2, cyclin D1, survivin, dihydropyrimidine dehydrogenase (DPD), microsatellite instability (MSI) and carcinoembryonic antigen (CEA) [26,27]. Kuremsky et al. suggested that EGFR, thymidylate synthase and p21 have potential utility while Spolverato et al. concluded that there is no single biomarker that is robust, sensitive and specific enough or that has been tested in a rigorous prospective study where treatment factors and response grading are standardised. This is perhaps not surprising given the multiple targets, feedback mechanisms and redundancy of these pathway modulators. Combinations of these biomarkers have yielded more promising results, with a study combining three elements Ki67, thymidylate synthase and Bax to have 83% sensitivity and 84% specificity in predicting tumour response [28].

Differing methodologies also pose challenges in interpreting study findings. Mutations found by gene sequencing of pre-treatment biopsies for p53 were higher in non-responders to neoadjuvant chemoradiation however, results using immunohistochemistry (IHC) were mixed and no conclusions could be drawn [29]. This review also surmised p21 to have reasonable predictive utility, with high levels post-treatment correlating with worse outcomes. Pre-treatment expression of COX-2 and EGFR appeared to correlate with poor response while high spontaneous or intrinsic apoptosis correlated with a good response.

Survivin is thought to be inversely related to the apoptotic index. Pre-treatment survivin expression correlated with tumour downstaging in a study of 54 patients, with high survivin correlating with less downstaging [30]. Bax, on the other hand, is a proapoptotic protein of the Bcl-2 family, and Bax expression was found to be significantly higher in the complete pathological response group compared to partial response [31]. Similarly, Smac is a pro-apoptotic protein, and in a study of 40 patients, high Smac correlated with tumour volume reduction [32].

It is also unclear how these biomarkers are altered by radiation, and not all studies compared pre- and post-treatment samples. Pre- and post-treatment samples were immunostained for p53 and its downstream targets of MDM2 and p21 [33]. p53 did not change with short-course hyperfractionated radiation (2 fractions delivered daily for 5 days), however MDM2 and p21 were moderately induced. The authors attributed the lack of p53 induction to loss of p53 function, which correlated with mutations in the p53 genes. Other studies demonstrated a decrease in Ki67 and p21, in conjunction with an increase in apoptosis [34–37]. A panel of molecular markers, including p53 and p21, DNA mismatch repair proteins (MLH-1 and MSH2), MIB-1, thymidylate synthase, EGFR and VEGF in 91 patients at biopsy and at time of surgery showed baseline over-expression for p53, p21, MLH-1, MLSH2, MIB-1, thymidylate synthase, EGFR and VEGF. Of all these biomarkers, only VEGF decreased after treatment [38].

Chemotherapy and radiotherapy may also have differential effects. Preoperative radiation increased COX-2, however the addition of chemotherapy blunted this effect [39]. The fibro-inflammatory reaction increased after addition of chemotherapy to radiation and correlated with T-stage downstaging. Higher thymidylate synthase levels were associated with higher tumour response (as per classification by Zlobec et al. [40]) in patients treated with chemoradiotherapy, but not in the group treated with radiation alone, raising the question of whether the addition of chemotherapy is purely synergistic [41]. Thymidylate synthase plays an important role in pyrimidine nucleotide synthesis and is a target enzyme for 5-FU which is the most common backbone chemotherapy agent in rectal cancer. Low thymidylate synthase levels have been shown to confer a poorer response to adjuvant therapy in colorectal cancer [42].

Biomarker changes early on during the treatment course can potentially classify patients into responders and those who will not derive a benefit from ongoing treatment, allowing stratification of therapy. p21 and apoptosis-positive biopsy specimens obtained 7 days post commencement of chemoradiation correlated with response based on regression, size reduction and T-stage downstaging [43].

Almost every study mentioned thus far has included a marker of proliferation versus apoptosis. Most commonly, Ki67 is the cell proliferation marker however, others include the proliferating-cell nuclear antigen (PCNA) and mitotic counts [37]. There also appears to be a distinction between

Table 1
Investigational biomarkers of response to therapy in locally advanced rectal cancer.

Biomarker	Methodology	Treatment (chemotherapy, duration of radiation)	Results
Ki67, Mcm3, Bax, Bcl-2, ssDNA, Grp78, TS, DPD, CD34, VEGF, nestin and L-type amino-acid transporter [28]	IHC	Long-course chemoradiation, with infusional irinotecan and oral S-1	Pre-treatment high Ki67, Bax, TS and low Grp78 correlated with tumour response (Dworak grades 3 and 4)
COX-2, EGFR, Ki67, p21, survivin, TS and VEGF [30]	IHC	Long-course chemoradiation, with 5-FU or capecitabine	Pre-treatment low survivin correlated with histological tumour downstaging
Bcl-2, Bax, Ki-67, Ku-70 and HDAC1 and mGluR4 [31]	IHC	Long-course chemoradiation, with concurrent 5-FU	Pre-treatment Bax expression correlated with tumour response (Dworak)
Ki67, VEGF and Smac [32]	IHC	Long-course chemoradiation, with 5-FU and oxaliplatin	Smac expression decreased after treatment, high Smac and low Ki67 or VEGF correlated with tumour response (tumour volume measurements)
M30 and Ki67 [34]	IHC	Short-course radiation and non-irradiated cases	M30 expression increased after radiation, but did not correlate with cancer-specific survival. High M30 in non-irradiated cases correlated with improved survival.
p53, p21, apoptotic index, Ki67 [35]	IHC for p53, Ki67 and p21; TUNEL for apoptosis	Radiation 20 Gy in combination with intraoperative radiation15 Gy	Pre-treatment negative p53 and positive p21 and apoptosis correlated with greater tumour shrinkage (barium enema) and regression (Rich et al.)
COX-2, Ki67 [36]	IHC	Long-course chemoradiation, with 5-FU	COX-2 increased and Ki67 decreased post radiation, no correlation with tumour downstaging
Ki67, PCNA and mitotic counts [37]	IHC and mitoses counted per 10 high-power field	Long course radiation +/− concurrent 5-FU +/− intra-operative radiation	Proliferative activity decreased post radiation, and was associated with more favourable pathological tumour stage, and improved DFS
P53, p21, MLH-1, MSH2, MIB-1, TS, EGFR and VEGF [38]	IHC	Long-course chemoradiation, with 5-FU	Low constitutive p21, absence of post-treatment EGFR were associated with improved DFS and OS; high post-treatment MIB-1 was associated with worse OS
CK18, Ki67 and COX-2, fibro-inflammatory reaction [39]	IHC and scoring of fibro-inflammatory changes as per Shia et al.	Long-course radiation +/− concurrent 5-FU	Addition of chemotherapy to radiation decreased COX-2 and Ki67 expression and increased inflammatory changes, the latter correlated with tumour downstaging
TS, p53, p21, VEGF, MLH1, MSH2 [41]	IHC	38 patients treated with radiation alone (40 Gy), 19 patients treated with radiation (45 Gy) with concurrent 5-FU and oxaliplatin	High TS levels correlated with tumour response in the chemoradiation group
P21, Ki67, apoptosis and p53 [43]	IHC	Long-course chemoradiation, with uracil-tegafur or S-1	p21 and apoptosis-positivity on biopsy specimens obtained a week post treatment correlated with tumour response (JCCC), tumour volume reduction, as assessed by barium enema +/− MRI, and tumour stage downstaging
TP, TS, VEGF, bax, p53, Nfkappa B, survivin, COX-2, PCNA, CD44, CD133, MMP-2 and MMP-9 [46]	Reverse transcriptase-PCR	Long-course chemoradiation, with 5-FU	Pre-treatment low CD44 mRNA correlated with tumour regression, PCNA correlated with pathological lymph node negativity
CD133 [47]	IHC	20–45 Gy radiation with concurrent 5-FU and tegafur-uracil	CD133 expression increased from 27.5% to 70% with chemoradiation, histological responders were lower with CD133 expression, no relation to survival
E-cadherin, mir200c, beta-catenin [51]	IHC for E-cadherin and beta-catenin, ISH for mir200c	Long-course chemoradiation, with 5-FU	Reduced E-cadherin and expression of mir200c, as well as increased beta-catenin were associated with non-response. E-cadherin and mir200c associated with inferior cancer-specific survival
MSH2, MLH1, p53, p21, p27, topo II, Ki67 and Bcl-2 [55]	IHC	Long-course chemoradiation, with 5-FU and CPT-11	MSH2 and MLH1 deficiency in biopsy samples, and p21-positivity, correlated with response

Table 1 (*Continued*)

Biomarker	Methodology	Treatment (chemotherapy, duration of radiation)	Results
MSI [56]	Fluorescent multiplex PCR-based method	Long-course chemoradiation, with 5-FU +/− cisplatin or oxaliplatin	8% (2/25) were MSI-high and did not respond (Dworak) to chemoradiation
COX-2, VEGF, EGFR, Ki67, CAIX and c-CK18, fibro-inflammatory changes [105]	IHC, stromal changes as described by Shia et al.	79 patients received chemoradiation 45 Gy with concurrent 5-FU; 20 patients received 30 Gy radiation	Ki67 and EGFR were downregulated, while c-CK 18 and COX-2 were upregulated with treatment; high VEGF in pre-treatment sample correlated with lower tumour response (Dworak), lower Ki-67 and c-CK18 in post-treatment samples correlated with better regression; fibrosis and COX-2 expression correlated with OS
MT isoforms, MCM3, Ki67 [106]	Real-time PCR for MT, IHC for MCM3 and Ki67	Short-course radiation	mRNA expression for MT increased post radiation, MCM3 expression decreased post radiation
COX-2 [107]	IHC	Long-course chemoradiation, with 5-FU	Pre-treatment COX-2 overexpression correlated with poor tumour response (5-point regression grade)
PLK1 [108]	IHC +/− Affymetrix HG133 microarray	Long-course chemoradiation, with capecitabine and oxaliplatin	Decreased pre-treatment PLK1 expression was associated with tumour regression (Dworak) and decreased tumour recurrence
Hypoxia-related proteins HIF-1α, CA-IX, VEGF, GLUT-1 [109]	IHC	Long-course chemoradiation, with 5-FU	Increased pre-treatment CA-IX expression correlated with decreased OS HIF-1α, VEGF, GLUT-1 may be co-modulated.
CA-IX [110]	IHC and confirmation by western blotting analysis	Long-course chemoradiation and short-course radiation both included	Negative or weakly positive tumour CA-IX expression correlated with improved DFS
EGFR, VEGF, HIF-α [111]	Quantitative real-time-PCR	Short-course chemoradiation with 5-FU and tegafur-uracil	Pre-treatment EGFR, VEGF, HIF-α correlated with response (JCCC) EGFR, VEGF levels correlated with improved DFS
GLUT-1, CXCR4, CA9 [112]	IHC	Long-course chemoradiation, with 5-FU	Pre-treatment CA9 expression was significantly lower in responders (Wheeler et al.)

Abbreviations: 5-FU, 5-fluorouracil, CA9, carbonic anhydrase 9, CA-IX, carbonic anhydrase-9, COX, cyclooxygenase, CXCR4, C-X-C chemokine receptor 4, DFS, disease-free survival, DPD, dihydropyrimidine dehydrogenase, EGFR, epidermal growth factor receptor, GLUT-1, glucose transporter-1, HDAC1, histone deacetylase, HIF-1α, hypoxia-induced factor 1-alpha, IHC, immunohistochemistry, ISH, `in situ` hybridisation, JCCC, Japanese classification of colorectal cancer, mGluR4, metobotropic glutamate receptor 4, MMP, matrix metalloproteinase, MRI, magnetic resonance imaging, MSI, microsatellite instability, MT, metallothionein, OS, overall survival, PCNA, proliferating-cell nuclear antigen, PCR, polymerase chain reaction, PLK1, Human polo-like kinase 1, ssDNA, single-stranded DNA, TP, thymidylate phosphorylase, TS, thymidylate synthase, TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling, VEGF, vascular endothelial growth factor.

intrinsic and extrinsic radiation-induced effects, with some reports demonstrating intrinsic apoptosis to be beneficial [29,34,43] while others reported radiation-induced effects correlating with better prognosis [39].

There has been increasing interest in stem cell markers in colorectal cancer, based on the cancer stem-cell theory that a sub-clone of cells has tumourigenic potential and drives disease progression [44,45]. CD44 is a stem cell marker and has been shown to be associated with resistance to radiation. Low CD44 was found to be correlated with tumour regression [46] and remained the only significant factor after multiple logistic regression with odds ratio (OR) 4.7 ($p = 0.03$). Prominin-1 or CD133 is another marker thought to originate from cancer stem cells. [47]. CD133 increased after chemoradiation and correlated with lower histological response, however this did not translate into survival differences. These stem cell markers may be useful in determining radioresistance and therapeutic strategies. Another study found higher CD133+CXCR4+ cells in rectal compared to colon cancer cases, and this correlated with liver metastases and poorer survival [48].

2.2. Biomarkers of emerging interest

2.2.1. The epithelial to mesenchymal transition (EMT)

EMT is a process whereby cancer cells lose their epithelial traits and become more mesenchymal, acquiring invasive capabilities and plasticity [49]. EMT is thought to predict for aggressive disease and metastasis. EMT is recognised as a biological hallmark in the Colorectal Cancer Subtyping Consortium (CRCSC) molecular subtypes of CRC, based on cross comparison of all the different studies inclusive of 4562 samples with collated gene expression data [50]. These were mainly stage I to III cancers, and included colon as well as rectal cancers. Colorectal molecular subtype (CMS) 4 is a tumour cohort defined as possessing mesenchymal

characteristics and carries the worst prognosis. Hence markers of EMT have been explored as biomarkers of response to neoadjuvant therapy. In a rectal cancer study, E-cadherin loss, micro-RNA-200c (mir200c) loss, increased beta-catenin and presence of tumour budding were associated with non-response [51]. Reduced E-cadherin and reduced mir200c were associated with reduced cancer-specific survival. This supports the hypothesis that EMT does play a role in rectal cancer progression and in treatment response.

2.2.2. Microsatellite instability (MSI)

MSI accounts for around 15% of colorectal cancers and around 2–8% of rectal cancers [52,53]. MSI is another hallmark in the CRCSC molecular subtyping [50,54]. MSI-high tumours consist of cells with mismatch repair deficiency that accumulate DNA errors throughout the genome and are associated with a superior outcome. However, its role in treatment response in locally advanced rectal cancer is controversial. Some studies have demonstrated MSI correlating with better response, while others have not [27,38,55]. A study found 8% of rectal cancer samples to be MSI-high and showed no response to chemoradiotherapy [56]. MSH-2 negative cell lines have shown increased death due to chromosomal damage in the S-phase of the cell cycle after ionising radiation [57]. Other studies have shown conflicting results, with baseline MLH-1 positive patients reported to achieve a higher pCR [38]. Microsatellite instability in biopsy specimens correlated with better survival in a study of 57 patients undergoing 5-FU, radiation and CPT-11, a topoisomerase I inhibitor [55].

3. Blood-based biomarkers

3.1. Carcinoembryonic antigen (CEA)

CEA is currently the only biomarker in the National Comprehensive Cancer Network (NCCN) guidelines, included as part of baseline staging and in post treatment surveillance. Studies have consistently demonstrated low pre-operative levels of CEA to be associated with more favourable tumour response, most using 5 ng/ml as the cutoff [17,58–60]. However, this has not been used to stratify treatment strategies as it lacks sensitivity and specificity. Studies that have adopted a non-operative approach post long-course chemoradiation have not included CEA in determining treatment plan [20].

3.2. Tissue-inhibitor of metalloproteinase-1 (TIMP-1)

TIMP-1 is a member of the natural matrix metalloproteinase inhibitor family, shown to predict treatment response in CRC in the metastatic setting [61]. However, data for rectal cancer is sparse. In a study of 33 patients with rectal cancer treated with neoadjuvant chemoradiotherapy (5-FU), post-treatment serum TIMP-1 (ELISA) increased by 13% [62]. A recent study demonstrated that lower post-chemoradiation TIMP-1 levels to be related to pCR (Mandard classification) [63]. Importantly, TIMP-1 was able to stratify the good clinical responders, as assessed by complete or partial response in radiologic imaging.

3.3. Haematological and inflammatory profiles

There is increasing evidence that the clinical effect of neoadjuvant chemoradiation is dependent on host conditions. A study of 73 patients undergoing long-course therapy found that high haemoglobin levels ($p = 0.001$) and low platelets ($p = 0.0008$) correlated significantly with tumour size reduction as assessed by barium enema [64]. Response was also high in cases with high lymphocytes and low neutrophils whilst high C-reactive protein and fibrinogen were predictors of poor response. These findings raise the issue of whether optimising the host systemic response may sensitise the tumour to treatment, with interventions such as pre-operative blood transfusions to reverse anaemia.

Another study showed that patients with good response to chemoradiation had higher pre-treatment levels of circulating lymphocytes, higher lymphocyte to total white cell count ratio and lower neutrophil to lymphocyte ratio [65]. Multivariate analysis revealed that lymphocyte to white cell ratio significantly correlated with regression ($p < 0.01$) as well as disease free survival and overall survival ($p = 0.04$ and 0.02 respectively). Similar trends were found in another study which included patients who received chemoradiation or radiation alone [66]. Ratio of lymphocytes to total white cell count was higher in the pCR group, and ratio of neutrophils lower. They also reported lymphocytes to be reduced significantly during treatment, which may provide an opportunistic microenvironment for tumour cells to proliferate.

Microarray profiling of these peripheral cells may provide further insight into the biological processes underlying treatment response. Pre-treatment peripheral blood mononuclear cells in 35 cases of locally advanced rectal cancers treated with long-course therapy were subjected to microarray gene profiling [67]. Differentially expressed genes were BC 035656.1, CIR, PRDM2, CAPG, FALZ, HLA-DPB2, NUPL2 and ZFP26. The FALZ locus codes transcription factors and overexpression leads to apoptosis, while ZFP36 induces VEGF degradation. FALZ gene expression determined by qRT-PCR (as validation) showed significant differences between responders and non-responders.

3.4. Interleukins and osteopontin

There is increasing focus on the role of inflammation in CRC [68,69]. Serum interleukin-6 (IL-6) is a pro-inflammatory cytokine while osteopontin is a glycophosphoprotein that is secreted in the context of malignancy or inflammation. Both were investigated in thirty patients treated with long-course chemoradiation [36]. IL-6 increased while osteopontin was unchanged post-treatment. There was a trend suggesting high osteopontin levels correlating with

development of metastases. Another study examined IL-6 and osteopontin in conjunction with IL-8 [70], and found that only IL-8 was significant in predicting pCR or downstaging after chemoradiation in multivariate analysis. This may be explained by a positive correlation between osteopontin and IL-8. IL-6 polymorphisms have also been investigated, with particular polymorphisms correlating with risk of colon cancer but this was not evident in the rectal cancer cases [71].

3.5. Vascular endothelial growth factor (VEGF)

VEGF can be activated in tumour cells by cytokines including IL-1 and IL-6 as well as hypoxia resulting in secretion of proteolytic enzymes and matrix metalloproteases. The effect of treatment on VEGF levels was investigated in tissue and in blood from 32 patients who received short-course neoadjuvant radiation prior to curative surgery, compared to matched patients who did not receive radiation [72]. VEGF expression by IHC in tissue was significantly increased post-treatment, however circulating VEGF (ELISA) was decreased. The latter was hypothesised to be due to a tumour reduction effect however this has not been studied with respect to tumour recurrence.

4. Tumour DNA, RNA and cells

4.1. MicroRNA (miRNA)

It is estimated that up to 30% of the human genome is regulated by miRNAs. miRNA have been found to be overexpressed in tumour tissue in colorectal cancer [73]. The rectal cancer microRNAome was described by Gaedcke et al. who screened 57 rectal cancer samples and matched controls [74]. Patients were treated with long-course radiation with concurrent 5-FU +/− oxaliplatin. Forty-nine miRNAs were found to be differentially expressed. Rectal-cancer specific miRNA included miR-492, miR-542-5p, miR-584, miR-483-5p, miR144, miR2110, miR-652, miR-375, miR-147b, miR-148a, miR-190, miR-26a/b and miR-338-3p. Another study used 904 miRNA gene chips to analyse expression in normal and cancerous rectal tissue. One hundred and six miRNAs were found to be aberrantly expressed in rectal cancer, of which 45 miRNAs were newly found to be overexpressed, including miR-18a, miR-135b, miR-21, miR-143 and miR-145 [75]. miR-135b had been found to be overexpressed while miR-143 and miR-145 downregulated in colorectal cancer [76–78]. A recent study found miR-21-5p to predict response to chemoradiation with a high accuracy, with an

Table 2
Investigational miRNAs in response to therapy in locally advanced rectal cancer.

Study and reference	Treatment (chemotherapy, duration of radiation)	Results
38 fresh rectal biopsy samples [113]	Neoadjuvant chemotherapy (oxaliplatin and 5-FU) and radiotherapy	14 miRNAs were differentially expressed in TRG1 patients compared to TRG > 1 (Mandard); 13 were confirmed by qRT-PCR Upregulated – miR-1183, miR-483-5p, miR-622, miR-125a-3p, miR-1224-5p, miR-188-5p, miR-1471, miR-671-5p, miR-1909, miR-630, miR-765 Down-regulated – miR-1274b, miR720 miR-622 and miR-630 had 100% sensitivity and 100% specificity
12 formalin-fixed paraffin embedded biopsy samples [114]	Unknown	3 miRNA transcripts miR-16, miR-590-5p and miR-153 predicted complete versus incomplete response (Dworak). 2 transcripts miR-519c-3p and miR-561 predicted good versus poor response with a median accuracy of 100%
20 fresh rectal biopsy samples [115]	Neoadjuvant chemotherapy (capecitabine or infusional 5-FU) and radiotherapy	miR-215, miR190b and miR-29b-2 were overexpressed in non-responders while let-7e, miR-196b, miR-450a, miR-450b-5-p and miR-99a were overexpressed in responders (Mandard)
85 formalin-fixed paraffin embedded post-treatment samples [116]	Neoadjuvant chemotherapy (capecitabine) and radiotherapy	miR-21, miR-99, miR-125b, miR-125b1, let-7c and miR-490 upregulation correlated with TRG 1-2 (Mandard). miR-21 and miR-125a-3p downregulation correlated with TRG 4
40 formalin-fixed paraffin embedded biopsy and post-treatment samples [117]	Neoadjuvant chemotherapy (5-FU) and radiotherapy	miR-21 was downregulated while miR-143 and miR-145 were upregulated with treatment. miR- 145 correlated with tumour regression (Schneider et al.)
103 formalin-fixed paraffin embedded post-treatment samples [51]	Neoadjuvant chemotherapy (capecitabine or infusional 5-FU) and radiotherapy	miR-200c loss was associated with non-response (Mandard)
27 biopsy specimens in training set and 17 in validation set [79]	Neoadjuvant chemotherapy (5-FU based) and radiotherapy	miR-21-5p was overexpressed in complete responders, with sensitivity and specificity of 78% and 86% respectively

Abbreviations: 5-FU, 5-fluorouracil, miR, micro RNA, qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction, TRG, tumour regression grade.

AUC (area under curve) of 0.94 [79]. Of note, they validated their findings in an independent group of patients.miRNA has been reported to be important in determination of radiosensitivity of rectal cancers to chemoradiotherapy. In vitro studies demonstrated the correlation between miRNA and radiosensitivity [80]. Transfection of let-7g, miR-224, miR-320a and miR-332 in colorectal cancer cell lines induced radiation sensitivity. Let-7g expression also appeared to correlate with better survival. Table 2 shows the miRNA candidates that have been investigated in response to treatment in locally advanced rectal cancer.

The advantage of miRNA expression profiling is the large number of candidate markers that can be obtained at any one time. Targeting these miRNA with oligonucleotides represents a potentially useful therapy [81,82]. However, the challenge lies in the interpretation of large amounts of data, given that most studies have little, if any, overlap in dysregulated markers.

4.2. Global gene expression

Microarrays screening a large array of genes have been increasingly utilised to develop a profile that will predict response [82–85]. Using tissue microarrays in 23 patients treated with chemoradiation, different expression levels were obtained for 54 genes with a sensitivity of 78% and specificity of 86% in responder versus non-responder (assessed by T downstaging) groups with locally advanced rectal cancer [84]. Lower levels of calmin, kinectin 1, copine III, villin-like, motilin, cdc42, mypsin IA, cyclin T1, IL12A, SMC1 and platelet-derived growth factor were found in the responders. In the responders assessed by tumour regression, four genes were differentially expressed including REGL, ACVR2B, SMARCC1 and ZNF134. Of note, many of the biomarkers mentioned previously such as p53, p21, p27, bcl2, Ki-67, MSH2, survivin, thymidylate synthase and PCNA were not differentially expressed. Another study also confirmed a gene set predicting for response with an accuracy of 87%. Thymidylate synthase and RAD23 were highly expressed in cases that had a complete response [86].

Genes that were able to discriminate between responders and non-responders with an accuracy of 82.4%, using the human U95Av2 Gene Chip, included genes involved in apoptosis [87]. Lumican, thrombospondin 2 and galectin-1, which are apoptosis inducers, were higher in responders.

4.3. KRAS mutational status and chromosomal alterations

Chromosomal copy number alterations may be associated with tumour response. Complete pathological response was associated with significantly fewer high copy gains and loss of 12p13.31 was associated with pCR [88]. KRAS mutations (codons 12, 13, 61) were reported to correlate with improved response to neoadjuvant chemoradiation in a small study [89]. Other studies investigating KRAS mutational status found no correlation with outcomes [90,91]. However, differences were found between the G12V and G13D mutations, with the former associated with greater regression.

4.4. Circulating tumour nucleic acid (ctNA)

ctNA has been used as a surrogate for circulating tumour cells (CTCs), however it is not possible to definitively identify the cell of origin of ctNA [92]. Candidate nucleic acid biomarkers are chosen based on their assumed derivation from cancer cells, and as markers of metastatic potential. Levels of hTERT and ctRNA were found to be higher pre-chemoradiation than post-treatment in responders [93]. Another small study of 26 patients investigated CK20 and CEA ctNA, with a positive rate (defined as both biomarkers being positive) of 32% pre-treatment [94]. Sixty-three percent of responders were positive pre-treatment while 18% of non-responders were positive. There was a significant decrease post-treatment in responders. Another study found that CK20 ctRNA correlated with inferior survival [95]. The utility of ctDNA measured by real-time PCR as a therapy monitoring marker has also been explored [96]. Serial sampling revealed that the median ctDNA was 4.2 ng/ml before chemoradiation, 1.0 ng/ml at the end of chemoradiation and 4.1 ng/ml postoperatively. There was a significant difference in postoperative ctDNA, with responders (ypT0-2) having 2.2 ng/ml and non-responders (ypT3-4) 5.1 ng/ml.

4.5. Circulating tumour cells (CTCs)

There is increasing evidence for the clinical utility of CTCs in colorectal cancer, with most studies confirming high CTC counts correlating with poorer prognosis [97]. CTCs are thought to be released from the primary tumour into the bloodstream and considered the initiating seed for metastasis at distant organ sites [98]. However, the majority of these studies do not focus on rectal cancers as a separate entity. Rectal cancers receiving neoadjuvant chemoradiotherapy comprised a small subgroup (12 patients) of one study, which enumerated CTCs based on EpCAM (epithelial cell adhesion marker) positivity using flow cytometry [99]. No differences were found in CTC counts between the colonic and rectal cancers, and no effect was found with neoadjuvant chemoradiotherapy. However, the small numbers and the isolation of CTCs based on EpCAM alone, may have affected the results, due to inability to capture cells that have undergone EMT and hence display more mesenchymal surface marker characteristics [100].

5. Constitutional patient factors

There is increasing interest in the potential for treatment-associated toxicities or symptom burden to function as a biomarker for treatment response or tumour burden. In a study of 54 patients, lower levels of fatigue at baseline and

at completion of chemoradiation were found to be significant predictors of pathological response, as gauged by tumour downstaging [101].

Obesity in rectal cancer has also been reported to be associated with inferior or similar survival outcomes [102]. The measure of visceral to subcutaneous fat areas was thought to be a more accurate indicator of abdominal or visceral adiposity and quantitative measures of visceral adiposity were correlated with clininopathological variables as well as survival outcomes in a retrospective study of patients treated with chemoradiation [103]. Patients who had higher visceral fat had less T-stage downstaging while patients with no or partial response and high adiposity comprised the worst group in terms of disease-free survival.

6. Conclusion

The approach of selecting and validating biomarkers relies on pre-existing knowledge of those same candidate biomarkers. The drawbacks of this approach include the limited pool of candidate biomarkers, given the known multiplicity and redundancy of carcinogenic pathways. More recently, microarrays and miRNA expression profiling have allowed the analyses of hundreds of potential biomarkers and selection of promising candidates [104]. This will have even greater potential if applied to circulating tumour cells or nucleic acids.

With regard to sampling time-point strategies, biomarkers measured post-treatment may be useful to decide on subsequent therapy, but the information it affords in addition to the current standard as defined on the surgical specimen, is uncertain. Baseline pre-treatment biomarkers will likely have higher clinical utility as they aid in the initial treatment decision making, prior to obtaining the surgical sample.

It is clear that currently a single biomarker will not be robust enough to have prognostic and predictive utility. Models that combine blood biomarkers, functional imaging with positron emission tomography (PET) and clinical data have shown an AUC of 0.81 for pCR, and 0.78 for tumour response based on downstaging. This is superior to the AUC of the individual markers [70]. With this information, nomograms can be constructed to incorporate all potentially useful biomarkers that on their own are not robust enough to have clinical relevance. Further, microarrays that can interrogate a tumour for multiple promising candidate markers at any one time can increase this yield. Moving forward, a non-static sample such as circulating tumour cells or circulating nucleic acids, has the potential to provide insight into tumour heterogeneity and treatment related mutational changes.

Conflict of interest

None declared.

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Biography

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