

Review

Biomarkers for Predicting the Response to Radiation-Based Neoadjuvant Therapy in Rectal Cancer

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Abstract

Locally advanced rectal cancer (RC) is treated with neoadjuvant chemoradiotherapy (nCRT) followed by radical surgery. Currently, organ-sparing approaches and/or “watch-and-wait” strategies other than unnecessary surgery have been suggested as the best option for patients who achieve complete regression after neoadjuvant treatment. However, patients respond differently to nCRT, hence the urgent need for effective methods to predict whether individual rectal cancer patients could benefit from this treatment. In this review, we summarize the biomarkers reported to be potential predictors of the therapeutic response of RC to nCRT. Biomarkers that are associated with genes, ribonucleic acid (RNA) and proteins are summarized and described first, followed by other types including immune and tumour microenvironment-related biomarkers, imaging biomarkers, microbiome-associated biomarkers, and blood-based biomarkers.

Keywords: rectal cancer; biomarker; neoadjuvant chemoradiation; response

1. Introduction

Colorectal cancer (CRC) is identified as the third most common malignancy worldwide and the second most common cause of cancer-related deaths [1]. Rectal cancer (RC) accounts for approximately 30% of CRC and has worse clinical outcomes than colon cancer [2]. Chemoradiotherapy followed by surgery is the current standard of care for locally advanced RC. To maximally resect the tumour, preserve the sphincter and improve local control, surgery is usually performed after a 6–8 week period of chemoradiotherapy and according to the principles of total mesorectal excision (TME) [3,4]. However, TME has been associated with high postoperative morbidity and mortality rates. In addition, TME-associated bowel, urinary and sexual dysfunctions result in poor long-term quality of life in these patients [2,3]. Therefore, more personalized and less invasive multimodal treatment strategies are urgently needed for RC patients. New strategies have received much attention in recent years, but regrettably there is a ceiling effect (approximately 20% of cases) on achieving a pathologic complete response (pCR). According to previous reports, the pCR rate of RC patients at the time of surgery is in the range of only 8% to 20%. Up to 40% of RC patients are resistant to neoadjuvant chemoradiotherapy (nCRT), with some experiencing a progression of disease and others showing a slight regression to stable disease [3–5]. Clearly, different responses to nCRT contribute to varying clinical out-

comes, including disease-free survival (DFS) and overall survival (OS) [2,3]. Organ preservation with no immediate surgery, otherwise referred to as the “watch-and-wait” strategy, is currently suggested as the preferred management for RC patients who have shown an adequate response [2]. In contrast, patients who are resistant to nCRT need more successful treatment strategies at an early stage. Although radiation therapy has been widely used for various tumours, little progress has been made in predicting treatment outcomes following radiation. The inability to accurately predict treatment outcome has also limited the use of personalized therapy at an individual level [6]. It is clearly desirable to have the ability to accurately determine treatment outcomes before the start of treatment, to identify individuals who would benefit most from nCRT, to know what dose should be given, and to know whether the therapeutic response could be improved by combining with other molecular-targeted strategies.

Hence, there is an urgent need to identify biomarkers that predict patient response to nCRT in the early phase, to develop alternative treatment strategies for non-responders and thus reduce the toxicity from ineffective nCRT, and to provide appropriate alternative treatments in a timely manner. The future management of RC patients is likely to be highly individualized, with a more rigorous treatment approach for high-risk patients and more flexible treatment principles for good responders. This review will focus on potential biomarkers to predict the response to radiation-



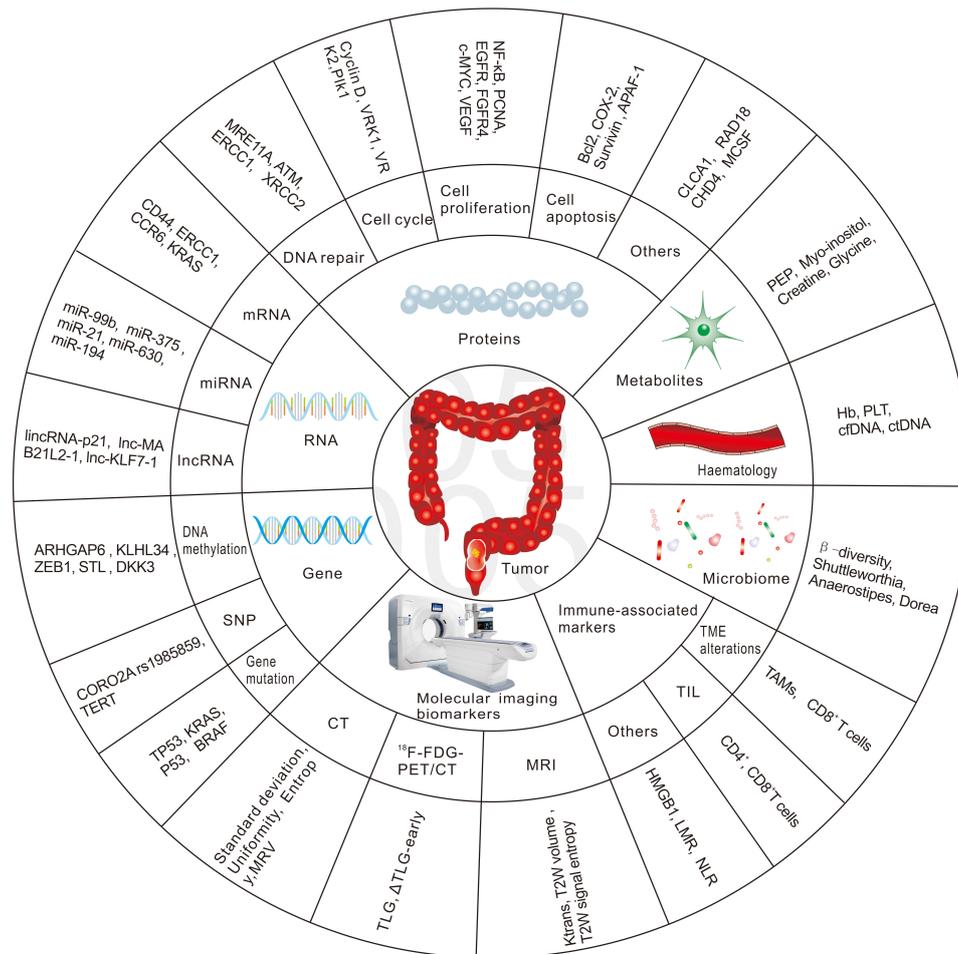


Fig. 1. The most common biomarkers in predicting response to nCRT in RC.

based neoadjuvant therapy in patients with RC. We have summarized the most common biomarkers in Fig. 1.

2. Traditional Assessment of the Response to Neoadjuvant Chemoradiotherapy

Previous reports have shown that the gold standard for evaluating the extent of tumour regression after nCRT is histopathological assessment of the surgically resected tumour and lymph node samples. However, these assessment criteria vary in different countries and centres, and scholars have yet to reach agreement on the best assessment method. With regard to the evaluation of nCRT response, the American Joint Commission on Cancer (AJCC) [7], Dworak/Rodel [8], Mandard [9], Becker [10], Ryan [11], and Memorial Sloan Kettering Cancer Center (MSKCC) [12] are currently the most commonly used tumour regression grading (TRG) systems [13,14]. These are described in Table 1 (Ref. [7–12]).

3. Genetic Biomarkers

DeoxyriboNucleic acid (DNA) is an extremely important biomolecule that stores genetic information used to

determine the formation of different cells, tissues and the whole organism [15]. The main mechanism of action of chemoradiotherapy is by damaging the cellular DNA [4]. Various DNA repair mechanisms are stimulated in DNA-damaged cells to arrest the cell cycle and allow repair enzymes to identify and repair the aberrant nucleotides, thus keeping the genome in good condition. Over time, irreparable damage can occur if the damaging events inside the cell and the activity of DNA repair machinery become unbalanced. This can be seen in the radiation therapy process, which finally leads to cell apoptosis. Previous studies have provided substantial evidence that some genetic characteristics are potential predictors of the therapeutic response to nCRT.

3.1 Chromosomal Alterations

It is well recognized that genomic copy number can change during the cell life cycle. For example, the amplification of oncogenes, the deletion of tumour suppressor genes, or some other rearrangements may result in alterations to gene transcription [15]. Chromosomal instability, which includes the amplification and deletion of chromosomal segments or entire chromosomes, is a common fea-

Table 1. Tumour regression grading systems used for colorectal cancer.

Grading system	Grading	Description	Reference No.
American Joint Commission on Cancer (AJCC)	0	No residual cancer (complete regression)	[7]
	1	single cells or small groups of cells	
	2	residual cancer with the desmoplastic response	
	3	minimal evidence of tumour response	
Dworak/Rodel scoring system	0	No regression	[8]
	1	Dominant tumour mass with fibrosis and/or vasculopathy	
	2	Dominantly fibrosis with few cancer cells or groups	
	3	few scattered cancer cells on fibrosis background	
Mandard scoring system	4	No tumour cells, only fibrotic mass (complete regression)	[9]
	1	No residual cancer (complete regression)	
	2	Rare residual cancer cells	
	3	Predominantly fibrosis, but increase of residual cancer cells	
	4	Residual cancer outgrowing fibrosis	
Becker classification	5	Absent of regressive changes	[10]
	1a	No residual tumour cells	
	1b	<10% residual tumour cells/tumour area	
	2	10–50% residual tumour cells	
Ryan	3	>50% residual tumour cells	[11]
	1	No or rare residual of cancer cells (complete regression)	
	2	Residual cancer cells with predominant fibrosis	
Memorial Sloan Kettering Cancer Center	3	No fibrosis and/or with extensive residual cancer cells	[12]
	1	complete regression	
	2	86%–99% of tumour remission	
	3	≤85% of tumour remission	

ture of most CRCs. In an exploratory study, Molinari *et al.* [16] analysed alterations of chromosomal copy number and found that non-responders had a specific alteration profile. These included more frequent changes at the 18q23, 17p13-12, 13q31-34, 13q12, 10p14-13, 16p13, 8q23-24, 7p22-21, 7q21, 7q36, 3q29 and 2q21 chromosomal regions. Insights into the profile of chromosomal alterations could thus provide helpful information in predicting the response to nCRT, leading to an optimized strategy for RC. Chen *et al.* [17] also reported that chromosomal copy number alterations (CNAs) were associated with the response to nCRT. In their prospective phase II study, they revealed that non-pCR patients showed more frequent loss of chromosomal region 15q11.1-q26.3 ($p < 0.00002$). In contrast, pCR patients showed more frequent loss of chromosomal region 12p13.31 ($p < 0.0003$). The same authors also found that some specific CNAs were significantly associated with persistent lymph node metastasis following chemoradiotherapy in RC, in particular the loss of chromosome 4 [18]. Subsequently, other researchers found that chromosome segregation errors affected the response of RC patients to nCRT. They noted that errors in chromosome segregation predicted an enhanced pathological response to nCRT (OR = 3.9; $p = 0.02$). Those errors caused downstream structural damage to chromosomes that in combination with defects

in DNA repair mechanisms enhanced the effect of DNA-damaging therapies [19]. These findings identified a potential mechanistic predictive marker of treatment response to nCRT and suggest that targeting of chromosomal instability may be an effective therapeutic strategy.

3.2 DNA Methylation

Various molecular pathways are involved in the development of CRC, of which DNA methylation is an important pathway. Although the development of RC has been widely studied, methylation profiling is seldom explored for its ability to predict response to nCRT. Molinari *et al.* [20] first analysed methylation profiles in biopsy samples from normal individuals and from RC patients and evaluated whether these could be used to predict nCRT. Only TIMP3 methylation status showed a significant difference within the four TRG classes ($p < 0.05$). Another study found that the methylation level was significantly reduced in post-treatment resection specimens (171.5 vs. 152.7, $p = 0.01$). T stage ($p = 0.005$) and tumour regression ($p < 0.001$) were strongly associated with the pre-treatment methylation level, which was also closely linked to complete and partial pathological responses ($p = 0.01$) [21]. Kim *et al.* [22] identified 7 hypermethylated CpG sites (ARHGAP6 cg07828380, KLHL34 cg01828474, ZEB1

cg04430381, STL cg00991794, DKK3 cg041006961, DZIP1 cg26886381, and DZIP1 cg24107021) that were able to predict nCRT response. These authors also showed that the methylation status of KLHL34 cg14232291 could predict radiosensitivity in patients who received nCRT. Pre-clinical studies have shown that cellular retinol-binding protein 1 (CRBP1) is related to radiation sensitivity. Following comprehensive molecular analysis, Yokoi *et al.* [23] found that hypermethylation of the CRBP1 promoter DNA resulted in epigenetic silencing and that histological responses in RC patients treated with nCRT were significantly associated with the quantitative methylation value of CRBP1 ($p = 0.031$). Recently, Canto *et al.* [24] analysed differentially methylated (DM) CpGs in 32 RC tissue samples and 5 normal rectal tissues. They found that three DM CpGs linked to the INSIG1, GPR1 and OBSL1 genes could be used in a classifier to sensitively and specifically identify patients who had a pCR.

3.3 Single-Nucleotide Polymorphisms (SNPs)

Global genomics research into single-nucleotide polymorphism (SNP) variations and the initial stages of human genomic haplotype mapping both contributed to the discovery of cancer-promoting genes. This led to the identification of specific molecular characteristics as potential biomarkers to predict therapy response and prognosis [25]. Several studies have reported that polymorphisms in DNA repair genes are associated with the sensitivity of cancer patients to nCRT. For example, several groups investigated the potential association between thymidylate synthase (TS) polymorphisms and the response of RC to nCRT, but no consensus has been reached [26–28]. A meta-analysis was conducted by Yang *et al.* [27] to determine whether TS polymorphisms could predict the response to nCRT in RC. They found that patients with the TS 2R/3R genotype showed a positive response and that patients with the 2R/3R or 2R/2R genotype could benefit more from nCRT than patients with other genotypes. However, both the 1494del6 and the 5'-untranslated region expression allele polymorphisms showed little predictive value. Lamas *et al.* [28] collected blood samples from 93 stage II-III RC patients and determined their genotypes for TS, excision repair cross-complementing group 1 (ERCC1) and X-ray cross-complementing group 1 (XRCC1). An overall tumour response rate of 47.3% was observed, and the authors found that XRCC1 G/G carriers were more likely to show a better response than G/A carriers (odds ratio (OR) 4.18; 95% confidence interval (CI): 1.62–10.74, $p = 0.003$). In addition, higher expression of TS linked to the 3G/3G, 3C/3G and 2R/3G genotypes was associated with a better treatment response rate than lower expression genotypes (OR= 2.65; 95% CI: 1.10–6.39, $p = 0.02$) [28]. Kim *et al.* [29] found 9 SNPs that were associated with nCRT response. In particular, the reference allele (C) of the SNP CORO2A rs1985859 was more likely to be associated with

a positive response than the substitution allele (T) ($p = 0.01$). In clinical analysis, the SNP FAM101A rs7955740 showed no relation to radiosensitivity, but dysfunction of FAM101A in RC cells *in vitro* was closely associated with early phase apoptosis and colony formation. Sebjo *et al.* [30] analysed polymorphisms in epidermal growth factor receptor (EGFR) and its ligands, TS, and DNA repair genes in 84 patients with stages II-III RC who underwent nCRT. They found the rs11615 C > T polymorphism in the ERCC1 gene and the rs11942466 polymorphism in the amphiregulin gene region (OR = 0.26; 95% CI: 0.06–0.79; $p = 0.014$) were significantly linked to pCR ($p = 0.023$). Moreover, the C/C genotype was associated with resistance to nCRT. Researchers have evaluated SNPs in RC patients who participated in the phase III trial ACCORD-12, with the Dworak score used to assess therapeutic response. Sixty-six germline SNPs were found in 10 candidate DNA repair genes. Boige *et al.* [31] concluded that 5 SNPs located in ERCC1, MTHFR, excision repair cross-complementing group 2 (ERCC2) and XPA were closely related to the Dworak score. XPA rs3176683 was valuable for predicting the response to nCRT, while the ERCC2 rs1799787 and ERCC1 rs10412761 variants were reported to be promising prognostic markers. Of note, the likelihood of response was decreased by 60% in the T/G haplotype of rs10412761 and rs1799787 ($p < 0.001$). Sclafani *et al.* [32] reported that a SNP (rs61764370, T > G base substitution) in the let-7 complementary site 6 of KRAS messenger RNA (mRNA) was a potential biomarker for predicting response to nCRT. The TG genotype was more likely to result in complete response (CR) ($p = 0.02$). Rampazzo *et al.* [33] analysed 8 SNPs (rs11742908, rs2736108, rs2736098, rs2736100, rs2736122, rs2735940, rs2853690 and rs35241335) located in the regulatory and coding regions of the telomerase reverse transcriptase (*TERT*) gene. Their results showed the rs2853690AA/GG and rs2736108CC genotypes were associated with less telomere erosion and lower levels of circulating TERT following nCRT. Both genotypes were also potential biomarkers for better response to nCRT [OR= 4.6 (1.1–19.1) and 3.0 (1.3–6.9), respectively]. Low levels of circulating TERT (\leq median) after nCRT were also good predictors of better treatment response. Chiang *et al.* [34] reported that E346A/rs867228 homozygosity of the formyl peptide receptor 1 (FPR1) was associated with poor 5-year OS ($p = 0.014$). They also found this FPR1 genotype could influence nCRT-elicited anticancer immunity in their animal model by decreasing T lymphocyte infiltration and migration. Moreover, the CC genotype of FPR1-E346A could independently predict the response to nCRT.

3.4 Gene Mutations

Studies have suggested that RC patients with or without p53 mutations respond differently to neoadjuvant radiation-based therapy. Whether p53 mutation is a reliable biomarker to predict therapeutic response to nCRT remains

controversial [35,36], with early studies reporting that it did not predict response to radiotherapy [35]. A meta-analysis published in 2012 showed that patients with wild-type p53 or with low expression levels of p53 protein were more likely to show pCR when treated with nCRT (poor response: RR = 0.85; 95% CI: 0.75–0.96; $p = 0.007$; good response: RR = 1.30; 95% CI: 1.14–1.49; $p = 0.001$; complete response RR = 1.65; 95% CI: 1.19–2.30; $p = 0.003$) [36]. Dululao *et al.* [37] screened for TP53 and KRAS mutations in pre-treatment tumour biopsies and in paired normal surgical tissue from 148 stage II–III RC patients treated with nCRT. They concluded that mutations in TP53 and in different KRAS codons could influence the response to nCRT. For example, wild-type KRAS patients were more likely to show pCR than those with any KRAS mutation ($p = 0.006$), while KRAS codon 13 mutations were negatively associated with pCR ($p = 0.03$). Nevertheless, the role of KRAS mutation in predicting the response to nCRT is still controversial [38]. TP53 mutation was reported to be associated with radioresistance, while patients with both TP53 and KRAS mutations were more likely to show less response to nCRT and to suffer lymph node metastasis [39,40]. Jiang *et al.* [41] analysed the genes of RC patients who underwent nCRT and found that BRAF and SMAD4 mutations were associated with positive response to chemoradiotherapy and better prognosis. Some researchers have attempted to construct models of predictive genotype signatures (PGS) to predict nCRT responses in RC. Xiao *et al.* [25] recently built a PGS model, based on target sequencing of 15 genes, whose predictive value was proved better than that of any clinical factor. Predictive models that contain multiple gene characteristics could therefore assist in the more accurate selection of patients who might benefit from nCRT, thus facilitating the personalization of treatment strategies.

4. RNA Biomarkers

4.1 mRNA

Messenger RNA (mRNA), transcribed from a strand of template DNA, is a class of single-stranded RNA that carries genetic information to guide protein synthesis, thus gene alterations caused by nCRT can change the expression profiles of mRNA. These may in turn also be potential biomarkers for predicting therapeutic outcomes in RC. Some newly reported mRNA-associated biomarkers are listed in Table 2 (Ref. [42–50]).

Huh *et al.* [42] analysed pre-nCRT biopsies of RC patients to determine the predictive value of 13 tissue biomarkers: nuclear factor-kappa B, survivin, proliferating cell nuclear antigen, TS, p53, vascular endothelial growth factor (VEGF), matrix metalloproteinase-9, matrix metalloproteinase-2, cluster of differentiation 44 (CD44), CD133, thymidine phosphorylase, cyclooxygenase-2 and BCL2-associated X protein. These authors concluded that only CD44 mRNA expression was significantly predictive of therapeutic response (OR = 4.69, $p = 0.030$), even though

it also correlated with expression of the other 12 markers (all $p < 0.05$) [42]. Overexpression of ERCC1 mRNA was later reported to be associated with poor response to FOLFOX (5-fluorouracil, leucovorin, and oxaliplatin)-based nCRT in RC patients [43]. Patients with a higher level of C-C motif chemokine receptor 6 (CCR6) mRNA were prone to a poor response ($p = 0.004$), suggesting that CCR6 may be a biomarker for radiosensitivity and may also be a promising target for radiosensitization [44]. Yan *et al.* [45] found that an E3 ubiquitin-linked enzyme named RAD18 was a promising predictive biomarker for the efficacy of nCRT in RC. Overexpression of RAD18 mRNA helped to identify nCRT-resistant patients with an accuracy of 65%. Wang *et al.* [46] reported that a high level of chromodomain helicase DNA-binding protein 4 (CHD4) mRNA showed 60% accuracy for predicting nCRT resistance in patients with RC, while *in vitro* studies demonstrated that knock-down of CHD4 mRNA increased the sensitivity to nCRT in microsatellite stable (MSS) CRCs. Flores *et al.* [47] analysed blood samples of RC patients and evaluated TS mRNA levels in circulating tumour cells (CTCs). These authors found that 100% of non-responders expressed TS mRNA in all samples ($p = 0.001$). Cho *et al.* [48] developed a multigene mRNA-based biomarker model to predict nCRT response in RC patients. They found that the model performed well in predicting the response to nCRT, with an AUC of 0.84. Moreover, to evaluate the prediction stability of the model, internal cross-validation among three cohorts resulted in AUC values of 0.808–0.909. This suggests that the multigene, mRNA-based biomarker model may be valuable for identifying patients who could benefit from nCRT. Hur *et al.* [49] measured the expression levels of 7 mRNAs and used these results to develop a prediction model to predict response to nCRT. They found that expression of p21, p53, CD133 and Ki67 at the mRNA level was closely related to pCR. Furthermore, the prediction model could discriminate pCR with a preferable accuracy [50]. Recently, Ferrandon *et al.* [50] reported that a higher level of coenzyme A synthase (COASY) mRNA expression might be associated with radioresistance in RC. This was confirmed both in CRC cell lines and in independent patient cohorts. The authors also suggested that by regulating DNA repair and the status of phosphoinositide 3-kinase (PI3K), the level of COASY could serve as a predictive marker of radiation response. Besides, Li *et al.* [51] identified that 5 hub genes (YES1, PPP2R5C, PPP2R1B, PDPK1 and KRAS) were associated with the response of RC patients to nCRT.

4.2 Noncoding RNA

4.2.1 MicroRNAs (miRNAs)

MicroRNA (miRNA) is a kind of short, single-stranded, non-coding RNA, which can regulate gene expression and physiological processes [13,14]. Owing to their stability in serum, plasma, and other biofluids, miRNAs have been suggested as promising biomarkers for

Table 2. mRNA biomarkers reported to predict radiation-based therapy response in RC patients.

Sample type	No. of samples	Treatment	Methods	Response assessment	Predictive biomarker	Performance	Main function	Reference no.
Pre-nCRT biopsy	123	Ncrt (leucovorin and 5-FU and radiotherapy)	RT-PCR	Rodel scoring: responder: grade 3–4 Non-responder: grade 1–2	CD44	$p = 0.030$	cell adhesion	[42]
Pre-nCRT biopsy	86	nCRT (FOLFOX-4 and radiotherapy)	immunohistochemistry	AJCC/UICC	ERCC1	$p < 0.0001$; OR 9.397; 95% CI 2.721–32.457	DNA damage repair	[43]
Pre-CRT biopsy	12	nCRT	RNA sequencing and IHC	Mandard scoring standard pCR: TRG 1	CCR6	$p = 0.004$	chemokine receptor	[44]
Pre- and post nCRT biopsy	83	nCRT	IHC	Dworak scoring	RAD18	$p < 0.05$	an E3 ubiquitin-linked enzyme	[45]
Pre-CRT biopsy	172	nCRT (5-FU and radiotherapy)	IHC	Dworak scoring	CHD4	$p = 0.001$	DNA-binding, DNA repair	[46]
Pre-nCRT and Pre-surgery blood	30	nCRT (5-FU/capecitabin and radiotherapy)	CISH	Not mentioned	TS	$p = 0.001$	DNA synthesis	[47]
Pre-CRT biopsy	184	nCRT (capecitabin or 5-FU + leucovorin and radiotherapy)	NanoString nCounter gene expression assay	Criteria from the Korean Society of Pathologists	ITGA7, FZD9, MMP3, HRAS, MECOM, NKD1, PRKCB, and PIK3CD	AUC: 0.846	a multi-gene mRNA-based biomarker model	[48]
Pre-CRT biopsy	120	nCRT (radiotherapy and 5-FU with leucovorin or xeloda only)	RT-PCR	Mandard scoring responder: grade 1–2 Non-responder: grade 3–5	p53, p21, Ki67, CD133	AUC 0.922 (95% CI: 0.841–0.999)	a predictive model	[49]
Pre-CRT biopsy	33	nCRT (5-FU and radiotherapy)	RT-qPCR	AJCC	COASY	AUC 0.827	a mitochondrial bi-functional enzyme	[50]

Abbreviations: nCRT, neoadjuvant chemoradiotherapy; RT-PCR, reverse transcriptase-polymerase chain reaction; AJCC/UICC, American Joint Commission on Cancer/International Union Against Cancer; 5-FU, 5-fluorouracil; ERCC1, excision repair cross-complementing group 1; IHC, immunohistochemistry; CCR6, C-C motif chemokine receptor 6; CHD4, chromodomain helicase DNA-binding protein 4; CISH, chromogenic in-situ hybridization; TS, thymidylate synthase; COASY, CoA Synthase; RT-qPCR, real time quantitative-polymerase chain reaction.

disease diagnosis, for predicting certain treatment responses, and for estimating the prognosis of various types of cancers [14]. Ionizing radiation can regulate the expression of miRNAs, while in turn the spectrum of diverse miRNAs can also affect the radiosensitivity of tumour cells and ultimately their response to radiation [52]. The biological functions of miRNAs have been widely investigated, in particular the potential as predictive biomarkers because of their peculiar features [53,54].

As reviewed in previous studies, various miRNAs have been investigated as predictive biomarkers of nCRT response, including miR-95, miR-99a, miR-99, let-7e, let-7c, miR-765, miR-720, miR-630, miR-622, miR-671-5p, miR-519c-3p, miR-590-5p, miR-1274b, miR-561, miR-490, miR-483-5p, miR-451a, miR-450a, miR-450b-5p, miR-21, miR-205-5p, miR-200c, miR-215, miR-345, miR-29b-2, miR-21-5p, miR-196b, miR-1909, miR-190b, miR-1471, miR-125-b1, miR-1183, miR-188-5p, miR-153, miR-16, miR-1246, miR-1290-3p, miR-130a, miR-1224-5p, miR-135b, miR-145, miR-125b and miR-125a-3p [13, 52,54,55]. Table 3 (Ref. [53,56–62]) summarized Some newly discovered miRNA-related biomarkers. Millino *et al.* [56] analysed gene expression and miRNA levels in biopsies of RC patients and found that the expression levels of 29 miRNAs and 256 genes were different between responders (R) and non-responders (NR). In particular, only the NR patients who had low levels of a single transcript, RAB5B, appeared to express miR-630. Moreover, 8 transcripts (BCL2L13, NRG, ITGA2, MYO1B, GTSE1, KLF7, TRAM1 and TMEM188) were strong predictors of nCRT response. In another study, Angelo *et al.* [53] found that miR-194 was significantly upregulated in responders ($p = 0.016$) and may be a predictive biomarker of response to nCRT. Campayo *et al.* [57] tested a nCRT-response signature in which miR-483-5p, let-7e, miR-375, let-7b, miR-328, miR-183, miR-99b and miR-21 were included in the preliminary screening. After validation, they found that the levels of miR-99b, miR-375 and miR-21 could predict the response to nCRT. After combining miR-375, miR-21 and miR-99b, they were able to predict the response to nCRT in RC. Baek *et al.* [58] analysed the serum samples and biopsy specimens from patients with RC before nCRT and found that overexpression of miR-199a-5p, miR-199b-5p and miR-199a/b-3p were associated with better therapeutic outcomes to nCRT. These workers also reported that exosomal miR-199b-5p was associated with the response to Ncrt. Notably, Machackova *et al.* [59] reported that 69 miRNAs were differentially expressed between non-responders (TRG 4, 5) and responders (TRG1, 2), with 21 miRNAs being overexpressed and 48 miRNAs expressed at low levels. A significantly higher level of miR-487a-3p expression was confirmed in non-responders (AUC = 0.766, $p < 0.0006$). Cristóbal *et al.* [60] studied miR-199b levels of RC patients. Following nCRT, low miR-199b level was associated with positive lymph nodes ($p = 0.005$), poor therapeutic

response ($p = 0.004$). Dereglulation of the miR-19b level in RC was suggested to be a potential biomarker for better clinical outcome to nCRT ($p < 0.001$), smaller tumour size post-CRT ($p = 0.003$), and no recurrence ($p = 0.001$) [61]. Recently, an integrated miRNA panel comprised of 8 miRNAs (Table 3) showed promising results for the prediction of response to nCRT in RC with a preferable accuracy [62]. Moreover, Li *et al.* [63] reported that radiation significantly altered the levels of 8 miRNAs in the plasma, and that plasma levels of miR-519d-3p, miR-342-5p and miR-374a-5p were closely associated with the response to radiotherapy. In summary, there are obviously many possible predictive miRNA biomarkers, all of which are also promising therapeutic targets. However, further research is required, as most studies have shown variable results for miRNAs and consensus has yet to be reached.

4.2.2 Long Noncoding RNA (lncRNA)

Long noncoding RNA (lncRNA) is a special type of RNA that is >200 nucleotides in length and does not directly code for proteins. Long intergenic noncoding RNA is a subtype of lncRNA [64]. lncRNA has recently attracted the attention of researchers, as progress in technology has allowed a more comprehensive understanding of molecular biology. Previous studies suggested that long intergenic noncoding RNA-p21 (lincRNA-p21) in CRC plays a limited role. Wang *et al.* [65] concluded that the expression level of lincRNA-p21 in RC was decreased in tissue samples and cell lines, leading to increased levels of β -catenin. They also found that the lincRNA-21 level in RC could be altered by radiation, which in turn increased the response to radiotherapy by facilitating the apoptosis of cancer cells. lincRNA is therefore a promising target to increase radiosensitivity in RC. Other preclinical studies have shown that lincRNA-ROR, which negatively regulates p53/miR-145, can increase the resistance to radiotherapy. Moreover, lncRNA OIP5-AS1 can increase the sensitivity of RC to radiation by accelerating radiation-induced cell apoptosis and by modifying the expression of DYRK1A through miR-369-3p. However, their role in predicting the response of RC to chemoradiotherapy needs further investigation [66,67]. Ferrando *et al.* [68] found differential expression of 11 lncRNAs between responders and non-responders, with a false discovery rate of <0.01. They also highlighted that lnc-MAB21L2-1, LINC00324 and lnc-KLF7-1 were the best predictors and that lncRNAs could thus be potential biomarkers for predicting nCRT responses in patients with RC. Zhang *et al.* [69] found that the levels of four hub lncRNAs (HSD52, FLJ33534, LINC00909 and DBET) in non-responders were significantly different to those of responders (all $p < 0.05$). Furthermore, they showed that overexpression of LINC00909 contributed to resistance to radiation and 5-FU *in vitro* and *in vivo* and was therefore suggested as a potential novel target that could strengthen the response to nCRT. More recently, Benitez *et al.* [64]

Table 3. miRNA biomarkers investigated for their ability to predict radiation-based therapy responses in RC patients.

Sample type	No. of samples	Treatment	Methods	Response assessment	Endpoint	Predictive biomarker	Performance	Reference no.
Pre-CRT biopsy	38	nCRT	RT-qPCR and ISH	Mandard scoring responder: grade 1–2 non-responder grade 3–5	responsiveness	miR-194	$p = 0.016$	[53]
Pre- and post-CRT biopsy	59	nCRT(5-FU or capecitabine ± oxaliplatin and radiotherapy)	one-color microarray technique	Mandard scoring responder: grade 1–2 non-responder grade 3–5	responsiveness	miR-630	Not reported	[56]
Pre-CRT biopsy	96	nCRT (5-FU and radiotherapy)	Recover All Total Nucleic Acid Isolation Kit	Dworak classification	responsiveness	miR-99b, miR-375 and miR-21	AUC 0.736 (0.62,0.85) $p = 0.00043$ sensitivity 60% specificity 82.9%	[57]
Pre-CRT biopsy and serum	65 biopsies and 89 serum samples	nCRT (capecitabine or 5-FU+leucovorin and radiotherapy)	RT-qPCR	Rodel scoring	responsiveness	miR-199b-5p	$p = 0.0397$	[58]
Pre-CRT biopsy	87	nCRT (5-FU and radiotherapy)	small RNA sequencing and qPCR	Mandard scoring responder: grade 1–2 non-responder grade 3–5	responsiveness	miR-487a-3p	$p < 0.024$ AUC = 0.766 sensitivity 78% specificity 60%	[59]
Pre-CRT biopsy	185	Ncrt (5-FU and radiotherapy)	RT-PCR	Ryan classification	responsiveness	miR-199b	$p = 0.004$	[60]
Pre-CRT biopsy	121	Ncrt (5-FU and radiotherapy)	Recover All Total Nucleic Acid Isolation Kit	Ryan classification	responsiveness	miR-19b	$p < 0.001$	[61]
Pre-CRT plasma specimens	106	Ncrt (5-FU or capecitabine and radiotherapy)	RT-qPCR	Mandard scoring responder: grade 1–2 non-responder grade 3–5	pCR	miRNAs: miR-33a-5p, miR-30e-5p, miR-210-3p, miR-130a-5p, miR-214-3p, miR-320a, miR-338-3p, and miR-1260a	0.82 (0.67,0.92) sensitivity 77% specificity 73%	[62]

Abbreviations: nCRT, neoadjuvant chemoradiotherapy; RT-PCR, reverse transcriptase-polymerase chain reaction; ISH, *in-situ* hybridization; RT-qPCR, real time quantitative-polymerase chain reaction; 5-FU, 5-fluorouracil.

collected pre-CRT tissue samples from 70 RC patients to analyse the level of RNAs. They found that lincRNA-p21 was overexpressed in stage III RC ($p = 0.007$) and was significantly related to poor down-staging ($p = 0.016$) and TRG response ($p = 0.027$). These authors concluded that lincRNA-p21 was a promising biomarker that independently predicted the response to nCRT ($p = 0.047$).

4.2.3 Small Nuclear Ribonucleic Acids (snRNAs) and Small Nucleolar RNAs (snoRNAs)

Small nuclear ribonucleic acids (snRNAs) and small nucleolar RNAs (snoRNAs) are short, non-protein-coding RNAs with complicated functions. These include guiding ribose methylation of ribosomal RNA and pseudouridylation of small nuclear RNAs at targeted nucleotide residues. Previous studies have reported that snRNAs and snoRNAs contribute to tumour development by stimulating cell proliferation, cell invasion and cell migration, as well as by inhibiting the apoptosis of RC cells [70]. It was previously reported that some types of snoRNAs, such as SNORD14E, SNORD67, SNORD12C, and SNORD17, can provide useful prognostic information on colon cancer [71]. However, the value of snRNAs and snoRNAs in predicting the response to chemoradiotherapy needs further exploration in prospective studies.

5. Protein and Metabolite Biomarkers

5.1 Cellular Protein Biomarkers

Proteins are the most sophisticated and crucial biomacromolecules in the human body and are the ultimate product of gene expression. Previous studies have reported that epigenetic and/or genomic mutations, as well as alterations in the transcription process can result in changes to protein expression. The level and spectrum of certain proteins might therefore indicate vital signs of the internal environment and of some diseases. Moreover, alterations in protein expression could be used as potential markers of the response to cancer treatment. Researchers have therefore explored the ability of proteins to predict the response to specific treatments. By using prior knowledge of genes and transcriptomics, it has been revealed that some proteins are linked to the radiosensitivity of RC and to clinical outcomes. Usually, researchers focus on a single protein or protein panel in which the expression level can be assessed by immunohistochemistry (IHC) or western blot (WB) analysis of biopsy tissues or serum samples, respectively.

Certain proteins with predictive potential are involved in the process of DNA repair. These include meiotic recombination 11 homolog A, ataxia telangiectasia mutated (ATM), proliferating cell nuclear antigen-associated factor 15 (Paf15), ERCC1, X-ray repair cross-complementing protein 2 (XRCC2), and cell cycle proteins such as cyclin D, vaccinia-related kinase-1 and -2, and polo-like kinase 1 (Plk1). Other proteins contribute to cell prolifer-

ation, including nuclear factor- κ B (NF- κ B), Golgi phosphoprotein 3 (GOLPH3), proliferating cell nuclear antigen, EGFR, fibroblast growth factor receptor 4 (FGFR4), c-MYC, VEGF, Ki67 and focal adhesion kinase (FAK). Yet other proteins participate in apoptosis, including B-cell CLL/lymphoma 2 (Bcl2), cyclooxygenase-2 (COX-2), BCL2-associated X protein (Bax), p53, p21, survivin and apoptotic protease-activating factor 1. Still other proteins serve as tumour biomarkers (carbohydrate antigen 19-9, carcinoembryonic antigen) or contribute to metabolism, such as 3-hydroxy-3-methylglutaryl coenzyme A synthase, transketolase, hydroxyacyl-CoA dehydrogenase, 17- β -hydroxysteroid dehydrogenase type 2 and vascular non-inflammatory molecule 1. All of these proteins have been widely studied and reviewed with regard to their predictive significance in RC. However, the results are controversial and no consensus has been reached [13,14,72]. Herein, we focus mainly on newly identified proteins from biopsies and blood which were mainly listed in Table 4 (Ref. [73–84]).

Pucci *et al.* [73] reported that aberrant Ku70, Ku80 and sClusterin (a partner of Ku70) expression were significantly associated with radioresistance and may form a potential “cluster” of predictive factors for nCRT response in patients with RC. Another study examined differential gene expression between RC and normal tissue and identified 8 genes with a >16-fold difference (TAP2, SLC39A7, PSMB8, PPP1R18, PPBP, KRBOX1, HSPA1B and B3GALT4). The expression of these proteins was closely associated with the therapeutic outcome of nCRT ($p < 0.0005$) [74]. Chen *et al.* [75] investigated the association between chloride channel accessory 1 (CLCA1) expression and nCRT response and found that high levels of CLCA1 expression predicted vascular invasion ($p = 0.028$), pre-treatment lymphatic metastasis ($p = 0.032$), and poor therapeutic response ($p = 0.042$). The transcription factor DEK is expressed in RC and is thought to be a reliable biomarker for achieving pCR following nCRT. DEK may be related to the pro-apoptotic factor P38 [76]. Expression of the transforming acidic coiled-coil protein-3 in RC patients was found to negatively correlate with sensitivity to nCRT (RR = 2.23, 95% CI: 1.44–3.45; $p = 0.001$), resulting in less pCR ($p = 0.001$) [77]. Yan *et al.* [45] reported that low expression of RAD18 (an E3 ubiquitin-linked enzyme) in pre-nCRT biopsies of RC patients correlated to better clinical outcomes after treatment. This may be linked to stimulation of the caspase-3- and caspase-9-mediated apoptotic pathway, leading to increased cell apoptosis. Using an animal model, these authors further demonstrated that dysfunction of RAD18 prevented the tumour from growing when exposed to 5-FU and/or irradiation *in vivo*. Besides, high expression of CHD4 was reported to be significantly associated with tumour regression grade ($p = 0.001$). *In vitro* studies revealed that CHD4 contributes to radioresistance in CRC patients with microsatellite instability-high (MSI-H) tumours, while a lack of CHD4 expression

Table 4. Protein biomarkers investigated for their ability to predict radiation-based therapy response in RC patients.

Sample type	No. of samples	Treatment	Methods	Response assessment	Predictive biomarker	Performance	Main function	Reference no.
Pre- and post-nCRT biopsy	23	nCRT (capecitabine and radiotherapy)	IHC	Dworack scoring Responder Grade 4 partial responder Grade 1–3	Ku70/80	$p < 0.001$	DNA repair, apoptosis regulator	[73]
Pre-CRT biopsy	62	nCRT	RNA sequencing and RT-PCR	Mandard scoring responder: grade 1–2 non-responder grade 3–5	PSMB8	$p = 0.001$	cell metabolism, immune modulation	[74]
Pre-CRT biopsy	172	nCRT (5-FU and radiotherapy)	IHC	Dworack scoring	CLCA1	$p = 0.042$	ion transporter, regulating chloride conductance	[75]
Pre-CRT biopsy	74	nCRT (5FU or FOLFOX and radiotherapy)	IHC	Ryan classification	DEK	$p = 0.023$	DNA damage repair	[76]
Pre- and post-nCRT biopsy	152	(capecitabine or CapOX or FOLFOX or 5-FU and radiotherapy)	IHC and WB	AJCC scoring responder: grade 0–1 non-responder: grade 2–3	TACC 3	$p = 0.001$	cell proliferation	[77]
Pre- and post-nCRT biopsy	256	(FOLFOX or CapOX and radiotherapy)	IHC	AJCC scoring	FOXK1, FOXK2	AUC = 0.80, $p < 0.01$ and AUC = 0.76, $p < 0.01$, respectively	cell proliferation, myogenic differentiation	[78]
Pre-CRT biopsy	172	nCRT (5-FU and radiotherapy)	IHC and WB	Dworak/Rodel scoring	BMI1	$p = 0.001$	cell proliferation, tumorigenesis	[79]
Pre-CRT biopsy	110	radiotherapy	IHC and WB	not mentioned	SGK1	$p = 0.0325$	transcription regulation, cell metabolism, cell differentiation	[80]
Pre-CRT biopsy	156	nCRT (capecitabine or 5-FU plus leucovorin and radiotherapy)	nCounter Pan-Cancer Pathway Panel	Mandard scoring responder: grade 1–2 non-responder grade 3–5	IL12A, GNA11, FGFR3, H3F3A, SPRY2, IL2RB, SGK2, NKD1 and IL1R1	specificity 79.4% accuracy 81.0% sensitivity 82.3%	/	[81]

Table 4. Continued.

Sample type	No. of samples	Treatment	Methods	Response assessment	Predictive biomarker	Performance	Main function	Reference no.
Pre-CRT biopsy	186	nCRT	RT-qPCR and WB	AJCC scoring	VSTM2L	$p = 0.03$	uncharacterized function	[82]
Pre-CRT biopsy	95	nCRT (5-FU and radiotherapy)	IHC	Mandard scoring CR: grade 1 non-CR: grade 2–4	CXCR4 and COX2	OR = 4.47; 95% CI: 1.15–17.4; OR = 3.21; 95% CI: 1.14–9.09, respectively	chemokine receptor; tumorigenesis	[83]
Pre-CRT biopsy	35	nCRT (5-FU plus leucovorin and radiotherapy)	RT-qPCR	Mandard scoring responder: grade 1–2 non-responder grade 3–4	SMAD7	$p = 0.014$	signaling cascade regulation; tumorigenesis	[84]

Abbreviations: nCRT, neoadjuvant chemoradiotherapy; IHC, immunohistochemistry; RT-PCR, reverse transcriptase-polymerase chain reaction; AJCC, American Joint Commission on Cancer; 5-FU, 5-fluorouracil; FOLFOX, 5-fluorouracil, leucovorin, and oxaliplatin; CLCA1, chloride channel accessory 1; TACC 3, transforming acidic coiled-coil protein-3; BMI1, B-cell-specific Moloney murine leukemia virus insertion site 1; VSTM2L, V-set and transmembrane domain containing 2 like; CXCR4, CXC chemokine receptor 4; SGK1, serum and glucocorticoid-regulated kinase 1.

enhances radio-sensitivity in microsatellite stable (MSS) CRC patients [46].

The forkhead box (FOX) family members FOXK1 and FOXK2 participate in cell proliferation and carcinogenesis. Recently, Zhang *et al.* [78] evaluated the expression of these proteins in RC and their ability to predict nCRT responses. They found that lower levels of FOXK1 and FOXK2 expression were detected in patients with pCR ($p < 0.05$). In another study, Hsu *et al.* [79] found that over-expression of B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI 1) was significantly associated with post-treatment tumour stage (T1–T2; $p = 0.015$), advanced pre-treatment nodal status (N1–N2; $p < 0.001$), poor therapeutic response ($p = 0.001$) and prognosis. In addition, the lack of BMI 1 expression could modulate the expression of Kruppel-like factor 4 to enhance the radio-sensitivity of MSS CRCs. The ATF3-driven overexpression of serum- and glucocorticoid-regulated kinase 1 (SGK1) leads to radio-resistance in non-pCR RC patients [80]. Park *et al.* [81] evaluated the gene expression of tissue samples in RC patients and found that a signature of 9 genes (Table 4) could identify responders with relatively good accuracy, specificity and sensitivity. This signature was also pertinent for clinical and pathological features and could serve as a useful biomarker for predicting the response to nCRT.

High expression levels for the V-set and transmembrane domain-containing 2 like protein in RC have been associated with the therapeutic results of nCRT ($p = 0.030$). These proteins are thought to contribute to radio-resistance in an IL-4-mediated signalling pathway by downregulating γ -H2AX expression and modifying the progression of cell apoptosis and proliferation [82]. Fratte *et al.* [83] investigated the expression of 11 tumour-related proteins (RAD51, CD44, HIF1, CXCR4, COX2, Ki67, GLUT1, CA-IX, VEGF, CXCL12, and MLH1) in pre-nCRT tissue biopsies of RC patients. These authors report that pre-treatment expression levels of RAD51, HIF1, CXCR4, COX2 and Ki67 were predictors of the response to nCRT. Moreover, Ki67 expression together with that of CXCR4 could increase the predictive ability. SMAD7 is a member of the SMAD family and a key mediator of the intracellular signalling cascade, especially the TGF- β pathway. SMAD7 expression was significantly elevated in the primary tumour tissue of responders to nCRT ($p = 0.014$) [84]. In addition, Koyama *et al.* [85] identified 350 differentially expressed genes, of which 199 were downregulated and 123 were upregulated in poor responders. These authors also found that inhibition of Akt activation improved the therapeutic response to CRT. Another genome-wide RNAi screening study identified FICD, NHP2, LDL-RAD2, SYNE3, NCAPH, and replication factor C subunit 4 (RFC4) as potential biomarkers of radio-resistance in RC. Further analysis revealed that RFC4 expression in tissue samples was associated with a poor response to nCRT in

RC patients and with worse prognosis. The authors speculate this may be due to the promotion of DNA repair by non-homologous end joining (NHEJ), including RFC4 and Ku70/Ku80 [86]. However, a recent gene expression-based study showed opposite results. Momma *et al.* [72] screened for potential markers of the response to nCRT by analysis of pre-CRT tissue samples from 53 responders and 61 non-responders. The researchers also employed a further six independent datasets that included 99 responders and 176 non-responders in order to validate the predictive values of the signatures. Surprisingly, they found that current signatures based on gene expression and identified through microarray platforms were not sufficiently robust to predict nCRT response and thus to influence decision-making in the treatment of RC.

5.2 Metabolic Biomarkers

Metabolites are generated by various types of biochemical reactions in the body and also participate in the pathophysiological process of tumourigenesis and progression. Recent work in laboratory medicine has applied metabolomics for the classification of tumours and for the evaluation of treatment response, recurrence and prognosis. This has allowed metabolomics to produce biomarkers in various fields [87]. Early studies showed that serum concentrations of phosphoenolpyruvic acid, hypoxanthine, myo-inositol, creatine, and glycine were potential metabolic biomarkers for predicting response to CRT and prognosis in RC [88,89]. A prospective cohort study (NCT03149978) by Jia *et al.* [87] found that RC patients who underwent nCRT could be discriminated between responders and non-responders by conducting metabolomic analyses of their serum. Potential responders could be identified using 15 differentially expressed metabolites. These results suggest that complete resection for all patients is not required in order to achieve the desired clinical and therapeutic outcomes. Using proper evaluation, potential non-responders could avoid unnecessary nCRT and oncologists could directly advise patients for whom surgery is the preferred option. Tomàs *et al.* [90] recently conducted a study of RC patients to identify biomarkers related to energy metabolism and to circulating levels of paraoxonase-1 that are able to predict nCRT responses. A low pre-nCRT level of plasma valine was associated with pCR (AUC = 0.826), while low concentrations of succinate were associated with relapse (AUC = 0.833). The content of serum exosomes has the potential to predict responses to neoadjuvant radiotherapy in RC. Recent work detected 129 metabolites in the plasma and exosomes, of which 23 differentially accumulated metabolites (DAMs) were present at significantly different levels between responders and non-responders. The exosome levels of pentadecanoic acid and of sucrose were higher in poor responders. Proteome components of serum-derived exosomes are also promising biomarkers, and the combination of proteomic and metabolomic biomarkers is

likely to be a fruitful area of investigation [91].

6. Immune and Tumour Microenvironment Biomarkers

It has been suggested that radiotherapy could stimulate the immune response process in a damage-associated molecular pattern (DAMP)-mediated manner, thus making it more efficient than chemotherapy. Non-irradiated tumour sites can sometimes also obtain a therapeutic response in what is known as the abscopal effect [92,93]. Hence, radiation may enhance the body's immune response through the initiation of immunogenic cell death (ICD), exposure of DAMPs, increased tumour-associated antigens (TAAS), and the recruitment of priming T lymphocytes and myeloid cells. This has also been considered to represent an *in situ* vaccination [34]. Substantial evidence indicates the tumour microenvironment is a vital factor and a reliable marker for predicting the response to certain treatments, the progression of tumours, and the clinical outcome of patients [94]. Thanks to rapid advances in next-generation sequencing technology over the past decade, the relationship between tumour microenvironment, immune regulation-associated biomarkers, and the response to chemoradiotherapy have all been areas of active investigation. The recently reported immune-associated biomarkers were listed in Table 5 (Ref. [95–103]).

6.1 Immune Biomarkers in Biopsy Tissue

6.1.1 Changes in the Tumour Microenvironment

The tumour microenvironment is composed of stromal fibroblasts, endothelial cells, immune cells and an array of bio-macromolecules in the surrounding tissues and inside the tumour cells. Increasing evidence shows that radiation can alter the tumour microenvironment according to the tumour histology, anatomic site, and various other clinical characteristics [104]. Kamran *et al.* [6] demonstrated that radiation could alter the tumour microenvironment. They analysed post-CRT tumour biopsy samples and observed significantly more CD8⁺ T cells ($p = 0.002$), resting mast cells ($p = 0.0007$), monocytes ($p = 0.01$), M2 macrophages ($p = 0.002$) and naïve B cells ($p = 0.044$) than pre-CRT. In pre-CRT tumour tissue, they found significantly more activated mast cells ($p = 0.006$) and memory B cells ($p = 0.04$) than post-CRT. The effects of radiotherapy on tumour-associated macrophages (TAMs), including pro-inflammatory (M1) and immunosuppressive (M2) types, were recently analysed by Stary *et al.* [105]. These authors suggested that radiation could transform TAMs into an M1-like pro-inflammatory phenotype that enhances the therapeutic effects of radiotherapy in RC patients. They also found that radiation increased the phagocytic activity and increased the expression of markers that stimulate T-cell activation. Immunofluorescence staining of tumour biopsies revealed that the phenotype and/or density of plasmacytoid dendritic cells, CD8⁺ T cells and 6-sulfo LacNAc-

expressing monocytes were significantly altered by nRCT. This can in turn influence the clinical response of RC patients to nCRT [106]. Wei *et al.* [107] demonstrated that local tumour irradiation could induce abscopal responses and trigger anti-tumour immunity in the body in a complicated process that involves blocking the PD-1 pathway, increasing the number of reprogrammable CD8⁺ T cells, enhancing the number of poly-functional intra-tumoural CD8⁺ T cells, and decreasing the number of intra-tumoural and dysfunctional CD8⁺ T cells. Meanwhile, nCRT was shown to boost various biomarker scores of immune-associated characteristics in cancer patients, including the immune signature, cytolytic activity and interferon- γ signature. nCRT therefore has the potential to strengthen the therapeutic response of RC patients to immunotherapy [94].

6.1.2 Tumour-Infiltrating Lymphocytes (TILs)

Tumour-infiltrating lymphocytes (TILs) are an important component of the body's primary immune response and have a major influence on the progression and survival of tumours, including the response to radiotherapy and chemotherapy [108]. Previous studies have shown that tumour *in situ* immune cell infiltration markedly influences the clinical outcome of patients with solid tumours [92]. Since chemoradiotherapy is known to induce cell death and immunogenic potential in CRCs, several studies have examined immune infiltration as a predictor of response to nCRT in patients with RC. Teng *et al.* [95] performed immunohistochemistry of CD33, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death 1 ligand 1 (PD-L1), CD11b, forkhead box protein 3 (FOXP3), CD4, CD56 and CD8 in pre-treatment biopsy specimens and in post-nCRT surgical tissue samples of RC patients. CD4⁺ and CD8⁺ TIL densities were significantly increased after nCRT ($p = 0.005$ and 0.004 , respectively). A higher density of CD4⁺ and CD8⁺ TILs in the tumours was also associated with a good response to nCRT ($p = 0.022$ and 0.022 , respectively), as was a lower density of myeloid-derived suppressor cell (MDSC)-TILs ($p = 0.005$). Anitei *et al.* [96] demonstrated that patients with a complete or partial response to nCRT displayed a higher infiltration of CD3⁺ cells in their biopsy samples compared to non-responders ($p = 0.015$). Furthermore, Patients whose biopsies had a higher infiltration of CD3⁺ and CD8⁺ lymphocytes were more likely to show a better response to nCRT (CD3⁺ cells; $p = 0.01$). The ability of pre-CRT CD8⁺ TIL density to predict nCRT response of RC patients was confirmed in other recent studies [97,108]. In contrast, another study reported that stromal CD8⁺ cell density was not related to the response to CRT [79]. Akiyoshi *et al.* [98] evaluated the expression of the T-cell receptor (TCR) repertoire in pre-nCRT tissue samples. These authors found that patients with low TCR diversity and low CD8⁺ TIL density (double-low) were more likely to show a poor response to nCRT than “double-high” patients (16.7% vs. 84.2%, respectively; $p < 0.0001$).

Table 5. Immune-associated biomarkers investigated for their ability to predict radiation-based therapy response in RC patients.

Sample type	No. of samples	Treatment	Methods	Response assessment	Endpoint	Predictive biomarkers	Performance	Reference no.
Pre- and post-CRT biopsy	62	nCRT (5-FU and radiotherapy)	IHC	Dworak scoring responder: grade 3–4 non-responder grade 0–2	responsiveness	CD8+ TILs, CD4+ TILs and MDSC-TILs	$p = 0.022$, $p = 0.022$ and $p = 0.005$, respectively	[95]
Pre-CRT biopsy	55	nCRT	IHC	Ryan classification	responsiveness	CD3+ TILs	$p = 0.01$	[96]
Pre-CRT biopsy	117	nCRT (FOLFOX or 5-FU and radiotherapy)	IHC and quantitative digital image analysis	Ryan classification CR grade 0–1 non-CR grade 2–3	pCR	CD8+ T-cell density	$p = 0.001$	[97]
Pre- and post-CRT biopsy	67	nCRT (5-FU and radiotherapy)	IHC and RT-PCR	Dworak scoring responder: grade 3–4 partial responder: grade 2 non-responder grade 1	responsiveness	TCR diversity index and CD8+ TIL density	$p = 0.049$, and $p < 0.001$, respectively	[98]
Pre- and post-CRT biopsy and blood	25	nCRT (capecitabine and radiotherapy)	Flow Cytometry Analysis	Ryan classification	responsiveness	HLA DR-/CD33+/CD16-/CD11b+ MDSC		[99]
Pre-CRT biopsy	50	nCRT (capecitabine and radiotherapy and/or CAPOX)	IHC	mrTRG	responsiveness	B cell infiltration	$p = 0.047$	[100]
Pre-CRT blood	295	nCRT	Chemoluminescence sequential immunometric assay	Not mentioned	responsiveness	IL-8	$p < 0.001$	[101]
Pre- and post-CRT blood	35	nCRT (5-FU and radiotherapy)	Procarta polystyrene bead-based multiplex immunoassay	The Japanese Classification of Colorectal Carcinoma	responsiveness	IL-6, sCD40L, CCL-5	/	[102]
Pre-CRT biopsy	267	nCRT	IHC	Dworak scoring good responder: grade 3–4	pCR	LCR and $N \times M$ value	$p = 0.016$ and $p = 0.005$, respectively	[103]

Abbreviations: nCRT, neoadjuvant chemoradiotherapy; IHC, immunohistochemistry; RT-PCR, reverse transcriptase-polymerase chain reaction; 5-FU, 5-fluorouracil; CD8, cluster of differentiation 8; CD4, cluster of differentiation 4; TILs, tumour-infiltrating lymphocytes; FOLFOX, 5-fluorouracil, leucovorin, and oxaliplatin; pCR, pathologic complete response; MDSCs, myeloid-derived suppressor cells; TCR, T-cell receptor; mrTRG, magnetic resonance tumour regression grade; IL-8, interleukin-8; IL-6, interleukin-6; sCD40L, soluble CD40-ligand; CCL-5, chemokine ligand-5; LCR, lymphocyte-to-C-reactive protein ratio; $N \times M$ value, neutrophil \times monocyte value.

6.1.3 Programmed Death Ligand-1 (PD-L1)

Programmed death ligand-1 (PD-L1) is a kind of transmembrane protein which is related to the inhibition of the immune system. The expression of PD-L1 was reported to be associated with therapeutic results of nCRT in RC. Chen *et al.* evaluated the density of CD8⁺ TILs and the expression of PD-L1 within the tumour microenvironment of both pre- and post-nCRT samples of RC. They again showed that nCRT can recruit CD8⁺ TILs and increase the expression of PD-L1 within the tumour microenvironment. A higher level of tumour PD-L1 expression in pre-nCRT biopsies was associated with better DFS and OS ($p = 0.003$ and $p = 0.045$, respectively), and similarly for post-nCRT biopsies ($p = 0.003$ and $p = 0.0001$, respectively) [109]. Although the role of PD-L1 in predicting nCRT response is still unknown, those studies provide a preliminary foundation for further exploration of the association between immune status and the response to nCRT.

6.1.4 High Mobility Group Box Protein 1 (HMGB1)

High mobility group box protein 1 (HMGB1) is a kind of damage-associated molecular pattern which is caused by radiation-associated ICD and that HMGB1 has a vital influence on antigen-specific, T-cell-mediated tumour immunity. Previous studies have reported that radiotherapy contributes to ICD, thus strengthening the radiation-mediated tumouricidal effect [93]. Huang *et al.* [110] reported that higher PD-1⁺ TIL density and cyto-HMGB1 expression levels in the tumour microenvironment before nCRT contributed to better therapeutic results of nCRT in RC. Moreover, translocation of cytosolic HMGB1 stimulated the maturation of DCs in a Toll-like receptor 4-mediated manner and enhanced the recruitment of PD-1⁺ TILs to the tumour microenvironment. This could be used as a substitute for immune scavenging (by providing effector cells) and immune surveillance (by emitting hazardous signals). Therefore, HMGB1 could be a promising predictive biomarker for the response to nCRT and also to follow the status of RC patients during nCRT.

In addition to the above-mentioned immune-related biomarkers for predicting response to nCRT in RC, others that have received attention include CD133, COX-2, CD56⁺ natural killer-like phenotype, CD68⁺ macrophages, stromal organization, and HLA-DR⁻/CD11b⁺/CD33⁺ myeloid-derived suppressor cells within the tumour [99,111–113]. Sendoya *et al.* [100] analysed the baseline genome and transcription characteristics of RC patients and found that patients with high expression levels of interferon signalling and of B cell genes were more likely to show a good response to nCRT ($p < 0.005$). They further confirmed the association between B-cell infiltration and responders ($p = 0.047$) by analysis of CD20⁺ cell expression. Other workers have investigated whether collagen features (CFs) in the tumour microenvironment are predictive of the nCRT

response. Jiang *et al.* [114] used a CF-support vector machine (SVM) classifier to predict therapeutic response in a multicentre retrospective analysis of 428 patients with RC. These authors concluded the CF-SVM classifier used in the tumour microenvironment was a potential biomarker for predicting response to nCRT. Moreover, by integrating clinicopathological characteristics with the CF-SVM classifier, the CF-based model could be a reliable tool to predict the response of individual RC patients to nCRT.

6.2 Host Immune Response

The regulation of immunity is complex, with many researchers investigating the connection between circulating bio-macromolecules and the immune system. Once validated, serum biomarkers could be more applicable clinically than biopsy biomarkers due to their non-invasive characteristics.

A prospective study analysed 9 biomarkers (25-OH-vitamin D, osteopontin, CA IX, IL-6, IL-8, CRP, LDH, CA19-9 and CEA) of RC patients who underwent CRT. The authors found that IL-8 (OR 0.94, $p = 0.036$) and CEA (OR= 0.97, $p = 0.029$) were significant predictors of response [101]. The levels of C-C motif chemokine ligand-5, TNF- α and soluble CD40-ligand were reported to be excellent markers of a good response to nCRT [102]. The inflammatory response was also found to be associated with the genesis and prognosis of numerous cancers [115]. The neutrophil-to-lymphocyte ratio (NLR) has been widely investigated for the prediction of nCRT response in RC. However, a consensus has yet to be reached on the time points to measure NLR and on the NLR cut-off values (used to determine whether a test is positive or negative) to be used. The time points for evaluation of pre-CRT and post-CRT outcomes were also limited [13,14]. In a retrospective study of 1052 RC patients, both a lower LMR ($p = 0.0001$) and a higher NLR ($p = 0.0001$) were associated with the response to nCRT. An NLR value of ≥ 3.11 was associated with a lower likelihood of achieving complete total mesorectal excision or of preserving the sphincter [116]. Elevated pre-nCRT NLR was also associated with poor therapeutic response to nCRT [116]. Dreyer *et al.* [117] evaluated the modified Glasgow prognostic score and the albumin, C-reactive protein and hemoglobin levels of patients with RC. These workers found that the body's inflammatory status prior to nCRT was associated with a poor response to nCRT [118]. More recently, the neutrophil \times monocyte (N \times M) ratio and the lymphocyte-to-C-reactive protein ratio (LCR) were found to be markers of a good therapeutic response to CRT ($p = 0.005$ and $p = 0.016$, respectively) [103]. These biomarkers are readily accessible and economically feasible, making them applicable to clinical practice. In combination with other validated biomarkers or clinicopathological characteristics, these biomarkers could be used to design more personalized treatment strategies for RC patients.

7. Haematological Biomarkers

Many blood-based cellular and secreted biomacromolecules have been studied over the past few years thanks to technical advances in the minimally invasive method of liquid biopsy. Several of these molecules have been reported as biomarkers that provide real-time and comprehensive information on tumour diagnosis, staging, progression, and even on the tumour micro- and macro-environment [119]. These biomarkers could also be used to predict the clinical response to specific therapies, therapy-related side-effects, and prognosis, thus improving the decision-making process [119]. Importantly, blood samples can be readily obtained before, during, and after nCRT. Additionally, the assessment of tumour biomarkers is not disturbed by the process of blood collecting. Therefore, haematological markers of therapeutic response and prognosis are now widely studied in clinical oncology.

7.1 Haemoglobin and Platelet Count

Haemoglobin is a kind protein responsible for carrying oxygen in higher organisms. The role of hemoglobin in predicting response of certain therapeutic strategies has been widely investigated. McGrane *et al.* [120] analysed blood samples from 273 patients with RC and treated with nCRT. They found that a hemoglobin level of <120 g/L (anemic) at presentation correlated with a higher regression grade and thus an inferior response to nCRT ($p = 0.006$). These workers also reported that patients with anemia had higher mortality rates than non-anemic patients (HR 1.73; 95% CI: 1.05–2.86). Multiple lines of evidence show that platelets (PLTs) have a strong influence on the progression and metastasis of tumours, with the effects mediated by a variety of mechanisms. PLTs promote epithelial-mesenchymal transformation, thus allowing cancer cells to escape immune surveillance, obstruct the microvasculature, and stimulate angiogenesis [113,121]. A retrospective study investigated the role of PLT count in predicting the clinical outcomes of nCRT. Patients with an elevated PLT count showed a lower pCR rate compared to those with lower counts (12.8% vs. 22.1%, respectively; $p = 0.001$) [122].

7.2 Circulating Lymphocyte Level

Lymphocytes are extremely sensitive to radiation. Radiation therapy therefore leads to exhaustion of lymphocytes in the hematopoietic system, with radiation doses of <1 Gy being sufficient to directly destroy lymphocytes in the circulatory system [123]. Radiation-induced lymphocytopenia (RIL) may counteract the anti-tumour effects of radiotherapy and has recently become a promising area of research. In solid tumours, RIL was shown to be significantly associated with the therapeutic response, PFS, and even OS [123,124]. Heo *et al.* [124] investigated whether the level of circulating lymphocytes during nCRT could predict therapy response in patients with RC. They found

a sustained blood lymphocyte count (lymphocyte count at 4 weeks/baseline lymphocyte count >0.35; OR = 8.33, $p = 0.02$) during CRT was significantly associated with pCR. Liu *et al.* [125] evaluated the absolute lymphocyte count (ALC) of RC patients during nCRT. They found that a high ALC nadir was associated with pathologic response (OR= 4.32, 95% CI: 1.22–15.26, $p = 0.023$) and suggested that ALC may serve as a stratification marker for RC patients scheduled to receive nCRT. Recent studies have suggested that micronuclei frequency (MNf) in peripheral blood lymphocytes may be a prognostic biomarker for monitoring the response of RC patients who undergo induction chemotherapy and then operation. However, it is not known whether MNf could also serve as a predictive biomarker of the response to chemoradiotherapy [126]. Other indexes based on changes to circulating lymphocytes have also been suggested as potential biomarkers of the response to nCRT. These include the platelet-to-lymphocyte ratio, neutrophil-to-lymphocyte ratio, lymphocyte-to-monocyte ratio, and lymphocyte-C-reactive protein ratio [127,128].

7.3 Circulating Tumour Cells (CTCs)

Circulating tumour cells (CTCs) are released into the bloodstream by primary tumours which then could metastasize to distant organ sites. Moreover, CTCs are a minimally invasive and preferred alternative to tumour biopsy [14,119]. The clinical utility of CTCs in RC has been widely investigated. Most studies have confirmed that a high level of CTC counts correlates with poor prognosis [119]. When integrated with tumour characteristics, CTC counts could be used as a substitute for primary tumour cells. Previous research demonstrated that cytokeratin 20-positive CTCs were predictors of the response to nCRT in RC patients [129]. Troncarelli *et al.* [47] reported that TS expression was completely absent in CTCs from patients with a pCR ($p = 0.001$). In contrast, CTCs from 83% of non-responders expressed TS ($p < 0.001$). Furthermore, RAD23 homolog B (RAD23B), an excision repair protein, was expressed in the CTCs of non-responders. These authors concluded that TS/RAD23B and/or TS mRNA expression in CTCs were possible biomarkers for predicting the response to nCRT.

7.4 Cell-Free DNA (cfDNA)

It has been hypothesized that cell-free DNA (cfDNA) results from cell lysis caused by apoptosis or necrosis of cells [14]. In a study of 34 RC patients, Sun *et al.* [130] analysed cfDNA for the methylation status of the O6-methylguanine-DNA methyltransferase (MGMT) promoter region and to screen for KRAS mutations. They concluded that MGMT promoter methylation status, the 400-/100-bp DNA ratio, and the 400-base pair DNA concentration in the baseline cfDNA were helpful for predicting the response to nCRT. Schou *et al.* [131] analysed cfDNA in plasma samples of RC patients treated with nCRT and surgery. These

authors concluded that cfDNA had the potential to improve pre- and post-nCRT risk assessment and thus promote the development of personalized therapy in RC.

7.5 Circulating Tumour DNA (ctDNA)

Circulating tumour DNA (ctDNA) represents 0.01%–10% of the total cfDNA and has become a promising biomarker in various types of cancers [118]. Numerous studies have shown the value of ctDNA genomic alterations or ctDNA concentrations in tumour diagnosis, monitoring of treatment response and of resistance, selection of targeted therapy, and the detection of residual disease [119,132,133].

A Japanese study investigated the clinical utility of ctDNA to predict nCRT responses and post-operative recurrence in RC patients. ctDNA levels at baseline and after nCRT were found to be significantly different ($p = 0.0003$). The authors concluded that the change in ctDNA was independently associated with pCR ($p = 0.0276$) [132]. A prospective multicentre Chinese trial further explored the value of ctDNA for monitoring tumour burden, predicting nCRT response, and predicting survival. Zhou *et al.* [134] found that baseline ctDNA levels were strongly associated with positive nCRT responses and that MFS was closely related to the median variant allele frequency (VAF) of mutations in the baseline ctDNA (HR 1.27, $p < 0.001$). They also demonstrated that ctDNA closely mirrored the tumour burden and served as a real-time monitoring indicator. A recent meta-analysis of 21 publications concluded that higher baseline levels of the longer fragments of cfDNA, certain methylated genes and tumour-specific mutations, and the integrity index were potential predictors of poor response to treatment. Furthermore, undetectable baseline ctDNA levels and a decrease in common RC mutations during nCRT (dynamic monitoring) may be robust indicators of pCR [133].

8. Molecular Imaging Biomarkers

8.1 Computed Tomography (CT)

Computed tomography (CT) is extremely important for diagnosing and staging cancers and for evaluating the efficacy of certain therapeutic strategies. Over the past few years, researchers have been exploring the role of CT imaging in determining the prognosis of RC and for predicting the response to nCRT. A retrospective study ($n = 95$) analysed the texture features of pre-nCRT CT images, including standard deviation, skewness, kurtosis, uniformity and entropy. Chee *et al.* [135] concluded that features of homogeneous textures were associated with better nCRT responses. Other studies have also suggested that kurtosis and fractal dimension (FD) are potential CT-derived biomarkers for predicting nCRT responses [136,137]. Perfusion CT imaging was also investigated in a prospective study of RC treated with nCRT. The results showed that hot-spot blood volume and a decline in hot-spot perme-

ability were significant predictors of pCR outcome ($p < 0.0001$) [138]. Texture analysis contributes to the assessment of heterogeneity of medical images by analysing grey-level intensities on a pixel-by-pixel basis. Some researchers have constructed prognostic models of RC by analysing pre-treatment, contrast-enhanced CT textures to identify patients with poorer down-staging prospects. These could then be offered more intensive treatment via a higher radiation dose or by using other strategies [139]. Another retrospective study evaluated the total subcutaneous, visceral, mesorectal and abdominal fatty tissue components based on the findings of CT images. Dilek *et al.* [140] demonstrated that a cut-off value of ≥ 69.4 for mesorectal fat tissue volume (MRV) was associated with positive nCRT responses. In ROC analysis, the specificity was 58.5% and the sensitivity was 82.9% (AUC = 0.75 (0.65–0.84), $p < 0.001$).

8.2 F-Fluorodeoxyglucose Positron Emission Tomography/Computed Tomography (18F-FDG-PET/CT)

F-Fluorodeoxyglucose Positron Emission Tomography/Computed Tomography (18F-FDG-PET/CT) is a helpful imaging tool used extensively in clinical oncology for disease diagnosis, staging, evaluation of therapeutic efficacy, diagnosis of relapse, identification of the underlying lesion, and long-term follow-up. Researchers have explored FDG-PET/CT-derived imaging markers for predicting the response of RC patients to nCRT by studying pre- and post-CRT radiological characteristics and correlating them with the response to nCRT. The most challenging aspect was to choose the best parameter for semi-quantitative analysis to assess response so that it could be used as a predictive biomarker. Previous studies in patients with RC have shown that changes in the standard uptake value (Δ SUV), SUVmax, or $\% \Delta$ SUV may predict positive nCRT responses [55]. Lovinfosse *et al.* [141] analysed PET/CT images obtained before nCRT in RC patients and evaluated the histogram-intensity features, total lesion glycolysis (TLG), metabolic tumoural volume (MTV), maximum and mean standard uptake values, and 11 regional and local textural features. These authors concluded that TLG could be a robust marker of good therapeutic responses to nCRT (TRG 3-4) [141]. Early total lesion glycolysis (TLG-early) and its percent change compared to baseline (Δ TLG-early) were also reported as potential biomarkers to discriminate responders from non-responders. Furthermore, Δ TLG-early exhibited the highest accuracy for response prediction, especially in high-risk RC patients treated with nCRT using bevacizumab [142].

Using baseline FDG-PET/CT and metric learning (ML), Wu *et al.* [143] built a novel artificial intelligence (AI) model to predict the response to nCRT in 236 newly diagnosed RC patients. ML determines the dissimilarity or similarity between objects in an AI-mediated manner based on a distance metric. The authors found that the model had a preferable accuracy, specificity and sensitivity in predict-

ing therapeutic response of nCRT. Using the AI-mediated ML model, baseline FDG-PET/CT images were concluded to be robust biomarkers for predicting nCRT responses in RC. The AI-based ML model is non-invasive and may allow significant progress in personalized, precision therapy.

8.3 Magnetic Resonance Imaging (MRI)

Magnetic resonance imaging (MRI) is also a helpful imaging tool used extensively in clinical oncology which has several desirable characteristics, particularly its exceptional resolution and contrast definition between the lesions and surrounding tissues. These have contributed to wide application of MRI in the disease setting, including the evaluation of response to nCRT in RC [55,144]. Although the alterations in tumour morphology occur later than other changes at the molecular and biological levels, MRI can still accurately assess tumour characteristics. The potential role of MRI in predicting response to nCRT in RC has been thoroughly investigated.

Dynamic contrast-enhanced MRI (DCE-MRI) has been employed extensively to obtain functional imaging. This can in turn be used to provide useful information on vascular and tissue permeability. Kim *et al.* [145] reported that a decrease in the tumour perfusion parameter (Ktrans) was significantly associated with good response to nCRT in RC ($p = 0.0007$). In contrast, none of the other parameters examined were effective at predicting the efficacy of nCRT [145]. Ciolina *et al.* [146] confirmed that pre-Ktrans predicted response to therapy, while wash-out and Kep measured before nCRT correlated with RC grading.

Diffusion-weighted MRI (DW-MRI) detects water proton mobility in tissues to provide information on microscopic structures. This technique has also been applied to monitor treatment and to predict nCRT response in RC. The apparent diffusion coefficient (ADC) in DW-MRI is a useful instrument for quantitative analysis owing to its various characteristics, including cellularity, tumour proliferation, tumour necrosis, tumour grade, extracellular space tortuosity and tissue organization [55]. However, the use of ADC as a predictive biomarker of nCRT response has been controversial, as discussed elsewhere [55,144]. A prospective study showed that texture features of RC on T2-weighted (T2w) magnetic resonance images were potential imaging biomarkers for the clinical outcome of nCRT. The authors found that pre-treatment medium texture-scale quantified as kurtosis, mid-treatment kurtosis without filtration, and changes of kurtosis in the pre-treatment and mid-treatment images differed significantly between the PR+NR and pCR patient groups ($p = 0.045, 0.038, \text{ and } 0.01$, respectively). In particular, the ROC value in pre-treatment kurtosis was significantly higher than all other parameters (0.907, $p < 0.001$) [147]. Another prospective observational cohort study found that a lower baseline tumour blood flow from dynamic susceptibility contrast MRI was associated with better response to nCRT ($p = 0.01$) [148].

Recently, a multivariable model incorporating mrT stage and quantitative parameters from baseline MRI, including T2w volume and T2w signal entropy, was used to distinguish responders from non-responders prior to nCRT in RC [149]. The combination of T2-weighted MRI volumetry, diffusion-weighted imaging (DWI), and 18F-FDG PET/CT obtained before CRT and before surgery was useful for predicting therapeutic results in RC [150]. Furthermore, quantitative imaging outperformed molecular markers for the prediction of response to nCRT. These findings may help physicians in selecting the most appropriate patients for organ preservation in RC cases.

8.4 Radiomics

Radiomics, which converts digital images into quantitative data, is based on the concept that radiological images are comprised of data that reflects underlying pathophysiology. Advances in science and technology have allowed the extraction of large amounts of quantitative data from tomographic images (CT, MR or PET images). The end goal of radiomics is to generate imaging biomarkers as decision support tools for clinical practice [151]. The implementation of radiomics has progressed rapidly as our understanding of tumour biology has improved. This has in turn contributed to the introduction of precision medicine. Recently, several studies evaluated the potential role of radiomics as a biomarker for predicting tumour responses in RC.

One study explored the clinical and pre-nCRT multi-parameter MRI features of 186 RC patients in order to develop and validate a radiomics model for predicting therapeutic response. Cui *et al.* [152] concluded that their pre-treatment radiomics-based model was of great value for predicting pCR and could potentially guide the selection of patients for a “watch-and-wait” policy. Another study showed that MRI and FDG-PET radiomics features could serve as potential biomarkers. A logistic regression model comprised of six second-order texture features (one from T2w MRI: T2w correlation and five from PET: metabolic volume, glycolytic volume, PET 10th percentile, PET homogeneity, PET contrast) gave the best results for predicting nCRT responses in RC patients (AUC = 0.86) [153]. By comprehensively analysing pre- and post-nCRT MRI data, Liu *et al.* [154] developed a radiomics model that showed excellent performance and could be employed as a non-invasive and individualized tool to predict cases of pCR in patients treated with nCRT. An international multi-centre study of RC patients who underwent nCRT and total mesorectal excision showed that pre-treatment, MRI-based radiomics of RC and/or the characteristics of the surrounding mesorectal compartment were able to predict TRG, the neoadjuvant rectal (NAR) score, and pCR. Both the mesorectal compartment and tumour provided useful and authentic information on therapeutic response and prognosis [155]. More recently, elevated heterogeneity in skew-

ness maps of baseline tumours in T2w-based radiomics has been shown to correlate with nCRT responses [156].

Variation in the collection and analysis of imaging data has impeded the clinical use of imaging biomarkers. Hence, standard and independent software is urgently needed to acquire original data and to further analyse the post-processing data. The primary obstacles with regard to radiomics are the optimal collection and combination of diverse multimodal data sources in a quantitative fashion, the variety of radiomic features, unbalanced datasets, and having few observations. Moreover, the study populations are usually small and limited to single institutions, and the reproducibility of techniques has seldom been investigated. Consequently, the usefulness of the above imaging biomarkers requires further validation and larger prospective studies are warranted before applying these biomarkers as predictive factors in clinical trials.

9. Microbiome Biomarkers

The microbiome is a complicated ecosystem that includes viruses, protozoa, fungi, bacteria and other microbes within the body. The disequilibrium of commensal microbes is thought to contribute to the genesis of cancers and has also been linked to treatment response and survival of different cancer types [157]. For example, a high level of *Fusobacterium nucleatum* was found to be associated with mutational characteristics in colorectal cancer [158]. Serna *et al.* [159] reported that pre-nCRT *F. nucleatum* levels were not reliable for predicting a pCR to nCRT, but the persistence of *F. nucleatum* post-nCRT was associated with a high relapse rate in RC. Jang *et al.* [160] further explored the predictive value of the gut microbiome for response to preoperative nCRT. These authors found a significant difference in β -diversity ($p = 0.028$) between PR and non-PR patients, but not in α -diversity. Moreover, *Bacteroidales* (Bacteroides, Rikenellaceae and *Bacteroidaceae*) was more abundant in non-responder patients than in complete responder patients. Another study analysed the microbiome of fecal samples from RC patients at the initiation of, and just after nCRT. Shi *et al.* [161] identified that responders were abundant in *Shuttleworthia*, while non-responders were rich in *Parabacteroides merdae* and some other bacteria, including *Lachnospiracea incertae sedis*, *Murimonas*, *Romboutsia*, *Oscillibacter*, *Blautia*, *Prausnitzii*, *Clostridium IV*, *Faecalibacterium* and *Ruminococcaceae*. Moreover, responders were also enriched in fatty acid metabolism-mediated pathways.

Recently, a prospective study explored the value of the gut microbiome for predicting the nCRT response. Yi *et al.* [162] reported that nCRT was associated with significant alterations in the microbiome, including a large increase in *Streptococcus* spp. and *Lactobacillus* and a decrease in pathogens associated with RC. They also found the microbiota of baseline samples varied between non-responders and responders. In the responders, *Anaerostipes*, *Dorea* and

Roseburia, which contribute to the production of butyrate, were overrepresented, whereas non-responders were rich in *Fusobacterium* and *Coriobacteriaceae*. The relationship between intestinal microbiota and radiotherapy warrants further investigation of a possible role for the microbiome in predicting the therapeutic efficacy of nCRT, and to evaluate the possibility of modifying the gut microbiome before nCRT. The transplantation of fecal microbiota to improve therapeutic efficacy should also be further investigated.

10. Conclusions

The identification and validation of specific and sensitive molecular biomarkers to select patients who may obtain a clinical benefit from nCRT is a research-intensive process. Although previous studies have shown some progress with the discovery of many potentially interesting biomarkers, several limitations still exist. First, clinical variations in the radiation dose, in the interval between nCRT and surgery, in the chemotherapy regimen used, and in the evaluation criteria may lead to different results between studies. Second, the majority of previous studies evaluated only one type of biomarker (e.g., gene alteration, RNA, protein) and did not use a holistic approach. Moreover, the lack of validation studies of potential biomarkers requires further large-scale prospective studies. Although most RC patients routinely receive nCRT as the main therapeutic strategy, they do not gain equal benefit from this treatment. Robust and confirmed predictive biomarkers should help to select RC patients who benefit the most from nCRT, thus ensuring the best and most appropriate treatment for each patient. Taking a “watch and wait” strategy could avoid high-morbidity surgery, prolong the surgery interval, and avoid the unnecessary side-effects of ineffective chemoradiotherapy. Further exploration of predictive biomarkers is necessary to identify new chemo-radiosensitizing targets and to provide further evidence in support of combined radiation-based therapy and immunotherapy.

Abbreviations

RC, rectal cancer; nCRT, neoadjuvant chemoradiotherapy; CRC, Colorectal cancer; TME, total mesorectal excision; pCR, pathologic complete response; DFS, disease-free survival; OS, overall survival; TRGs, tumor regression grading systems; MSKCC, Memorial Sloan Kettering Cancer Center; DNA, Deoxyribonucleic acid; aCGH, array Comparative Genomic Hybridization; CNAs, chromosomal copy number alterations; CRT, chemoradiotherapy; CRBP1, cellular retinol-binding protein 1; SNPs, Single-nucleotide polymorphisms; TS, thymidylate synthase; pCRT, preoperative chemoradiotherapy; XRCC1, X-ray cross-complementing group 1; RR, relative risk; OR, odds ratio; HR, hazard ratio; CI, confidence interval; CR, complete response; PFS, progression-free survival; FPR1, formyl peptide receptor 1; AUC, area under the curve;

mRNA, messenger RNA; TERT, telomerase reverse transcriptase; CTCs, circulating tumor cells; VEGF, vascular endothelial growth factor; RT-PCR, reverse transcriptase-polymerase chain reaction; AJCC/UICC, American Joint Commission on Cancer/International Union Against Cancer; 5-FU, 5-fluorouracil; ERCC1, excision repair cross-complementing group 1; IHC, immunohistochemistry; CCR6, C-C motif chemokine receptor 6; CHD4, chromodomain helicase DNA-binding protein 4; CISH, chromogenic in-situ hybridization; COASY, CoA Synthase; RT-qPCR, real time quantitative-polymerase chain reaction; miRNAs, microRNAs; lncRNA, long noncoding RNA; XRCC2, X-ray repair cross-complementing protein 2; ERCC1, excision repair cross-complementing group 1; ATM, ataxia telangiectasia mutated, MRE11, meiotic recombination 11 homolog A; PCNA, proliferating cell nuclear antigen; FGFR4, fibroblast growth factor receptor 4; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; FOLFOX, 5-fluorouracil, leucovorin, and oxaliplatin; CLCA1, chloride channel accessory 1; TACC 3, transforming acidic coiled-coil protein-3; BMI1, B-cell-specific Moloney murine leukemia virus insertion site 1; VSTM2L, V-set and transmembrane domain containing 2 like; CXCR4, CXC chemokine receptor 4; SGK1, serum and glucocorticoid-regulated kinase 1; DAMPs, damage associated molecular patterns; TAAS, tumour-associated antigens; TAM, tumor-associated macrophages; TILs, Tumor-infiltrating lymphocytes; PD-L1, programmed death ligand-1; HMGB1, high mobility group box protein 1; ICD, immunogenic cell death; CD8, cluster of differentiation 8; CD4, cluster of differentiation 4; FOLFOX, 5-fluorouracil, leucovorin, and oxaliplatin; MDSCs, myeloid-derived suppressor cells; TCR, T-cell receptor; mrTRG, magnetic resonance tumor regression grade; IL-8, interleukin-8; IL-6, interleukin-6; Scd40l, soluble CD40-ligand; CCL-5, chemokine ligand-5; LCR, lymphocyte-to-C-reactive protein ratio; $N \times M$ value, neutrophil \times monocyte value; cfDNA, Cell-free DNA; Circulating ctDNA, tumor DNA; CT, Computed Tomography; MRI, Magnetic Resonance Imaging; PET/CT, Positron Emission Tomography/Computed Tomography; DCE-MRI, Dynamic contrast-enhanced MRI; DW-MRI, diffusion-weighted MRI.

Author Contributions

ZL (Zhaojun Li) and ZL (Zhengyin Liao) contributed to the conception and idea of this article. YC, BY and MC contributed to the literature search. YC and BY wrote the manuscript. All authors have read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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