Network Mapping of Molecular Biomarkers Influencing Radiation Response in Rectal Cancer

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Abstract

Preoperative radiotherapy (RT) plays an important role in the management of locally advanced rectal cancer (RC). Tumor regression after RT shows marked variability, and robust molecular methods are needed to help predict likely response. The aim of this study was to review the current published literature and use Gene Ontology (GO) analysis to define key molecular biomarkers governing radiation response in RC. A systematic review of electronic bibliographic databases (Medline, Embase) was performed for original articles published between 2000 and 2015. Biomarkers were then classified according to biological function and incorporated into a hierarchical GO tree. Both significant and nonsignificant results were included in the analysis. Significance was binarized on the basis of univariate and multivariate statistics. Significance scores were calculated for each biological domain (or node), and a direct acyclic graph was generated for intuitive mapping of biological pathways and markers involved in RC radiation response. Seventy-two individual biomarkers across 74 studies were identified. On highest-order classification, molecular biomarkers falling within the domains of response to stress, cellular metabolism, and pathways inhibiting apoptosis were found to be the most influential in predicting radiosensitivity. Homogenizing biomarker data from original articles using controlled GO terminology demonstrated that cellular mechanisms of response to RT in RC—in particular the metabolic response to RT—may hold promise in developing radiotherapeutic biomarkers to help predict, and in the future modulate, radiation response.

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Introduction

Total mesorectal excision represents the surgical standard of care in rectal cancer (RC) and has led to significant improvements in local control over the past 3 decades. The additive benefit of radiotherapy (RT) in reducing local recurrence in locally advanced RC (T3/T4 and/or node-positive tumors) has been confirmed by several landmark randomized controlled trials (RCTs). In the majority of patients, RT leads to clinically meaningful tumor regression. However, there is considerable variability in terms of tumor response, such that up to 20% of tumors undergo minimal regression, while an estimated 15% exhibit a complete radiologic response. The remainder have an intermediate response, with varying degrees of residual viable tumor seen in the postoperative resection specimen. Correspondingly, patients will fall broadly into 3 groups: (1) those in whom RT has led to complete tumor destruction; in this group of patients, there is growing interest in the feasibility of organ preservation6,7; (2) those in whom RT will result in at least a moderate degree of tumor regression, which in turn may enhance the likelihood of R0 resection and/or sphincter preservation; and (3) those in whom preoperative RT will lead to negligible tumor shrinkage. Paradoxically, for patients in the last category, the delay in proceeding to tumor excision while completing RT may increase the likelihood of distant metastases. Thus, the development of reliable methods to predict response at the pretreatment phase represents a critical unmet need in order to personalize locally advanced RC treatment algorithms.

Current understanding of response to RT is limited by the lack of a unifying interpretation of the molecular pathways implicated in response mechanisms. Additionally, systematic reviews in this context have served little to no role in expanding current understanding...
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understanding of this topic, as they typically evaluate data as a narrative series of disconnected molecular entities, with varying experimental methodology and clinical outcome measures and no wider appreciation of biological interconnectivity. While biomarkers, commonly oncogenes, are important in cancer diagnosis, such genes do not occur in isolation, and therefore mechanisms such as those governing tissue response to RT are likely to involve multiple biological pathways and processes at genomic, transcriptomic, and metabolomic levels. Thus, it is desirable to map potential biomarkers more holistically, in such a way as to allow integration of heterogeneous molecular data and intuitive visualization of the resulting network in order to identify areas with greatest translational potential.

Here we present a systematic review and network analysis of biomarkers implicated in RC radiosensitivity, with the aim of mapping key molecular processes involved, statistically estimating their relative importance, and permitting visualization of how these are interconnected.

Methods

An electronic literature search of the Medline and Embase databases was performed for English-language articles published between January 2000 and November 2015, utilizing Boolean logic and controlled vocabularies (Medical Subject Headings [MeSH] and Emtree). No review protocol was published in advance of this review. The following keywords were used, with wildcards used to maximize article capture: rectal, cancer, neoplasm, response, pathological complete response, tumor response, radiotherapy, chemoradio*, neoadjuvant, predict*, biomarker, *omic, RNA, DNA. Both human in vivo and human colorectal cancer (CRC) cell-based in vitro studies were included. Inclusion criteria were studies examining potential biomarkers (specific genes, protein products, or metabolites) correlated with a documented tumor regression grade (TRG) after RT (either long-course chemoradiotherapy [LCCR] or short-course RT [SCRT]). Studies were excluded if they evaluated targeted monoclonal antibody therapy, gene expression panels obtained through microarrays, micro-RNAs (miRNA) where the gene targets were not defined, or pure animal model–derived data. miRNA studies were initially screened but were excluded from this review primarily because the corresponding gene targets of respective miRNAs were not defined in any of the studies. Irrespective of this, with the potential for a single miRNA to target multiple genes, the inability to assign each miRNA to a single gene classifier was deemed incompatible with the objectives of the present study. Similarly, there was poor congruence between studies that used tissue microarrays, and the resultant outputs from these were almost universally reported as panels of multiple genes providing an in silico means for predicting response, and as such, these data were noncompliant with our objectives.

The following information was extracted from each publication: (1) studied biomarker, (2) analytical methodology used, (3) patient numbers, and (4) statistical parameter used by the authors to categorize discovery as significant (or not), according to correlation with TRG (including P values and odds ratios [ORs] for univariate tests, and sensitivity and specificity for multivariate tests). Each biomarker was then classified according to the system proposed by the Gene Ontology (GO) Consortium into 1 of 5 domains of molecular function or biological purpose (cell cycle, cell death, response to stimuli, cell signaling, and cellular metabolism). The GO approach represents a relatively recent development in biological computational science that offers a means of annotating and grouping molecular products of upstream gene expression according to functional/biological commonality. We chose to utilize a GO approach here in order to generate a biologically more interpretable panorama of processes governing radiation response.

Depending on the statistical parameter used in the original study, significance was binarized (significant or not) based on P values (P < .05), OR (> 1), or sensitivity and specificity (> 60%). A score summarizing the significance of a given biomarker was in turn calculated as a ratio of significant biomarkers relative to the total number of biomarkers under a given ontology heading. Nonunique biomarkers reported by different studies were included in this scoring, as the magnitude of supporting or conflicting evidence for a given biomarker by different studies needs to be represented. For each ontology node, a second score was computed to represent the total number of biomarkers and publications investigating this domain. This was calculated as the number of unique biomarkers for a given GO term (unlike in the first score, where nonunique biomarkers were considered). The 2 scores (each ranging between 0 and 1) represent 2 dimensions on the reported biomarkers we identified through systematic review: relative significance and popularity.

Results

A total of 452 studies were identified after removal of articles that did not include full text (mostly conference proceedings) and manual deduplication. The abstracts of these articles were further screened, and 373 were excluded because they did not address the question of tissue biomarkers predicting response to RT in RC, or because there was insufficient information on methodology or statistical analysis. A further 11 excluded articles comprised systematic reviews and meta-analyses, the bibliographies of which were hand searched, providing a further 5 articles (Figure 1). This left 74 articles that provided sufficient methodologic and statistical detail for both systematic review and to enable integration into a node-based biological network of tissue-based biomarkers. For ease of discussion, biomarkers evaluated in these studies were analyzed on the basis of biological and molecular function according to GO, and clustered under broad, highest-level biological function using controlled language, as previously described.*,**,† For many biomarkers with multiple GO terms fell under different domains of biological function and so were recorded as separate entries in a data sheet of hierarchical ontology terms for each biomarker. It should be noted, however, that many of the biomarkers assessed have multiple functions and GO annotations; this is taken into account in the network-driven analytics.

The data obtained from the review process were extracted in such a way as to assign an identifier to each biomarker assessed by each study (Table 1). This generated a list of biomarkers with multiple entries for those that had been the subject of investigation for more than one study. Equally, to account for statistical power, separate entries were made for each statistical test (univariate or multivariate) applied to each biomarker. The full data extraction table is provided in the Supplemental Material.
By using GO terms, we were able to build a hierarchical classification for each biomarker that allowed for clustering under 1 of 5 subheadings (cell cycle, cell death, response to stimuli, cell signaling, and cellular metabolism). A network model was then developed (Figure 2). In this model, nodes are annotated according to their GO term and are clustered in spokes around the root term “biological process.” Nodes are sized according to a significance score for that ontology term, calculated by taking into account the various statistical tests applied to each study (most commonly chi-square association or OR between biomarker and TRG in univariate and multivariate analysis, respectively). The size of the node represents the number of biomarkers investigated under that ontology term, and the color scale indicates the relative significance level. Therefore, a large red node would represent an ontology term with both a large number of biomarkers and high statistical significance.

The GO-based analytical approach used here shows that when the vast number of assessed biomarkers in the literature are
clustered according to controlled language, it is possible to begin to identify patterns that may aid direction of future study by highlighting areas where there has been success to date. Figure 2 indicates that the biological function spoke "cell cycle" demonstrates a number of biomarkers that have shown negative results in experimental work. Thus, despite this being a fundamental component of cancer regulation, cell-cycle-based molecular dynamics may have little to do with radiation response. We
found that the greatest numbers of significant biomarkers lie in the domains of “response to stimulus” and the interplay of this domain with “cell death.” In addition, while a relatively small number of biomarkers responsible for cellular metabolism have been evaluated to date, many of these demonstrate a high level of correlation with TRG.

Discussion

Cell Cycle

Numerous genes involved in cell-cycle regulation and cellular proliferation have been implicated in cancer development and progression. The p53 tumor suppressor network has been extensively studied and is ubiquitously implicated in almost all cancer subtypes. At its core, p53 can be broadly considered a tumor suppressor activated in response to stress, with subsequent effects on apoptosis, cell-cycle arrest, and senescence, with loss of p53 function being associated with decreased apoptosis.16,73 Although certain radioresistant CRC cell lines demonstrate decreased expression of p53, multiple immunohistochemical (IHC) studies aimed at identifying it as a candidate biomarker for radioresistance have not shown a significant association between isolated p53 expression and TRG.14,16-19,45 One 2014 study in which IHC was performed for a panel of biomarkers including p53, revealed a correlation between low p53 expression and pathologic complete response, but not with TRG,42 whereas Kelley et al31 found that intact p53 with deficiency in caspase-8 expression predicted inferior TRG.

These conflicting findings may in part relate to the complex interactions of the p53 network. In a study of 112 patients, both univariate and multivariate analysis found that p53 expression in pretreatment biopsy specimens did not correlate with RT response, whereas p21 expression—the product of which is a cyclin-dependent kinase (CDK) inhibitor acting through p53-dependent and independent pathways—was significantly associated with both TRG and disease-free survival.14 By contrast, 2 further studies examining both p21 and p53 did not demonstrate any correlation between p21 expression and TRG.15,16

Downstream, a cross-regulatory network exists between p53 and protein kinase B (Akt), balancing the apoptotic regulation by p53 with the effects of the Akt signaling pathway. Phosphorylated Akt exerts subsequent effects on cell-cycle progression, metabolism, and inhibition of apoptotic pathways through Bad74 although its expression was not found to be predictive of response to RT when evaluated with IHC.21 Other CDK inhibitors, notably p27, have also failed to demonstrate any correlation with response to RT.16,20 Markers of cell-cycle progression are, however, useful surrogate markers of proliferation, and both Ki-67 and securin (pituitary tumor transforming gene 1, PTTG1) have been evaluated for correlation with response to RT. Whereas Ki-67 is detected in all active phases of the cell cycle, securin peaks in the G2/M phases. Significant correlation has been demonstrated between Ki-67 expression and response to RT.22-24 Although securin did not demonstrate a predictive capacity for TRG, expression levels in general were shown...
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to decrease after RT; furthermore, for those tumors with high securin expression after treatment, overall prognosis was poorer.26

The regenerating islet-derived gene 4 (REG4) is one of a family of genes (Reg) belonging to the calcium-dependent lectin superfamily, encoding a group of small secretory proteins. These have been found to be constitutively expressed in cell lines of the gastrointestinal tract with characteristic up-regulation in RC at both messenger RNA (mRNA) and peptide levels.75,76 In cellular models, it has been shown to activate cell proliferation, in addition to in vitro migration and invasion of CRC cells.77 Increased expression of REG4 in radioresistant RC cell lines led to a subsequent up-regulation of NEIL2 and survivin (BIRC5), suggesting a network correlation of expression of these genes and their products with radiosensitivity.11 Additionally, IHC for REG4 expression in 172 clinical specimens revealed increased REG4 expression to be a surrogate marker for radioresistance.25 Survivin acts as both a controller of mitotic progression and inhibition of apoptosis, and it has been demonstrated to play a role in the molecular pathogenesis of CRC.78 Conflicting evidence exists, however, for survivin as a putative biomarker for radioresistance. Whereas McDowell et al12 did not find survivin alone to be a predictor of response when assessed by both mRNA and protein expression, other studies have found a significant correlation in both in vivo and in vitro models with established radioresistant cell lines, including SW480 and HCT-15.10,11,13

Although the full role and molecular function of the glycoprotein YKL-40 (chitinase-3-like-1) is yet to be elucidated, it is understood to regulate a number of cellular processes, including cellular proliferation, by exerting effects on the tumor microenvironment to stimulate angiogenesis and tissue remodeling in CRC.79,80 A multicenter study utilizing IHC to verify YKL-40 expression in biopsy specimens found a high positive predictive value for radioresistance. This predictive value was increased to 94% when evaluating for biomarker coexpression with the c-Met protooncogene.27

Cell Death

Cell death covers a range of biological processes by which the cell ceases to function, either as a result of an intrinsic, programmed pathway or in response to extrinsic stressful stimuli such as exposure to cytotoxic agents or RT. A number of mediators of the apoptotic pathway have shown potential as predictors of radiosensitivity.2

A critical step in apoptotic signaling is the release of cytochrome c from mitochondria, which in turn activates apoptosis protease-activating factor 1 (APAF-1/apoptosome). After the activation of the initiator caspase-9, a series of reactions ensue that result in apoptosis and that are regulated by a balance of pro- and anti-apoptotic mediators. APAF-1 expression in pretreatment biopsy samples has been shown to correlate significantly with an improved TRG. Evidence for downstream mediators such as Bax (proapoptotic) and Bcl-2 (antiapoptotic) as predictive biomarkers has been inconclusive.14,16,30 although one group found a significant association between Bcl-2 and TRG in both univariate and multivariate analysis.37 Furthermore, programmed cell death 4 (PDCD4) was first shown to be up-regulated in apoptosis by Shibahara et al41 and is now known to act as a nuclear mediator of apoptosis having interactions with factors downstream of Akt. In a recent study PDCD4 expression was shown to correlate positively with TRG on multivariate analysis.35

Although some correlation has been observed between apoptotic rate in pretreatment CRC biopsy samples and response to RT,12,29 expression levels of M30 (caspase-cleaved keratin 18 cytoskeletal protein) have not been shown to be predictive of response to RT.34 Similarly, annexin V—an established marker for apoptosis, as it binds to phosphatidylserine molecules on the cell surface (an event observed in apoptotic cells)—was not found to be predictive of response.28 In spite of this, there is increasing evidence that the presence of higher levels of complex lipids is not only characteristic of the tumor microenvironment but may also contribute to radioresistance. Levels of phosphatidylethanolamine binding protein 4 (hPEBP4) have been shown to correlate strongly with increased resistance to RT in a cohort of prospectively recruited patients who received SCRT.53 Functionally, hPEBP4 is a secreted glycoprotein that has been shown to be involved in the activation of Akt.82 Overexpression of hPEBP4 in SW480 cell lines has been shown to enhance clonogenic survival, a finding mirrored in mouse models subjected to ionizing radiation. Inhibition of Akt activation additionally appeared to reverse the radioresistance effect of hPEBP4.52

Response to Stimuli

Ionizing radiation leads to DNA strand breaks and ultimately cell death. Mediators involved in maintaining cellular homeostasis and responding to changes in the tumor microenvironment have shown variation in expression levels in relation to radiosensitivity. The presence of relative tumor hypoxia has been implicated in radioresistance in a number of malignancies. Central to the regulation of over 100 genes involved in the adaptive response to hypoxia is hypoxia-inducible factor 1 (HIF-1).53,82 The effects of this transcription factor, stabilized at low oxygen tensions, include regulation of apoptosis, angiogenesis, glycolysis, and the cell cycle. A number of these downstream genes and products have subsequently been investigated as putative biomarkers for radiation response. Using quantitative PCR to evaluate HIF-1 expression in pretreatment tumor biopsy samples, Toiyama et al39 demonstrated not only significantly lower levels of HIF-1 in those with significant tumor regression but also downstream induced factors epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) on both univariate and multivariate analyses.

The effects of hypoxia include further biochemical changes in the tumor microenvironment. CA9 (carbonic anhydrase 9), coding for carbonic anhydrase isoenzyme 9, plays an important role in regulating extracellular pH under both hypoxic and normoxic conditions, and has been found to be underexpressed in tumors with a pathologic complete response.40 This study broadly examined a number of mechanisms of the adaptive response to hypoxia, including glucose transporter 1 (GLUT-1) expression, which had been shown in an earlier study to be significantly associated with radioresistance. Conversely, GLUT-1—negative tumors were found to have a 70% probability of a good response to RT. These findings were not corroborated by Guedj et al,46 however, who found no significant association between GLUT-1 expression and TRG. It is worth noting that these 2 studies used differing intervals between RT and surgery, and also used different TRG grading systems.

Figure 3 summarizes the factors identified through this review that have been found to correlate with response to RT, based on
individual variations. Pathway A outlines how HIF-1 relates to other markers identified through these studies in the presence of hypoxia. The pathways directed at mounting a local response to ionizing radiation, repairing DNA double-strand breaks and stabilizing protein structures, or activating apoptosis are summarized in pathways B and C.

The primary aim of RT is to cause lethal double-stranded DNA (DS-DNA) breaks resulting in cell death. A number of genes involved in base-excision repair pathways have already been well studied. NEIL2 belongs to a family of DNA glycosylases that initiates the first step in base excision repair. NEIL2 has been shown to be up-regulated in radioresistant RC cell lines as well as in pretreatment clinical biopsy samples of patients whose disease had a poor response to RT. Equally implicated in the base excision repair pathway, polymorphisms within the hOGG gene—specifically the hOGG1 1245C > G polymorphism—have been shown to be associated with inferior TRG when extracted from peripheral blood and RC tissue in 238 RC patients. It is thought that the 1245C > G polymorphism codes for a low-functioning isoform of the protein, resulting in less DS breaks as part of excision repair, leading to a smaller proportion of cells undergoing cell death as part of lethal DS-DNA breaks.

The X-ray repair cross-complementing proteins 2 and 3 (XRCC2 and XRCC3) are involved in a separate DNA repair pathway
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(homologous recombinant repair) and have been separately evaluated as markers of radiosensitivity. Knockdown of XRCC2 in vitro results in impaired repair of DS-DNA breaks, and Qin et al.\textsuperscript{22} concluded that intact XRCC2 proteins resulted in radioresistance by facilitating DNA repair after RT. In addition to nuclear repair mechanisms, DNA damage signals the release of cytochrome c and activated Smac proteins from the mitochondria, with subsequent activation of the apoptosome, as previously described. As well as preventing the inhibition of apoptotic promotores, it is postulated that Smac could also play a role in both activation and enhancement of caspase-3. Correspondingly, increased expression of Smac has been found to correlate with improved TRG.\textsuperscript{24,38} Within the cytosol, a number of small stabilizing proteins (heat-shock proteins, Hsps) have been found to have roles in stabilizing protein structures and maintaining cellular homeostasis. The gene DNAJC12 codes for a 40 kDa Hsp, increased expression of which was shown to be significantly associated with inferior TRG as well as with increased vascular and perineural invasion and posttreatment nodal involvement,\textsuperscript{47} with selected Hsps being correlated with radioresistance. Increased Hsp42 expression, verified by protein separation with 2-D gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, was associated with radioresistance in 17 clinical specimens sampled before RT.\textsuperscript{28} Similarly, increased expression of Hsp90 in pretreatment biopsy samples has been shown to correlate with a poor response to RT.\textsuperscript{21}

**Cell Signaling**

Many of the factors already discussed play a role in both cell—cell signaling and intracellular signal cascade mechanisms. Cell-surface signaling molecules have long been established as molecular fingerprints for cancer immunotyping, such as the family of clusters of differentiation (CD). This large class of molecules act as receptors or ligands, which either initiate a signaling cascade once activated or play a role in processes such as cell—cell adhesion,\textsuperscript{10,14,37,40,56,57} although the evidence for these correlating with radiosensitivity has been inconclusive (Table 2). CXCL10 (CXC motif chemokine 10) is the ligand for the CXCR3 receptor, although this is found predominantly in T cells.\textsuperscript{85} Presence of increased expression of CXCL10 mRNA in pretreatment RC biopsy samples was shown to be predictive of sensitivity to RT, although IHC did not corroborate this.\textsuperscript{41} CXCL10 has been shown to act as an angiostatic, inhibiting VEGF;\textsuperscript{41} but the correlation between mRNA expression and protein expression in this case, and hence the implication for CXCL10 to act as a surrogate biomarker is not clear.

Although numerous cell-surface adhesion molecules play a major role in the interaction between tumor and neighboring microenvironment, only the glycoprotein thrombospondin 2 (THBS2) has been shown to be significantly associated with response to RT.\textsuperscript{14} THBS2 demonstrates antiangiogenic effects upon interaction with stromal endothelial cells, and low or absent levels of THBS2 expression in CRC biopsy samples has been shown to be linked to inferior TRG. In this study, however, 118 of the 172 specimens examined fell into an intermediate TRG (Dworak TRG 2-3), with a near 50-50 split between high and low expression of THBS2 on IHC.\textsuperscript{61}

As with the cellular response mechanisms to stress, expression levels of a number of receptor tyrosine kinases (RTKs) have been shown to increase in RC and may be implicated in radioresistance. The downstream effects of activation of RTKs and the mitogen-activated protein kinase (MAPK) pathway on gene regulation, cellular proliferation and apoptosis as described, have highlighted these pathways as potential players in radioresistance. IGFAP1 (Ras GTPase-activating-like protein) has been evaluated as a potential biomarker on the basis of its being an essential scaffold protein in extracellular signal-regulated kinase signaling, which is a key step in the MAPK cascade.\textsuperscript{50} It is known that MAPKs are activated—and MAPK signaling up-regulated—in response to a variety of stimuli.\textsuperscript{57} Although this study did not evaluate levels of IGFAP1 in pretreatment biopsy samples as a stand-alone predictor of response to RT, the authors found that apical cell IGFAP1 expression increased most dramatically before and after treatment in those patients with a poor TRG (< 50% regression).\textsuperscript{58}

Components of other signaling pathways activated by membrane receptor binding (notably the Wnt and transforming growth factor [TGF]-β signaling cascades) have failed to demonstrate conclusive results. SMAD3 transcriptionally regulates TGF-β target genes upon accumulation in the nucleus, and SMAD3 mutations in murine models have been linked to the development of CRC.\textsuperscript{88} The phosphorylated isoform of SMAD3 is considered as a hallmark for SMAD3 activation, increased expression of which has been shown to be associated with a poor TRG.\textsuperscript{60} β-Catenin can be detected at the membranous, cytoplasmic, and nuclear levels, representing its function within the Wnt cascade, and counts activation of genes linked to the APC gene among its downstream effects. A shift in treatment response has been demonstrated that is dependent on nuclear versus cytoplasmic expression of β-catenin, with increased nuclear expression being associated with radioresistance.\textsuperscript{55} However, conflicting evidence was provided in 2 other studies; no significant association was found between membranous β-catenin expression and Dworak TRG, but when correlated with the Cologne grading system, statistical significance was reached on univariate analysis.\textsuperscript{54} A further multivariate analysis of 130 samples also demonstrated no significant correlation of β-catenin with Mandard TRG.\textsuperscript{35} By contrast, in a single study, expression of T-cell factor 4 (TCF4)—another downstream mediator of the Wnt cascade and implicated in the adenoma—adenocarcinoma sequence—demonstrated inverse correlation with TRG.\textsuperscript{68}

EGFR is one of a group of the RTKs that are activated and up-regulated by the stabilization of HIF-1. As with a number of other studies, conflicting results abound, with only one IHC study out of 3 identified demonstrating a link between low EGFR expression and complete pathologic response.\textsuperscript{14,15,22} Evaluation of gene expression levels with quantitative PCR corroborated this finding, demonstrating a significant association between low expression levels of the EGFR gene and increased tumor regression.\textsuperscript{50}

Growth-hormone-releasing hormone (GHRH), when bound to its receptor, stimulates local tissue proliferation via release of insulin-like growth factor 1 (IGF-1) and has been demonstrated as a feature of tumor aggressiveness, with effects on both malignant transformation and metastasis.\textsuperscript{14} Work on RC cell lines has demonstrated that when administered in combination with a cytotoxic agent, GHRH antagonists can induce S-phase arrest and thus apoptosis in part by inhibition of these mechanisms.\textsuperscript{90} Correspondingly, increased expression of the GHRH receptor in pretreatment RC biopsy samples has been found to correlate with radioresistance.\textsuperscript{21}
**Cellular Metabolism**

The cytotoxic backbone of the majority of chemoradiotherapeutic regimens is 5-fluorouracil (5-FU), which functions as an antimetabolite and pyrimidine analog, irreversibly inhibiting the action of thymidylate synthase. Thymidylate synthase in turn plays a key role in nucleic acid metabolism, catalyzing the conversion of deoxyuridine monophosphate (dUMP) to deoxyuridine TMP, a key nucleoside required in DNA replication. Most groups have found a correlation between low-level thymidylate synthase expression and enhanced TRG, although this has not been universally reported. Microarray analysis of published RC transcriptomic data sets has identified candidate genes involved in both nucleotide and amino acid metabolism that may be implicated in radioresistance, including CPS1 (carbonyl phosphate synthetase 1), an enzyme involved in glutamine metabolism and in catalyzing the initial steps in ammonia detoxification. CPS1 was identified as the most significantly up-regulated gene in the glutamine pathway in radioresistant RC; additionally, IHC staining of clinical specimens demonstrated significant correlation between CPS1 expression and radioresistance.

Another enzymatic marker being explored is asparaginase synthetase (ASNS), which has recently been found to serve functionality beyond amino acid synthesis; accumulation of both aspartate and glutamine in ASNS-deficient tissues leads to an increase in nucleotide synthesis and thus cellular proliferation. Consequently, a significant correlation between low ASNS expression and radiosensitivity was observed in RC specimens, after identification of both the ASNS and PAH genes as being differentially expressed between response and nonresponse in a transcriptomic data set.

As described, the distinguishing features of invasive cancer involve a multitude of genetic and proteomic anomalies, which in turn have profound downstream effects on normal cellular metabolism and bioenergetics. Metabolic phenotyping approaches (metabonomics/metabolomics) are showing increasing promise in...
elucidating the metabolic factors that govern radiosensitivity. Modifications to lipid biosynthetic pathways and lipid metabolism are now established as hallmark features of cancer, and intracellular lipid mediators have now been implicated in mechanisms of response to ionizing radiation and cytotoxicity. Through analysis of the gene expression database of RC tissue, HSD17B2 and HMGCS2 were identified as key lipid biosynthesis-associated genes with a predictive capacity for response to RT. The enzyme product HSD17B2 (hydroxysteroid 17-beta dehydrogenase 2) is known to catalyze the reduction of E2 estrogens, testosterone to androstenedione, and 5a-androstenediol to dehydroepiandrosterone. HMGCS2 (3-hydroxy-3-methylglutaryl-CoA synthase 2) catalyses the formation of HMG-CoA from both acetyl-CoA and aceto-acetyl coenzyme A (CoA), a key early step in the malonate and ketogenesis pathways. This is in keeping with the Warburg phenomenon consistently displayed by solid cancers, where a propensity for anaerobic fermentation of ketone bodies is displayed, even in the presence of abundant oxygen. Overexpression of both genes and their products was found to be associated with poor response to RT.

Cyclooxygenase-2 (COX-2; prostaglandin endoperoxidase synthase) converts arachidonic acid to prostaglandin H2, which is the precursor to all other prostanoids that act principally as inflammatory mediators. For this reason, and because arachidonic acid is released from the cell membrane after direct damage by ionizing radiation, a number of groups have evaluated the role of COX-2 in radiosensitivity. In addition to the functional effects of these bioactive lipids (angiogenesis, increased vascular permeability, and chemotaxis: Figure 2), COX-2 has also been shown to directly stimulate VEGF synthesis. This supports a theory of COX-2 playing an important role in the response to the cellular injury sustained after RT, although whether this is purely due to an increased amount of substrate (ie, arachidonic acid) as a result of cell membrane damage or an additional up-regulated pathway remains to be fully elucidated. Moreover, a cell-wide inflammatory response mechanism, portended by up-regulation of nuclear factor kappa B (NF-κB) in the nucleus, which in turn acts as a transcription factor for (among others) COX-2, TGF-β, VEGF, and EGF, has been proposed. However, evaluation of NF-κB as a predictive biomarker reveals conflicting results. Nuclear IHC staining for NF-κB in 74 pretreatment samples demonstrated up-regulation of NF-κB correlated with poorer overall survival, but not with TRG. This may have been partly due to a type I error, however, as a subsequent larger study found that lower NF-κB expression correlated with improved TRG. Furthermore, despite previous evidence that annexin 1 (ANXA1) is an endogenous inhibitor of NFkB, Sheu et al found that high levels of expression of ANXA1 correlated with a poor TRG. ANXA1 exerts its effects as both a potent anti-inflammatory mediator and a calcium ion/phospholipid binder that has effects on tumourigenesis and progression.

Conclusion
Locally advanced RC represents an excellent case model for personalized multimodality therapy, although to date molecular phenotyping has had little direct impact on the RC treatment algorithm. Currently an estimated 15% to 20% of patients with locally advanced RC experience complete tumor regression after up-front RT. The value of radical surgery in these patients is increasingly challenged, and selected patients in this group could instead be offered organ-preserving treatment, such as local excision or a watch-and-wait approach. A model predictive of the RT response, applied at the pretreatment stage, will be critical to personalizing RC treatment and would facilitate organ preservation, perhaps even in patients for whom up-front RT would not otherwise be routinely considered. Molecular biomarker discovery offers the strongest opportunity for development of a radiation response predictive model. However, the inherent complexities of radiation oncobiology, coupled with the vast heterogeneity of existing works in the literature, have made it difficult to precisely define the molecular drivers of radiosensitivity. In the present study, we sought to present the RC radiation response molecular landscape as holistically as possible; we used a GO approach to group biomarkers together according to biological commonality for ease of interpretation. A more targeted search for relevant biomarkers in this context is essential, and as this work appears to demonstrate, there are several key areas that appear to be showing genuine promise and others—perhaps surprisingly—that do not.

Insofar as analyzing a heterogeneous landscape of biomarkers, the generation of a knowledge-based GO network is a concise way of displaying data acquired from a comprehensive systematic review while providing both an appreciation of the statistical analyses of the original studies and insight into potential overall biological impact. We believe the work presented here is the first such analysis of its type and offers an intuitive method of visually appraising statistical data gathered from published experimental work by mapping identified biomarkers using GO terms. One benefit of such analysis is that it can take into account all published data, including nonsignificant findings, and can aid in identifying gaps in knowledge and potential targets for future work. We have shown that by clustering biomarkers according to GO terms and integrating them into a network analysis based on biological function, the ontologies under "stress response" and "cellular metabolism" appear to be of greatest significance. Conversely, biomarkers of cell-cycle regulation and progression have been found to be of low relative significance despite large numbers of studies. The work presented here should allow for more targeted direction of enquiry for future RT biomarker discovery studies, and the biostatistical methodology we used will undoubtedly be applicable to other clinical questions in which a biomarker-driven end point is the ultimate aim.

The data presented herein are subject to a number of inherent limitations. Although the methodology used has sought to mitigate the impact of these, it is important to highlight them. First, it is possible that subtle differences exist in terms of the molecular mechanisms that govern response to SCRT (25 Gy, 5 Gy in 5 fractions) and LCCR (50.4 Gy, 1.8 Gy in 28 fractions), and studies evaluating both approaches have been included in the present review. Unfortunately, the current literature on biomarkers in RC radiation response does not permit these distinctions to be defined. However, despite differences in timing and fractionation, the recent Stockholm III RCT demonstrated equivalent oncologic outcomes after SCRT and immediate surgery, SCRT and delayed surgery, and LCCR. This suggests that in spite of methodologic variability, different approaches lead to relatively homogeneous oncologic end points, which most likely is because the central constituent of each
approach is pelvic RT. We suggest that this fundamental radiobiological commonality will compensate for differences in approach, and therefore all eligible studies evaluating LCCR \( (n = 67) \), SCRT \( (n = 5) \), and a combination of these \( (n = 2) \) were included in the present study. Finally, multiple tumour regression grading systems were used across studies, and this lack of congruence may lead to difficulties in interpreting data (Table 3).

In the context of LCCR, 5-FU–based chemotherapy was almost universally used for radiosensitization, combined with fractionated long-course RT. However, we acknowledge that a minority of included studies also included use of oxaliplatin and/or leucovorin as induction agents.\(^{10,22,24,30,35,37,57,64}\) Although we appreciate that this represents a further source of data heterogeneity, it is interesting to note that a number of the biomarkers assessed in these papers (eg, EGFR, Smac, and Ki-67) demonstrated congruent results with other studies where 5-FU alone was used as the radiation sensitizer.\(^{22,24,35,57}\) In this context, we believe that the data were sufficiently comparable for collective inclusion in the present review.

Although previous studies have suggested that radiation response per se is not a reliable surrogate endpoint in recent clinical trials, we would argue that the development of reliable methods with which to predict radiation response will allow more precise treatment planning in the future for locally advanced RC.\(^{103,104}\) It is feasible to surmise that were a favorable phenotype to be revealed through biomolecular profiling, an individual would be offered pelvic RT followed by radical surgery (conventional practice) or organ-preserving treatment (in the case of a complete response). In the patient in whom an unfavorable phenotype (ie, disease likely to be poorly responsive or nonresponsive to RT) is found, a different treatment approach would be required, potentially involving either beyond total mesorectal excision surgery (without preoperative therapy) or neoadjuvant chemoradiotherapy, which is the subject of the ongoing Prospect multicenter RCT (ClinicalTrials.gov NCT01515787). Furthermore, it is important to acknowledge that the clinical landscape is evolving in RC; the introduction of watch-and-wait strategies, given our currently limited understanding of the biology underpinning radiation response, is somewhat controversial. The ability to robustly predict response to RT based on tumor biology will be an essential stratifier to guide patient selection for organ-preserving treatment. The final point to consider in terms of the value of radiation response characterization is the potential to modulate radiation response once the key molecular drivers are defined.

In conclusion, what has been demonstrated by this work is that the goal of a single biomarker remains both out of reach and unrealistic based on current evidence. However, as for the implications of radiation response biomarkers going forward, one can posit a number of clinical end points. Foremost, as debates on watch and wait and selection of optimal up-front therapy continues (chemoradiation vs. chemotherapy), the ability to accurately predict radiation response (and more importantly to be able to adequately survey the patient in a longitudinal fashion) would solidify the oncobiological credibility of these approaches. For practical purposes, this would obviously require a clinically translatable, bedside, or office-based test (rather than tissue biopsy), and work would need to be guided to establish how tissue-based biomarkers may be reflected in a more readily accessible liquid biopsy source, be that from stool, blood, or urine.

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**Disclosure**

The authors have stated that they have no conflict of interest.

**Supplemental Data**

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