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REVIEW

Genotypic characteristics of resistant tumors to pre-operative ionizing radiation in rectal cancer

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Abstract

Due to a wide range of clinical response in patients undergoing neo-adjuvant chemoradiation for rectal cancer it is essential to understand molecular factors that lead to the broad response observed in patients receiving the same form of treatment. Despite extensive research in this field, the exact mechanisms still remain elusive. Data raging from DNA-repair to specific molecules leading to cell survival as well as resistance to apoptosis have been investigated. Individually, or in combination, there is no single pathway that has become clinically applicable to date. In the following review, we describe the current status of various pathways that might lead to resistance to the therapeutic applications of ionizing radiation in rectal cancer.

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Key words: Ionizing radiation; DNA double-strand break; Non-homologous end-joining pathway; DNA-PKcs; Ku proteins; Complete pathological response; Radiation therapy; Apoptosis; Angiogenesis

Core tip: Treatment of locally advanced rectal cancer stage Ⅱ and Ⅲ includes neoadjuvant chemo-radiation followed by surgery if clinically feasible. A strategy of observing patients without an operation has been proposed by some surgeons, but this is still the center of much debate. Moreover, the therapeutic effect of ionizing radiation in treatment of rectal cancer varies significantly from one person to another. This has led investigators to identify the molecular targets and pathways in rectal tumors resistant to ionizing radiation in a bid to improve the therapeutic effect of radiation by advanced biomedical and genetic engineering.

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INTRODUCTION

There are approximately 40340 patients diagnosed with rectal cancer annually in United States[1]. Cancer of the colon and rectum combined claimed 51690 deaths in 2012[1]. Rectal cancer, though staged similarly to colon cancer, is managed differently due to the pelvic location of the rectum. The rectum is in close proximity to the urogenital organs and anal sphincters. Hence, surgery for rectal cancer is associated with complications ranging from 15% to 70%[2]. Moreover, many patients will have local as well as distant metastasis during post-op surveillance[3,4]. Hence, careful and methodical planning

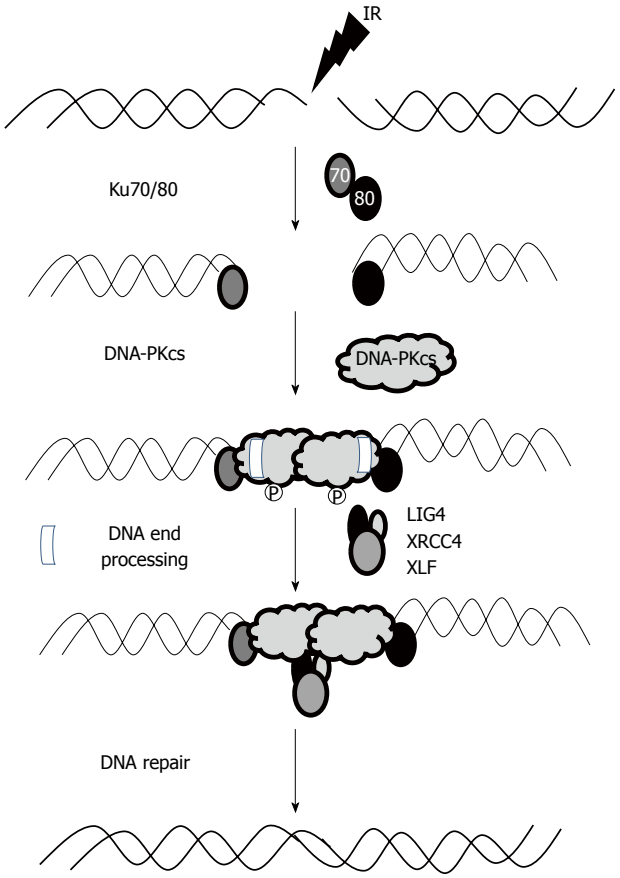

Figure 1 Schematic representations of double-strand break repair by non-homologous end-joining mechanism. The KU proteins are the initial participants in this process as they rapidly bind to broken DNA segments. Another major function of the KU proteins is the active recruitment of DNA-PKcs. DNA-PK activation assists with the recruitment of other proteins involved in the limited DNAend-processing (Artemis, pol m, pol l, and TDK) required to generate ligatable DNA ends. Ligation is mediated by the LIG4/XRCC4 complex and is assisted by the ligation mediator XLF. Once this process is completed, DNA integrity is maintained.

is required to avoid unnecessary surgery with potential short and long term complications. Recent studies have underscored the importance of ionizing radiation (as neoadjuvant therapy) in patients with stage Ⅱ and Ⅲ rectal cancer. There are many benefits to the use of IR in the neoadjuvant compared to the adjuvant setting[5]. Additionally, in some cases, this approach allows the tumors to be down-staged resulting in complete pathological response (pCR, *i.e.*, complete obliteration of the tumor following preoperative chemoradiation at laparotomy) or complete clinical response (cCR, *i.e.*, complete obliteration of the tumor following preoperative chemoradiation during repeat colonoscopy or other diagnostic modalities such as MRI).

However, the benefit from preoperative radiation varies significantly in trials with a substantially wide pCR (9%-37%)[6-10]. Patients who achieve a pCR have better outcomes compared to patients who do not[11]. Some surgeons have elected a watchful waiting approach for patients who achieve cCR[12-17].

The logical clinical and pre-clinical question is to devise methods by which we can personalize treatment for rectal cancer, such that the most effective therapy with the least side effect profile can be offered consistently to patients affected by rectal cancer. In order to achieve this objective, extensive research has been performed over the last few decades to identify biological markers and genetic phenotypes that can predict successful response to radiation and translate into improved survival. We present a review of the current status of these markers.

THE THERAPEUTIC EFFECTS OF IR

The NHEJ pathway of DNA repair

The therapeutic effect of IR is largely the result of double stranded DNA breaks that result from IR-induced DNA damage. DNA breaks are difficult to repair and typically result in apoptosis. DNA double-strand break (DSB) can be repaired by one of the following three pathways: homologous recombination[18], non-homologous end-joining (NHEJ) pathway, or an alternate NHEJ pathway (characterized by larger deletions and translocations)[19]. The details behind the selection and execution of these pathways are not entirely clear, but it seems that NHEJ is the major pathway as it is the only one that occurs in all stages of cell cycle.

The NHEJ pathway is essential for DSB repair and is also important for V (D) J recombination during T and B cell lymphocyte development. The catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) is an integral part of the NHEJ pathway. The actual mechanism of this pathway is rather complex (Figure 1), but can be broadly classified into three steps. In the first phase, Ku 70/80 heterodimer identifies DSB, facilitates the activation and recruitment of DNA-PKcs, and then ties the DNA ends in a synaptic complex[20]. The next step involves enzymatic processing of the DNA ends followed by ligation (by DNA ligase Ⅳ) in the last phase. The order and timing of this sequence of events is not well defined; however, it is widely regarded that Ku 70/80 protein is the most important and integral part of this sequence as it recruits DNA-PKcs as well as interacts with a host of other important proteins. Moreover, Ku has lyase activity allowing it to process DNA ends during NHEJ[21].

Following successful DNA repair, the cell might undergo back to the normal cell cycle. If some error occurs during the repair, the cell might undergo genomic instability and if the cell is unable to repair the radiation-induced damage, it undergoes apoptosis (Figure 2)[22]. Thus, a logical place to begin investigating marker of radioresistance is by interrogating the NHEJ pathway of DNA repair in cancer cells.

Role of DNA-PKcs

DNA-PKcs has multiple roles in DNA repair and carcinogenesis. DNA-PKcs facilitates DSB repair, thus ensuring stability and integrity of genetic chromosomes. Hence, low levels of DNA-PKcs might result in muta-

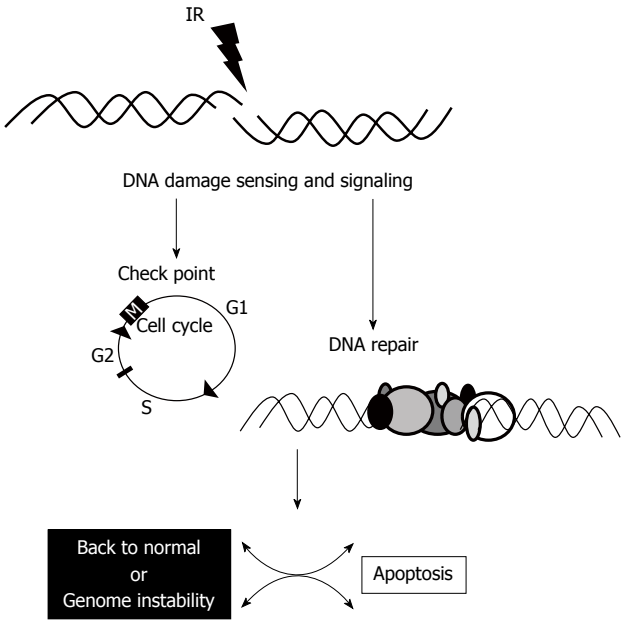

Figure 2 Schematic representation of the events that occur following IR-induced DNA damage. Sensing mechanisms and signaling first stop the cell cycle that allows the cell to repair the DNA damage. If unsuccessful, apoptosis ensues. If the repair is nearly complete, the cell might continue to replicate with genome instability.

tions promulgating the cascade of carcinogenesis. A cell with low levels of DNA-PKcs might be unable to repair the DNA damage incurred by IR and destine the cell for apoptosis. In this scenario, low levels of DNA-PKcs should be a surrogate for radiosensitivity.

On the other hand, cancer cells might contain higher DNA-PKcs levels induced by the rapid cell turnover. In this scenario, increases in DNA-PKcs activity will enhance cancer cell resistance and decrease susceptibility to chemotherapy and ionizing radiation[23-26].

Pre-clinical studies have demonstrated that DNA-PKcs deficient Chinese hamster ovary cells showed profound cell death following treatment with IR compared to the DNA-PKcs complimented V3-YAC cells[27]. Colon cancer HCT-116 DNA-PKcs$^{-/-}$ cells and xenografts were exquisitely sensitive to IR[28,29]. Unfortunately, the role of DNA-PKcs activity in development of various cancers has been investigated in multiple studies and has shown conflicting results in carcinogenesis as well as being a poor predictor of a response to IR, but more data is needed in this area (Table 1).

Significant increases of DNA-PKcs activity have been observed in certain gastrointestinal cancers such as colorectal cancer[30,31], esophageal cancer[32], nasopharyngeal cancer, and non-small cell lung cancer[33]. Conversely, loss of DNA-PKcs expression has been linked to gastric tumors correlating with signs of invasion and poor survival[34,35].

Levels of DNA-PKcs in cancer cells before treatment (radiation or chemotherapy) has been compared to levels after treatment, and have shown mixed results. The expression of DNA-PKcs was noted to be directly proportional to a favorable response with radiation in esophageal and early breast cancer but not in nasopharyngeal cancer[36-38]. On the other hand, studies have revealed increased levels of DNA-PKcs and Ku proteins in residual tumors after radiation treatment, suggesting a means of survival and a marker of radioresistance in recurrent tumors[39].

While the cellular status of the DNA-PKcs as a predictor of IR remains to be investigated, DNA-PKcs inhibition might have a therapeutic role in rectal cancer. Pre-clinical studies showed that pharmacological inhibition of DNA-PKcs led to substantial chemo- and radiosensitization[27,40-42]. The effect of DNA- PKcs inhibitors has been examined in mouse xenograft tumor models with favorable results. There has been significant tumor growth delay and improved survival in mice treated with combined DNA-PKcs inhibition and ionizing radiation. The combination treatment reduces levels of cell proliferation marker Ki67 and increases activity of certain proteins known for its anti-tumor properties[43,44].

Inhibitors of DNA-PKcs have been shown to have a synergistic effect along with cisplatinum/platinum based drugs in treatment of ovarian, colon, and breast cancer[45-47]. Multiple DNA-PKcs kinase activity inhibitors are not only in various stages of development but a few are being tested in clinical trials (Table 2). Similarly, new *in vivo* substrates of DNA-dependent protein kinase (Akt1/PKBa, Hsp90a, NR4A[48-52]), which can be induced by ionizing radiation have been identified.

Furthermore, additional DNA-PKcs inhibitors have been developed such as anti-DNA-PKcs ScFv 18-2 (derived from an existing anti-DNA PKcs monoclonal antibody)[53], and anti-DPK3-scFv (selected from a humanized semi-synthetic scFV library)[44]. These anti-DNA PKcs sensitize cells to radiation induced injury[44,54,55] in a similar fashion to RNA inhibition of DNA-PKcs transcripts[56-58].

The interaction between epidermal growth factor receptor (EGFR) and the DNA-PKcs has also been explored. This interaction is required for radiation induced nuclear AKT phosphorylation and cell survival[52,59,60]. Similarly, blockage of EGFR signaling pathway with a monoclonal antibody can inhibit DNA-PKcs activation and thereby decrease DNA repair capacity. This could enhance sensitization and susceptibility of cells to ionizing radiation[61,62].

Clinically, deficiency in DNA-PK activity led to sensitivity to nitrogen mustards in patients with chronic lymphocytic leukemia[25]. The drug 2-N-morpholino-8-dibenzothiophenyl-chromen-4-one (NU7441) is a potent and specific DNA-PK inhibitor[63]. Treatment with NU7441 and topoisomerase inhibitors combined with IR caused potent chemo-radio sensitization in SW620 colorectal cancer cells as well as xenografts[27]. The various mechanisms by which DNA-PKcs inhibitors facilitate radiation induced death include apoptosis[64,65], acceleration of senescence, induction of mitotic catastrophe, and autophagy[43,66,67].

Studies evaluating expression of DNA-PKcs in pe-

Table 1 Association between DNA-PKcs activity and cancer development from clinical investigations

Tumor type	Assay	Specimen	Sample size	DNA-PKcs activity	Interpretation
Nasopharyngeal cancer	IHC	Tumor	66	↑ in 70% of tumor tissue	No association with locoregional control and survival
Nasopharyngeal cancer	IHC	Tumor	223	↑ in 37% of tumor tissue	Overexpression associated with advanced stage and poor survival
Esophageal cancer	IHC, IB, Kinase activity	Tumor, normal	13 paired	↑ in tumor tissue	NA
Gastric cancer	IHC	Tumor	279	↑ in 73% of tumor tissue	Loss of expression associated with lymphatic invasion, lymph node metastasis, advanced pathological stage, and poor survival
Gastric cancer	IHC	Tumor, normal	791	↑ in 80% of tumor tissue	Loss of expression associated with intratumoral neutrophils, microsatellite instability, mutations in DNA-PKcs and poor survival
Colorectal cancer	RT-PCR, IB, kinase activity	Tumor, normal	12 paired	↑ in tumor tissue	NA
Colorectal cancer	IHC, IB	Tumor, normal	359 (35 paired)	↑ in 64% of tumor tissue	Overexpression associated with clinical stage, lymphatic invasion, distant metastasis and poor survival
Non-small cell lung cancer	IHC	Tumor	113	↑ in 89% of tumor tissue	Overexpression associated with tumor grade
Non-small cell lung cancer	IHC	Tumor	86	↑ in 87% of tumor tissue	No association with clinical characteristics or outcome
Non-small cell lung cancer	RT-PCR	Tumor, normal	140 paired	↑ in tumor tissue	Overexpression associated with poor survival
Non-small cell lung cancer	IHC	Tumor, normal	116 (12 paired)	↑ in 75% of tumor tissue	No association with clinical characteristics or outcome
Glioma	Kinase activity	Tumor	36	↑ in tumor tissue	Hyperactivity correlates with rumor grading
Ovarian cancer	IHC	Tumor, normal	100	↓ in 40% of tumor tissue	loss of expression associated with tumor progression, advanced clincal stage, and lymph node metastasis
ALL, CLL, lymphoma, multiple myeloma	IHC, IB	Lymphoid tissue	86	↑ During lymphoid development and in lymphoid malignancies	Overexpression associated with higher lymphoma grading and degree of maturation in lymphoid malignancies other than multiple myeloma
B-cell CLL	IB, kinase activity	Lukemia cells	54	↑ in del(17p) and del(11q)	Overexpression associated with shorter treatment free interval
B-cell CLL	RT-PCR	Lukemia cells	50	↑ in del(17p)	Overexpression associated with poor survival
Cancer of breast, cervix, head and neck esophageal and lymphoma	Kinase activity	PBLs	167	↓ in advanced stage	Hypoactivity associated with advanced stage and distant metastasis
Radiation response					
Esophageal cancer	IHC	Tumor	67	↑ in 54% of tumor tissue	Overexpression predicts better response to chemoradiation
Oral squamous cell carcinoma	IHC	Tumor	42	↑ in residual tumor after RT	Not predictive of radiation response
Cervical cancer	IHC	Tumor	22	↑ in residual tumor after RT	No association with clinical characteristics
Breast cancer	IHC	Tumor	224	↑ in 43% of tumor tissue	Overexpression predicts better locoregional control of radiation alone versus chemotherapy alone in early stage
Cancer risk					
Lung cancer	Kinase activity	PBLs	Cancer 41/healthy 41	↓ in cancer patients	Hypoactivity associated with cancer of the lung
Breast, cervix, head and neck, esophagus and lymphoma	Kinase activity	PBLs	Cancer 93/healthy 41	↓ in cancer patients	Hypoactivity associated with chromosomal instability and cancer of breast and cervix

Adapted with permission[148]. ALL: Acute lymphocytic leukemia; CLL: Chronic lymphocytic leukemia; IHC: Immunohistochemistry; PBLs: Peripheral blood lymphocytes; RT-PCR: Reverse transcription polymerase chain reaction; ↑: Indicates increase activity; ↓: Indicates decrease activity.

ripheral blood lymphocytes (PBLs) as a marker of host immunity and cancer development have shown an additional role in cancer development as it relates to host immunity. Data from multiple studies demonstrated that cancer patients have a lower level of DNA-PKcs activity in PBLs[23,68,69], suggesting impaired ability to recognize cancer cells leading to a poor prognosis. Whether this is mediated by activation of natural killer (NK) cells or release of pro-inflammatory cytokines is not clearly understood[70]. Destruction of NK cells leading to increases

Table 2 Non-homologous end-joining inhibitors

Inhibitor	Mechanism/comments
A12B4C3	PNKP inhibitor, sensitizes cells to camptothecin
BTW3	A small peptide DNA-PK inhibitor, proposed to compete for DNA-PKcs autophosphorylation
KU0060648	DNA-PK and P13K inhibitor
NU7441/KU57788	DNA-PK inhibitor, competitive with ATP
ScFv 18-2	An antibody-derived DNA-PK inhibitor that can bind to an epitope unique to DNA-PKcs
ZSTK474	DNA-PK and P13K inhibitor, competitive with ATP; in phase 1 clinical trials (NCT01280487 and NCT01682473)
CC-115	Dual inhibitor of DNA-PKcs and mTOR, in phase 1 clinical trials
CC-122	DNA-PK inhibitor, in phase 1 clinical trials

Reprinted with permission from Elsevier[149]. P13K: Phosphatidyl inositol 3 kinase.

in spontaneous tumor development in mouse models[71] leans in favor to the former hypothesis. Moreover, an inverse association between DNA-PKcs activity in PBLs and stage of cancer was also observed in patients who were treated with radiotherapy for advanced cancer, displaying poorer prognosis and higher frequency of distant metastasis[68].

In addition to its role in NHEJ pathway, DNA-PKcs regulates the DNA damage repair mechanisms by a variety of mechanisms. These include DNA interstrand crosslink (ICL) repair[72,73], AKT activation, EGFR nuclear translocation, or activation/mobilization of chromatin remodeling factor structure-specific recognition protein 1 (SSRP1) from nucleolus[60,74,75]. Biomedical engineering aiming to mimic some of the activities of the DNA-PKcs has been instrumental in developing novel agents that might be useful for cancer therapeutics.

It is clear that the status of the DNA-PKcs plays a fundamental role in ionizing radiation-induced cell death. Many aspects of its role in cancer therapeutics are currently under investigation. In rectal cancer, the role of DNA-PKcs is still in its infancy. As markers of a response to ionizing radiation, the role of the DNA-PKcs is complicated by the fact that there is paucity of high quality data. In rectal cancer, our group demonstrated counter-intuitive results with regards to the role of DNA-PKcs in the response to IR (discussed below). In prostate cancer, nuclear positivity for DNA-PKcs was associated with chemical recurrence[76]. Further studies are required to shed more light into these issues.

The Ku proteins

Ku70 and Ku80 proteins are essential components of the NHEJ pathway. These proteins serve as a medium by which multiple other DNA-repair proteins can be attached to the pathway cascade[77]. Importantly, the Ku proteins have a high affinity for broken DNA strands and rapidly bind to them. This initial process also recruits DNA-PKcs for DNA repair, though the exact mechanism is still unknown[78,79]. Additionally, Ku proteins play a major role in recruitment of XRCC4[80,81], XLF[82], APLF (APTX and PNK-like factor)[83] to DSBs helping with the repair process and promoting NHEJ. Moreover, Ku has the ability to enzymatically process DNA ends during NHEJ using the 5'-deoxyribose-5-phosphate (5'-dRP)/AP lyase activity[21]. Ku also excises abasic sites near DSBs suggesting a potential role in repairing damage by IR[21].

Intuitively, tumors that express high levels of Ku proteins should be able to repair the damage induced by IR more efficiently and thus become more resistant to therapy. *In vitro* studies have failed to show an association between the Ku proteins and radiosensitivity[84]. *Ex vivo* studies have also interrogated the role of the Ku proteins as surrogates of a response to IR.

Lack of Ku70 immunoreactivity correlated with radiosensitivity in patients with carcinoma of the cervix. In these patients, survival was better in tumors that had lower nuclear expression of Ku70[84]. In squamous cell carcinoma of the head and neck, Ku80 over expression was an independent predictor of regional recurrence and mortality in patient treated with IR[85]. Similarly, in rectal cancer low levels of Ku70 and Ku80 were associated with pCR. Ku70 was associated with down-staging. Disease free survival was 42% in patients with high Ku70 expression compared to 78% in patients with low expression of the same protein. Similar results were observed for Ku80[86]. Elevated levels of Ku proteins occur in high grade lymphoid malignancies[87]. The Ku70/Ku80 heterodimer DNA end-binding activity was 2- to 3-fold higher in the resistant B-CLL cell subset compared with the sensitive B-CLL cell subset[88], highlighting a possible mechanism behind increased DNA-PKcs activity in resistant CLL cells. The authors showed that novel DNA-dependent protein kinase (DNA-PK) inhibitor, NU7026 (2-(morpholin-4-yl)-benzo[h]chomen-4-one), and the phosphatidylinositol 3 (PI-3) kinase inhibitor, wortmannin, restored sensitivity to DNA damage-induced apoptosis of otherwise resistant cells.

Ku proteins can be upregulated after radiation treatment[39,89]. In one such study, expression of DNA-PK complex proteins (including Ku 70 proteins) increased after radiation treatment in residual tumors, and the increased values correlated with the tumor radiation resistance[89]. Various mechanisms have been postulated behind the role of Ku proteins in radioresistance. A distinct cell-interdependent signal is conveyed through gap junctions during chemotherapy with cisplatin, mediated by

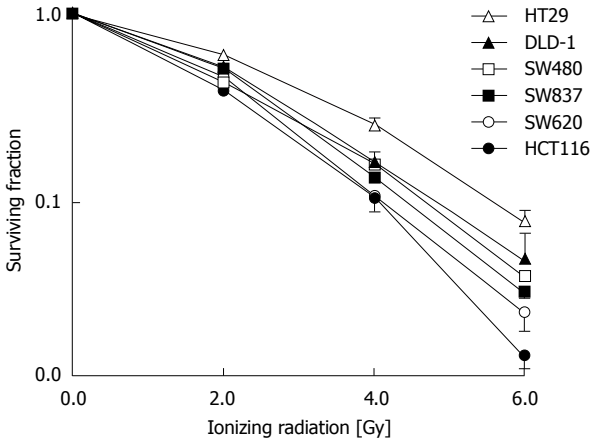

Figure 3 Response to ionizing radiation in several colorectal cancer cell lines subjected to various doses of ionizing radiation. There is a variable response to the same doses or ionizing radiation (Gy).

the kinase function of Ku70, Ku80 and DNA-dependent protein kinase complex. This communication may explain the resistance to cisplatin-induced death of cancer cells[90]. It is also possible that the role of Ku proteins and DNA-PKcs in DNA damage repair depends upon the extent and complexity of damage by IR. Studies have revealed that simple DSBs induced by laser irradiation are repaired rapidly involving Ku70/80 and XRCC4/Ligase IV/XLF. In contrast, DSBs with greater chemical complexity are repaired slowly and requires additional use of DNA-PKcs[91].

While these data seem compelling, more research is required prior to establishing the role of the Ku proteins in a response to radiation in rectal cancer. Current data on this subject, while promising, is currently limited and not clinically available. In rectal cancer, our group demonstrated counter-intuitive results with regards to the role of DNA-PKcs in the response to IR (discussed below).

ANALYSIS OF GENOTYPIC ORIGINS OF RADIORESISTANCE *IN VITRO* AND *IN VIVO* MODELS OF RECTAL CANCER

Examination of factors leading to radioresistance can practically be approached *in vitro*. Analysis of five colon cancer cell lines (HT29, DLD-1, SW480, SW620, and HCT116) as well as one rectal cancer cell line (SW837) have demonstrated a similar pattern of response to a group of patients treated for rectal cancer with pre-operative IR (Figure 3)[92]. The cell lines that have been treated with IR and examined originate from patients with different characteristics.

SW480 cells were derived from a primary Duke's stage B colon adenocarcinoma from a 50-year-old Caucasian male, while the SW620 cell line was cultured from a lymph node metastasis from the same patient at a later time. The DLD-1 cell line was established from an adult male with adenocarcinoma of the colon. The SW837 cell line was derived from a 53-year-old Caucasian male with rectal cancer. HCT-116 cells were cultured from an adult male with colon cancer. HT-29 cells were derived from a 44-year-old Caucasian woman with colorectal adenocarcinoma. All of these cells have mutations of the p53 gene, except for HCT-116 cells (p53-Wt). HT-29 cell have mutations of both alleles of the p53 gene (p53-null)[92]. HCT-116 cells display microsatellite instability.

These cells have been extensively studied and a number of properties are known. Analysis of these factors and a response to IR has not yielded any uniform patter of predictability that could be surrogate markers in *ex vivo* studies. For instance, the inhibitor of apoptosis, survivin, has been shown to play a significant role in resistance to IR (discussed below)[93]. Analysis of this model of rectal cancer *in vitro* (Figure 3) has not consistently corroborated this finding. For instance, survivin was expressed in higher levels in the radiosensitive SW620 compared to the relative more radioresistant SW480 cell line. Interestingly, these two cells originated from the same patient one at the time of stage Ⅱ colon cancer (SW480) and the second one from a lymph node metastasis (SW620) such that these two cell lines contain similar genetic background.

Analysis of these cell lines is representative of the response that was observed in 117 patients who were treated with preoperative ionizing radiation and underwent surgical resection (Figure 4). A pivotal question is to determine what causes these differences in patients and cell lines receiving the same treatment. A simple approach in the laboratory is to take the more radioresistant and the more radiosensitive cells and analyze specific differences. This approach has been undertaken *in vitro* and *in vivo*. HCT-116 cell and xenografts are substantially more sensitive to IR compared to HT-29 cells and xenografts (Figure 5).

DNA repair in this model

Analysis of DNA induced damage (by γH2AX) indicated that the radioresistant HCT-116 cells suffer more DNA damage when exposed to IR and that this damage persists over time indicating a poor ability of the cells to repair the DNA affected by IR (Figure 6)[94]. Predictably, HT-29 cells should be able to repair DNA more effectively and should have increased levels of DNA-PKcs and Ku proteins. In fact, the opposite results have been observed in our studies. Our results showed that compared to HCT-116 cells, HT-29 cells expressed lower levels of DNA-PKcs and Ku proteins[95].

Cell cycle kinetics in this model

Examination of cell cycle kinetics demonstrates that the radiosensitive HCT-116 cells substantially accumulate in the G-2 phase of the cell cycle. HT-29 cells proceed through the cell cycle in spite of receiving the same dose of IR (Figure 7)[22,28,92,94,96,97]. According to these observations, there should be differences in cell cycle regulators and apoptotic factors that could be used to predict a response to IR.

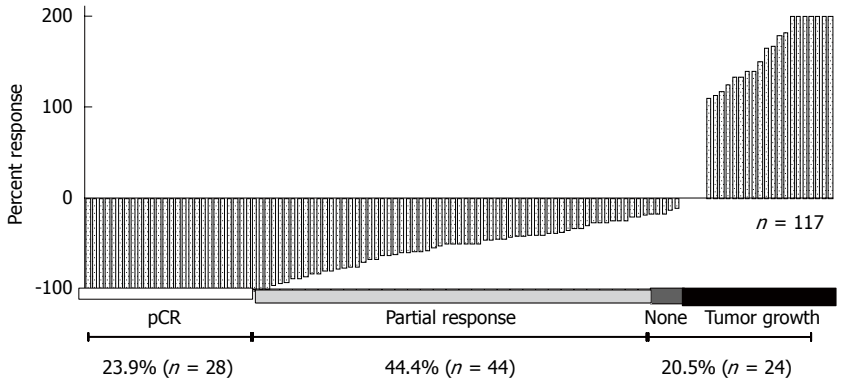

Figure 4 There is a high variability of a response to ionizing radiation in rectal cancer patients treated with pre-operative ionizing radiation. Each bar on the X-axis represents an individual patient. The Y-axis represents the clinical response to pre-operative ionizing radiation. Nearly one fourth of patients achieve a pCR, but close to another fourth do not respond to the same form of treatment, while the rest of patients have achieve a partial response.

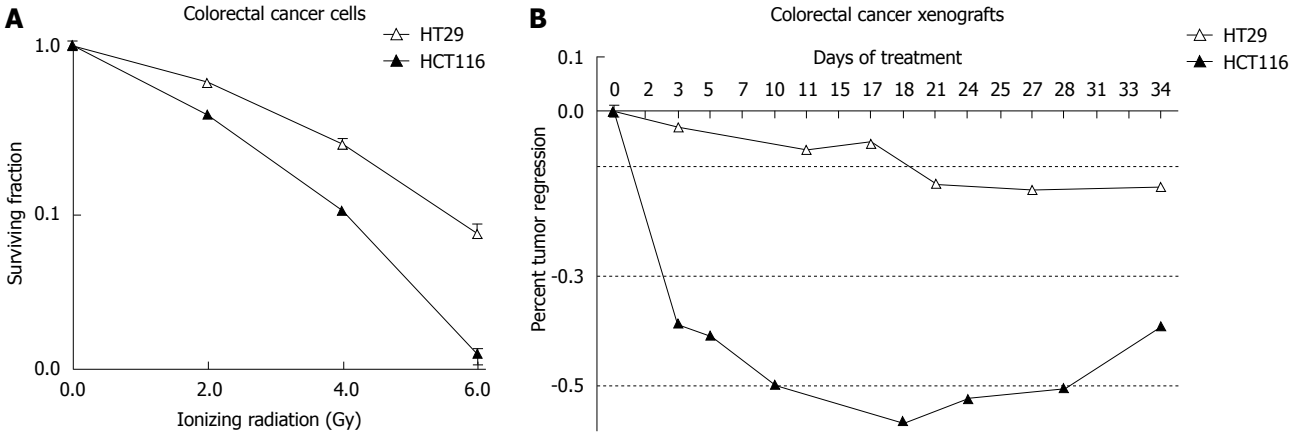

Figure 5 Analysis of the most radiosensitive (HCT116) and the most radioresistant (HT29) cells (A), a similar response has been noted in cells implanted in immune compromised mice bearing xenografts of these cells (B).

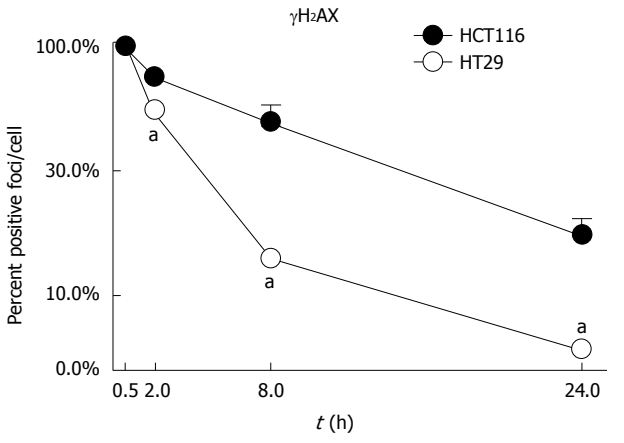

Figure 6 Analysis of DNA-induced damaged by ionizing radiation as determined by γH2AX. HCT116 cells undergo more pronounced damage when treated with the same dose of ionizing radiation compared to HT29 cells. The damage induced in HCT116 cells persists over time. [a]$P < 0.05$ *vs* HCT116.

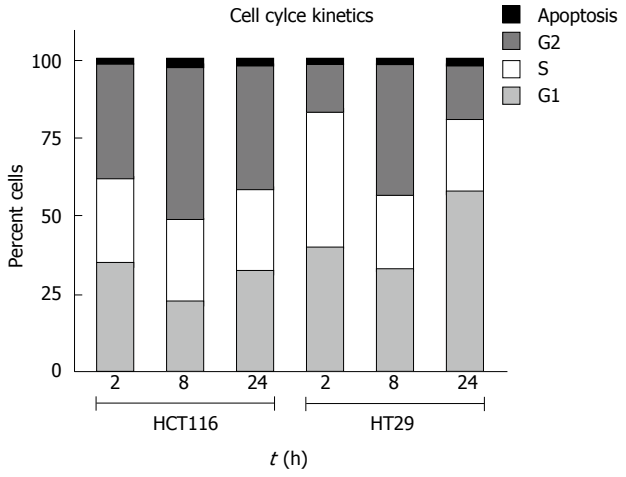

Figure 7 Cell cycle kinetics of HCT116 and HT29 cells treated with 2.0 Gy ionizing radiation. There is a pronounced accumulation of cells in G2 in HCT116 cells. HT29 cells continue through the cell cycle in spite of receiving the same dose of ionizing radiation.

Apoptosis in this model

Analysis of this model with regards to the central mediators of apoptosis (as depicted in Figure 8) has demonstrated the following in HCT116 (*vs* HT29 cells): marked over expression of p21, decreased expression of p53, Bax, Bcl-2 and survivin[92]. Examination of these findings is intuitive in some areas while counterintuitive in oth-

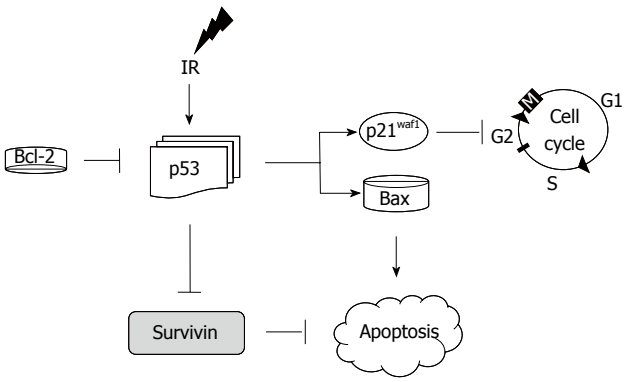

Figure 8 Schematic representation of molecular events following the cellular response to ionizing radiation-induced damage. Ionizing radiation causes an up-regulation of p53. p53 then directly activates the cyclin dependent kinase inhibitor p21. Cell cycle progression stops until the cell repairs the damaged induced by ionizing radiation. If the cell is unable to repair itself, it undergoes apoptosis. Bcl-2 inhibits p53 up-regulation, while p53 inhibits the inhibitor of apoptosis: survivin.

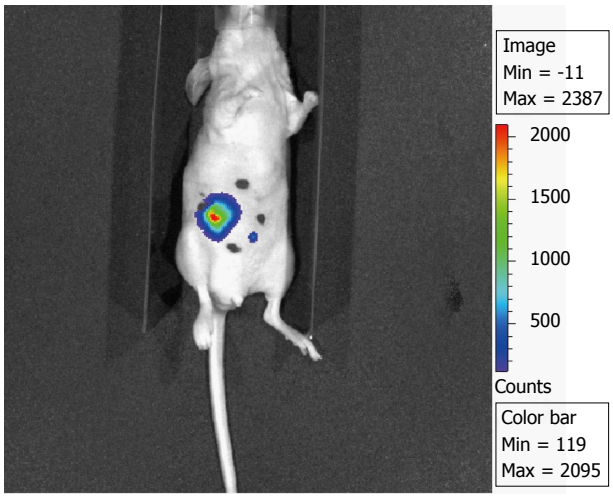

Figure 9 Orthotopic model for the study of rectal cancer. This model has the following characteristics: (1) cecal transplantation of tumors with a known response to ionizing radiation; (2) attachment of the cecum to the lateral abdominal wall with a permanent suture for the administration of ionizing radiation; and (3) transfection of cells with luciferase before tumor implantation for the assessment of the chemoradiotherapeutic interventions over time by bioluminescence imaging before the end of the study. This technique allows targeted delivery of ionizing radiation in an intraperitoneal tumor.

ers. For instance, p21 elevation in response to IR is an expected response of these radiosensitive cells. This was associated with an appropriate response of p53 leading to activation of p21 culminating in apoptosis as demonstrated by an elevation of the cleaved PARP-1. In HT29 cells, on the other hand, p53 was markedly elevated. This is the result of the mutated status of p53 in HT29 cells. However, the results with regards to Bax and survivin are not clear in these experiments as a decrease in survivin and Bax was expected in these radioresistant cells.

In separate *in vitro* studies, analysis with colorectal cancer cells with stable knock out (KO) of genes responsible for apoptosis from IR-induced injury was undertaken. This demonstrated that the p21 and the Bax KO genotypes were associated with radiosensitivity rather than radioresistance (Figure 6)[28]. The results with regards to p21 have been previously reported and indicate that it is mitotic catastrophe that leads these cells to undergo cellular death rather than becoming more radioresistant. The Bax KO genotype leading to a more radiosensitive phenotype as opposed to radioresistance was partly mediated by apoptosis inducing factor (AIF) and not to caspase mediated apoptosis[28]. AIF is an important mediator of cellular death that requires further studies as a predictor of a response to IR in rectal cancer[98].

These observations *in vitro* have been noted *in vivo* models of rectal cancer as well. However, one of the limitations of the studies *in vivo* is that these studies have relied on xenograft models of rectal cancer. We have previously described an orthotropic model in which cells have been implanted in the cecum and then the cecum was secured to the abdominal wall for targeted IR. Because these cells can be labeled with luciferase, the response to IR can be followed over time by biluminescence imaging (Figure 9). However, this model requires further validation[97].

In summary, observations from these studies demonstrate that there are good models for the study of rectal cancer in response to IR *in vitro* and *in vivo*. We have identified some molecules that can be used to predict a response to IR in HT-29 and HCT-116 cells. Application of these factors to the rest of the cells as depicted in Figure 3 has yielded mixed results. There is no unifying pathway that has been identified to date. Moreover, identification of predictors for a response to IR remain at large. For instance, many inhibitors of apoptosis examined (IAPs; survivin, XIAP, cIAP 1/2) were all increased in the more radiosensitive SW620 cells compared to the SW480 cells. Survivin, in response IR in colorectal cancer cells (0, 2, 4, and 6 Gy) was expressed in the following order in several cells: SW620 > HT-29 > HCT-116. Apoptosis was interrogated by PARP-1 cleavage and demonstrated that apoptosis in response to IR occurred in the following pattern: DLD-1 > HCT-116 > SW480 > HT-29 > SW480. p27 demonstrated the following pattern: HT-29 > HCT-116 > SW480. There was no particular pattern of expression of these factors nor was there a correlation to a response to IR noted. Thus, there is further need for identification of a unifying pathway that could be used to determine a response to IR.

The additional advantage of the current *in vitro* and *in vivo* models is that they can be utilized for the study of radiosensitizing agents and some of these have demonstrated promising results[92,94]. The effects of the radiosensitizing agents on specific pathways can also be explored in this fashion.

We then proceeded with a review of literature to determine how these observations compared to other studies. The result of this review have been previously documented to some extent and are presented and updated in the following discussion[22,99].

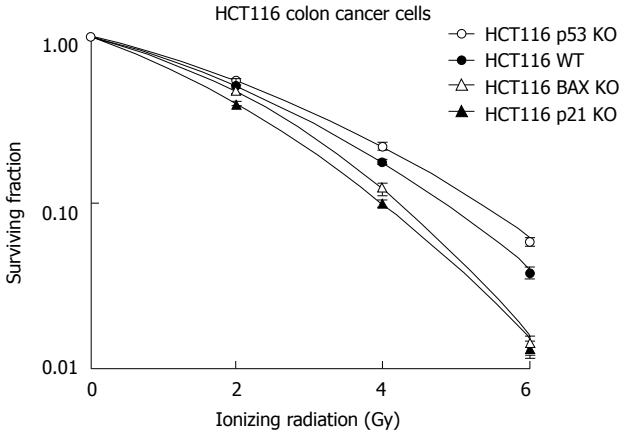

Figure 10 Analysis of HCT116 cells with stable KO genotypes for p53, p21, and Bax compared to wild-type. Cells deficient in p53 are more radioresistant, while p21 and Bax deficient cells are more radiosensitive compared to wild-type.

FACTORS THAT LEAD TO A RESPONSE TO IR: A REVIEW OF THE LITERATURE

Apoptosis

If cells are unable to repair the damage induced by IR, the cell is destined to undergo programmed cell death. In the classical pathway, the stressed cell leads to an up-regulation of p53, which then stops the cell cycle *via* induction of the cyclin depended kinase inhibitor p21. Failure to repair the damage causes BAX to induce apoptosis[22,100] (Figure 8).

It is conceivable that defects in any of these molecules (apoptotic or cell cycle proteins) alone or in combination could serve as a surrogate to predict a response to IR in rectal cancer. *In vitro* studies with colon cancer cells exposed to radiation have been in agreement with the classical response to apoptosis with p53, but not uniformly with p21 and BAX (as discussed in the previous section)[28] (Figure 8).

Apoptotic proteins: p53, p21, BAX, Bcl-2, survivin, and SMAC/Diablo

p53: *In vitro*, HCT-116 cells deficient of p53 are more radioresistant compared to HCT-116 wild-type cells. Tumor xenografts derived from the same cells demonstrated a similar effect[28]. These results have been mirrored in models of colorectal cancer *in vitro* and *in vivo*[101,102], but in disagreement with others[103-105]. Other studies have suggested that p53 mutations may render cells more radiosensitive owing to a reduction in p53-dependent DNA repair mechanisms[106]. Thus, *in vitro* and *in vivo* studies with regards to p53 have shown mixed results. *In vitro*, data indicates that lack of p53 leads to radioresistance. However, the mutational status of p53 is important to consider in all analyses examining p53[22].

Ex vivo studies have demonstrated a number of heterogeneous findings as well. Some studies have shown that mutated p53 leads to radioresistance in rectal cancer tissues[107]. Nuclear expression of p53 in rectal cancers predicted treatment failure and signified resistance to preoperative IR[96]. Other studies have demonstrated no usefulness of p53 as a marker of a response to IR[108,109]. To date, *ex vivo* studies have failed to provide usefulness as a marker of a response to IR. This might be the result of the low number of subjects included in the studies, the wide range of techniques utilized to detect p53, or the ability of the antibody to recognize the mutated *vs* the wild-type form of p53[22].

Cell cycle factors such as p53 and the cyclin dependent kinase inhibitors (CDKIs) (p21 and p27) have been studied as possible candidates to predict a response to ionizing radiation in rectal cancer. p21 is the classical CDKI and is activated by p53[110,111]. Irradiated colon cancer DLD-1 cells expressed low levels of p21[112]. The expected response to IR in cells and tumors deficient of p21 would be a radioresistant phenotype. Recent studies have shown that HCT-116 cell deficient of p21 are, in fact, more sensitive to ionizing radiation compared to wild-type HCT-116 cells[28,113]. Tumor xenografts deficient of p21 demonstrated more tumor regression compared to the wild-type genotype treated with the same dose of ionizing radiation[28].

p21: *Ex vivo* studies demonstrated the p21 positive tumors had a good response to IR[114]. Another study showed that p21 expression correlated with good pathological response and tumor radiosensitivity[115]. Similarly, a reduction by 50% in post-irradiated rectal tissue compared to pre-irradiated one was associated with radioresistance[116]. Another study did not find p21 useful as a predictor of a response to IR[117].

p27: This study found that p27 positive tumors had a better response to IR with an OR of 3.3[117]. Similarly, the absence of p53 and p27 prior to treatment was associated with poor response to IR in rectal tumors[118].

Bax: Bax is a pro-apoptotic protein that leads to the release of cytochrome c from the intermitochondrial membrane[100]. It may be anticipated that Bax deficiency would be associated with radioresistance. *In vitro* and *in vivo* studies have demonstrated the opposite phenotype to IR (Figure 10)[28]. While a few studies demonstrate that Bax deficient cells are resistant to chemotherapeutic agents[119-121], evidence indicating the response of Bax deficient colorectal cancer cells to IR in pre-clinical studies is lacking. Limited *ex vivo* studies have shown that Bax tumor expression had a positive response to chemoradiation in patients treated for rectal cancer[122,123].

Bcl-2 inhibits cellular apoptosis and is overexpressed in many colorectal tumors[124]. BAX is the apoptogenic counter part of Bcl-2. Current studies have failed to demonstrate the association of Bcl-2 as a marker of response to IR[22,123,125].

Survivin: Survivin is one of eight inhibitors of apopto-

sis (IAPs) that are generated *via* induction of NFκB[100]. Survivin binds and inactivates caspases 3, 7 and 9[100]. *In vitro* and *in vivo* data showed that the NFκB-IAPs axis is a predictor of a poor response to IR when over expressed[22]. *Ex vivo* data supports the role of survivin in raidoresistance[93]. Furthermore, the five year survival of patients with survivin positive stage Ⅱ colon cancer tumors was 41% lower than patients with survivin negative tumors[126]. The role of other r IAPs (*i.e.*, XIAP, cIAP, *etc.*) and a response to IR remains at large.

The role of the IAPs in response to IR has been further interrogated by directly inhibiting the inhibition of the IAPs *via* augmentation of an antagonistic factor to the IAPs: SMAC/Diablo.

SMAC/Diablo: Pro-apoptotic molecules with the ability to reduce the functional activity of the inhibitors of apoptosis might have potential therapeutic applications. Compounds that mimic the action of SMAC/Diablo (Smac-mimetics) are under study for their ability to chemo- and radiosensitize tumor cells[127]. The Smac mimetic JP-1201 radiosensitized HT-29 colorectal cancer cells and xenografts by a marked augmentation in apoptosis, which was associated with a reduction in the levels of the IAP XIAP[94].

Proliferation markers and mitotic index as markers: A few studies have reported high Ki-67 staining correlated with a positive response to IR[128,129]. In contrast, most studies have demonstrated that proliferating nuclear antigen labeling index does not correlate with response to IR[115,125,130].

Apoptotic index: Evaluation of apoptosis in cancer cells has shown that patients with higher pre-radiation level of apoptosis (apoptotic index) had lower rate of recurrence and longer disease free period after radiation[131].

Logically, tumors that have an intact machinery to undergo apoptosis should respond better to ionizing radiation rather that those with mutation of one or more pro-apoptotic factors or activation of anti-apoptotic factors. Caspase mediated apoptosis has been shown to play a promising role in predicting a response to IR. A high spontaneous apoptotic index in pretreated tumor tissue was associated with a superior rate of response to radiation[132]. Furthermore, in a large study including 465 pre-irradiated biopsies tumors underwent immunohistochemistry staining against the active form of caspase 3. This study showed that tumors with a high apoptotic index had less recurrence and a higher disease free survival[131].

While these results seem promising, uniformity across studies has not been established nor substantial reproducibility or adoption to clinical practice. The practical usefulness of this approach is limited by the dynamic process of apoptosis and by the wide variety of measurements and laboratory standardizations. The individual evaluation of specific molecules in the process of apoptosis either as a single factor or in combination with others seems to suffer from the same issues.

Hypoxia and angiogenic factors

Hypoxia: Lack of oxygen supply to cancer cells has been linked to poor response to radiation. This premise was tested in patients undergoing neoadjuvant therapy for rectal cancer with the assistance of positron emission tomography using the copper-60-diacetyl-bis (N4-methylthiosemicarbazone (^{60}Cu-ATSM), an agent that accumulates in tissues lacking adequate oxygenation. Tumors with higher baseline tumor-muscle activity ratios (suggesting hypoxia) in the pre-treatment PET scan were shown to have a poor response to radiation[133]. Other agents tested in different studies have been less useful probably as a result of technical limitations[134].

Further evidence of the role of hypoxia in response to IR was demonstrated by the fact that higher levels of HIF-1 (hypoxia inducible protein factor 1, a protein that increases in oxygen deprived tissues) predicts poor response to neoadjuvant chemotherapy in patients with rectal cancer[135]. Additionally, HIF-1 correlates with increased levels of pro-angiogenic vascular endothelial growth factor (VEGF), a marker of angiogenesis for tumor growth[136].

VEGF: Low levels of VEGF have been associated with improved response to radiation[135,137,138], and vice versa[135,137-139]. Therefore, VEGF inhibition with the antibody bevacizumab has shown beneficial effects in treating cancers with neoadjuvant chemoradiotherapy[137,140,141]. Various mechanisms by which VEGF inhibition causes this effect may include reducing vascular density within a tumor, decreasing interstitial tumor pressures, improving global oxygenation status, vascular normalization and thus increasing responsiveness of endothelial cells to radiation[137,141,142]. It seems logical that if bevacizumab were to be used as a neoadjuvant agent in combination with IR for the treatment of patients with rectal cancer, these should have a higher rate of pCR compared to standard treatments. However, this observation has not been validated in clinical trials[143].

EGFR signaling: Initial reports revealed that combination of radiation and EGFR inhibition exerted a synergistic cytotoxic effect and hence raised interest in developing EGFR inhibitors. Hence, multiple EGFR inhibitors (*e.g.*, cetuximab and panitumumab) were developed and tested and have demonstrated promise in patients with KRAS wild-type tumors. However, with regards to the usefulness in EGFR signaling as a predictor of a response to IR, the data is lacking. Similarly, data pertaining to the usefulness of inhibiting the EGFR signaling pathway as a radiosensitizing modality has also demonstrated disappointing results[144].

High-throughput analyses

Microarray analysis: Single molecules as independent factors or in combination with other molecules of specific pathways (*i.e.*, apoptosis or angiogenesis) have not provided to be clinically useful to date. A major limita-

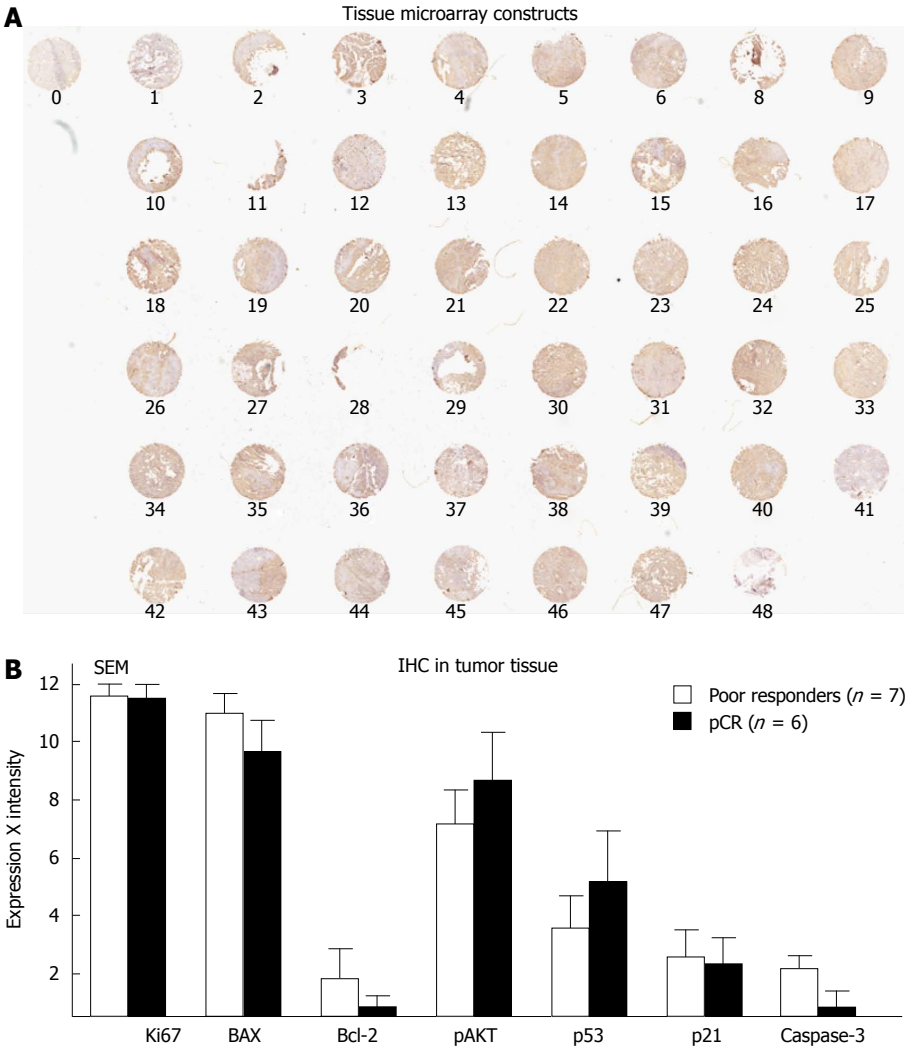

Figure 11 Tissue Microarray Constructs were created with 48 patients with rectal cancer that received pre-operative radiation. A: Of these 48 patients (each dot represents a patient), six had a pCR and seven did not respond to treatment; B: The differences in various tumor markers comparing these two groups. IHC: Immunohistochemistry; SEM: Scanning electron microscope.

tion of examining a specific pathway had to do with the dynamics of the process and the particular point in time at which it is being measured. Further, many tumors are heterogeneous in terms of mutations and alterations. Thus, interrogating several genes or proteins simultaneously is a logical approach in terms of elucidating origins of radioresistance in rectal cancer. In the era of personalized care, these tumor "fingerprints" not only make sense, but is the direction of the future.

Unfortunately, as appealing as it might seem, current efforts have been unsuccessful. Two studies have independently performed RNA arrays to analyze radioresistant and radiosensitive tumors. These studies have had limited genes and have had different results[145,146].

Tissue microarray: Tissue microarray is another technique to assess multiple proteins with a single experiment with tissues handled in a similar fashion. In one study, tissue microarray was performed with the goal of predicting survival and recurrence in patients treated with chemoradiation. In this study, Cox-2 emerged as a potential predictor of survival[147]. In a second study, our group subjected rectal cancer tissue to tissue microarray and tested eight different antibodies. MIB was the only independent predictor of a response to chemoradiation[8]. In our analysis, we examined tissue microarray in 48 patients who were treated with preoperative IR. We then divided all of these patients in two groups: patients who achieved a pCR (n = 6) compared to those who did not respond to IR or patients who experienced tumor growth (n = 7) in spite of pre-operative chemoradiation. We stained the tissue microarrays with seven antibodies and demonstrated no particular protein that could be used to differentiate these groups (Figure 11)[8].

CONCLUSION

Rectal cancer is the ideal clinical problem where personalized treatment could be investigated. This theory stems from the fact that a select patient population obtains an excellent response from the same form of chemoradiation, while others do not. Despite putting forward multiple mechanisms of tumor death from ionizing radiation and various possible causes of radioresistance, there

has not been a unifying pathway that can reliably predict a response to IR *in vitro*, *in vivo* or *ex vivo*. It is difficult to explain the reasons behind a clear discrepancy in the current observations in the literature. However, differences in tumor biology, genotypic profiling or phenotypic characteristics are some of these factors. There are currently good *in vitro*, *in vivo*, and *ex vivo* models for the study of rectal cancer and the trend seems optimistic in developing a predictive finger print for patients with rectal cancer that might respond well to IR. Recent data has shown that DNA-PKcs and Ku proteins (as vital players in NHEJ pathway allowing DSB repair) may have a central role in radiation induced cell death. Nevertheless many facets of its function in conjunction with the complex and intricate details of the pathway are still under investigation. More data is required before we can formulate one unified explanation for the heterogeneity noted in therapeutic effect of ionizing radiation. Until then, the hope of developing novel therapies for rectal cancer and improving the therapeutic yield of ionizing radiation with radiosensitizers remains a challenging clinical problem. The findings so far should not be viewed in a pessimistic fashion. There are several pathways that have provided potential targets for chemoradiotherapeutic interventions. We need to continue to investigate potential molecules predictive of a response to IR. As we dwell into the future, we need to remember that markers predictive of an aggressive behavior are currently in clinical practice such as testing for BRCA or RET proto-oncogene mutations. A view into the future also includes investigating base line characteristics of patient's genotypic background in normal tissue compared to tumor tissue after IR. It is important to determine if a patient starts with high levels at base line, but a particular gene is not activated then the base line levels are not as predictive. In the opposite scenario, we might have a patient with a molecule that at base line is low, but it is activated substantially with IR. In that scenario, we might consider those features as more predictive. The future, therefore, should be viewed with optimism.

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