HIF-1α and Resistance to Ionizing Radiation in Rectal Cancer

Kelly Harris[1,2], Xiaohuan Gao[1,2], Sara Huerta-Yepez[2], Payal Kapur[2] and Sergio Huerta[1,3*]

[1]VA North Texas Health Care System, USA

[2]Department of Pathology, University of Texas Southwestern, USA

[3]Department of Surgery, University of Texas Southwestern, USA

[*]**Corresponding author**: Sergio Huerta, MD, FACS, VA North Texas Health Care System, Department of Surgery (112), 4500 S. Lancaster Road, Dallas, TX 75216, USA, Tel: 75390-9159; E-mail: Sergio.Huerta2@VA.gov

Abstract

Introduction: There is a wide range of responses to pre-operative chemoradiation (CRT) in patients with rectal cancer. Factors that dictate tumors response have not been identified. The objective of this study was to determine the role of hypoxia-inducible factor 1-alpha (HIF-1α) in *in vitro* and *ex vivo* models of rectal cancer.

Methods: Levels of HIF-1α were assessed in radiosensitive (HCT-116) and a radioresistant (HT-29) cells. Radioresistant HT-29 cells were exposed to a potent radiosensitizing agent (a nitric oxide donor: DETANONOate [1 mM]) and IR followed by Western blot analysis with anti- HIF-1α antibodies. Patients with rectal cancer that were treated with CRT were separated based on good responders (≥ 50% reduction in tumor by comparing tumor size pre-operatively and postoperatively) compared to those that did not respond (<50% reduction in tumor). Blocks were stained for HIF-1α. Tissues collected from normal colonic epithelium from all these patients were subjected to the same treatments.

Results: *In vitro*, radiosensitive HCT-116 colorectal cancer cells demonstrated low levels of HIF-1α following treatment IR. Western blot analysis showed high protein levels of HIF-1α in radioresistant HT-29 cells subjected to IR with induction at 2.0 and 4.0 Gy. Protein levels of HIF-1α were attenuated by treatment of cells HT-29 with the radiosensitizing agent DETANONOate following exposure to IR. *Ex vivo*, tissue microarrays demonstrated no difference in HIF-1α expression between patients with a poor response to CRT (418.1 ± 32.6) vs. good responders (371.1 ± 31.5; p=0.3). However, examination tumor tissue compared to normal epithelium showed an increase in HIF-1α (1.5 fold; p<0.001) in patients who responded poorly to IR. This effect was not observed in the cohort of patients who responded well to IR.

Conclusions: The results of this study further support the potential role of HIF-1α in resistance to IR. This study implicates it a potential role of HIF-1α in rectal cancers.

Keywords: Rectal cancer; HIF-1 alpha; Chemoradiotherapy response; Ionizing radiation

Introduction

The management of rectal cancer has experienced a drastic evolution over the past three decades. The introduction of total mesorectal excision and neoadjuvant chemoradiation (CRT) has made substantial advances in the management of adenocarcinoma of the rectum [1-3]. Neoadjuvant chemoradiation for the management of patients with stage II and III rectal cancer introduced a fascinating phenomenon in that some patients treated with this modality achieve a clinical complete response (cCR), which is defined as the lack of detectable rectal tumor with diagnostic modalities [i.e. endorectal ultrasound (EUS), magnetic resonance imaging (MRI), digital rectal exam (DRE), or proctoscopy]. The fraction of patients treated with CRT who achieve a cCR is highly variable depending on the study (10-40%) [4]. Following surgical intervention, some patients will have no evidence of residual cancer in the specimen. This is defined as a pathological complete response (pCR). Patients who achieve a pCR have better outcomes compared to patients who do not [5]. An

intriguing concept of observing patients who achieve a cCR without surgical intervention has also been proposed [6-8].

It is, therefore, pivotal in the management of rectal cancer to identify factors that lead to resistance to pre-operative CRT, as this may lead to robust selection of patients who may avoid surgical intervention. Examination of tumors from patients who received pre-operative CRT showed that Ki-67 (a marker of cell proliferation), Bcl-2 (an anti-apoptotic mediator), Bax (a pro-apoptotic mediator), and p53 were associated with Pcr [4]. However, a systematic analysis of the literature failed to indicated that Ki-67, p53, and Bcl-2 were likely to predict a response to CRT [9]. These markers were selected in our previous studies and by systematic reviews by other groups because they are central mediators of apoptotic cell death following radiation-induced DNA damage [10]. There are currently inconsistent and inconclusive results regarding various molecular pathways that lead to a radioresistant phenotype in rectal cancer [11].

An important pathway implicated in a response to IR is the one activated once the cells undergo hypoxia. Lack of oxygen supply to cancer cells leads to a poor response to radiation. For instance, tumors with higher baseline tumor-muscle activity ratios (suggesting hypoxia)

in the pre-treatment PET scans were shown to have a poor response to radiation [12]. Hypoxia prevents degradation of the HIF-1α protein subunit, increasing the concentration of this important transcription factor. Radiation also increases HIF-1α activity in response to oxidative stress [13]. The multifactorial roles governed by HIF-1α include angiogenesis, apoptosis resistance and, in general, tumor survival in response to radiation. Moeller's work has demonstrated a complex and pleiotropic role of HIF-1α in response to IR. The role of HIF-1α as a marker of resistance to CRT in rectal cancer remains unclear.

In previous studies, we have developed in vitro, *in vivo* [14] and *ex vivo* [4] models for the study of a response to ionizing radiation (IR) in rectal cancer. We hypothesized that HIF-1α was elevated in radioresistant cells and in tissues of patients with radioresistant tumors. We tested this hypothesis by: (1) investigating the expression of HIF-1α in radiosensitive HCT-116 cells compared to radioresistant HT-29 cells. (2) Determining the effects of a potent radiosensitizing agent (DETANONOate) on HIF-1α expression and (3) by determining the expression of HIF-1α in tissue microarrays of patients with poor response to chemoradiotherapy compared to good responders.

Methods

Cell culture

HCT-116 (CCL-247) and HT-29 (HTB-38) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown and handled as previously described [14]. All cell lines were maintained at 37°C in ATCC-formulated McCoy's 5a Medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), and 1% (v/v) L-glutamine. HCT-116 cells were originally cultured from an adult male with colon cancer. HT-29 cells were derived from a 44 years old Caucasian woman with colorectal adenocarcinoma.

Clonogenic survival assays

The clonogenic assays were performed on single-cell suspension of mid-log growing cells as previously described [15]. HCT-116, and HT-29 cells were trypsinized and counted using a Coulter Counter (Casy1, Schärfe System, Reutlingen, Germany). Cells were then diluted serially to appropriate concentrations, plated in complete McCoy's 5a growth medium into 60 mm culture dishes in triplicate per data point and irradiated using a ^{137}Cs irradiator (J.L. Shepherd and Associates, Glendale, CA) at room temperature at a dose rate of 3.6 Gy/min. After 10-14 days, colonies were stained with 0.5% crystal violet solution for 30 minutes and counted under light microscopy. Survival fraction (SFs) was calculated as colonies counted/cells seeded×(plating efficiency). Plating efficiency (PE) was defined as (colony counted in un-irradiated control)/(cells seeded) as previously described [15]. All experiments were repeated at least three times.

Western Blot analysis and in HT29 and HCT116 cells

Western blot analysis of total protein extracts from HT-29 and HCT-116 cells were performed as previously described with minor modification [15]. Specifically, monolayer cells were washed twice with ice-cold PBS, scraped into RIPA buffer (Sigma-Aldrich, St. Louis, MO) supplemented with complete Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN), incubated for 5 min on ice with intermittent vortexing. After a brief centrifugation, the supernatant

was mixed with Laemmli sample buffer. Protein concentration was determined by a DC protein assay kit (Bio-Rad, Hercules, CA). An aliquot of total protein lysate (25 μg) was loaded in SDS-PAGE at 10%, transferred onto nitrocellulose membranes (BioRad Laboratories, Hercules, CA), and blocked with 5% nonfat dry milk. Membranes were then exposed to primary antibodies: rabbit anti- HIF-1α 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA).

After the incubation, the membranes were exposed to horseradish peroxidase-conjugated secondary antibody 1:4000 (anti-IgG, Bio-Rad, Hercules, CA). These membranes were then developed with Lumiglo reagent and Peroxide reagent (Cell Signaling Technology, Danvers, MA) and exposed to X-ray film (Amersham Biosciences, Little Chalfont, United Kingdom). Levels of beta-actin (Santa Cruz Biotechnology, Santa Cruz, CA) were used to normalize the proteins levels. Relative concentrations were assessed by densitometric analysis of digitized autographic images, using the public domain NIH Image J Program (available on the internet) as we have previously described [14]. All westerns were repeated a minimum of three times. A representative sample of each Western blot is presented in the results section.

Treatment of Cells with DETANONOate

The nitric oxide donor, (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl) amino] diazen-1-ium-1,2-diolate (DETANONOate; DETA) was obtained from ALEXIS Biochemicals (San Diego, CA). HT-29 were exposed to DETA (1000 μM) for 16 h (DETA pre-treatment). Following pre-treatment cells were subjected to ionizing radiation at doses of 0 (control), 2 Gy, 4 Gy and 6 Gy. Cells were then allowed to grow for clonogenic assays and compared to cells untreated with DETA. Treated and untreated cells were also collected for total protein extraction and Western blot analysis and performed as described above as previously described [14].

Tissue Microarrays

We have previously reported the use of tissue microarray constructs [4]. Briefly, all Hematoxylin and eosin (H&E) stained section from each specimen were reviewed by a staff pathologist (PK) to select representative areas of the tumor from which to acquire cores for microarray analysis. Two samples, for each patient, one from the tumor and one from normal colonic epithelium adjacent to the cancer, were identified and circled on the H&E stained slides. Tissue microarrays were built using a semiautomatic arraying instrument (Beecher Instruments, Silver Spring, MD) that uses two separate core needles for punching the donor and recipient blocks and a micrometer-precise coordinate system for assembling tissue samples on a block. For each case, single 0.6 mm (diameter 0.6 mm, height 3-4 mm) core diameter samples were obtained from the circled areas of tumor and/or normal colonic mucosa from each "donor" block and placed on separate a "recipient" TMA block (45x20 mm, 0.7 mm center). All samples were spaced 0.5 mm apart as previously described [4].

Immunohistochemistry of Tissue Microarrays

Serial slices of the microarray were deparaffinized and hydrated. Antigen retrieval was performed using sodium citrate (0.01 M, pH 6.0). Endogenous peroxidase activity was inhibited with methanol and 3% hydrogen peroxide. Antibody-tissue non-immune binding was blocked by immersing them in universal blocking solution and 1%

bovine serum albumin for 60 minutes. Tissue slices were incubated overnight at room temperature with anti-HIF-1α (Novus Biologicals. Southpark way.Co). After washing the slides, they were incubated with the universal Link biotinylated antibody (Dako) and then with streptavidin conjugated with horseradish peroxidase (HPR); color was generated by adding DAB (diaminobenzidine). The reaction was stopped and samples were counterstained with hematoxylin. Tissues were dehydrated and the preparations were covered with resin and dried at room temperature. Expression analysis of each microarray protein was determined by digital pathology with a ScanScope CS digital processor (Aperio, San Diego, CA, USA).

Digital image analysis

Immunohistochemically stained sections were digitized at 40X magnification utilizing an Aperio Scanscope CS (Aperio, Vista, CA). The Aperio Scanscope CS obtains 40X images with a spatial resolution of 0.45 μm/pixels. Images were reviewed utilizing an ImageScope (Aperio). Once the areas were annotated, they were sent for automated image analysis utilizing Spectrum Software (Aperio). Within Tissue IA, an algorithm was developed to quantify nuclear and total HIF-1α expression. The output from the algorithm returns a number of quantitative measurements, namely the intensity, concentration and percentage of positive staining present. Quantitative scales of intensity and percentage were categorized to 4 and 5 classes, respectively, after cut-off values were determined. The intensity of staining was categorized as 0 (no staining), 1+ (moderate), 2+ (strong), and 3+ (intense). Final IHC score was calculated from a combination of intensity and percentage score.

Statistical Analysis

All data is expressed as means ± SE. PRISM statistical analysis software (GraphPad Software, Inc., San Diego, CA) was used for contingency table analysis and Analysis of the Variance (ANOVA). Densitometry levels for the exposure of cells to DETANONOate was evaluated by ANOVA. Statistical significance for tissue microarray results was determined by contingency table analysis (X^2).

Results

In vitro, we have previously characterized one colorectal cancer cell line that was highly radiosensitive (HCT-116) and one that was substantially radioresistant (HT-29); survival fraction at 2 Gy (SF-2) = 38.9% ± 2.5 vs. 61.1% ± 3.7; respectively [14]. In this study, we performed Western blot analysis with antibodies specific for HIF-1α as described in the methods.

HCT-116 colorectal cancer cells demonstrated low levels of HIF-1 α following treatment with IR (0, 2, 4, and 6 [Gy]). On the other hand, HT-29 cells demonstrated high protein levels of HIF-1α expression that was induced with 2 and 4 Gy IR (Figure 1).
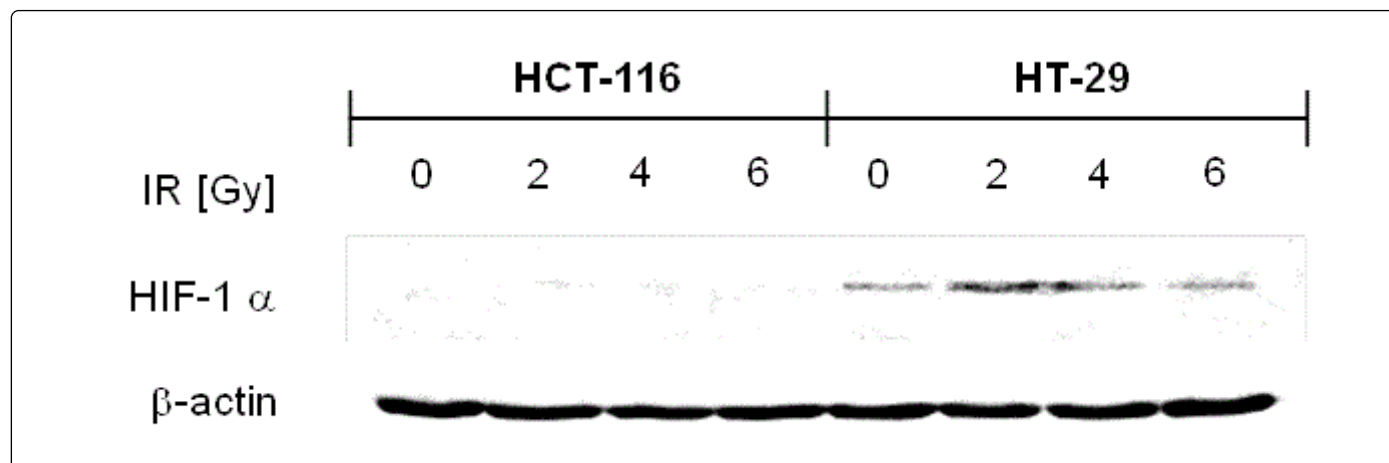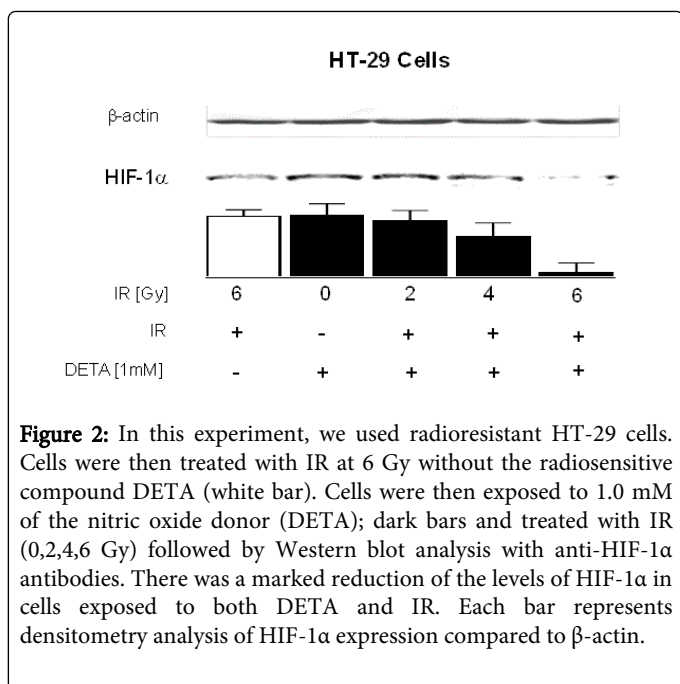

Figure 1: In this experiment two colorectal cancer cell lines were exposed to various doses of ionizing radiation. Radiosensitive HCT-116 and radioresistant HT-26 cell lines were exposed to (0-6 Gy or IR) and allowed to grow by colony forming essays. Cells were then collected at the end of the experiment and protein was extracted. Western blot analysis of radiosensitive (HCT-116) and radioresistant (HT-29) cell lines with antibodies specific for HIF-1α. HCT-116 cells demonstrated poor levels of HIF-1α. HT-29 showed higher expression of HIF-1α at base line and its levels were induced at 2 and 4 Gy.

In previous studies, we have demonstrated that HT-29 cells and xenografts were profoundly radiosensitized when exposed to 1.0 mM of the nitric oxide donor DETANONOate (DETA) [14]. In this study, we exposed HT-29 cells to DETA (1.0 mM) and treated them with IR followed by Western blot analysis with antibodies specific for HIF-1α.
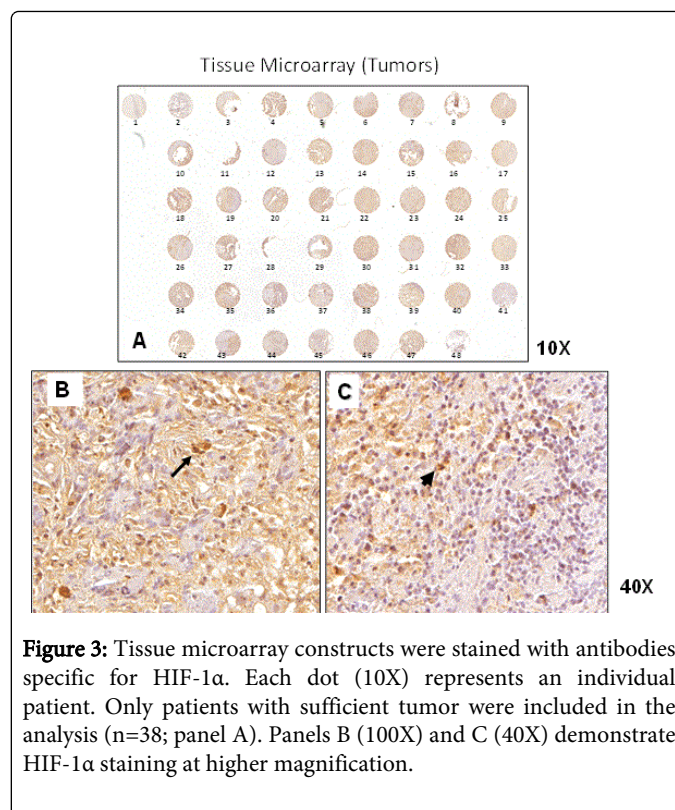
Densitometry analysis in Western blot analyses in cells subjected to HIF-1α compared to β-actin demonstrated a relative expression of 62.5 ± 5.7 in cell treated with 6.0 Gy IR without DETA. Cells treated with 1.0 mM DETA demonstrated expression of 63.4 ± 10.1, 58.7 ± 9.0, 44.9 ± 11.9, and 14.2 ± 83.3 following IR treatment at 0, 2.0, 4.0, and 6.0 Gy (Figure 2). This represents a 4.4-fold attenuation of HIF-1α in cells treated with DETA and 6.0 Gy IR compared to cells treated with the same dose of IR without DETA.

Figure 2: In this experiment, we used radioresistant HT-29 cells. Cells were then treated with IR at 6 Gy without the radiosensitive compound DETA (white bar). Cells were then exposed to 1.0 mM of the nitric oxide donor (DETA); dark bars and treated with IR (0,2,4,6 Gy) followed by Western blot analysis with anti-HIF-1α antibodies. There was a marked reduction of the levels of HIF-1α in cells exposed to both DETA and IR. Each bar represents densitometry analysis of HIF-1α expression compared to β-actin.

Tissue Microarrays

We have previously developed tissue microarray (TMA) constructs in patients from archived stage II and III rectal cancer tumors and matching adjacent mucosa (2 cm from the tumor; n=38) [4]. There were 20 patients in whom the tumor did not respond to therapy (poor responders) and 18 in which the tumors decreased by more than 50% (good responders). Assessment of tumor growth had been determined as previously described [4]. Briefly, patient's tumors were measured preoperatively via MRI, EUS, or proctostocpy. This tumor size was then compared to the tumor size at the time of pathologica examination following treatment with neoajuvant CRT and surgical intervention. In this study, we retrieved these TMA blocks and stained them with antibodies specific for HIF-1α (Figure 3).

Figure 3: Tissue microarray constructs were stained with antibodies specific for HIF-1α. Each dot (10X) represents an individual patient. Only patients with sufficient tumor were included in the analysis (n=38; panel A). Panels B (100X) and C (40X) demonstrate HIF-1α staining at higher magnification.

Immunohistochemistry

Immunohistochemistry of tissue microarrays demonstrated no difference in HIF-1α between patients with a poor response to IR (418.1 ± 32.6) vs. good responders (371.1 ± 31.5; p=0.3). In poor responders, there was a significant increase in HIF-1α in tumor tissue compared to adjacent epithelium following CRT (a 1.5 fold increase; p=0.001). Patients who responded well IR demonstrated no significant difference between tumor tissue and adjacent epithelium (Table 1).

	Tumor	Adjacent Epithelium	Fold-change Tumor vs. Adjacent Epithelium
Poor Response to IR (mean ± SEM)	418.1 ± 32.6	276.2 ± 30.9	1.5 (p<0.001)
Good Response to IR (mean ± SEM)	371.1 ± 31.5	328 3 ± 24.0	1.1 (p=0.2)
Fold-change Tumor in good responders vs. poor responders	p=0.3	p=0.2	

Table 1: Analysis of tissue microarray constructs in tissues of patients with rectal cancer who responded well to IR vs. poor responders and adjacent normal epithelium of matching controls

Discussion

A mechanism by which ionizing radiation exerts its therapeutic effects is by the creation of double stranded DNA breaks and free radical formation. The creation of free radicals is dependent on the availability of oxygen. Further, oxygen radiosensitizes cells by stabilizing DNA lesions. Radiolysis of water provides the reactive free radicals to lead to DNA damage [15]. Thus, hypoxic tissue responds poorly to ionizing radiation treatment.

An important goal of the management of patients with rectal cancer is to identify the cohort of patients that would respond appropriately to neoadjuvant therapy. At our institution, 25% of patients achieved a pCR [4]. We have investigated factors leading to a response to CRT with unyielding clinical applicability [4,10,14-16]. The pivotal role of hypoxia in tumor tissue led us to investigate the role of HIF-1α in our *in vitro* and *ex vivo* models of rectal cancer.

HIF-1α is a nuclear transcription factor directly activated by hypoxic conditions, oncogenes and other forms of stress [17]. *In vitro*,

human oral squamous cell carcinoma cell lines exposed to 5-Gy IR were protected from apoptotic cell death in cells that had high mRNA base-line HIF-1α expression compared to similar cells that had low level of HIF-1α mRNA at base line [18]. Studies that transiently inhibited HIF-1α by siRNA in radioresistant human head and neck cancer cells demonstrated that HIF-1α played a role in the up regulation of two molecules responsible for a radioresistant phenotype: Plasminogen Activator Inhibitor Type-1, and VEGF [19].

The role of HIF-1α in a response to IR is complex and pleomorphic [20]. Extensive work by Moeller's group underscores this issue. For instance, p53 and HIF-1α in combination to a hypoxic environment are all required for IR-induced, caspase-mediated apoptosis as well as loss of clonogenicity [20]. This work as also demonstrated that endothelial cell survival is the result of activation of survival factors mediated by an increase of HIF-1α in cells that have been re-oxygenated via IR mediated effects which then leads to the formation of reactive oxygen species [20]. Thus, the mechanisms HIF-1α in normoxic and hypoxic conditions as a result of radiotherapy in HCT-116 and PC-3 cell has been well established by this group [20].

We have worked on models of rectal cancer that have a known radiosensitive (HCT-116 cells) and radioresistant (HT-29 cells) phenotype as well as known patients that responded well to neoadjuvant (CRT) and patients that did not. Given the involvement of HIF-1α in a response to radiation, we elected to interrogate our models with anti- HIF-1α antibodies.

Clinically, the role of HIF-1α has been previously implicated in the response to CRT in several studies involving rectal cancer [21]. For instance, in rectal cancer specimens of patients treated with neoadjuvant therapy, high levels of HIF-1α were associated with decreased disease-free survival, suggesting that it is a negative prognostic factor [22]. In other studies, elevated levels of HIF-1α have independently predicted poor prognosis to radiotherapy [23,24].

In rectal cancer, much more substantial evidence of the involvement of HIF-1α might be extracted by its close link to VEGF. HIF-1α correlated with increased levels of pro-angiogenic vascular endothelial growth factor (VEGF) [21]. Decreased levels of VEGF led to a decrease in micovessel count and an increase in necrosis in radiosensitive Bax-/- HCT-116 cells [10]. Since Bax is a pro-apoptotic molecule, the knock out phenotype should have rendered cells more radioresistant rather than the observed radiosensitivity in the HCT-116 Bax wild-type counterpart cells, suggesting that the survival factors in cell death were down-regulated [10]. Angiogenesis is critical to tumor propagation. High levels of VEGF have been associated with an aggressive phenotype in numerous tumors, including colorectal cancer [25]. Responsiveness to ionizing radiation correlated with low levels of VEGF in one study [26]. Another study showed no difference in cell survival regardless of VEGF. However, VEGF knockout genotype in xenografts correlated with radiosensitivity to ionizing radiation [27].

The role of VEGF in rectal cancer response to CRT is further supported by preclinical and early clinical studies demonstrating the pharmacologic inhibition of VEGF, with the antibody bevacizumab, reduced vascular density within tumors, decreased interstitial tumor pressure, and improved global oxygenation status as opposed to inhibiting it. This led to an increased likelihood of cell death by radiation, providing support for the notion of vascular normalization by anti-VEGF treatment [28-30]. Additionally anti-VEGF treatment might increase responsiveness of tumor vessel endothelial cells to radiation [27].

Pharmacological inhibition of VEGF and other components of the angiogenesis axis is an active and promising in the area of rectal cancer treatment. Multiple studies have incorporated bevacizumab within the neoadjuvant treatment regimen, with some encouraging response rates [30,31]. However, more clinical trials are needed to determine the efficacy of bevacizumab and its side effect profile in comparison with standard regiments. A recent study, treating patients with rectal cancer with pre-operative chemotherapy only (5-fluorouracil, oxaliplatin [FOLFOX, which is the current standard treatment for patients with stage III colon cancer in the adjuvant setting] and bevacizumab) showed a similar response in pCR compared to patients treated with IR and standard chemotherapy [32].

The result of this study supports the role of HIF-1α in response to radiation and its potential as a candidate tumor maker in patients with rectal cancer. In this study, we first found that radiosensitive HCT-116 cells had low levels of HIF-1α compared to radioresistant HT-29 cells. The findings with regards to HCT-116 and HIF-1α shown by Moller's experiment under normoxic conditions also demonstrated that HIF-1α protein levels by Western blot analysis in HCT-116 cells were also low [20]. Further, in HT-29 cells, our observations suggested an induction of HIF-1α in combination with IR at 2 and 4 Gy. Additionally, we demonstrated that the potent radiosensitizing agent DETANONOate [16] might lead to its profound radiosenstive effects *in vitro* and *in vivo* via, at least in part, by attenuation of HIF-1α.

In the last set of experiments, we interrogated tissue microarrays in patient who achieved a good response to CRT compared to poor responders. There was no difference in patients who achieved a good response vs. patients that did not. However, observation of HIF-1α levels in patients who did not achieve a good response demonstrated a significant increase in their tumor tissue compared to the adjacent normal mucosa from matching controls.

Because our goal is to be able to characterize tumors that will respond to CRT and because previous studies have been unyielding, the notion of comparing molecular pathways in normal tissue compared to tumor tissue of the same patient might explain the inconclusive results in the literature regarding current molecules understudy for resistance to CRT.

The set of the experiments in this study were not designed to elucidate specific pathways, which designates these results as observational and constitutes the major limitation of this study. The number of subjects (n=38) in our tissue microarray studies is small and might lead to type II errors in the analysis of HIF-1α in poor responders compared to good responders of tumor tissues analyzed. We also limited our study to two cell lines. More cell lines as well as pathways need to be investigated to provide more conclusive results. However, taken together with previous evidence, our results support the potential role of HIF-1α in the response to ionizing radiation in rectal cancer.

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