Comparison of cellular lethality in DNA repair-proficient or -deficient cell lines resulting from exposure to 70 MeV/n protons or 290 MeV/n carbon ions

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Abstract. Charged particle therapy utilizing protons or carbon ions has been rapidly intensifying over recent years. The present study was designed to jointly investigate these two charged particle treatment modalities with respect to modeled anatomical depth-dependent dose and linear energy transfer (LET) deliveries to cells with either normal or compromised DNA repair phenotypes. We compared cellular lethality in response to dose, LET and Bragg peak location for accelerated protons and carbon ions at 70 and 290 MeV/n, respectively. A novel experimental live cell irradiation OptiCell™ *in vitro* culture system using three different Chinese hamster ovary (CHO) cells as a mammalian model was conducted. A wild-type DNA repair-competent CHO cell line (CHO 10B2) was compared to two other CHO cell lines (51D1 and xrs5), each genetically deficient with respect to one of the two major DNA repair pathways (homologous recombination and non-homologous end joining pathways, respectively) following genotoxic insults. We found that wild-type and homologous recombination-deficient (RAD51D) cellular lethality was dependent on both the dose and LET of the carbon ions, whereas it was only dependent on dose for protons. The non-homologous end joining deficient cell line (Ku80 mutant) showed nearly identical dose-response profiles for both carbon ions and protons. Our results show that the increasingly used modality of carbon ions as charged particle therapy is advantageous to protons in a radiotherapeutic context, primarily for tumor cells proficient in non-homologous end joining DNA repair where cellular lethality is dependent not only on the dose as in the case of more common photon therapeutic modalities, but more importantly on the carbon ion LETs. Genetic characterization of patient tumors would be key to individualize and optimize the selection of radiation modality, clinical outcome and treatment cost.

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Introduction

Charged particle therapy research and its clinical application has been expanding since its introduction in the early 1960's. Today, proton therapy is the prevailing form of charged particle therapy with 37 facilities around the world treating patients with various types of cancers including uveal melanoma, unresectable sarcomas, and basal skull or paraspinal tumors that require a significantly higher dose of ionizing radiation (1-4). Proton therapy is also considered advantageous relative to conventional forms of photon radiotherapy in cases where precise localization of the radiologic effects to a tumor is imperative during treatment. Quintessential examples of the types of tumors where proton therapy is the most advantageous in treatment include prostate cancer and pediatric neoplasms (5-7).

In the 1990's, the advantages of proton therapy eventually led to an expansion of the field of charged particle therapy to include carbon ion radiotherapy. Today, carbon ion radiotherapy centers at a limited number of locations worldwide are currently treating patients for the same types of cancers commonly treated elsewhere with protons (8-14). Carbon ions are typically accelerated at energies between 140 and 400 MeV for applications in a clinical setting whereas energies between 65 and 200 MeV are most commonly utilized when accelerating protons (11,15). Proton and carbon radiotherapy are both effective for precisely treating and delineating a localized tumor during treatment with ionizing radiation. This is due to the beam of accelerated particles gradually depositing increasing amounts of energy along a path in the biological

tissue (16,17). At a certain depth in living tissue and organs, the majority of a particle beam's energy (and therefore dose) is deposited along a relatively short traversal of the beam path termed the 'Bragg peak'. This narrow region is known as an area of high linear energy transfer, or LET, and is where a significant amount of energy from a particle beam is deposited into the tissue. This property enables the majority of a significant dose of ionizing radiation to be controllably localized to a relatively small tumor volume when treating oncology patients with these charged particle beams in clinical settings.

The therapeutic value of these charged particle therapies is in part, defined through their relative biological effectiveness, or 'RBE', which is defined as the ratio of a given dose of charged particles at a specific depth passing through air, water, or a biological tissue relative to the dose of X-rays required (or known) to produce an equal biological effect of a given dose of charged particles at a specific depth within air, water, or biological tissue. The RBE incident along particle beam paths, and most notably, at the Bragg peak for protons and carbon ions in radiotherapy has been a key factor when comparing these two types of charged particle therapies. Bragg peak RBE values of proton beams determined experimentally range between 1.0 and 2.1, and therapeutic values are estimated to be of 1.0 or 1.1 depending on the treatment center (15,18-20). Reported RBE values for carbon ions in radiotherapy have, in contrast, varied considerably, in part due to the limited number of facilities where these charged particle beams are available to patients worldwide. While empirical values for carbon ion RBEs at the Bragg range between 2.3 and 5 in a basic research setting, a consensus has yet to be reached on a definitive value to apply clinically for this particular type of radiotherapy (21-25). In both carbon and proton radiotherapy, the Bragg peak of a particle beam can be manipulated throughout the entirety of a tumor in order to deliver a maximum dose of radiation to all malignant cells. When this technique is applied to a tumor, a spread out Bragg peak, or 'SOBP', can be delivered to the malignancy in its entirety.

Due to the large overlap in applications for these two similar types of therapies, we investigated the cellular lethality of protons accelerated at 70 MeV/n and carbon ions accelerated at 290 MeV/n preceding, beyond, and located at the Bragg peak using Chinese hamster ovary (CHO) cells as a mammalian cell model utilizing particle accelerators at the National Institute of Radiological Sciences (NIRS) in Japan. Wild-type, homologous recombination mutant 51D1 (RAD51D mutant), and non-homologous end joining mutant xrs5 (Ku80 deficient) cell lines were used to evaluate cell lethality per dose at discrete points along a continuous path of ionizing radiation of X-rays, γ-rays, protons, or carbon ions tracking through an Opticell™ stacked culture cell system. We found that all forms of radiation were dependent on dose as evaluated by cellular lethality, however, only carbon ions produced cellular lethality that was dependent on LET at the Bragg peak. Among the various cell lines used, xrs5 cells alone displayed cellular lethality that was completely dependent on dose regardless of the type of radiation exposure. Conversely, wild-type cells, and to a lesser extent, 51D1 cells were most sensitive to carbon ion exposure, least sensitive to γ-ray exposure, and showed intermediate sensitivity to protons. Collectively, our findings suggest that carbon ion therapy is advantageous over proton therapy in light of carbon ion irradiation characteristics. Most noteworthy are the higher LET values at the Bragg peak and the fact that LET levels themselves in combination with dose are primary determinants of cellular lethality when treating a tumor. Ultimately, the specific genetics of any given malignancy with respect to its DNA damage repair proficiency affects the final extent of therapeutic advantage gained through the use of carbon ion beams in patients treated at charged particle therapy centers.

Materials and methods

Radiation conditions. Particle-based irradiation experiments were carried out at the NIRS in Chiba, Japan. Carbon ions were accelerated to 290 MeV/n using the Heavy Ion Medical Accelerator (HIMAC) synchrotron and protons were accelerated at 70 MeV/n using the NIRS-930 cyclotron delivery port in C-8. Dose rates for carbon ions and protons were set at 1 Gy/min. γ-ray irradiation experiments were carried out at a dose rate of ~2.5 Gy/min at Colorado State University (Fort Collins, CO) using a Model Mark I-68A (SS0056) 6,000Ci ^{137}Cesium sealed source model (J.L. Shepherd, Carlsbad, CA). X-ray irradiation experiments were carried out at the NIRS using a Titan X-ray generator (Shimadzu, Japan) with a peak tube/voltage potential of 200 kVp, a tube intensity of 20 mA, 0.5 mm of aluminum and copper filters, at a dose rate of 1 Gy/min. Irradiations were carried out at room temperature.

Cell culture. Original Chinese hamster ovary epithelial wild-type cells (CHO 10B2) were kindly supplied by Dr Joel Bedford (Colorado State University, Fort Collins, CO). DNA repair-deficient CHO mutant cell lines for the: i) homologous recombination pathway (51D1 cells; CHO AA8 RAD51D mutant cell lines) and ii) the non-homologous end-joining pathway (xrs5 cells; Ku80 gene deficient) were kindly supplied by Dr Larry Thompson (Lawrence Livermore National Laboratory, Livermore, CA)(26,27). All cells were grown and maintained in α-MEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO), 1X antibiotics and antimycotics (anti-anti, Invitrogen), at 37˚C in CO_2 incubators at 5% CO_2 and 100% humidity. Doubling times were ~12 h for all cell lines.

Irradiation procedure and cell survival assays. Cultured cells were trypsinized and re-suspended into growth medium containing α-MEM with 10% FBS and antibiotics (anti-anti, Invitrogen). Once re-suspended, 10 ml of medium containing between 500 and 700 cells were placed into each individual Opticell™ (Thermo Scientific, Rochester, NY) cell culture container ~1 h prior to irradiation. All samples were then appropriately organized and irradiated. Radiation physics quantitative values including dose distribution and LET distribution for both of the proton and carbon beams used is summarized in Fig. 1. Immediately following radiation, all cells were incubated at 37˚C with 5% CO_2 humidity for 7-10 days. After this culturing period, tissue culture vessels were then washed with 0.9% NaCl, fixed in 100% ethanol, and stained with 0.1% crystal violet. Colonies containing >50 cells were scored as a surviving colony. A minimum

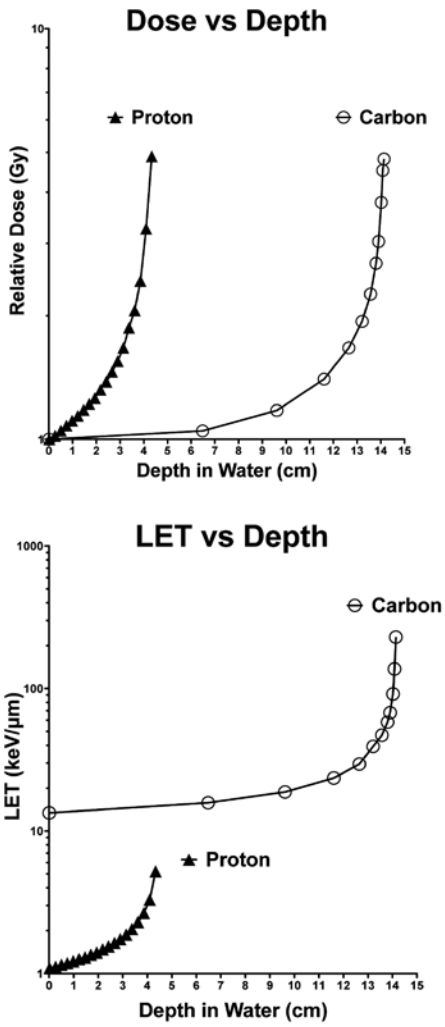

Figure 1. Depth dependence of dose and linear energy transfer (LET) spectra of carbon ions and protons (modeled using an *in vitro* Opticell™ stacked culture system). Dose and LET distribution values were calculated for corresponding depths in water for the 70 MeV/n accelerated protons and 290 MeV/n accelerated carbon ions. Both protons and carbon ions delivered a maximum dose of ~4.8 Gy to cultured wild-type cells located in 4 and 14 cm of water, respectively. LET values for carbon ions were significantly higher than values for the protons at all depths, and reached a maximum value located at a depth in water of 14 cm. Protons delivered a maximum LET value at a depth of 4 cm. Data points representing LET and dose values at different depths in water for carbon ions and protons are depicted as open circles and closed triangles, respectively.

of three independent experiments was carried out for each type of radiation studied. Survival curves were drawn for individual data points plotting a given Opticell container's 'depth' from doses calculated according to values presented in Fig. 1.

Data treatment and statistical analysis. All experimental data were analyzed using the Prism 5™ software. Standard errors of the means for all data points were calculated for all experimental data points and are depicted in each figure.

Results

Radiation physics parameters. Depth distribution values for the dose and LET were calculated and plotted against corresponding depths in water for the 70 MeV/n accelerated protons and 290 MeV/n accelerated carbon ions (Fig. 1). These maximum doses were delivered at depths of ~4 and 14 cm (in water), for protons and carbon ions, respectively. In regard to LET distribution values, carbon ions displayed higher LET values at all depths (in water) relative to the protons, and delivered a peak LET at a depth of ~14 cm (in water). Proton LET values were significantly lower relative to those for carbon ions and a peak LET at a depth of ~4 cm (in water).

Dose-depth distribution effect of proton and carbon beam cellular lethality. Stacked Opticell culture system cell survival assays were carried out using CHO wild-type xrs5 and 51D1 cells exposed to 70 MeV/n accelerated protons and doses of 290 MeV/n accelerated carbon ions. Carbon ions yielded lower survival fractions relative to protons at their respective Bragg peaks for wild-type and 51D1 cells at equal and lower doses (Fig. 2). xrs5 cells had comparable survival values for proton and carbon ions at doses of 0.5 and 1 Gy (Fig. 2).

Determination of dose-dependent cell survival. Cell survival was evaluated in response to the dose for all three cell lines using γ-rays, 70 MeV/n accelerated protons, and 290 MeV/n accelerated carbon ions. Both wild-type and 51D1 cells were most sensitive to carbon ions, and displayed responses in cell survival that were dependent on both the dose and LET for this particular form of radiation. In contrast, xrs5 cells displayed relatively comparable cell survival responses to dose for all types of radiation exposure, including exposure to high LET (defined as LET values >30 keV/μm) carbon ions (Fig. 3).

RBE values for wild-type cells. RBE values were calculated based on the average D10 values representative of a particular form of radiation. High LET 290 MeV/n carbon ions had the highest average RBE of 2.03, followed by low LET 290 MeV/n carbon ions with an RBE of 1.29 and 70 MeV/n protons with an RBE of approximately 1.

Discussion

Our results show that the degree of cell killing assessed via cell survival assays was dependent on the dose for all types of radiation used in our study (Fig. 3). Of the various types of radiation used, carbon ions produced cell survival levels that were dependent on both the dose and amount of LET exposure (Fig. 3). The LET values of carbon ions near Bragg peak are about one hundred times higher than protons and other low LET radiation values (Fig. 1). In regards to notable differences observed in survival responses between the different cell lines used, wild-type and homologous recombination mutant 51D1 cells tended to be the most sensitive to carbon ion radiation, especially at regions of high LET (Figs. 2 and 3). On the other hand, xrs5 cells displayed essentially the same sensitivity to the three types and LET radiation used in our study (Figs. 2B and 3). At lower doses (of 1 Gy) in particular, non-homologous end joining deficient cells trended towards a higher sensitivity to either carbon ions or protons than did homologous recombination mutants, which is consistent with

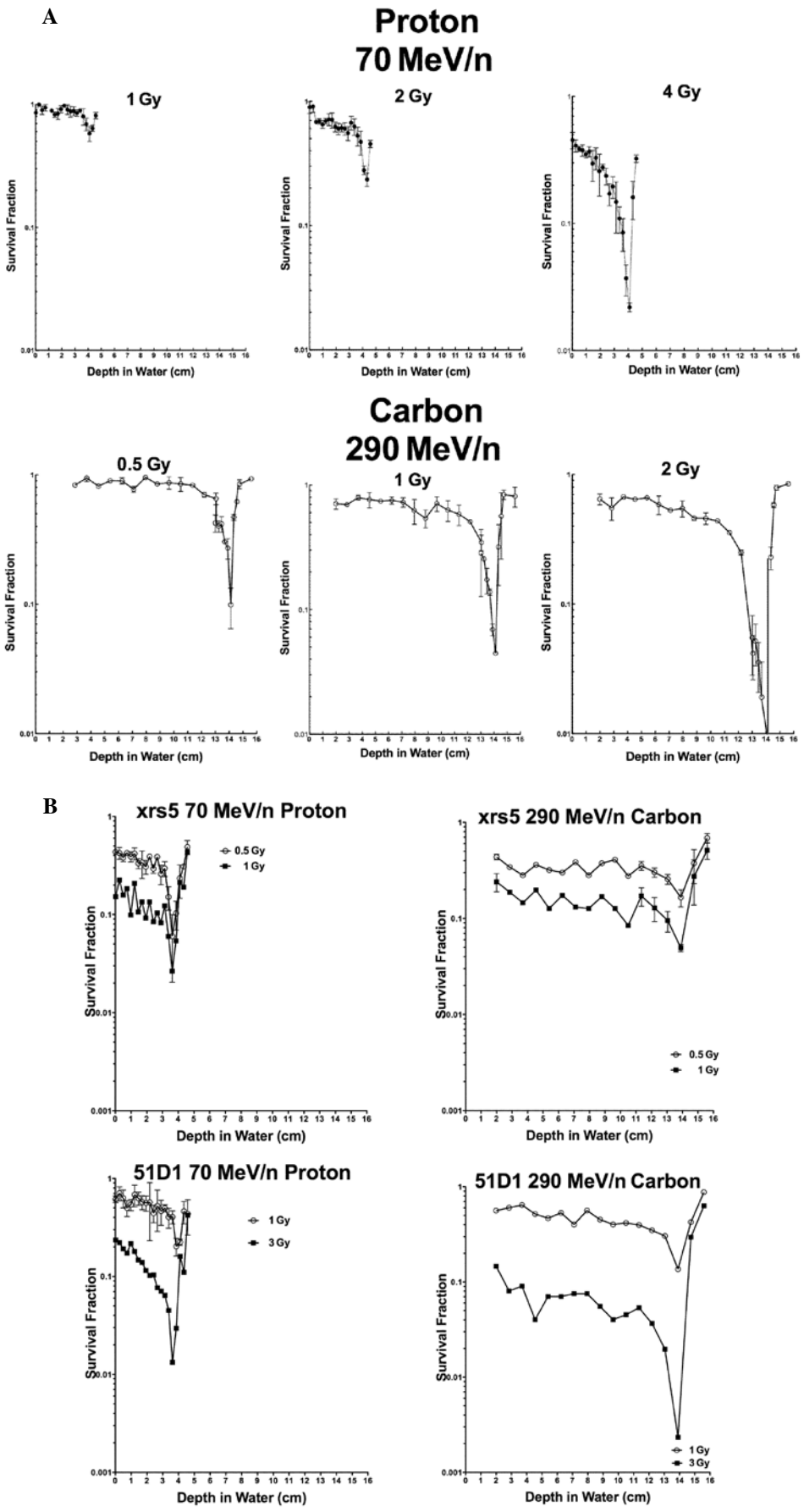

Figure 2. (A) DNA repair-competent cell survival vs. depth in water. Wild-type mammalian epithelial CHO 10B2 cells were exposed to 70 MeV/n accelerated protons and 290 MeV/n accelerated carbon ions. Carbon ion exposure resulted in notably lower survival fractions at Bragg peak regions relative to protons at entering doses of 1 and 2 Gy. (B) DNA repair-deficient cell survival vs. depth in water 51D1 and xrs5 repair-deficient cell lines were exposed to 70 MeV/n accelerated protons and 290 MeV/n accelerated carbon ions. Both beams yielded similar survival fractions at doses of 0.5 and 1 Gy at their respective Bragg peaks for xrs5 cells. 51D1 cells were considerably more sensitive to carbon ions at Bragg peak regions when compared to protons at entering doses of 1 and 3 Gy.

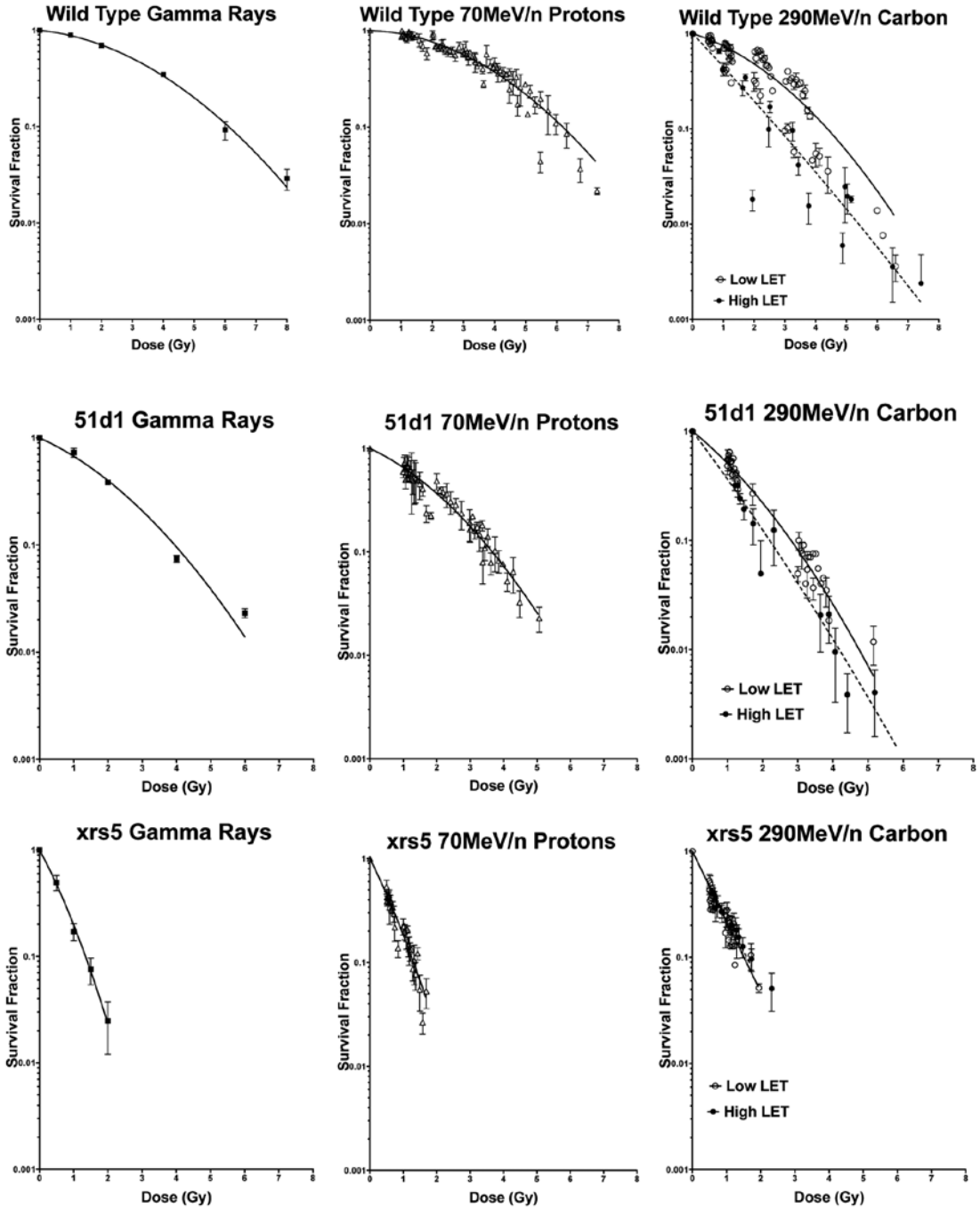

Figure 3. Influence of cellular DNA repair proficiency, radiation quality and dose on cell survival. Of the DNA repair cellular phenotypes and the radiation qualities studied, wild-type and 51D1 cells both displayed the highest sensitivity to carbon ions, particularly within regions of exposure to high LET (defined as LET values >30 keV/μm depicted as circles in graphs) within Bragg peaks. In contrast, xrs5 cells displayed comparable survival responses to the three beam qualities and the carbon LET's studied.

the relative roles of these two repair pathways in double-stranded DNA damage (Figs. 2B and 3).

When comparing the effectiveness between carbon ion radiotherapy and proton radiotherapy, our study suggests that carbon ions are advantageous to protons in the sense that cellular lethality is dependent on both dose and LET values for carbon ions (for cells proficient in non homologous end joining DNA repair), whereas cell survival for protons is dependent only on dose. Additionally, carbon ions in our study displayed LET values that were significantly higher than protons at the Bragg peak regions, which is also advantageous, especially when considering that an SOBP of high LET radiation is actually administered to a tumor as a whole.

A previous study reported findings similar to ours when evaluating the cellular lethality for xrs5 cells in response to increasing LET exposure of carbon ions (28). Concurrently, these two studies suggest that loss of non-homologous end joining DNA repair capacity undermines the carbon ion cellular lethality dependence on both the quality of radiation and quantity of LET exposure, and that only patients diagnosed with NHEJ DNA repair-competent tumors will maximally benefit from carbon ion therapy. To the best of our knowledge,

our present study is one of the first to investigate whether cells deficient in the other major cellular DNA repair pathway and incapable of homologous recombination also display this phenomenon. Results from the present study demonstrate that homologous recombination-deficient cells demonstrate a dependence on the quality of radiation and quantity of LET when cell survival is measured. However, investigative efforts are still required to definitively reiterate or reinforce this finding.

Another finding from our study that is worth mentioning are the RBE values derived from D10 values representing our monoenergetic proton and carbon beams. We discovered average RBE values calculated from D10 doses to be 2.03 at high LET Bragg peak regions and 1.29 at low LET regions outside the Bragg peak for wild-type cells exposed to carbon ions. The average calculated RBE for protons was approximately 1.0 for wild-type cells. From the perspective of RBE in our study, carbon ions may be considered advantageous to protons when using this wild-type *in vitro* model. RBE values for wild-type cells in our study are comparable to those depicted in previous studies (15,29,30).

In light of our findings, it would be significant to determine if carbon ions remain advantageous over protons in terms of a LET deposition and cell survival dependence on both dose and LET deposition at lower energy levels where a Bragg peak for accelerated carbon ions is located at the same depth in water as a comparative proton beam. Additionally, a future investigative effort evaluating the degree of synergy between inhibitors of various DNA repair pathways (i.e. homologous recombination and non-homologous end joining repair) combined with either proton or carbon radiation exposure could shed light on our findings. Further research involving proton and carbon *in vitro* experiments and more effective particle therapy treatment modalities are required for types of localized cancers where these types of radiation are applicable.

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