Caloric restriction augments radiation efficacy in breast cancer

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Dietary modification such as caloric restriction (CR) has been shown to decrease tumor initiation and progression. We sought to determine if nutrient restriction could be used as a novel therapeutic intervention to enhance cytotoxic therapies such as radiation (IR) and alter the molecular profile of triple-negative breast cancer (TNBC), which displays a poor prognosis. In two murine models of TNBC, significant tumor regression is noted with IR or diet modification, and a greater regression is observed combining diet modification with IR. Two methods of diet modification were compared, and it was found that a daily 30% reduction in total calories provided more significant tumor regression than alternate day feeding. At the molecular level, tumors treated with CR and IR showed less proliferation and more apoptosis. cDNA array analysis demonstrated the IGF-1R pathway plays a key role in achieving this physiologic response, and multiple members of the IGF-1R pathway including IGF-1R, IRS, PIK3ca and mTOR were found to be downregulated. The innovative use of CR as a novel therapeutic option has the potential to change the biology of tumors and enhance the opportunity for clinical benefit in the treatment of patients with TNBC.

Introduction

Caloric restriction (CR) is an intervention that has been shown to extend life and reduce age-related and chronic diseases, such as cardiovascular disease and cancer.[1-3] Although the positive benefits of CR were first described nearly a century ago,[4] there now is renewed interest in CR with an, increasing body of literature demonstrating a direct connection between dietary practices, cancer outcomes and obesity. One such cancer that has been shown in vivo to respond to CR is breast cancer.[5] Caloric restriction has been shown to not only decrease the incidence, but also the progression of breast cancers.[6,7] Most recently CR has been shown in vivo to decrease metastases from breast cancer.[7] Human epidemiologic studies indicate the relevance of dietary intake on breast cancer incidence and treatment. Population studies on obese patients have shown increase rates of breast cancer mortality, while those assessing anorexia nervosa have found a ~50% decreased risk of breast cancer compared with the normal population.[8]

At the molecular level, CR has optimal characteristics to combat cancer, as it has been shown to increase apoptosis and decrease the rate of proliferation, angiogenesis, hormones levels and growth factors.[9] In fact, many novel therapeutic agents for cancer treatment are targeted against individual molecules known to also be targets of CR, such as IGF1-R/Akt pathway, mTOR and AMP-K.[10] These observations have led to investigations into CR as a potential treatment for cancer. To date, CR has proven to be very important, because it can inhibit mTOR[11,12] and can increase the effectiveness of chemotherapy[13,14] while protecting normal tissue.[12,15,16] Interestingly, many of those same molecular targets, including mTOR, are also altered with radiation therapy.[17-20] Therefore, in this study we sought to determine if CR could be used in conjunction with radiation therapy to render cancer cells more susceptible to treatment.
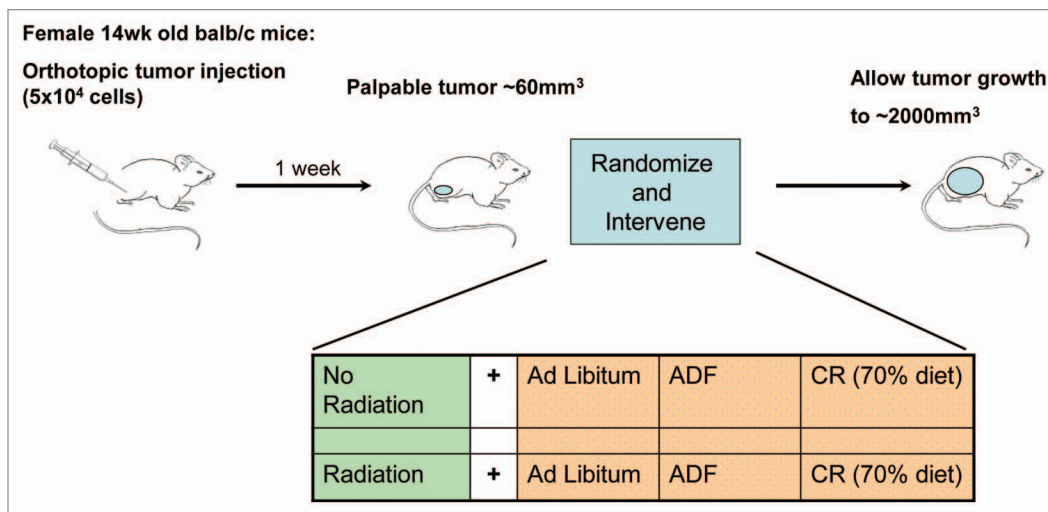
Figure 1. Experimental design. Tumors were generated by injecting either 67NR or 4T1 cells into the mammary fat pad of balb/c mice. Once tumors were palpable, mice were treated with IR to the primary tumor alone, treated with a dietary intervention of either ADF or CR or treated with a combination of the two and compared with control mice given no IR and an ad libitum diet.

The use of caloric restriction to augment radiation treatment has not been previously investigated. In this study, an in vivo model of breast cancer was used to determine if CR could increase the efficacy of radiation on established tumors. This is the first study to report the use of CR as a novel therapy in combination with radiation for breast cancer.

Results

Physiologic response of dietary intervention. Dietary caloric intake has a strong positive correlation for the incidence of many human cancers,[8,21] and spontaneous tumor development in mouse models.[22,23] Studies have also shown that large reductions (40–60% reduction) in caloric intake can slow the growth of implanted tumors in mouse models.[7,24,25] Using an in vivo model of breast cancer that mimics the clinical presentation in patients, we sought to determine if diet modification could slow established tumor growth with and without radiation (**Fig. 1**).

Mice were implanted orthotopically with one of two triple-negative breast cancer (TNBC) cell lines: 4T1, which are highly metastatic, or 67NR, which are locally aggressive. When palpable tumors formed, feeding changes were instituted; when tumors reached 100 mm³, radiation was administered, and tumor growth was recorded.

Mice with both types of TNBC tumors were treated with one of four conditions: control, which was ad libitum feeding (AL), alternate day feeing (ADF), IR or ADF + IR. Alternate day feeding was used, since it has previously been reported to cause a 25–30% reduction in caloric intake over time.[17] Measurement of food intake over the course of the experiment showed an average intake of 3.71 g per day per mouse for ad libitum fed animals, whereas ADF mice consumed an average of 6.78 g per fed day or 3.39 g per day per mouse, about a 9% decrease in caloric intake. The difference in our observed decrease in intake from

published reports for ADF may be due to the relatively short timeline of the experiment (38 d).

ADF did cause a decrease in tumor growth rate when compared with AL controls (**Fig. 2A and B**) for both 4t1 and 67NR implants. At 1,000 mm³, ADF caused an average growth delay of 16% (NS) for 67NR tumors and 30% (p value 1.37E-06) for 4t1 tumors. We also sought to determine how dietary restriction interacts with ionizing radiation (IR). To determine the proper dose of radiation to use for each cell line-derived tumor in vitro, clonogenic expansion assays were performed, and the radiation dose that achieved 1 log cell kill was determined to be 6 Gy for 67NR cells and 8 Gy for 4t1 cells (**Fig. S1**). IR alone caused a delay of 14% (NS) for 67NR tumors and 11% (p value 9.64E-07) for 4T1 tumors, and ADF combined with IR caused an average growth delay of 25% for 67NR tumors (p-value 0.0069) and 45% for 4T1 tumors (p value 1.32E-07).

Since these results seemed encouraging, but the 20–30% reduction in calories was not achieved, another cohort of mice were then treated after palpable 4T1 tumors were generated with caloric restriction (CR) that constituted controlled daily feeding of 70% of normal intake, and an additional arm which received both CR and IR. Compared with AL fed mice at 1,000 mm³, CR provided a growth delay of 56% (p value 2.29E-06), while the CR + IR group yielded a more than additive growth delay of 82% (p value 1.18E-05) (**Fig. 2C**). All growth delays were considered significant if p < 0.05 by Student's t-test.

The weight of each mouse was measured three times a week for the duration of the experiment and recorded (**Figs. 2D–F**). Minor and temporary weight loss was noted with ADF treated mice that was not observed in AL cohorts (**Fig. 2D and E**). Weight loss was observed in CR-treated mice, with an average weight loss of 12% (**Fig. 2F**).

Molecular response of dietary interevention. The molecular changes responsible for the physiologic tumor regression noted were explored. Histologic evaluation on hematoxylin and eosin
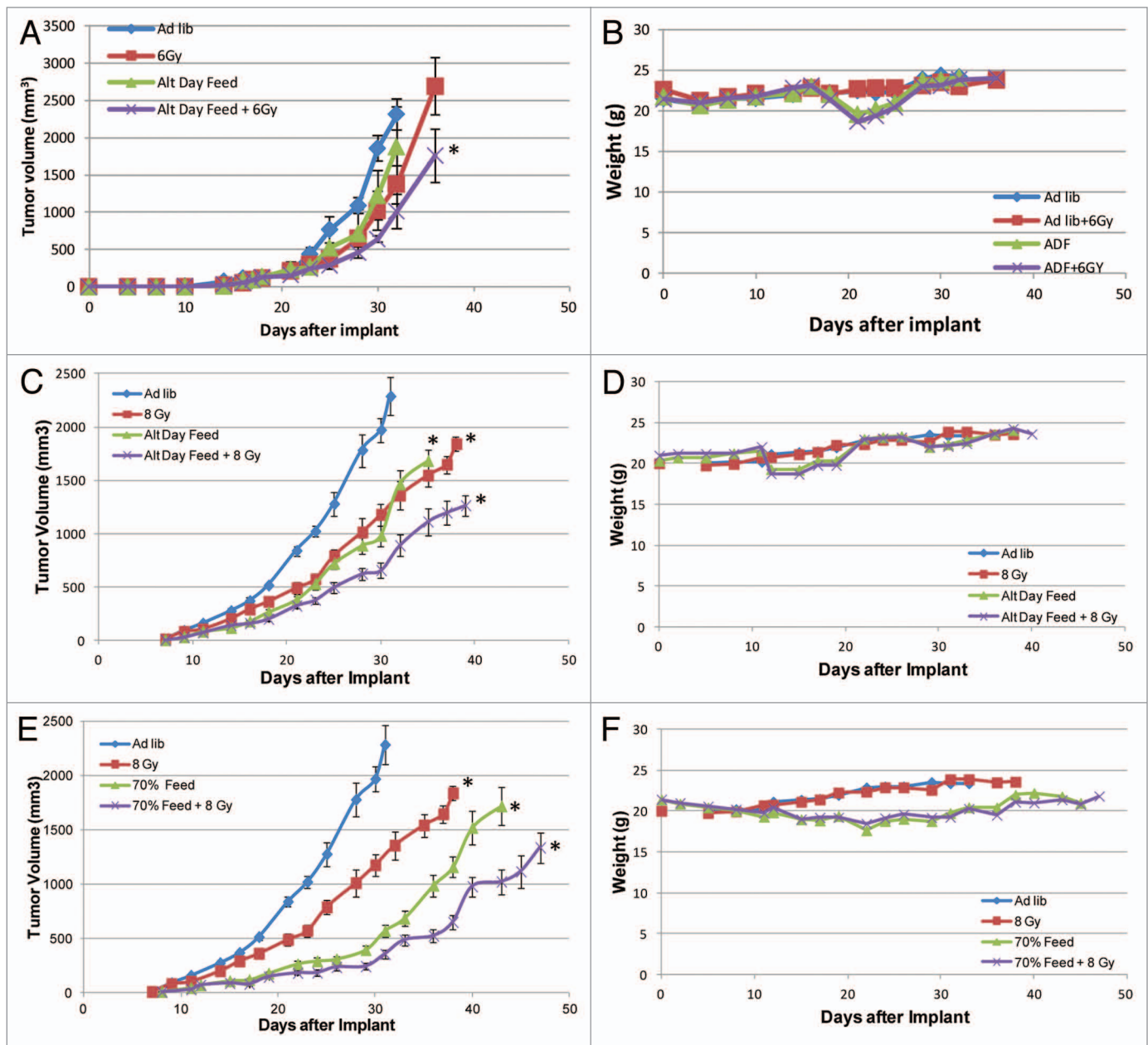
Figure 2. Tumor re-growth-delay curves for tumors created from two TNBC cell lines: 4T1 and 67NR cells using two different methods of caloric restriction. Mice were treated with each of the following conditions for 67NR (**A**) cell lines: ad libitum (AL) diet, radiation (RT), alternate day feeding (ADF) or ADF + RT, and their weights were recorded (**B**). From the reference of the AL cohort for 67NR tumors at 1,000 mm^3, RT gave a 16% growth delay, ADF 14%, ADF + RT 25%. From the reference of the AL cohort for 4T1 and weights were tumors at 1,000 mm^3 (**C**), RT gave a 23% growth delay, ADF 30%, ADF + RT 45% also recorded for this cohort (**D**). Using the 4T1 tumors, the ADF method was then compared with a 30% reduction in calories (CR) (**E**) using: AL, RT, CR and CR + RT. From the reference of the AL cohort for 4T1 tumors at 1,000 mm^3, RT gave a 23% growth delay, CR 56%, CR + RT 82% with an average weight reduction (**F**) of 12%. Based on this, the optimal method for performing future studies would be a constant reduction of 30% of the caloric intake. *Denotes $p < 0.05$ by Student's t-test at 1,000 mm^3.

for all treatment conditions revealed high-grade tumors with central necrosis. Tumors were noted to have decreased proliferation with CR alone, IR alone and an even further reduction with CR + IR when Ki-67 was assessed (**Fig. 3A**). Apoptosis was increased with each treatment alone, and when CR was combined with IR, even more apoptosis was noted, as measured with bcl-2 (**Fig. 3B**).

Evidence demonstrates that caloric restriction provides a benefit in breast cancer by acting on multiple molecular targets, such as the insulin-like growth factor-1 receptor (IGF-1R) pathway, inflammatory pathways and estrogen and leptin signaling. To determine the pathway most important in our triple-negative breast cancer models, cDNA arrays were done for the 4T1 tumors exposed to AL, IR, CR and CR + IR. After analyzing by GO-Elite
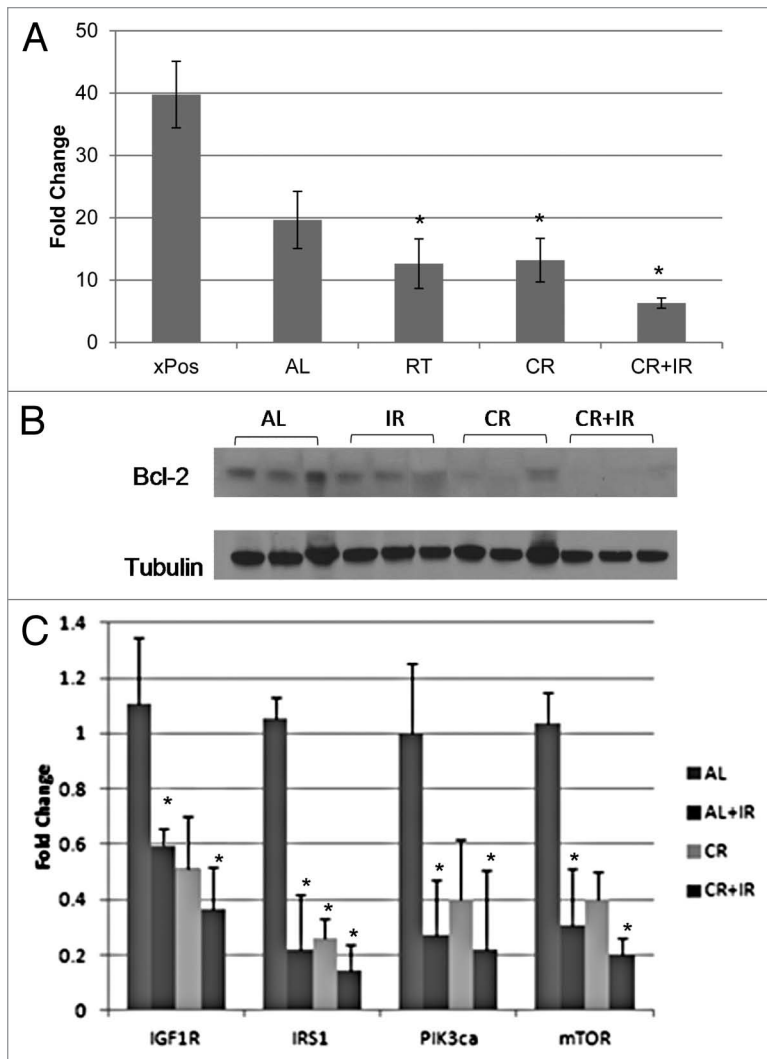
Figure 3. Molecular effects of treatment. Proliferation was evaluated by Ki-67 (**A**), and bcl-2 was used to evaluate apoptosis (**B**) for all conditions: Ad lib, CR, IR and CR + IR. Combination therapy was noted to decrease proliferation and increase apoptosis in tumors. qRT-PCR of IGF-1R pathway molecules shows downregulation (**C**) with either CR, IR or both. *Notes statistical significance with a p < 0.05.

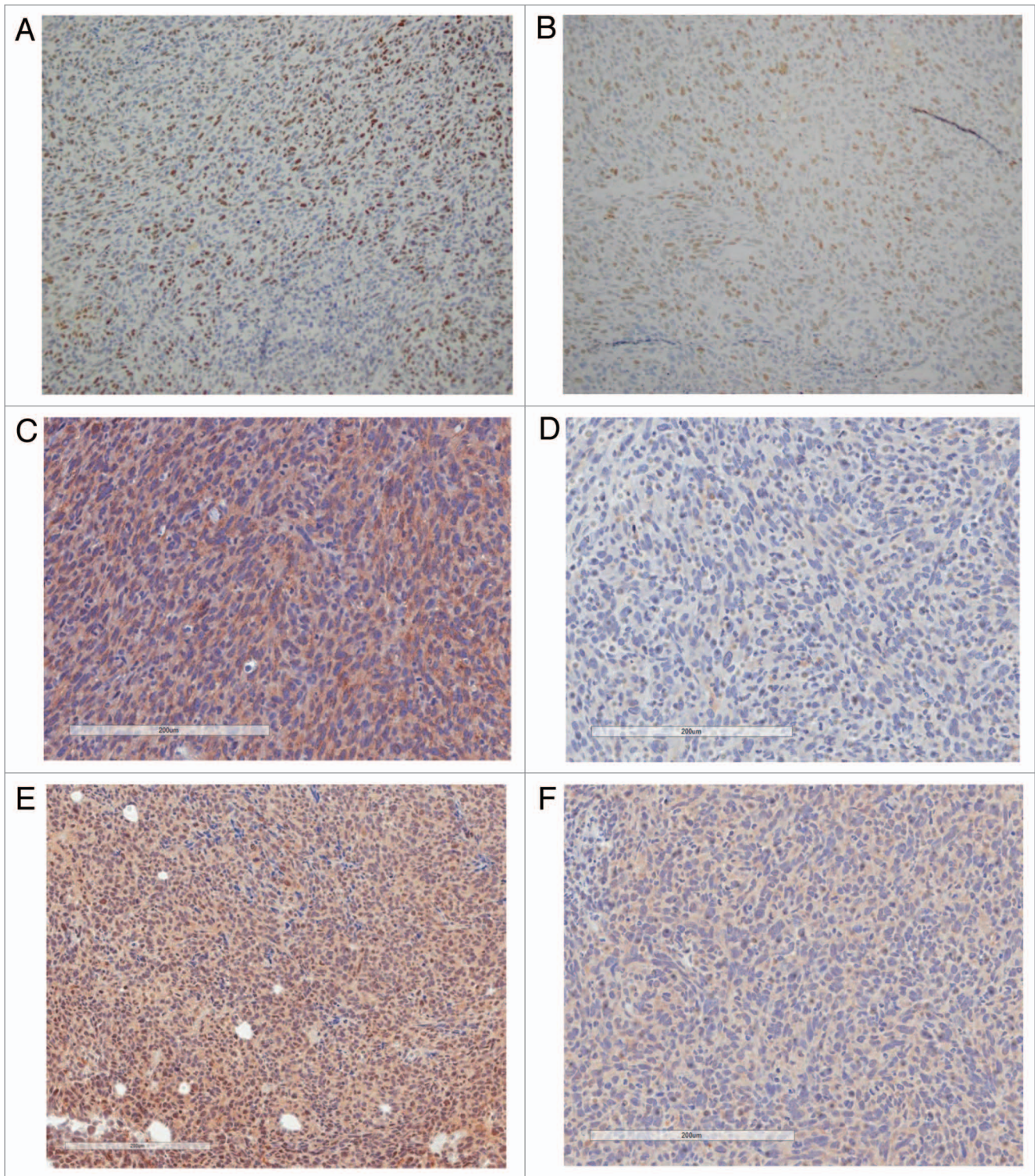stained the ad libitum samples with 3+ intensity and stained the CR + IR with 1+ intensity.

Mice were euthanized for 2,000 mm³ tumors or for humane endpoints such as shortness of breath due to the metastatic tumor burden in the lung. The tumor regression curves demonstrate that breathing difficulties were reached significantly later in ADF or CR mice than in AL fed controls, indicating that metastatic growth is delayed with dietary restriction.

Discussion

Triple-negative breast cancers (TNBC) account for nearly 20% of breast malignancies and are more aggressive, have poor prognosis and a higher recurrence rate. Caloric restriction (CR) has been shown to decrease the incidence and progression of spontaneous breast cancers in murine models and protect normal tissue during chemotherapy. Our results show that a nutritional intervention represses tumor growth in an additive manner when combined with radiation in two different aggressive murine models (4T1 and 67NR) of breast cancer. The average growth delay noted was 15–30% when treated with ADF alone and greater than 50% when treated with CR alone. In combination with IR, a more than additive growth delay was noted at greater than 80%. The significant increase in growth delay observed with combination therapy is a promising result, as it points to CR as a viable adjuvant therapy for breast cancer patients receiving radiation. Local control of disease is clearly affected, but it is also intriguing that the survival of the mice is increased with combination therapy.

Concern has been raised regarding the use of a dietary intervention with standard cancer therapy. Cancer patients, particularly those with advanced disease may be at risk of weight loss or cachexia due to the molecular properties of advanced disease. In addition, cancer patients may be on weight-altering chemotherapeutic drugs. In our study, two different regimens of dietary intervention were evaluated including ADF and CR. This demonstrated that mice placed on a regimen of alternate day feeding do not lose weight, while the mice on a CR diet, lost an average of 12% (4 g), which was a temporary loss. Similarly, other published data has not shown irreversible weight loss or cachexia caused by caloric restriction in cancer models.[30] While one human trial examining the effects of caloric restriction in combination with radiation has just begun, and effects are therefore unknown, a series by Longo et al. shows that ten patients were able to undergo short-term fasting during chemotherapy, and this treatment actually decreased the typical side effects of the drugs used.[15] This effect has also been shown in vitro.[12,16] Several other clinical trials testing the effects of ketogenic and low-carbohydrate diets on brain, lung and even cancers known to be associated with cachexia, including pancreatic cancer and

version 1.2.5 (www.genmapp.org/go_elite), we used the GOID system to compare the CR + IR condition with controls; one of the five most significant pathways included the IGF pathway (**Table 1**). Since the IGF-1R pathway has been implicated as a possible therapeutic target for TNBCs, because its downregulation leads to apoptosis, this seemed like an appropriate pathway to query. In addition, CR[26,27] and IR[28,29] are both independently noted in the literature to decrease members of the pathway.

Expression of members of the IGF-1R pathway, including IGF-1R, IRS, PIk3ca and mTOR, were evaluated. qRT-PCR reactions showed a reduction in all members assessed (**Fig. 3C**), which implicates this pathway in mediating the tumor regression noted. Immunohistochemical evaluation confirmed these results with representative images shown for IGF-1R (**Fig. 4B**) and GSK-3β (**Fig. 4C**), demonstrating that both of these proteins

Figure 4. Immunohistochemical investigation of proliferation and IGF-1R pathway. Proliferation was also evaluated by immunohistochemistry (IHC) with Ki-67 staining, which was noted to have higher proliferation in the ad libitum tumors (**A**) compared with the tumors treated with CR and IR. IGF-1R was noted to have positive staining (3+) in AL tumors and negative (1+) in CR + IR samples while the downstream target of GSK-3B revealed positive staining (3+) in AL tumors and 2+ staining in CR + IR tumors.

metastatic cancers, are currently enrolling patients.[31,32] Carefully selecting proper eligibility criteria to enroll patients onto trials with a dietary intervention will be crucial to their success.

At the molecular level, CR has been noted to increase apoptosis while augmenting anti-proliferative effects and decreasing DNA synthesis.[33-36] Our results are congruent with that, and

Table 1. Top 5 GO categories significantly altered with CR and RT combined

GO name	Z score	AVG-log fold change	STDEV-log fold change	p value
Gluconeogenesis	8.385544	1.21299	0.188822	< 0.001
Insulin-like growth factor binding	8.385544	1.400017	0.182701	< 0.001
Peptide cross-linking	7.797834	1.585857	0.574637	< 0.001
Extracellular region part	6.449937	1.208581	0.759949	< 0.001
Extracellular region	5.283875	1.287505	0.774612	< 0.001

show that combination therapy with CR and IR cause even more apoptosis and less proliferation. The ability of CR to slow tumor growth is likely attributable to the induction of a number of molecular changes on targets such as inflammation, adipokines, mTOR[11,12] and in the insulin-like growth factor (IGF-1R) pathway.[30,37-39] Longo et al. have implied that short-term starvation, or 48 h fasting (comparable to ADF), reduces IGF-1 levels by 70%, reduces glucose by 60% and increases IGFBP-1 11-fold.[2] Previous studies have suggested that fasting induces several metabolic changes; the major change being a shift to alternative nutrient sources and the reduction of growth factors such as IGF-1.[33,40,41]

Our cDNA array analysis confirmed the importance of the IGF-1R pathway in our model. Components of this pathway play an important role in carcinogenesis, and elevation of pathway components are prognostic for poor outcomes.[37,42-44] Consistent with that notion, downregulation of the IGF-1R signaling cascade leads to widespread apoptosis of cancer cells through the PI3K/AKT pathway.[45-48] In addition, it is also known that breast tumors often express higher IGF-1 levels than normal breast tissues counterparts.[34,49,50] Genotoxic therapies for breast cancer, including radiation, are reported to decrease components of the IGF-1R signaling pathway.[35,36,51,52] Our results at the molecular level demonstrate that various components of the IGF-1R pathway are downregulated with CR and RT used separately and are further decreased by the combination of treatments. This suggests that this pathway might be a driving force in the anti-proliferative effect noted.

Our data suggests that nutrient restriction could be used in combination with radiation therapy to confer a clinical benefit. In fact, many novel therapeutic agents for cancer treatment target molecules known to also be targets of CR, such as IGF1-R, the PI3K/Akt pathway, mTOR and AMP-K.[2,9,10,39,45,46,53] However, while clinical studies to test these new drugs seem promising, further studies may be limited by overlapping toxicity profiles of the agents targeted at members of those pathways being used.[54] Although CR has never been used as a treatment in oncologic clinical trials,[15] CR is being successfully implemented with diligent dietary counseling and behavior modification for other diseases in trials throughout the country.[44,47,55,56] CR may be an ideal complementary treatment modality for cancer, because it might alter many molecular targets concurrently, and it is free of adverse effects associated with multiple targeted agents.

Materials and Methods

Mice and tumor cell injections. Eighty female Balb/c mice were obtained from NCI Frederick at 6–8 wk of age and treated on a NCI Animal Care and Use Committee approved protocol. At 14-wk-old, 20 mice were implanted with 67NR tumor cells (triple-negative locally advanced cells) to determine efficacy of the treatment intervention (gift from Dr Fred Miller, Prentis Cancer Research Center, Karmanos Cancer Institute). Sixty mice were then implanted with 4T1 tumor cells, which are highly metastatic and randomized to a treatment arm (gift from Dr Patricia Steeg, NCI). All cells were cultured in DMEM supplemented with 10% FBS and pen/strep.

For implantation, mice were anesthetized, shaved, and a small incision (~0.5 cm) was made through the skin just anterior to the rear leg in order to visualize the #4 mammary fat pad, so that tumors could be created in situ. Five × 10^4 4T1 cells in 50 μL of sterile PBS were injected into the fat pad and the site closed with a staple. Tumors were measured three times per week using manual calipers, and their volumes were determined by the formula: 1/2 length × width^2. Mice were euthanized when the tumor reached 2,000 mm^3 or earlier if required by humane endpoints.

67NR pilot intervention. A pilot trial was first performed to determine if tumor regression in a locally aggressive triple-negative cell line could be noted with a dietary modification. To do this, after implanting 67NR cells and waiting for palpable primary tumors with an average size of 60 mm^3 to develop, mice were placed in four treatment cohorts of five mice each. The conditions for the cohorts included (1) mice given ad libitum (AL) feeding; (2) mice treated with radiation (IR) to the primary tumor; (3) mice given alternate day feeding (ADF) and (4) mice treated with IR to the primary tumor and given ADF.

Average daily food intake per animal was determined for all groups by weighing food 2–3 times per week. Intake baseline was established at least 2 wk prior to implantation and/or altering feeding protocol. All animals had unrestricted access to water regardless of feeding protocol. Ad libitum fed (AL): AL animals were given unrestricted access to standard NIH31 mouse diet for the duration of the experiment. Alternate day feeding (ADF): The dietary intervention of ADF is noted in the literature to be equivalent to a global reduction of 30% calories. Therefore, for this pilot intervention, animals were fasted and fed in alternating 24 h cycles starting when tumors became palpable around 1 wk after implant. During the fed state, mice were given ad libitum access to NIH31 mouse diet. Ionizing radiation (IR): Mammary fat pad tumors in the IR groups were irradiated when the tumors were palpable with an average size of 100 mm^3. Mice were sedated, placed into a restraint jig with shielding to isolate the tumor, and the tumor with margin was treated with a 6 Gy dose for tumors generated from the 67NR cells using a Pantak H-320 (320 kV) Precision X-Ray.

4T1 tumor intervention. Since benefit was noted with a pilot trial of a locally aggressive cancer, a larger study was undertaken to look at the efficacy of dietary intervention in the 4T1 model, which is also a triple-negative cell line, but is highly metastatic. We chose to evaluate radiation in conjunction with two different

dietary interventions including ADF and caloric restriction, instituting a 30% reduction in calories to determine if both regimens would have equivalent efficacy as previously reported. Therefore, 6 cohorts of 10 mice were evaluated in this portion of the study: (1) AL feeding; (2) mice treated with (IR) to the primary tumor; (3) mice given ADF; (4) caloric restriction (CR) or a 30% reduction in their daily intake; (5) mice treated with IR to the primary tumor and given ADF and (6) mice treated with IR to the primary tumor while on a CR diet. Caloric restriction (CR): At 10-wk-old, each animal was singly housed, and baseline average daily food intake of NIH31 mouse diet was calculated per individual over 2 wk. Starting 2 d after tumor implant, each animal was fed once per day a weighed portion equivalent to 90% of its individual average daily intake. Over an 18 d period, animals were stepped down in 5% increments every 4–5 d, ensuring weight loss was stabilized before each step until the animals were calorically restricted to 70% of baseline. IR: As described above 12 d after implant, however, tumors generated from the 4T1 cells were treated to 8 Gy using a Pantak H-320 (320 kV) Precision X-Ray.

Clonogenic survival curves. To determine an equivalent dose of radiation to treat mice implanted with two different cell types, 4T1 or 67NR cells were trypsinized and plated in 6-well plates at the appropriate densities and then incubated for 6–8 h at 37°C. Cells were then irradiated with a dose of 0, 2, 4, 6 or 8 Gy. The plates were then incubated for 10 d and colonies were stained with crystal violet. Colonies of 50 or more cells were counted, and the fraction of surviving cells at each dose was calculated. A dose of radiation was chosen to achieve a 90% cell kill in vitro. This tuned out to be 6 Gy for 67NR cells and 8 Gy for 4T1 cells as denoted in **Figure S1**.

RNA isolation and RT-PCR. Tumors were grossly dissected and immediately stabilized by placement in RNAlater (Ambion) per manufacturer's instructions. Tissue was placed in TRIzol reagent (Invitrogen) with lysing matrix D (MP Biomedicals) and homogenized using manufacturer's suggested settings for a FastPrep-24 machine (MP Biomedicals). Following lysis, extraction was performed using a standard phenol-chloroform method. Following extraction, 1 μl of 20 mg/ml glycogen (Fermentas) and 1.5 volumes of 100% ethanol was added to each sample. Total RNA was then purified using RNeasy Mini columns (Qiagen) using standard manufacturer's protocol. RNA concentration was determined and integrity was confirmed by viewing 18S and 28S by denaturing agarose gel electrophoresis. mRNA expression for IGF-1R, IRS-1, PIk3ca, mTOR, were assessed using the high capacity cDNA reverse transcription kit (ABI) as per manufacturer's instructions. RT-PCR reactions were performed using 20 ng of cDNA.

cDNA microarrays. RNA from primary tumors treated with AL, CR, IR and CR + IR were labeled with Cy3-dUTP and compared with the reference RNA (Stratagene Universal Reference), which was labeled with Cy5-dUTP. The method was previously described by Khan et al.[57] Microarray slides were obtained from the Radiation Oncology Sciences Program Microarray Lab at the National Institutes of Health.[58] Slides were 8 k human slides printed on site using a Named Genes clone set from Research Genetics, spotted onto poly-L-lysine coated slides using an OmniGrid arrayer (GeneMachines). Hybridization and washing were done as previously described and slides were then scanned at 10 microns intervals using a Genepix® 4000 scanner (Axon Instruments), and images and data were stored in a database (mAdb) maintained by the Center for Information Technology, National Institutes of Health.[59] The microarray data have been deposited in National Center for Biotechnology Information Gene Expression Omnibus.[60]

Data analysis. Data was extracted into two categories using the mAdb system: "all genes" and "outliers." "All genes" were extracted excluding spots flagged as Bad/Not found, and spots with target diameters less than 50 μm or greater than 300 μm. Only spots with signal-to-background ratios of > 2, a minimum background corrected signal of 250 counts and 80% of pixels in the spots with intensity greater than a SD plus background were used excluding spots flagged as Bad/Not found. Local Mean Across Element Signal Intensity (LOESS) was applied to dye-normalization. Ratio data were analyzed by LIMMA statistics to obtain significant genes with selection criteria p ≤ 0.01, Bonferroni correction by using R version 2.11.1 and BioConductor package limma version 3.4.3. To identify biological pathways, including miRNA which altered by IR or/and 70% calorie restriction, pathway analysis was conducted using GO-Elite and GenMAPP-CS.

Tissue staining and scoring. Formalin-fixed, paraffin-embedded tissue sections were cut at 6 μm and deparaffinized by standard techniques. Antigen retrieval was performed by heating the sections in 10 mmol/L citrate buffer pH 6.0 for 50 min with the use of a pressure cooker. A rabbit monoclonal antibody for Ki-67 at 1:600 dilution (AbCam; catalog no. ab16667) for 60 min at room temperature. The immune complexes were visualized with Mouse ABC (Vector Laboratories, Inc) and the chromogenic substrate Dako Liquid DAB_Substrate-Chromogen Solution (Dako North America, Inc; catalog no. K3468; diaminobenzidine tetrahydrochloride) for 3 min. Ki-67 labeling index was determined by counting ≥ 500 nuclei in areas of the section with the highest labeling rates and was considered high when ≥ 10% of tumor cells were stained.[61] The scoring was performed by an experienced staff pathologist and was also interpreted with the Aperio Image analysis system in order to obtain the number and percentage of positive cells in each tumor.

Statistical analysis. Statisitical significance between tumor growth curves was calculated as previously described.[62] Briefly, tumor growth data were fit using an exponential growth equation; the tumor growth time (days) for control animals was calculated and then subtracted from all treated groups. SDs were derived and used to calculate the Student's t-test and p values for the differences between the various groups. Comparisons comparing statisitical significance for RT-PCR anaylsis was made by the two-tailed Student's t-test. A difference between groups of p < 0.05 was considered significant.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cc/article/25016

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