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α6 Integrin Cleavage: Sensitizing human prostate cancer to ionizing radiation

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Abstract

Purpose—The goal was to determine if prostate tumor cells containing a mutant α6 integrin would be defective in tumor re-population following clinically relevant fractionated ionizing radiation (IR) treatments.

Material and methods—Human prostate cancer cells derived from PC3N cells were used which conditionally expressed a cleavable, wild type form of α6 integrin (PC3N-α6-WT) or a mutated non-cleavable form of α6 integrin (PC3N-α6-RR). The resulting tumor growth before, during and after fractionated doses of IR (3 Gy × 10 days) was analyzed using the endpoints of tumor growth inhibition (T/C), tumor growth delay (T-C), tumor doubling time (Td) and tumor cell kill (Log_{10} cell kill).

Results—The T/C values were 36.1% and 39.5%, the T-C values were 20.5 days and 28.5 days and the Td values were 5.5 and 10.5 days for the irradiated PC3N-α6-WT and PC3N-α6-RR cells, respectively. The Log_{10} was 1.1 for the PC3N-α6-WT cells and 0.8 for the PC3N-α6-RR cells. The tumor response to IR was altered in tumors expressing the mutant α6 integrin as indicated by a significant increase in tumor growth inhibition, an increase in tumor growth delay, an increase in tumor doubling time and an increase in tumor cell kill.

Conclusions—Blocking integrin cleavage *in vivo* may be efficacious for increasing the IR responsiveness of slow growing, pro-metastatic human prostate cancer.

Keywords

Prostate cancer; ionizing radiation; tumor growth inhibition; growth delay; integrin; tumor doubling time; tumor cell kill

Introduction

Prostate cancer is the most common cancer in men and it is the second most common cause of cancer-related deaths (Jemal et al. 2007). Approximately 60 – 70% of men with newly diagnosed adenocarcinoma of the prostate have organ confined disease. Conventional treatments include radical prostatectomy, external beam radiation therapy, interstitial brachytherapy, hormonal therapy and active surveillance. The apparently inherent radio-resistance of prostate tumors is a major challenge with ionizing radiation (IR) therapy, requiring the balance of minimizing normal tissue toxicity to the bladder, urethra and rectum while

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delivering effective therapeutic radiation (Livsey et al. 2004, Hummerich et al. 2006). Prostate cancer being treated in a curative manner requires external beam radiation doses of 75 – 80 Gy for effective tumor control and exhibit a lower α to β ratio, potentially as low as 1.5 Gy as compared to other tumor types (Kupelian et al. 2000, Valdagni et al. 2005). Several centers have initiated preliminary trials using higher doses per fraction, called aggressive hypo fractionation (Parker & Patrocinio 2005). Recent work has indicated that a high dose per fraction IR is efficacious for increasing tumor response rates of human prostate cancer implanted in a mouse (Lotan et al. 2006). These studies underscore that human prostate cancer is both a radiation resistant tumor type and is capable of tumor repopulation following therapeutic doses of IR.

Epithelial malignancies such as prostate cancer in general have a resistance to the killing effects of damage inducing agents that we and others have called cell adhesion mediated drug resistance (CAM-DR) (Damiano 2002, Cordes 2006, Kremer et al. 2006, Hehlgans et al. 2007). Others have found that epithelial malignancies also have an adhesion mediated resistance to ionizing radiation, called CAM-IR (Cell Adhesion Mediated Ionizing Radiation Resistance) (Damiano 2002, Cordes & Meineke 2003, Cordes 2006, Kremer et al. 2006, Hehlgans et al. 2007). Both of these types of resistance are dependent upon integrin functions and can be overcome with cell adhesion disruption strategies.

Invasive and migrating human prostate cancers express on their surfaces $\alpha 6\beta 1$ and $\alpha 3\beta 1$ integrins (Schmelz et al. 2002, Demetriou et al. 2004). The $\alpha 6$ integrin on the tumor cell surface is cleaved at specific residues resulting in a variant called $\alpha 6$p that is missing over half of the extracellular segment; a domain associated with cell adhesion to the extra-cellular ligand, laminin (Davis et al. 2001). Using site directed mutagenesis and a transfection strategy, we created stable prostate cancer cell lines that expressed either the uncleavable form of the integrin $\alpha 6$ (PC3N-$\alpha 6$-RR) or the wild type receptor (PC3N-$\alpha 6$-WT) (Pawar et al. 2007). Our goal in the present study was to determine if human tumors containing the integrin mutant were defective in tumor repopulation after IR.

Methods

Culture conditions and cell lines

All cell lines were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were grown in Iscove's Modified Dulbecco's Medium plus 10% fetal bovine serum, penicillin/streptomycin, 100 U/ml (Invitrogen, Carlsbad, CA, USA). PC3N cells (Tran et al. 1999), a variant of the human PC3 prostate carcinoma cell line were transfected as previously described to generate the PC3N-$\alpha 6$-wt and PC3N-$\alpha 6$-RR (Pawar et al. 2007). Briefly, the R594 and R595 codons of the integrin $\alpha 6$ gene were mutated to alanines. Both WT and RR mutant genes were cloned into the T-REx-pcDNA6/T system (Invitrogen, Carlsbad, CA, USA) and transfected into PC3N cells.

Antibodies and chemicals used

Anti-$\alpha 6$ integrin rabbit polyclonal antibody AA6A was produced by Bethyl Laboratories Inc. (Montgomery, TX, USA). While AA6A is specific for the last 16 amino acids (EIHAQPSDKERLTSDA) in the cytoplasmic region of the human $\alpha 6$A sequence it will recognize full-length $\alpha 3$ due to amino acids QPS (Tamura et al. 1990). The rabbit anti-actin antibody, AAN01 was obtained from Cytoskeleton, Denver, CO, USA. Doxycycline was purchased from Sigma, St Louis, MO, USA. Urokinase was purchased from Chemicon, Temecula, CA, USA and consisted of a mixture of single chain and double chain forms of uPA (Urokinase Plasminogen Activator).

Immunoblotting

Cells were lysed in RIPA (RadioImmunoPrecipitation Assay) buffer and the resulting protein solubilized in non-reducing sample buffer and 30 μg of protein was analyzed by a 10% SDS – PAGE (Sodium Dodecyl Sulfate – PolyAcrylamide Gel Electrophoresis) gel. Proteins resolved in the gel were electrotransferred to Millipore Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA), incubated with anti-integrin α6 antibody AA6A and detected using a secondary antibody conjugated to horseradish peroxidase. Membranes were probed with the anti-actin rabbit polyclonal antibody from Cytoskeleton (Denver, CO, USA) as loading control. Visualization was by chemiluminescence (ECL Western Blotting Detection System, Amersham Biosciences, Piscataway, NJ, USA).

Severe Combined Immunodeficiency (SCID) mice and establishment of subcutaneous tumors

BALB-c/B-17/IcrACC SCID mice (Arizona Cancer Center SCID Colony) were maintained in a specific-pathogen-free environment in compliance with U.S. Public Health Service guidelines. Cells were harvested using trypsin, washed and resuspended into Dulbecco's Phosphate Buffered Saline (D-PBS) (Invitrogen, Carlsbad, CA, USA) at a concentration of 8.0×10^7 cells/ml. Eight-week-old male SCID mice were each inoculated subcutaneously in the flank with 8×10^6 cells by injection of 0.1 ml of cell/D-PBS mixture with a 27 g needle. Mice were maintained on sterile Doxycycline Diet (Bio-Serv, Frenchtown, NJ, USA) containing 200 mg/kg of doxycycline for proper gene expression in the T-REx expression system. Tumor width and length were measured by calipers and volume was estimated with the following equation: $\frac{1}{2}\times w^2\times l$. 1 mm^3 was presumed to be equal to 1 mg.

Radiation Treatments

Tumors were established for 18 days to an average size of 700 mm^3 prior to radiation treatment. Mice were placed in a plastic tube apparatus with the flank tumor protruding from a slot in the apparatus. Lead shielding was placed over the apparatus with holes in the shielding exposing the tumors. Daily radiation treatments of 3 Gray (Gy) were delivered using a Co^{60} irradiator at a dose rate of 0.6 Gy/min. Mice were irradiated a total of 10 times over 2 weeks on a MTWTF schedule for a total dose of 30 Gy. The animal care was performed according to protocol #04-076 as approved by the Institutional Animal Care and Use Committee. Radiation conditions and data measurements were provided through the Experimental Mouse Shared Service at the University of Arizona Cancer Center.

Tumor harvesting and RT-PCR (Reverse-transcriptase – Polymerase Chain Reaction)

Mice were sacrificed when tumor mass was approximately 2.0 g. Tumors were excised and frozen and stored at −80°C until use. RNA (Ribonucleic Acid) was extracted from approximately 0.4 g of minced tumor with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as per manufacturer's protocol. 18S and 28S rRNA integrity was evaluated by ethidium bromide staining on a 1% agarose/formaldehyde gel. RT-PCR was performed according to manufacturer's instructions with a cMaster Rtplus PCR system kit from Eppendorf (Westbury, NY, USA). The primers to detect the mutation (R594, R595 to A594, A595) were 5′-CTC TGC TGC GCG AGT GAA TTC-3′and 5′-TGT CTT GAT TTC CTT CTC GGG T-3′ (150 bp). The GAPDH (glycer-aldehyde-3-phosphate dehydrogenase) primers used were 5′-TGG TAT CGT GGA AGG ACT CAT GAC-3′ AND 5′-AGT CCA GTG AGC TTC CCG TTC AGC-3′ (181 BP). PCR products were electrophoresed on a 2% agarose gel and visualized with ethidium bromide.

End points for assessing solid tumor activity

Four different parameters were evaluated and include the following: Tumor growth inhibition (T/C Value); Tumor Growth Delay (T-C Value); Tumor-doubling time (Td) and Tumor Cell Kill (Log_{10} cell kill) as previously defined (Bissery et al. 1991).

Immunohistochemistry

Freshly obtained surgical samples of mouse tumor tissue were fixed in formalin and embedded in paraffin, sectioned, and examined using hematoxylin and eosin (H&E) staining.

Results

The PC3N cells were transfected with wild type and mutated integrin α6 constructs to make PC3N-α6-WT and PC3N-α6-RR cells respectively as described earlier (Pawar et al. 2007). The transfected protein sequence is expressed in these cells by doxycycline in a concentration dependent manner (Figure 1A). Over-expression of the integrin α6 is observed in cells treated with 0.001 and 0.01 μg/ml Doxycycline and is proportional to the concentration of Doxycycline. The minimum amount of Doxycycline for induction of integrin α6 (i.e., 0.001 μg/ml) was used in subsequent experiments. Figure 1B demonstrates that as expected, the integrin α6 expressed in PC3N-α6-WT is cleaved by uPA as compared to the mutated, non-cleavable integrin α6 expressed in the PC3N-α6-RR cells.

Tumor cells expressing the mutant integrin demonstrated an increased growth delay following radiation treatment as compared to the cells expressing the wild type integrin. BALB-c/B-17/IcrACC SCID mice were subcutaneously inoculated with cells containing the wild type integrin (PC3N-α6-WT) and cells containing the mutated integrin (PC3N-α6-RR cells). Comparable histology of the resulting tumors were observed in all mice by day 14 before the onset of treatment (Figure 2B, 2C). Radiation treatment was initiated at day 18 on 8 mice bearing PC3N-α6-WT tumors and 8 mice bearing PC3N-α6-RR tumors. All mice were irradiated a total of 10 times over two weeks for a total dose of 30 Gy. By day 25 the median tumor weight of untreated tumors in mice injected with PC3N-α6-WT cells had increased to approximately 1300 mg. In contrast, tumors containing the PC3N-α6-RR cells had reached approximately 800 mg. It was interesting to note that the median weight of irradiated tumors dropped to approximately 500 mg in both PC3N-α6-WT and PC3N-α6-RR injected groups (Figure 2A). The maximum median tumor mass was observed on day 28 in the group of mice that had not been exposed to radiation and the measurements were terminated at this point since the group size decreased to less than 6 mice due to sacrificing mice bearing tumors greater than 2000 mm^3 (Table I). Measurements of the median tumor mass in the radiation treated PC3N-α6-WT and PC3N-α6-RR tumor bearing mice was continued till day 47 and 53, respectively. The median tumor mass of nearly 1300 mg was reached on day 47 in the radiation treated PC3N-α6-WT injected mice as compared to the 25 days in the untreated group. Similarly the radiation treated PC3N-α6-RR injected group of mice contained a lesser median tumor mass as compared to the untreated group (Table I and Figure 2).

Tumors were dissected and RNA was harvested from the specimens. PCR was performed on the cDNA generated from the RNA to detect the mutated integrin α6. GAPDH amplification was carried out as control. The mutant transcript was retrieved in radiation treated and untreated tumors induced by PC3N-α6-RR and was absent in PC3N-α6-WT induced tumors (Figure 2, inset).

The growth of untreated PC3N-α6-WT and PC3N-α6-RR cells was log-linear up to a mean tumor size of approximately 1.5 g, at which point the mice were humanely sacrificed (Table I). This allowed for calculation of tumor growth indices using the standard NCI criteria (Bissery

et al. 1991). The tumor doubling time (Td), of untreated mice receiving the PC3N-α6-WT cells was 5.5 days and this resulted in 40% larger median tumor sizes on day 25 compared to the median PC3N-α6-RR tumor size on day 25 (Table II). There was substantial tumor growth delay in the groups treated with radiation when compared to the untreated PC3N-α6-WT cells; radiation produced % T/C values of 36.1% for the PC3N-α6-WT cells and 39.5% for the PC3N-α6-RR cells. Of note, according to NCI standards, a tumor growth inhibition %T/C value $\leq$42% is the minimum level denoting an 'active' treatment in a solid tumor model in mice (Bissery et al. 1991). Thus, the radiation treatments induced a significant degree of tumor growth inhibition in each cell type compared to the untreated mice receiving the PC3N-α6-WT cells. The addition of radiation also produced greater than one-log $_{10}$ tumor cell kill in each cell line *in vivo*, demonstrating that radiation did not just impede tumor growth, but actually induced significant tumor cell killing (far right column, Table II). The growth of untreated PC3N-α6-RR cells (Td = 10.5 days), was slower as compared to that of untreated PC3N-α6-WT cells *in vivo* (Td = 5.5 days). A comparison of radiation effects in the two groups of mice receiving the PC3N-α6-RR cells showed that radiation induced a T/C % of 56% and a $\log_{10}$ cell kill of 0.8. This suggests that radiation was less effective at blocking tumor cell growth and division in the PC3N-α6-RR cell line compared to the PC3N-α6-WT cell line.

Discussion

The results of radiation therapy of human prostate cancer cells growing in SCID mice showed that radiation effectively reduced the tumor burden independent of the type of integrin expressed by the tumor cells. Both the PC3N-α6-WT and the PC3N-α6-RR containing tumors responded to radiation therapy with an increased tumor cell kill. The degree of radiation induced tumor growth inhibition reached NCI standards for an 'active regimen' in both cell types *in vivo*.

After the radiation treatments ceased, there was significant growth delay in both tumor types. Interestingly, the tumors containing the mutated integrin had a significantly increased growth inhibition after the radiation treatment (%T/C = 39.5) as compared to the tumors containing the wild type integrin (%T/C = 36.1). The tumor growth delay was increased in the mutant integrin containing tumors (20.5 in PC3N-α6-WT as compared to 28.5 in PC3N-α6-RR) as was the tumor doubling time (5.5 in PC3N-α6-WT as compared to 10.5 in PC3N-α6-RR). Taken together, these data suggest that strategies to block α6 integrin cleavage *in vivo* may be useful for increasing the radiation responsiveness of slow growing human prostate cancer. Alternatively, detection of integrin cleavage in tumors may suggest a more radiation resistant phenotype.

While tumor repopulation after RT requires tumor cell survival (i.e., resistance), it also requires a productive interaction of the tumor cells with their microenvironment. It is well known that the extra-cellular matrix (ECM) environment changes dramatically following IR treatment. Radiation rapidly and persistently alters the soluble and insoluble components of the ECM (Barcellos-Hoff et al. 2005). In model systems, cells in the ECM such as fibroblasts, respond to IR by increasing the production or remodeling of collagen (type I and III) and fibronectin (Barcellos-Hoff 1993, Remy et al. 1991). Fibroblasts isolated from post-radiation biopsies in patients with recurrent breast cancer produce dramatically increased levels of fibronectin (Brouty-Boye et al. 1991).

The work presented here indicates that the ability of the tumor cells to repopulate an extracellular area altered by IR is affected by the status of the integrin on the tumor cell surface (i.e., whether it can be cleaved). Stated another way, we infer that the ability of residual tumor cells to repopulate a new fibronectin and collagen I rich environment may be significantly influenced by the cleavage of a laminin binding integrin. The removal of the ligand binding

domain on the integrin $\alpha 6\beta 1$ on the tumor cell surface may enable tumor repopulation in a fibronectin and collagen I enriched environment.

Since integrin cleavage on the tumor cell surface is specific to the laminin binding integrin, $\alpha 6\beta 1$, this also opens the interesting possibility of functional cross-talk between laminin and fibronectin or collagen I binding integrins for tumor cell repopulation or reseeding. The functional cross-talk between adhesion receptors has been previously reported between cadherin and integrin molecules. Cell adhesion molecules mediate cell-cell and cell-extracellular matrix adhesions, and coordination between these molecules is essential for tissue formation and morphogenesis. Crosstalk between integrins and cadherins results from a physical response to integrin-mediated adhesion, complex cell differentiation processes, or direct signaling pathways linking the two adhesion systems (Chen & Gumbiner 2006). The possibility that integrins may crosstalk to each other after IR and during tumor repopulation, has not been previously explored and represents a new class of cellular damage responses. Current experiments are underway to determine if cleavage of a laminin binding integrin $\alpha 6\beta 1$ does in fact promote the function of fibronectin ($\alpha 51\beta$) or collagen binding integrins ($\alpha 3\beta 1$, $\alpha 2\beta 1$).

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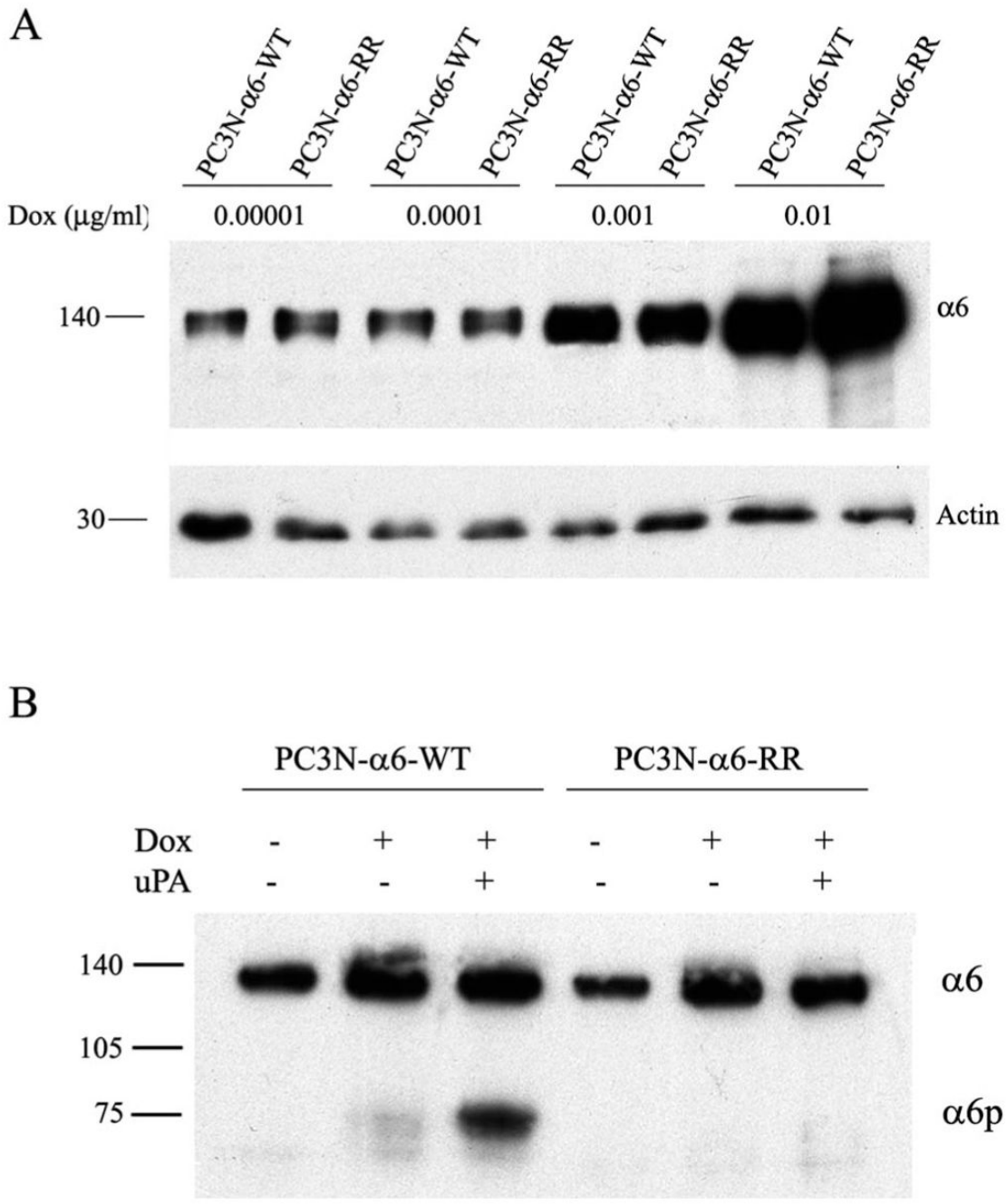

Figure 1.

Doxycycline concentration dependent expression of cleavable and non-cleavable integrin α6. (A) PC3N-α6-WT and PC3N-α6-RR cells were treated with 0.00001 – 0.01 μg Doxycycline per ml of media. Cell lysates were analyzed for expression levels of integrin α6. The α6 integrin was detected in whole cell lysate using the AA6A rabbit polyclonal antibody on a Western blot. Actin was used as a loading control. (B) PC3N-α6-WT and PC3N-α6-RR cells were left untreated or treated 0.001 μg/ml Doxycycline alone or with 20 μg/ml uPA and the lysates analyzed by PAGE. The integrins α6 (140 kDa) and α6p (~75 kDa) were detected using the AA6A rabbit polyclonal antibody by Western blot analysis.

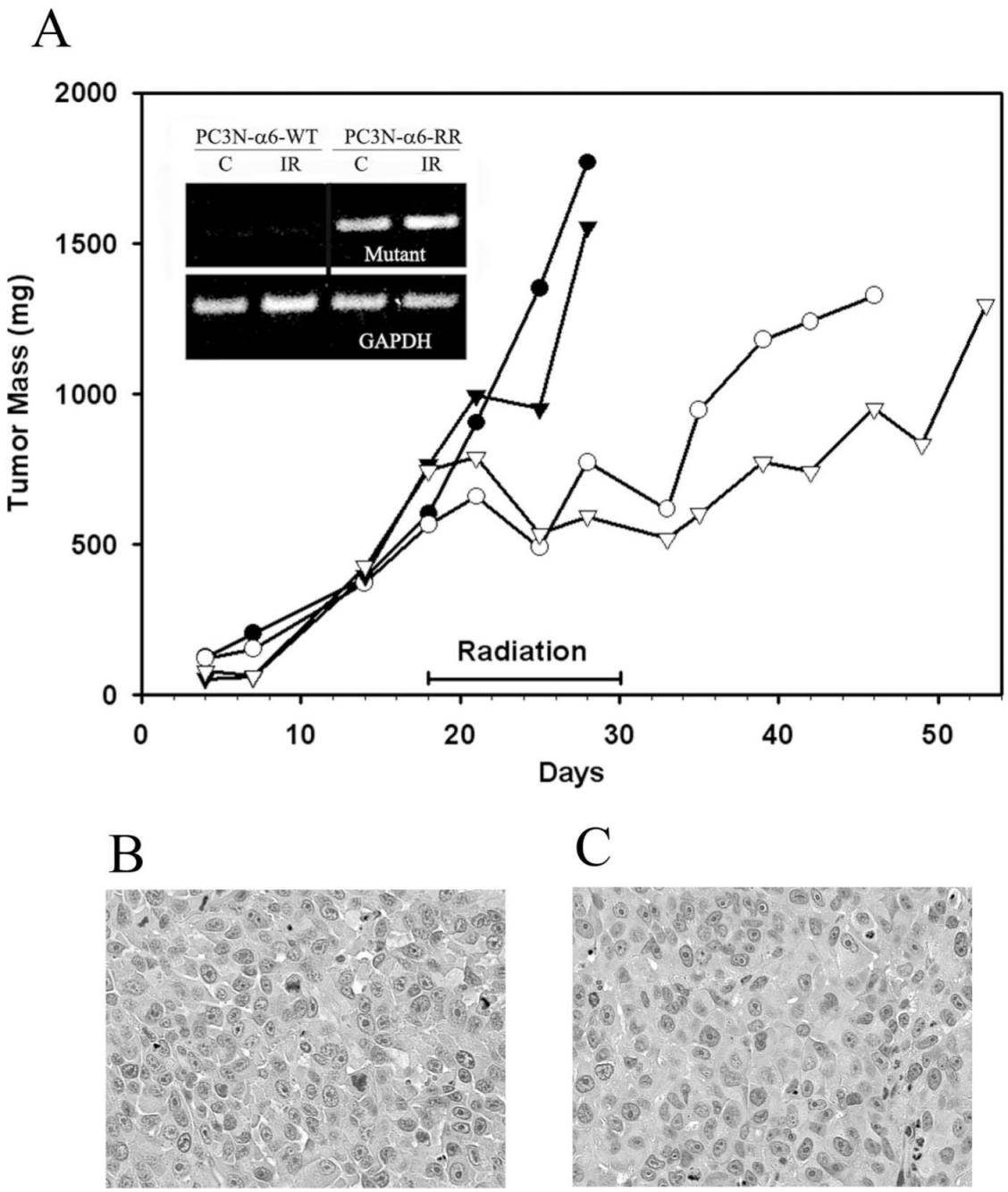

Figure 2.

Radiation response and histological appearance of PC3N-*α*6-WT and PC3N-*α*6-RR induced tumors. Four groups of 8 mice were subcutaneously inoculated with either PC3N-*α*6-WT or PC3N-*α*6-RR cells. Tumors were irradiated over 10 days for a total dose of 30 Gy. (A) The graph demonstrates median tumor mass (mg) over 53 days in untreated (●) and radiation treated (○) PC3N-*α*6-WT induced tumors and untreated (▼) and radiation treated (▽) PC3N-*α*6-RR induced tumors. Inset: Expression of the mutated integrin *α*6 in tumors formed using PC3N-*α*6-RR cells. RNA was extracted from PC3N-*α*6-WT and PC3N-*α*6-RR radiation treated (IR) and untreated (C) tumors. PCR was done on the cDNA generated from the RNA to detect and

amplify the mutated integrin α6. GAPDH amplification was carried out as control; (B) H&E staining of subcutaneous tumor from mouse injected with PC3N-α6-WT cells before radiation treatment; (C) H&E staining of subcutaneous tumor from mouse injected with PC3N-α6-RR cells before radiation treatment.

Table I

Radiation response of prostate tumor cells

Day	PC3N-α6-WT	PC3N-α6-WT + RT	PC3N-α6-RR	PC3N-α6-RR + RT
18	601 (319 – 1008)	564 (369 – 961)	763 (244 – 890)	745 (284 – 1009)
21	905 (327 – 1320)	658 (513 – 784)	995 (652 – 3025)	788 (505 – 1149)
25	1353 (359 – 1938)	489 (303 – 970)	953 (727 – 1615)	534 (316 – 1065)
28	1770 (603 – 2193)	773 (175 – 889)	1554 (696 – 2088)	593 (200 – 779)
33	—	615 (253 – 723)	—	518 (227 – 909)
35	—	947 (171 – 1481)	—	601 (211 – 1138)
39	—	1181 (259 – 1757)	—	773 (209 – 973)
42	—	1241 (423 – 2301)	—	741 (157 – 1160)
46	—	1326 (564 – 3058)	—	953 (322 – 1477)
49	—	—	—	832 (630 – 1807)
53	—	—	—	1298 (505 – 1895)

Numerical values represent the median and range (n = 8) of tumor mass (mg). Mice that receive radiation treatment (RT) were given a dose of 3 Gy per day for 10 days on a MTWTF schedule over 2 weeks. Mice were sacrificed when measured tumor mass exceeded 2000 mg. Measurements of tumor volume ceased when animal groups dropped below 6 mice.

Table II

Comparison of control and irradiated PC3N cell variants

Cell line and treatment	Median tumor wt [mg] on day 25 (*p* value)]	% T/C, day 25	Median time for tumor wt to reach 1300 mg in days (*p* value)	T-C (days)	Td (days)*	Log_{10} cell kill total
PC3N-*α*6-WT	1353	—	24.5 –	—	5.5	—
PC3N-*α*6-WT + RT	489 (*p* = 0.006)	36.1	45 (0.01)	20.5	—	1.1
PC3N-*α*6-RR	953 (*p* = 0.51)		26.5 (0.63)		10.5	
PC3N-*α*6-RR + RT	534 (*p* = 0.009)	39.5	53 (0.001)	28.5	—	1.6
PC3N-*α*6-RR + RT[t]	534 (*p* = 0.003)	56	53 (<0.001)	26.5	—	0.8

*Estimated from log-linear plot of data in Table I;

[t]PC3N-*α*6-RR + RT values calculated against PC3N-*α*6-RR values;

% T/C = (Median Tumor Weight of Treated/Median Tumor Weight of Control) × 100;

T-C is tumor growth delay based upon the median time in days required for the treatment group (T) and the untreated control group (c) tumor to reach 1300 mg;

Td = Tumor doubling in days from log-linear portion of the tumor growth curve for untreated tumors;

Log Cell Kill = T-C/(3.32 × Td).

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