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Disruption of estrogen receptor α -p53 interaction in breast tumors: a novel mechanism underlying the anti-tumor effect of radiation therapy

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Abstract

Inactivation of tumor suppressor p53 is one of the most frequent events in cancer. Unlike many other cancers, however, p53 gene mutations are infrequent in breast cancers, as about 80% of breast tumors contain wild type p53. The mechanisms underlying functional inactivation of wild type p53 in breast cancer have remained elusive. Besides, how p53 gets activated in breast tumors subjected to radiation therapy remains unknown. We recently reported that in MCF-7 breast cancer cells, estrogen receptor alpha (ER α) directly binds to p53 and represses its function. Furthermore, the ER α -p53 interaction was disrupted by ionizing radiation. These observations have important translational implications especially as there are no reliable cellular or molecular criteria for rational radiotherapy for breast cancer. Here we report our studies towards addressing this important issue, using an MCF-7 breast cancer xenograft model in mice. Radiation effectively inhibits growth of these tumors and stabilizes p53, but has no observable effect on ER α protein level. Importantly, chromatin immunoprecipitation (ChIP) assays demonstrated that ER α interacts with p53 bound to endogenous target gene promoters in tumors in vivo, and this interaction is considerably reduced in response to radiotherapy although p53 level is increased. Concomitant with its effect on ER α -p53 interaction, radiation increases p53-mediated transcriptional activation of several target genes and increases p53-mediated transcriptional repression of *survivin*. Our studies show that disruption of ER α -p53 interaction in vivo resulting in restoration of functional p53 is a cellular response to radiation. Radiation could be affecting ER α and/or p53 directly or it could be influencing other proteins associated with the ER α -p53 complex. To the best of our knowledge, this is the first report on analysis of DNA–protein–protein interaction occurring on endogenous gene promoters in vivo in breast tumor tissues. These findings suggest that alleviating the inhibitory effect of ER α on p53 could be one of the molecular mechanisms underlying activation of p53 by radiation in breast tumors, and therefore, could be exploited to develop more effective ways of combining radiation therapy with systemic therapies such as hormonal therapy and chemotherapy.

Keywords

Breast cancer; Chromatin immunoprecipitation; Estrogen receptor α ; Ionizing radiation; p21; Radiation therapy; Real-time PCR; Survivin; Tumor growth; Tumor suppressor p53; Xenograft mouse model

Introduction

Tumor suppressor p53 plays a crucial role in various physiological processes, including cell cycle progression, apoptosis, and maintenance of genome integrity. Mutations in p53 have been found in approximately 50% of all human cancers [1–3]. By contrast, only about 20% of breast cancers have p53 mutations [4]. In cancers which express wild type p53, its functions are also frequently inactivated by various mechanisms. For example, overexpression or amplification of MDM2, a major negative regulator of p53, is observed in various cancers, especially in soft tissue sarcomas [5–7]. However, overexpression or amplification of MDM2 is a rare event in breast cancers [8, 9]. Mechanisms underlying the inactivation of p53 functions in breast cancers are not fully understood.

Estrogen receptor alpha (ER α) regulates growth and development of various tissues and promotes proliferation of breast cancer cells [10, 11]. Recently our laboratory demonstrated that ER α binds to p53 and represses its functions in breast cancer cells, suggesting that inhibition of p53 functions by ER α contributes to the inactivation of p53 [12, 13]. We had reported ER α -p53 interaction at the p53 binding sites on the endogenous p21 and survivin gene promoters in MCF-7 cells and that ER α cannot access these promoter sites in the absence of p53. We had shown this both by knocking down p53 in MCF-7 cells and by looking at the recruitment of exogenously expressed ER α to these promoters in p53-null cells. In both cases ER α was unable to bind to the promoter sites. Importantly, the interaction between ER α and p53 in MCF-7 cells was disrupted by ionizing radiation [12]. Radiation therapy, a common treatment modality for breast cancer both in the absence of, and in conjunction with, adjuvant systemic therapy, has been shown to reduce local recurrences and overall mortality in breast cancer patients [14–18]. However, the molecular mechanisms that dictate response to radiation therapy remain unclear. At the cellular level, it is well documented that radiation elicits complex cellular responses, among which activation of the p53 signaling pathway is critical [19]. Radiation-induced p53 phosphorylation and disruption of MDM2-p53 interaction results in p53 stabilization and activation [20], which ultimately leads to cell cycle arrest or apoptosis. However, the effect, if any, of radiation on other protein-protein interactions that regulate p53 in vivo, especially in tissues where MDM2 is not over-expressed, is not known. The mechanism(s) underlying induction of p53 activity in tumors subjected to ionizing radiation remains unknown. To address this important issue, we investigated the effect of ionizing radiation on tumor growth and its effect on ER α -p53 interaction using the MCF-7 breast cancer cell xenograft in mice as a model system. Our experiments have revealed ER α -p53 interaction as a major target of ionizing radiation and this finding could have translational significance in improving breast cancer therapy.

Materials and methods

Cell culture

MCF-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen) at 37°C under 5% CO₂.

Preparation of 17- β estradiol (E2) implants and measurement of plasma E2 levels

To prepare E2 implants, E2 was mixed with cholesterol at a mass ratio of 1:9 and injected by syringe into Silastic tubing with an inner diameter of 1.57 mm (Dow Corning, Midland, MI). The tubing was sealed with A-100-S Type A Medical Adhesive (Factor II, Inc., Lakeside, AZ). The final E2 implant was 0.8 cm in length. Plasma E2 concentrations were measured using the Estradiol EIA Kit (Cayman Chemical, Ann Arbor, MI).

Animals and radiation treatments

Female athymic NCr-nu/nu mice bilaterally ovariectomized at 3 weeks of age were obtained from the National Cancer Institute at Frederick Animal Distribution System. Animals were housed in the institutional Department of Laboratory Animal Resources. At 5 weeks of age, E2 pellets were implanted s.c. in the interscapular area. One week later, 5×10^6 MCF-7 cells suspended in matrigel were injected s.c. into the region adjacent to the fourth mammary gland. The matrigel was from the EHS sarcoma as we had described previously [21]. When the xenograft tumors reached approximately 6 mm in diameter (usually 5 weeks after MCF-7 cell inoculation), radiation treatments were performed using a Philips RT 250 Orthovoltage X-ray machine. The area surrounding the tumor was shielded to achieve tumor-specific irradiation. The tumors were exposed to a total of 15 Gy radiation in 5 fractions of 3 Gy for 5 consecutive days. Animals for mock irradiation were placed in the irradiator exactly as those for irradiation except that the X-ray was not turned on. After radiation, tumor size was measured twice a week with electronic digital caliper to monitor tumor growth. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the Roswell Park Cancer Institute.

Immunohistochemistry (IHC) of p53 and ER α

For IHC studies, the animals carrying the xenograft tumors were sacrificed by CO₂ inhalation 4 h after the final dose of radiation on the fifth day and the resected tumors were fixed in 10% formalin. IHC staining was performed by the Pathology Core Facility at Roswell Park Cancer Institute as follows: formalin fixed paraffin sections were cut at 5 μ m, placed on charged slides and dried in a 60°C oven for 1 h. Slides at room temperature were then deparaffinized in three changes of xylene and rehydrated using graded alcohols. Endogenous peroxidase was quenched with aqueous 3% H₂O₂ for 10 min and washed with phosphate buffered saline-Tween20 (PBS/T). Antigen retrieval was performed with citrate buffer for 10 min (near boil) in a microwave oven with a 15 min cool down followed by rinsing in PBS/T. Slides were then stained in a DAKO autostainer under the following conditions: Non-specific sites were blocked with 0.03% casein (in PBS/T) for 30 min. The primary antibodies against p53 (E26; from Abcam) and ER α (MC-20, from Santa Cruz) at a 1:100 and 1:4,000 dilution, respectively, were applied to slides for 1-h. An isotype-matched

control IgG was used on a duplicate slide in place of the primary antibody as a negative control. A PBS/T wash was followed by the biotinylated secondary antibody (goat anti-rabbit) for 30 min. Slides were washed with PBS/T followed by the ABC Elite reagent (Vector Lab) for 30 min. The slides were further washed with PBS/T and the chromagen DAB was applied for 5 min (color reaction product—brown). The slides were then counterstained with hematoxylin, and dehydrated, cleared and cover-slipped. To determine the percentage of p53 and ER α positive cells, 2,000 cells were screened in each group.

Chromatin immunoprecipitation (ChIP)

The animals carrying the xenograft tumors were sacrificed by CO₂ inhalation 4 h after the final dose of radiation on the fifth day and the resected tumors were minced with a razor blade in the presence of an aqueous solution of protease inhibitor cocktail (Roche Diagnostics). The tissue was then cross-linked with 1.5% formaldehyde at room temperature for 15 min. Cross-linked samples were washed twice with ice cold PBS and homogenized in ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1). Homogenized samples were incubated on ice for 30 min and then subjected to sonication. Subsequent ChIP assay steps were performed as described previously [12, 13].

Quantitative real time-PCR (qRT-PCR)

The *qRT-PCR* assay was performed as previously described [12, 13]. Essentially, total RNA from MCF7 xenograft tumors resected from animals sacrificed 4 h after the final dose of radiation on the fifth day was extracted using the “Absolutely RNA Miniprep Kit” (Stratagene). For analyzing RNA levels of p53 regulated genes, 500 ng of total RNA was reverse-transcribed in 20 μ l of reaction using the “iScript cDNA Synthesis Kit” (Bio-Rad). One μ l of the resulting cDNA was used in a total volume of 25 μ l of PCR reaction. Real-time PCR was carried out in an Applied Biosystems Prism 7300 Sequence Detection System using iTaq SYBR Green Supermix (Bio-Rad). The relative RNA levels in radiation treated MCF-7 xenograft versus control were calculated using the C_t method with the endogenous β -actin RNA as control. Sequences of primers used are shown in Table 1.

Western blot analysis

The xenograft tumors resected from animals sacrificed 4 h after the final dose of radiation on the fifth day were homogenized and lysed in Laemmli buffer. Protein concentrations were measured using the RC DC method (Bio-Rad). Sixty μ g protein was separated by 10% SDS polyacrylamide gel electrophoresis and then transferred to PVDF membrane. Primary antibodies against ER α (HC-20), p21 (C-19), p53 (C-19), and survivin (FL-142) were obtained from Santa Cruz Biotechnology. Antibody against β -actin (A2066) was obtained from Sigma.

Statistical analysis

Unpaired Student *t*-test was used to compare the tumor size of the radiation treated xenograft to the sham-radiated group. A *P* value <0.05 was considered statistically significant.

Results

Radiation inhibits MCF-7 xenograft tumor growth

We used the MCF-7 xenograft model to study radiation responses in breast cancer. Growth of xenograft was maintained by continuous supply of E2. With the E2 implant, which resulted in a plasma E2 concentrations of 35.3 ± 4.9 pg/ml ($n = 4$) 3 weeks after E2 pellet implantation, a 100% tumor incidence was observed in this model. Xenografts were subjected to a total dose of 15 Gy ionizing radiation in 5 fractions of 3 Gy for 5 consecutive days. Compared to the sham-radiation group, significant inhibition ($P < 0.01$) of tumor growth by radiation was observed from 2 weeks onwards after completing the radiation treatment (Fig. 1).

Radiation disrupts ER α -p53 interaction on the p21 and survivin gene promoters

Previously we reported that ER α directly binds to p53 on the p21 and survivin gene promoters and that the ER α -p53 interaction was disrupted by radiation in MCF-7 cells [12, 13]. To determine if radiation had a similar effect in MCF-7 xenografts, we analyzed the ER α -p53 interaction on the p21 and survivin gene promoters (Fig. 2a) by ChIP assay. Consistent with our previous observation in MCF-7 cells, we detected ER α -p53 interaction at the p53-binding sites on both the p21 and survivin gene promoters (Fig. 2b). This interaction was greatly diminished in response to radiation (Fig. 2b, compare lanes 5 & 10), in spite of increased p53 protein level in irradiated tumors (Figs. 4 and 5). Neither p53 nor ER α -binding were detected on the non-specific (NS) site on either promoter. Similarly, as a negative control, there was no binding when IgG was used for immunoprecipitation.

Radiation increases expression of genes that are transcriptional activation targets for p53 and decreases expression of the survivin gene, a target for transcriptional repression by p53

As a transcription regulator, p53 activates transcription of a variety of genes in response to genomic stress. We used quantitative Real Time-PCR (qRT-PCR) to examine if radiation enhanced p53-activated transcription of target genes. Exposure to radiation increased transcription levels for several p53-activated genes (Fig. 3a). The RNA level of the CDK inhibitor, p21, was increased more than 10 fold. The increased p21 RNA level was in agreement with its protein level (Fig. 5). Another cell cycle related gene BTG2 was increased 3-fold. Although a moderate increase was also observed for apoptotic genes including FAS, DR5, PUMA, and NOXA (Fig. 3), no evident apoptosis was detected by PARP cleavage and TUNEL assays (data not shown). Besides activating transcription, p53 is also known to repress transcription of a number of genes such as survivin. To determine whether radiation affects transcription of target genes repressed by p53, we analyzed survivin RNA and protein levels in response to radiation. Survivin is a member of the inhibitor of apoptosis (IAP) family and is expressed in most types of cancer, in contrast to most normal adult tissues where its expression is very low. Survivin can inhibit apoptosis and as well as positively regulate progression through the cell cycle [22, 23]. Our results showed that both the survivin RNA and protein levels were considerably reduced by radiation (Figs. 3b, 5).

Radiation stabilizes p53 protein

Radiation usually induces p53 stabilization and accumulation in tumor cells. Since we observed effective inhibition of tumor growth by radiation in MCF-7 xeno-grafts, it was important to determine whether radiation stabilized p53 in vivo. Using IHC, we detected 15.2% p53 positive cells in radiation treated samples, compared to 8.2% in the control group (Fig. 4). Consistently, increased p53 protein level was also detected by western blot analysis (Fig. 5). By contrast, radiation did not affect ER α protein level as monitored by IHC (Fig. 4) or by western blotting (Fig. 5).

Discussion

Radiation has been shown to elicit complex cellular responses in various cancer cells, which results in inhibition of cell growth or apoptosis. Because of its ability to kill cancer cells, radiation therapy has been a common treatment modality for cancer patients [19, 24]. However, cellular and molecular mechanisms underlying the response to radiation therapy of tumors have not been investigated rigorously. Understanding the cellular responses to radiation in vivo in animal models will be very helpful for optimizing radiation therapy to elicit maximal antitumor effects while reducing bystander effects on normal cells. Towards this goal, we investigated the response of MCF-7 xenograft tumors in mice to ionizing radiation. As reported [25], we observed that radiation effectively inhibited the growth of MCF-7 xenografts. Radiation responsiveness of MCF-7 xenografts could be due to the elevated estrogen levels supplied by the constant estrogen-releasing pellets, as it has been reported that estrogen treatment enhances response to radiation [26]. Radiation sensitivity of breast cancer cells is also affected by their p53 status, i.e. cells expressing wild type p53 are more sensitive to radiation [4]. Activation of tumor suppressor p53 signaling pathways by radiation has been demonstrated in many model systems. It has been shown that p53 elicits cell- and tissue-specific gene expression profiles in response to radiation, but induction of p21 by radiation has been consistently observed in different model systems [27–30]. In the xenograft tumor model, we observed that radiation stabilized p53 and enhanced transcription of target genes activated by p53, especially in the case of p21 where the response was dramatic. In addition, radiation also increases expression of apoptotic genes such as FAS and PUMA. However, TUNEL and PARP cleavage assays did not reveal any apoptosis (data not shown), suggesting that the inhibitory effect of radiation on tumor growth probably was due to cell cycle arrest rather than apoptosis. Based on the earlier reports that induction of p21 by ionizing irradiation results in G1 and G2 arrest and inhibition of apoptosis in U2OS osteosarcoma cells [31] and in MCF-7 cells [32–34], it is likely that the transcriptional activation of p21 leads to blockage of apoptosis in the xenograft tumors.

Interestingly, we observed that survivin, a target gene repressed by p53, was drastically down-regulated by radiation. Survivin has been shown to play an important role in radiosensitivity in tumor cells [23, 35], with its high levels associated with resistance to radiotherapy. Down regulation of survivin by radiation has been reported in human umbilical vein endothelial cells and A549 cells [36, 37], and shown to sensitize tumor cells to chemotherapeutic agents [38, 39]. Thus, our results suggest that down regulation of survivin combined with upregulation of p21 may play an important role in the radiation

response of the xenografts. Future studies should reveal whether these changes in response to radiation can sensitize the tumor to chemotherapeutic agents.

Intriguingly, hormonal therapy has been known to interact with radiation therapy to substantially improve local tumor control [40] as well as to decrease distant metastases and improve patient survival [41, 42]. One interesting possibility could be that such tumors containing wild type ER α and p53 may be benefited by radiation therapy because of its ability to disrupt ER α -p53 interaction leading to restoration of functional p53. Radiation-induced changes in expression of target genes activated or repressed by p53 play a critical role in inhibiting tumor growth. The effect of radiation on interactions between p53 and regulatory proteins could also contribute to p53 activation and radiation-induced inhibition of tumor growth. For example, disruption of MDM2-p53 interaction by radiation leads to p53 stabilization and activation [20]. Several proteins have been reported to interact with p53 and modulate its functions. However, whether radiation affects those protein-protein interactions is not yet known. Previously, we reported that ER α binds to p53 and represses its functions and radiation disrupts the ER α -p53 interaction in MCF-7 cells in culture. We show here that radiation also disrupts ER α -p53 interaction in the in vivo MCF-7 xenograft model, demonstrating that disruption of ER α -p53 interaction is a cellular response that occurs in vivo in tumors in response to radiation. In addition, our studies have shown that disruption of the ER α -p53 interaction in tumors is associated with radiation-induced activation of p53 and inhibition of tumor growth. It is likely that there could be one or more other proteins in the complex that allows ER α to interact with p53 in these cells, and such interactions could be influenced by radiation. Those multi-protein interactions regulated by temporal and spatial cues in the tumor and could affect response to radiation. Radiation itself could alter or modify such interaction generating a two-way influence. Future studies with the help of advances in technologies should address these complexities. We had previously reported that disruption of the ER α -p53 interaction by knocking down ER α sensitizes cultured MCF-7 cells to staurosporine-induced apoptosis [13]. Since radiation alleviates the inhibitory effect of ER α on p53 and down regulates survivin expression, radiation therapy could sensitize the tumor to other chemotherapeutic agents. Restoration of p53 functions has been an attractive cancer therapeutic strategy [43, 44]. One approach is to remove inhibitory factors that inactivate p53. For example, nutlin is a small molecule that specifically prevents MDM2 binding to p53 and induces apoptosis [45]. Since ER α is a major inhibitor of p53 in breast cancer cells [12, 13], our current demonstration that radiation disrupts the ER α -p53 interaction and enhances p53 functions in vivo in tumors suggests that radiotherapy may be more effective in ER-positive breast cancers expressing wild type p53. Future clinical studies should address this interesting possibility. In addition, understanding the molecular determinants of effects of radiotherapy on tumors should help in developing more effective ways of combining radiation therapy with systemic therapies such as hormonal therapy and chemotherapy.

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Institute (R01CA79911), and the NCI Cancer Center Support Grant to the Roswell Park Cancer Institute (CA016056).

Abbreviations

ERα	Estrogen receptor α
E2 17-β	Estradiol
ChIP	Chromatin immunoprecipitation
DAB	3, 3'-Diaminobenzidine tetrahydrochloride
EHS	Engelbreth-Holm-Swarm
IHC	Immunohistochemistry
PARP	Poly(ADP-ribose)polymerase
PBS/T	Phosphate buffer saline/Tween
qRT-PCR	Quantitative real time polymerase chain reaction
s.c.	Sub-cutaneous
TUNEL	Terminal transferase dUTP nick end labeling

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Fig. 1.

Radiation inhibits tumor growth in the MCF-7 xenograft model. MCF-7 xenografts were subjected to mock ($n = 5$) or radiation ($n = 6$) treatment. After radiation, average tumor diameters were measured twice a week for 5 weeks. Data represents mean $\pm$ SE. From 14 days post-irradiation, significant difference ($P < 0.01$) was observed

Fig. 2. Radiation disrupts ERα-p53 interaction in vivo in MCF7 xenograft tumors in mice. **(a)** Schematic representation of human *p21* and *Survivin* gene promoters. The horizontal arrows represent primers used for PCR following ChIP. The transcription start site is denoted by a bent arrow. **(b)** ChIP assay products were run on a 1.5% agarose gel and stained with ethidium bromide. NS, non-specific site without any p53-binding sequence; MlgG and RlgG, mouse and rabbit IgG, respectively. Data shown are representative of two independent ChIP assays from tissue samples from two animals

Fig. 3. Radiation enhances p53-mediated transcriptional regulation. Total RNA was extracted from mock and radiation treated xenografts. RNA levels of p53-regulated target genes were analyzed by qRT-PCR using SYBR green method. Relative RNA level in mock treated group was normalized to 1. Data shown represent mean $\pm$ SD of independent measurements from tumors from two animals. **(a)** Effect of radiation on genes activated by p53. **(b)** Effect of radiation on survivin, an example of genes repressed by p53

Fig. 4.

Radiation stabilizes p53 but does not alter ERα in MCF-7 xenografts. Representative IHC staining for p53 and ERα. Arrows indicate examples of p53 positive and ERα positive cells. Among 2,000 cells screened, 8% and 15% cells were positive for p53 in mock and radiation treated tumors, respectively. For ERα, 42% and 40% of the cells were positive in control and radiation treated tumors, respectively. Photographs were taken under the 40× objective

Fig. 5. Radiation increases p21 and decreases survivin protein levels in xenograft tumors in mice. Proteins were extracted from mock and radiation treated xenografts. Protein levels were analyzed by western blotting. Data shown are representative of independent western blotting analysis of samples from two animals

Table 1

Sequences of primers used for quantitative real time- PCR

Gene name	Forward primer (5' → 3')	Reverse primer (5' → 3')
Actin	ATGGGTCAGAAGGATTCCTAT	AAGGTCTCAAACATGATCTGGG
BAX	TGGAGCTGCAGAGGATGATTG	CCAGTTGAAGTTGCCGTCAGA
BID	TCCTTGCTCCGTGATGTCTTT	GTCCGTTCAAGTCCATCCCATT
BTG2	GTGAGCGAGCAGAGGCTTAAG	GAGCCCTTGGACGGCTTT
DR5	CCCAGTGGATGGAACATCCT	CACAAACGGAATGATCCAGACA
FAS	GGACCCTCCTACCTCTGGTTCTT	TCATGATGCAGGCCTTCCA
GADD45	CTCAACGTCGACCCCGATAA	GCCTGGATCAGGGTGAAGTG
NOXA	GGAGGTGCACGTTTCATCAA	TGTATTCCATCTTCCGTTTCCA
p21	GAGACTCTCAGGGTCGAAAACG	GATGTAGAGCGGGCCTTTGA
PIDD	TCCAGAAATGCCCAGACTGTT	CCGATAGCGGATGGTGATG
PIG3	TCACCAAAGGTGCTGGAGTTAA	GAGAACCCATCGACCATCAAG
PTEN	GTTGCAGCAATTCAGTGTAAAGCT	TAGGGCCTCTTGTGCCTTTAAA
PUMA	ATGCCTGCCTCACCTTCATC	TCACACGTCGCTCTCTCTAAACC
Survivin	TAGCCTGCCAACAGCCATCT	AGTCTATCTCAGGCCGACTCAGA