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Low doses ionizing radiation enhances the invasiveness of breast cancer cells by inducing epithelial–mesenchymal transition

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ABSTRACT

Epithelial–mesenchymal transition (EMT) is a process cellular morphologic and molecular alterations facilitate cell invasion. We hypothesized that low dose ionizing irradiation (LDIR) enhances the invasiveness of breast cancer cells by inducing EMT. The effects of LDIR on cellular morphology and the EMT markers of MCF-7 breast cancer cells were analyzed by western blot/RT-PCR and migration/invasion was examined using the transwell assay. We found that LDIR led to the phenotypic changes of EMT in MCF-7 cells and down-regulation of epithelial differentiation markers and transcriptional induction of mesenchymal markers. Furthermore, the radiated cells demonstrated enhanced migration/invasion MCF-7 cells compared with non-radiated cells. In summary, LDIR promotes the invasiveness of breast cancer cells through epithelial to mesenchymal transition. These findings may ultimately provide a new targeted approach for improving the therapeutic effectiveness of radiation in breast cancer.

1. Introduction

Breast cancer remains the most common female malignancy around the world, with an international incidence of more than 1.2 million. Advances in the multi-disciplinary approach to treatment with improved combinations of surgery, chemotherapy, radiotherapy and endocrine therapy have led to significant improvements in outcomes. Despite these advancements, more than half a million women will die of breast cancer annually. Thus within each treatment modality, ongoing investigations to improve therapeutic benefits continue.

Radiation therapy is an important component of definitive treatment for both early-stage and locally advanced breast cancer. Multiple studies have now demonstrated the benefits of radiation in reducing local–regional recurrence risk and improve survival in both the breast conserving and post-mastectomy setting [1–4]. Notwithstanding the therapeutic benefits of radiation, there are emerging data implying that ionizing radiation may have cause changes in the microenvironment that produce anti-tumor effects [5]. Clinical and laboratory data suggest that ionizing radiation may promote the metastatic ability of cancer cells and may elicit changes in the host microenvironment that may facilitate tumor progression and development of second malignancies [5–8]. Recently, several investigations have attempted to identify the potential mechanisms by which ionizing radiation activates cellular targets that may be facilitating tumor invasion and metastasis. The increased expression of matrix metalloproteinases (MMPs) [9] and enhanced angiogenesis [7] have been suggested as possible contributors in increasing cellular metastatic potential, however, the mechanisms of action remain unclear. Our group and others have previously demonstrated that epithelial to mesenchymal transition (EMT) plays an important role in tumor progression and metastasis [10–13]. In the current study, we hypothesize that low doses ionizing irradiation enhances the invasiveness of breast cancer cells by inducing EMT. We investigated the effects of low dose radiation on breast cancer cells to determine if induction of EMT increased the potential for cellular invasion, to further elucidate the mechanism of the anti-tumor effects of radiation.

2. Materials and methods

2.1. Materials

The reagents utilized were as follows: Dulbecco's Modified Eagle's Medium [DMEM] (Gibco-BRLRockville, IN, USA), Fetal bovine serum [FBS] (Haoyang Biological Manufacturer Co. Ltd., Tianjin, China), anti-E-cadherin/anti-Snail antibody/anti-vimentin antibody (Cell Signaling Technology, Beverly, MA, USA), anti-mouse IgG horseradish peroxidase [HRP] antibody (ZhongShan Goldenbridge, Beijing, China). The Pro-lighting HRP agent for western

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blotting detection was purchased from Tiangen Biotech CO. LTD (Beijing, China) and the remaining cell lysis buffers and reagents from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise specified. The MCF-7 breast cancer cell line (American Type Culture Collection ATCC, Rockville, MD, USA) and the NIH3T3 mouse fibroblast cell line (American Type Culture Collection Rockville, MD, USA) were utilized for this study. The MCF-7 and NIH3T3 cells were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS Gibco-BRL and 100 units of penicillin–streptomycin at 37 °C with 5% CO2 in a humidified incubator.

2.2. Radiation technique

The cells were irradiated at room temperature using a Faxitron Cabinet X-ray System (Faxitron, Wheeling, IL, USA). The radiation was filtered through a 0.5 mm aluminum filter resulting in a dose rate of 0.4 Gy/min.

2.3. Quantitative reverse-transcription PCR (RT-PCR) analysis

The (RT-PCR) analysis was performed as previously described [14]. Briefly, total RNA were extracted with TRIzol reagents according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from 1 μg of total RNA by PrimerScript RT Reagent Kit (TaKaRa, Shiga, Japan). Real-time quantitative RT-PCR (QRT-PCR) was performed using a SYBR green PCR mix in Applied Biosystems StepOne and StepOnePlus Real-Time PCR Systems (Applied Biosystems, Inc., Foster City, CA). The gene expression of mRNAs from each sample was calculated by normalizing with endogenous control beta-actin. The experiments were repeated in triplicate to confirm findings.

2.4. Western blot analysis

The cells were washed twice with cold phosphate-buffered saline (PBS) and lysed in RIPA buffer (Shennengbocai, Shanghai, China) (1% NP40, 0.1% SDS, 5 mM EDTA, 0.5% sodium deoxycholate, 1 mM sodium vandate) containing protease and phosphatase inhibitors (1 mM PMSF, 1 mM sodium fluoride). The samples were incubated on ice for 30 min and centrifuged at 12,000*g* for 15 min at 4 °C. The supernatants were collected, and the protein concentration was measured by the BCA Protein Assay Kit (Merck, Darmstadt, Germany). Equal amount of protein were separated by sodium laurylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE), electrotransferred to polyvinylidene fluoride membranes (ImmobilonP; Millipore, Bedford, MA) and blocked in 5% nonfat dry milk in Tris-buffered saline, pH 7.5 (100 mM NaCl, 50 mM Tris and 0.1% Tween-20). Membranes were immunoblotted overnight at 4 °C with with primary antibodies, followed by their respective horseradish peroxidase conjugated secondary antibodies (KPL, Gaithersburg, MD, USA). Signals were detected by enhanced chemiluminescence. Beta-actin was used as the loading control.

2.5. Invasion and migration assay

The invasion assays were performed in 24-well transwell chambers (Corning, Acton, MA, USA) containing polycarbonate filters with 8-μm pores coated with matrigel (BD Biosciences, Bedford, MA, USA). Initially, the chambers were rehydrated with DMEM (serum free) for 2 h at 37 °C in 5% CO2 atmosphere. Five hundred microlitres of balanced mixture of the conditional medium from NIH3T3 fibroblasts and the complete medium was added to the lower compartment as the chemotactic factor. Then 1×10^5 cells in serum-free DMEM were added to the upper compartment of the chamber. Each cell group was plated in 3 duplicate wells. After incubation for 24 h, the noninvasive cells were removed with a cotton swab. Cells that had migrated through the membrane and stuck to the lower surface of the membrane were fixed with methanol and stained with crystal violet. Finally, the cells in the lower compartment of the chamber were counted under a light microscope in at least ten random visual fields. The migration assay was similar to the invasion assay described above, except that the upper side of the membranes was not coated with the matrigel.

2.6. Statistical analysis

Statistical analysis was carried out using the software SPSS 16.0 (Chicago, IL, USA). Student's *t*-test was chosen to analyze the statistical difference. Results were presented as mean ± stand error of mean (SEM). $P < 0.05$ was considered statistically significant. Each trial was individually analyzed and then compared to assess for variance.

3. Results

3.1. The effects of low dose radiation on cellular morphology

When irradiating the parental MCF-7 cells with fractioned doses of 1 Gy/day and 2 Gy/day respectively, the radiated cells demonstrated changes in their morphology when receiving a total threshold dose of 20 Gy or greater. As shown in Fig. 1, the parental MCF-7 cells typically exhibit a cobblestone-like appearance and tight cell–cell junction which is characteristic of the epithelial phenotype, but when the cells were radiated to doses of >20 Gy, they developed a spindle-like morphology, loss of cell-to-cell contact and an increase in the cell scattering, which is more characteristic of fibroblast. These morphologic changes were maintained at doses above 20 Gy. While the same experiment was conducted with T47D cells, the degree of cellular changes were less prominent compared to the MCF-7 cells (data not shown), and thus, the MCF-7 cells were utilized for the remainder of the study.

3.2. Effects of low dose radiation on mesenchyal and epithilial markers

The expression of EMT markers was conducted in the irradiated cells that had received a total dose of 20 Gy. RT-PCR demonstrated that the epithelial markers E-cadherin and CK-18 were down-regulated in radiated cells while the mesenchymal markers vimentin and fibronectin were upregulated (Fig. 2A). The results of the western blot to detect the expression of E-cadherin and vimentin is shown in Fig. 2B.

A number of transcriptional repressors including Snail, Twist and Slug could inhibit the EMT by targeting the E-cadherin transcription during progress of cancers growth and invasion [15,16]. The transcriptional repressors (Snail, Twist and Slug) were analyzed and as expected, the Snail and Twist ($p = 0.0097$ and $P = 0.0012$ respectively) were significant elevated while the Slug did not significantly changed ($p = 0.2973$), further demonstrating the effects of low dose radiation on breast cancer cells to produce morphologic changes from epithelial to mesenchyal cell types (Fig. 2A).

3.3. Effects of low dose radiation on cellular migration and invasion

The effects of low dose radiation on cellular migration and invasion in MCF-7 cells was evaluated by assessing motility using a two-chamber assay. As shown in Fig. 3, the ability of low dose radiation to increase the invasive capability of breast cancer cells was demonstrated by the significant difference in the absolute number of cells migrating and invading through the membrane into the lower compartment of the chamber in the radiated cells

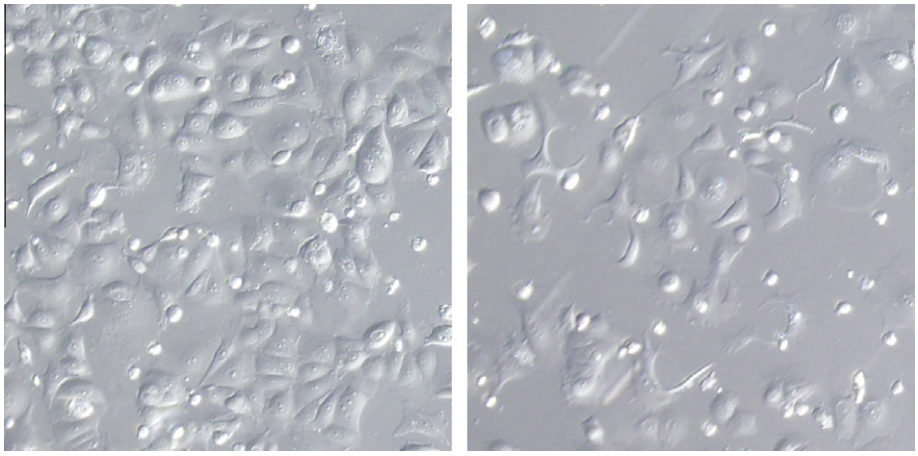

Fig. 1. Low doses ionizing radiation induces phenotypic changes. Cells were examined with phase contrast microscopy. The left panel shows the parental (non-radiated) MCF-7 cells while right panel shows the irradiated MCF-7 cells.

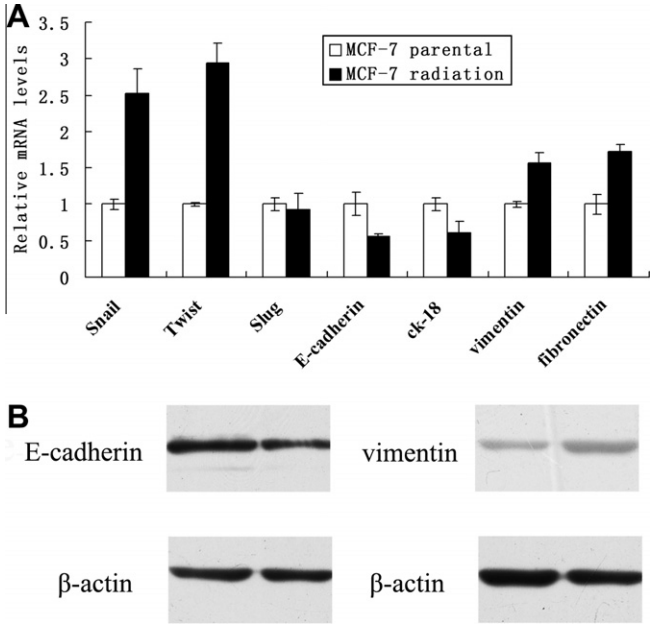

Fig. 2. Changes in EMT markers induced by low doses ionizing radiation. (A) Expression of EMT markers (E-cadherin, CK-18, vimentin, fibronectin) and transcription factors (Snail, Twist, Slug) examined by reverse transcription real-time PCR (data shown as mean ± SED). (B) Expression of epithelial marker E-cadherin and mesenchymal marker vimentin by western blot analysis.

(65.8 ± 5.7 and 24.3 ± 2.6 respectively, $p = 0.0039$) compared with the parental MCF-7 cells (47.2 ± 3.2 and 6.2 ± 1.4 respectively $p = 0.0002$).

4. Discussion

Radiotherapy is an integral part of definitive treatment for breast cancer. Despite recent advances, radiation resistance remains a challenge in some patients and thus an understanding of the molecular mechanisms that potentiate radio-resistance is critical prior to the development of new radiotherapeutic strategies to overcome them [17,18].

The epithelial to mesenchymal transition (EMT), defined as the observed change in cellular morphology from epithelial to mesenchymal phenotype [19], has been observed in radiated breast cancer cells. EMT can lead to increased cellular adhesion, apical-basal polarity, cellular motility, increasing the potential for invasion/metastasis. This phenomenon is characterized by the loss of cell–cell adhesion molecules, down-regulation of epithelial differentiation markers, and transcriptional induction of mesenchymal markers [20]. We and other groups have reported that EMT plays an important role in tumor progression and metastasis [10–14]. Also, EMT is reported to be related to radioresistence in many cancers including breast cancer [21–23]. In the present study, we have demonstrated that delivering low doses of radiation to breast cancer cells induces the cells to exhibit the classic hallmarks of EMT which ultimately promote increased cellular invasiveness. We demonstrated that doses of 20 Gy or greater induce a morphologic change in the breast cancer cells from cobblestone-like epithelial morphology to the spindle-shaped mesenchymal cells. Confirming these visual changes on a molecular level, we demonstrated that the epithelial markers E-cadherin and CK-18 were down-regulated while the mesenchymal markers vimentin and fibronectin were up-regulated, consistent with the biology of EMT. Further supporting these findings, we found that the transcription repressors Snail and Twist that are critical regulators of EMT were upregulated in the radiated breast cells [10,12,16,24]. Lastly, our *in vitro* assays found significantly more migration/invasion in the radiated cells. All of these findings suggest that that low dose ionizing radiation induces changes in the cellular micro-environment that lead to mesenchymal changes which significantly enhance the ability of breast cancer cells for migration and invasion.

It is important to note that these findings of radiation-induced enhancement of invasiveness was observed at doses that are clinically utilized for standard fractionated breast radiation treatment, thus these laboratory findings are clinically meaningful. While previous reports have acknowledged Snail and Twist as transcription factors associated with EMT induction [25,26] and EMT has been postulated to be one of the critical steps in the progression of malignancy [22,27–29], our current study confirms the occurance of these processes in breast cancer cells treated with standard fractionated doses of radiation, providing further insight into the mechanisms of radio-resistance. It is possible that concomitant use of an agent that can potentially reverse the EMT process during radiation therapy may prove to enhance the effectiveness of radiotherapy for breast cancer.

To the best of our knowledge, this is the first study to demonstrate that the low dose radiation enhances the potential for invasion of breast cancer cells, specifically through changes in the radiated micro-environment that induce EMT. We have

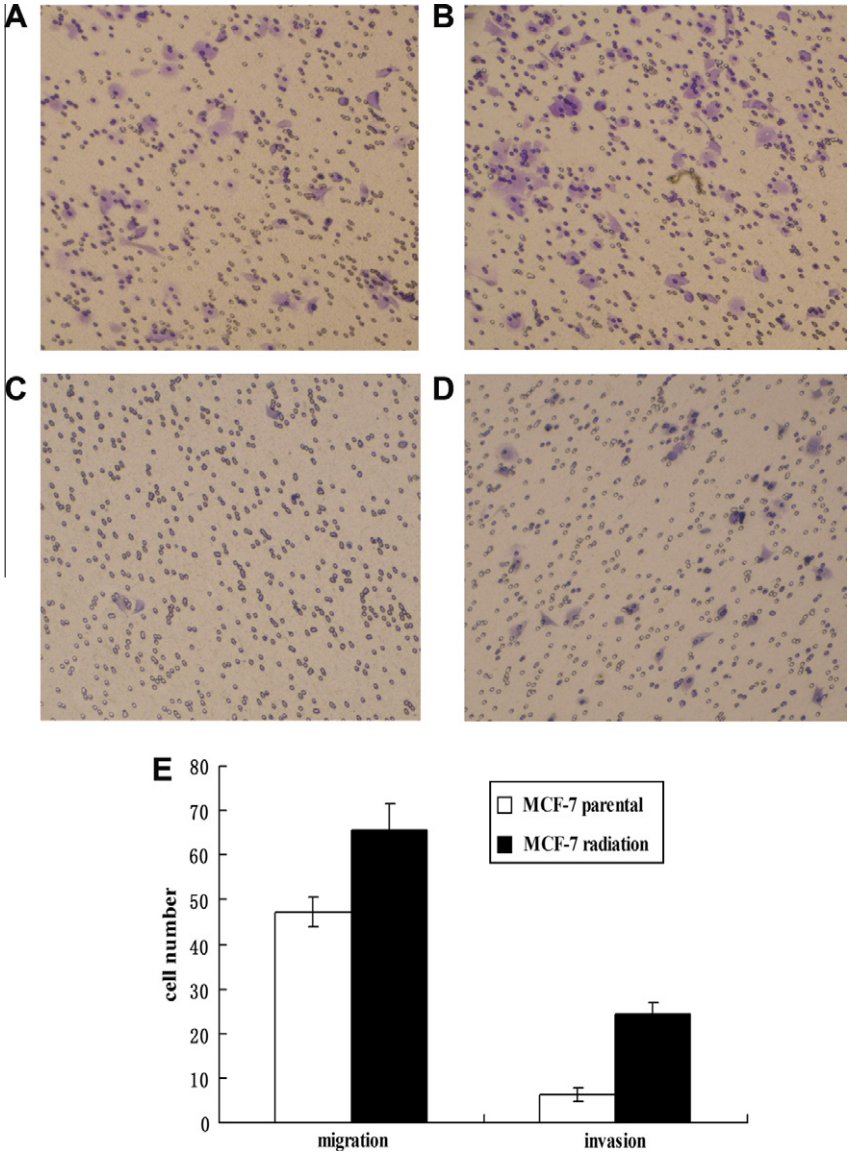

Fig. 3. Low doses ionizing radiation promoted cell mobility. (A) Migration assay of the parental (non-radiated) MCF-7 cells and the irradiated MCF-7 cells; (B) invasion assay of the parental MCF-7 cells; (C) invasion assay of the irradiated MCF-7 cell; (D) crystal violet stained cell counts >10 representative fields; (E) summary graphs for migration and invasion, respectively (data presented as mean ± SED).

confirmed the radiation induced EMT changes both morphologically and on a molecular level, and been able to demonstrate the enhanced invasiveness in radiated breast cancer cells. Elucidating potential mechanisms for radio-resistance may allow for effective targeted therapy such as inhibition of EMT-associated transcription factors that may further enhance the effectiveness of radiotherapy for breast cancer in the future.

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