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Centrosome Clustering Is a Tumor-selective Target for the Improvement of Radiotherapy in Breast Cancer Cells

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Abstract. *Background/Aim: Owing to the frequent observation of centrosome amplification in human cancers, cancer cells have a unique mechanism to suppress detrimental multipolar division by clustering multiple centrosomes into two functional spindle poles, known as centrosome clustering. This study investigated whether inhibition of centrosome clustering enhances the radiation sensitivity of breast cancer cells. Materials and Methods: In this study, inhibition of centrosome clustering was examined by using various centrosome-declustering agents and KIFC1 siRNA in three breast cancer cell lines and two normal fibroblast cell lines. The combination effect of radiation and centrosome declustering was evaluated by cell viability, clonogenic, immunofluorescence assay. Results: This study showed that targeting centrosome clustering enhanced the efficacy of radiotherapy of breast cancer cells with less damage to normal cells. Ionizing radiation induced centrosome amplification in breast cancer cells, but not in normal fibroblast cells. Notably, we showed that centrosome declustering efficiently radiosensitized the centrosome-amplified breast cancer cells through induction of multipolar spindles but did not affect the viability of normal fibroblasts in response to irradiation. Furthermore, KIFC1 mediated the radiosensitivity of the centrosome-amplified breast cancer cells. Conclusion: Our data provided the first evidence that centrosome clustering is a tumor-selective target for the improvement of radiotherapy in breast cancer cells.*

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Centrosomes, the major microtubule-organizing centers, act as bipolar spindle poles for accurate chromosomal segregation during mitosis (1). Centrosome amplification, frequently observed in human cancers, contributes to chromosomal instability and tumorigenesis through the induction of abnormal mitosis in mammals (1-3); moreover, it is tightly associated with clinical aggressiveness and poor prognosis in several tumors, including breast cancer (4). In addition, centrosome amplification is also efficiently induced by DNA damage, such as due to radiation, in cancer cells, which are usually defective in DNA damage checkpoints such as the loss of p53 and chk1 (1, 5, 6). Extra centrosomes during mitosis can lead to the formation of multiple spindles. However, multipolar cell division is detrimental to cell viability and leads to cell arrest or apoptosis of most of the progeny derived from multipolar mitosis (1). To escape detrimental multipolar divisions, cancer cells have a unique mechanism to suppress multipolar spindles, known as centrosome clustering, which clusters multiple centrosomes into two functional spindle poles (7, 8). Therefore, recent studies (7, 8) suggested that the inhibition of centrosome clustering selectively inhibited cancer cells with extra centrosomes, rather than normal cells which have two centrosomes.

Radiotherapy is used for the treatment of approximately 50% of all patients with cancer (9). For breast cancer, radiotherapy is recommended for most patients to optimize local control after breast conserving surgery, as well as after mastectomy in patients who are at a higher risk of recurrence (10, 11). However, the induction of several side-effects, including pneumonitis and fibrosis, by radiation damage to adjacent normal cells and the radioresistance of tumor cells during/after radiotherapy in patients with recurrent cancer are major problems for successful radiotherapy. Extensive studies over several decades have suggested that many factors, including survival signal transduction pathways (*e.g.*, EGFR, PI3K, and AKT) and tumor microenvironment (*e.g.*, hypoxia and tumor vasculature) are associated with radiation

sensitivity in tumor or adjacent normal tissues. However, the clinical applications of the combination of these targeted drugs with radiotherapy is often limited by the various toxic side effects (9). Therefore, the development of a tumor-selective target that induces less damage to normal cells is an urgent medical need for the improvement of radiotherapy.

As radiotherapy induces centrosome amplification in cancer cells and centrosome clustering is an essential mechanism for the survival of cancer cells with supernumerary centrosomes, this study investigated whether the inhibition of centrosome clustering enhanced the efficacy of radiotherapy in breast cancer cells. This study demonstrated that centrosome-declustering agents enhanced the radiation sensitivity of breast cancer cells through the induction of radiation-induced extra centrosomes but spared normal fibroblast cells. In addition, centrosome clustering protein Kinesin Family Member C1 (KIFC1), also known HSET, mediated the radiosensitizing effect of centrosome declustering in breast cancer cells. Therefore, our data have provided the first evidence of the novel strategy to target centrosome clustering in radiotherapy.

Materials and Methods

Cell culture and treatment. All human cancer cells and two human fibroblast cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). MCF7 and Hs68 cells were cultured in DMEM (Corning, NY, USA). MDA-MB-231 and BT-549 cells were cultured in RPMI (Welgene, Daegu, Korea). BJ cells were cultured in MEM (Welgene, Daegu, Korea). All culture media were supplemented with 10% fetal bovine serum (FBS; Corning) and 1% penicillin/streptomycin (GeneDepot, Barker, TX, USA). The cells were incubated at 37˚C in a humidified 5% CO_2 incubator, preserved and passaged in accordance with ATCC protocols for a maximum of 2 months, and tested weekly for mycoplasma infection by using a PCR method.

Immunofluorescence. Immunofluorescence was performed as previously described (12). In brief, cells fixed with 4% paraformaldehyde were permeabilized and blocked with 0.2% Triton X-100 and 5% FBS in PBS. The fixed cells were incubated with primary antibodies against acetyl-α-tubulin (Sigma-Aldrich, St. Louis, MO, USA) and pericentrin (Abcam, Cambridge, MA, USA) and a secondary anti-mouse Alexa-488 antibody (Molecular Probes, Eugene, OR, USA). Images were obtained by using a confocal laser-scanning microscope (LSM 880; Carl Zeiss, Inc., Jena, Germany). Multiple focal planes were acquired and the planes containing centrioles were processed as maximum projections to show that all centrosomes were imaged. The software ImageJ was used for counting the number of centrosomes. For each experiment, the centrosome area was determined through the measurement of the fluorescence signal for pericentrin images.

Clonogenic assay. The clonogenic assay was performed as previously described (13). The cells were seeded in 60-mm cell culture dishes and allowed to adhere overnight. After 24 h, DMSO (Sigma-Aldrich), griseofulvin (Sigma-Aldrich), PJ34 (Tocris, Ellisville, MO, USA), or CW069 (Selleckchem, Houston, TX, USA) were treated at the indicated concentrations and incubated for 2 weeks. At the end of the incubation, the cells were fixed, stained with 0.4% crystal violet/40% methanol solution for 15 min, and the number of colonies was counted.

Cell viability assay. The cells were treated with the indicated compounds in 96-well plates. Cell proliferation or viability was determined by using the WST-8 assay (Cyto X™ cell viability assay kit; LPS solution, Daejeon, Korea) in accordance with the manufacturer's protocol. The absorbance at 450 nm was measured by using a Versamax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Antibodies and western blot analysis. The following antibodies were used: mouse monoclonal antibodies against acetylated-α-tubulin (Sigma-Aldrich), KIFC1 (M-63, Santa Cruz, CA), and β-actin (C4; Santa Cruz); and rabbit polyclonal antibody against pericentrin (Abcam). Western blotting was performed as previously described (11, 14). In brief, the proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane, and incubated with the appropriate antibodies. The HRP-conjugated secondary antibody was detected by using the enhanced chemiluminescence detection system (Amersham Life Science, Piscataway, NJ, USA) and the bands were imaged by using the Amersham Imager 600 system (GE Healthcare).

RNA interference. The following sequence was used for RNA interference: KIFC1, 5'-CUCUACGCUUUGCCUCCAA-3' (Bioneer, Daejeon, Korea). Non-silencing siRNA (Bioneer) was used as the negative control. The siRNAs (20 nM KIFC1 siRNA for BT-549 cells or 50 nM KIFC1 siRNA for MDA-MB-231 cells) were transfected by using G-Fectin (Genolution, Seoul, Korea) in accordance with the manufacturer's protocol.

Statistical analysis. A two-tailed Student's *t*-test was performed to assess the differences between groups. *p*-Values of less than 0.05 were considered to be statistically significant. Statistical analyses were computed by Excel and XLSTAT softwares.

Results

Ionizing radiation induces centrosome amplification in breast cancer cells, but not in normal fibroblast cells. To examine the difference in radiation-induced centrosome amplification between cancer cells and normal cells, three breast cancer cell lines, MCF7, MDA-MB231, and BT-549, and two normal fibroblast cells, BJ and Hs68, were used. Irradiation effectively induced centrosome amplification (*i.e.*, >2 pericentrin signals) in the three breast cancer cell lines (Figure 1A-C), whereas radiation-induced centrosome amplification was not observed in the two normal fibroblast cells lines (Figure 1D-F). Thus, these results suggested that irradiation preferentially induced centrosome amplification in breast cancer cells than in normal fibroblast cells.

Centrosome-declustering agents radiosensitize breast cancer cells. As irradiation preferentially induced centrosome amplification (*e.g.* extra centrosomes) in breast cancer cells

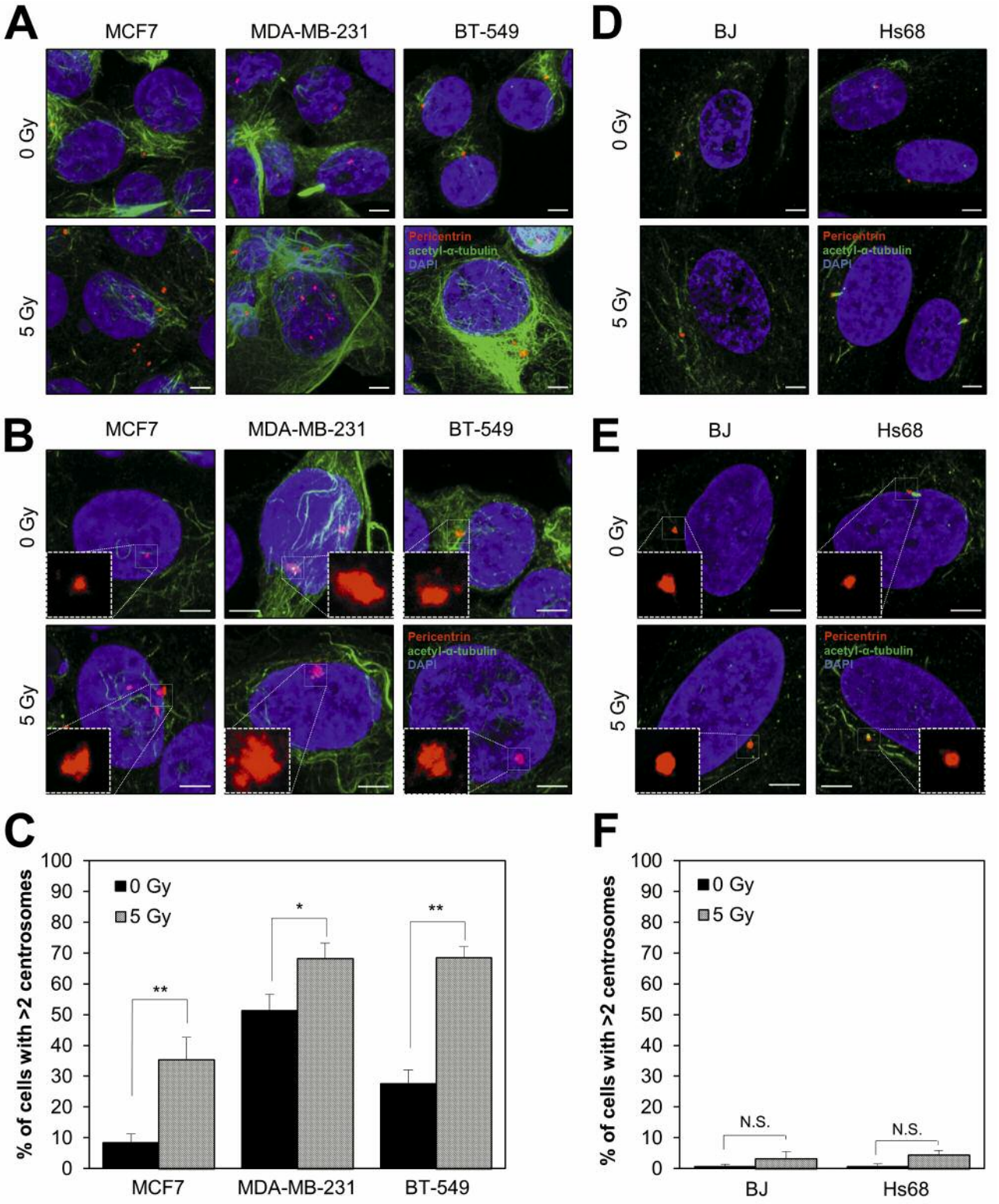

Figure 1. *Ionizing radiation induces centrosome amplification in breast cancer cells, but not in normal fibroblast cells. A-F: MCF7, MDA-MB-231, BT-549, BJ, and Hs68 cells were treated with 5 Gy radiation for 72 h. The cells were stained with anti-acetyl-α-tubulin (green), anti-pericentrin (red), and DAPI (blue) (A, B, D, and E). C and F: The number of centrosomes in interphase cells was counted by using image J software from >120 cells per dataset. Scale bar=5 μm. The data represent typical results and are presented as the mean±standard deviation of three independent experiments; p<0.01 (**) and p<0.05 (*). N.S.: Not significant.*

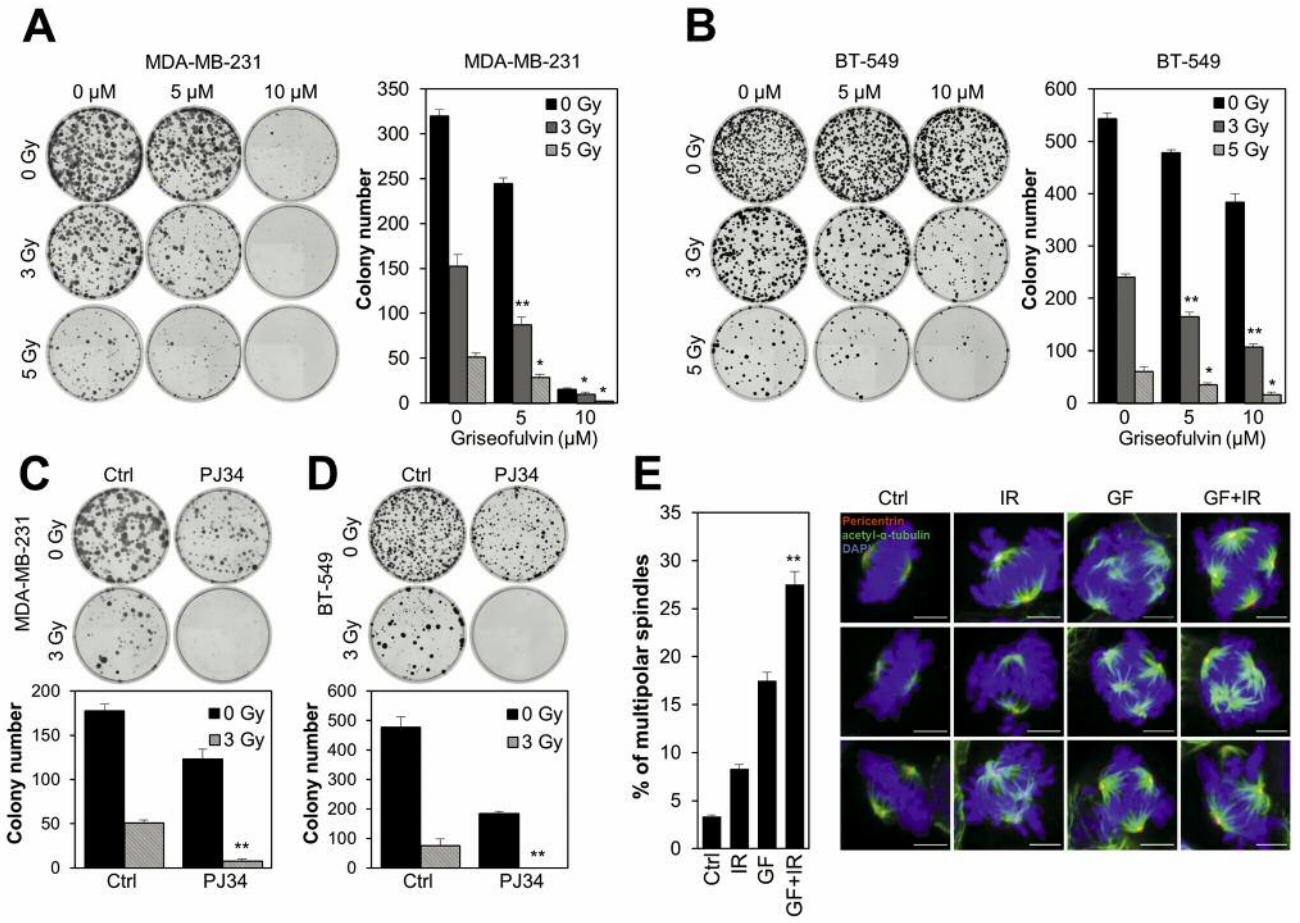

Figure 2. *Griseofulvin radiosensitizes breast cancer cells. A-D: MDA-MB-231 and BT-549 cells were treated with griseofulvin (5 or 10 μM) or PJ34 (5 μM) in combination with the indicated dose of radiation for 2 weeks. The representative images of the colony formation assay staining with crystal violet (A and B, right panels; C and D, upper panels). The number of colonies was counted by using the ImageJ software (A and B, left panels; C and D, lower panels). E: BT-549 cells were treated with 10 μM griseofulvin and 3 Gy radiation for 24 h. The cells were stained with anti-acetyl-α-tubulin (green), anti-pericentrin (red), and DAPI (blue). The percentage of multipolar spindle was determined using mitotic cells (>120 cells per data point). Scale bar=5 μm. The data represent typical results and are presented as the mean±standard deviation of three independent experiments; p<0.01 (**) and p<0.05 (*).*

and centrosome clustering is a crucial mechanism for cell division and the survival of cancer cells with extra centrosomes (7), it was hypothesized that centrosome clustering could be a novel target for the enhancement of radiotherapeutic efficacy. To test this hypothesis, the known centrosome-declustering agent griseofulvin was used, that is a non-toxic antifungal drug (15). The clonogenic assay indicated that the combination of griseofulvin treatment with radiation significantly enhanced the radiation sensitivity of MDA-MB231 and BT-549 cells (Figure 2A and B). In addition, another centrosome-declustering agent, PJ34 (16), also enhanced the radiation sensitivity of MDA-MB231 and BT-549 cells (Figure 2C and D). Significant increases in multipolar spindles in the cells treated with griseofulvin and radiation compared with the cells treated with griseofulvin or radiation alone were observed (Figure 2E), which suggested that centrosome clustering may be a target for radiotherapy through the induction of multipolar spindles in breast cancer cells. Therefore, these results suggested that the inhibition of centrosome clustering enhanced the radiosensitivity of breast cancer cells.

Centrosome-declustering agents do not affect the viability of normal fibroblast cells in response to irradiation. As our data (Figure 1D-F) and other reports (5) have shown that irradiation did not induce centrosome amplification in normal fibroblasts and centrosome declustering did not affect the viability of the cells with two centrosomes (7), it was hypothesized that centrosome-declustering agents may not affect the viability of normal fibroblast cells in response to

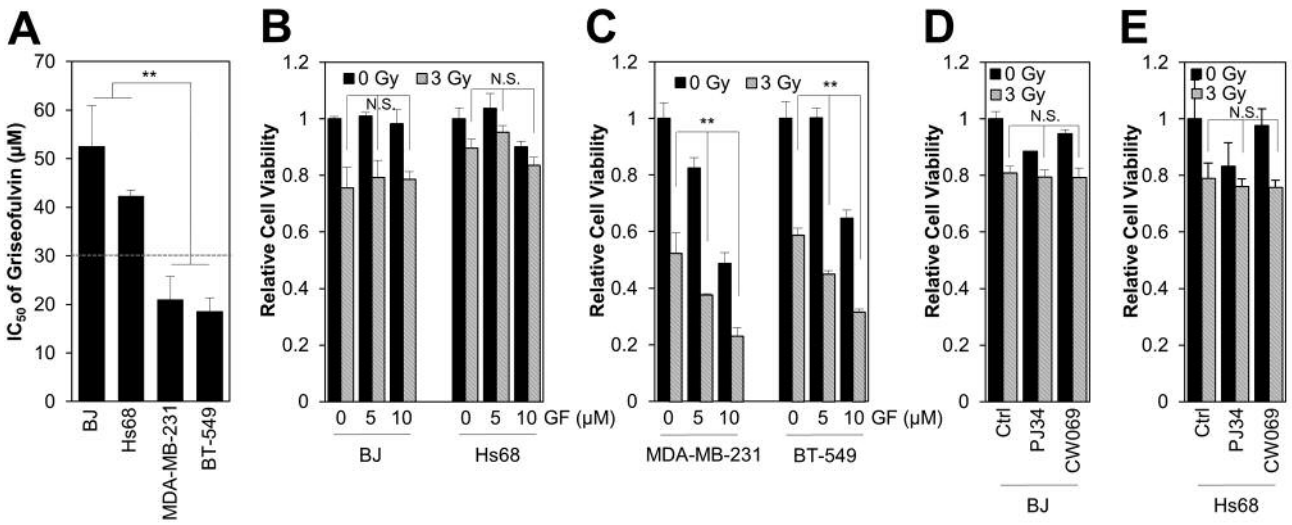

Figure 3. *Centrosome-declustering agents do not affect the viability of normal fibroblast cells in response to irradiation. A: The IC_{50} of griseofulvin was determined in BJ, Hs68, MDA-MB-231, and BT-549 cells by using Softmax pro 6.5 program. B and C: BJ, Hs68, MDA-MB-231 and BT-549 cells were treated with DMSO (Ctrl) or griseofulvin (5 μM and 10 μM) combined with 3 Gy radiation for 48 h. Cell viability was determined by using WST-8 assay. D and E: BJ and Hs68 cells were treated with DMSO (Ctrl), PJ34 (5 μM), CW069 (75 μM) combined with 3 Gy radiation for 48 h. The cell viability was determined by WST-8 assay. The data are presented as the mean±standard deviation of three independent experiments; p<0.01 (**) and p<0.05 (*). N.S.: Not significant.*

irradiation. To test this possibility, the cytotoxic effect of centrosome-declustering agents on the viability of two normal fibroblast cell lines was examined. As expected, normal fibroblasts, including BJ and Hs68 cell lines, were less sensitive to griseofulvin treatment (IC_{50}>40 μM) than breast cancer cells, such as MDA-MB231 and BT-549 cells (IC_{50}<20 μM) (Figure 3A). Interestingly, the combination treatment of griseofulvin and radiation did not affect the viability of the two normal fibroblast cell lines (Figure 3B) but inhibited the viability of the two breast cancer cell lines (Figure 3C). In addition, the two normal fibroblast cell lines were less sensitive to other centrosome-declustering agents, including PJ34 and CW069 (16, 17), and their viability was unaffected by irradiation (Figure 3D and E). Collectively, our data suggested that the combination treatment of radiation and centrosome-declustering agents did not affect the viability of normal fibroblasts, providing evidence that the inhibition of centrosome clustering was nontoxic to normal cells which have two centrosomes.

Centrosome clustering protein KIFC1 mediated the radiation sensitivity of breast cancer cells. As it is known that KIFC1 is a key regulator in the process of centrosome clustering of the centrosome-amplified cancer cells (7), we examined whether KIFC1 mediated the radiation sensitivity of breast cancer cells. First, the expression levels of KIFC1 were evaluated in normal fibroblast cell lines (BJ and Hs68), a mammary epithelial cell line (MCF10A), and breast cancer cell lines (MCF7, MDA-MB231, and BT-549). Interestingly, KIFC1 expression was very low in both normal fibroblast cell lines and mammary epithelial cells, but high in breast cancer cells (Figure 4A). The role of KIFC1 in the radiation sensitivity of MDA-MB231 and BT-549 cells was subsequently examined. The siRNA-mediated depletion of KIFC1 enhanced the radiation sensitivity of two breast cancer cell lines (Figure 4B-D). In addition, the treatment with CW069, a KIFC1 inhibitor (17), in combination with radiation also significantly enhanced the radiation sensitivity of two breast cancer cell lines (Figure 4E). Therefore, the centrosome clustering protein KIFC1 mediated the radiation sensitivity of breast cancer cells.

Discussion

Although radiotherapy is widely used in the treatment of patients with cancer, the main problems that must be resolved for successful radiotherapy include adjacent normal cell damage and tumor radioresistance. Our study showed that inhibition of centrosome clustering enhanced the radiation sensitivity of breast cancer cells, whereas normal fibroblast cells were sufficiently tolerant of the combination treatment of radiation and centrosome-declustering agents. Furthermore, the centrosome clustering protein KIFC1 mediated the radiation sensitivity of centrosome declustering in breast cancer cells. Our data provided the first evidence that centrosome clustering is a tumor-selective target for the improvement of radiotherapy in breast cancer.

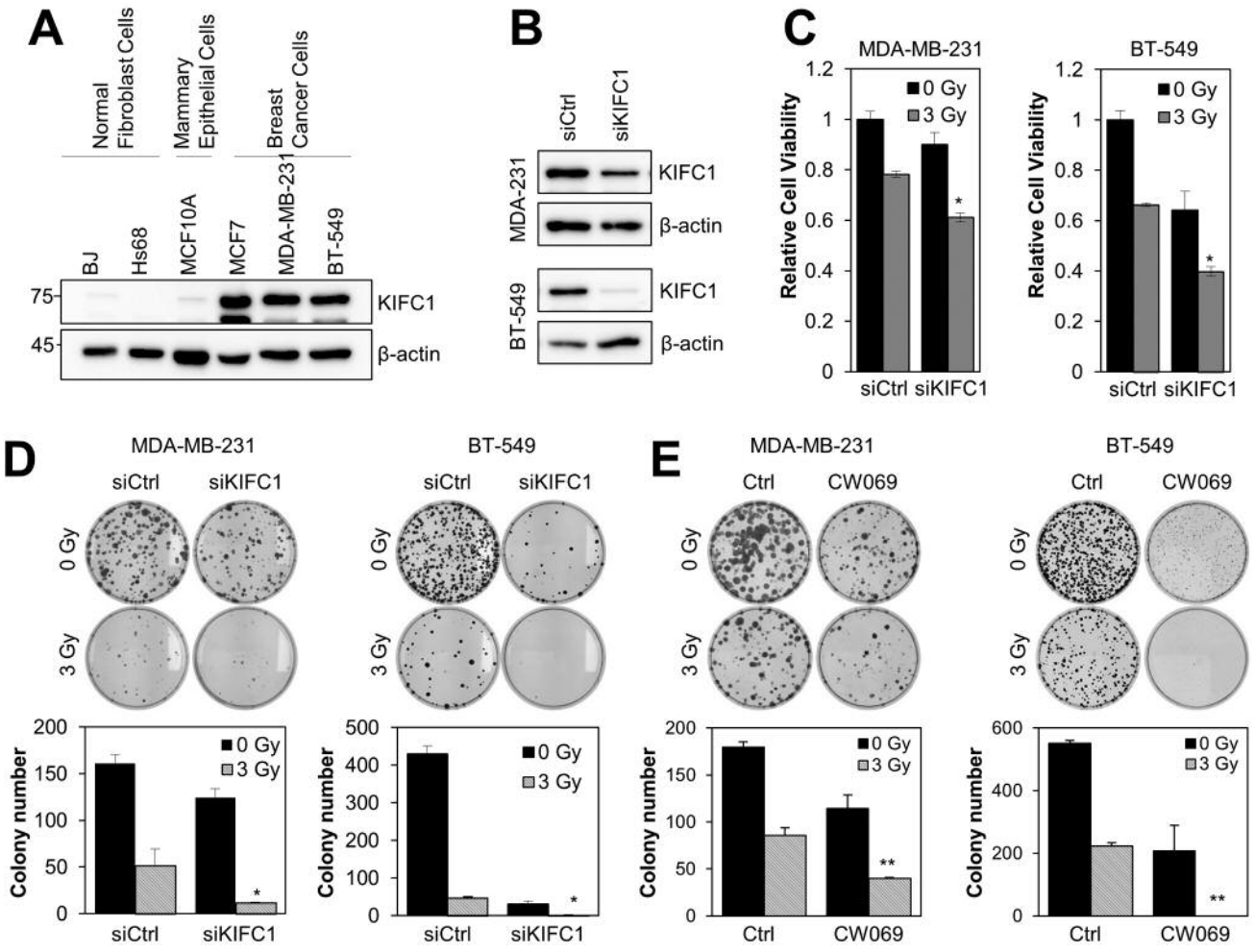

Figure 4. *KIFC1 regulates the radiation sensitivity of breast cancer cells. A: The cell lysates of BJ, Hs68, MCF10A, MCF7, MDA-MB-231, and BT-549 cells were analyzed by immunoblotting with an antibody to KIFC1. B-D: MDA-MB-231 and BT-549 cells were transfected with control siRNA or KIFC1 siRNA for 48 h, respectively. The cell lysates were analyzed by immunoblotting with the KIFC1 antibody (B). β-Actin was used as the loading control (A and B). The cells transfected with siRNAs were further treated with 3 Gy radiation for 48 h (C) or 2 weeks (D). Cell viability was determined by WST-8 assay (C). E: MDA-MB-231 and BT-549 cells were treated with CW069 (75 μM) combined with 3 Gy radiation for 2 weeks. The representative images of the colony formation assay staining with crystal violet (D and E, upper panels). The number of colonies was counted using ImageJ software (D and E, lower panels). The data represent typical results and are presented as the mean±standard deviation of three independent experiments; $p<0.01$ (**) and $p<0.05$ (*).*

It is well known that irradiation efficiently induces centrosome amplification in cancer cells (6, 18), although it is relatively poorly understood whether irradiation induces centrosome amplification in non-transformed normal cells. Notably, radiation induced centrosome amplification in breast cancer cells, but not in normal fibroblast cells. Similarly, Kawamura *et al*. (5) showed that irradiation did not induce centrosome amplification in normal fibroblast cells. However, Bourke *et al*. (19) demonstrated that irradiation induced centrosome amplification in lymphoblastoid cells. Lymphoblastoid cell lines are established by DNA tumor virus genes, including Simian virus 40 large T antigen (20), and the suppression of p53 protein may occur in these transformed cell lines. As it is known that DNA damage-induced centrosome defects activate the p53 pathway in mammal cells (1), centrosome amplification in cancer cells might be involved with the status of p53. Indeed, Kawamura *et al*. (5) showed that p53 depletion induced centrosome amplification in normal fibroblast cells in response to irradiation. Similarly, our data also showed that radiation-induced centrosome amplification was more frequently observed in MDA-

MB231 and BT-549 cells, which are p53 deficient cells, than in MCF7 cells, which are p53 proficient cells (Figure 1A-C). Therefore, it may be possible that the evaluation of p53 functional status and the centrosome amplification of tumor patients could be useful for decisions on the combination treatment of radiation and centrosome-declustering agents.

Recent studies (7, 8) suggested that inhibition of centrosome clustering protein KIFC1 selectively kills cancer cells with extra centrosomes and was well-tolerated in normal cells and cancer cells with two centrosomes, suggesting that centrosome clustering is a cancer cell-selective target for anticancer treatment. However, not all cancer cells have amplified centrosomes. For example, the percentage of MCF7 cells with extra centrosomes is relatively low; accordingly, the inhibition of KIFC1 only slightly reduced the viability of MCF7 cells (7). Our data showed that radiation increased centrosome amplification in breast cancer cells and implied that the irradiated centrosome amplified cells were more susceptible to the inhibition of centrosome clustering. Indeed, centrosome-declustering agents enhanced susceptibility to irradiation through an increase in multipolar spindles in breast cancer cells but spared normal fibroblast cells. In addition, radiotherapy is more effective for controlling the localized primary tumor than chemotherapy. Therefore, radiotherapy is a valuable modality to increase susceptibility to the inhibition of centrosome clustering in cancer cells. In addition, recent studies (15, 21) showed that griseofulvin and its analogs preferentially inhibited cancer cells than non-malignant cells. Since it is a Food and Drug Administration-approved drug with a long track record of safety, griseofulvin and its analogs may be potential radiosensitizers in a combination with radiotherapy as a treatment for breast cancer. However, further preclinical studies are required before this can be applied in a clinical setting.

In conclusion, targeting centrosome clustering enhanced the efficacy of radiotherapy in breast cancer cells, causing less damage to normal cells. Our work provides the strategy for the targeting of centrosome clustering in radiotherapy.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Acknowledgements

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