Deoxycytidine kinase participates in the regulation of radiation-induced autophagy and apoptosis in breast cancer cells

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Abstract. Deoxycytidine kinase (dCK) is a rate limiting enzyme critical for the phosphorylation of endogenous deoxyribonucleosides and for the anti-tumor activity of many nucleoside analogs. dCK is activated in response to ionizing radiation (IR) and it is required for the G2/M checkpoint induced by IR. However, whether dCK plays a role in radiation-induced autophagy and apoptosis is less clear. In this study, we reported that dCK decreased IR-induced total cell death and apoptosis, and increased IR-induced autophagy in SKBR3 and MDA-MB-231 breast cancer cell lines. A molecular switch exists between apoptosis and autophagy. We further demonstrated that serine 74 phosphorylation was required for the regulation of autophagy. In dCK wild-type (WT) or dCK S74E (mutant) MDA-MB-231 cell models, the expression levels of phospho-Akt, phospho-mammalian target of rapamycin (mTOR) and phospho-P70S6K significantly decreased following exposure to IR. Moreover, the ratio of Bcl-2/Beclin1 (BECN1) significantly decreased in the S74E mutant cells; however, no change was observed in the ratio of Bcl-2/BAX. Taken together, our findings indicate that phosphorylated and activated dCK inhibits IR-induced total cell death and apoptosis, and promotes IR-induced autophagy through the mTOR pathway and by inhibiting the binding of Bcl-2 protein to BECN1.

Introduction

Breast carcinoma (BC) is the most prevalent cause of cancer-related mortality in women worldwide (1,2), both in low- and middle-income countries and approximately 1.67 million new cancer cases were diagnosed in 2012 (25% of all cancers) (3). At present, the treatment of breast cancer mainly involves surgery, radiotherapy and chemotherapy; 75% of patients with breast cancer require radiotherapy. Although the early treatment effects are good, the treatment effects in general remain poor, the main reason being the fact that the sensitivity of breast cancer to radiation is not ideal. Therefore, the identification of methods with which to improve the sensitivity to radiation of breast cancer are of utmost importance.

Apoptosis (programmed cell death, type I) refers to a constellation of characteristic changes leading directly to cell death (4). Surface death receptors, through the mitochondrial release of cytochrome c, cellular stress and some treatments can trigger apoptosis (5). Apoptosis was previously considered the primary mechanism of radiation-induced cell death (6).

Autophagy is a lysosomal-dependent self-digestion process (7) which promotes cells survival under certain types of stress, such as nutrient starvation, reactive oxygen species (ROS), hypoxia, DNA damage and the unfolded protein response (8). However, excessive cell damage is beyond the limit of repair under certain physiological conditions, and in this case, autophagy turns into the programmed cell death mechanism (type-II) (9). Therefore, autophagy is considered to be a ‘double-edged sword’ in the process of tumor development.

Deoxycytidine kinase (dCK) is an enzyme critical for the phosphorylation of natural deoxyribonucleic acid (10-13). This reaction is the first and rate-limiting step in deoxyribonucleoside salvage, which produce and maintain a balanced
pool of deoxyribonucleoside triphosphates (dNTPs) for DNA synthesis (14). dCK also promotes the phosphorylation of ara-C, CNDAC and other nucleoside analogues; these drugs can only be activated after phosphorylation, and then inhibit tumor growth (15-18). The phosphorylation of dCK and post-translational modification is crucial for its enzymatic activity (19). dCK protein has four phosphorylation sites, Thr-3, Ser-11, Ser-15 and Ser74 (20-23). dCK activity can be increased by Ser-74 phosphorylation (24,25) and this phosphorylation is required for the initiation of the G2/M checkpoint (26). It has been previously demonstrated that nucleoside analogs that exhibit synergistic activity with radiotherapy are activated by dCK (11). In our previous study, we demonstrated that dCK regulated radiation-induced cell death through apoptosis and autophagy in HeLa cells (27).

In this study, we aimed to analyze the roles of dCK in ionizing radiation (IR)-induced apoptosis and autophagy in breast cancer cells, in order to determine whether dCK participates in the regulation of cell death induced by IR and to elucidate the main underlying mechanisms.

Materials and methods

Cell line, antibodies and reagents. The human breast cancer cell lines, SKBR3 (HER2-like: estrogen receptor-negative/progesterone receptor-negative, ErbB2-positive, ATCC® HTB-30™) and MDA-MB-231 (basal-like: estrogen receptor/ progesterone receptor/ErbB2-negative, ATCC® HTB-26™), were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) in glass petri dishes at 37°C in a 5% CO2 incubator.

Anti-dCK (Cat. no. ab96599; diluted 1:500) antibody was purchased from Abcam Inc. (Cambridge, MA, USA). Anti-MAPL3C (Cat. no. 4108; diluted 1:500), anti-p-mammalian target of rapamycin (p-mTOR; Cat. no. 2971; diluted 1:500), anti-p-Akt (Cat. no. 9271; diluted 1:500), anti-p-P70S6K (Cat. no. 9205; diluted 1:500) and anti-poly(ADP-ribose) polymerase (PARP; Cat. no. 9542; diluted 1:1,000) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-GAPDH (Cat. no. 5174; diluted 1:1,000) antibody was obtained from Cell Signaling Technology (Beverly, MA, USA). Peroxidase-conjugated anti-mouse IgG (p-mTOR; Cat. no. 2971; diluted 1:500), anti-p-Akt (Cat. no. 9271; diluted 1:500), anti-p-P70S6K (Cat. no. 9205; diluted 1:500) and anti-poly(ADP-ribose) polymerase (PARP; Cat. no. 9542; diluted 1:1,000) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-GAPDH (Cat. no. 5174; diluted 1:1,000) antibody was obtained from Cell Signaling Technology (Beverly, MA, USA). Peroxidase-conjugated anti-mouse IgG (Cat. no. 7056; diluted 1:1,000) and peroxidase-conjugated anti-rabbit IgG (Cat. no. 7054; diluted 1:1,000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fetal bovine serum (FBS), 3-methyladenine (3-MA, used for treatment at 20 mM for 48 h) and rapamycin (used for treatment at 200 nM for 48 h) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), ZVAD-FMK (used for treatment at 20 µM for 48 h) was purchased from Selleckchem (Houston, TX, USA) and the pSUPER retroviral vector was obtained from OligoEngine (Seattle, WA, USA).

Radiation. An X-ray generator (X-RAD 320 ix, Precision X-ray Inc., North Branford, CT, USA) was utilized to deliver radiation at a dose rate of 1.0 Gy/min. All the cells were exposed to IR for 8 min.

Plasmids. Wild-type (WT; dCK-WT), dCK-S74A, dCK-S74E mutants were kind gifts from Dr Bo Xu (Southern Research Institute, Birmingham, AL, USA). shRNAs were designed according to the instructions provided on the website www.idtdna.com. The shRNAs were synthesized, denatured, ligated to the pSUPER vector at the BglIII and HindIII sites. The primers used were as follows: dCK-WT forward, 5'-tactagtag ggcacccgcgccagaaag-3' and reverse, 5'-agctctgataaaagttcatactt-3'; dCK-S74A forward, 5'-ctcaattctgcaagaa aagtgttg-3' and reverse, 5'-ccacacttttgttctgcaattga-3'; dCK-S74E forward, 5'-ctcaattgaaagaaatgttg-3' and reverse, 5'-ccacacttttgttctgcaattga-3'; and dCK-shRNAs forward, 5'-gtatgcttgctgtgctgtgcaattg-3' and reverse, 5'-agcttgaaagtgttgcat ggacttatctta-3' and reverse, 5'-agcttgaaagtgttgcatggacttatctta-3' and reverse, 5'-agcttgaaagtgttgcatggacttatctta-3'.

Establishment of cells in which dCK was silenced. The pSUPER-dCK and the vector with a scrambled sequence, i.e., pSUPER, were constructed in our laboratory. The plasmids were transfected into 293T packaging cells (Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China) by calcium phosphate co-precipitation, to produce pseudovirus particles [Ampho Pack plasmid 10 µg, PsupershRNA plasmid 10 µg, 2 mol/l CaCl2, 31 µl, ddH2O to 250 µl and 2X HEPES buffer salt solution (HBS) 250 µl]. Supernatant containing pseudovirus particles was collected after 72 h and then used to infect the SKBR3 cells and MDA-MB-231 cells together with polybrene (8 µg/ml). Positive stable cell clones were selected by growing the cells with puromycin (0.8 µg/ml) for 1 week.

Western blot analysis. The cells were harvested and lysed in RIPA lysis buffer [HEPES (50 mM), sodium chloride (NaCl; 150 mM), ethylenediaminetetraacetic acid (EDTA; 1 mM), egtazic acid (EGTA; 2.5 mM), sodium fluoride (NaF; 10 mM), dithiothreitol (DTT; 1 mM), sodium orthovanadate (SV; 1 mM), phenylmethylsulfonyl fluoride (PMSF; 1 mM), Nonidot P-40 (NP-40; 1%)] and sodium dodecyl sulphate (SDS; 0.1%), and a 2 ml aliquot was mixed with 20 µl protease inhibitor cocktail and the lysates was laid on ice for 5 min, followed by sonication.

The supernatant was then removed to another tube following centrifugation at 15,000 x g for 10 min and lysate was mixed with 5X SDS loading buffer (BioTeke, Beijing, China) and heated to 95°C for 5 min. Of the total protein, 50 µg were separated by SDS-PAGE, and transferred onto nitrocellulose membranes which were then blocked in 5% non-fat dried milk in Tris-buffered saline (TBS) and Tween-20 (10 mmol/l Tris, pH 7.5, 100 mmol/l NaCl and 0.1% Tween-20) at room temperature for 1.5 h, and then incubated with appropriate primary antibody (anti-dCK antibody, anti-MAPL3C antibody, anti-p-mTOR antibody, anti-p-Akt antibody, anti-p-P70S6K antibody, anti-PARP antibody and anti-GAPDH antibody) overnight at 4°C, and horseradish peroxidase-conjugated secondary antibodies (anti-mouse IgG or anti-rabbit IgG) at room temperature for 1 h. Finally, the signals were visualized by using the Pierce chemiluminescence detection system according to the manufacturer's instructions (Santa Cruz Biotechnology); GAPDH protein was used as a loading control. The intensity of the protein bands
Figure 1. dCK decreases radiation-induced cell death. (A) Establishment of isogenic cell lines with stable dCK knockdown. SKBR3 and MDA-MB-231 cell lines were stably transfected with pSUPER control or dCK shRNA. Individual clones were obtained under puromycin selection. The effects of dCK knockdown were confirmed by western blot analysis. (B) The cells in the pSUPER and dCK knockdown groups were treated with 8 Gy radiation for 48 h, and the cells were then stained with trypan blue and analyzed by flow cytometry. (C and D) Radiosensitivity was assessed by the colony formation assay in both the cells in which dCK was silenced and in the control cells following radiation treatment (0-8 Gy). (E) Vector control, dCK wild-type, dCK S74A mutant or S74E mutant were re-introduced into the SKBR3 cells and MDA-MB-231 cells in which dCK was knocked down. The overexpression of different dCK genotypes is shown in the representative western blots. (F and G) The cells with different dCK genotypes were treated with 8 Gy radiation. After 48 h, the cells were stained with trypan blue and analyzed by flow cytometry. Data are presented as the means ± SD of 3 independent experiments. *P<0.05 vs. control group (0 Gy).
was quantified using image software (Quantity One) and ratios of specific bands to the loading control were analyzed.

**Flow cytometric analysis.** The cells were plated in 6-well plates and exposed to IR. The cells were then collected at 48 h following IR and washed 3 times in PBS. For apoptosis detection, the cells were stained with PI (Sigma-Aldrich Chemical Co.) and FITC-labeled Annexin V (Sigma-Aldrich Chemical Co.). For cell death analysis, the cells were stained by trypan blue (Sigma-Aldrich Chemical Co.). The stained cells were detected using a flow cytometer (BD Biosciences, San Jose, CA, USA). The data were analyzed using CellQuest software (BD Biosciences) and FlowJo software (Treestar Inc., Ashland, OR, USA).

**Colony formation assay.** The cells were seeded in 6-well plates in triplicate using standard culture medium. After 24 h, the cells were irradiated (0, 2, 4, 6, and 8 Gy). After 2 weeks, the cells were fixed with 4% formaldehyde, and stained with 0.5% crystal violet (Merck, Darmstadt, Germany). The number of colonies (>50 cells) was counted using a TC20™ Automated Cell Counter (Bio-Rad Laboratories, Shanghai, China) and normalized to the corresponding non-irradiated control. Cell survival curves were made by the multitarget click model of GraphPad Prism 5.0 (Systat Software, San Jose, CA, USA).

**Data calculation methods.** For all figures, we took the comparison between the pSUPER and dCK knockdown groups or we took the comparison between the vector, dCK-WT, dCK-S74A and dCK-S74E groups into consideration. For example, in Fig. 1B, we first want to detect the rate of cell death induced only by IR. Therefore, the effects of IR on mortality were determined by the mortality of the IR (8 Gy) group minus the mortality of the control (0 Gy) group and then by dividing the control group. We then compared the difference between the pSUPER and dCK knockdown group to examine the role of dCK in IR-induced cell death.

**Statistical analysis.** All data were obtained from at least 3 independent experiments. Statistical evaluations are presented as the means ± SE. The significance of the differences between groups was determined by one-way ANOVA, and a value of P<0.05 was considered to indicate a statistically significant difference.

**Results**

**dCK decreases radiation-induced cell death.** In order to elucidate the roles of dCK in radiation-induced cell death, dCK expression was knocked down in the SKBR3 and MDA-MB-231 cells (Fig. 1A). We then determined that the knockdown of dCK enhanced IR-induced cell death by 53% in the MDA-MB-231 cells and by 28% in the SKBR3 cells as compared with the control cells (Fig. 1B). Colony formation assays demonstrated that dCK knockdown increased radiosensitivity (Fig. 1C and D). dCK S74A has a serine 74 to alanine substitution, which abrogates phosphorylation, and dCK S74E has a serine 74 to glutamic acid substitution, which mimics phosphorylation. We re-introduced the empty vector, wild-type dCK, dCK S74A or the S74E plasmid into the cells in which dCK was knocked down (Fig. 1E). IR increased the rate of cell death by 129, 90, 137 and 65% in the SKBR3 cells transfected with vector, dCK WT, dCK S74A, dCK S74E respectively, and by 367, 320, 356, 196% in MDA-MB-231 cells transfected with vector, dCK WT, dCK S74A, dCK S74E respectively. Thus, compared to the control cells exposed to IR, the re-introduction of dCK WT, dCK S74A and dCK S74E decreased IR-induced cell death by 39, -8 and 64% in the SKBR3 cells, and by 47, 11 and 171% in the MDA-MB-231 cells, respectively, suggesting that dCK protects breast cancer cells from IR-induced cell death (Fig. 1F and G).

Autoapthagy and apoptosis can contribute to cell death following exposure to IR. We found that IR increased cell death by 488% in the MDA-MB-231 cells. However, the knockdown ATG5 and Beclin1 (BECN1; data not shown) only increased IR-induced cell death by 247 and 211% (data not shown). Furthermore, we inhibited autophagy and apoptosis with 3-MA and ZVAD-FMK, respectively, and induced autophagy with rapamycin. We found that ZVAD-FMK decreased IR-induced cell death by 21% and rapamycin increased IR-induced cell death by 104% (Fig. 2A). Moreover, 3-MA and ZVAD-FMK did not affect IR-induced cell death and rapamycin increased IR-induced cell death by 104% (Fig. 2A).
death by 51 and 43%, and rapamycin increased IR-induced cell death by 53% in the MDA-MB-231-pSUPER cells. 3-MA and ZVAD-FMK decreased IR-induced cell death by 49 and 40%, and rapamycin increased IR-induced cell death by 40% in the MDA-MB-231 cells in which dCK was silenced (Fig. 2B). Thus, these data indicated that both autophagy and apoptosis contributed to IR-induced cell death (Fig. 2).

dCK suppresses IR-induced apoptosis. To confirm that dCK contributes to IR-induced apoptosis, we knocked down dCK and then examined the expression of PARP and cleaved-PARP in the MDA-MB-231 and SKBR3 cells (Fig. 3A and B). In Fig. 3A and B, P0, P8, d0 and d8 represent pSUPER-transfected cells exposed to 0 Gy IR, and pSUPER-transfected cells exposed to 8 Gy IR, cells in which dCK was knocked down and exposed to 0 Gy IR, and cells in which dCK was knocked down cells and exposed to 8 Gy IR, respectively. Our results revealed that the knockdown of dCK increased the IR increased the ratio of cleaved PARP/PARP by 247% in the MDA-MB-231 and by 81% in the SKBR3 cells, suggesting that...
dCK was involved in IR-induced apoptosis. Furthermore, empty vector, dCK-WT, dCK-S74A and dCK-S74E plasmids were re-introduced into the cells in which dCK was knocked down and the rate of apoptosis was detected. In the MDA-MB-231 cells, compared to the empty vector group, the re-introduction of dCK-S74A, dCK-WT and dCK-S74E decreased IR-induced apoptosis (early apoptosis and late apoptosis) by 171, 273 and 383%, respectively. In addition, in the SKBR3 cells, compared to the empty vector group, the re-introduction of dCK-S74A, dCK-WT and dCK-S74E decreased IR-induced apoptosis by -6, 37 and 59%. It was thus suggested that phosphorylated dCK suppresses IR induced-apoptosis (Fig. 3C and D).

dCK promotes IR-induced autophagy. As is already known, the excessive induction of autophagy, or when cell damage is beyond the limit of repair, autophagy then turns into a programmed cell death mechanism (type-II) (9,28-30). In this study, we first used autophagy inhibitors to examine the role of dCK in IR-induced autophagy. We found that 3-MA markedly decreased LC3-II expression in the cells exposed to IR (data not shown). Ammonium chloride (NH4Cl) is a lysosomal inhibitor which can block organelle acidification and enable the assessment of the autophagic flux (31). In this study, western blot analysis revealed that LC3-II expression increased in a time-dependent manner (data not shown), reaching peak levels at 72 h following exposure to IR and the signal was much higher in the MDA-MB-231 cells treated with NH4Cl and IR (data not shown).

To confirm that dCK contributes to IR-induced autophagy, we knocked down dCK and then examined the expression of LC3-I and LC3-II in the MDA-MB-231 and SKBR3 cells. The results revealed that compared with the pSUPER-transfected cells, the knockdown of dCK decreased the expression of LC3-II (Fig. 4A). In addition, in order to investigate whether dCK S74 phosphorylation is associated with IR-induced autophagy, we re-introduced dCK constructs into the cells in which dCK was knocked down (Fig. 4B). Western blot analysis revealed that in the SKBR3 cells, only dCK S74E increased the level of LC3-II protein by 65% following exposure to IR compared to the vector group. In the MDA-MB-231 cells, the re-introduction of dCK-WT, dCK-S74E and dCK-S74A increased the levels of LC3-II by 306, 336 and 150% following exposure to IR, respectively and there was no significant change with IR treatment in the empty vector group cells. These results thus indicate that dCK S74 phosphorylation is involved in IR-induced autophagy.

Crosstalk between apoptosis and autophagy following exposure to IR. Given that dCK plays an important role in both IR-induced apoptosis and autophagy, we focused on the potential association between apoptosis and autophagy. Cisplatin, a first class anti-tumor drug widely used in the treatment of various types of cancer, targets DNA to induce apoptosis through the mitochondrial death pathway or Fas death pathway (32,33). We thus used cisplatin as a positive control and found that cisplatin-induced apoptosis was reversed by ZVAD-FMK (data not shown).

In the SKBR3 cells in which dCK was knocked down, rapamycin increased IR-induced apoptosis by 20% compared to the control group; however, 3-MA and ZVAD-FMK failed to affect apoptosis. There was also no significant difference in IR-induced apoptosis in the cells transfected with the pSUPER control and treated with rapamycin, 3-MA or ZVAD-FMK (Fig. 5A). In the MDA-MB-231 cells transfected with the pSUPER control, 3-MA and ZVAD-FMK decreased IR-induced apoptosis by 21 and 8%, while rapamycin increased apoptosis by 13%. In the MDA-MB-231 cells in which dCK was knocked down, 3-MA and ZVAD-FMK decreased the rate of apoptosis by 17 and 20%, while rapamycin increased it by 26% compared to the control group (Fig. 5B). IR increased the ratio of cleaved-PARP/PARP by 17% in the SKBR3 cells in which dCK was silenced and this ratio did not change in the pSUPER control-transfected cells. 3-MA decreased the cleaved-PARP/PARP ratio in SKBR3-pSUPER and SKBR3-dCKRI cells following exposure to IR. When the
cells were treated with ZVAD-FMK, the cleaved-PARP/PARP ratio decreased. Of note, the ratio of cleaved-PARP/PARP was much lower in the cells treated with 3-MA + ZVAD-FMK than in the cells treated with 3-MA or ZVAD-FMK alone (Fig. 5C). It was suggested that the suppression of autophagy decreased IR-induced apoptosis.

Figure 5. Suppression of autophagy/apoptosis decreases the apoptosis/autophagy induced by ionizing radiation (IR) in MDA-MB-231 cells. (A and B) The cells transfected with pSUPER and the cells in which dCK was knocked down were incubated with 3-MA (2 mM), ZVAD-FMK (20 µM) or 3-MA (2 mM) + ZVAD-FMK(20 µM) for 1 h, and then exposed to IR (8 Gy) and incubated for 48 h. The cells were collected, stained with PI and Annexin V-FITC and analyzed by flow cytometry. (C and D) Western blot analysis was performed to detect the expression of PARP, cleaved PARP and LC3I/II; GAPDH was used as an internal standard. (C and D) *P<0.05 vs. the control group.
In the MDA-MB-231 cells, IR increased the expression of LC3-II by 26% in the pSUPER control group; however, there was no change in the cells in which dCK was knocked down. 3-MA decreased LC3-II expression by 36% in the pSUPER-transfected cells and by 6% in the cells in which dCK was knocked down following exposure to IR. When the cells were treated with ZVAD-FMK, LC3-II expression decreased in MDA-MB-231 cells in the pSUPER and dCK knockdown group. Of note, the ratio of LC3-II was much lower in the cells (in which dCK was knocked down cells) treated with 3-MA + ZVAD-FMK compared with the cells treated with 3-MA or ZVAD-FMK alone in (Fig. 5D). These findings suggested that the suppression of apoptosis decreased IR-induced autophagy. This suggests that a crosstalk exists between apoptosis and autophagy following exposure to IR.

**dCK regulates IR-induced autophagy through the mTOR pathway.** Since dCK plays an important role in IR-induced autophagy, we wished to determine whether the Akt/mTOR/p70S6K signaling pathway is involved in this process. As shown in Fig. 6A, in the SKBR3 cells, only dCK-S74E decreased the expression of p-mTOR by 77% following exposure to IR. In the MDA-MB-231 cells, dCK-WT significantly decreased the level of p-Akt by 54%, that of p-mTOR by 82% and that of p-P70S6K by 54%. dCK S74E significantly decreased the level of p-Akt by 57%, that of p-mTOR by 87% and that of p-P70S6K by 64%. dCK-S74A slightly decreased the level of p-Akt, p-mTOR and p-P70S6K by 19, 38 and 7%, respectively following exposure to IR. However, the empty vector only decreased the levels of p-Akt and p-mTOR by 9 and 20%, respectively (Fig. 6B). These data suggest that activated dCK inhibits the Akt/mTOR/P70S6K pathway and promotes autophagy in breast cancer cells exposed to IR.

**Activation of dCK regulates the binding of Bcl-2/BECN1 following exposure to IR.** The complex formed by the autophagy-related protein, BENC1, and the anti-apoptotic protein, Bcl-2, leads to the inhibition of autophagy-associated cell death (34-37). In this study, we thus determined the effect of dCK activation on the interaction between Bcl-2 and BECN1.
following exposure to IR. As shown in Fig. 7, dCK-S74E decreased the ratio of Bcl-2/BECLN1 compared with the control group in the SKBR3 and MDA-MB-231 cells; however, there was no change in the ratio of Bcl-2/BAX, suggesting that activated dCK inhibits the binding of Bcl-2/BECLN1 and promotes autophagy following exposure to IR.

Discussion

Breast cancer is one of the most common types of cancer worldwide and is the leading cause of cancer-related mortality among females (38,39). Radiotherapy is a major strategy in the treatment of breast cancer. It is known that many nucleoside analogs are used in combination with radiotherapy, and dCK is required for the anti-tumor activity of these nucleoside analogs (11,40). IR can activate dCK and the phosphorylation of dCK is crucial for its enzymatic activity (41).

In this study, we found that the knockdown of dCK increased IR-induced cell death and apoptosis in both the MDA-MB-231 and SKBR3 cells. We found that the MDA-MB-231 cells were more sensitive to IR than the SKBR3 cells. This may be due to the fact that the SKBR3 cells express HER2, but the MDA-MB-231 cells are HER2-negative. HER2 overexpression promotes DNA damage repair and results in resistance to radiation (42). In this study, we re-introduced different dCK constructs into the SKBR3 and MDA-MB-231 cell lines in which dCK was knocked down. dCK phosphor-mimetic S74E reversed IR-induced cell death and apoptosis after the silencing of dCK, while dCK S74A failed to do so, confirming the important role of dCK S74 phosphorylation in cell death and apoptosis under IR treatment.

Autophagy was traditionally considered to be a protective mechanism, important for the removal of damaged proteins and organelles, and conferring stress tolerance and enhancing cell viability under adverse conditions (43-45). However when cell damage is beyond the limits of repair under certain physiological conditions, autophagy turns into the programmed cell death mechanism (type-II) (9,45,46). BECN1 is a key mediator of autophagy. It interacts with several co-factors that regulate autophagy and its dysfunction has been implicated in several disorders, including many types of human cancer (36). Moreover, Atg5 is covalently bound to Atg12 (47), which is essential for the occurrence of autophagy (48). In this study, we found that the knockdown of ATG5 and BECN1 decreased IR-induced cell death compared with the control group in MDA-MB-231 cell models (data not shown). Apoptosis and autophagic cell death are the most important mechanisms of cell death. By using 3-MA and ZVAD-FMK to inhibit autophagy or apoptosis and rapamycin to induce autophagy, our data indicated that both the autophagy inhibitor and apoptosis inhibitor led to a decrease in IR-induced cell death in the cells transfected with the pSUPER control vector and in the cells in which dCK was knocked down. Rapamycin increased IR-induced cell death significantly in the cells in which dCK was knocked down, indicating that both apoptosis and autophagy contribute to IR-induced cell death.

We provide evidence that dCK increases LC3-II expression in response to IR. Moreover, S74E noticeably increased LC3-II expression compared with WT or S74A in response to IR, indicating that dCK S74 phosphorylation promoted IR-induced autophagy. It is very interesting that S74E inhibited IR-induced total cell death and apoptosis and increased autophagic cell death in the MDA-MB-231 cell line, suggesting that apoptosis plays a more important role than autophagy in contributing to cell death. We found that IR-induced apoptosis was inhibited by 3-MA and IR-induced autophagy was inhibited by ZVAD-FMK. Treatment with 3-MA + ZVAD-FMK decreased apoptosis and autophagy more markedly than 3-MA or ZVAD-FMK alone, confirming the existence of a crosstalk between apoptosis and autophagy following exposure to IR.

In conclusion, dCK was found to affect IR-induced apoptosis and autophagy, and there was a switch from autophagy to apoptosis in the SKBR3 and MDA-MB-231 cell lines. The phosphorylation of dCK at serine 74 increased autophagy through the Akt/mTOR/p70S6K signaling pathway, and inhibited the binding of Bcl-2/Beclin in response to IR in the MDA-MB-231 cell line.

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Competing interests
The authors declare that they have no competing interests.

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