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Original Article

Adenovirus-mediated expression of UHRF1 reduces the radiosensitivity of cervical cancer HeLa cells to γ-irradiation

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Aim: An *in vitro* study was carried out to determine the effect of UHRF1 overexpression on radiosensitivity in human cervical cancer HeLa cells using adenovirus-mediated *UHRF1* gene transfer (Ad5-UHRF1).
Methods: Cell survival was evaluated using the clonogenic survival assay and the MTT assay; apoptosis and cell cycle distribution were monitored by flow cytometry. Protein levels were measured by Western blotting. Silencing XRCC4 expression was performed by transfection of small interfering RNA (siRNA).
Results: Increased expression of UHRF1 by Ad5-UHRF1 significantly reduced the radiosensitivity of HeLa cells. The UHRF1-mediated radioresistance was correlated with increased DNA repair capability and increased expression of the DNA damage repair protein, XRCC4. Knocking down XRCC4 expression in the cells using XRCC4 siRNA markedly reduced the UHRF1-mediated radioresistance.
Conclusion: These results provide the first evidence for revealing a functional role of UHRF1 in human cervical cancer cells as a negative regulator of radiosensitivity.

Keywords: UHRF1; HeLa cells; radiosensitivity; XRCC4; DNA repair
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Introduction

Cervical cancer is the second most common female cancer worldwide. Radiotherapy may reduce local recurrences after surgery for patients with early stages of cervical cancer and is the most effective therapy for advanced cervical cancer. Resistance of advanced and invasive cervical cancer to radiotherapy is one of the reasons for treatment failure; moreover, the mechanisms underlying this radioresistance are not fully understood[1–3].

UHRF1 (ubiquitin-like protein containing PHD and RING domains 1) was first cloned and isolated in 1998[4]. As a 95-kDa nuclear protein consisting of 782 amino acids (18 exons, spanning 60 kb), it has also been named NP95 and is assigned to chromosome 19p13.3. The UHRF1 open reading frame contains an unusual N-terminal domain that bears a striking resemblance to ubiquitin, a leucine zipper motif, a zinc finger motif, a potential ATP/GTP binding site, a putative cyclin A/E-cdk2 phosphorylation site, retinoblastoma protein (Rb)-binding motifs (^{331}LMCDE^{335} and ^{725}LCCQE^{729}), a ring finger domain, and an SRA-YDG domain[4]. Mouse UHRF1 is strongly expressed in the testis, spleen, thymus, and lung tissue, but not in the brain, liver, or skeletal muscles[4]. Although the biological functions of UHRF1 are unknown, previous studies have suggested that (i) UHRF1 does not have a direct role in DNA replication as part of the DNA synthesizing machinery, like PCNA, but is presumably involved in other DNA replication-linked nuclear events[5]; (ii) UHRF1 may be a growth-regulated gene because its expression is regulated during the cell cycle, required for the G_1/S transition, and specifically induced by E1A, which can force post-mitotic cells to proliferate, and it is a chromatin-associated ubiquitin ligase[6, 7]; (iii) UHRF1 mRNA levels are also increased in transformed BALB/3T3 cells, suggesting that it may participate in the maintenance of the transformed phenotype[8]; (iv) UHRF1 might help recruit DNA-cytosine-5-methyltransferase 1 (DNMT1) to hemimethylated DNA to facilitate faithful maintenance of DNA methylation[9, 10]; and (v) mouse *Np95*-null (*Np95*$^{-/-}$) embryonic stem cells are more sensitive to X rays, UV light, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, and hydroxyu-

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rea than wild-type ($Np95^{+/+}$) or $Np95^{+/-}$ embryonic stem cells[11]. Furthermore, several stable transformants from HEK293 and WI-38 cells that had been transfected with antisense human *NP95* cDNA were more sensitive to X rays, UV light and hydroxyurea than the corresponding parental cells[12]. Additionally, there was no significant redistribution of UHRF1 foci shortly after DNA damage by γ-irradiation, but nodular UHRF1 foci characteristically seen in the G_2 phase were also detected in G_2-arrested cells following γ-irradiation[6]. These results indicate that UHRF1 may play an important role in the regulation of radiosensitivity. Taken together, the available studies indicate that UHRF1 may be a putative oncogene, which would represent a new target for cancer treatment[13].

The current study was designed to determine the effectiveness of adenoviral-mediated transduction of UHRF1 (Ad5-UHRF1) on the radiosensitivity of human cervical cancer HeLa cells. Ad5-UHRF1 significantly reduced HeLa cell sensitivity to γ-irradiation, which is associated with a reduction in radiation-induced apoptosis and G_2 arrest. The radioresistance is correlated with increased DNA repair and increased expression of the DNA damage repair protein XRCC4. Decreased expression of XRCC4 by XRCC4 siRNA significantly reduced the UHRF1-mediated radioresistance. These results provide the first evidence that UHRF1 is an important mediator of radiosensitivity in human cervical cancer cells.

Materials and methods

Cell culture and irradiation The human cervical carcinoma cell line HeLa was originally obtained from American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% FCS, 10 mmol/L glutamine, a mixture of non-essential amino acids, 100 unit/mL penicillin G, and 100 μg/mL streptomycin at 37 °C in 95% air/5% CO_2 (Sigma-Aldrich, St Louis, MO, USA). Irradiation was performed with a ^{137}Cs γ-source at a dose rate of 3.5 Gy/min.

Generation of recombinant adenoviruses The E1-deleted adenovirus-β-gal (Ad-β-gal) was obtained from Introgen Therapeutics, Inc (Houston, TX, USA). A recombinant adenovirus (pAd/CMV/V5-DEST, Invitrogen, Carlsbad, CA, USA) containing full-length human UHRF1 cDNA (Ad5-UHRF1) was prepared as previously described[14]. These adenoviral vectors were propagated in 293 human embryonic kidney (HEK) cells (Invitrogen, Carlsbad, CA, USA) using the Stratagene MBS Mammalian Transfection Kit with a modified calcium phosphate transfection protocol. The transfected cells were incubated at 37 °C for 7 d and then harvested and subjected to four freeze (liquid nitrogen)/thaw (a 37 °C water bath) cycles. Cell lysates were centrifuged at 12 000×*g* for 10 min at 4 °C, and the supernatant (primary virus stock) was transferred to a fresh screw-cap mini-centrifuge tube and stored at -80 °C. Recombinant adenoviruses were further amplified using the same procedure, and the cell lysates were centrifuged on cesium chloride step gradients at 60 000×*g* at 4 °C for 2 h to separate viruses from defective particles and empty capsids. Recovered virus bands were dialyzed against PBS. Viruses were aliquoted in a buffer containing 10 mmol/L Tris, pH 7.4, 10 mmol/L $MgCl_2$, and 10% *v/v* glycerol and stored at -80°C. Under these conditions, there was no precipitation of virus particles or loss of virus infectivity due to inactivation or aggregation. To control for the biological effect of the virus *per se*, the vector Ad5.CMV.Null (Ad5), as a negative control, was constructed in a similar manner but without subcloned gene sequences between the CMV promoter (P cmv) and the polyadenylation signal (TK pA).

For adenovirus infection, 2.5×10^4 cells/well in each well of a six-well dish were infected with the appropriate amount of replication-defective adenoviruses with Ad-β-gal and incubated with gentle shaking for 2 h at 37 °C. X-gal (1 mg/mL) was used to stain cells for β-gal, and the cells were incubated overnight. The cells were then fixed in 10% formalin, washed in PBS, and kept in PBS at 4 °C. Blue-stained cells were considered infected with Ad-β-gal. Afterward, fresh growth medium was added to each dish. To monitor UHRF1 expression, the infected cells were further incubated at 37 °C for various time periods and UHRF1 protein expression was then determined by Western blotting. To examine radiosensitivity, the infected cells at multiplicities of infection (MOI) of 50 or 100 plaque-forming units/cell were incubated at 37 °C for 48 h before γ-ray irradiation.

Silencing XRCC4 using small interfering RNA (siRNA) The XRCC4-siRNA (sc-37406) and control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For siRNA treatments, subconfluent proliferating cells were transfected with 50 nmol/L of siRNA using the siPORT Amine transfection reagent (Ambion, Austin, TX, USA). None of the siRNAs caused significant cytotoxicity based on cell morphology and MTT assays.

Western blotting Protein expression was detected using Western blotting, as previously described[14]. As indicated in respective figures, infected and/or irradiated cells were harvested by trypsin and centrifugation, washed twice with ice-cold PBS, and lysed with a protein lysis buffer containing

Tris-HCl (50 mmol/L, pH 7.4), NP-40 (1%), Na-deoxycholate (0.25%), NaCl (150 mmol/L), EDTA (1 mmol/L), PMSF (1 mmol/L), Na_3VO_4 (1 mmol/L), NaF (1 mmol/L), and a protease inhibitor cocktail [containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin, and aprotinin; P8340, Sigma-Aldrich, St Louis, MO, USA]. After centrifugation, the supernatant was transferred into new tubes. The protein concentration was determined using the Bio-RAD protein assay (Invitrogen, Carlsbad, CA, USA). Total proteins were separated on SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes (Millipore, Billerica, MA). The membranes were then incubated in blocking solution (5% nonfat-milk in 20 mmol/L Tris-HCl, 150 mmol/L NaCl, and 0.1% Tween-20) (TBS-T), followed by overnight incubation with the appropriate primary antibodies. Afterward, the membranes were completely washed with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotech, Santa Cruz, CA, USA) for 1 h. Finally, immunocomplexes were developed with an enhanced horseradish peroxidase/luminol chemiluminescence reagent (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. Primary antibodies: a rabbit polyclonal anti-UHRF1 serum was raised against the purified full-length myc-His_6-tagged UHRF1 protein and affinity purified before use (1:1000 dilution). A rabbit polyclonal anti-Ku80 antibody (1:500 dilution) was purchased from Sigma (St Louis, MO, USA). Mouse monoclonal anti-Ku70 antibody (A-9, 1:1000 dilution), mouse monoclonal anti-DNA-PKcs antibody (G-4, 1:1,000 dilution), goat polyclonal anti-XRCC4 antibody (D-18, 1:500 dilution), mouse monoclonal anti-ATM antibody (5C2, 1:1000 dilution), and goat polyclonal anti α-actin antibody (I–19, 1:2000 dilution) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein bands were quantitated by densitometry, and the values were expressed relative to α-actin (control for loading and transfer).

Clonogenic assay The effectiveness of the combination of Ad5-UHRF1 and ionizing radiation was assessed by clonogenic survival assays. Exponentially growing HeLa cells in six-well tissue culture dishes were infected with Ad5-UHRF1 or Ad5 vector for 48 h and then exposed to different doses of γ-radiation. Following irradiation, the cells were trypsinized and counted, and 2×10^5 cells were seeded in 100-mm culture dishes in two sets of triplicates for each radiation dose, which ensured that >50 macroscopic colonies would appear in each plate after about two weeks. Colonies were finally fixed and stained with 0.5% gentian violet solution. The colonies containing 50 cells were scored. The plating efficiency (PE) was calculated as follows: PE=(colonies formed/cells seeded)×100%.

The radiation doses were chosen so that the survival of the cells extended over a range of 3 logs. Survival curves, which included at least five doses per experiment and three dishes per dose, were fitted to the data using the single-hit multitarget model. The percentage plating efficiency for each dish was calculated by dividing the number of colonies by the number of cells plated and multiplying by 100. The surviving fraction for each radiation dose was normalized by the plating efficiency of un-irradiated controls (40%–60% for HeLa). The values of D_0 or Dq were derived from least-squares regression applied to the logarithm of surviving fractions below 0.1.

Flow cytometry assay Cell cycle and apoptosis analysis was performed using flow cytometry. The culture medium was collected into centrifuge tubes. The cells removed by trypsin were poured into the same tubes. Cells were centrifuged for five minutes at 1800×*g*. The supernatants were poured out, washed once with 1×phosphate-buffered saline and centrifuged for five minutes again. The cells were finally fixed by 5 mL of pre-cooled 70% ethanol for at least 4 h. The fixed cells were centrifuged and washed with 1×phosphate-buffered saline. After centrifugation, the cell pellets were re-suspended in 500 μL propidium iodine (10 μg/mL) containing 300 μg/mL RNase (Sigma-Aldrich, MO, USA). The cells were then incubated on ice for 30 min and filtered using a 53-μm nylon mesh. The cell cycle distribution was calculated from 10 000 cells with ModFit LT™ software (Becton Dickinson, CA, USA) using FACScalibur (Becton Dickinson, CA, USA).

Determination of apoptosis by TUNEL assay Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) was performed using the APO-BRDU kit (PharMingen, San Diego, CA, USA) to quantify induction of apoptosis following the manufacturer's instructions. Briefly, cells were fixed in 1% (*w/v*) paraformaldehyde in ice-cold PBS and incubated on ice for 15 min. Then, the cells were washed twice with PBS and stored in 70% (*v/v*) ethanol overnight. About 1×10^6 cells/treatment, in duplicate, along with positive and negative controls, were counted, pelleted, washed twice with washing buffer, and subjected to a labeling reaction using terminal deoxynucleotidyl transferase overnight at room temperature. At the end of the reaction, the cells were rinsed twice before treatment with fluorescein-labeled anti-BrdU antibody solution in the dark for 30 min. The cells were stained with propidium iodide/RNase solution for 30 min in the dark and analyzed by flow cytometry (Epics XL-MCL, Beckman Coulter Corp, Miami, FL, USA).

Host cell reactivation assay Ten thousand cells were plated in each well of six-well plates. The cells were infected with Ad5-UHRF1 or Ad5 at an MOI of 100 followed by a 48-h incubation. The cells were then infected with Ad-β-gal (1×10^3 vp/cell) that had been irradiated with 0–4000 Gy of γ-ray irradiation and incubated for an additional 24 h. This dose of 4000 Gy was necessitated by the small genome size of the adenoviral vector compared with a mammalian cell. Calculations indicated that this dose would produce about 1-2 DSBs/vector particle. The cells were then stained with X-gal following the procedure described above and fixed in 10% formalin. β-gal-positive (blue) cells were scored under high power (×40) of a light microscope. The data are presented as a percentage of control.

Statistics All experiments were repeated at least three times Results are expressed as the mean±SEM (standard error of mean). Statistical analysis was performed with a two-tailed Student *t*-test when two treatment regimens were compared. A confidence level of 95% ($P<0.05$) was considered statistically significant.

Results

Increase in UHRF1 protein expression by the adenovirus-transduced UHRF1 gene In this study, HPV-positive cervical cancer HeLa cells, which lack normal RB and p53 functions, were used for investigating the biologic activities of UHRF1. To detect the expression of UHRF1 protein, HeLa cells were infected with Ad5 (as a negative control) or Ad5-UHRF1 at an MOI of 50 or 100. At 48 h after infection, total cellular proteins were extracted and subjected to Western blotting. As shown in Figure 1A, HeLa cells contained a low level of the endogenous UHRF1 protein, because an approximately 95 kDa protein band was detectable. Ad5 infection did not cause any significant alteration in UHRF1 protein expression. However, the expression of the UHRF1 protein was significantly elevated in the cells infected by Ad5-UHRF1 in a dose-dependent manner, with about a 4-fold increase at an MOI of 50 and a 6-fold increase at an MOI of 100.

Adenovirus-mediated *UHRF1* gene expression reduces radiosensitivity of HeLa cells As shown in Figure

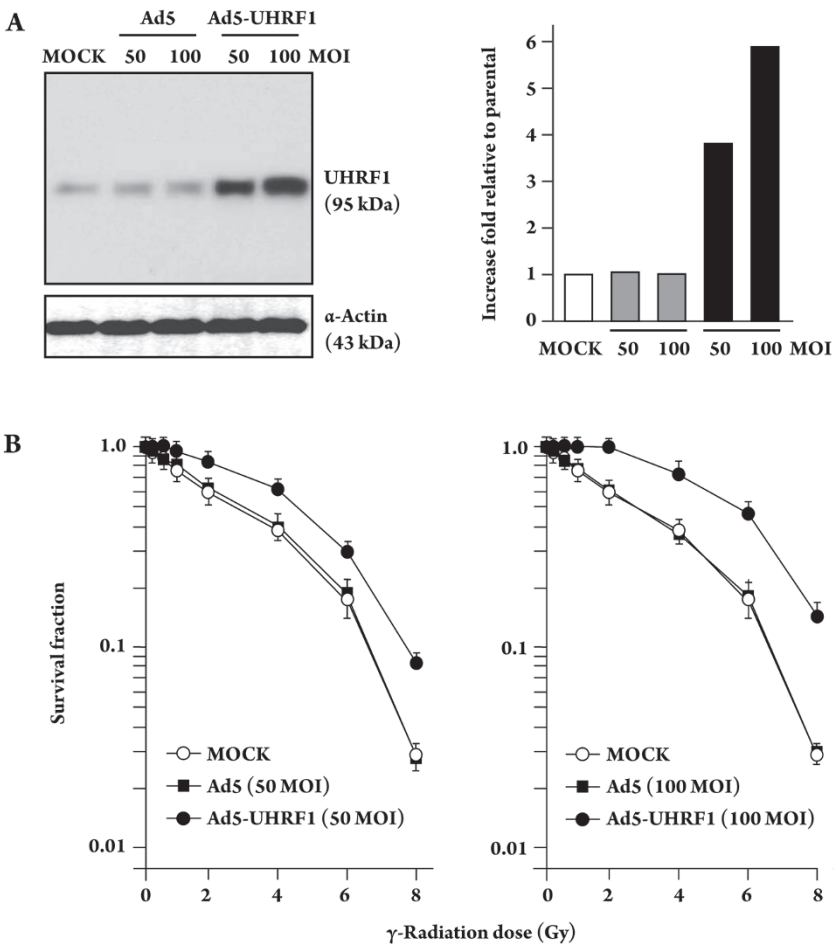

Figure 1. Ad5-UHRF1 reduces radiosensitivity of HeLa cells. (A) Exponentially growing cells were infected with PBS (MOCK) or infected with Ad5 or Ad5-UHRF1 at an MOI of 50 or 100 for 48 h, collected and then subjected to Western blotting. Each lane was loaded with 50 μg of total protein, and α-actin was used as a loading control. (B) Exponentially growing cells were uninfected or infected with an MOI of 50 (left panel) or 100 (right panel) for 48 h, exposed to 0–8 Gy of γ-radiation and subjected to the clonogenic assay. Data shown are means±SEM of three independent experiments.

1B, in PBS-treated (MOCK) HeLa cells (HeLa/MOCK), doses of 2, 4, and 6 Gy of irradiation killed about 39%, 61%, and 92% of cells, respectively. HeLa cells infected by Ad5 at an MOI of 50 (HeLa/Ad5) showed a similar survival curve to that of HeLa/parental cells after irradiation, *ie*, Ad5 did not change radiosensitivity. However, 2, 4, and 6 Gy of γ-irradiation killed approximately 18%, 38%, and 80% cells, respectively, after infection with an MOI of 50 of Ad5-UHRF1.

A shoulder of the survival curve signifying the cellular repair capacity was observed in irradiated HeLa cells infected with Ad5-UHRF1 at an MOI of 50, as indicated by an increased value of D_0 at 2.3 versus 0.9 for MOCK cells and Ad5-infected cells. The value of *D*q was 1.7 Gy for MOCK cells and Ad5-infected cells, whereas the value was 0.9 Gy for the Ad5-UHRF1 infected cells. An MOI of 100 of Ad5-UHRF1 caused a further reduction in radiosensitivity (Figure 1B), with a D_0 value of 0.9 and a *D*q value of 2.1. Infection by Ad5-UHRF1 at an MOI of 100 plaque-forming units/cell did not produce a significant toxic effect or reduction in plating efficiency of HeLa cells up to 72 h after infection. These results indicated that the Ad5-UHRF1-mediated radiosensitivity appeared not to be a result of the cytotoxicity of high UHRF1 expression.

Ad5-UHRF1 reduces apoptosis caused by radiation The flow cytometry assay was used to determine whether Ad5-UHRF1 led to radiation-induced apoptosis and cell cycle arrest in HeLa cells. The data presented in Figure 2 are representative of three independent observations following 6 Gy irradiation. Radiation alone induced apoptosis in MOCK cells, as indicated with an accumulation of the sub-G_1 population. No impact on the radiation-induced apoptosis was observed after Ad5 infection compared with MOCK cells. However, Ad5-UHRF1 potently reduced radiation-induced apoptosis, as indicated by a significantly decreased sub-G_1 population following irradiation. Furthermore, as determined by the TUNEL assay, 6 Gy alone induced about 18%–20% apoptosis over the background level in MOCK cells and Ad5-infected cells, and Ad5-UHRF1 led to a reduction of about 3% apoptosis over the background. Ad5-UHRF1 blocked radiation-mediated apoptosis induction 48 or 72 h following γ-irradiation as well (data not shown). These results indicate that increased expression of UHRF1 reduces apoptosis induced by radiation.

We noted that radiation alone also caused a significant G_2-M arrest, except for the sub-G_1 accumulation, in control MOCK and Ad5 cells. However, an increased G_1 arrest was observed in Ad5-UHRF1 cells, which was accompanied by a reduction in the sub-G_1 and G_2-M populations (Figure 2).

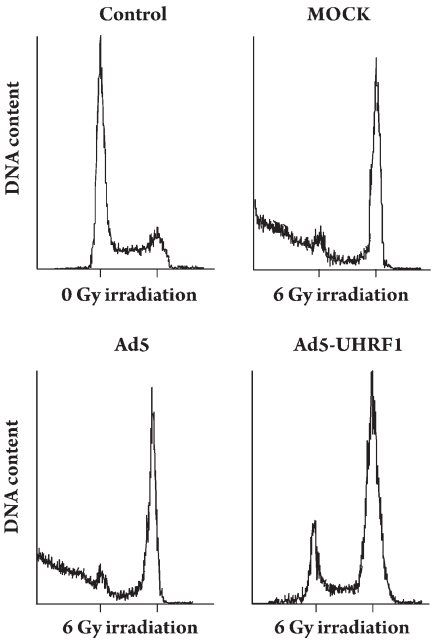

Figure 2. Effect of UHRF1 overexpression on apoptosis and cell cycle arrest caused by radiation. Cells were either PBS (MOCK)-infected or infected with Ad5 or Ad5-UHRF1 at an MOI of 100, incubated for 48 h, and then irradiated with 6 Gy. The cells were harvested 24 h after irradiation and subjected to flow cytometry. The "sub-G_1 peak" representing apoptotic cells is separated from the G_1 peak of the nonapoptotic cell population.

These results indicate that UHRF1 overexpression not only reduces apoptosis induction, but also alters the cell cycle distribution after radiation.

UHRF1 increases the DNA repair capacity of HeLa cells We also performed a host cell reactivation assay to determine whether the effect of Ad5-UHRF1 on radiosensitivity was due to an enhancement of the DNA repair capacity. For this, HeLa cells pre-infected with either PBS (MOCK) or Ad5 or Ad5-UHRF1 at an MOI of 100 for 48 h were subsequently infected with Ad-β-gal that had been irradiated with 4000 Gy of γ-radiation. The ability of the HeLa cells to reactivate the irradiated Ad-β-gal based on β-gal expression was then determined 24 h later. As shown in Figure 3, the capacity to reactivate irradiated Ad-β-gal was significantly enhanced in HeLa cells infected with Ad5-UHRF1 compared with HeLa cells receiving either MOCK or Ad5 infection, with an approximately 2-fold increase ($P<0.05$). These results indicate that UHRF1 overexpression increases the DNA damage repair capacity of HeLa cells.

UHRF1 increases DNA repair protein XRCC4 expression Several mammalian non-homologous end joining proteins have been identified in DNA damage repair, includ-

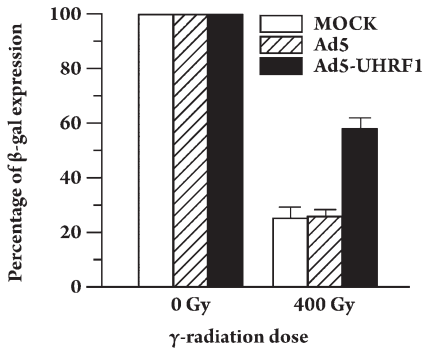

Figure 3. Effect of UHRF1 overexpression on DNA repair capacity. Exponentially growing cells were seeded in each well of six-well tissue culture dishes, mock-infected or infected with Ad5 or Ad5-UHRF1 at an MOI of 100 for 48 h, and then re-infected with irradiated (0 or 4000 Gy) Ad-β-gal at 1000 vp/cell for another 24 h. The cells were finally stained for β-gal, as described under Materials and Methods. The β-gal-positive cells were counted. Data shown are means±SEM as a percentage of unirradiated Ad-β-gal from three independent experiments.

ing the ataxia telangiectasia mutated (ATM) protein, the Ku protein (Ku70–Ku80 heterodimer), the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), X-ray-sensitive complementation group 4 (XRCC4). As shown in Figure 4, the endogenous expression levels of the proteins ATM, Ku70, Ku80, and DNA-PK were not altered within MOCK cells, Ad5-infected cells, and Ad5-UHRF1-infected cells. γ-Irradiation alone caused a significant increase in ATM, DNA-PK, and XRCC4 protein expression but not in Ku70 and Ku80 protein expression, results were consistent among MOCK cells, Ad5-infected cells and Ad5-UHRF1 infected cells. However, an increased expression of the endogenous XRCC4 protein, but not the other proteins, was detectable in HeLa cells infected with Ad5-UHRF1. Furthermore, another accumulation of XRCC4 protein caused by radiation was enhanced in Ad5-UHRF1 infected cells. These results indicate that, among the DNA damage repair proteins examined here, only XRCC4 was affected by UHRF1 overexpression.

Knocking down XRCC4 attenuates UHRF1 mediated radiosensitivity To further determine the possible role of XRCC4 in UHRF1-mediated radiosensitivity, we employed UHRF1 siRNA to down-regulate XRCC4 expression and measured the radiosensitivity in Ad5-UHRF1-infected cells. As illustrated in Figure 5A, XRCC4 protein expression was significantly reduced in HeLa cells transfected with UHRF1 siRNA relative to cells transfected with control siRNA. The reduced expression of XRCC4 protein by XRCC4 siRNA was observed to be time-dependent, because the expression was significantly inhibited at 24 h and not detectable at 72 h following transfection.

As determined by MTT assay, the reduced radiosensitivity caused by UHRF1 overexpression was significantly inhibited in HeLa cells transfected with XRCC4 siRNA (Figure 5B). For example, an approximately 49% cell viability was obtained in control siRNA+Ad5-UHRF1 cells, whereas only 23% cell viability was obtained in XRCC4 siRNA+Ad5-UHRF1 cells (P<0.01). An increased radiosensitivity was observed in the cells transfected with XRCC4 siRNA compared with the cells transfected with control siRNA (P<0.05). Similar results have also been obtained by employing a clonogenenic assay (Figure 5C). In addition, we also

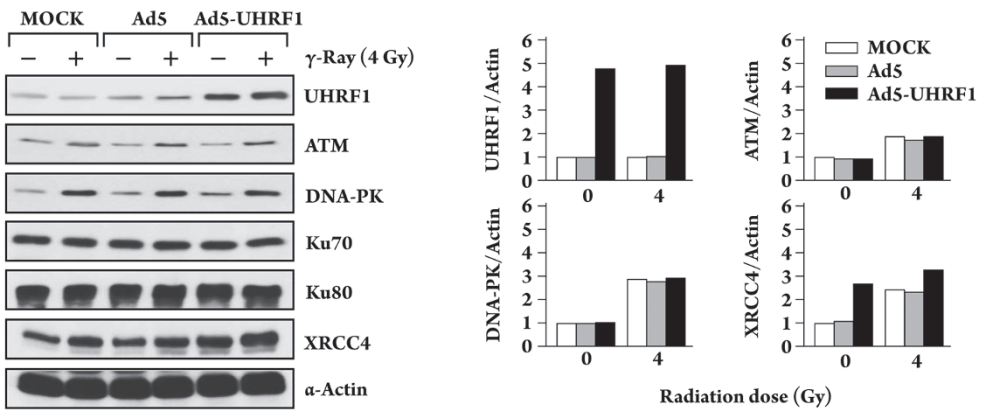

Figure 4. Effect of UHRF1 overexpression on expression of DSB repair proteins. Exponentially growing cells were mock treated or infected with Ad5 or Ad5-UHRF1 at an MOI of 100 for 48 h, followed by exposure to 0 or 4 Gy. The cells were then harvested and lysed 24 h after irradiation, and 100 μg protein was electrophoresed and subjected to Western blotting. α-Actin was used as a loading control. For comparison, the densitometry value for Ad5-infected and un-irradiated cells was assigned a value of 1, and values for all treatments were normalized to 1 (fold change versus Ad5 control).

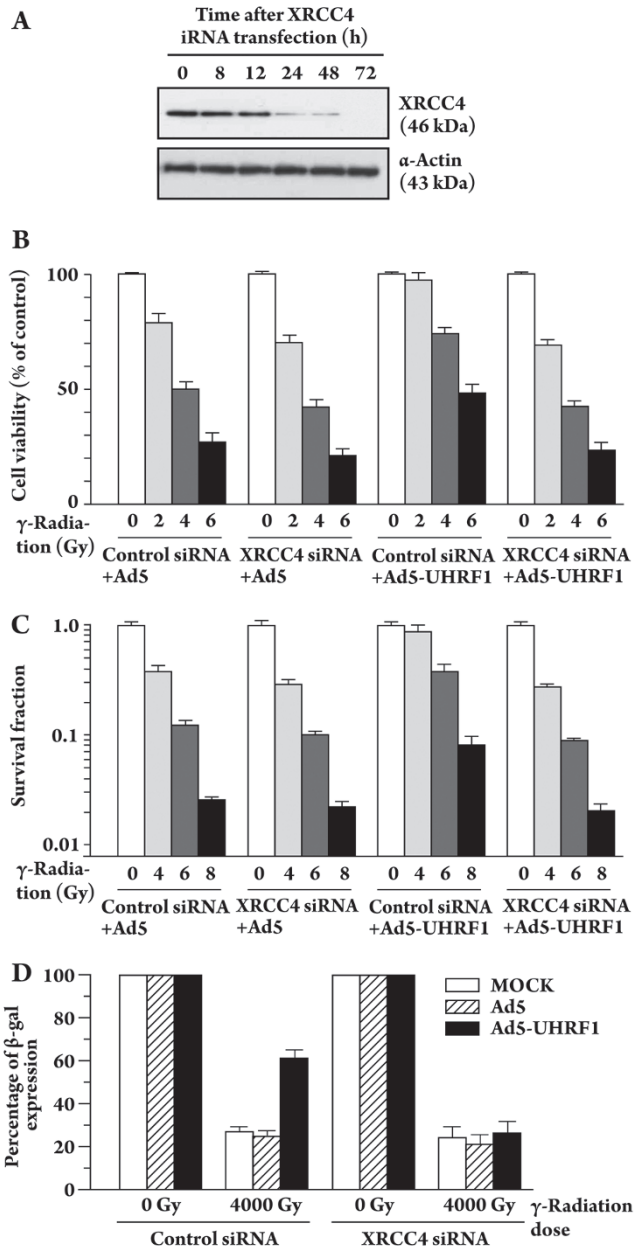

Figure 5. Effect of XRCC4 siRNA on UHRF1-mediated radioresistance and DNA damage repair. Exponentially growing HeLa cells were transfected with control siRNA or XRCC4 siRNA for 24 h, MOCK-infected or infected with Ad5 or Ad5-UHRF1 at an MOI of 100 for 48 h and finally irradiated (0, 2, 4, or 6 Gy). The cells were subjected to the MTT assay 24 h after radiation as described under "Materials and Methods." (A) Western blotting was performed to determine XRCC4 expression at different times after XRCC4 siRNA transfection. Each lane was loaded with 50 μg total protein, and α-actin was used as a loading control. (B) Data shown are means±SEM of three independent experiments. (C) Exponentially growing HeLa cells were transfected with control siRNA or XRCC4 siRNA for 24 h, MOCK-infected or infected with Ad5 or Ad5-UHRF1 at an MOI of 100 for 48 h, and finally irradiated (0, 4, 6, or 8 Gy). The cells were subjected to a clonogenic assay after radiation as described under "Materials and Methods." (D) Exponentially growing HeLa cells were transfected with control siRNA or XRCC4 siRNA for 24 h, MOCK-infected or infected with Ad5 or Ad5-UHRF1 at an MOI of 100 for 48 h, and infected with Ad-β-gal that was either unirradiated or irradiated with 0 or 4000 Gy at an MOI of 100 for another 24 h. Cells were then stained for β-gal. The β-gal-positive cells were counted and recorded independently by two investigators and visualized. The percentage of positive cells was normalized to the controls for comparison. Results are expressed as means±SEM of 3 independent experiments with similar results.

found that silencing XRCC4 expression significantly blocked the UHRF1 reduction of DNA repair ability, although the reduced expression of XRCC4 alone only slightly affected the cell capability of DNA damage repair (Figure 5D). These results suggest that XRCC4 is a radiosensitivity mediator and may be a critical target in UHRF1 mediated radiosensitivity and DNA damage repair.

Discussion

Radiotherapy is used to kill cancer cells through the use of high-energy X or γ-rays or particles and is still one of the best therapies to treat cervical cancer. Although advanced and invasive cervical cancers become resistant to radiotherapy, the mechanisms of this phenomenon still need to be discovered. The present study was designed to examine the effect of human UHRF1, a newly isolated gene, on the radiosensitivity of human cervical cancer HeLa cells using adenovirus-mediated expression of human UHRF1. Ad5-UHRF1 significantly reduced the radiosensitivity of HeLa cancer cells in a dose-dependent manner as determined based on clonogenic survival assays (Figure 1). Infection with the control

Ad5 vector caused a negligible impact on the cell radiosensitivity, indicating that the effect of Ad5-UHRF1 on radioresistance is mediated by the expression of UHRF1 and not due to a nonspecific effect of the vector. These results are consistent with a previous report in which the down-expression of human or mouse UHRF1 was shown to increase cellular susceptibility to ionizing and UV radiation[12]. Moreover, this reduced radiosensitivity by UHRF1 is independent of the p53 and RB tumor suppressor genes. Both of these genes play important roles in the regulation of radiosensitivity, because HeLa cells contain inactivated p53 and RB function due to the repression of the HPV E6 and E7 genes.

The cell cycle checkpoint is an essential control mechanism for maintaining genome stability. Radiation-induced DNA damage is one of the most important initiation signals of cell cycle arrest. Apoptosis is a form of cell death designed to eliminate unwanted host cells through activation of a coordinated, internally programmed series of events affected by a dedicated set of gene products. Apoptosis is induced by ionizing radiation, which is one of the most common mechanisms of cell death. As shown in Figure 2, γ-irradiation caused a typical G_2 cell cycle arrest and apoptosis induction in parental HeLa cells and Ad5-infected HeLa cells (Figure 2). However, HeLa cells infected with Ad5-UHRF1 exhibited a significant reduction of the G_2 population and apoptosis caused by radiation. It was noted that radiation increased the G_1 population in Ad5-UHRF1-infected cells, although the reason for this phenomenon is unknown. p53 and RB are inactivated in HeLa cells due to HPV E6 and E7; hence, the radiation-induced G_1 arrest in Ad5-UHRF1-infected cells may occur via a p53- and RB-independent mechanism. Further study on the role of p53 or RB in the UHRF1-mediated radiosensitivity is underway in our laboratory.

Double strand breaks of DNA are the most critical DNA damage induced by ionizing radiation. Defects in DNA damage repair and/or DNA processing lead to genomic instability and, in turn, to increased susceptibility to ionizing radiation[15]. Alterations of DNA repair-associated genes are therefore expected to show abnormal cell responses to radiation. Ad5-UHRF1 cells characterized as radioresistant by clonogenic survival assays showed an increased viral recovery from radiation-induced (lethal) damage as determined by a sensitive host cell reactivation technique; ie, HeLa cells with Ad5-UHRF1 infection increased their ability to restore reporter gene expression using an irradiated Ad-β-gal vector by two-fold when compared with control cells with Ad5 infection (Figure 3). The enhanced host cell reactivation of the radiation-damaged reporter was not detected in control Ad5-infected cells. The data regarding host cell reactivation indicate that Ad5-UHRF1 cells may carry increased capability to repair DNA damaged by γ-irradiation.

In eukaryotes, double strand breaks caused by radiation are repaired by either homologous recombination or non-homologous end joining[16]. The non-homologous end joining is predominant in the G_0, G_1, and early S phases of cells and is exclusively required for V(D)J recombination[16]. Several proteins have been identified to play essential roles in non-homologous end joining[17–19], including ATM, the Ku protein (Ku70-Ku80 heterodimer), DNA-PKcs, XRCC4 and others. To study the impact of UHRF1 on these proteins, we determined the expression of these proteins after Ad5-UHRF1 infection compared with the control Ad5 infection and found significantly increased expression of XRCC4 protein in Ad5-UHRF1 cells (Figure 4). Moreover, a further enhancement of the radiation-induced increase in the XRCC4 protein was observed in Ad5-UHRF1 cells. The alterations of other proteins, including Ku70, Ku80, and DNA-PKcs were not detected in Ad5-UHRF1 cells in contrast to MOCK cells and Ad5 cells. The down-regulation of XRCC4 by XRCC4 siRNA significantly reduced Ad5-UHRF1 mediated radioresistance (Figure 5).

The XRCC4 and ligase IV proteins form a complex *in vivo* for DNA repair by binding cooperatively to DNA, bridging two damaged DNA ends for ligation, and interacting with the Ku protein already present at the ends[18]. Disruption of the XRCC4 gene in mice led to embryonic lethality and accompanied a primary defect in severe apoptosis and neurogenesis[18, 19]. The early development of B-cell lymphomas was observed in $XRCC4^{-/-}$ and $p53^{-/-}$ mice due to an increased likelihood of chromosome translocations and gene amplifications[18]. Thus, XRCC4 and the associated NHEJ factors appear to play a critical role not only in DNA damage repair and maintaining the integrity of the genome, but also in guarding against cancer. More recently, Jones KR *et al*[20] reported that the adenovirus expressing the truncated XRCC4 protein sensitized breast cancer cells to ionizing radiation, presumably through interference with the functional activity of ligase IV, leading to inhibition of the final ligation step in end joining of DNA damage caused by radiation. In accordance, our XRCC expression data provide further evidence of a connection between cellular radioresistance and a common signal(s) affecting DNA repair and DNA processing after irradiation in Ad5-UHRF1 cells. Although the exact mechanism by which UHRF1 reduces the radiosensitivity of cells is still not fully elucidated, taken together the data suggest that XRCC4 may be one of the most important DNA damage repair proteins targeted by UHRF1 in radioresistance.

In summary, we have shown that the enforced expression of functional UHRF1 by adenoviral-mediated transduction of UHRF1 may reduce the radiosensitivity of cervical cancer HeLa cells by enhancing the DNA damage repair pathway, reducing apoptosis induction and altering cell cycle progression. Increased expression of the DNA repair protein XRCC4 by UHRF1 may be one of mechanisms for the enhancement of DNA repair in radioresistance. A complete understanding of the role of XRCC4 in UHRF1-mediated radiosensitivity requires future studies. However, our present results suggest the need for continued development of strategies for sensitizing human cervical cancer cells to radiotherapy by targeting UHRF1 expression.

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Author contribution

Sai-jun FAN designed research; Xin-li LI and Qing-hui MENG performed research; Xin-li LI and Qing-hui MENG contributed new analytical tools and reagents; Xin-li LI and Qing-hui MENG analyzed data; Sai-jun FAN wrote the paper.

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