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Radiosensitization by targeting radioresistance-related genes with protein kinase A inhibitor in radioresistant cancer cells

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Abbreviations: DNA-PK, DNA-dependent protein kinase; DSBs, double strand breaks; H-87, N-[2-(p-bromo cinnamyl methyl amino)ethyl]-5-isoquinoline sulfonamide; H-89, N-[2-(p-bromo cinnamyl amino)ethyl]-5-isoquinoline sulfonamide; HR, homologous recombination; IR, ionizing radiation; MDR, multidrug resistant; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; NHEJ, nonhomologuous end-joining; PKA, protein kinase A; PMSF, phenyl methyl sulphonyl fluoride

Abstract

Here we determined which radiation-responsive genes were altered in radioresistant CEM/IR and FM3A/IR variants, which showed higher resistance to irradiation than parental human leukemia CEM and mouse mammary carcinoma FM3A cells, respectively and studied if radioresistance observed after radiotherapy could be restored by inhibition of protein kinase A. The expressions of DNA-PKcs, Ku70/80, Rad51 and Rad54 genes that related to DNA damage repair, and Bcl-2 and NF-κB genes that related to antiapoptosis, were up-regulated, but the expression of proapototic Bax gene was down-regulated in the radioresistant cells as compared to each parental counterpart. We also revealed that the combined treatment of radiation and the inhibitor of protein kinase A (PKA) to these radioresistant cells resulted in synergistic inhibition of DNA-PK, Rad51 and Bcl-2 expressions of the cells, and consequently restored radiosensitivity of the cells. Our results propose that combined treatment with radiotherapy and PKA inhibitor can be a novel therapeutic strategy to radioresistant cancers.

Keywords: cyclic AMP-dependent protein kinases; gene expression profiling; gene expression regulation, neoplastic; radiation

Introduction

Radiation therapy is an effective modality for the treatment of many tumors (Rosen *et al*., 1999). However, its therapeutic efficacy is often hindered by acquirement of radioresistant phenotype of cancer cells (Pirollo *et al*., 1997). Ionizing or ultraviolet radiation-induced cellular stress signaling pathways induce insensitivity of cancer cells to radiotherapy (Gupta *et al.,* 2003; Chastel *et al*., 2004; Willers *et al*., 2004). Therefore, small molecules designed to specifically target key components of this signaling network are now being developed for clinical use as single therapeutic agents and/or in combination with other forms of therapy to overcome the resistance.

Molecular events leading to the formation of a radioresistant phenotype in cancer cells treated with ionizing radiation (IR) have not been studied extensively. Many genes have been found to be inducible by stress conditions such as radiation, and some of the stress-responsive genes have been implicated in cells adapting to or being protected from radiation (Vallis *et al*., 2002). The identification of many proteins that modulate apoptosis within a cell has provided insights into the molecular events important for mediating radiation-induced apoptosis. The deregulation of DNA damage repair (Ader *et al*., 2002), induction of cell cycle arrest (Kang *et al*., 2004; Vavrova *et al*., 2004) and apoptosis resistance (Snyder and Morgan, 2004; Soderlund *et al*., 2005) may be responsible for radioresistance, even though

the mechanism of radioresistance is not fully understood. Therefore, the proteins controlling the processes of repair of DNA double strand breaks (DSBs), cell cycle arrest and apoptosis after IR-induced DNA damage are attractive candidates for generating variation in radiosensitivity.

Two major pathways for repairing DNA DSBs including nonhomologuous end-joining (NHEJ) and homologous recombination (HR) have been identified in mammalian cells, and are induced by a number of DNA-damaging agents including IR and radiomimetic drugs (Fukushima *et al*., 2001). NHEJ dominates during G1 to early S phase of the cell cycle, and HR is used in late S to G_2 phases. If left unrepaired, they can lead to chromosome loss and cell death. The NHEJ pathway requires the activity of DNA-dependent protein kinase (DNA-PK), a multimeric serine-threonine kinase composed of a catalytic subunit (DNA-PKcs) and regulatory subunits (Ku autoantigens: Ku70/80) (Smith and Jackson, 1999; Jackson, 2002). In contrast, Rad51 and Rad54 proteins play a key role in HR in eukaryotes (Essers *et al*., 2000; Allen *et al*., 2003). Altered expression of DNA repair protein has been implicated in the radioresistant phenotype observed in cell population that had been treated with multiple dose of radiation (Marples *et al*., 2002). In addition, many other stress-responsive genes are up-regulated in gene expression profiles obtained from radiation-treated cells (Chastel *et al*., 2004). Accumulating evidences suggest that the antiapoptotic factors such as Bcl-2 (Condon *et al*., 2002; Streffer *et al.,* 2002) and NF-κB (Chen *et al.,* 2002; Tamatani *et al.,* 2004) could be major contributors to radioresistance, and also cross-talk between the DNA-PK and NF-κB could contribute to acquire drug resistance of cancer cells (Um *et al*., 2001).

Agents that target various signaling pathways are being used in combination with radiotherapy to improve the therapeutic index without a clear understanding of how these agents may affect radiosensitization (Tenzer *et al*., 2002). It has been reported that the protein kinase A (PKA) signal pathway regulates cell proliferation, differentiation and apoptosis of cancer cells (Tortora *et al*., 2002) and proposed that inhibition of PKA could down-regulate NF-κB and Ku activities, both of which play important roles in drug resistance, and it lead to enhance the effectiveness of anticancer drugs against multidrug resistant (MDR) cells with high activities of NF-κB and Ku (Um *et al*., 2001).

In this study, we determined which radiation-responsive genes were altered in radioresistant CEM/IR and FM3A/IR variants, which showed higher resistance to irradiation than parental human leukemia CEM and mouse mammary carcinoma FM3A cells, respectively and studied if radioresistance observed after radiotherapy could be restored by inhibitor of protein kinase A.

Materials and Methods

Materials

The following reagent was obtained from the listed source and used at the concentrations indicated in the text. N-[2-(p-bromo cinnamyl amino) ethyl]-5-isoquinoline sulfonamide (H-89) was purchased from Calbiochem (San Diego, CA). N-[2-(p-bromo cinnamyl methyl amino) ethyl]-5-isoquinoline sulfonamide (H-87) was kindly donated by Dr. Hidaka (University of Nagoya, Japan). All other materials were obtained in the highest grade.

Development of the radioresistant sublines

The human lymphoblastic leukemia CCRF-CEM (CEM) cells were kindly donated by Dr. Beck (University of Illinois). The cells were maintained in RPMI 1640 (GIBCO Invitrogen Corp., Carlsbad, CA) containing 10% fetal bovine serum (FBS; GIBCO) in a 5% CO_2 atmosphere at 37°C. The cells were irradiated with 6 Gy fraction per cycle using ^{137}Cs gamma cell 3000 Elan Exactor (MDS Nordion, Canada) radiation source at a dose rate of 5 Gy/min and rested for 2 weeks between cycles. This interval was chosen to give the cells enough time to carry out repair processes. Ten cycles of radiation with a total dose of 60 Gy was given over a period of 3 months to produce the CEM/IR subline, which was used without cloning. The FM3A mouse mammary carcinoma cells were cultured in RPMI1640 supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 g/ml). The FM3A/IR cells were derived after fractionated irradiation of FM3A cells with a total dose of 54 Gy (6 Gy/fraction/two weeks).

Growth inhibition assay

Cells (2×10^4 cells/ml) were seeded in 96 well plates and treated with radiation in the presence or absence of H-89 or H-87, the PKA inhibitor. After irradiation, the cells were incubated at 37 °C for 5 days. It followed by the addition of 100 l of MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] solution (5 mg/ml) and the plates were incubated in the dark room for 4 h (Kim *et al*., 2004). The water insoluble MTT-formazan crystals were dissolved in dimethyl sulfoxide, and the reduction of MTT was determined at 570 nm using ELISA reader (Bio-Tec Instruments).

Cell extract preparation and electrophoretic mobility shift assay (EMSA)

Cells (1×10^6 cells/ml) were exposed to graded single doses in the presence or absence of PKA inhibitor for the indicated times, nuclear extracts of the cells were prepared from the cells (Um *et al*., 2004). In brief, the cells were washed with cold phosphate buffered saline and harvested quickly and then resuspended in 300 l of lysis buffer [10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM phenyl methyl sulphonyl fluoride (PMSF)]. The cells allowed swelling in ice for 10 min. After 0.05% Nonidet P40 was added, the tube was vigorously mixed 3 times for 3 s on a vortex, and centrifuged at $250 \times g$ for 10 min to pallet the nuclei. The nuclear pellet was resuspened in 30 l of ice-cold nuclear extraction buffer (5 mM HEPES, pH 7.9, and 26% glycerol (v/v), 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF). Then this pellet was incubated on ice for 30 min with intermittent mixing, and centrifuged at 24,000 $\times$ *g* for 20 min at 4°C. The nuclear extract was either used immediately or stored at -70°C for later use. Ten g of nuclear extract was incubated with ^{32}P-labeled double-stranded oligonucleotide, 5'-AGTTGAGGG-GACTTTCCCAGGC-3' for Ku binding (Santa Cruz Biotechnology) in binding buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 4% glycerol) containing 50 g/ml of poly (dI-dC). The DNA-protein complex was separated from free oligonucleotide on 4% nondenaturing polyacrylamide gel using 0.5 $\times$ TBE buffer (44.4 mM Tris-HCl, pH 8.0, 44.5 mM boric acid, 1 mM EDTA) for 3 h at 120 V. The gels were dried and autoradiographed.

Western blot analysis

Whole cell lysates or nuclear extracts containing an equal amount of protein were separated by SDS-PAGE and blotted to nitrocellulose membrane (Hybond-ECL, Amersham Biosciences, Buckinghamshire, England). The membrane was incubated with antibody as specified, followed by secondary antibody conjugated with horseradish peroxidase. Specific antigen-antibody complexes were detected by enhanced chemiluminescence (Pierce Biotechnology, Inc., Rockford, IL). The following antibodies were used in this study. The purified antibodies specific to the 70- and 86-kDa Ku protein, Rad51, Rad54, p53, MDM2, NF-κB, Bcl-2, Bax and β-tubulin were from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. The anti-DNA-PKcs antibody was from Neomarkers (Lab Vision Corp., Fremont, CA). Secondary antibodies were obtained from Amersham Biosciences.

DNA-PK assay

The kinase activity of whole DNA-PK complex was determined using the Signa TECT^{TM} DNA-dependent Protein Kinase Assay system from Promega Corp. (Madison, WI). In brief, 10 g of nuclear extract of radioresistant and its parental cells was incubated with activator DNA, a biotinylated p53-derived peptide substrate, and [^{32}P] ATP for 5 min at 30°C. The sample was terminated by adding termination buffer. Each termination reaction sample was spotted onto SAM^{2TM} Biotin Capture Membrane and then washed with 2 M NaCl and 2 M NaCl in 1% H_3PO_4. The SAM2^{TM} membrane squares were analyzed using Molecular Imager System (Bio-Rad Laboratories, Inc., Model GS 525, Hercules, CA).

Apoptosis assay

The cells (1×10^6 cells/well) treated with radiation (1-4 Gy) in the presence or absence of H-89 were washed in incubation buffer (10 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$). After 24 h of incubation, cells were centrifuged and resuspended in 500 l of annexin V buffer provided with the annexin V FITC kit (BD pharmigen). Five l of annexin V was added to each well and the cells were incubated with at room temperature for 15 min. Cells were then transferred to tubes and samples were analyzed by flow cytometry on a FACScan system (Becton Dickinson).

Results

Isolation of radioresistant variants of CEM and FM3A cells and determination of gene expression in response to radiation

To assess radioresistance of CEM/IR cells, the cell survival after graded single doses of γ-irradiation was compared between CEM/IR and parental CEM cells (Figure 1A). CEM/IR cells were markedly resistant to radiation than CEM cells. Similar result was observed in FM3A/IR cells, showing significant radioresistance in comparison with parental FM3A cells (Figure 1B). Therefore, to establish the mechanism how cancer cells acquire radioresistant phenotype, the expression of a variety of genes commonly associated with responsiveness to radiation in CEM/IR cells was determined by Western blot analysis and their cellular levels compared with those in CEM cells. As shown in Figure 2A, the expression of DNA-PKcs, Ku70/80, Rad51 and Rad54 genes that related to DNA damage repair, and Bcl-2 and NF-κB genes that related to antiapoptosis, was up-regulated, but the expression of proapototic Bax gene was down-regulated in CEM/IR cells as compared to

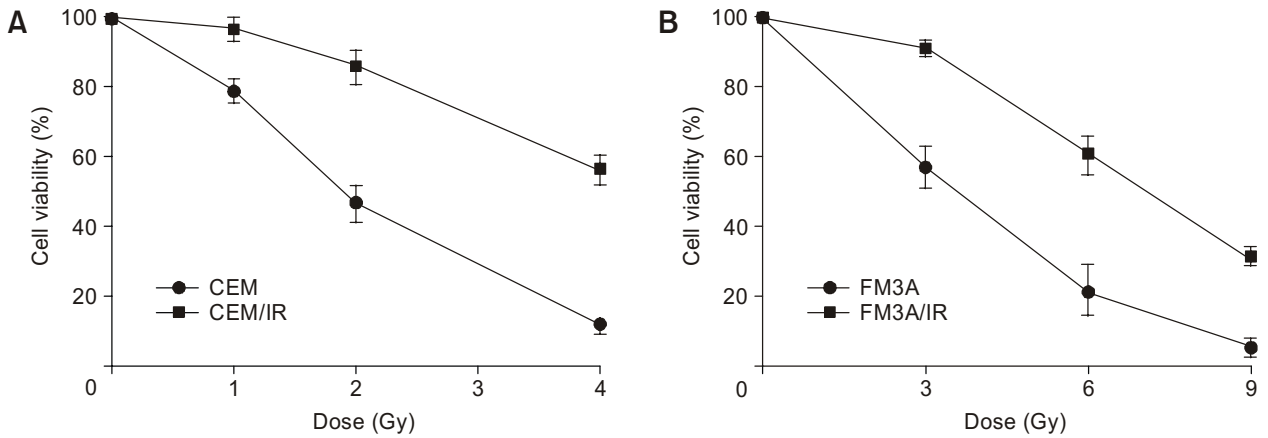

Figure 1. Determination of radiosensitivity in radioresistant variants of cancer cells. The radioresistant CEM/IR (A), FM3A/IR (B) cells and each parental counterpart were exposed to the indicated doses of γ-irradiation. At 5 days after irradiation, the cell viability relative to untreated control cells was determined using the MTT assay.

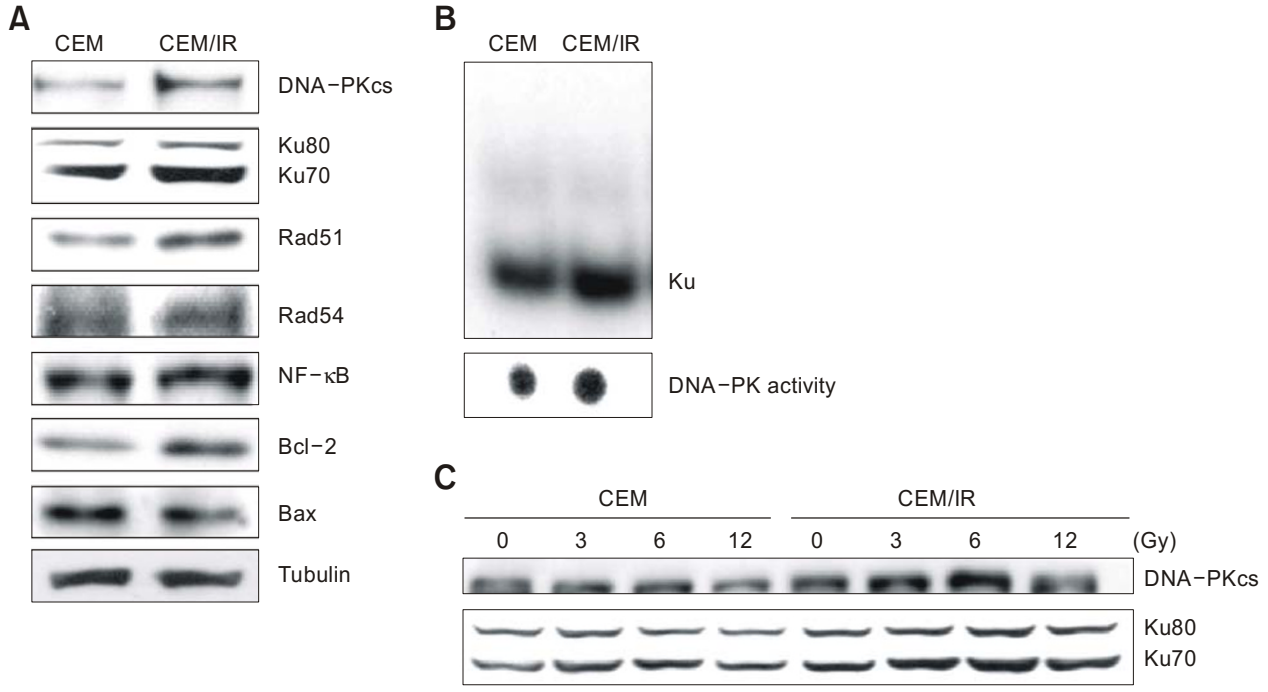

Figure 2. The change in levels of radiation-responsive proteins between CEM and CEM/IR cells. The basal levels of radiation-responsive proteins were assayed by Western blot analysis using whole cell lysates or nuclear extracts isolated from CEM/IR and its parental CEM cells. β-tubulin (tubulin) was used as a loading control (A). Ku-DNA binding and kinase activity of DNA-PK were determined by electrophoretic gel mobility shift assay (EMSA) (B, upper panel) and DNA-PK Kinase Assay system, respectively (B, lower panel). At 4 h after exposure to the indicated doses of γ-irradiation, expression of DNA-PK components was determined by Western blot analysis (C).

those in the CEM cells. In agreement with elevated protein levels of DNA-PKcs and Ku70/80 in CEM/IR cells, the Ku-DNA binding activity and the kinase activity of whole DNA-PK complex in the cells were increased in comparison with the CEM cells (Figure 2B).

To investigate difference in cellular response to IR between CEM/IR and CEM cells, the modulation of DNA-PK, the sensitive markers of DNA damage repair, was determined at 4 h after exposure to various doses of γ-irradiation. The basal levels of Ku70/80 and DNA-PKcs were higher in CEM/IR cells

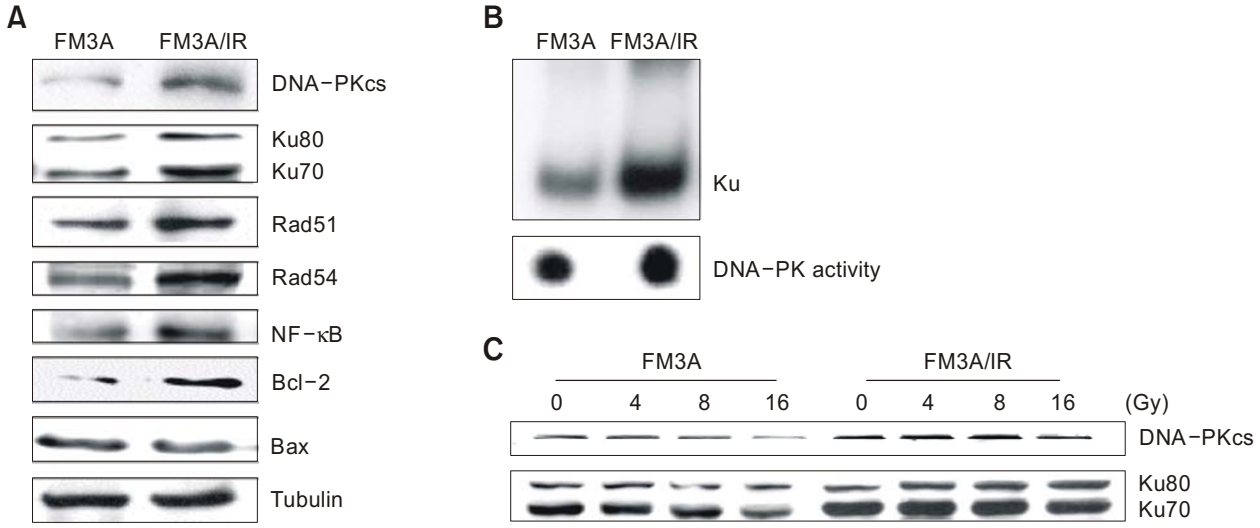

Figure 3. The altered expression of DNA repair- and apoptosis-related genes in FM3A/IR cells. The basal levels of DNA repair and apoptosis-related proteins were assayed by Western blot analysis using whole cell lysates or nuclear extracts isolated from FM3A/IR and its parental FM3A cells. β-tubulin (tubulin) was used as a loading control (A). Ku-DNA binding and kinase activity of DNA-PK were determined by EMSA (B, upper panel) and DNA-PK Kinase Assay system (B, lower panel), respectively. At 10 h after exposure to the indicated doses of γ-irradiation, alteration of DNA-PK expression was determined by Western blot analysis (C).

than CEM cells. Moreover, the radioresistant cells showed remarkable induction of DNA-PKcs and Ku proteins after low doses (3-6 Gy) of γ-irradiation compared with its parental cells (Figure 2C). Therefore, the radioresistant cells might be more resistant to radiation-induced DNA damage than the parental cells. However, at higher dose (12 Gy) of γ-irradiation, the DNA-PK was not induced probably due to serious cellular injury. This result suggests that increased response to γ-irradiation may reflect radioresistance in CEM/IR cells. Similar phenomenon was observed in other radioresistant FM3A/IR cells. The up-regulation of DNA-PKcs, Ku70/80, Rad51, Rad54, Bcl-2 and NF-κB genes and the down-regulation of Bax gene were observed in FM3A/IR cells as compared to FM3A cells (Figure 3A). Concurrently, the Ku-DNA binding activity and the kinase activity of whole DNA-PK complex in FM3A/IR cells were more increased than those in the FM3A cells (Figure 3B). As shown in Figure 3C, a significant increased expression of DNA-PKcs and Ku80 in FM3A/IR cells after irradiation (4-8 Gy) was observed, whereas the expression of these DNA-PK components in FM3A cells was decreased at same irradiation condition, suggesting differential response to IR between FM3A/IR and FM3A cells. These observations suggest that increased levels of the proteins controlling the processes of DNA damage repair and apoptosis after IR-induced DNA damage could contribute to the acquisition of radioresistant phenotype of cancer cells.

Suppression of DNA damage repair- and antiapoptotis-related genes in the radioresistant cells by PKA inhibitor

It has been reported that the activated PKA pathway plays an important role in DNA damage repair, and PKA could be activated by radiation (Tortora *et al*., 2003). We also previously reported that PKA inhibitor markedly potentiates cytotoxicity of anticancer drugs via inhibition of increased NF-κB and Ku activities in MDR cells (Um *et al*., 2001). Therefore, the levels of DNA-PKcs, Rad51 and Bcl-2 and Ku-DNA binding activity were compared between FM3A/IR and FM3A cells after treatment with various doses of PKA inhibitor, H-89, for 8 h (Figure 4A). H-89-induced down-regulation of Ku-DNA binding activity, DNA-PKcs, Rad51 and Bcl-2 was more prominent in FM3A/IR cells than FM3A cells. Therefore, to determine whether PKA inhibitor could affect the radiation-induced the expression of anti-apoptotic molecules, FM3A/IR and FM3A cells were exposed to two doses of IR in the presence or absence of H-89 (Figure 4B). Radiation-induced DNA-PKcs and Ku70/80 expressions of FM3A/IR cells were significantly reduced by H-89 treatment. Concurrently, co-treatment of radiation and H-89 to the cells resulted in synergistic inhibition of Bcl-2 and rad51 expressions and Ku-DNA binding activity in the cells, whereas FM3A cells were less responsive to the combined effect of radiation and PKA inhibitor under the same condition. Other radioresistant cells showed similar results. The levels of

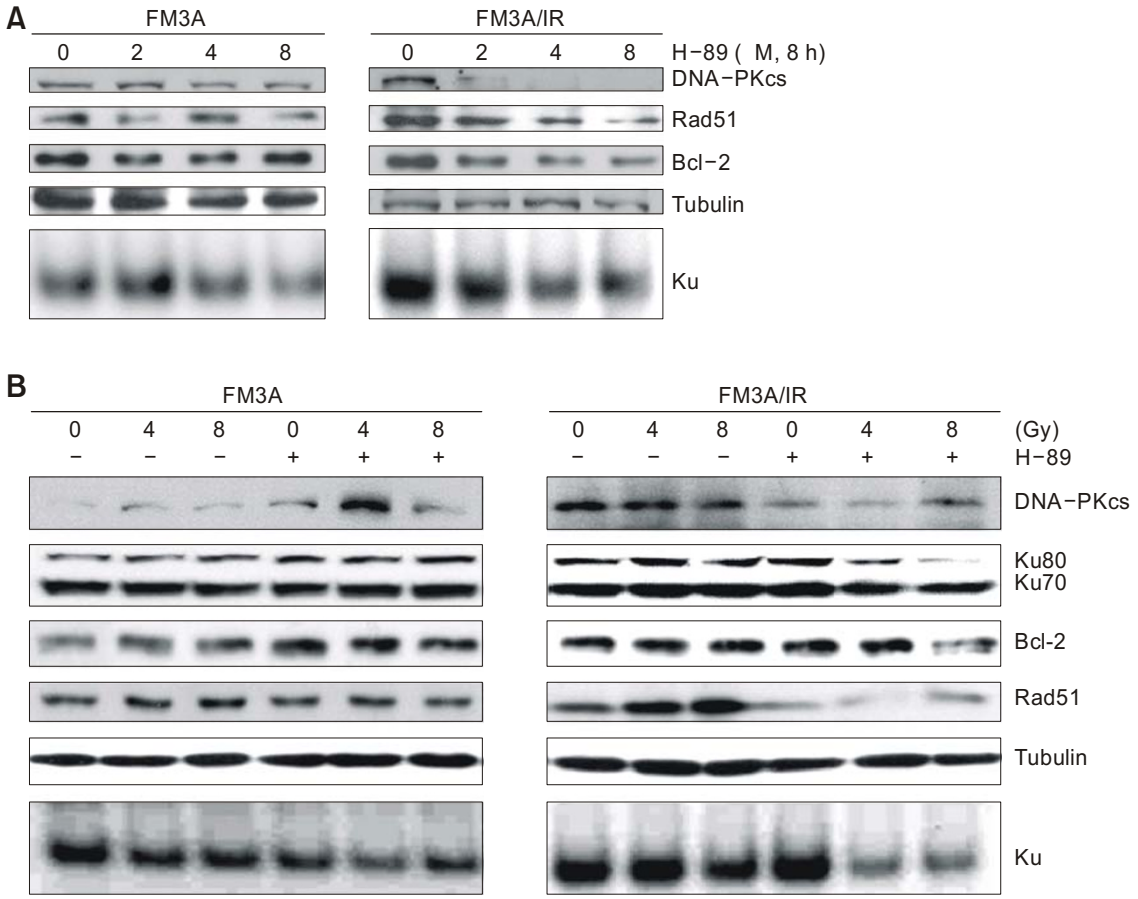

Figure 4. Inhibition of DNA repair-related and Bcl-2 gene expression by PKA inhibitor and its combination effect with radiation in FM3A/IR cells. The cells were treated with the indicated doses of H-89 for 8 h (A) or were exposed to the indicated doses of radiation for 6 h in the presence or absence of 4 M H-89 (B). The expression of DNA-PKcs, Rad51 and Bcl-2 and the activity/level of Ku were measured by Western blot analysis and EMSA, respectively.

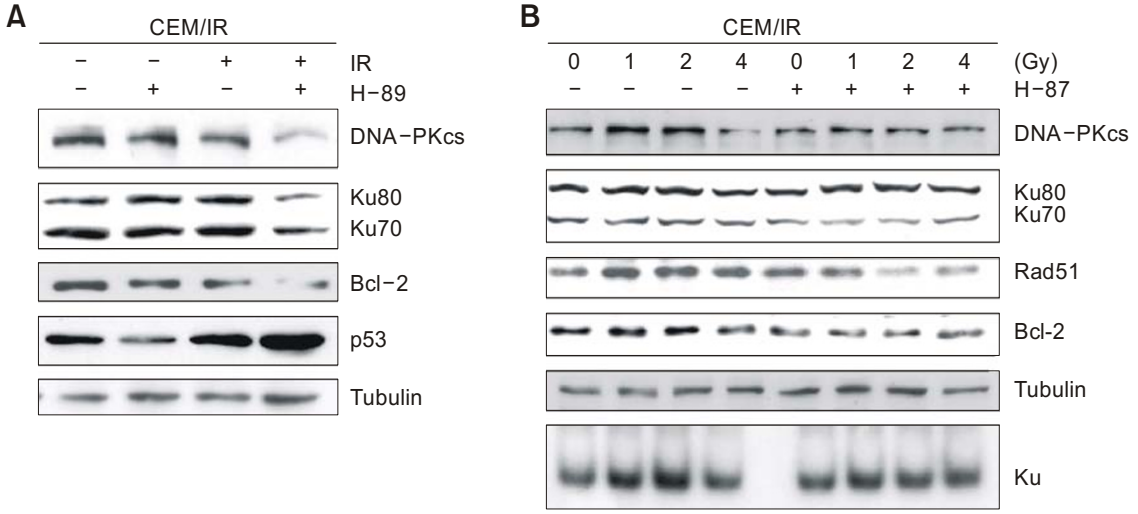

Figure 5. Effect of PKA inhibitor on the expression of DNA repair- and apoptosis-related genes in radiation-treated CEM/IR cells. The cells were exposed to radiation (IR, 6 Gy) in the presence or absence of 4 M H-89 (A) or exposed to the indicated doses of radiation in the presence or absence of 4 M H-87 (B). The levels of DNA repair- and apoptosis-related proteins and Ku-DNA binding activity were measured by Western blot analysis and EMSA, respectively.

DNA-PKcs and Ku70/80 of radiation-treated CEM/IR cells were significantly decreased by H-89 treatment and it was followed by an increase of p53 and a decrease of Bcl-2 level (Figure 5A), suggesting that the PKA pathway could modulate the proteins controlling the processes of repair of DNA DSBs and apoptosis after irradiation and consequently inhibition of PKA may enhance radiosensitivity of radioresistant cancer cells.

Moreover, another isoquinoline sulfonamide compound H-87, the specific PKA inhibitor, also significantly suppressed stress-responsive genes such as DNA-PKcs, Ku70/80, rad51 and Bcl-2 and also Ku-DNA binding activity induced by radiation in CEM/IR cells (Figure 5B). These results indicate the possibility that inhibition of PKA signaling pathway could enhance the cytotoxic effects of IR against radioresistant cells via inhibition of prosurvival signaling molecules such as Bcl-2 and DNA-PK.

Enhancement of radiosensitivity by PKA inhibitor

To determine whether suppression of prosurvival factors by PKA inhibitors leads to enhance cytotoxic response of radioresistant cells to radiation, and thereby restore the radiosensitivity of radioresistant cells, FM3A/IR and FM3A cells were treated with various dose of irradiation in the presence or absence of H-89, and growth inhibition was determined by MTT assay after 5 days. PKA inhibitor potentiated the cytotoxicity of radiation against radioresistant cells. Treatment of 2 M H-89, a nontoxic concentration, significantly sensitized FM3A/IR cells to irradiation compared with FM3A cells (Figure 6A). In the CEM/IR cells, H-87 also showed the high combination effect with radiation at a nontoxic concentration of 2 M. By contrast, CEM cells were less responsive to the combined effect of radiation and PKA inhibitor under the same condition (Figure 6B). These results indicated that PKA inhibitor, which suppressed the expression of prosurvival factors including Bcl-2 and DNA-PK, could be effective in restoring radiosensitivity of the radioresistant cells and thus it may be good candidates for the development of agents for circumventing radioresistance of cancer cells.

In addition, apoptotic cell death was analysed with annexin V, which can detect early stage of apoptosis (Martin *et al.,* 1995). Apoptotic phenotype measured by annexin V staining was observed in CEM cells at 24 h after exposure to even 1 Gy irradiation (Figure 7A), while CEM/IR cells did not show significant apoptotic phenotype at 24 h after exposure to even 4 Gy irradiation (Figure 7C). Apoptosis could be accelerated by pretreatment of cells with H-89 in the radiation-treated CEM/IR cells (Figure 7D) but not in the CEM cells (Figure 7B). These results demonstrated that the synergistic cytotoxic effect of radiation and PKA inhibitor was associated with induction of apoptosis.

Discussion

Although radiotherapy is a powerful tool for the treatment of cancer, the repeated treatments received during fractionated irradiation may promote the change from a transient to a constitutive pattern of

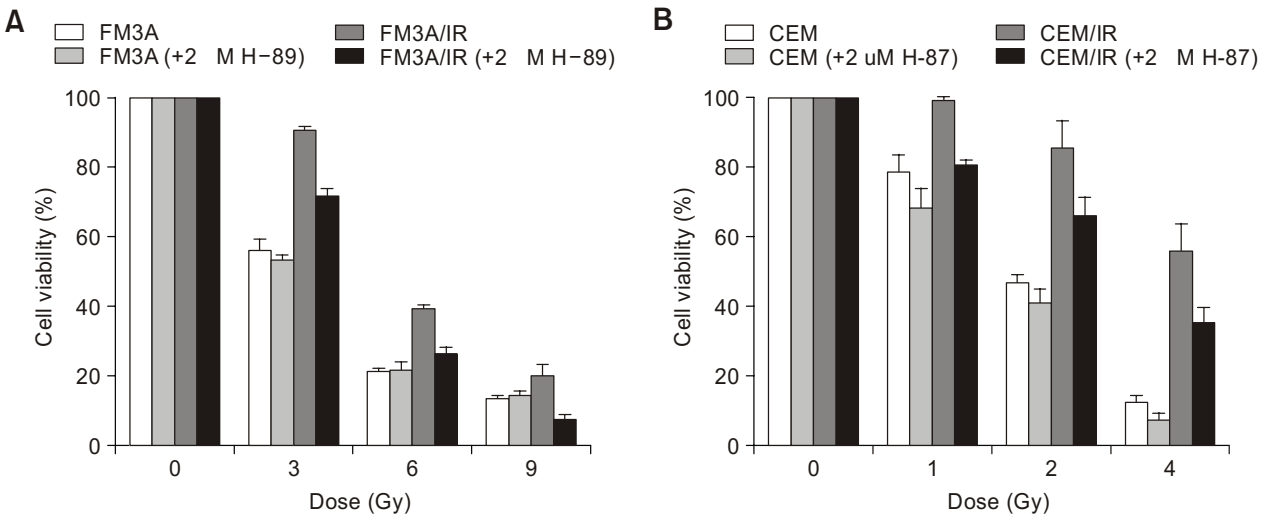

Figure 6. Enhancement of radiosensitivity by PKA inhibitor in the radioresistant variants of cancer cells. FM3A/IR (A) and CEM/IR cells (B) were treated with indicated doses of radiation in the presence or absence of 2 M H-89 or 2 M H-87, respectively. After 5 days, growth inhibition was determined with MTT assay.

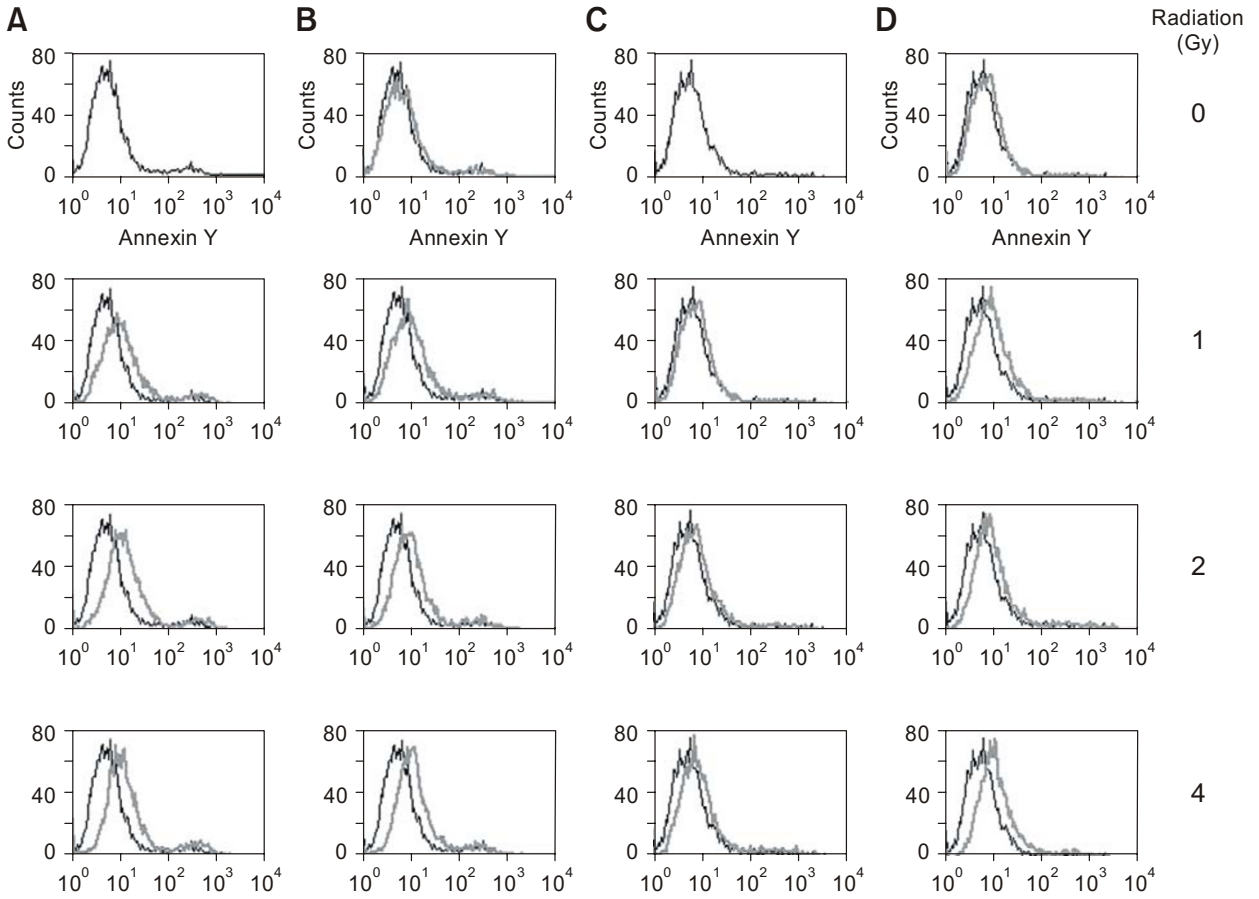

Figure 7. Effect of PKA inhibitor on radiation-induced apoptosis of radioresistant cells. CEM (A, B) and CEM/IR cells (C, D) were exposed to radiation with 1, 2 or 4 Gy in the presence (B, D) or absence (A, C) of 4 M H-89. After 24 h quantification of radiation-induced apoptosis based on annexin V staining was analyzed by flow cytometry. Solid line: untreated cells, gray line: cells treated with radiation and/or H-89.

gene expression and it could lead to acquire radioresistant phenotype of the cancer cells (Fukuda *et al.,* 2004; Hennesse *et al*., 2004). Therefore, understanding the molecular mechanisms involved in the response of tumors to IR and acquisition of radioresistance are important for improving radiotherapy and overcoming the radioresistance.

In this study, we revealed that radioresistance of cancer cells with the enhanced levels of radiation-responsive proteins, which are able to prevent radiation-induced apoptosis, and may contribute to multifactorial radioresistance phenotype. In the radioresistant CEM/IR and FM3A/IR cells, the expressions of DNA-PKcs, Ku70/80, Rad51 and Rad54 genes that related to DNA damage repair, and concurrently Bcl-2 and NF-κB genes that related to antiapoptosis, were up-regulated, but the expression of proapototic Bax gene was down-regulated in comparison with each parental counterpart, suggesting that altered levels of the proteins controlling the processes of DNA damage repair and apoptosis in cancer cells could contribute the acquisition of radioresistant phenotype.

It has been reported that the many cancer therapeutics target and disrupt DNA function through adduct formation or by causing single- and double-chain scissions, and thereby DNA-PK, which participates in the repair of DNA DSBs by activating the NHEJ pathway, can play an important role in conferring cells resistance to ionizing radiation (IR) or DNA-damaging anticancer drugs (Shen *et al*., 1998; Kim *et al*., 2000; Maacke *et al*., 2000; Marples *et al*., 2002; Um *et al.,* 2004). Also, the overexpression of Rad51, another DSBs repair protein, can contribute to acquire the radioresistant phenotype (Yanagisawa *et al*., 1998). Our data showed that the radioresistant CEM/IR and FM3A/IR cells exhibited higher constitutive expression of Ku/DNA- PKcs, Rad51 and Rad54 and DNA-PK activity than each parental counterpart. These results suggested that

the increased expression/activity of DNA damage repair genes might be an important feature of radioresistant cancer cells.

Although repair mechanisms such as NHEJ (DNA-PK) and HR (Rad51 and Rad54) are important mammalian responses to double-strand DNA damage, Bcl-2 and NF-κB expressions are perhaps important determinants of radiosensitivity. In fact, it was reported that NF-κB and Bcl-2 have been implicated in resistance to radiation as well as anticancer drugs in many types of tumors, and thereby the suppression of NF-κB and Bcl-2 expressions could overcome resistance to radiation of prostate cancer cells (Inayat *et al*., 2002; Dorai and Aggarwal, 2004). Our previous study also showed that multidrug resistant CEM/VLB_{100} cells exhibited markedly increased constitutive DNA-PK and NF-κB activity, although it was not known why both NF-κB and Ku activities were constitutively up-regulated in the cells and how NF-κB or Ku activity regulate each other activity mutually (Um *et al.,* 2001). In the present study, CEM/IR and FM3A/IR cells showed higher basal levels of antiapoptotic proteins such as Bcl-2 and NF-κB that could contribute to acquire radioresistant phenotype, than each parental counterpart. Therefore, selective inhibition of these radioresistance-related genes would result in improved radiosensitivity and thereby these molecules would be good candidates for radiosensitization.

It has been reported that the activation of cAMP/PKA signaling pathway is required for cancer cell survival and the inhibition of PKA has a cooperative antitumor effect with a selected class of cytotoxic drugs and radiotherapy (Tortora and Ciardiello, 2003) and causes inactivation of Bcl-2 which eventually leads to induction of apoptosis (Tortora and Ciardiello, 2002). Since the FM3A/IR and CEM/IR cells have higher PKA activity (1.6-1.9 folds: data not shown) than each parental counterpart, and the down-regulation of PKA involved in enhancing radiation-induced apoptosis (Findik *et al*., 1995), we determined whether PKA inhibitor could modulate the over-expression of DNA damage repair- and antiapoptotic proteins in the radioresistant cells, and subsequently affect radiation-induced cytotoxicity of the cells. Our results revealed that the expression of Ku and Bcl-2 in FM3A/IR cells was significantly decreased by the treatment of PKA inhibitor H-89. Moreover, the combined treatment of PKA inhibitor and radiation resulted in synergistically decreased level of DNA-PKcs/Ku, rad51 and Ku DNA-binding activity and concurrently reduced levels of Bcl-2 and enhanced p53 in radioresistant cells. Similar effect observed with other specific PKA inhibitor H-87. Our finding revealed that PKA inhibitor markedly potentiated cytotoxicity of radiation and the radiosensitizing effect of PKA inhibitor was higher in radioresistant cells than their parental cells.

Previously, we revealed that PKA inhibitor markedly suppressed constitutive and drug-induced activities of NF-κB and Ku in CEM/VLB_{100} cells and subsequently potentiated the cytotoxic activity of anticancer drugs (Um *et al.,* 2001). Therefore, PKA inhibitor appeared to enhance the radiosensitivity against radioresistant cells via inhibition of cellular DNA repair and antiapoptotic proteins, both of which play important roles in radioresistance. Therefore, combination of radiotherapy with PKA inhibitor, as a new class of radiosensitizer, can be a novel therapeutic strategy for treatment of radioresistant cancer cells.

At present, we do not have sufficient data to explain inter-related regulation among radioresistance-associated molecules by PKA signaling pathway, and thus further experiments will be required to clarify the precise mechanisms of modulation of these molecules by PKA inhibitor.

Acknowledgement

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References

Ader I, Muller C, Bonnet J, Favre G, Cohen-Jonathan E, Salles B, Toulas C. The radioprotective effect of the 24 kDa FGF-2 isoform in HeLa cells is related to an increased expression and activity of the DNA dependent protein kinase (DNA-PK) catalytic subunit. Oncogene 2002;21:6471-9

Allen C, Halbrook J, Nickoloff JA. Interactive competition between homologous recombination and non-homologous end joining. Mol Cancer Res 2003;1:913-20

Chastel C, Jiricny J, Jaussi R. Activation of stress-responsive promoters by ionizing radiation for deployment in targeted gene therapy. DNA Repair (Amst) 2004;3:201-15

Chen X, Shen B, Xia L, Khaletzkiy A, Chu D, Wong JY, Li JJ. Activation of nuclear factor kappaB in radioresistance of TP53-inactive human keratinocytes. Cancer Res 2002;62: 1213-21

Condon LT, Ashman JN, Ell SR, Stafford ND, Greenman J, Cawkwell L. Overexpression of Bcl-2 in squamous cell carcinoma of the larynx: a marker of radioresistance. Int J Cancer 2002;100:472-5

Dorai T, Aggarwal BB. Role of chemopreventive agents in cancer therapy. Cancer Lett 2004;215:129-40

Essers J, van Steeg H, de Wit J, Swagemakers SM, Vermeij M, Hoeijmakers JH, Kanaar R. Homologous and non-homologous recombination differentially affect DNA damage repair in mice. EMBO J 2000;19:1703-10

Findik D, Song Q, Hidaka H, Lavin M. Protein kinase A inhibitors enhance radiation-induced apoptosis. J Cell Biochem 1995;57:12-21

Fukuda K, Sakakura C, Miyagawa K, Kuriu Y, Kin S, Nakase Y, Hagiwara A, Mitsufuji S, Okazaki Y, Hayashizaki Y, Yamagishi H. Differential gene expression profiles of radioresistant oesophageal cancer cell lines established by continuous fractionated irradiation. Br J Cancer 2004;91: 1543-50

Fukushima T, Takata M, Morrison C, Araki R, Fujimori A, Abe M, Tatsumi K, Jasin M, Dhar PK, Sonoda E, Chiba T, Takeda S. Genetic analysis of the DNA-dependent protein kinase reveals an inhibitory role of Ku in late S-G2 phase DNA double-strand break repair. J Biol Chem 2001;276:44413-8

Gupta AK, Cerniglia GJ, Mick R, Ahmed MS, Bakanauskas VJ, Muschel RJ, McKenna WG. Radiation sensitization of human cancer cells in vivo by inhibiting the activity of PI3K using LY294002. Int J Radiat Oncol Biol Phys 2003;56: 846-53

Inayat MS, Chendil D, Mohiuddin M, Elford HL, Gallicchio VS, Ahmed MM. Didox (a novel ribonucleotide reductase inhibitor) overcomes Bcl-2 mediated radiation resistance in prostate cancer cell line PC-3. Cancer Biol Ther 2002;1: 539-45

Jackson SP. Sensing and repairing DNA double-strand breaks. Carcinogenesis 2002;23;687-96

Kang CM, Cho HN, Ahn JM, Lee SS, Jeoung DI, Cho CK, Bae S, Lee SJ, Lee YS. Alteration of gene expression during radiation-induced resistance and tumorigenesis in NIH3T3 cells revealed by cDNA microarrays: involvement of MDM2 and CDC25B. Carcinogenesis 2004;25:123-32

Kim SH, Um JH, Kim DW, Kwon BH, Kim DW, Chung BS, Kang CD. Potentiation of chemosensitivity in multidrug-resistant human leukemia CEM cells by inhibition of DNA-dependent protein kinase using wortmannin. Leuk Res 2000;24:917-25

Kim YS, Jhon DY, Lee KY. Involvement of ROS and JNK1 in selenite-induced apoptosis in Chang liver cells. Exp Mol Med 2004;36:157-64

Henness S, Davey MW, Harvie RM, Banyer J, Wasinger V, Corthals G, Davey RA. Changes in gene expression associated with stable drug and radiation resistance in small cell lung cancer cells are similar to those caused by a single X-ray dose. Radiat Res 2004;16:495-503

Maacke H, Jost K, Opitz S, Miska S, Yuan Y, Hasselbach L, Luttges J, Kalthoff H, Sturzbecher HW. DNA repair and recombination factor Rad51 is over-expressed in human pancreatic adenocarcinoma. Oncogene 2000;19:2791-5

Marples B, Cann NE, Mitchell CR, Johnston PJ, Joiner MC. Evidence for the involvement of DNA-dependent protein kinase in the phenomena of low dose hyper-radiosensitivity and increased radioresistance. Int J Radiat Biol 2002;78: 1139-47

Martin SJ, Reutelingaperger CP, McGahon AJ, Rader JA, van Schie RC, LaFace DM, Green DR. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of bcl-2 and abl. J Exp Med 1995;182: 1545-56

Pirollo KF, Hao Z, Rait A, Ho CW, Chang EH. Evidence supporting a signal transduction pathway leading to the radiation-resistant phenotype in human tumor cells. Biochem Biophys Res Commun 1997;230:196-201

Rosen EM, Fan S, Rockwell S, Goldberg ID. The molecular and cellular basis of radiosensitivity: implications for understanding how normal tissues and tumors respond to therapeutic radiation. Cancer Invest 1999;17:56-72

Shen H, Schultz M, Kruh GD, Tew KD. Increased expression of DNA-dependent protein kinase confers resistance to adriamycin. Biochim Biophys Acta 1998;1381:131-8

Smith GC, Jackson SP. The DNA-dependent protein kinase. Genes Dev 1999;13:916-34

Snyder AR, Morgan WF. Gene expression profiling after irradiation: clues to understanding acute and persistent responses ? Cancer Metastasis Rev 2004;23:259-68

Soderlund K, Perez-Tenorio G, Stal O. Activation of the phosphatidylinositol 3-kinase/Akt pathway prevents radiation-induced apoptosis in breast cancer cells. Int J Oncol 2005;26:25-32

Streffer JR, Rimner A, Rieger J, Naumann U, Rodemann HP, Weller M. BCL-2 family proteins modulate radiosensitivity in human malignant glioma cells. J Neurooncol 2002;56:43-9.

Tamatani T, Azuma M, Ashida Y, Motegi K, Takashima R, Harada K, Kawaguchi S, Sato M. Enhanced radiosensitization and chemosensitization in NF-kappaB-suppressed human oral cancer cells via the inhibition of gamma-irradiation- and 5-FU-induced production of IL-6 and IL-8. Int J Cancer 2004;108:912-21

Tenzer A, Zingg D, Riesterer O, Vuong V, Bodis S, M. Pruschy. Signal transduction inhibitors as radiosensitizers. Curr Med Chem Anti-Canc Agents 2002;2:727-42

Tortora G, Ciardiello F. Protein kinase A as target for novel integrated strategies of cancer therapy. Ann N Y Acad Sci 2002;968:139-47

Tortora G, Ciardiello F. Antisense targeting protein kinase A type I as a drug for integrated strategies of cancer therapy. Ann N Y Acad Sci 2003;1002:236-43

Um JH, Kang CD, Lee BG, Kim DW, Chung BS, Kim SH. Increased and correlated nuclear factor-kappa B and Ku autoantigen activities are associated with development of multidrug resistance. Oncogene 2001;20:6048-56

Um JH, Kang CD, Bae JH, Shin GG, Kim MJ, Kim DW, Kim DW, Chung BS, Kim SH. Association of DNA-dependent protein kinase with hypoxia inducible factor-1 and its implication in resistance to anticancer drugs in hypoxic tumor cells. EXP Mol Med 2004;36:233-42

Um JH, Kwon JK, Kang CD, Kim MJ, Ju DS, Bae JH, Kim DW, Chung BS, Kim SH. Relationship between antiapoptotic molecules and metastatic potency and the involvement of DNA-dependent protein kinase in the chemosensitization of metastatic human cancer cells by epidermal growth factor receptor blockade. J Pharmacol Exp Ther 2004;311:1062-70

Vallis KA, Chen Z, Stanford WL, Yu M, Hill RP, Bernstein A. Identification of radiation-responsive genes in vitro using a gene trap strategy predicts for modulation of expression by radiation in vivo. Radiat Res 2002;157:8-18

Vavrova J, Rezacova M, Vokurkova D, Psutka J. Cell cycle alteration, apoptosis and response of leukemic cell lines to gamma radiation with high- and low-dose rate. Physiol Res 2004; 53:335-42

Willers H, Dahm-Daphi J, Powell SN. Repair of radiation damage to DNA. Br J Cancer 2004;90:1297-301

Yanagisawa T, Urade M, Yamamoto Y, Furuyama J. Increased expression of human DNA repair genes, XRCC1, XRCC3 and RAD51, in radioresistant human KB carcinoma cell line N10. Oral Oncol 1998;34:524-8