

Mitophagy in the A549 lung cancer cell line, radiation-induced damage, and the effect of ATM and PARKIN on the mitochondria

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ABSTRACT

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Background: Non-small cell lung cancer (NSCLC) is the most commonly diagnosed cancer, and radiotherapy (RT) is used for the cancer therapy. RT affects DNA and causes DNA double-strand breaks which are repaired by DNA repair protein ataxia telangiectasia mutated (ATM). RT also affects the mitochondria which is a key player in mediating the radiation response in tumors and removing damaged mitochondria through mitophagy. During mitophagy, PARKIN accumulates on defective mitochondria to mediate the clearance of damaged mitochondria. This study examines the effect of radiation on mitophagy using PARKIN and ATM antibodies on the human NSCLC A549 line. **Materials and Methods:** A549 cells were treated with 2, 4, 6 and 8 Gy of radiation were analyzed on days 1 and 3 after a single dose of radiotherapy. PARKIN and ATM expressions of A549 cells were examined by using immunohistochemical technique. **Results:** In the control groups, weak immunoreactivity of ATM and PARKIN was observed on both days 1 and 3. The most intense ATM expression was seen in the 6 and 8 Gy groups after day 1. The most intense PARKIN expression was seen after the days 1 and 3 in the 2 Gy groups. PARKIN immunoreactivity decreased due to increasing radiation dose. **Conclusion:** It must be considered that mitophagy mechanisms are activated in RT applications. It must be considered that the activation of mitophagy mechanisms in RT and A549 lung cancer cell lines may provide hemostasis in cancer cells. Molecules targeting mitophagy must be developed for use with radiotherapy.

INTRODUCTION

Radiotherapy (RT) directly affects the DNA structure and repair mechanisms of cancer cells. DNA creates double-strand breaks (DSBs) and produces therapeutic effects on tumor cells through apoptosis, necrosis, senescence, abnormal mitosis and autophagy^(1, 2). The ataxia-telangiectasia mutation (ATM) protein is a precursor of the DSB repair protein and is displaced from the cytoplasm in the nucleus after irradiation. RT activates the ATM kinase enzyme which has a role in the cellular response to any DNA hazard or radiation damage⁽³⁾. The presence of ATM monomers in the nucleus indicates that it induces phosphorylation of H2AX histone variants (γ H2AX), an early sensor of DSB recognition by the non-homologous end-joining (NHEJ) pathway.

RT also damages cell organelles such as mitochondria and endoplasmic reticulum. It plays an important role in the effects of radiation. Unwanted damaged cytoplasmic structures are removed by lysosomal enzymes called autophagy, which is an

important way of cleaning and repairing the cell⁽⁴⁾. Mitophagy refers to the selective degradation of mitochondria by autophagy. During mitophagy, PTEN-derived putative kinase protein 1 (PINK1) accumulates in the outer membrane of depolarized mitochondria, resulting in PARKIN (Parkinson's juvenile disease protein 2). Activated PARKIN, in turn, ubiquitinates the scores of outer mitochondrial membrane proteins of depolarized mitochondria and subsequent recruitment of multiple autophagy cargo proteins. Cargoes directly bind to LC3 in the autophagosome, leading to degradation of all mitochondria within autophagolysosomes⁽⁵⁾. PARKIN is a ~52 kDa (426 amino acid) enzyme protein encoded by PARKINSON disease, PARKIN RBR [Zinc binding domain called Really Interesting New Gene (RING)-in-between (B)-Really Interesting New Gene (RING)]. The E3 ubiquitin-protein ligase (PARK2) gene is located on chromosome 6q. PARKIN displaces depolarized mitochondria and induces mitophagy⁽⁶⁾. PARKIN is normally found in the cytoplasm and it induces the degradation of various

membrane proteins that are destroyed by autophagy and come to mitochondria to clean mitochondrial damage by mitophagy^(7, 8). PARKIN is activated to promote proteasomal degradation of mitochondrial outer membrane proteins and selective elimination of damaged mitochondria by mitophagy.

The ATM protein has recently been suggested to play critical roles in response to mitochondrial dysfunction by initiating mitophagy. ATM may directly regulate mitochondrial homeostasis by responding to ROS or by regulating mitochondrial quality control genes such as PINK1 or PARKIN⁽⁹⁾. The ATM mutation causes increased basal autophagy but defects in mitophagy induced by mitochondrial membrane decoupling agents. The absence of ATM led to radiation sensitivity due to the inoperability of mitophagy and cell death⁽¹⁰⁾. The clearance of damaged mitochondria in mitophagy was mediated by PINK1 accumulation in damaged mitochondria⁽¹¹⁾.

This study aims to investigate the effects of single doses of 2, 4, 6 and 8 Gy on ATM and PARKIN expression of the A549 cell line to clarify the role of the mitochondrial mitophagy system during acute radiation effects. The novel idea of this study is to give some new data on mitophagy, which contributes to the control of mitochondrial dynamics and cell death during radiotherapy.

MATERIALS AND METHODS

Cell culture and ionizing radiation

The human NSCLC A549 cell line (CCL-185) was purchased from the American Type Culture Collection. Cells were cultured by using RPMI-1640 (Gibco, USA) medium including 10% fetal bovine serum (FBS) (Biosera, USA), 100 U/ml penicillin, 100 µg/ml streptomycin (Biosera, USA), and 2 mM L-glutamate (Biosera, USA) in a humidified incubator at 37°C with 5% CO₂. Cells were incubated and then exposed to increasing doses of ionizing radiation (0, 2, 4, 6, and 8 Gy). The control (C) group was untreated. Radiotherapy was administered in vitro using a 4 MeV electron beam linear accelerator (Elekta, Stockholm, Sweden) at the Department of Radiation Oncology, Faculty of Medicine, Manisa Celal Bayar University.

Clonogenic survival assay

In order to analyze colony formation, 1×10^4 cells were plated in a 6-well plate before 2, 4, 6, and 8 Gy irradiation. The cells were removed after being cultured under a static condition for 14 days. Clones were fixed with 99% methanol for 30 minutes and then stained with 0.1 % crystal violet for 20 minutes at room temperature. The clones were counted under the microscope by two independent researchers and only clones containing more than 50 cells were considered colonies. SF (surviving fraction) = Colony

count/ (inoculated cells × plating efficiency). We used SF to calculate D0 (mean lethal dose).

Immunocytochemistry

Added A549 cells were fixed with 4% paraformaldehyde and washed twice with PBS on days 1 and 3 after 2, 4, 6 and 8 Gy irradiation to assess ATM and PARKIN immunoreactivity. The cells were incubated with anti-ATM (Cat No: 1:100; sc-; Santa Cruz Biotechnology, Dallas, TX) and anti-PARKIN polyclonal antibody (1:100; sc-30130; Santa Cruz Biotechnology, Dallas, TX) for 18 hours at 4°C. Samples were then washed three times (5 min each) in PBS, followed by incubation with biotinylated IgG and streptavidin peroxidase (Histostain plus Kit; 87-9999; Zymed Laboratories, Waltham, MA).

After the excess secondary antibodies were washed with PBS (three times, 5 min each), the immunohistochemical reaction was visualized by staining with the 3,3'-diaminobenzidine (DAB) Liquid Substrate System (ACK125; ScyTek Laboratories, West Logan, UT) and Mayer's hematoxylin (HMM999; ScyTek Laboratories) was used for counterstaining. Normal IgG instead of primary antibody was used as negative control. All samples were then covered with a mounting medium (107961.0500; Merck Millipore, Burlington, MA) and observed under a light microscope (BX-40; Olympus, Tokyo, Japan).

Immunostaining of ATM and PARKIN in A549 cells was semi-quantitatively evaluated by using H-SCORE analysis. Immunostaining intensities were scored as follows: 0 (no staining), 1 (weak but detectable staining), 2 (moderate staining), and 3 (intense staining). For each sample, an H-SCORE value was obtained by multiplying the sum of the percentage of cells stained in each intensity category by the corresponding score, using the formula $H\text{-SCORE} = \sum Pi (i+1)$, where i is the intensity.

Statistical analysis

Data are shown as mean ± SD for normal distribution parameters and median for non-normal distribution parameters. Results from each group were evaluated by H-score according to the degree of staining with antibodies. The obtained values were statistically compared with control groups using one-way ANOVA and Tukey tests. All analyses were performed in SPSS 22.0 and differences were considered significant if $p < 0.05$.

RESULTS

Clonogenic survival assay

The antiproliferative effect of radiotherapy was investigated in A549 cell line by clonogenic survival assay method. As shown in figure 1, the survival

fractions of A549 cells after 2, 4, 6 and 8 Gy of ionizing radiation decreased with increasing radiation doses compared to control cells ($P < 0.05$).

Immunohistochemical staining

Immunohistochemical staining of ATM and PARKIN of A549 cells revealed that these proteins were predominantly underexpressed on days 1 and 3 in the control group (279.8 ± 6.374 ; 189.5 ± 6.294 , respectively). Moderate staining was observed in the A549 cell line after 24 hours (306.1 ± 13.65 ; 313.4 ± 3.806 , respectively) and 3rd day (284.5 ± 9.652 ; 304.5 ± 11.46 , respectively) radiation of 2 and 4 Gy against ATM primary antibody. After 2 and 4 Gy irradiation for 24 hours, ATM expression in the A549 cell line was not significant compared to the control group. However, ATM immunoreactivity increased at day 1 (388.4 ± 3.806 ; 363.9 ± 8.373) at 6 and 8 Gy, respectively. On day 3, depending on the radiation dose, ATM expression was evident in the A549 cell line in the radiation groups, and ATM immunoreactivity was evident at 6 Gy (344.8 ± 9.578) and peak at 8 Gy (373.9 ± 6.332) compared to the control group (figures 2 and table 1).

It was determined that the highest PARKIN immunoreactivity expression level in A549 cells was reached on days 1 and 3 (391.5 ± 3.923 ; 378.1 ± 13.06 , respectively) after 2 Gy of radiation application but PARKIN expression was low in both on days 1 and 3 in the control group (142.4 ± 22.20 ; 205.3 ± 10.00 , respectively). It was remarkable that PARKIN expression decreased by 4.6.8 Gy (318.7 ± 13.06 ; 293.7 ± 26.08 ; 230.7 ± 14.61 , respectively) on day 1 and day 3 with increasing dose (figures 3 and table 1).

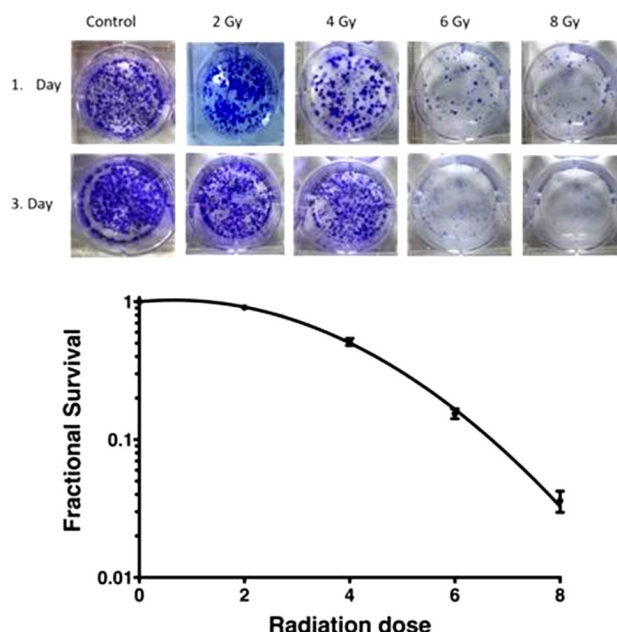


Figure 1. For A549 cells treated with ionizing radiation, the clonogenic assay showed that increasing doses of radiation (2, 4, 6 and 8 Gy) raised cell death and decreased the colony forming ability of A549 cells.

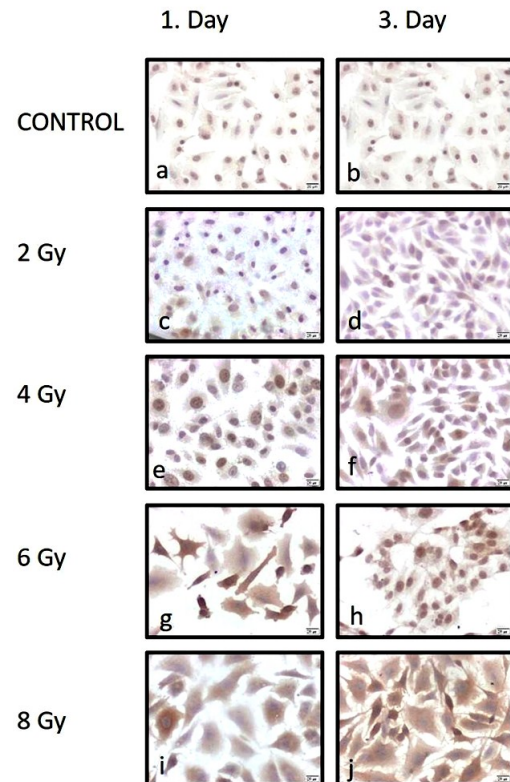


Figure 2. Immunohistochemistry staining, ATM expression in irradiated A549 cells. Weak immunoreactivity of ATM was seen in the control groups at both 24 and 72 hours (a, b). After 24 hours, the most intense ATM expression was observed in the 6 and 8 Gy groups (g, j). Objective 20X.

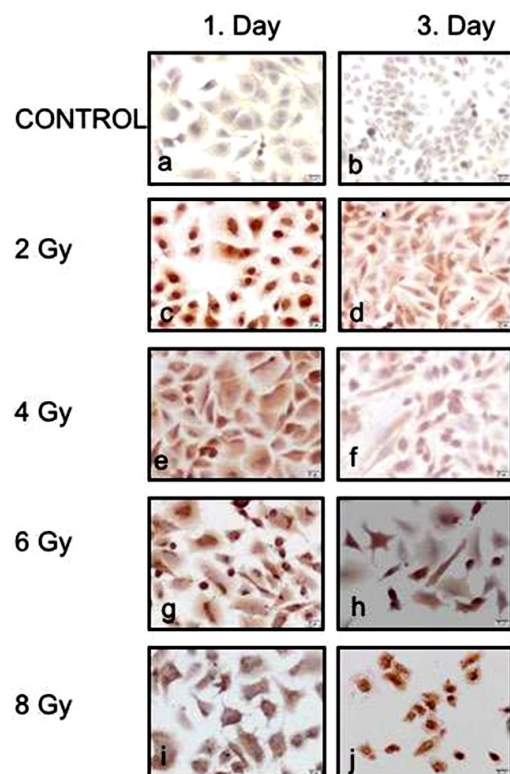


Figure 3. Immunohistochemistry staining, expression of PARKIN in irradiated A549 cells. Very weak PARKIN immunoreactivity was seen in the control groups at both 24 and 72 hours (a, b). The most intense PARKIN expression was seen in 2 groups (c, d) after both 24 and 72 hours of radiation exposure. PARKIN immunoreactivity decreased depending on the radiation dose. Objective 20X.

Table 1. H-Score values of ATM and PARKIN expression in cells A549.

	CONTROL	2 GY	4 GY	6 GY	8 GY
ATM	279.8±	306.1±	313.4±	388.4±	363.9±
1. Day	6.374	13.65***	3.806***	3.806***	8.373***
ATM	189.5±	284.5±	304.5±	344.8±	373.9±
3. Day	6.294	9.652***	11.46****	9.578***	6.332***
PAKIN	142.4±	391.5±	350.3±	302.3±	260.3±
1. Day	22.20	3.923***	8.26****	11.24***	18.92***
PAKIN	205.3±	378.1±	318.7±	293.7±	230.7±
3. Day	10.00	13.06***	13.06***	26.08***	14.61***

* p< 0.05, **p< 0.01, ***p< 0.001. Data are expressed as mean ± SD (Standard deviation). Different groups were compared with control groups using one-way ANOVA and Tukey tests. A p-value of <0.05 was considered statistically significant. ****p<0.0001.

DISCUSSION

RT causes DNA damage and is commonly used to destroy cancer cells. RT also affects cell organelles like mitochondria. Mitochondrial damage induced by radiation leads to metabolic oxidative stress that randomly interacts with intracellular biomacromolecules, resulting in fatal cellular damage and cell death. Damaged mitochondria are eliminated by mitophagy by using different components of the mitochondrial structure via various autophagy receptors (12). Dysregulated mitophagy is closely related to many physiological and pathological processes such as aging, neurodegenerative diseases and cancer (13). However, rapid activation of mitophagy mechanisms can abolish the damaged mitochondria and restore homeostasis in both normal cells and cancer cells (14). This study focuses on the function of mitophagy during RT and new aspects in cancer therapy.

Radiotherapy activates the death mechanisms of cells as well as vital mechanisms through adaptive response to mitochondrial stress. ATM, a DNA stress marker, activates compensatory mechanisms and regulates vital functions (3). In this study, low ATM expression was observed in A549 lung cancer cells of the control group due to the absence of DNA damage. On the other hand, the A549 cell line treated with RT had an increase in ATM immunoreactivity on day 1 and increased reactivity with more radiation dose was seen on days 1 and 3. It was thought that a single dose of radiotherapy caused the increase in ATM expression in lung cancer cells due to DNA damage. There is a relationship between DNA damage repair and ATM increase after lung radiotherapy. It has been reported that ATM inhibition creates a radiosensitizing effect on G2 arrest and apoptosis, and this effect provides therapeutic benefit in cancer therapy (15). Moreover, activated ATM phosphorylates many substrates including Chk2 and p53. Therefore, this propagates the damage signal to multiple cellular pathways and can cause cell growth arrest (16).

In addition to DSBs, other types of cellular stress activate ATM and the deletion of ATM interactor (ATMIN) protein rescued the proliferative defects and premature senescence of Nbs1-deficient cells

which suggests that loss of Nbs1 resulted in ATM IN-ATM-mediated activation of p53 signaling (17). In this study, ATM expression of the A549 cell line was similar after 6 and 8 Gy of radiation for 24 hours but ATM immunoreactivity increased with the most intense 8 Gy within 3 days, depending on the radiation dose. These results were considered as an indicator of radiation-induced DNA damage. ATM transmits DNA damage signals to p53, a tumor suppressor protein, and plays an important role in apoptotic responses. P53 is a transcription factor for cell cycle regulatory genes, including p21 and GADD45a, and GADD45a (known as DNA damage protein) plays a key role in cell cycle arrest-induced apoptotic cell death (18).

ATM plays an important role in promoting IR-induced autophagy through the MAPK14 pathway, mTOR pathway, and Beclin1/PI3K III complexes (19). The role of ATM kinase in cancer cell mitophagy has been reported recently (20), but the relationship between mitophagy and ATM is not fully known. Mitophagy is a complex process and PINK1 accumulates in the outer membrane of depolarized mitochondria and takes up the cytosolic ubiquitin ligase PARKIN and phosphorylates both PARKIN and ubiquitin (21). Activated PARKIN, in turn, ubiquitinates the scores of mitochondrial outer membrane proteins of depolarized mitochondria and subsequent recruitment of multiple autophagy cargo adapters such as OPTN and NDP52 (22). Finally, all these cargoes directly bind to LC3 in the autophagosome leading to the degradation of all mitochondria in autophagosomes (23).

PARKIN is an autophagic marker and a tumor suppressor gene. Its inactivation may play an important role in non-small cell lung cancer tumorigenesis. In this study, PARKIN expression of the A549 cell line was low in A549 lung cancer cells but increased expression was observed in irradiated lung cancer cells. Maximum expression of PARKIN was detected on days 1 and 3 treated with 2 Gy. PARKIN expression decreased with increasing doses (4, 6 and 8 Gy), suggesting that radiation performs the function of rescuing the cell by autophagy. However, a single 8 Gy high-dose radiotherapy was evaluated as direct and/or indicative of autophagy collapse and killing function. However, there is no complete consensus on the applications of radiotherapy to eliminate cells by the autophagy mechanism.

In addition to the studies suggesting that radiotherapy is an adaptive response that allows cancer cells to survive and grow (24), many studies indicate that excessive autophagy stimulation causes cell death (25). It was thought that the presence of both ATM and PARKIN expression in A549 lung cancer cells with 2-4 Gy is a marker of exerted mitophagy function. There is no complete consensus between ATM and PARKIN; Salkar et al. reported that PARKIN is expressed in the absence of ATM in AT cells while ATM ablation triggers loss of endogenous PARKIN

expression in MCL cell lines (20).

According to the observations, ATM may play a role in maintaining PARKIN stability and thus contribute to mitophagy. We also observed a low number of colony assays in the high-dose radiation groups and suggested that high-dose radiation causes cell death by DNA damage without mitophagy. However, low-dose radiation causes mitochondrial damage, and this damage removes damaged mitochondria by mitophagy as indicated by increased PARKIN expression.

We observed that ATM and PARKIN expressions were weak in the control groups and the expressions increased after the days 1 and 3 but the PARKIN immunoreactivity decreased with increasing radiation doses. Moreover, our immunohistochemical and colony-forming assay results indicated that ATM and PARKIN have an important role in the radiation effect on lung cancer cells and the changes in their expressions may contribute to mitophagy. Understanding mitophagy and developing targeted research in RT applications may increase success in cancer therapy. It should be considered that the activation of mitophagy mechanisms in RT and A549 lung cancer cell lines can provide hemostasis in cancer cells.

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