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# Studies on Cells with Depletion or Deficiency of DNA Repair Proteins

Mitra Etemadikhah

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Biology Education Centre and Rudbeck Laboratory, Department of Radiology, Oncology and Radiation Science, Uppsala University
Supervisors: Bo Stenerlöw and Ann-Sofie Gustafsson

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## Abbreviations

| | |
|---|---|
| ATM | Ataxia-telangiectasia mutated |
| ATR | Ataxia- and Rad3-related |
| BSA | Bovine serum albumin |
| CPM | Counts per minute |
| CTR | Control |
| DNA | Deoxyribonucleic acid |
| DNA-PK | DNA-dependent protein kinase |
| DNA-PKcs | DNA-dependent protein kinase catalytic subunit |
| DSB | Double-strand break |
| FBS | Fetal bovine serum |
| HR | Homologous recombination |
| L-glut | L-glutamine |
| mTOR | Mammalian target of rapamycin |
| NHEJ | Nonhomologous end joining |
| PBS | Phosphate buffered saline |
| PE | Plating efficiency |
| PEST | Penicillin-streptomycin |
| PFGE | Pulsed-field gel electrophoresis |
| PI3K | Phosphatidylinositol 3-kinase |
| PIKKs | Phosphatidylinositol 3-kinase-related kinases |

| | |
|---|---|
| RT | Room temperature |
| SF | Survival fraction |
| siRNA | Small interfering RNA |

# Introduction

## Radiation Therapy

Depending on factors such as tumor type and location in the body, the sensitivity of surrounding tissue and patient`s general health condition, different radiotherapies may be used as a part of cancer treatment. In external beam radiotherapy, the source is a machine that provides high energy radiation in form of x-rays or gamma rays. For internal radiotherapy, which is also known as brachytherapy, the radioactive material is placed near or inside the tumor. The applied dose for external or internal exposure is variable and it is determined by several factors including the purpose of treatment that can be either curative or palliative. Radiotherapy kills cancer cells by inducing DNA damage which can be caused directly or indirectly via providing charged particles inside the cell [1, 2, 13].

## DNA Double-Strand Breaks (DSBs) and Repair Pathways

DNA double-strand breaks (DSBs) are threats that become highlighted when we talk about genome integrity and transfer of genetic information to next generation. This kind of damage can be considered as the most hazardous of DNA lesions since they can cause irreversible alterations to cell genome and possibly cell death in case of not getting repaired. DNA DSBs can be induced to cells through exogenous sources like ionizing radiation and chemotherapeutic agents, or via endogenous sources like reactive radicals of oxygen that are produced during cellular metabolism. Homologous recombination (HR) and nonhomologous end joining (NHEJ) are the two main repair pathways applied by eukaryotic cells to deal with this kind of breaks. As can be understood from the name, HR needs a homologous pair as a template and therefore it is applicable during the S and G2 phases of cell cycle. In contrast, the NHEJ is based on resealing the two ends of the break without need of template and subsequently it can be used at any step of cell cycle [3, 4, 14, 15, 19].

## NHEJ and DNA-dependent Protein Kinase Catalytic Subunit

NHEJ is the most important and predominant DNA DSB repair pathway which is able to religate the broken ends when lacking a homologous pair as a template. There are several enzymes that participate in this repair pathway and each of them facilitates access to the breakage site for the next protein. Of these proteins, DNA-dependent protein kinase (DNA-PK) plays the key role. It

consists of two subunits: the heterodimer Ku 70/80 regulatory subunit, which recognizes the broken ends through its high affinity to DNA open ends, and the serine/threonine kinase, which is known as DNA-dependent protein kinase catalytic subunit (DNA-PKcs). The catalytic subunit applies the heterodimer Ku 70/80 as a scaffold to join the breakage site itself and recruit ligase IV and its cofactor XRCC4 to the complex. The lack of homologous template in NHEJ may cause the deletion of a tumor suppressor region, the amplification of a potentially-oncogenic part of the chromosome, or even translocation. All these probable risks can lead to tumorigenesis [3, 4, 14, 15].

### Aim of the Study

Studies have shown that partial deficiency of DNA-PKcs may contribute to an increased risk of cancer [9, 11, 18]. On the other hand, this partial deficiency may enhance radiosensitivity of tumor cells [10, 16]. This study focuses on the key role of DNA-PKcs and its importance in repair of DNA DSBs while cells are deficient or depleted of this crucial protein, either by knocking-down the related gene with siRNA or by inhibition of DNA-PKcs with inhibitory drugs Nu7026, Nu7441, or NVP-BEZ.

## Materials and Methods

### Cell lines and culturing

Two different cell lines were used in this study. A-431, which is a human epidermoid carcinoma cell type established from the solid tumor of an 85-year-old woman with the epithelial-like, adherent morphology growing in monolayers. Ham's F10 culture medium (BIOCHROM AG) supplemented with 10% fetal bovine serum (FBS), 5 ml penicillin-streptomycin (PEST), and 5 ml L-glutamine (L-glut) was used as complete growth medium. To reach the confluency of 60% to 70%, this cell line was subcultured 1:10 every three days in 25 $cm^2$ flask containing complete growth medium. HCT 116 is a human colorectal carcinoma with same morphology as A-431. McCoy`s 5A medium (BIOCHROM AG) supplemented with the same amount of FBS, PEST, and L-glut was used as the complete growth medium. It is subcultured 1:3 to 1:8 for 75 $cm^2$.Both cell lines were incubated at $37^oC$ with 5% $CO_2$ in air atmosphere.

**The Kinase Inhibitors**

NU7441 ($C_{25}H_{19}NO_3S$) and NU7026 ($C_{17}H_{15}NO_{3)}$, two highly potent and selective DNA-PK inhibitors, and NVP-BEZ235 ($C_{30}H_{23}N_5O$), a potent dual inhibitor of phosphatidylinositol 3-kinase (PI3K) and the mammalian target of rapamycin (mTOR), are drugs that were examined in terms of inhibitory effect on mentioned cell lines. 50 µM were used as final concentration for all drugs.

**Antibodies**

The following antibodies were used in this study: Monoclonal anti-β-actin, 42 kDa (1:10000) (Sigma), Monoclonal antibody to DNA-PKcs ~ 460 kDa (1:1000) (Abcam) as primary antibodies. Anti-rabbit (1:30000) (Invitrogen), anti-mouse (1:10000) (Invitrogen) were used as secondary antibodies.

**Irradiation Gamma Source $^{137}$Cs**

Cells were irradiated with $^{137}$Cs γ-ray photons (Gammacell 40 Exactor, MDS Nordion, Kanata, Canada) at a dose rate of 0.9948- 1.014 Gy per minute. Due to half-time of the $^{137}$Cs, the dose-rate decreases with time. The exposure time is calculated based on this dose-rate.

**siRNA Transfection**

*Procedure*

Cells were seeded in antibiotic-free complete medium one day before transfection. All calculations and dilutions to down-regulate the DNA-PKcs gene using Thermo Scientific DharmaFECT Transfection Reagents were done based on instruction from Dharmacon. To have 50 nM final siRNA concentration in each dish, siRNA (target and non-target) was diluted from stock (20 nM) using 5× siRNA buffer (DHARMACON), MQ water, and serum-free medium in tube 1. DharmaFECT transfection reagent was also diluted by serum-free medium in tube 2 to have 2.5 µL per dish final concentration. The content of each tube was gently pipetted and incubated for 5 minutes at room temperature. Then, the contents of tube 1 and tube 2 was mixed by pipetting and incubated for 20 minutes at room temperature. After the intended incubation time, the transfection medium was completed by adding antibiotic-free complete medium. Then, 2 ml per dish was distributed and all dishes were incubated at 37$^{o}$C with 5% $CO_2$ for 48-96 hours.

**Double-Strand Breaks (DSBs) Analysis with Pulsed-Field Gel Electrophoresis (PFGE)**

*Background*

To improve the ordinary technique of DNA separation, which has size limit up to ~ 50 kbp, pulsed field gel electrophoresis was developed by Schwartz and Cantor in 1984 [5]. The procedure is almost the same as standard gel electrophoresis except for the periodically-changed electric field direction which is applied to the gel and enables large DNA molecules up to 10 Mbp to migrate through the agarose gel matrix based on their size [6]. The following program consisting of five phases was run for 45 hours and 40 min with 56 V in this study.

3 h with 10-minutes pulses,

5:20 h with 20-minutes pulses,

8 h with 30-minutes pulses,

9:20 h with 40-minutes pulses, and finally

20 h with 60-minutes pulses

In order to protect large DNA fragments from being severed easily in this technique, cells are fixed in plug form by using fluid agarose after treatment. Consequently, these agarose plugs are treated by two-step lysis procedure to produce naked DNA and loaded to distinct wells in a gel matrix. Step 1: ESP buffer, a conventional lysis buffer (for 1 ml ESP buffer per plug: 15 ml 0.5 M EDTA + 4 ml 2% N-lauroylsarcosine (“SIGMA) + 1 ml of 1 mg/ml proteinase K (ROCHE), pH 8.0). Step 2: HS-buffer, a high-salt buffer (1.85 M NaCl + 0.15 M KCL + 5 mM $MgCl_2$ + 2 mM EDTA + 4 mM Tris + 0.5% Triton X-100, pH 7.5). The pulsed-field gel electrophoresis can be used to study the induced DNA double-strand breaks and their repair in eukaryotes. One of the DNA bases was labelled by a radioactive probe which was added to the growth medium and cells were incubated in it for two doubling times. The amount of radiation is measured by a radiation detector, scintillation counter, as counts per minutes (CPM) and the DSBs repair is quantified by following formula which is referred to as the fraction of activity that is released from total DNA in plug (FAR) [7].

$$FAR = \frac{\text{CPM of radioactivity of the gel piece with DNA < 5.7 Mbp in the lane}}{\text{CPM of total radioactivity in the lane}}$$

*Procedure*

To analyze DNA DSBs repair in desired cell line with PFGE, $5 \times 10^4 - 10 \times 10^4$ of HCT116 cells or $\geq 10 \times 10^4$ A431 cells, labelled with 2kBq/ml thymidine [methyl- $^{14}C$] (PERKINELMER), were seeded in dishes that were categorized in three groups as control, target siRNA plus inhibitory drug, and nontarget siRNA plus inhibitory drug. Each group included four dishes with 0 min, 15 min, 1 hour, and 4 hours repair time (table 1).

**Table1.** The schema of PFGE experiment

| Repair time and content of each dish | | |
|---|---|---|
| 0-min/CTR | 0-min/DrugTarget | 0-min/DrugNontarget |
| 15-min/CTR | 15-min/DrugTarget | 15-min/DrugNontarget |
| 60-min/CTR | 60-min/DrugTarget | 60-min/DrugNontarget |
| 240-min/CTR | 240-min/DrugTarget | 240-min/DrugNontarget |

Cells were incubated with $^{14}C$ at 37$^{o}$C with 5% $CO_2$ for two doubling times, about 80-100 hours. The day after seeding, transfection medium was added and all dishes were incubated at 37$^{o}$C with 5% $CO_2$ for 48-96 hours. After the intended incubation time, the dishes except controls were incubated with drug (50 µM/ 2ml) for one hour before irradiation by 40 Gy. The $^{14}C$-free drug solution was warmed-up for 1 hour before adding to cells at 37$^{o}$C with 5% $CO_2$. After 1 hour incubation with drug, dishes with variable repair time (15 min, 1 hour, and 4 hours) were kept on ice before irradiation, 20-25 min for A431 and 10 min for HCT116. Meanwhile, the agarose plugs for CTR and 0-min were prepared. The medium was discarded and cells dish were washed with room temperature PBS, and then, incubated with trypsin at 37$^{\circ}$C heater to be detached. The trypsinization time must be included in repair time; therefore for example, plug-making for 15-min dishes was started after 10 min incubation with trypsin. After cell detachment, 1.2 % agarose (LONZA) warmed-up at 37$^{\circ}$C was added and resuspended. To make 2 plugs in each casting form, one as CTR and the other as 0-time, 90 µl of the mixture was added in each well and

incubated at 4°C for 20 min. while these plugs were solidified, the rest dishes were irradiated with 40 Gy on ice. After 20 min, 0-min (no repair) plug was transferred to serum-free containing dish on ice to be irradiated, and CTR plug to tube containing ESP buffer. For dishes that were allowed to be repaired, plugs were prepared with same procedure after the intended repair time. The medium for 1 hour and 4 hours dishes was replaced with the warmed-up drug-containing medium to start repair right after irradiation. On following day, the ESP buffer was replaced by 2 ml per plug HS-buffer and plugs were incubated at 4°C for 10-20 hours. Due to low concentration of EDTA in this step, it should not extend over 25 hours. It`s possible to change it to 0.5 M EDTA and store in fridge for weeks. After the intended incubation time, the HS-buffer was removed and plugs were washed in 0.1 M EDTA (1 ml per plug) twice for one hour and in 0.5× TBE (1 ml per plug) once for one hour before loading in PFGE gel. The *Schizosaccharomyces pombe* genomic DNA, consisted of three chromosomes that were used as size markers, was also kept in 0.5× TBE for 1 hour at the same time as the plugs. Then all were loaded in agarose gel wells which was casted by 1.44 g agarose powder (Seakem Gold) dissolved in 180 ml 0.5x TBE-buffer. Wells were sealed with fluid agarose and the gel tray was kept in the PFGE machine containing running buffer to be equilibrated for 30-60 minutes prior to run. Then the program consisting of the five mentioned phases was run for 45 hours and 40 min with 56 V. When the program was finished, the gel was taken out and transferred to a staining-bath containing 50 µl sybersafe (INVITROGEN) in 500 ml distilled water over night. Subsequently, it was de-stained by rinsing in distilled water twice for reduction of background fluorescence and cut on a trans-illuminator table using UV light (302 nm). Each lane was divided into two pieces according to the DNA size marker, the first piece included the plug and the DNA molecules larger than 5.75 Mbp (smaller piece), and the second one included all DNA shorter than 5.75 Mbp (larger piece). Two pieces of sample-free gel were also removed as background. All pieces were placed in separate scintillation vials and 1 ml HCl, 0.2 M, was added to all vials and 2.5 ml distilled water was just added to those vials containing smaller piece (> 5.75 Mbp ) to provide same volume in all. The caps of vials were closed and all were heated at +95° C for 1-2 hours. The samples were cooled down over-night. On following day, 5 ml of scintillation fluid was added and all vials were vortexed one by one. After a couple of resting hours, the vials were put in the scintillation counter to measure the amount of $^{14}C$ in gel segments and the data was quantified by the FAR formula which explained in background part.

**Western Blot**

*Procedure*

Western blot according to the schema in table 2 was performed on cells that were seeded and treated in the same way as the dishes in pulsed-field gel electrophoresis experiment

**Table 2.** The schema of western blot experiment

| Content of each dish and irradiation dose | | |
|---|---|---|
| CTR | Drug/Target | Drug/Nontarget |
| $CTR_{4Gy}$ | $Drug/Target_{4Gy}$ | $Drug/Nontarget_{4Gy}$ |

To prepare lysate after intended treatments, all dishes were rinsed with ice-cold PBS once and incubated with lysis buffer (1% Tween-20, 20mM Tris (pH 8.0), 137 mM NaCl, 10% Glycerol, 2mM EDTA, 1mM activated sodium orthovanadate at 95$^{o}$C ($Na_3VO_4$), Protease inhibitor) on ice for at least 10 min while shaking slowly on a shaker. Then the lysate were transferred to pre-marked eppendorf tubes on ice and centrifuged 15 min at 15000 rpm (+4$^{\circ}$C). The supernatants were transferred to new pre-marked eppendorf tubes on ice and stored in freezer. To run electrophoresis with NuPAGE Novex Tris-Acetate Mini Gels, lysate were thawed on ice and NuPAGE LDS Sample Buffer 4× (Invitrogen) was warmed-up at 70$^{\circ}$C to dissolve better. Then 20 µl of lysate was mixed with 10 µl of NuPAGE LDS Sample Buffer (4×) in separate eppendorf tubes and warmed-up at 70$^{\circ}$C for 10 min. Then, 10 µl of Himark Pre-Stained ladder (Invitrogen) and 10 µl of each sample were loaded in separate wells of NuPAGE 3-8% Tris-Acetate gel (Invitrogen) and the gel was run at 150 V for about 1 hour. To do immunoblot, proteins were transferred to transfer membrane (Millipore) using cold transfer buffer (100 ml 10× electrophoresis buffer (RT) + 200 ml MeOH (RT) + 700 ml $dH_2O$) and through running wet transfer at 20 V over-night. The day after, membrane was blocked in blocking solution (1.5 g bovine serum albumin (BSA) (=5%, +4$^{\circ}$C) (Sigma-Aldrich) + 30 ml PBS-Tween 20 (RT)) for 1 hour and then incubated with primary antibody for 1-2 hours in room temperature on a shaker. After three times washing with PBS-Tween 20, each for 5 min, the membrane was incubated with a secondary antibody just for 1 hour in room temperature. After three times wash in PBS-Tween 20, each for 5 min, the membrane was incubated in chemi-luminescent solution

(Millipore) for 1 min and bands were visualized in a CCD camera (Super CCD HR, Fujifilm, Japan).

**Clonogenic Assay**

*Background*

Clonogenic assay is a method which examines the ability of every single cell to form a colony, consisting of at least 50 cells after special types of treatment, mostly ionizing radiation. Although it is mainly an in vitro assay, it has also been tested in vivo in several studies that provided very clear information of sensitivity of cells in normal tissues to radiation or chemotherapeutic drugs. In general, there are two main procedures to run this assay; the one that is used to quick screen the result of treatment, in which the cells are plated in proper dilution series for a couple of hours and then be treated. In the other option, treatment is performed before plating the cells in dilution series. In both techniques, cell suspension in stock is diluted so the number of cells per ml is about the same as the number of cells that shall be grown at the actual dose. After the intended period, the colonies are fixed, stained, and counted to determine the plating efficiency (PE), which is got from cells that were not exposed to any kind of treatment, and the surviving fraction (SF), which is the estimation of survival for cells after treatment and calculated by following formula [8].

$$\text{PE} = \frac{\text{no.of colonies formed}}{\text{no.of cells seeded}} \times 100\ \%$$

$$\text{SF} = \frac{\text{no.of colonies formed after treatment}}{\text{no.of cells seeded} \times \text{PE}}$$

*Procedure*

After removing the medium from A431 donor culture, cells were incubated with 0.5 ml trypsin-EDTA for 5-15 min at 37°C to be detached from the stock culture. Then, 10 ml complete medium was added to stop trypsinization and have a single-cell suspension and the number of cells was determined by a cell counter. Afterwards, dilution series were prepared and certain amounts of cells were plated in 25 $cm^2$ culture flasks. All samples were incubated at 37°C with 5% $CO_2$ and humidified air for 2 hours to attach to the flask and then irradiated by 1, 2, and 3 Gy using a $^{137}Cs$ gamma source. After 9 days, when colonies consisting of at least 50 cells were formed,

cells were fixed by removing media, a onetime wash with PBS, and incubation with 99.5 % ethanol in room temperature. Cells were stained with Mayer`s Haematoxylin (Histolab) for 15-20 min. It was followed by removing the stain and rinsing flasks in distilled water. To calculate the platting efficiency and survival fraction, colonies were counted manually and the quantified data were plotted.

## Results

### DNA Repair Analysis by PFGE in Cells Treated by DNA-PKcs Inhibitors

To study the ability of the cells to repair DNA double-strand breaks (DSBs) while they are depleted or deficient of key protein DNA protein kinase catalytic subunit (DNA-PKcs) due to treatment with cancer drugs NU7026 and NU7441, as specific DNA-PKcs inhibitors, and NVP-BEZ235, as dual inhibitor, DNA repair analysis by PFGE was done. HCT116 and A431 were treated with inhibitory drugs 1 hour before irradiation by 40 Gy and the final results were illustrated as it can be seen in Figure 2 for HCT116 and figure 4 for A431. The related agarose gel image of HCT116 cells after treatment with NU7026, NU7441, and NVP-BEZ235 drugs can be seen in figure 1. Figure 3 shows the agarose gel image of A431 after treatment with NU7026 and NU7441 drugs. The content of each lane has been explained below gel images.

**HCT116**

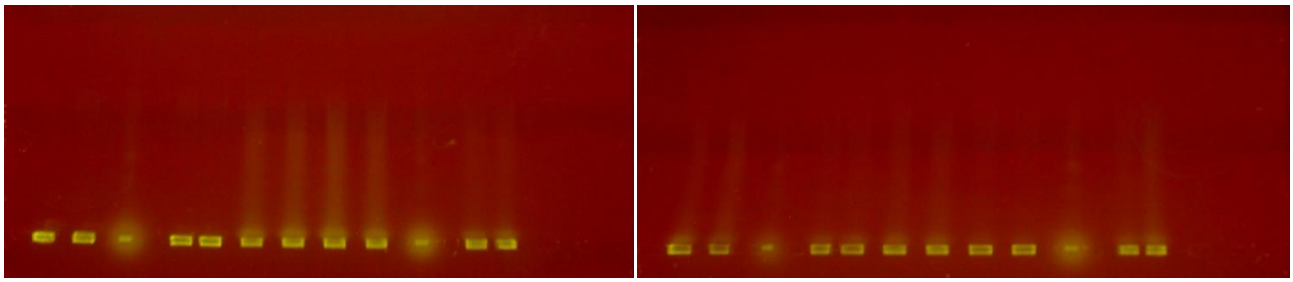



**Figure 1:** Agarose gel for HCT116 stained with SYBR safe DNA gel stain (50 µl in 500 ml distilled water). The lanes marked as M show *S. Pombe* chromosomal DNAs used as ladder with approximate sizes of 5.7, 4.6, and 3.5 megabases from bottom to top respectively. The content of each lane at the left side: lane 1, control; lane 2, control NU7026; lane 3, control NU7441; lane 4, control NVP-BEZ235; lane 5, irradiated control, no repair; lane 6, irradiated NU7026, no repair; lane 7, irradiated NU7441, no repair; lane 8, irradiated NVP-BEZ235, no repair; lane 9, irradiated control, 15 min repair; lane 10, irradiated NU7026, 15 min repair. The content of each lane at the right side: lane 1, irradiated NU7441, 15 min repair; lane 2, irradiated NVP-BEZ235, 15 min repair; lane 3, irradiated control, 1 h repair; lane 4, irradiated NU7026, 1 h repair; lane 5, irradiated NU7441, 1 h repair; lane 6, irradiated NVP-BEZ235, 1 h repair; lane 7, irradiated control, 4 h repair; lane 8, irradiated NU7026, 4 h repair; lane 9, irradiated NU7441, 4 h repair; lane 10, irradiated NVP-BEZ235, 4 h repair.

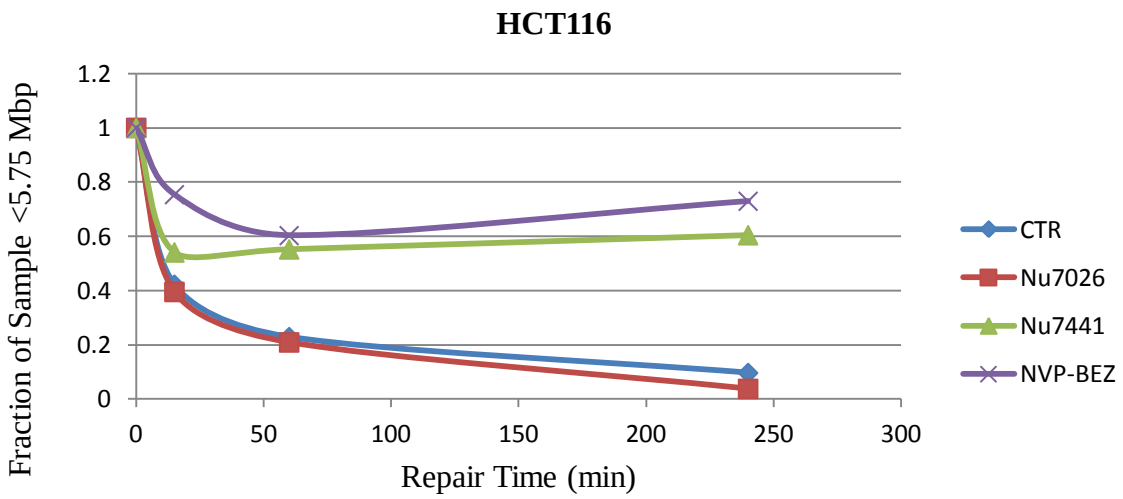



**Figure 2:** The fraction of activity that is released from total DNA in plugs containing HCT116 cells (FAR). Cells were treated by inhibitory drugs Nu7026, Nu7441, and NVP-BEZ (50 µM) 1 hour before irradiation by 40 Gy and FAR was calculated at no repair, 15 min, 1 h and 4 h repair. This data is related to agarose gel image in figure 1.

**A431**

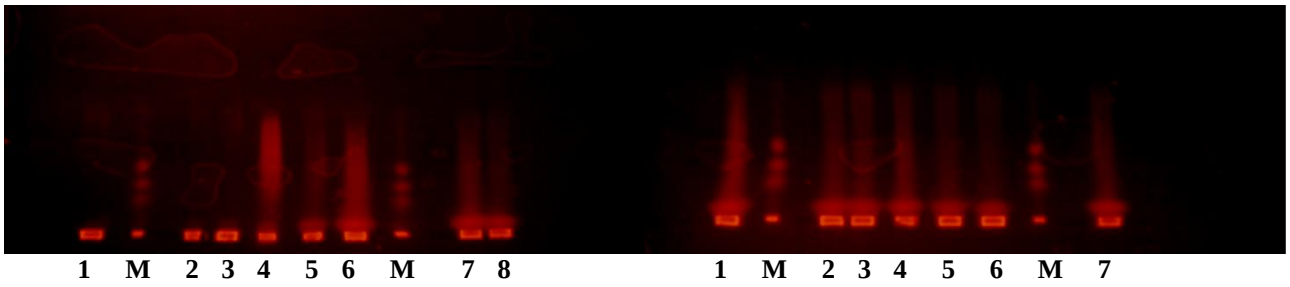



**Figure 3:** Agarose gel for A431 stained with SYBR safe DNA gel stain (50 µl in 500 ml distilled water). The lanes marked as M show *S. Pombe* chromosomal DNAs used as ladder with approximate sizes of 5.7, 4.6, and 3.5 megabases from bottom to top. The content of each lane at the left side: lane 1, control; lane 2, control NU7026; lane 3, control NU7441; lane 4, irradiated control, no repair; lane 5, irradiated NU7026, no repair; lane 6, irradiated NU7441, no repair; lane 7, irradiated control, 15 min repair; lane 8, irradiated NU7026, 15 min repair. The content of each lane at the right side: lane 1, irradiated NU7441, 15 min repair; lane 2, irradiated control, 1 h repair; lane 3, irradiated NU7026, 1 h repair; lane 4, irradiated NU7441, 1 h repair; lane 5, irradiated control, 4 h repair; lane 6, irradiated NU7026, 4 h repair; lane 7, irradiated NU7441, 4 h repair.

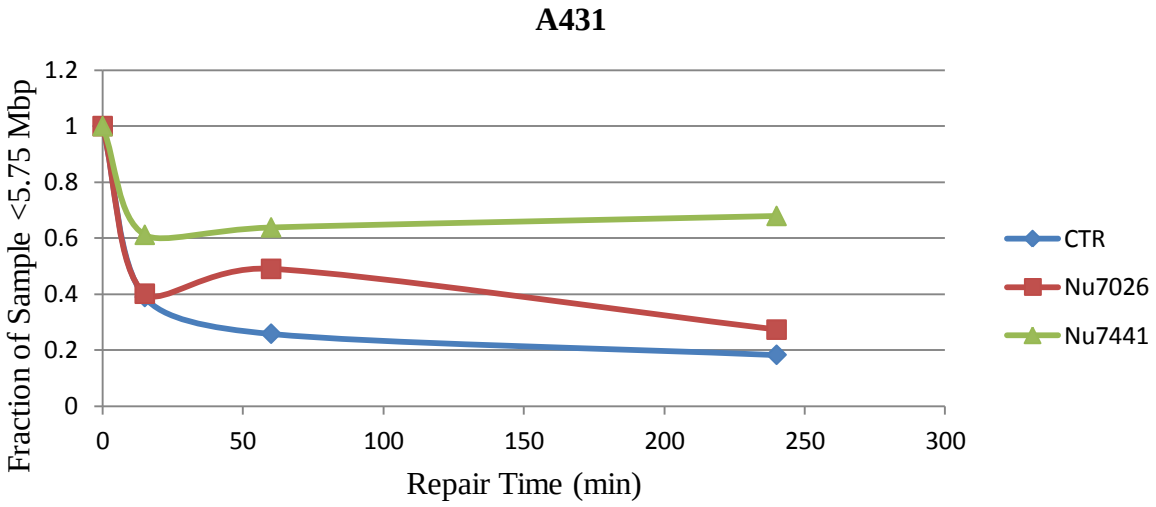



**Figure 4:** The fraction of activity that is released from total DNA in plugs containing A431 cells (FAR). Cells were treated by inhibitory drugs Nu7026 and Nu7441 (50 µM) 1 hour before irradiation by 40 Gy and FAR was calculated at no repair, 15 min, 1 h and 4 h repair. This data is related to agarose gel image in figure 3.

The agarose gel image in figure1 and plotted data in figure 2 show that the amount of unrepaired double-strand breaks, induced by 40 Gy irradiation in HCT116 cells, is much higher in cells that have been treated with the NU7441 and NVP-BEZ235 drugs than control samples, even after 4 hours of repair. Referring to agarose gel image 3 and plotted data in figure 4, the same result was seen for A431 cells that were treated by NU7441 compared to control and those that have been treated by NU7026. Based on these results, NU7441 was chosen for further studies on both cell lines and were accompanied with transfection of DNA-PKcs by small interfering RNA (siRNA) to induce higher depletion and deficiency of this key protein in cells.

**DNA Repair Analysis by PFGE in Cells Treated by Both Specific DNA-PKcs Inhibitor and siRNA Transfection Reagents**

Based on the results in figures 2 and 4, the NU7441 was chosen for further studies on both cell lines. The same PFGE experiments were run accompanied with transfection of DNA-PKcs by siRNA to induce a high level of deficiency in both HCT116 and A431 cells and examine their ability to repair. Therefore cells were treated by siRNA transfection reagents one day after seeding when they reached the confluency of ~ 70 %. After 48 hours, they were also treated with warmed-up drug, 1 hour before irradiation by 40 Gy. Then they were analyzed by PFGE and the quantified data was plotted in figure 5 for HCT116 and figure 6 for A431.

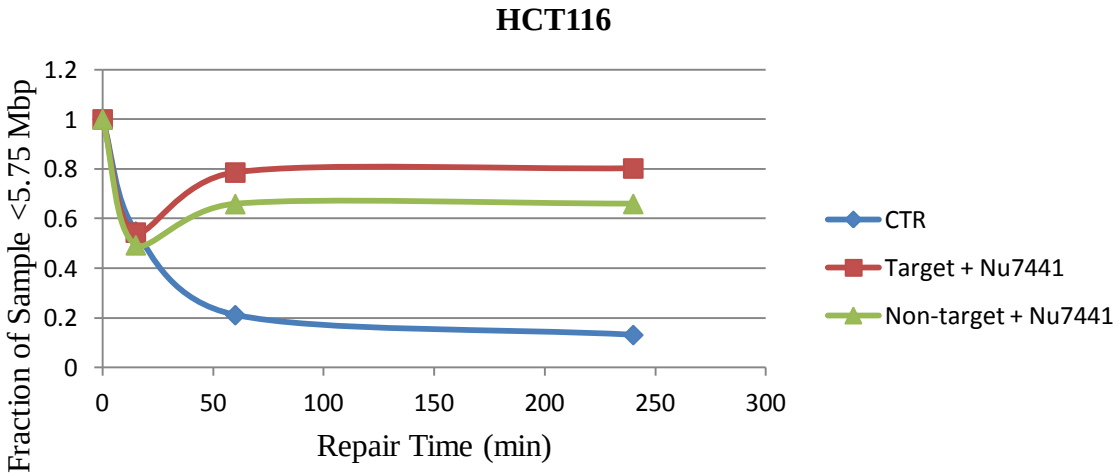



**Figure 5:** The fraction of activity that is released from total DNA in plugs containing HCT116 cells (FAR). Cells were treated by siRNA one day after seeding and by NU7441 (50 µM) 1h before irradiation by 40 Gy. FAR was calculated at no repair, 15 min, 1 h and 4 h repair.

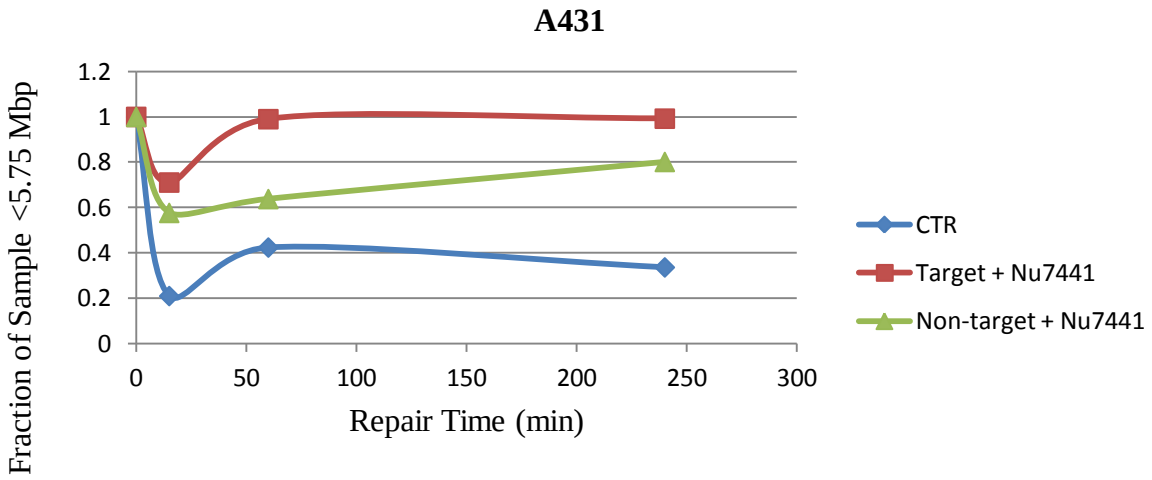
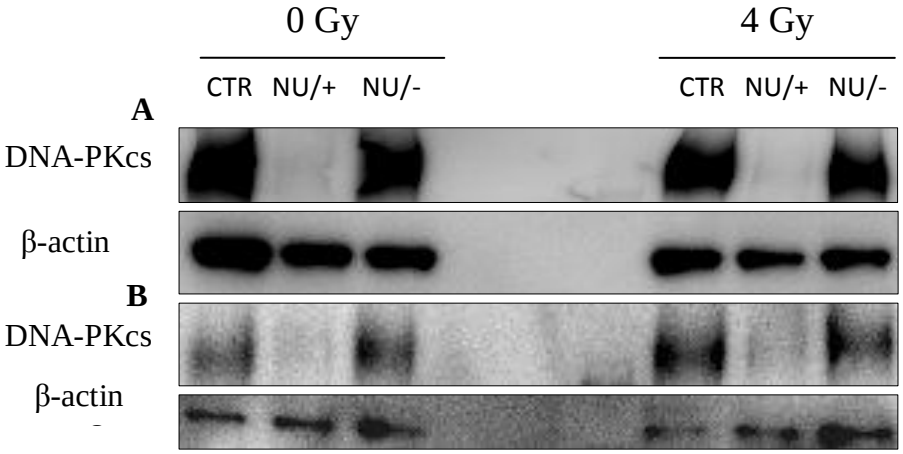



**Figure 6:** The fraction of activity that is released from total DNA in plugs containing A431 cells (FAR). Cells were treated by siRNA one day after seeding and by NU7441 (50 µM) 1h before irradiation by 40 Gy. FAR was calculated at no repair, 15 min, 1 h and 4 h repair.

**Examination of DNA-PKcs Protein Level by Western Blot after Transfection with siRNA**

To see whether the transfection of DNA-PKcs has been done properly or not, a western blot experiment according to the scheme in table 2 was done. All dishes were treated in the same way as PFGE. Dishes with NU7441 and irradiation were treated with drug 1 hour before irradiation with 4 Gy, and then be incubated at humidified 37˚C with 5 % $CO_2$. At the same time, drug was added to rest dishes with no irradiation and all were incubated at same condition for 1 hour before lysate preparation. The results were as follows:



**7:** (A, left) HCT116 cells treated with NU7441 (50 µM, 60 min) and siRNA against DNA-PKcs (NU/+) or with NU7441 and nonsilencing siRNA as control (NU/-). (A, right) HCT116 cells were irradiated by 4 Gy following the same pretreatment as part (A, left). (B) A431 cells with same treatment as part (A).

As can be seen in figure 7, DNA-PKcs transfection using silencing siRNA was done properly in both HCT116 and A431 cells.

**Determination of Radiosensitivity in Cancer Cells by Clonogenic Assay**

To determine the radiosensitivity of A431 cells and their ability to form a colony after irradiation, cells were seeded in 25 $cm^2$ flasks in triplicates for each radiation dose 1, 2, and 3 Gy and irradiated after 2 hour incubation at humidified 37°C with 5 % $CO_2$. The number of colonies was counted 9 days later and the quantified result was plotted as in figure 8.

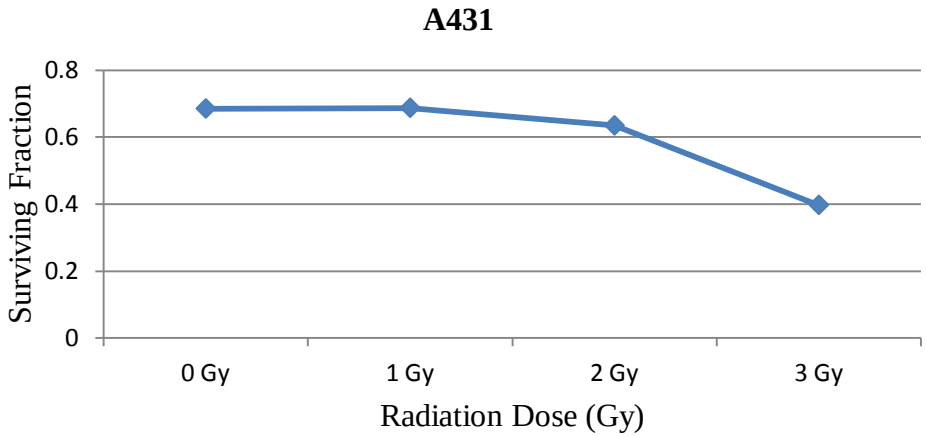



**Figure 8:** Clonogenic survival fraction for three different doses (1, 2, and 3 Gy) for A431 cells. Cells were seeded in triplicate for each radiation dose and irradiated 2 h after. Colonies were counted after 9 days.

As can be seen in figure 8, the survival fraction for A431 cells was decreased by induction of higher radiation dose. In this experiment, the survival fraction decreased to 40% at a dose 3 Gy which is ~ 30 % less compared to control.

## Discussion

The phosphatidylinositol 3-kinase-related kinases (PIKKs) are a family of proteins, three members of which have critical role in response to different DNA damage; ataxia-telangiectasia mutated (ATM), ataxia- and Rad3-related (ATR), and DNA-dependent protein kinase catalytic subunit (DNA-PKcs). DNA-PKcs plays a key role in NHEJ repair pathway in response to DNA DSBs, either induced by endogenous sources like reactive oxygen radicals produced by the cellular metabolism or by exogenous sources like IR [3, 4, 16]. The Ser2056 and Thr2609 are two well-known residues of DNA-PKcs that are phosphorylated as a response to DNA damage, either by DNA-PKcs itself or by other proteins like ATM and ATR. In response to IR-induced DSBs, DNA-PKcs is activated by autophosphorylation of Ser2056 and ATM-mediated phosphorylation of Thr2609 [9, 16]. This study demonstrates that the ability of DNA-PKcs-deficient cells to repair IR-induced DSBs decreases, which in turn can improve the effect of radiotherapy in tumor cells with low DNA-PKcs expression. The deficiency of DNA-PKcs in this study was induced by either DNA-PKcs inhibitors or siRNA transfection. Both cell lines, HCT116 cells after treatment with DNA-PKcs specific inhibitor, NU7441, and NVP-BEZ235, dual inhibitor of PI3K and mTOR, and A431 after treatment with NU7441, showed considerable decrease in ability of DSBs repair compared to control (Fig. 2 and 4). The effect of DNA-PKcs inhibition by these drugs in increase of radiosensitivity of human cancer cells has also been demonstrated with similar studies [12, 17, 18, 20]. The reduction of DSBs repair ability became more significant when the siRNA transfection was also added to the experiment to reduce the DNA-PKcs level to higher extent (Fig. 5 and 6). The importance of DNA-PKcs level in DSBs repair has also been shown in another study in which various levels of this protein were produced by use of siRNA knock-down in human lymphoblastoid cells [10]. Beside the function of DNA-PKcs in DSBs repair, reports have shown that this protein is also vital to operate a properly-regulated mitosis cell cycle through the formation of normal spindle apparatus. Cells with depleted DNA-PKcs form abnormal spindles which lead to misalignment of chromosomes and consequently chromosomal instability, the hallmark of cancer cells, and it finally leads to mitotic catastrophe in cells with DNA damage [9, 11]. These results show that, although DNA-PKcs deficiency increases the risk of cancer, it can also be used to enhance the radiosensitivity in tumor cells.

To further assess of our cell lines` ability to form a colony, the clonogenic assay in this study can be continued by combination of irradiation and DNA-PKcs inhibitors treatment in future experiments.

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