Inhibition of Iron Regulatory Protein 2 Enhances Sensitivity to Irradiation by Regulating the DNA Damage Response (DDR) in Breast Cancer Cells

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Inhibition of Iron Regulatory Protein 2 Enhances Sensitivity to Irradiation by Regulating the DNA Damage Response (DDR) in Breast Cancer Cells

Directed by Professor Sang Joon Shin

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The Graduate School
Yonsei University

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ABSTRACT

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Radiation therapy (RT) is a standard treatment of various tumor types, but many studies on RT are continued for enhancing radio-sensitivity and overcoming the radiation resistance.

Iron is well known as an essential element for cell metabolism, including DNA damage response (DDR), the major mechanism of occurring therapeutic resistance. Iron regulatory protein 2 (IRP2) is a key factor for regulating iron homeostasis and anti-tumor effect of iron deficiency has been demonstrated. In this study, we found that the correlation between IRP2 expression and irradiation. We observed that IRP2 exhibited contrasting responses to radiation exposure in radiation sensitive (RS) group and relatively radiation insensitive (RR) group of breast cancer (BC) cells. The expression of IRP2, IRP1 and TFR were upregulated only in RR group, therefore, we hypothesized that the upregulation of IRP2 represents a 'resistance' response to radiation. Therefore, combined treatment of IRP2 inhibitor and RT proposed effective method for sensitizing radiation response.

This study focuses on KS20226, a modified compound of KS20073 as a first-in-class IRP2 inhibitor, which exhibited anti-tumor effects in BC cells and potential as a ‘radio-sensitizer’. Through RNA-sequencing analysis of BC cells treated with KS20226, we confirmed that KS20226 induced mitochondrial dysfunction through the downregulation

of DNA repair and the G2/M checkpoint pathway. *In vitro* experiments also revealed the suppression of RAD51 and BRCA1/2. Considering previous studies on BC cells and RT, which suggest that DDR and cell cycle arrest are key mechanisms leading to radiation resistance, we propose that KS20226 could enhance radiation sensitivity and overcome radiation resistance in BC. Therefore, we suggest that combining KS20226 treatment with RT could be a potential therapeutic strategy for overcoming radiation resistance in BC cells.

Key words :radiation, IRP2, iron hoemostasis, DDR, radioresistance, mitochondrial dysfunction, BC

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I. INTRODUCTION

Breast cancer (BC) is one of the most common malignancies in women, characterized by its various subtypes based on hormone receptors such as ER, PR, and HER2, alongside growth factors. This diversity necessitates a tailored approach for each subtype, optimizing treatment effectiveness [1]. Radiation therapy (RT) serves as a frequently employed therapeutic modality in BC, effectively treating up to 83% of early-stage cases. However, challenges arise due to the diverse characteristics of BC and the development of radiation-resistant mechanisms in some patients, leading to metastasis and recurrence post-RT. Thus, a profound understanding of RT and its interactions with BC is vital for advancing cancer treatment [2].

Radiation impacts all cellular constituents. Historically, research in radiation biology primarily focused on the genotoxic effects of radiation, involving DNA, and the molecular mechanisms of DNA damage induction, signaling, regulation, and repair. Recent molecular biology studies have expanded to explore how radiation perturbs cellular networks, affecting metabolism homeostasis, growth, and proliferation. Notably, radiation interferes with genetic control mechanisms involving DNA, and also with epigenetic control, particularly through mitochondrial functions that influence transcription. Mitochondria, crucial for maintaining cellular integrity and functions, play a significant role in cell metabolism, bioenergetics, signaling, and genomic stability post-RT [3,4].

Iron is pivotal for normal cell growth, and even more so for cancer cells, which require rapid growth and division. It contributes to key mechanisms such as reactive oxygen species (ROS) production, DNA synthesis, DNA repair, and cell cycle regulation. Iron deficiency, shown to inhibit tumor growth, has been utilized in both animal studies and human clinical trials for cancer treatment. Furthermore, studies have identified higher levels of iron in BC tissues compared to normal breast tissue [5,6].

Fe-S clusters are integral to the function of mitochondrial and cytosolic proteins involved in various cellular processes, including the regulation of iron metabolism. Iron regulatory protein 1 (IRP1), a master regulator of cellular iron status, is an Fe-S cluster-containing protein. Under iron-replete conditions, IRP1 functions as cytosolic aconitase with an intact Fe-S cluster. In contrast, iron limitation leads to the disassembly of this cluster, and IRP1 assumes RNA binding activity. In this state, it binds to iron-responsive elements (IREs) in the untranslated regions (UTRs) of target mRNAs like transferrin receptor (TFR), ferritin, and ferroportin. IRP2, another key regulator of intracellular iron, similarly responds to cellular iron levels and regulates iron homeostasis post-transcriptionally. It has been well-established that IRP2, particularly overexpressed in breast cancer cells, plays a crucial role in cellular iron regulation by stabilizing TFR, thereby increasing iron uptake and intracellular iron levels. Consequently, IRP2 has emerged as a vital therapeutic target in impeding the growth of breast cancer cells.

In this study, we aim to elucidate the mechanism by which the IRP2 inhibitor KS20226, a modified compound of KS20073 as a first-in-class IRP2 inhibitor , triggers apoptosis in BC cells in final step. We hypothesize that KS20226 induces mitochondrial dysfunction through regulating the DNA damage response (DDR) and cell cycle via iron deficiency, thereby enhancing radiation sensitivity and offering a potential strategy to overcome radiation resistance (RR) in BC cells.

II. MATERIALS AND METHODS

1. Cell culture

Human breast cancer cells MDA-MB-453, MDA-MB-231, SK-BR-3, MCF-7 and T47D were purchased from the Korean Cell Line Bank (Seoul, South Korea). The MDA-MB-468 cell was purchased from ATCC (Manassas, VA, USA). MDA-MB-453, MDA-MB-231, SK-BR-3, and MCF-7 cells were cultured in RPMI 1640 medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum(FBS), and 100 U/mL penicillin, and 100 μg/mL streptomycin at 37℃ in a 5% CO2 atmosphere. MDA-MB-468 cells was cultured in DMEM medium (HyClone Laboratories Inc, Utah, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37℃ and with 5% CO2.

2. Cell viability assay

Cell viability was assessed CCK-8 (Dojindo, Kumamoto, Japan) kit. The cells were seeded on 96-well culture plates 1.5 x 10^4 cells/well. After incubated until cells were adhered to the bottom of the plate, KS20226, radiation was applied to individual wells and incubated at 37℃. After 48hr, CCK-8 reagent (10uL/well) were added per well and incubated for 3-4hr at 37℃. Absorbance was measured by a ELISA reader (VERSA Max, Molecular devices) at wavelength of 450nm. IC50 values were calculated by Graph Pad Prism software (San Diego, CA, USA).

3. Western blot

Protein expression was measured by the Western blot assy. Cells were harvested using cold-PBS through scrapping. For extraction of the proteins, we lysed total cell with RIPA (radioimmunoprecipitation assay, ELPIS-BIOTECH, Daejeon, South Korea) buffer with phosphatase and protease inhibitors (GenDEPOT, Texas, USA).

4. Flow cytometry

To analyze the apoptotic cells (%), 0.8 x 10^6 cells were seeded in 60mm cell culture dish and incubated at 37℃. After 24hr, cells were treated with KS20226 and radiation alone or combined them. Cells were collected through trypsization and centrifugation. Cells were resuspended in 100μl of 1X binding buffer, and stained with the PE Annexin V Apoptosis Detection Kit (Thermo Scientific, USA), according the manufacturer's protocol. They were gently vortexed and incubated for 15 min at room temperature in dark. After incubation, 3~400μl of 1X binding buffer was added. For cell cycle analysis, after cells collecting, cells were washed with PBS and resuspended with propidium iodide (PI) and RnaseA staining buffer (Becton, Dickinson and Company, New Jersey, USA) for 15min at roon temperature in the dark. Stained cells were then analyzing using BD FACSymphony A5 (Becton Dickinson company). We used the flowing sofrware (Turku Bioscience, Turku, Finalnd) for data analysis.

5. Colony formation assay

For colony formation assay, we seeded 5 x 10^3 to 2 x 10^4 BC cells in 60mm culture dish and incubated them for 2~3 weeks until colony formed absence or presence of treatment. The medium was changed every 2~3 days after colonies stabilized onto plates enough, and the colonies were counted only with >40 cells. After colonies formation, cells were washed gently using 1X PBS, fixed and stained using crystal violet mixture including 4% paraformaldehyde and 100% methanol for 30 min at room temperature. The number of colonies was analyzed by Image J (National Institutes of Health, Bethesda, MD, USA). To normalize BC cells, have different plating efficiencies, we calculated the plating efficiency (PE) for estimating surviving fraction (SF). The following is the formula used for the analysis.

$$\text{Surviving fraction (SF)} = \frac{\text{Number of colonies formed after treatment}}{\text{Number of cells seeded x 100\%}}$$

$$\text{Plating efficiency (PE)} = \frac{\text{Number of colonies formed for untreatment cells}}{\text{Number of cells seeded x 100\%}}$$

6. Transfection

Cells were seeded and cultured in 0.8 x 10^6 into 60mm culture plates. After cells reached 60-70% confluency, they were transfected with targeted siRNA using lipofectamine RNAiMAX (Invitrogen, USA) according to the manufacturer's protocol for 24hr. Cells were treated with KS20226 or radiation after transfection. siRNA was synthesized by Bioneer (Daejeon, South Korea).

7. RNA extraction

Cells were seeded and cultured in 60mm culture plates (0.8 x 10^6 cells). They were treated with KS20226 and Radiation alone or combined. After 48hr of 72hr, total RNA was extracted using the Ribospin2 kit (Geneall, seoul, South Korea) according to the manufacturer's instructions. The quality and concentration of RNA were measured by nano drop 1000 spectrophotometer (Thermo Scientific, USA).

8. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

1000ng of extracted RNA in previous step was reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., MA, USA) according to the manufacturer's protocol. Quantitative PCR was performed by Quantstudio3 (Applied Biosystems.) with SYBR Green master mix (Applied Biosystems Inc.) following the manufacturer's instruction. β-actin was used as the endogenous control for normalization.

Table 1. The sequence of primers used in qRT-PCR

Name	Direction	Sequence (5' to 3')
IRP1	Forward	CGC TGT GGT TGA CTT TGC TGC AAT
	Reverse	ATC TAT TAC AAG ATC AGC AGG GCA G
IRP2	Forward	GGC TGC AGA GCT GTA CCA GAA AGA A
	Reverse	CGG TCC TTT GGC AGC CCA GTC TCT
TFR	Forward	ACT TGC CCA GAT GTT CTC AG
	Reverse	GTA TCC CTC TAG CCA TTCAGT G
RAD51	Forward	TTT GGA GAA TT CGA ACT GG
	Reverse	TAC ATG GCC TTT CCT TCA C
BRCA1	Forward	GCT CGT GGA AGA TTT CGG TGT
	Reverse	TCA TCA ATC ACG GAC GTA TCA TC
BRCA2	Forward	CAG AAG CCC TTT GAG AGT GG
	Reverse	TCC ATC TGG GCT CCA TTT AG
ATM	Forward	GGT ATA GAA AAG CAC CAG TCC AGT ATT G
	Reverse	CGT GAA CAC CGG ACA AGA GTT T
ATR	Forward	AGT AGC TTC CTT TCG CTC CAA A
	Reverse	ACT GAC TCC GGC CAC TCC AT
β-actin	Forward	TTG CCG ACA GGA TGC AGA AG
	Reverse	AGG TGG ACA GCG AGG CCA GG

9. Differential Gene Expression Analysis by RNA sequencing

We performed RNA sequencing with total RNA samples from BC cells treated with KS20226 for 48hr. The basal levels of cell lines and changes in mRNA expression upon treatment with KS20226 were analyzed using DEG analysis, and gene enrichment plots was evaluated using GSEA software.

10. Spheroid assay

3D cell viability was assessed by spheroid assay. 96 U-bottom plates (Greiner Bio-One, Kremsmunster, Austria) were pre-coated with 60uL of Polly 2-hydroxyethyl methacrylate (poly-HEMA) (Sigma Aldrich, Missouri, USA) were dissolved with 95% ethanol at a final concentration of 20mg/ml. Coated plates were dried for 2-3hr in sterile environment, and dried overnight at 65℃. 1 x 10^3 cells/well of MDA-MB-468 and MDA-MB-453 cells were seeded onto plate, and total volume of seeded cells were 100μl. The plate was centrifuged at 216g for 10min and incubated 37℃. After 4 days, treated with KS2026 every 2 days. At 11 days, the spheroids were stained with Hoechst33342 33μM, Calcein AM 2μm, and EthD-1 3μm (Thermo Fisher Scientific Inc., Massachusetts, USA) and incubated for 3hr at 37℃. Fluorescence imaging was observed with Operetta CLS (PerkinElmer Inc., Massachusetts, USA).

11. JC-1 staining assay

The cells were plated and incubated on 96-well plate for JC-1 staining. Before staining, cells were washed with PBS gently. And add pre-warming staining solution including 0.2mg/mL of JC-1 and 0.1mg/mL of Hoeschst. Then cells were incubated for 20~30 min in dark at 37℃, 5% CO_2. After staining, washed cells gently with PBS and replaced with fresh PBS. Images were taken using the operetta CLS (PerKinElmer Inc, USA). For Red/Green ratio calculation, using intensity analysis in Harmony software.

12. Statistical analysis

All statistical analysis was performed in GraphPad v5.01 software for each experiment. The data included at least three replicates for each experiment, and presented as the mean+-S.D. Statistical comparisons were analyzed using an unpaired two-tail t-test and one-way ANOVA, and significance in the graphs as follows: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

III. RESULTS

1. IRP2 Expression Patterns and Their Influence on Radiation Response in Breast Cancer.

The role of Iron Regulatory Protein 2 (IRP2) in modulating radiation sensitivity is critical in breast cancer (BC) treatment. Given IRP2's known influence on DNA repair, synthesis, and cell cycle mechanisms, we hypothesized a potential interplay between IRP2 and radiation response. To test this, we classified BC cells into two groups based on their radiation sensitivity. The relatively radio-resistant (RR) group, comprising MDA-MB-468 and MDA-MB-453, and the radio-sensitive (RS) group, including SK-BR-3 and MCF7, were differentiated by evaluating the surviving fraction post-exposure to radiation doses ranging from 0 to 8 Gy (Fig 1A). Notably, cell viability in the RR group remained above 85% even at 6 Gy (Fig 1B). Furthermore, a dose-dependent upregulation in the expression of IRP2, along with related proteins IRP1 and TFR, was observed in MDA-MB-468 and MDA-MB-453. In contrast, SK-BR-3 and MCF7 cells from the RS group exhibited a negative correlation between IRP2 expression and irradiation levels (Fig 1C).

To elucidate the differences in radio-sensitivity, pathway analysis comparing the RR and RS groups was conducted (Fig 1D). Radiation response pathways were markedly upregulated in the RR group (MDA-MB-468 and MDA-MB-453) compared to the RS group (SK-BR-3 and MCF7). Particularly, RAD51 expression was significantly higher in MDA-MB-453 than in SK-BR-3. The gene expressions of BRCA1 and BRCA2, associated with homologous recombination (HR) repair, as well as ATM and CHEK2, markers of DNA damage, were elevated in both cell lines of the RR group. Additionally, we noted increased expression of BCL2, an anti-apoptotic marker commonly overexpressed in BC cells, in the RR group. This overexpression of BCL2 could potentially contribute to radiation resistance in cancer treatment.

Given the clear disparity in radiation response-related gene expression, we propose that DNA repair and BCL2-related genes are primary factors in the reduced radiation sensitivity observed in the RR group (MDA-MB-468, MDA-MB-453). Consequently, our findings suggest that the response of IRP2 to irradiation varies depending on the cell's inherent radio-sensitivity, potentially leading to radiation resistance. Therefore, targeting IRP2 in BC cells emerges as a promising approach to overcome radiation resistance.

2. Targeting IRP2 to Enhance Radio-Sensitivity in Breast Cancer Cells.

Exploring the potential of IRP2 as a selective target for enhancing radiation sensitivity in breast cancer (BC) cells, we employed the DepMap data analysis. This analysis revealed a positive correlation between increased expression of IREB2 (the gene encoding IRP2) and the enhancement of DNA repair and anti-apoptotic gene expressions within BC cells (Figure 1E). Specifically, IRP2 expression was found to be positively associated with key genes such as RAD51, BRCA2, BRCA1, and XIAP. This implies that inhibiting IRP2 could disrupt DNA repair mechanisms and promote apoptosis, making it a viable target for radio-sensitization.

To validate the role of IRP2 as a potential radio-sensitizer in BC cells, we investigated its direct impact on radiation sensitivity. Using siRNA transfection, we demonstrated that the knockdown of IRP2 led to the downregulation of TFR, RAD51, and anti-apoptotic markers including BCL-2, BCL-XL, and XIAP (Fig 1F), particularly in MDA-MB-468 and MDA-MB-453 cells. Notably, pre-treatment with siIRP2 in MDA-MB-453 cells before irradiation significantly inhibited colony formation (Fig 1G), underscoring the potential of IRP2 targeting as a strategy to enhance radiation response in BC treatments.

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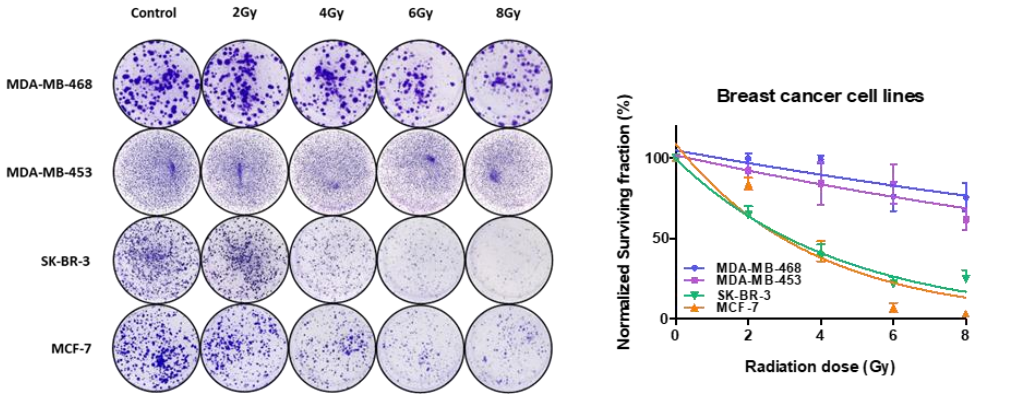

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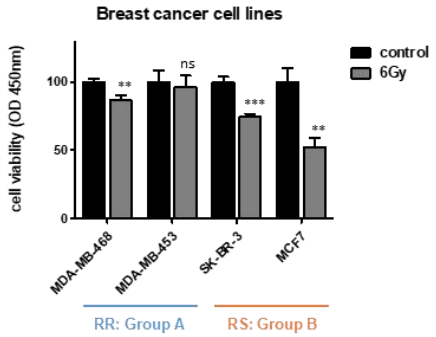

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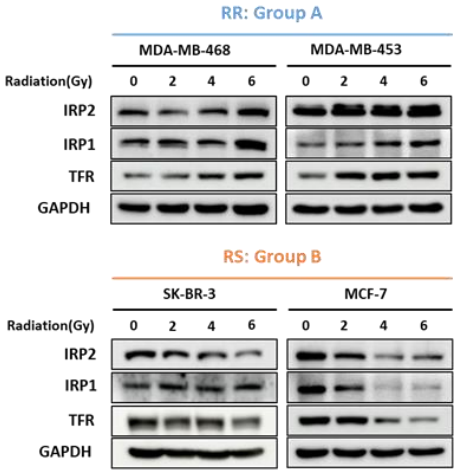

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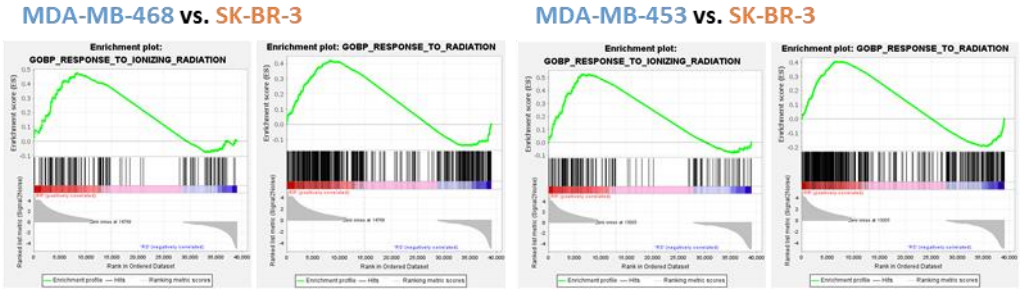

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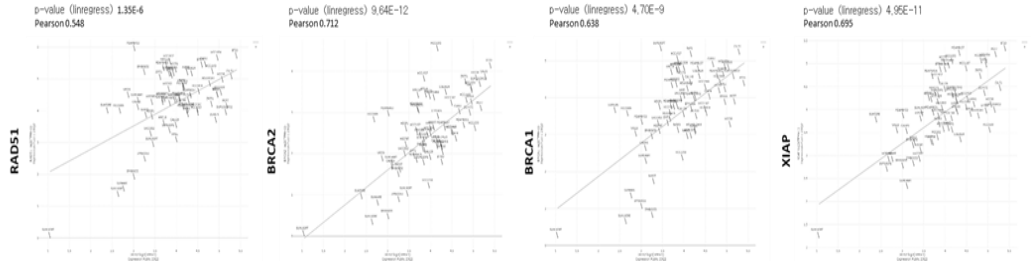

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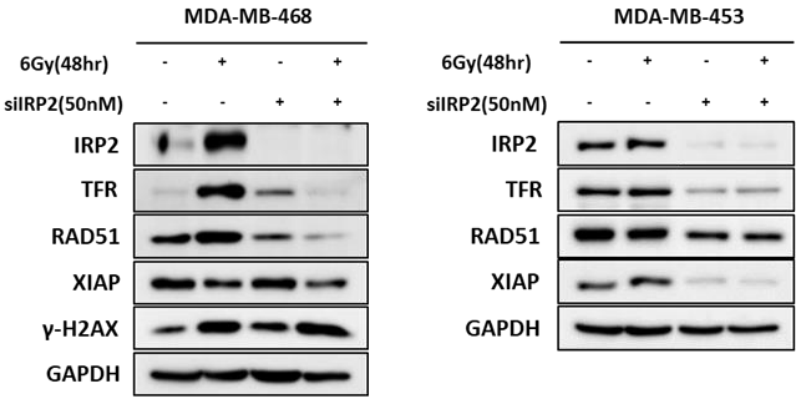

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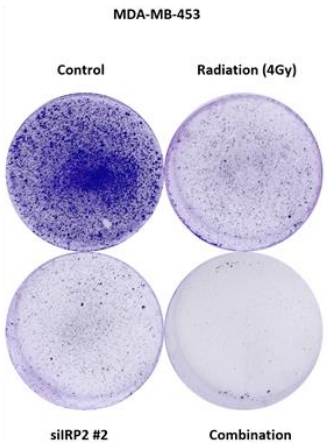

Figure 1. IRP2 is the direct target of induction of radiation sensitivity in breast cancer cells.

(A) (Left) Colony formation of BC cells in dose range of 0 to 8Gy irradiation, and (Right) the normalized surviving fraction. (B) Cell viability analysis upon treatment with radiation for 48hr by CCK-8 assay. (C) Western blot showed protein levels of IRP2, IRP1, and TFR in Group A and B treated with radiation 72hr in dose dependent manner. (D) Results of enrichment plot comparing groups A and B for response to radiation. (E) Correlation of gene expression between IRP2 and HR repair genes (RAD51, BRCA2 and BRCA1) and anti-apoptotic marker (XIAP) in BRCA, Pearson Correlation Coefficient and p-value are above each figures. (F) Western blot analysis of MDA-MB-468 and MDA-MB-453 cells with Radiation 48hr after 50nm of siIRP2 24hr. (G) Colony formation of MDA-MB-453 (Group A) cells treated with radiation and siIRP2 alone or combination.

3. An IRP2 Inhibitor, KS20226, Inducing Mitochondrial Dysfunction through Regulating Cell cycle and DNA Damage Response.

Building on our understanding of iron homeostasis and cancer metabolism, we explored the role of IRP2 deficiency in breast cancer (BC) cell growth. Using siIRP2, we demonstrated that inhibiting IRP2 enhances sensitivity to irradiation. Extending this research, we evaluated the anti-tumor efficacy of KS20226, a derivative of KS20073, in BC cells prior to combined treatment with RT.

We assessed BC cell viability upon KS20226 treatment (Fig 2A). KS20226 significantly inhibited the viability of various BC cells, with its IC50 values correlating with the basal IRP2 expression levels. Notably, SK-BR-3 cells, with the highest IRP2 expression, were most sensitive to KS20226. In contrast, MCF7 cells, expressing lower IRP2 levels, demonstrated markedly reduced sensitivity (Figure 2B).

To delve deeper, we performed RNA-sequencing analysis on MDA-MB-468, MDA-MB-453, SK-BR-3, and MCF7 cells treated with KS20226. Gene set enrichment analysis (GSEA) indicated a significant downregulation of gene sets related to DNA repair and the cell cycle, particularly the G2M checkpoint, in all four BC cell lines post-treatment (Figure 2C). These results were further substantiated by cell cycle analysis, where KS20226 induced G2/M arrest in a dose-dependent manner (Fig 2D). Post-KS20226 treatment revealed a decrease in IRP1, IRP2, and transferrin receptor (TFR) levels across the BC cell lines (Fig 2E), suggesting effective induction of iron deficiency. Correspondingly, RAD51 expression decreased, aligning with our gene pathway analysis results (Fig 2C). Additionally, increased levels of LC3B, an autophagy marker, and cleaved caspase-3 were observed, indicating enhanced autophagy and apoptosis (Fig 2E).

Further GSEA insights revealed a downregulation of mitochondria-related genes in BC

cells treated with KS20226, mirroring trends observed in previous studies on KS20073 and KS20226 in colorectal cancer cells [9]. Mitochondria gene sets were significantly downregulated in all treated BC cell lines (Fig 2F). Consistent with these results, mitochondrial membrane potential was assessed using JC-1 staining, revealing an increase in green fluorescence (JC-1 monomers) and a decrease in red fluorescence (JC-1 aggregates) as KS20226 concentration increased, indicating mitochondrial dysfunction (Fig 2G).

These findings suggest that KS20226 induces mitochondrial dysfunction by impairing DNA repair and promoting G2/M arrest, consequently inhibiting cell growth by triggering autophagy and apoptosis in BC cells.

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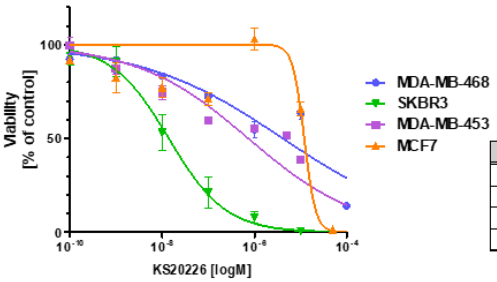

Breast cancer cell lines	IC50(μM)
MDA-MB-468	4.16
MDA-MB-453	0.74
SKBR3	0.014
MCF7	12.02

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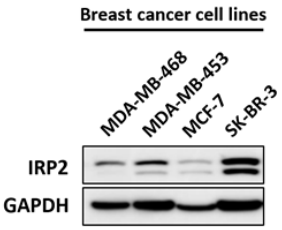

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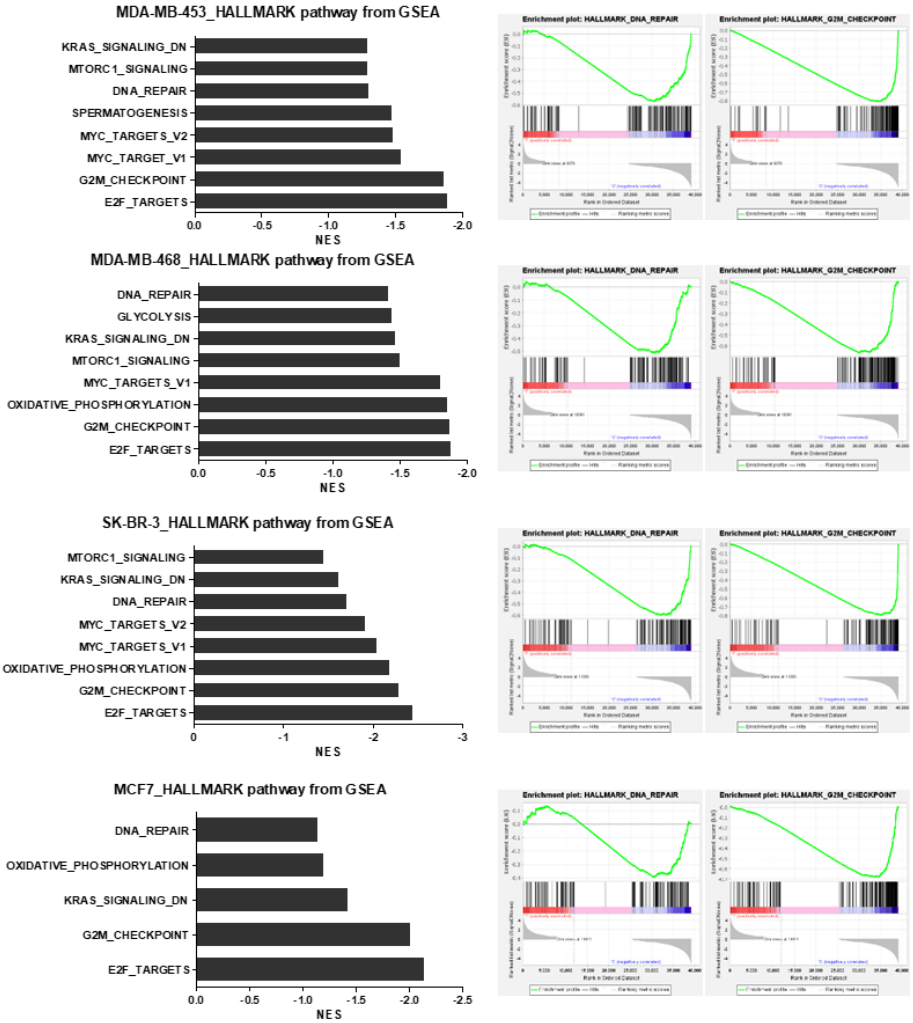

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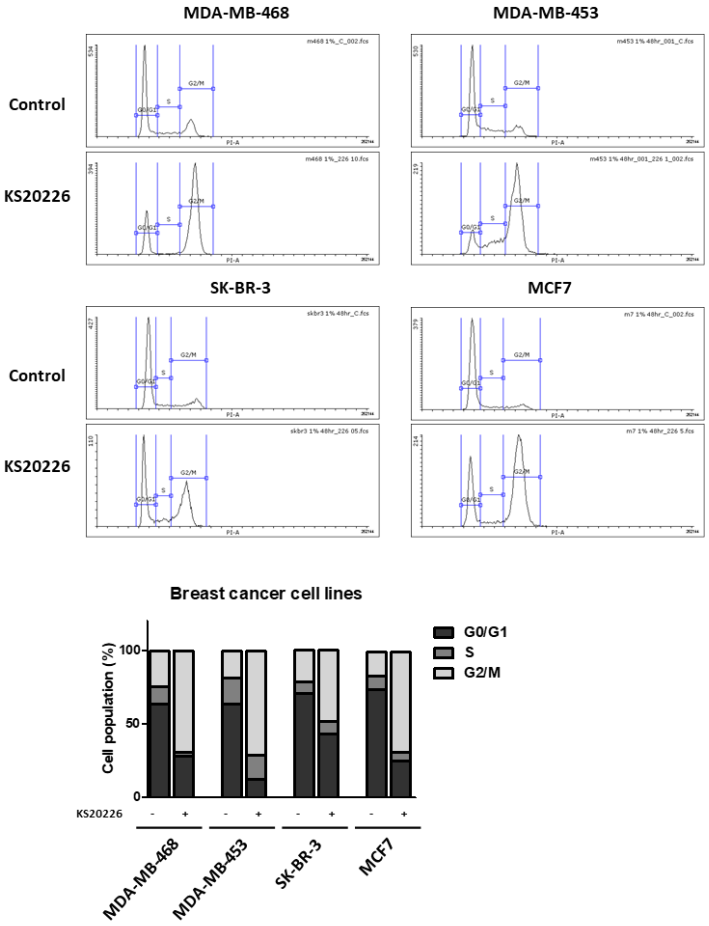

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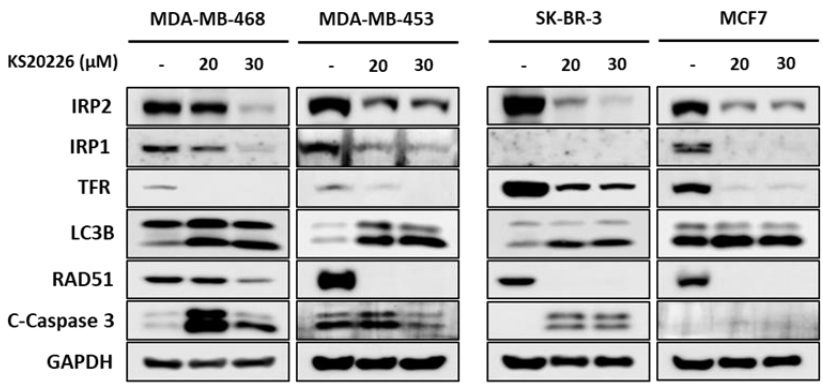

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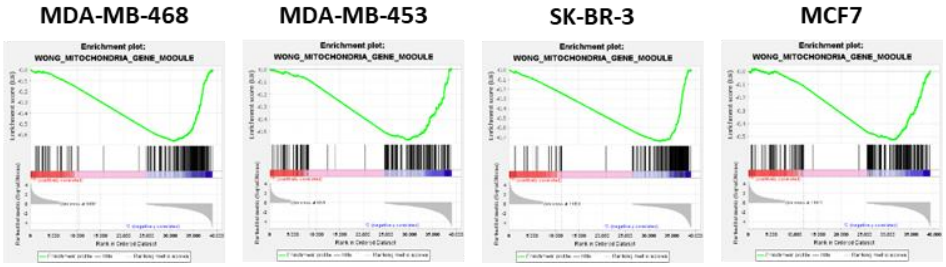

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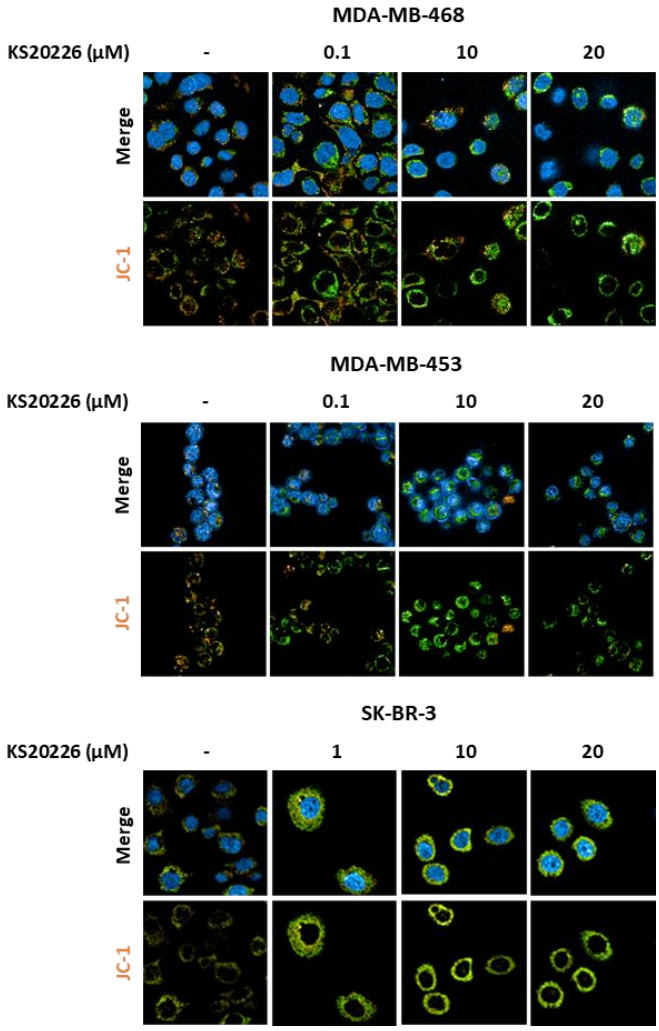

Figure 2. KS20226 triggered BC cells death via inhibition of IRP2.

(A) Breast cancer cells were exposed to various concentration of KS20226 then subjected to CCK-8 assay. IC50 of each cell was shown in the right table. (B) The protein expression of IRP2 of BC cells by western blot. (C) GSEA pathway analysis for HALLMARK pathway (Left) and GSEA results of Enrichment plot related with DNA repair and G2M checkpoint pathway (Right) in MDA-MB-468, MDA-MB-453, SK-BR-3. (D) Cell cycle analysis of BC cells after treatment of KS20226 for 48hr with PI staining and flow cytometry. (E) MDA-MB-468, MDA-MB-453, SK-BR-3 and MCF-7 cells were treated with 20 or 30uM of KS20226 72hr. Western blotting was performed with the following lysates with GAPDH acting as positive control. (F) Enrichment plot of BC cells about mitochondria gene module treated with KS20226. (G) JC-1 staining showed change of mitochondrial membrane potential followed by KS20226 treatment for 48hr of BC cells.

4. Combining KS20226 with Radiation to Enhance Radio-Sensitivity in Breast Cancer Cells through Induing Mitochondrial dysfunction via Regulating Cell cycle and DNA Damage Response.

In our quest to enhance radiation sensitivity in breast cancer (BC) cells, we investigated the combined effect of KS20226 and radiation therapy (RT) on cell cycle regulation and DNA damage response (DDR). We specifically focused on MDA-MB-468 and MDA-MB-453 cells, categorized as the radio-resistant (RR) group, to assess the efficacy of this combined treatment.

Our initial investigations centered on the capacity of KS20226 to induce cell cycle arrest. In the RR group cells, the proportion of cells in the G2/M phase remained around 50% when treated with radiation alone. However, when KS20226 was combined with radiation, there was a significant increase in the G2/M cell population, reaching up to 92% in MDA-MB-468. A similar trend was observed in MDA-MB-453 cells (Fig 3A). This combination treatment also notably reduced the protein expression levels of IRP2, TFR, and RAD51, which were otherwise upregulated by radiation treatment alone (Fig 3B). Additionally, the DNA damage marker γ-H2AX exhibited prolonged presence in the combination treatment compared to radiation alone. A corresponding pattern in mRNA levels of IRP2, IRP1, and TFR was also noted with the treatments of RT, KS20226, and their combination (Fig 3C).

Further exploring the DNA repair mechanisms, we examined changes in mRNA expression with the co-treatment of KS20226 and radiation. Notably, the mRNA expressions of RAD51, BRCA1, and BRCA2 were downregulated with the treatment of KS20226 alone and in combination, unlike in radiation-only treatment. Moreover, the expression of ATM and ATR, key regulators of DDR, was significantly upregulated in both KS20226-only and combination treatments (Fig 3D). Employing the JC-1 staining assay, we demonstrated that KS20226 reduced mitochondrial membrane potential (MMP),

inducing mitochondrial dysfunction in the combination treatment with radiation. In the RR group, radiation alone was insufficient to reduce MMP; however, the addition of KS20226 led to a notable increase in green fluorescence intensity in both cell types (Fig 3E). The quantified Red/Green ratio further substantiated the role of KS20226 as a significant radiation sensitizer (Fig 3E).

These findings highlight the potential of KS20226, when combined with radiation, to enhance radio-sensitivity in BC cells through mechanisms involving mitochondrial dysfunction and the modulation of DNA damage response pathways.

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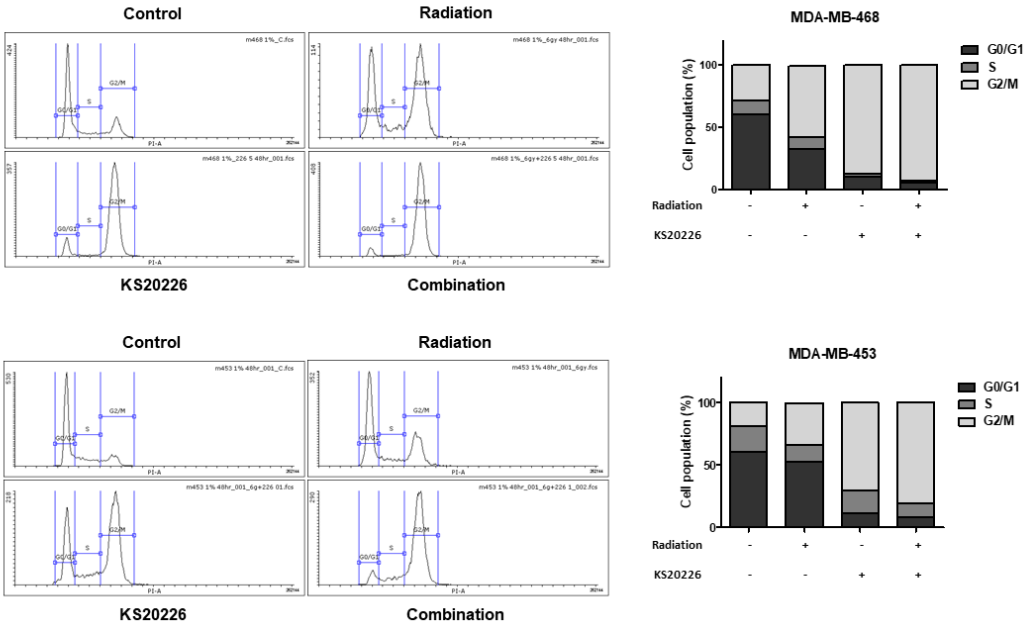

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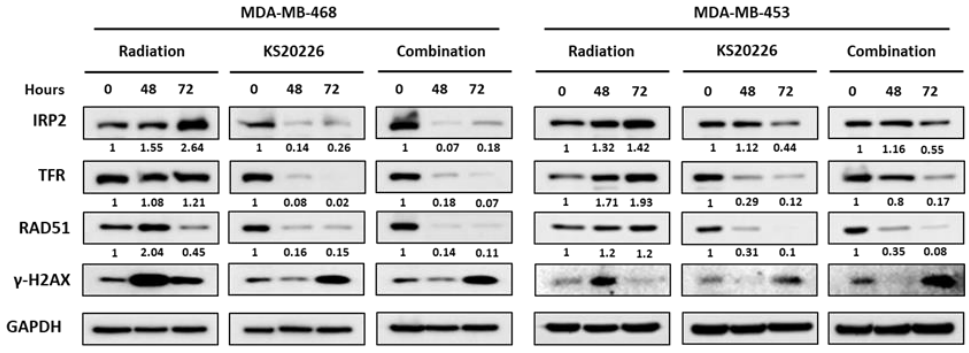

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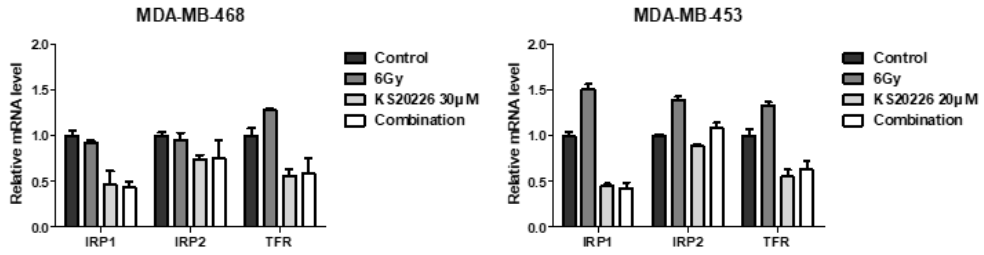

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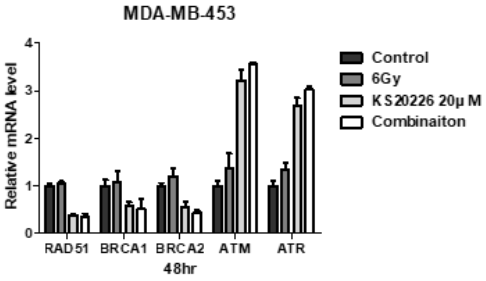

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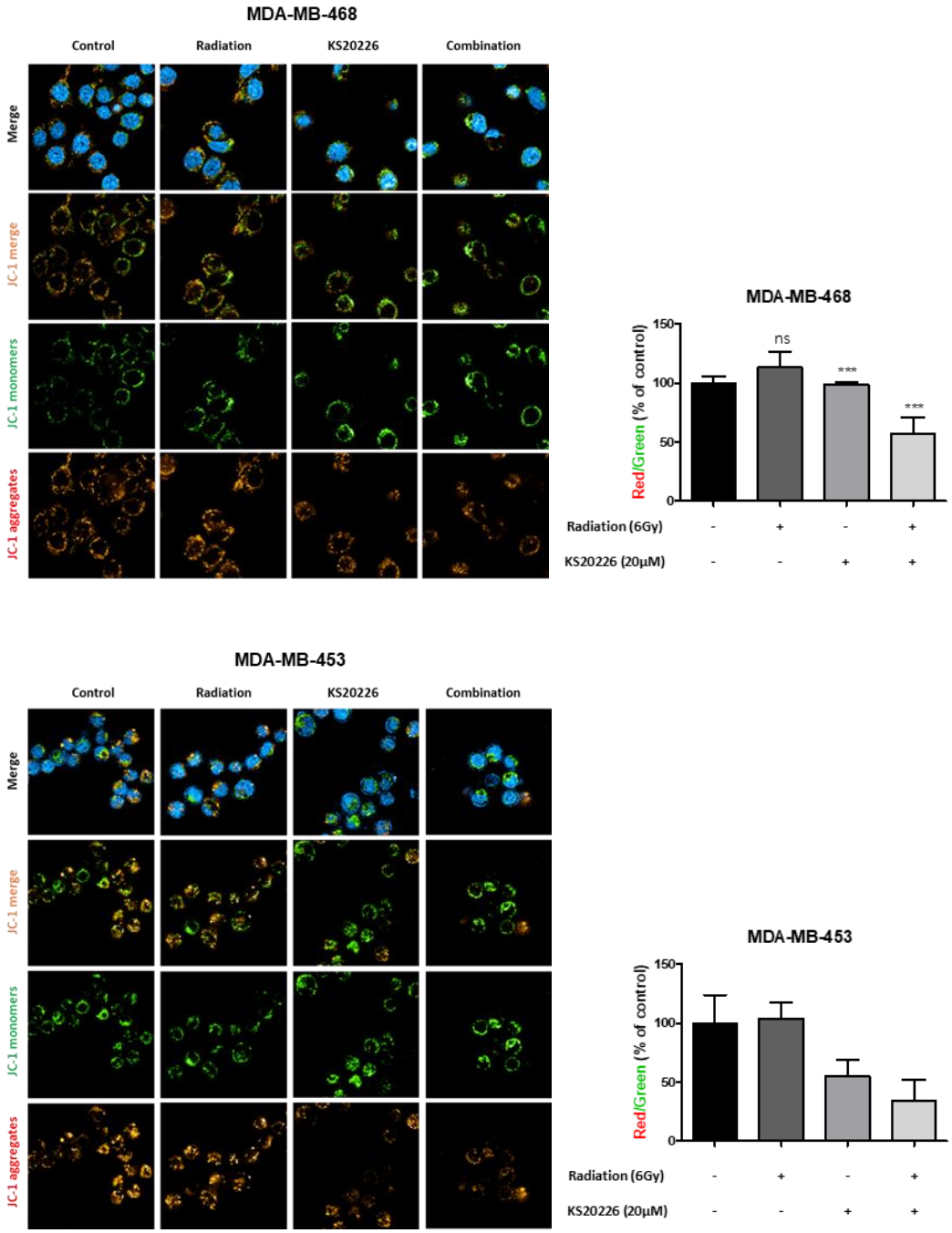


Figure 3. Combination treatment of KS20226 and radiation induced mitochondrial dysfunction via cell cycle arrest and inhibition of DNA damage response.

(A) Cell cycle analysis after treatment of KS20226 and radiation for 48hr with PI staining by flow cytometry. (B) Protein levels of IRP2, TFR, RAD51, and r-H2AX in MDA-MB-468 and MDA-MB0453 cells after treatment of KS20226 and/or radiation in 48hr and 72hr. (C) qRT-PCR analysis of mRNA expression of IRP2, IRP1 and TFR after treatment of KS20226 and/or radiation in and 72hr. (D) qRT-PCR results for DNA repair related gene after combination of KS20226 and radiation in MDA-MB-468 and MDA-MB-453. Anti-apoptotic and apoptosis markers of Group A treated with KS20226 and radiation. (E) (up) The confocal image and (down) triplicated graph about ration of JC-1 aggregates and monomers of MDA-MB-468 and MDA-MB-453 treated with KS20226 and radiation.

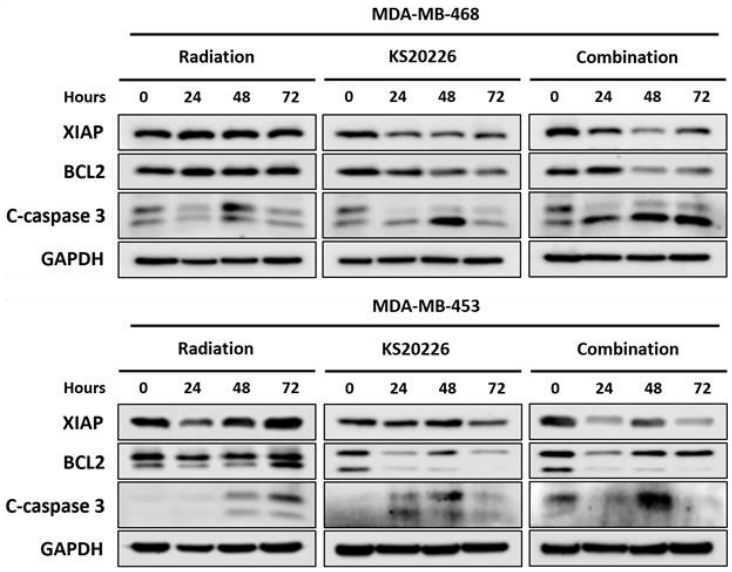
5. Enhancing Radiation Sensitivity in Radio-Resistant Breast Cancer Cells Through KS20226-Induced Apoptosis.

In our investigation into the potential of KS20226 as a radiation sensitizer, we focused on its ability to induce mitochondrial dysfunction through regulation of the cell cycle and DNA damage response (DDR). We found that KS20226 sensitizes breast cancer (BC) cells to irradiation, not only by inhibiting factors causing radiation resistance (RR) but also by prolonging DNA damage. Significantly, KS20226 downregulated XIAP, BCL2, BCL-XL, and induced apoptosis, as evidenced by the upregulation of cleaved-caspase 3 (Fig 4D). This suggests that KS20226 induces iron deficiency via IRP2 inhibition, ultimately leading to enhanced DNA damage, disruption of DDR, and apoptosis.

Our results indicate that MDA-MB-468 and MDA-MB-453 cells, which exhibit low sensitivity to radiation, respond significantly to IRP2 (Fig 1). Moreover, we observed notable regulation of DNA repair and cell cycle in these cells when treated with a combination of KS20226 and radiation (Fig 3). Additionally, our data demonstrate that this combined treatment effectively inhibits cancer cell growth. Initially, we observed decreased protein levels of XIAP and BCL2, coupled with an increase in cleaved caspase 3 in the combined treatment (Fig 4A). Importantly, apoptosis was more pronounced with the combined KS20226 and radiation treatment than with RT alone (Fig 4B). These findings indicate that co-treating KS20226 with radiation disrupts cell proliferation and leads to cell death through the inhibition of DDR and the induction of apoptosis.

In cell viability assays, the combination of KS20226 with radiation sensitized the cells to RT, resulting in significant inhibition of cell viability in both MDA-MB-468 and MDA-MB-453 cells compared to individual treatments (Fig 4C). Additionally, the combined treatment produced a synergistic effect on the surviving fraction in both cell types (Fig 4D).

A



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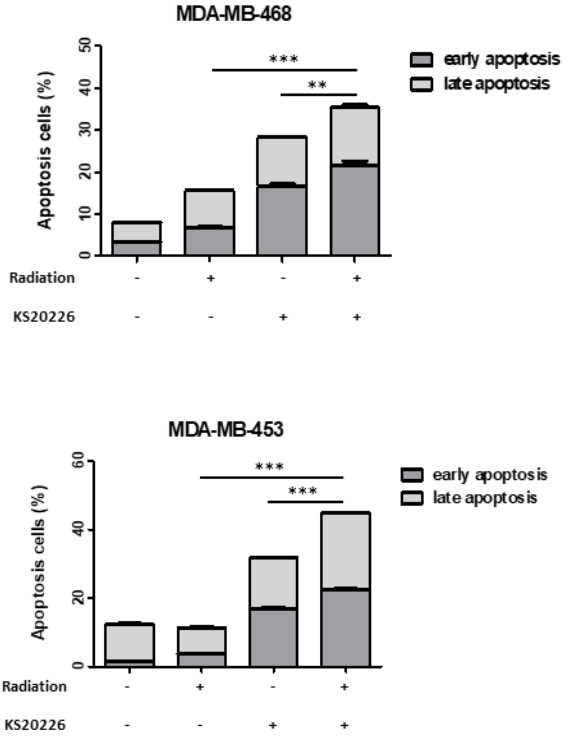

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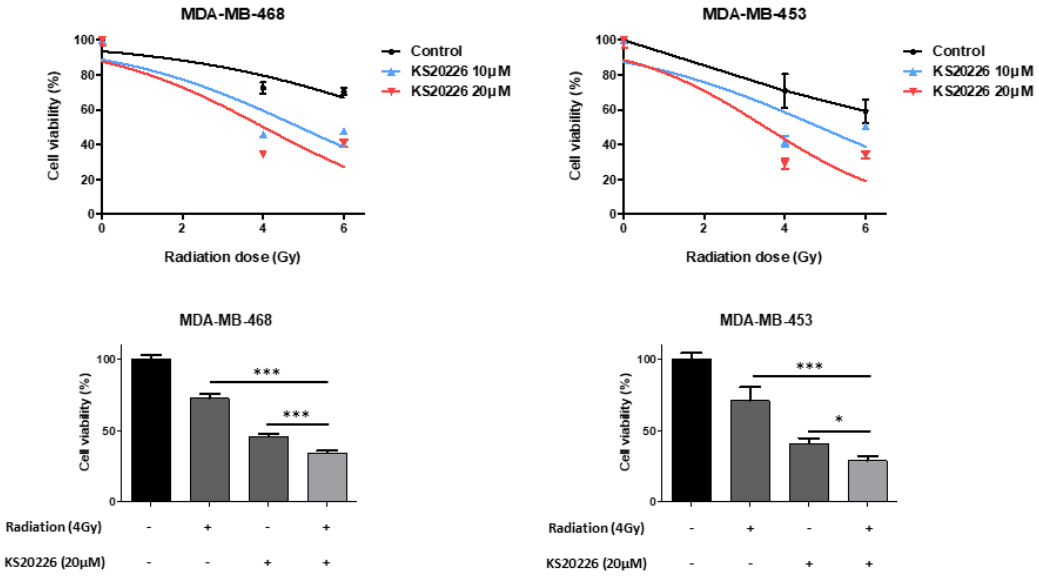

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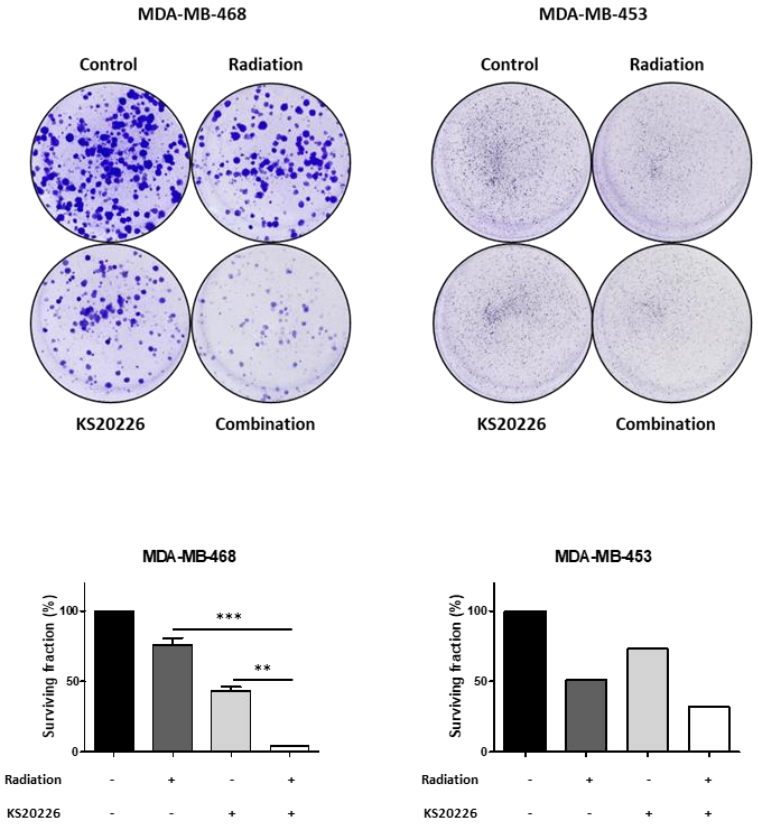


Figure 4. KS20226 enhanced the sensitivity to irradiation through inducing apoptosis in BC cells.

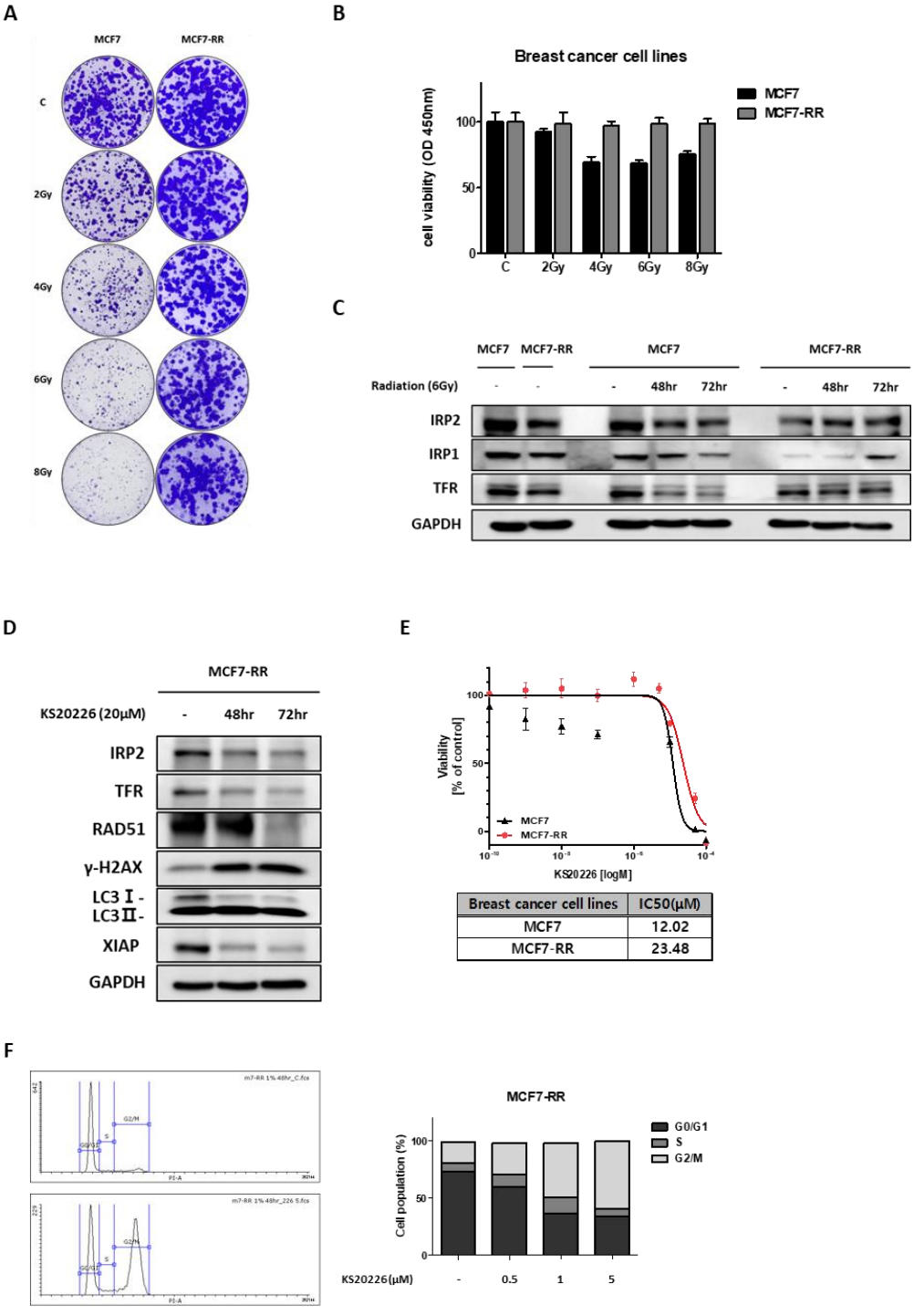
(A) Anti-apoptotic and apoptosis markers of Group A treated with KS20226 and 6Gy of radiation. (B) The percentage of apoptosis of MDA-MB-468 and MDA-MB-453 cells were treated with radiation or KS20226 or combination for 48hr or 72hr, then subjected to flow cytometry. (C) Cell viability (CCK-8) in Group A of cells (MDA-MB-468, MDA-MB-453) incubated with increasing concentrations of RT and KS20226 for 48hr. (D) Clonogenic survival assay in MDA-MB-468 and MDA-MB-453cells treated with IR alone (4Gy), KS20226 alone (0.5uM), and IR + KS10076 (4Gy and 0.5 μM). Data are presented as mean ± SD; n = 3. P values were determined using one-way ANOVA; ns not significant.

6. KS20226 Overcomes the Radiation Resistant of Breast Cancer cells through IRP2 inhibition.

In this part of our study, we investigated the effectiveness of KS20226 in addressing radiation resistance (RR) in breast cancer (BC) cells. We developed radiation-resistant BC cell lines and compared the IRP2 expression response to irradiation in both parental and RR cells. To confirm the establishment of resistance, we performed colony formation assays on parental and RR MCF7 cells post-radiation treatment (Fig 5A) and assessed cell viability (Fig 5B). Consistent with our expectations, IRP1, IR2, and TFR exhibited a reverse response to radiation. In parental MCF-7 cells treated with 6 Gy of radiation, IRP1, IRP2, and TFR were downregulated in a time-dependent manner, aligning with our previous findings (Fig 1C). Conversely, their expression was significantly upregulated in MCF-7-RR cells (Fig 5C), underscoring the role of IRP2 as a key factor in inducing RR.

Further investigations revealed that KS20226 could effectively suppress the increased IRP2 levels in RR cells. We observed that KS20226 substantially downregulated IRP2, TFR, RAD51, and XIAP, while upregulating γ-H2AX and LC3B (Fig 5D). A cell viability assay conducted on MCF7-RR cells allowed us to calculate the IC50 of KS20226 (Fig 5E). In addition, KS20226 induced G2/M arrest of MCF7-RR in a dose-dependent manner (Fig 5F), and it is also consistent with our previous results (Fig 2).

These finding indicate that KS20226 not only induces iron deficiency in RR cells but also holds significant potential in overcoming radiation resistance in breast cancer treatment.



Breast cancer cell lines	IC50(μM)
MCF7	12.02
MCF7-RR	23.48

Figure 5. KS20226 significantly inhibits the cell viability of radiation resistant cells.
(A) Colony formation of parental and radiation resistant MCF-7 cells in dose range of 0 to 8Gy irradiation. (B) CCK-8 assay of parental and radiation resistant MCF-7 cells in dose range of 0 to 8Gy irradiation of 48hr. (C) Western blot analysis of parental and radiation resistant MCF-7 cells treated with 6Gy of irradiation. (D) Protein level of parental and MCF7-RR after treatment of KS20226. (E) Cell viability of MCF7 and MCF7-RR cells about KS20226. (F) Cell cycle analysis on MCF7-RR treated with KS20226 48hr in dose-dependent manner.

IV. DISCUSSION

In this study, building upon previous research indicating the effectiveness of regulating iron homeostasis related with radiation therapy in breast cancer treatment.

First, we assessed the respective radiation sensitivity of BC cells. RT is employed as a treatment for BC, helping control cancers by either destroying cancer cells or inhibiting their growth. However, some cancers initially respond to radiation but may later develop radiation resistance [10]. RR in BC may cause from diverse molecular biological mechanisms within cancer cells. Mechanisms involving DNA damage and repair, cell cycle regulation, and cell death processes are known to be associated with RR [11]. And there are various studies about relationship between mitochondrial function and radiation sensitivity [12,13,14]. Because iron homeostasis was well known as a crucial point of cell metabolic pathway, including mitochondrial function, our study is focused on sensitizing RT and overcoming RR through targeting IRP2 protein [15]. Combining inhibition of IRP2 with RT is considered a strategy to overcome RR and enhance treatment effectiveness [16].

To investigate the relationship between IRP2 levels and radiation sensitivity on BC cells, we classified four BC cells into two groups based on their radiation sensitivity [17]. Relatively radiation resistant (RR) group (MDA-MB-468, MDA-MB-453) and relatively radiation sensitive (RS) group (SK-BR-3, MCF7) were classified. Then we observed the IRP2 protein levels treated with irradiation on two groups. Interestingly, the protein level of IRP2, IRP1 and TFR, a key protein of iron homeostasis, was increased in only RR group in dose dependent manners while RS group showed decrease [15]. Furthermore, knockdown of IRP2 downregulated RAD51 and effectively inhibited BC cell growth treated with radiation. In this regard, we hypothesized that IRP2 was directly related with radiation, and could be a key marker as a radio-sensitizer on cancer treatment [18, 19].

Next, the cancer cell viability inhibitory efficacy of the IRP2 inhibitor KS20226 was estimated in various BC cells. We demonstrated that KS20226 caused mitochondrial

dysfunction via inducing the inhibition of DNA repair (RAD51, BRCA1, BRCA2) and G2/M arrest through the pathway analysis and various in vitro experiments [20].

Through the combination treatment of KS20226 with radiation, we observed that KS20226 could inhibit the increase of IRP2 protein in RR group. As we purposed, KS20226 effectively induced mitochondrial dysfunction, G2/M arrest, and inhibited the DNA damage response in the RR group (MDA-MB-468, MDA-MB-453) treated with RT. Moreover, when KS20226 and RT were administered in combination, they exhibited significantly better cell growth inhibition compared to each monotherapy [21].

Our continuing study demonstrated that KS20226 not only serves as a radiation sensitizer but also has the potential to overcome radiation resistance. We confirmed that KS20226 effectively worked on MCF7-RR cells which was established by MCF7, included in RS group (Fig 1). We observed that inhibition of cell viability, DDR and induced cell cycle arrest on MCF7-RR cells treated with KS20226.

The ability that we demonstrated on our study of KS20226 about DDR, cell cycle, and mitochondrial dysfunction on BC cells was revealed clear. And theses mechanisms were well known as a cause inducing radiation resistance. In addition, our future research will focus on elucidating whether the cell cycle arrest and DNA repair inhibitory effect of KS20226 if KS20226 demonstrates effective cell growth inhibition in various radiation-resistant cells in vitro and in vivo experiments [22]. Finally, we propose the role of KS20226 as a strong anti-cancer drug for overcoming radiation resistance.

V. CONCLUSION

In conclusion, Inhibition of IRP2 improves the radiation sensitivity of radio-resistance breast cancer cells by causing mitochondrial dysfunction via inducing cell cycle arrest and inhibition of DNA damage repair. The decrease in RAD51 delays DDR after RT, and prologued the damage on RR cells. Thereby impeding the recovery from radiation-induced damage and ultimately increasing radiation sensitivity. Our study demonstrated that IRP2 inhibitor KS20226 effectively regulated mechanisms related with radiation resistance. KS20226 downregulated intracellular iron levels through inhibition of IRP2, TFR on BC cells. Therefore, mitochondrial dysfunction was occurred via inducing G2/M cell cycle arrest, DNA damage repair and DNA synthesis inhibition (Fig 6). Finally exhibited a synergistic effect of KS20226 on combination treatment with radiation on BC cells, therefore we suggested its clinical availability as a radio-sensitizer. Based on the ability on inhibition of radio-resistance mechanisms, the combination of KS20226 with radiation therapy may prove to be a potential novel therapeutic strategy in the treatment of breast cancer.

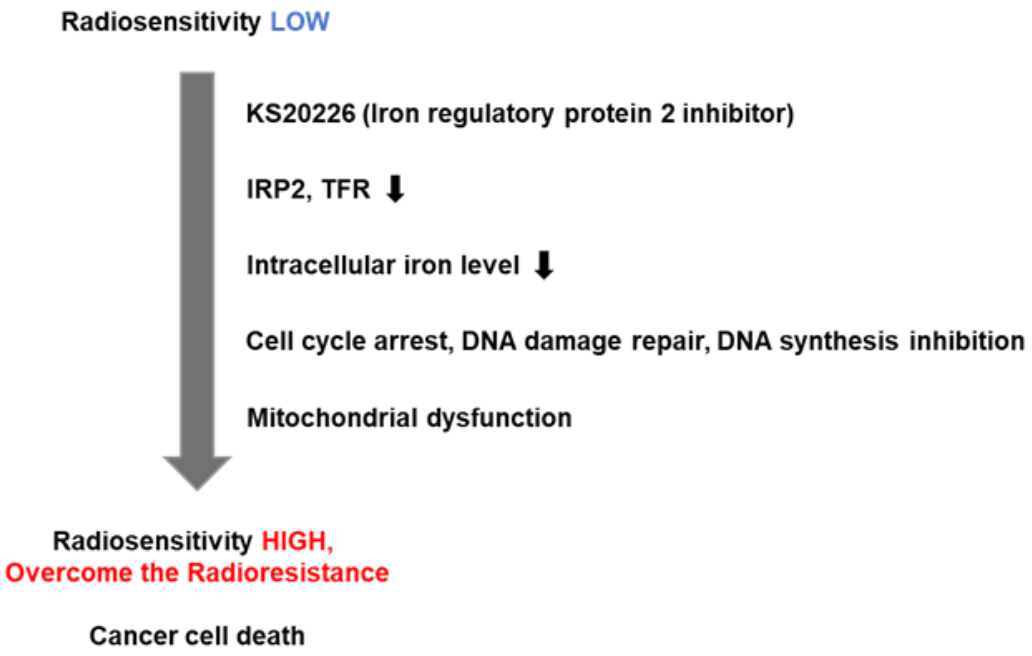


Fig 6. The main flow about KS20226 as a radio-sensitizer.

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ABSTRACT (IN KOREAN)

유방암에서 iron regulatory protein 2 억제를 통한 DDR 조절과 방사선 민감도 강화

<지도교수 신상준>

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정예영

방사선 치료(RT)는 다양한 종류의 종양에 대한 표준 치료 방법이지만, 방사선 감수성의 향상과 방사선 저항성 극복을 위한 다양한 연구가 여전히 진행되고 있다. 철은 세포 대사, 특히 DNA 손상 응답 (DDR)을 포함한 주요 치료 내성 메커니즘의 필수 요소로 잘 알려져 있으며, iron homeostasis를 조절하는 핵심 요소인 iron regulatory protein 2 (IRP2)의 결핍에 대한 항암 효과가 입증되었다.

본 연구에서는 먼저 IRP2 발현과 방사선 간의 상관관계를 규명하였다. 방사선에 민감한(RS) 그룹과 상대적으로 방사선에 덜 민감한 (RR) 그룹의 유방암(BC) 세포에서 IRP2는 방사선 조사에 대해 대조적인 반응을 보였다. IRP2, IRP1 및 TFR의 발현은 RR 그룹에서만 상승하였기 때문에 IRP2의 상승은 방사선에 대한 '저항성' 반응을 유발한다고 가정했다. 따라서 IRP2 억제제와 RT의 병용 치료를 방사선 감수성을 증가시킬 수 있는 효과적인 방법으로 제시하였다.

우리는 이전의 연구에서 최초의 IRP2 억제제인 KS20073과 이의 항암 효과를 입증한 바가 있다. 나아가 이번 연구에서 KS20073의 modified compound인 KS20226이 BC 세포에서 항암 효과를 나타내고, 'radiation sensitizer'로의 잠재적 역할을 할 수 있다는 가능성을 보여주었다. KS20226로 처리된 BC 세포의 RNA-sequencing 분석을 통해 KS20226이 DNA repair 및 G2/M checkpoint 경로를 억제한다는 것을 확인했다. BC 세포와 RT에 관한 이전

연구들을 고려하면 DDR 및 세포주기 정지가 방사선 저항성 발생으로 이어지는 핵심 메커니즘으로 작용할 수 있으며, KS20226이 BC의 방사선 감수성을 증진시키고 방사선 저항성을 극복할 수 있는 치료제로서 사용될 수 있다. 따라서 KS20226와 RT의 병용 치료를 BC 세포에서 방사선 감수성을 증가시키고, 방사선 저항성을 극복하는 잠재적인 치료 전략으로서의 가능성으로 제시한다.

핵심되는 말: 방사선, IRP2, 철 대사, DDR, 방사선 저항성, 미토콘드리아 기능 장애, 유방암