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c-Met inhibitor SU11274 enhances the response of the prostate cancer cell line DU145 to ionizing radiation

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ABSTRACT

Hormone-refractory prostate cancer shows substantial resistance to most conventional therapies including radiotherapy, constitutes a key impediment to curing patients with the disease. c-Met overexpression plays a key role in prostate cancer tumorigenesis and disease progression. Here, we demonstrate that c-Met inhibition by SU11274 could significantly suppress cell survival and proliferation as well as enhance the radiosensitivity of DU145 cells. The underlying mechanisms of the effects of SU11274 on DU145 cells may include the inhibition of c-Met signaling, depolarization of the mitochondrial membrane potential, impairment of DNA repair function, abrogation of cell cycle arrest, and enhancement of cell death. Our study is the first to show the effectiveness of combining c-Met inhibition with ionizing radiation to cure hormone-refractory prostate cancer.

1. Introduction

Prostate cancer is the most frequently diagnosed malignancy in adult men from western countries such as the United States and certain countries in Europe; the incidence in Asian countries has also been increasing in the past decades [1]. Radiotherapy (RT) is an important modality for treating prostate cancer, serving as either a primary radical treatment or an adjuvant therapy after radical prostatectomy or chemical castration regimen. The effectiveness of RT has been well established in the past decades [2,3]; however, when primary prostate cancer proceeds to the hormone-refractory prostate cancer (HRPC) stage, it shows substantial resistance to most conventional therapies including RT [4]. Thus, the radioresistance of HRPC constitutes an important impediment to RT in curing patients of prostate cancer.

Although the precise molecular mechanisms underlying the transformation of prostate cancer from the primary androgen-sensitive to the androgen-insensitive status along with the gain of radioresistance remain largely unknown, lines of evidence have shown that signaling by the receptor tyrosine kinase (RTK) c-Met played a key role in it. Firstly, an inverse correlation between the expression of androgen receptor (AR) and c-Met has been observed in prostate epithelium and prostate cancer cells [5,6]. Secondly, AR signaling suppressed c-Met transcription, while the removal of androgen increased c-Met expression [7]. Thirdly, it is observed that c-Met expression is high in late stage and bone metastatic prostate cancer [5]. Further more, a recent study has demonstrated that c-Met expression has a close relationship with the cellular radiosensitivity [8]. Based on the above observations, we hypothesized that c-Met signaling regulates the radiosensitivity of prostate cancer cells and that the inhibition of c-Met signaling could reverse the radioresistance of HRPC cells.

In this study, we demonstrate the effectiveness of c-Met pathway inhibition using a selective small molecule c-Met inhibitor, SU11274, in radiosensitizing DU145, which is a frequently used androgen-insensitive prostate cancer cell line with high level of c-Met expression [5–7]. Furthermore, the underlying mechanisms of the therapeutic effects of SU11274 on DU145 cells were also studied. To the best of our knowledge, this is the first study focused on using the combination of c-Met pathway inhibition with IR in treating HRPC cells.

2. Materials and methods

2.1. Cell lines and cell culture

The DU145 prostate carcinoma cell line was kindly provided by the Urinary Surgery Department of the First Affiliated Hospital of Peking University. Cells were maintained in RPMI 1640 medium (M&C Gene Technology, Beijing, China) supplemented with 10% fetal bovine serum (FBS, Gibco, Auckland, New Zealand), at 37 °C in humidified air containing 5% carbon dioxide. SU11274 (Calbiochem, San Diego, CA, USA)-treated DU145 cells were

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obtained by culturing the cells with various concentrations of SU11274 for 24 h. Based on our results from Western blotting and cell growth assays, treatment with 5 μM of SU11274 resulted in a significant inhibition of c-Met phosphorylation and complete inhibition of the long-term survival of DU145 cells. The concentration of 5 μM was then selected to investigate the mechanisms underlying the radiosensitization effect of SU11274 in subsequent studies.

2.2. Cell survival and proliferation assay

Cell proliferation was assessed using the Alamar Blue (Invitrogen, Carlsbad, CA, USA) assay, according to the manufacturer's instructions. To evaluate the effect of SU11274 on long-term cell survival, cells were plated at a density of 200 cells/well in 6-well plates and cultured to quiescence for 14 days in medium containing 0.1% DMSO (control) or 1 μM, 2 μM, 5 μM SU11274. The resulting cell colonies were fixed with methanol, stained with crystal violet, and photographed.

2.3. Ionizing radiation treatment

Irradiations were performed by a medical linear accelerator (Varian Clinic 23EX, Varian Medical Systems, USA) using 6 MV photons with an absorption dose rate of 4 Gy/min. All IR treatments were performed in the Radiotherapy Department of Peking University First Hospital.

2.4. Clonogenic assay

Radiobiological survival curves were obtained by pretreating DU145 cells with 0.1% DMSO or 5 μM SU11274 medium for 24 h. Cells were then exposed to different dosages of radiation (0, 2, 4, 6 and 8 Gy), seeded in 6-well plates, and grown to quiescence for 14 days. At the end of 14 days, the cells were fixed with methanol and stained with crystal violet, and colonies containing more than 50 cells were counted.

2.5. Western blot analysis

Protein expression and phosphorylation in DU145 cells treated with either DMSO (control) or SU11274 were evaluated using Western blotting. Briefly, DU145 cells were seeded in 6-well plates and cultured to 50% confluence. The cells were then treated with medium containing 0.1% DMSO or 2, 4, 6 and 8 μM of SU11274 and incubated for an additional 24 h. Following the indicated treatments, cell protein extracts were prepared. Western blots were performed with 100 μg of protein extract, as described elsewhere.

2.6. Mitochondrial depolarization assay

DU145 cells were seeded in 35 mm^2 plastic dishes and cultured to 60% confluence. The cells were then treated for 30 min with 10 μg/mL JC-1 probe (Beyotime Biotechnology, Jiangsu, China), a cationic dye that exists as green-fluorescent monomers at low membrane potential or red-fluorescent "J-aggregates" at greater concentrations associated with greater membrane potentials. Immediately after loading, the cells were washed with ice-cold PBS three times and visualized by fluorescence microscopy (IX81, Olympus, Japan).

2.7. Immunofluorescence assay

DU145 cells were plated onto 35 mm^2 plastic dishes, pretreated with 0.1% DMSO or 5 μM SU11274 for 24 h, and then exposed to 4 Gy of IR. Cells were collected at 1 and 24 h after IR treatment, fixed in 4% paraformaldehyde (Sigma–Aldrich, St Louis, USA) for 10 min at room temperature and permeabilized in 0.1% Triton X-100 (Sigma–Aldrich, St Louis, USA) for 5 min. Thereafter, cells were blocked with 5% bovine serum albumin (BSA, Beyotime Biotechnology, Jiangsu, China) for 3 h and then incubated with a primary antibody against γH2AX (Ser139, Cell Signaling Technology, MA, USA) overnight at 4 °C. The cells were rinsed three times with PBS for 5 min per wash before incubation with an Alexa Fluor 488-conjugated secondary antibody (Cell Signaling Technology, MA, USA) for 1 h. The cells were then exposed to 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI, Beyotime Biotechnology, Jiangsu, China) for nuclear DNA staining, rinsed three times with PBS for 5 min per wash, and then visualized using confocal fluorescence microscopy (Leica Microsystems, Germany).

2.8. Cell cycle analysis by flow cytometry (FCM)

Cells from DMSO-treated control and 5 μM SU11274-pretreated groups were exposed to 4 Gy of IR and then harvested at 6, 12 and 24 h after irradiation. Harvested cells were fixed in 75% v/v ice-cold ethanol overnight, stained with propidium iodide, then counted by FCM. The results were analyzed using ModFit software (Verity Software House, Topsham, MN).

2.9. Cell death detection by DAPI nuclear staining

DU145 cells were plated in 35 mm^2 plastic dishes and cultured to 50% confluence. The cells were then incubated in medium containing 5 μM SU11274 for 24 h, exposed to 4 Gy IR, or underwent both treatments. After the indicated treatment, cells were cultured for another 48 h in complete medium. The cells were then fixed with 4% paraformaldehyde for 10 min at room temperature and subsequently stained with 1 μg/mL DAPI for 10 min. The cells then were rinsed three times with PBS and visualized by fluorescence microscopy (IX81, Olympus, Japan). Cells with abnormal nuclear morphologic changes such as fragmentation, chromatin condensation and micronucleation [9] were considered dead and counted.

2.10. Statistical analysis

Numerical results are presented as the mean ± S.D. The *t*-test was performed to compare the means of different groups using Prism 5.0 software (GraphPad Prism). Factorial design variance analysis was performed to identify the significance of the synergistic effect of SU11274 and IR using SPSS 19.0 software (IBM SPSS Statistics). For all statistical analyses, a p value less than 0.05 was considered statistically significant.

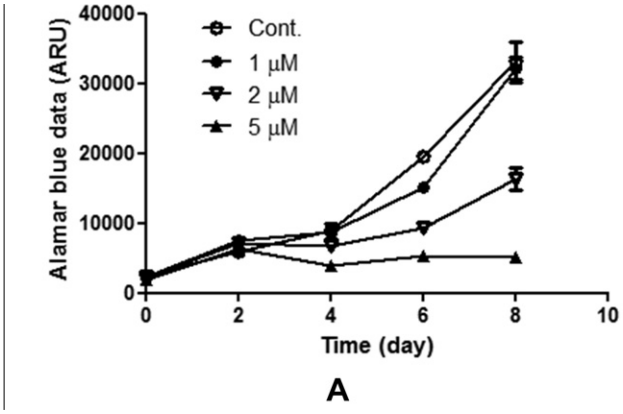
3. Results

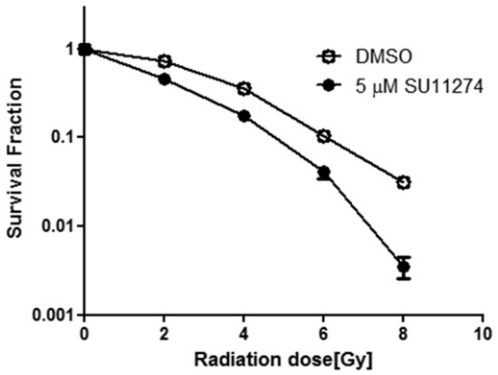
3.1. SU11274 suppresses survival and proliferation of DU145 cells

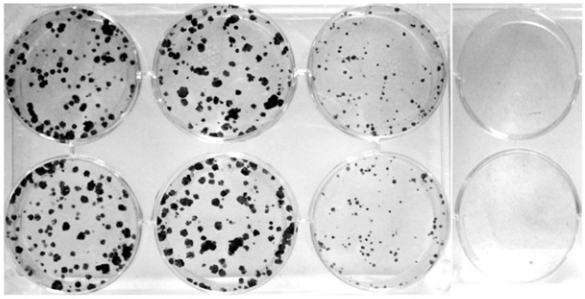
Our results show that SU11274 can suppress the proliferation of DU145 cells in a dose-dependent manner. As shown in Fig. 1A, an increase in the dose of SU11274 from 1 μM to 5 μM suppressed cell proliferation gradually. The 5 μM dose resulted in a complete inhibition of cell proliferation. Similarly, based on the result of clonogenic formation assay, a dose-dependent inhibition of cell long-term survival was also observed on treating DU145 cells with increasing doses of SU11274, as observed in Fig. 1B.

3.2. Targeting the c-Met pathway with SU11274 radiosensitizes DU145 cells

To assess the effect of c-Met pathway inhibition by SU11274 on the radiosensitization of DU145 cells, the clonogenic assay was







SU11274 Cont. 1 μM 2 μM 5 μM

B

	D_0 (Gy)	D_q (Gy)	N
DMSO group	1.93	2.12	3.00
5μM SU11274 group	1.78	0.81	1.58

C

Fig. 1. The effects of c-Met inhibitor SU11274 on cell proliferation, survival, and radiosensitivity of DU145 prostate cancer cells. (A) SU11274 could suppress DU145 cell proliferation in a dose-dependent manner. (B) The inhibitory effect of SU11274 on cell survival was determined by clonogenic assay. The number of colonies formed in the wells decreased as the SU11274 concentration increased. (C) Dose-survival curve derived from the results of clonogenic assay; the radiobiological parameters D_0, D_q and N are also listed.

performed. The dose-survival curves are shown in Fig. 1C. The surviving fraction (SF) was calculated using the following formula: SF = number of colonies formed/(number of cells seeded × plating efficiency of the control group), where plating efficiency was calculated as the ratio of colonies observed and the number of cells plated in the 0 Gy group. Dose–survival curves were plotted on a log-linear scale. The SF data were fitted into the single hit multi-target model formula:

$$SF = 1 - (1 - e^{-D/D_0})^N.$$

The "quasi-threshold dose" or D_q, which is a parameter to measure the width of shoulder of the survival curve, was also calculated. As interpreted elsewhere, high D_0, D_q and N values indicate increased radioresistance of cells. From our experiments, the parameters D_0, D_q and N in the DMSO-treated control group were 1.93, 2.12 and 3.00, respectively, while in the SU11274-pretreated group, the parameters were 1.78, 0.81 and 1.58, respectively. Thus, pretreating DU145 cells with SU11274 resulted in a significant radiosensitization of the cells.

3.3. The inhibitory effect of SU11274 on c-Met phosphorylation and its downstream signaling pathway

SU11274 is a highly specific inhibitor of c-Met. We studied its inhibitory effects on c-Met phosphorylation and signaling downstream of c-Met in DU145 cells. Because the Ras–MAPK and PI3K–AKT pathways are the major downstream mediators of c-Met signaling and are tightly associated with cell viability and proliferation [10,11], we examined the phosphorylation status of c-Met, MAPK and AKT by Western blotting. As shown in Fig. 2A, treatment with SU11274 for 24 h resulted in a dose-dependent suppression of the phosphorylation of c-Met, MAPK and AKT. Our data confirmed that SU11274 could efficiently abrogate c-Met phosphorylation and subsequent downstream signaling.

3.4. c-Met inhibition by SU11274 causes a significant disruption of the mitochondrial transmembrane potential in DU145 cells

The cellular mitochondrial membrane potential of cultures in the 5 μM SU11274-pretreated group and the DMSO-treated control group was monitored by fluorimetry using the cationic probe JC-1 [12]. The fluorescence microscopic images of JC-1 staining are presented in Fig. 2B. As shown in the DMSO-treated control group, orange JC-1 fluorescence indicates fully polarized mitochondria, whereas in the 5 μM SU11274-pretreated group, diffuse green fluorescence is indicative of significantly depolarized mitochondria.

3.5. c-Met inhibition results in a suppression of DNA damage repair ability and DNA damage accumulation following IR

To assess the effect of SU11274 on DNA repair function in DU145 cells, we measured the expression of γH2AX foci, which is considered a sensitive molecular marker of DNA damage and repair [13]; the γH2AX foci formation was measured in the DMSO-treated group and in the 5 μM SU11274-pretreated group before IR and 1 or 24 h after 4 Gy of IR. As shown in the immunofluorescence micrographs of Fig. 3A, at 24 h after IR, the number of γH2AX foci that formed in cellular nuclei in the

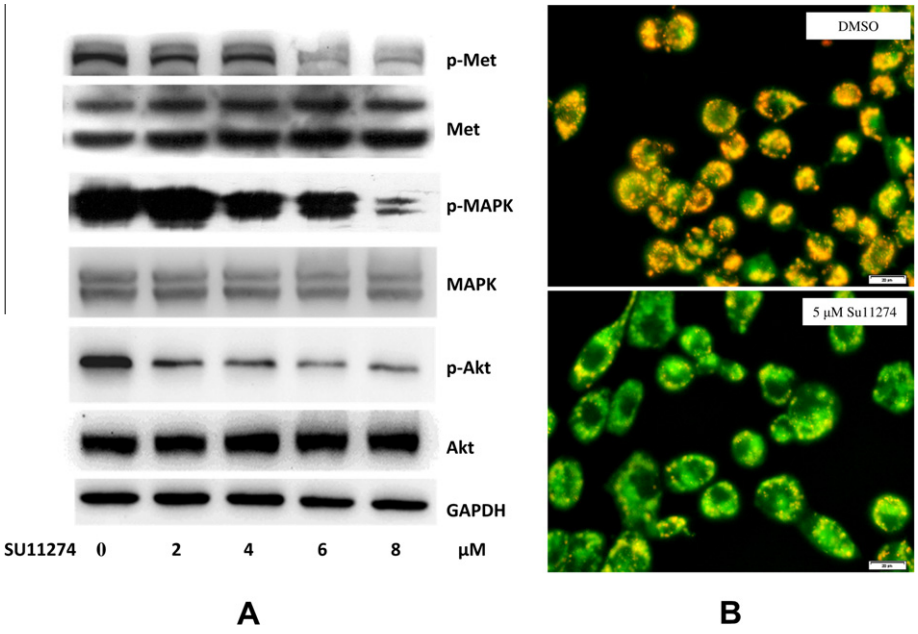


Fig. 2. SU11274 inhibits phosphorylation of c-Met and its key downstream pathway components as well as disrupts mitochondrial membrane potential in DU145 cells. (A) Western blotting results show that SU11274 inhibits the phosphorylation of c-Met and its key downstream pathway components MAPK and AKT. (B) SU11274 treatment significantly disrupts mitochondrial transmembrane potential in DU145 cells; orange JC-1 fluorescence indicates fully polarized mitochondrial membrane potential in the DMSO-treated control group, whereas diffuse green fluorescence indicates depolarized mitochondrial membrane potential in the SU11274-treated group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

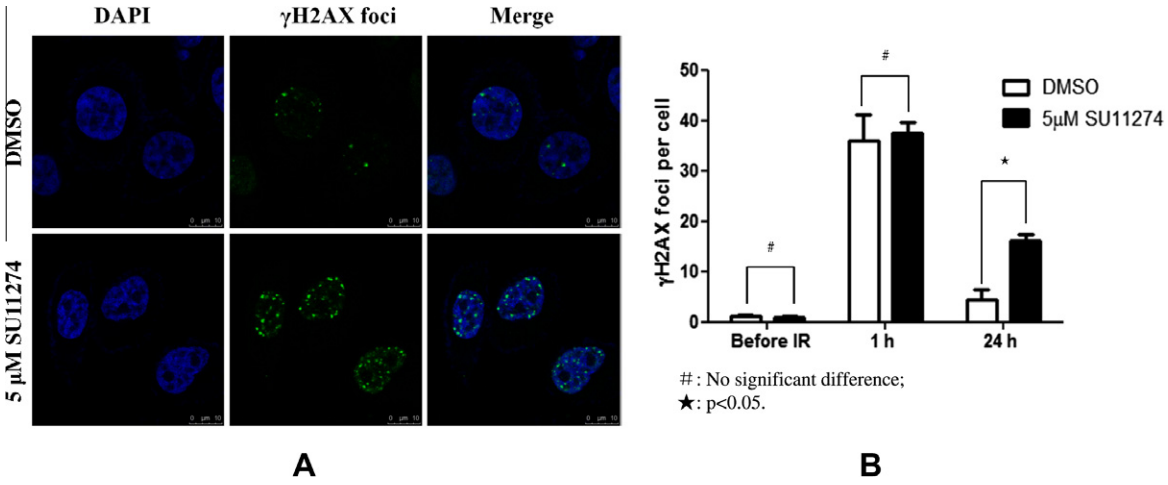


Fig. 3. The effect of c-Met inhibition by SU11274 on DNA damage repair function in DU145 cells. (A) Representative immunofluorescence micrographs of γH2AX foci formation in SU11274- and DMSO-treated DU145 cells at 24 h after 4 Gy of irradiation. (B) Counting the number of γH2AX foci observed in micrographs at the indicated time points demonstrated a significantly prolonged expression of γH2AX foci in SU11274-treated DU145 cells after irradiation.

SU11274-pretreated group were significantly more than those in the DMSO-treated control group. The dynamic changes of γH2AX foci expression in the DMSO-treated control and SU11274-pretreated group are shown in Fig 3B. Our experiments showed a significantly prolonged expression of γH2AX foci at 24 h after IR. The mean number of γH2AX foci per cell in the SU11274 treated group was significantly greater ($p < 0.05$) than the mean number of foci in the DMSO-treated control group 24 h after IR.

3.6. SU11274 abrogates IR induced G2/M cell cycle arrest and synergistically enhances IR induced cell death

We examined the cell cycle distribution of DU145 cells cultured with DMSO (control) or with 5 μM SU11274 before IR and at 6, 12 and 24 h after 4 Gy of IR. As shown in Fig. 4A, compared to the DMSO-treated control group, treatment of DU145 cells with 5 μM SU11274 for 24 h increased the percentage of cells in the G1 phase (56.8–62.7%), decreased the percentage in S phase (28.4–21.1%), and did not significantly affect the percentage in G2/M phase (14.8–16.2%). The cell cycle regulatory effect of SU11274 was more significantly manifested after the exposure of cells to 4 Gy IR. As shown in Fig. 4B, a statistically significant suppression of G2/M arrest was observed at 12 and 24 h after treatment with IR. Especially, at 24 h after IR, 47.6% of cells were arrested in the G2/M phase in the DMSO-treated group, however, treatment of the cells with 5 μM SU11274 suppressed the effect of IR on G2/M arrest, with only 30.4% of the SU11274-treated cells arresting in G2/M. Thus, based on the above observations, we can

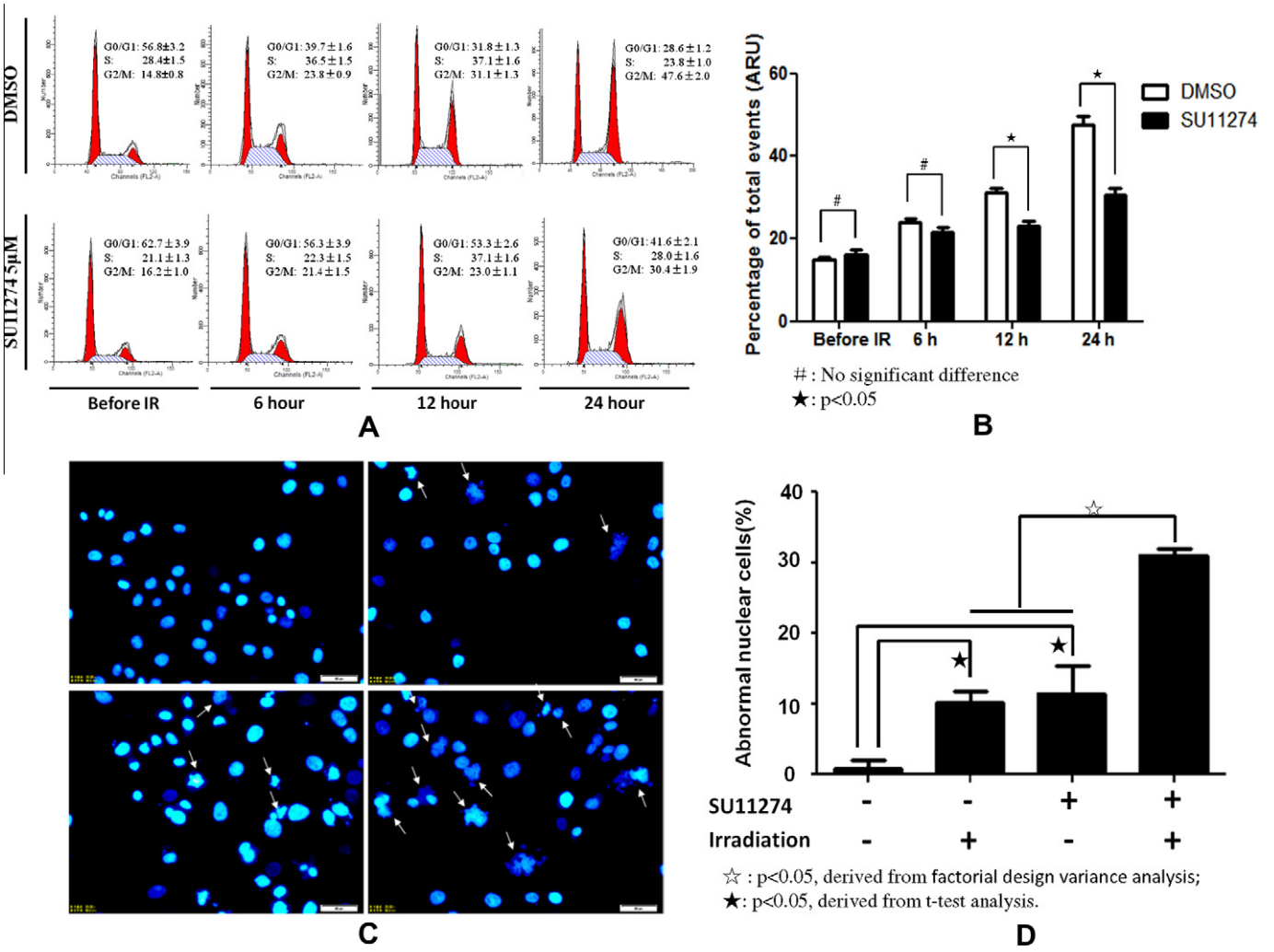


Fig. 4. Effect of SU11274 and irradiation on cell cycle arrest and cell death of DU145 cells. (A) The cell cycle distribution of DU145 cells treated with 5 μM SU11274 or DMSO (control) before and after 4 Gy of irradiation was determined. (B) In the SU11274-treated group, a sharp decrease in the fraction of cells in the G2/M phase compared to the DMSO-treated control group was observed; statistically significant differences were observed at 12 h and 24 h time points. (C) Micrographs of DAPI nuclear staining of DU145 cells in the DMSO-treated control group (upper left), 5 μM SU11274-treated group (lower left), 4 Gy IR-treated group (upper right) and the group treated with both SU11274 and ionizing radiation (lower right). The arrowheads indicate cells with abnormal nuclei. (D) A significant synergistic effect on cell death was observed in the SU11274 and irradiation combination treatment group; the rate of cell death in the combination treatment group was significantly greater than that in the two individual groups added together.

conclude that the pretreatment of DU145 cells with SU11274 could significantly abrogate the IR-induced G2/M arrest.

As shown in Fig. 4C, dead cells were detected using DAPI staining, which allows the identification of cell death via nuclear morphological changes. Fig. 4D shows the quantitation of the observed cell death. As shown, cell death was rarely observed in the untreated DU145 cells, while in cells incubated with 5 μM SU11274 alone, cell death was observed in 11.4 ± 2.3% of the cells. In cells treated with 4 Gy IR alone, death was observed in 10.1 ± 0.9% of the cells; however, a statistically significant synergistic effect ($p < 0.05$) was observed in the cells treated with the combination of 5 μM SU11274 and 4 Gy IR, as cell death rates reached 31.0 ± 0.5%, which was greater than the two individual groups combined.

4. Discussion

The studies of RTKs in solid tumors have achieved huge success through molecular targeted therapies [14]. c-Met, a unique member of the RTK family, has gained increasing interest in recent years for its universal involvement in tumor cell survival, growth, metastasis and multi-treatment resistance [8,10]. Recently, a number of c-Met targeted inhibitors have been developed, and dozens of clinical trials evaluating the efficiency of the inhibitors for treating solid tumor are ongoing [10]. Of these, a Phase II clinical trial evaluating the c-Met inhibition strategy in the treatment of advanced HRPC showed promising results, with a 68% disease control rate and 21 weeks of progression-free survival versus only 6 weeks for the placebo group [15]. However, there is no data available for trials evaluating the efficacy of combining c-Met inhibition with radiotherapy for HRPC treatment. Our preliminary results, based on *in vitro* studies, showed for the first time that the combination of c-Met inhibition and IR is effective in treating the refractory prostate disease.

In the present study, we showed that SU11274 inhibits DU145 cell survival and growth in a dose-dependent manner. Importantly, IR in combination with SU11274 produced a significant synergistic effect on inhibiting the survival of DU145 prostate cancer cells, indicating the significant radiosensitization effect of SU11274 on DU145 cells.

SU11274 augments the response of DU145 cells to IR by multiple mechanisms. These may involve inhibition of survival signaling pathways downstream of c-Met, depolarization of the mitochondrial membrane potential, impairment of cellular DNA repair ability, regulation of cell cycle arrest, and increases in radiation-induced cell death.

The ability to repair DNA is vital for cellular radiosensitvity; greater radioresistance appears to be correlated with more efficient repair of DNA damage. Therefore, targeting DNA repair pathways emerges as an attractive path to enhance tumor cell radiosensitivity [16]. Our data indicate that the radiosensitization effect of SU11274 on DU145 cells was associated with a prolonged expression of γH2AX foci after IR, suggesting that the cellular DNA repair ability declined upon c-Met inhibition. Although the rationale for c-Met inhibition to impair DNA repair ability has not been well documented, it has been reported that the signaling pathways downstream of RTKs intersect with the DNA repair mechanisms to modulate cellular response to IR. Of these signaling pathways, two pathways in particular, namely, the PI3K/AKT and Ras/Raf/MEK/MAPK pathways, are most commonly studied [17]. Our results show that the selective c-Met inhibitor SU11274 could efficiently inhibit the phosphorylation of AKT and MAPK, which are two key components of the c-Met signaling pathways, and thus, regulate the DNA repair ability in DU145 cells.

Mitochondria are the cell's powerhouse, and they conduct both vital and lethal functions in physiological and pathological conditions of the cell. Mitochondrial function status may also contribute to the regulation of cellular radiosensitivity. Not only do they present the largest target volume after nuclei for IR, but they also participate in adenosine triphosphate (ATP) production, redox regulation, calcium homeostasis and apoptosis [18]. We therefore studied the effect of c-Met inhibition by SU11274 on the mitochondria in DU145 cells. Our results indicate that c-Met inhibition by SU11274 can cause significant depolarization of mitochondrial membrane potential, and this effect may consequently contribute to growth inhibition and radiosensitization in the cells.

It has been well recognized that cell cycle distribution regulates cellular radiosensitivity. Cells are most sensitive to IR during the G2/M phase, less sensitive in G1, and least sensitive during the latter part of S phase [19]. Our results show that addition of 5 μM SU11274 alone can cause a G1 arrest and a decrease in the number of cells in S phase in DU145 cells. This type of cell cycle redistribution can predispose cells to a radiosensitive state. More importantly, after exposure of cells to IR, G2/M arrest is an important protective mechanism that allows cells time to repair damaged DNA, maintain genetic stability and avoid delivering mutations to their daughter cells. Agents that abrogate the G2/M checkpoint after IR lead to cell radiosensitization [19,20]. This finding is consistent with our results; upon treatment with 5 μM SU11274, a significant abrogation of G2/M arrest and radiosensitization effect was observed after exposure of DU145 cells to IR. Thus, abrogation of cell cycle arrest may serve as an important mechanism for SU11274-mediated radiosensitization in DU145 cells.

Exposure to IR results in cells death through a variety of mechanisms. Accumulating evidence suggests that multiple cell death modalities, including apoptosis, mitotic catastrophe, necrosis, autophagy, and cell senescence, contribute to the tumor cell eradication effect of IR [21–24]. The dominant form of cell death after IR depends on the cell type, the environmental context, and radiation quality. Apoptosis is the major cell death modality observed in response to IR in cells naturally prone to apoptosis such as hematopoietic cells and their malignant counterparts; conversely, for most solid cancers, especially those of epithelial origin, mitotic catastrophe is the primary mode of cell death following radiotherapy [21,23]. Of these cell death modalities, abnormal morphological changes in the nucleus are commonly observed characteristics [9]. Thus, nuclear DAPI staining was an ideal method to identify radiation induced cell death. Our results based on nuclear DAPI staining show a significant synergistic effect of c-Met inhibition by SU11274 in combination with IR in inducing DU145 cell death.

Of note, the significant inhibitory effect of SU11274 on cell survival and growth was found to be confined to those cells with high levels of c-Met expression and activation; in cells that had low c-Met expression or did not express the protein at all, SU11274 had only a modest effect if any on cell proliferation at the doses used [25–28]. Therefore, it is reasonable to speculate that these beneficial effects of c-Met inhibition in treating malignancies are cancer-type specific and c-Met expression status specific. Moreover, our results are based on *in vitro* studies, and the biological effect of c-Met inhibition *in vivo* may be even more complicated. Further studies are required to investigate the beneficial effect of targeting c-Met in HRPC radiotherapy treatment.

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