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Inhibition of checkpoint kinase 1 sensitizes lung cancer brain metastases to radiotherapy

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ABSTRACT

The most important therapeutic tool in brain metastasis is radiation therapy. However, resistance to radiation is a possible cause of recurrence or treatment failure. Recently, signal pathways about DNA damage checkpoints after irradiation have been noticed. We investigated the radiosensitivity can be enhanced with treatment of Chk1 inhibitor, AZD7762 in lung cancer cell lines and xenograft models of lung cancer brain metastasis. Clonogenic survival assays showed enhancement of radiosensitivity with AZD7762 after irradiation of various doses. AZD7762 increased ATR/ATM-mediated Chk1 phosphorylation and stabilized Cdc25A, suppressed cyclin A expression in lung cancer cell lines. In xenograft models of lung cancer (PC14PE6) brain metastasis, AZD7762 significantly prolonged the median survival time in response to radiation. Depletion of Chk1 using shRNA also showed an enhancement of sensitivity to radiation in PC14PE6 cells. The results of this study support that Chk1 can be a good target for enhancement of radiosensitivity.

1. Introduction

Almost a half of cancer patients will have metastases either at diagnosis or during disease course. Many cancer patients, especially lung cancer patients harbor multiple brain metastases. In these circumstances, whole-brain radiation therapy (WBRT) has been used in the management of brain metastases [1]. Chemotherapeutic agents have been studied for metastatic brain tumors (MBTs), but the clinical availability and effectiveness are still limited. Therefore, radiation therapy or stereotactic radiosurgery tend to be available tools in the cases of multiple MBTs. Nonetheless, resistance to radiation is the important cause of recurrence or treatment failure. Thus, we have sought to improve therapy for MBTs by combining additional agents with radiation.

DNA damage checkpoints are signal-transduction pathways that delay or arrest cell cycle progression in response to the damage. Originally, checkpoints aid in maintaining genomic integrity and cell survival. However, these mechanisms can be used to escape the DNA injury, resulting in acquiring resistance to irradiation [2]. There are several reports suggesting that the G_2 checkpoint can be exploited to enhance radiosensitivity. The radiosensitivity of Ataxia telangiectasia fibroblasts is related to the lack of G_2 arrest [3]. Caffeine enhances the radiosensitivity of cells primarily through abrogation of the G_2 checkpoint [4].

The checkpoint response to double-stranded breaks (DSBs) is initiated by ATM, whereas the checkpoint response to base adducts and inhibitions of replication are induced by ATR [5]. Although ATM and ATR share overlapping substrates, there is specificity in their signaling to the transducer kinases; ATM uniquely phosphorylates Chk2, while ATR phosphorylates Chk1 [6]. Studies of cells, which are functionally defective in components of DNA damage checkpoint pathways show cell cycle checkpoint defects, and an increased sensitivity to ionizing radiation (IR) and other DNA damage agents [2,7]. This latter observation highlights components of these DNA damage checkpoint pathways as potential therapeutic targets for enhancing the sensitivity of tumor cells to the radiotherapeutic/chemotherapeutic agents [8,9]. Tumor cell specific checkpoint mechanisms for DNA damage in response to IR would be the clue to solve the resistance.

Upon DNA damage, Chk1 is activated by ATM/ATR-mediated phosphorylation and then phosphorylates Cdc25 phosphatases,

Abbreviations: DSB, double-stranded DNA break; Chk1, checkpoint kinase 1; ATM, ataxia-telangiectasia mutated; ATR, ATM and Rad3-related; IR, ionizing radiation; MBT, metastatic brain tumor; shRNA, small hairpin RNA.

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thereby preventing the activation of Cdk1 kinase. Based on data demonstrating that Chk1 is an effective target for sensitization to chemotherapy and radiotherapy [10–12], small molecule Chk1 inhibitors have been developed for clinical use to enhance killing tumor cells by cytotoxic drugs and by radiation [13–15]. One Chk1 inhibitor, CEP3891 has previously been shown to increase cytotoxicity after irradiation *in vitro* [16]. A role for Chk1 in radiation resistance of cancer stem cells has recently been proposed [17]. Recently, a novel Chk1 inhibitor, AZD7762 was shown to enhance the cytotoxicity of DNA-damaging chemotherapy agents by abrogation of the cell cycle arrest [18]. In the present study, we show that the radiosensitivity can be enhanced with the treatment of Chk1 and Chk2 inhibitor, AZD7762 in lung cancer cell lines and brain metastasis xenograft model. Depletion of Chk1 in lung cancer cell (PC14PE6) using shRNA also showed an enhancement of sensitivity to radiation *in vitro*. The current study shows that Chk1 can be a good target for enhancement of radiosensitivity and that AZD7762 is a potent radiation sensitizer.

2. Material and methods

2.1. Cell culture and cell lines

Human lung cancer cell lines H23 (American Type Culture Collection) were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 Ug/ml streptomycin, and PC14PE6 was maintained in Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.2. Cell viability and Cell survival studies

Cell counting kit-8 (CCK-8) (Dojindo Molecular Technology, Gaithersburg, MD, USA) assay was used to determine cellular mitochondrial dehydrogenase activity which reflects initial cell death. Cells were plated in 96-well plates at a density of 0.4×10^4 cells/well and 100 U/ml for every well. After the incubation, cells were treated with 10 μl CCK-8 solution every well for 2 h and the absorbance at 450 nm was measured. Results were expressed as optical density (OD) compared to control cells, indicating the loss of cell growth and viability. To assess the effects of ionizing radiation (IR) on cells, several doses of radiation under ^{137}Cs γ-radiation source were used. After being incubated for 1–3 days, cells were subjected to the CCK-8 assay. For cell survival studies, cells were plated (8×10^5 cells/100 mm culture dishes) and incubated for 16 h at 37 °C. AZD7762 (Axon Medchem, Groningen, Netherlands) was added to the exponentially growing cells 1 h before radiation. Twenty-four hours after radiation and drug treatment, cells were trypsinized, counted, plated and incubated for 10–14 days. Colonies were fixed with methanol/acetic acid (3:1) and stained with 0.1% crystal violet in 50% ethanol. Colonies with over 50 cells were scored and cell survival was determined after correcting for the plating efficiency and for AZD7762 cytotoxicity alone.

2.3. Western blotting

Exponentially growing cells were exposed to radiation without or with AZD7762. After treatment cells were harvested, lysed, and prepared for Western blotting analysis, as previously described [19]. Cells were processed 1 h after IR (10 Gy). Anti-Chk1, anti-Chk2, anti-Cdc25A, anti-Cyclin A, and anti- α-tubulin antibodies were purchased from Santa Cruz Biotechnology. Anti-Chk1 phospho-Ser317 and anti-Chk2 phospho-Thr68 antibodies were obtained from Cell Signaling Technology.

2.4. Stable cell line

For generation of stable cell lines of tetracycline-regulated expression of a short hairpin RNA, PC14PE6 cells were transfected with plenti6/TR vector (Invitrogen) containing the TetR gene for constitutive, high-level expression of the Tet repressor under the control of a CMV promoter and selected under blasticidin. Next, pENTR/H1/TO vector (Invitrogen) containing a short hairpin RNA of interest gene was transfected into TetR-expressing cells and Zeocin-resistant colonies were picked. In the absence of tetracycline, the Tet repressor expressed from pLenti6/TR binds with extremely high affinity to each TetO2 sequence in the promoter of the pENTR/H1/TO construct. Binding of the Tet repressor homodimers to the TetO2 sequences represses transcription of shRNA. Upon addition, tetracycline binds with high affinity to Tet repressor and causes a conformational change in the repressor that renders it unable to bind the Tet operator. The Tet repressor: tetracycline complex then dissociates from the Tet operator and allows induction of transcription of shRNA. The human Chk1 sequence used for construct shRNA was 5′-AAGCGTGCCGTAGACTGTCCAGAAA-3′.

2.5. Orthotopic lung cancer brain metastasis xenograft studies

Male athymic nude mice, 8 weeks of age were used for this study. All experiments were conducted in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and within the protocols approved by the appropriate Institutional Review Boards at the Samsung Medical Center (Seoul, Korea). To produce orthotopic lung cancer brain metastasis models, mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine i.p. PC14PE6 cells (1.0×10^4) in 5 μl HBSS were stereotactically injected into the left striata of mice (coordinates; AP + 1.0, ML + 1.7, DV-3.2 mm from Bregma). To induce inhibition of Chk1, mice were administered with AZD7762, which was formulated in 0.9% saline, by i.v. injection on 2 weeks after tumor cells implantation at a dose of 25 mg/kg. For radiation treatment, mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine i.p. at 1 h after injection of AZD7762. Whole brain irradiation was delivered using a blood irradiator (IBL 437C Blood Irradiator, CIS US, Inc., Bedford, MA) at a dose rate of 2.3 Gy/min and a shielding device for the rest of the body (Lead, Custom-made for the mouse).

3. Results

3.1. Radiosensitization of lung cancer cells by the treatment of Chk1 inhibitor, AZD7762

We assessed radiosensitivity of lung cancer cell lines (PC14PE6 and H23) after treating them with various doses of radiation. The clonogenic survival assays (Fig. 1A) and CCK-8 assays (Fig. 1B) revealed that PC14PE6 cells were the most radioresistant, and that H23 cells were more sensitive to radiation. Functional defect in DNA damage checkpoint pathways showed an increased sensitivity to radiation and other DNA damage agents [2,7]. This observation suggests a possibility that components of these DNA damage checkpoint pathways as potential therapeutic targets for enhancing the radiosensitivity of tumor cells. We assessed radiation survival of lung cancer cell lines (PC14PE6 and H23) after treating them with Chk1 inhibitor, AZD7762 to test the possibility of enhancing the radiosensitivity. The activation of Chk1 by radiation was rapid and persisted for several hours postradiation. To ensure Chk1 inhibition, AZD7762 (30–100 nmol/L final concentration) was added to cells 1 h before radiation and left on for 24 h after radiation. We found that AZD7762 significantly sensitized both

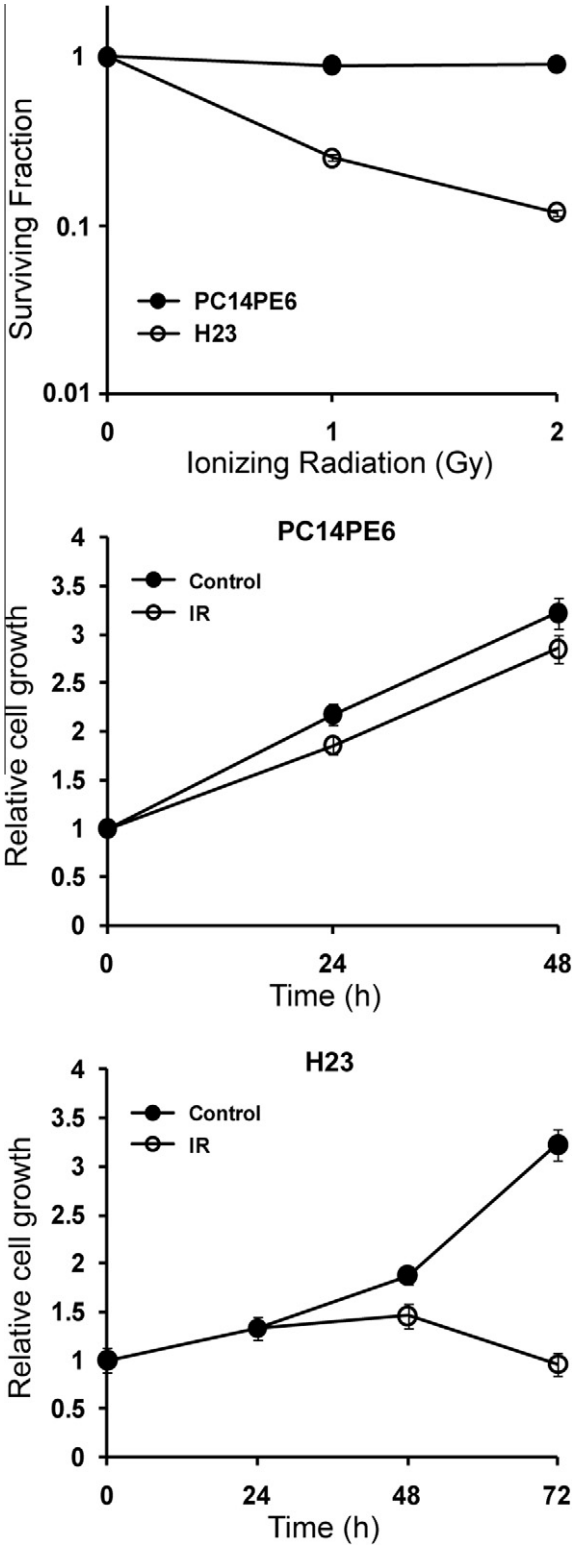


Fig. 1. Radiosensitivity of lung cancer cell lines (PC14PE6 and H23). (A) Clonogenic survival assays were performed with treatment of ionizing radiation (IR) as indicated. (B) CCK-8 assays were performed after treatment of IR (20 Gy). PC14PE6 cells are highly radioresistant and H23 cells are sensitive to IR.

radioresistant (PC14PE6) and radiosensitive (H23) cells to IR (Fig. 2A and B). AZD7762 cytotoxicity was minimal for all cell lines used. These results show that AZD7762 is a good radiosensitizer.

To confirm that AZD7762 inhibits Chk1/2 in our models, we analyzed Chk1 and Chk2 signaling in PC14PE6 and H23 cell lines after IR (Fig 2C). ATM and ATR-mediated phosphorylation of Chk1 (S317) and Chk2 (T68) were increased by the addition of AZD7762 to IR. AZD7762 stabilized Cdc25A and suppressed cyclin A expression. Taken together, these results show that AZD7762 inhibits Chk1/2 in PC14PE6 and H23 cells.

3.2. Chk1 inhibition enhances radiosensitivity in xenograft models

Based on the efficacy of AZD7762 as a radio-sensitizer *in vitro*, we hypothesized that AZD7762 would be an effective radio-sensitizer in lung cancer brain metastasis models. We produced orthotopic lung cancer brain metastasis models with injection of radioresistant PC14PE6 cells into athymic nude mice. We estimated the effects of Chk1 inhibition on the survival of PC14PE6 lung cancer brain metastasis xenograft model in response to IR. Tumor-bearing mice were treated with AZD7762 and/or IR as illustrated (Fig. 3A). As shown in Fig. 3B compared with vehicle control (median survival; 30 days), AZD7762 treatment (median survival; 35 days) had no effect on survival of mice. Radiation treatment (15 Gy) expanded survival rate (median survival; 40 days), significantly ($p = 0.020$, compared with Control). And combination of AZD7762 and IR (median survival; 51 days) further enhanced the survival rate ($p = 0.05$, compared with IR). AZD7762 treatment alone or in combination with radiation showed no toxicity. The addition of AZD7762 with radiation resulted in a significantly prolonged median survival time. These results show that AZD7762 sensitizes to radiation in lung cancer brain metastasis xenograft models.

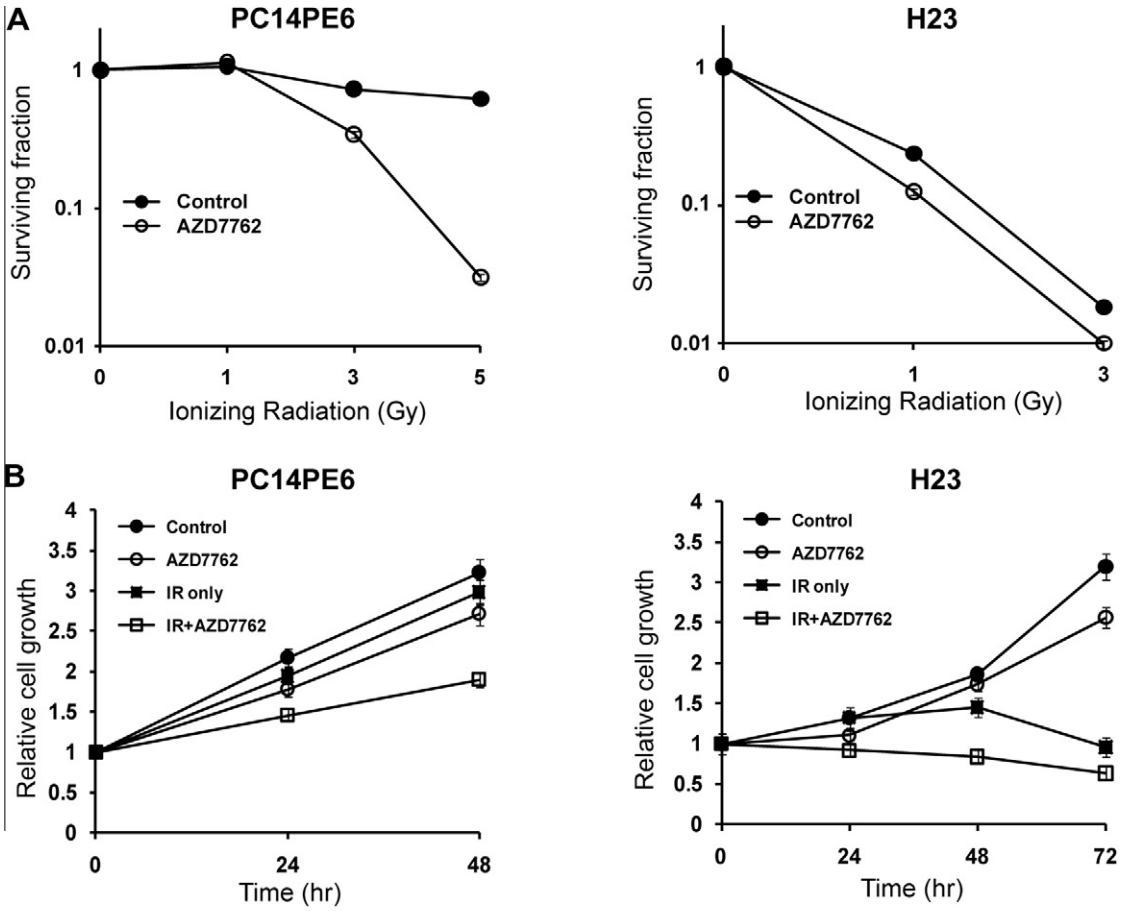
3.3. Depletion of Chk1 enhances radiosensitivity in vitro

To address the relative contribution of inhibition of Chk1 by AZD7762 to radiosensitization, we made inducible Chk1 shRNA expression cell line to selectively deplete Chk1 from PC14PE6 cells. Chk1 expression level in PC14PE6 (shChk1) cells was decreased according with induction of Chk1 shRNA by tetracycline (Fig. 4A). Relative to nonspecific shRNA expression cells, PC14PE6 (shChk1), Chk1-depeleted lung cancer cells were sensitized to radiation similarly, consistent with radiosensitization of inhibition of Chk1 by AZD7762 (Fig. 4B). These results suggest that radiosensitization is mediated by Chk1 depletion and Chk1 is a good target for an enhancement of sensitivity to radiation.

4. Discussion

In this study, we have shown that Chk1/2 inhibition by AZD7762 enhances radiation sensitivity in lung cancer cells and xenograft model of lung cancer brain metastasis. ATM and ATR are key upstream players in checkpoint pathways [20]. ATM responds principally to the occurrence of DSB in the genome. By contrast, ATR plays a distinct role in the detection of stalled DNA replication forks, but it also participates in DNA damage responses [20–22]. Activation of these kinases leads to activation of the effector kinases, Chk1 and Chk2. The activated effector kinases are then able to transiently delay cell cycle progression so that DNA can be efficiently repaired. The ATM/Chk2 pathway predominantly regulated the G_1 checkpoint and the ATR/Chk1 pathway the S and G_2 checkpoints. Functional defects of cell cycle checkpoint showed an increased sensitivity to IR and other DNA-damaging agents [7]. The compound inhibited the ATM signal transduction pathway, disrupted cell cycle checkpoint function, and sensitized tumor cells to IR [23].

Because AZD7762 is an inhibitor of both Chk1 and Chk2, we made Chk1 or Chk2 shRNA expression stable cell lines to analyze which kinase are involved in radiosensitization. We found that depletion of Chk1 with shRNA increased radiosensitivity in



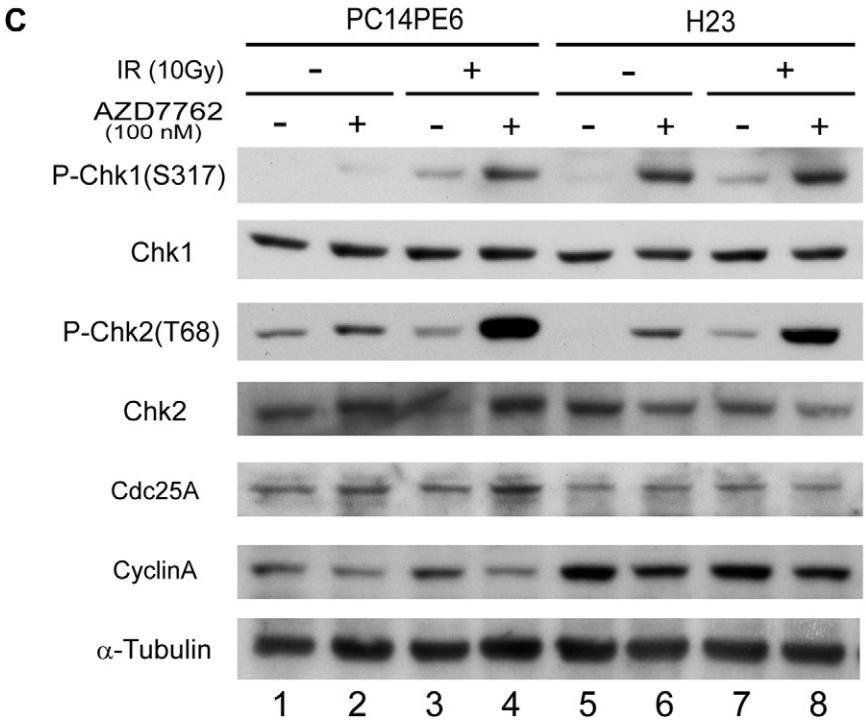


Fig. 2. Radiosensitization in response to Chk1 inhibition. Clonogenic survival (A) and CCK-8 (B) assays with or without AZD7762 treatment were performed for cell lines (PC14PE6 and H23). For clonogenic survival assays, AZD7762 (100 nmol/L) were treated for 1 h before and 24 h postradiation. CCK-8 assays were performed after treatment of IR (20 Gy) with or without AZD7762 (30 nmol/L). (C) PC14PE6 and H23 cells were treated with IR (10 Gy) in the presence or absence of AZD7762 (100 nmol/L). Cell lysates were subjected to SDS–PAGE followed by immunoblotting with indicated antibodies.

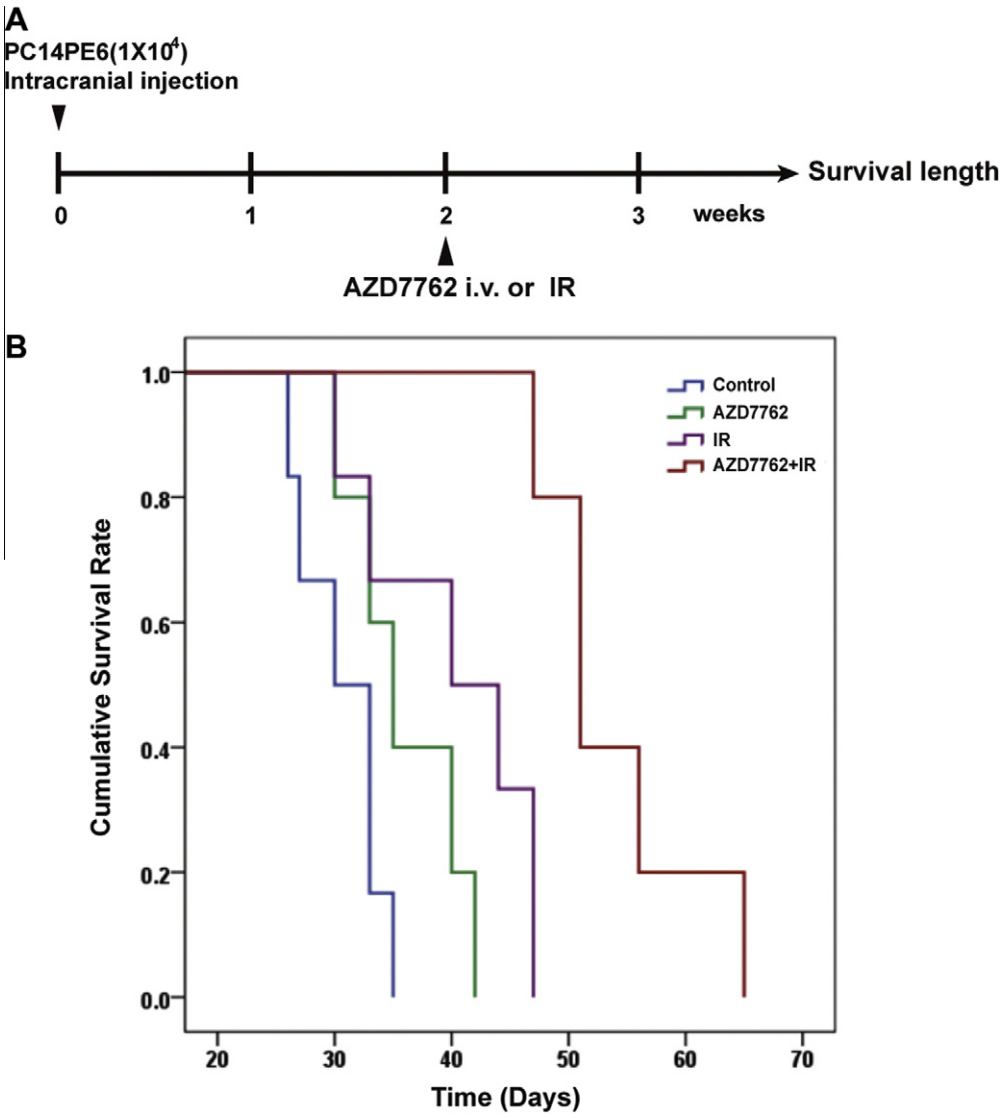


Fig. 3. Effects of AZD7762 on PC14PE6 brain metastasis xenograft in response to IR. (A) Schedule of treatment. Whole brain irradiation (15 Gy) was performed at 1 h after intravenous injection of AZD7762 (25 mg/kg) (B) Kaplan–Meier plot comparing survival of PC14PE6 xenografts treated as control (vehicle), AZD7762 alone (25 mg/kg, i.v.), radiation alone (IR; 15 Gy), and combined AZD7762 (25 mg/kg, i.v. injection prior to radiation) and IR (15 Gy).

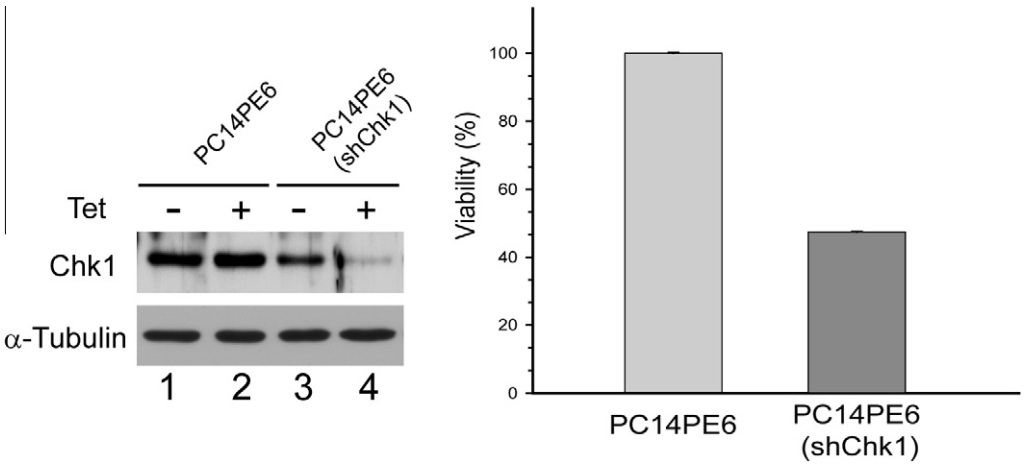


Fig. 4. Depletion of Chk1 enhances radiosensitivity of PC14PE6 cells *in vitro*. (A) Chk1 protein was selectively depleted in Chk1 shRNA expressing PC14PE6 cells with treatment of tetracycline (Tet) (1 μg/ml). PC14PE6 and shRNA expressing stable cell lines were plated 24 h before tetracycline treatment for shRNA induction. Cell lysates were subjected to immunoblotting with indicated antibodies after induction (48 h). (B) CCK-8 survival assays were performed with treatment of IR (20 Gy) after shRNA induction (48 h).

PC14PE6 cells, but depletion of Chk2 did not enhance the radiosensitivity (data not shown). Other recent studies described checkpoint kinase inhibitors such as XL-844 and PF0047736 (100-fold more selective for Chk1 than Chk2) have shown *in vitro* radiosensitizing comparable with AZD7762 [24,25]. Multiple studies using Chk2 siRNA have shown a lack of effect of Chk2 inhibition on radiosensitization [11,12]. Because Chk2 does have a role in checkpoint signaling especially in response to radiation, the exact mechanism of radiosensitization by depletion of Chk1, not Chk2 is not well understood. Taken together, these results suggest that sensitization by AZD7762 is mediated by inhibition of Chk1.

It has been established that activation of Chk1 in response to IR also depends upon upstream regulation by ATM [26–28]. This observation implied that certain DNA structures rely on ATM to elicit the ATR-dependent phosphorylation of Chk1. In more recent studies, TopBP1 has been identified as a direct activator of ATR [22]. TopBP1 has a direct and essential role in the pathway that connects ATM to ATR specifically in response to the occurrence of DSBs in the genome [29]. Claspin has also an essential role for ATR-dependent Chk1 activation [30]. Because Chk1 inhibition by AZD7762 showed enhancement of radiation sensitivity, it is worth to analyze the effect of disruption of Chk1 upstream (TopBP1 or Claspin) on radiosensitivity.

In closing, we have shown that the treatment of Chk1/2 inhibitor, AZD7762 enhanced the radiosensitivity in human lung cancer cell lines and xenograft model of lung cancer brain metastasis. AZD7762 enhanced the activity of DNA-damaging agents [18]. AZD7762 in combination with radiation and DNA-damaging agents may play an important role in improving both adjuvant therapy and the treatment of metastatic disease. DNA damage checkpoint pathways can also be a good target to improve the effect of radiation therapy.

5. Conflicts of interest

None declared.

Acknowledgment

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