A Doctoral Dissertation

Development of the Anti-cancer Therapeutic Modalities Overcoming Radio-resistance in Metastatic Brain Tumors

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Abstract

Brain metastases are the most common form of adult central nervous system (CNS) tumors, outnumbering primary brain tumors by 10:1. Brain metastases occur late in the progression of multiple types of cancer and are associated with poor patient survival. These metastases most commonly arise from cancers of the lung, breast, and skin (melanoma). Standard treatment options include symptomatic therapy with corticosteroids and whole-brain radiotherapy (WBRT), and more aggressive approaches such as surgery or radiosurgery are used in a subset of patients. The most successful therapeutic tool in brain metastases has been radiation therapy. However, resistance to radiation is a major cause of recurrence or treatment failure. Imperative goals for future research include optimizing the efficacy of radiation therapy against metastatic tumor cells compared with normal brain cells, and preventing the cognitive losses that a proportion of patients suffer. In this study, we seek to find functional radio-sensitization targets to translate to the clinic.

Recently, signal pathways about DNA damage checkpoints after irradiation and correlation of c-Met and radio-resistance have been noticed. However, it is insufficient of clues to translate to the clinic in metastatic brain cancers. Therefore, we investigated whether Chk1, one of the components of DNA damage checkpoint signaling and c-Met, a hepatocyte growth factor receptor, could be targeted for radiosensition in lung cancer brain metastases and breast cancer brain metastases, respectively.

We proved that there is a correlation between Chk1 activation and prognosis of brain metastases from patients with metastatic brain tumor and Chk1 is highly activated in respond to radiation in radio-resistant lung cancer cells. To prove a role for Chk1 as a target for radio-sensitization, we investigated radio-sensitizing effects of the Chk1 inhibitor, AZD7762 in lung cancer cell lines and xenograft models of lung cancer brain metastases. Clonogenic survival assays showed enhancement of radiosensitivity with AZD7762 after irradiation of

various doses. AZD7762 increased ATR/ATM-mediated Chk1 phosphorylation, stabilized Cdc25A and suppressed cyclin A expression in lung cancer cell lines. In xenograft models of lung cancer (PC14PE6) brain metastases, AZD7762 significantly prolonged the median survival time in response to radiation. Depletion of Chk1 using shRNA showed an enhancement of sensitivity to radiation in PC14PE6 cells. Also, life span was significantly increased by depletion of Chk1 in combination with radiation treatment in a radio-resistant lung cancer brain metastatic xenograft model. The results of this study strongly support our hypothesis that Chk1 is a good target for radio-sensitivity enhancement.

To find out the correlation between c-Met and radio-resistance, we investigated c-Met protein intensity and c-Met mRNA level after radiation in different breast cancer cell lines. In MDA-MB-435 and MDA-MB-231 cell lines, c-Met protein intensities and c-Met mRNA levels were increased after radiation. Also, c-Met was elevated in the radio-resistant clone, which was acquired from xenograft models of breast cancer brain metastases. A combination of c-Met silencing, using shRNA and radiation, showed regression of tumor growth in the breast cancer orthotopic xenograft model and prolonged median survival and decreased tumor mass volume in the brain metastatic xenograft model. Also, a combination of c-Met silencing and radiation significantly induced apoptosis in the breast cancer brain metastatic model. The results of this study support our hypothesis that c-Met is a good target for radiosensitivity enhancement.

Taken together, we suggest that Chk1 and c-Met can be functional targets in lung and breast cancer brain metastases for developing radio-sensitizers, which can be translated to the clinic.

List of Abbreviations

A-T	Ataxia-telangiectasia
ANOVA	One-way analysis of variance
ATM	A-T, mutated
ATR	ATM and Rad3 related
ATRIP	ATR interacting protein
BBB	Blood-brain barrier
CCK-8	Cell counting kit-8
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CI	Confidence interval
CNS	Central nervous system
DNA-PK	DNA-dependent protein kinase
DSBs	DNA double-strand breaks
EMT	Epithelial-to-mesenchymal transition
ER	Oesterogen receptor
ERKs	Extracellular signal-regulated kinases
GAPs	GTPase activating proteins
GBMs	Glioblastomas
GRB2	Growth factor receptor-bound protein 2
GSCs	GBM stem cells
HER2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
HRR	Homologous recombination repair

IHC	Immunohistochemical
IKK	Nuclear factor-κB inhibitor-α kinase
IR	Ionizing radiation
IκB	Nuclear factor-κB inhibitor-α
JNKs	Jun amino-terminal kinases
LSD	Least significant difference
MAPK	Mitogen-activated protein kinase
MBTs	Metastatic brain tumors
MEM	Minimal essential medium
MRN	MRE11:Rad50:NBS1
MTX	Methotrexate
NER	Nucleotide excision repair
NF-κB	nuclear factor-κB
NSCLC	Non small cell lung cancer
OD	Optical density
OS	Overall survival
p120	p120 Ras-GAP
PFS	Progression-free survival
PI3K	Phosphatidylinositol 3-kinase
PR	Progesterone receptor
ROS	Reactive oxygen species
RPA	Replication protein A
RPTK	Receptor protein tyrosine kinase
SH2 domain	Src-homology-2 domain
SOS	Son of sevenless
STAT3	Signal transducer and activator of transcription 3

Tet	Tetracycline
TIC	Tumor initiating cell
TopBP1	Topoisomerase IIβ binding protein 1
UV	Ultraviolet light
WBRT	Whole-brain radiation therapy

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General Introduction

Overview of metastatic brain tumors

Metastases, the spread of cancer from the site of primary tumor growth to distant organs, are a leading cause of cancer morbidity and mortality [1]. Brain metastases represent some of the most frequent neurological complications of systemic cancer, being important causes of morbidity and mortality [2,3]. Brain metastases are the most common forms of adult central nervous system (CNS) tumors, outnumbering primary brain tumors by 10:1 [4]. They occur late in the progression of multiple types of cancer and are associated with poor patient survival [5].

Incidences of brain metastatic disease appear to be rising as a result of multiple factors. First, there is an increase in the aged population. Second, improvements in systemic chemotherapy have increased the number of metastatic patients either responding to treatment or with stable disease that are at risk of brain progression. Third, the increased awareness of the warning signs and risk factors has led to more frequent screenings for the disease [6].

Brain metastases most commonly arise from cancers of the lung, breast, and skin (melanoma), but also occur at a reduced frequency in patients with diverse cancer types [7].

Incidences of brain metastases are highest in patients with lung tumors. Approximately 10-25% of patients with lung cancer have also been diagnosed with brain metastases and another 40-50% develops them during the course of their disease, with an even greater incidence detected at autopsy [8]. Brain metastases conferred an inferior overall survival to patients with non-small-cell lung cancer (NSCLC), particularly to those who had a limited number of systemic (liver, bone, and other organs) metastases [9]. For cancers of the breast, brain metastases occur after the diagnosis of systemic metastases. In patients with metastatic disease whose tumors fall into two categories – tumors with amplification of receptor tyrosine kinase ERBB2 (ERBB2+; also known as HER2+) or triple-negative (oestrogen

receptor (ER) and progesterone receptor (PR)-negative and normal levels of expression of ERBB2) tumors –incidences of brain metastases can exceed one-third of the patients. Incidences of brain metastases occur less frequently in patients with ER-positive (ER+) metastatic tumors.

Once metastases to the brain are diagnosed, the median survival period of untreated patients is 1-2 months. In patients treated with surgery, chemotherapy, and radiation therapy, the median survival period can be extended to only 4-6 months. The poor prognoses in these patients are due primarily to resistance to chemotherapy and the recurrent growth of tumors at the site of resected lesions, as well as the development of metastases in other areas of the brain [10,11].

In most patients with brain metastases, whole brain radiation therapy (WBRT) is the mainstay of treatment and efforts to improve the outcome of WBRT continue. Radiosensitizers are chemical or pharmacologic agents that increase the lethal effects of radiation when co-administered. In an attempt to improve outcomes, studies have examined the use of whole brain radiotherapy in combination with radiosensitizers [12]. However, multiple agents with preclinical radiosensitizing properties have failed to show benefit in randomized controlled clinical trials, including lonidamine, metronidazole, misonidazole, motexafin gadolinium, bromodeoxyuridine, and efiproxaril [13,14,15,16,17,18,19].

DNA damage response (DDR) pathway

The human autosomal recessive disease ataxia-telangiectasia (A-T) has a complex clinical phenotype, including progressive cerebellar ataxia, oculocutaneous telangiectasias, immune deficiency, hypogonadism, growth retardation, premature aging, radio-sensitivity, and cancer predisposition [20]. The defective gene in A-T was identified as ATM (A-T, mutated) and encodes a 350-kDa protein that belongs to the phosphatidylinositol 3-kinase (PI3K) family of proteins [21].

Cells are often exposed to various forms of DNA damage from sources including reactive oxygen species, ultraviolet light (UV), background radiation, and environmental mutagens [22]. Three kinases involved in the detection, signaling and repair of DNA double-strand breaks (DSBs) are ataxia-telangiectasia mutated (ATM), ATM and Rad3 related (ATR) and DNA-dependent protein kinase (DNA-PK) [23,24].

ATM is activated by DSBs. In an unperturbed cell, ATM exists as an inactive dimer (or higher-order oligomer), but the introduction of DNA double-strand breaks (DSB) by ionizing radiation (IR) or other insults activates the ATM kinase by intermolecular autophosphorylation and dimer dissociation [25,26]. The monomers of ATM are recruited to DSBs via interations with the MRE11:Rad50:NBS1 (MRN) sensor complex, which fully activates ATM, allowing it to act on downstream substrates [22]. Once ATM is activated, ATM phosphorylates several downstream substrates that contribute to the proper regulation of IR-induced arrests in G1 phase (e.g., p53, Mdm2, and Chk2), S phase (e.g., Nbs1, Smc1, Brca1, and FancD2), and G2 phase (e.g., Brca1 and Rad17) of the cell cycle [27].

ATR is activated most strongly when DNA replication is interrupted, such as nucleotide depletion or replication-blocking DNA damage lesions often induced by UV light [23,28]. The activity of ATR requires its association with the ATR interacting protein (ATRIP) and Topoisomerase IIβ binding protein 1 (TopBP1) proteins, and association with replication protein A (RPA)-coated single-strand DNA activates the kinase [29]. The activation of ATR does not require autophsphorylation or posttranslational modification [23]. Although it has been suggested that ATR is only activated in S phase following fork stalling, ATR-dependent γ-H2AX can form following UV irradiation of non-replicating cells at ssDNA generated during nucleotide excision repair (NER). Phosphorylation of checkpoint proteins has also been reported following NER [30].

The kinase substrates of the DNA damage sensors, ATR and ATM, include checkpoint kinase 1 (Chk1) and checkpoint kinase 2 (Chk2), respectively. Both of these checkpoint kinases

tightly regulate the cell cycle checkpoint [31,32]. Temporary arrest of the cell cycle allows for repair and prevention of the replication of damaged DNA or, alternatively, the induction of apoptosis or terminal cell cycle arrest in the event of heavily damaged cells [33,34,35].

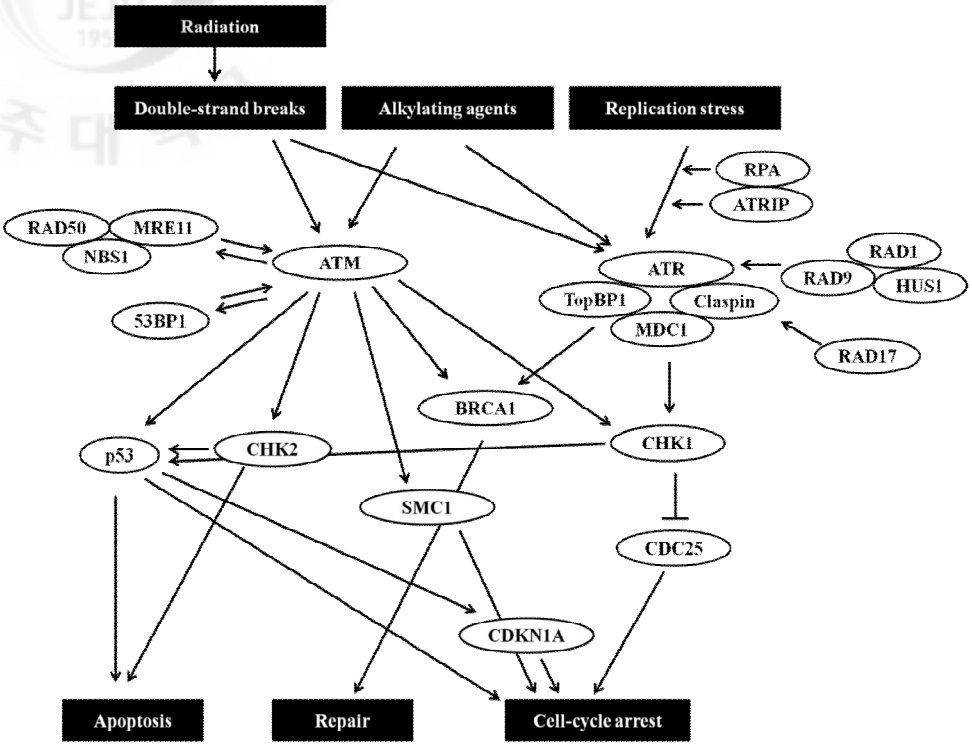

Fig. 1. Schematic diagram of the DNA damage response signal transduction network.

HGF and c-Met signaling

c-Met is a transmembrane receptor protein tyrosine kinase (RPTK) for which the primary stimulatory ligand is hepatocyte growth factor (HGF), which is also known as scatter factor (SF). c-Met triggers several downstream pathways [36]. First, the mitogen-activated protein kinase (MAPK) cascades consist of three subfamilies, each of which comprises by the Ras small GTPase, which in turn is switched on by son of sevenless (SOS) and switched off by GTPase activating proteins (GAPs), such as p120 Ras-GAP (p120). SOS can be activated

and p120 can be inhibited in response to c-Met. The terminal effectors include extracellular signal-regulated kinases (ERKs), Jun amino-terminal kinases (JNKs) and p38s. These translocate to the nucleus, where they influence the activity of various transcription factors. Second, phosphoinositide 3-kinase (PI3K) is a lipid kinase that associates with the multifunctional docking site of c-Met and catalyses the formation of phosphatidylinositol (3,4,5)-triphosphate (PtdIns(3,4,5)P_3). Production of PtdIns(3,4,5)P_3 creates a docking site for Akt. Once compartmentalized at the inner side of the plasma membrane, Akt becomes activated and phosphorylates several substrates involved in cell proliferation, survival and the regulation of cell size. Third, signal transducer and activator of transcription 3 (STAT3) monomers bind to c-Met through their Src-homology-2 domain (SH2 domain) and become trans-phosphorylated. Thereafter, each STAT3 moiety homodimerizes, using its SH2 domain to recognize the phosphotyrosine of its partner, and translocates to the nucleus to operate as a transcription factor. Fourth, in response to c-Met stimulation and the ensuing activation of PI3K- and Src-dependent pathways, nuclear factor-κB inhibitor-α kinase (IKK) is activated, and phosphorylates the nuclear factor-κB inhibitor-α (IκB) proteins (which are bound to nuclear factor-κB (NF-κB)). This triggers the ubiquitin-dependent degradation of IκB, resulting in the nuclear translocation of NF-κB and transcription.

The c-Met has been implicated in the development and progression of several human cancers, such as hepatocellular carcinoma, osteosarcoma, colorectal cancer, and glioblastomas (GBMs) [37]. C-Met involved in multiple pathways linked to cancer – such as cell migration, invasion, proliferation, and angiogenesis – is upregulated in a large number of human cancer [38].

Part Ⅰ. Inhibition of DNA damage checkpoint signaling sensitizes lung cancer brain metastases to radiotherapy

Introduction

Almost a half of cancer patients will have metastases either at diagnosis or during the 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 [39]. 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 have been used cases of multiple MBTs. Nonetheless, resistance to radiation is the most 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 acquired resistance to irradiation [40]. 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 [20,40]. 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 [41,42]. Tumor cell specific checkpoint mechanisms for DNA damage in response to IR would be the clue to solve the resistance.

In this report, to find possible targets for radio-resistance, we have investigated the biological association between DNA damage checkpoint and disease progression in metastatic brain tumors. Immunohistochemical analyses for the proteins were performed in the tumor tissues and the correlations between the expression level of the components and patients' survival were analyzed.

Upon DNA damage, Chk1 is activated by ATM/ATR-mediated phosphorylation and then phosphorylates Cdc25 phosphatases, thereby preventing the activation of Cdk1 kinase. Based on data demonstrating that Chk1 is an effective target for sensitization to chemotherapy and radiotherapy [43,44,45], small molecule Chk1 inhibitors have been developed for clinical use to enhance killing tumor cells by cytotoxic drugs and by radiation [46,47,48]. One Chk1 inhibitor, CEP3891 has previously been shown to increase cytotoxicity after irradiation *in vitro* [49]. A role for Chk1 in radiation resistance of cancer stem cells has recently been proposed [50]. Recently, a novel Chk1 inhibitor, AZD7762 was shown to enhance the cytotoxicity of DNA-damaging chemotherapy agents by abrogation of the cell cycle arrest [51]. In the present study, we show that the radio-sensitivity can be enhanced with the treatment of the Chk1 and Chk2 inhibitor AZD7762, in lung cancer cell lines and a brain metastases 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 radio-sensitivity enhancement and that AZD7762 is a potent radiation sensitizer.

Materials and Methods

Patient and tissue collection

Thirty-four surgical samples of 34 patients with MBTs were included in this study. All patients underwent surgery at Samsung Medical Center between February 2004 and August 2007. Tumor samples were reevaluated by two neuropathologists to confirm the diagnosis according to the World Health Organization criteria. Tumor samples were obtained during surgical treatment and were embedded in paraffin for histological studies. Written informed consent was obtained from all patients, and tissue collection was approved by the institutional review board. Immunohistochemical studies were performed in a blinded manner, without prior knowledge of clinical outcome.

Immunohistochemical (IHC) study

Four-micrometer-thick sections sliced from paraffin-embedded specimens were prepared on the slide. Sections were immunostained with antibodies for ATM (1:50, Santa Cruz Biotechnology), Rad17 (1:50, Santa Cruz Biotechnology), TopBP1 (1:50, Bethyl Laboratories, Inc.), phosphrylated-Chk1 (S317) (1:5, Cell Signaling Technology), Chk1 (1:50, Santa Cruz Biotechnology), Chk2 (1:50, Santa Cruz Biotechnology), and p53 (1:5,000, Santa Cruz Biotechnology). Tumor-containing sections were baked at 56°C for 30 minutes, deparaffinized in xylene, and rehydrated in graded concentrations of ethanol. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in methanol and with heat-induced antigen retrieval (for p53, 10 mM citrate buffer [pH 6.0] for 25 minutes in a vegetable steamer). Immunostaining involved sequential applications of primary antibody for 16 hours at 4°C, followed by biotinylated secondary antibodies (Vector Laboratories, Orton Southgate, UK) at 1:200 for 1 hour and avidin-biotin complex (Vector Laboratories)

for 1 hour. Negative control slides received normal horse and goat serum (Dako Corporation, Carpinteria, CA) as the primary antibody. Diaminobenzidine tetrahydrochloride was used as the enzyme substrate to observe the specific antibody localization, and Harris hematoxylin was used as a nuclear counterstain.

Sections were examined for immunoreactivity for the proteins by an observer who was unaware of the histologic diagnoses, outcomes, or clinical features. Tumors were categorized as following: grade 0 for those expressing no protein, Grade 1 for those expressing in <25% of cells, Grade 2 for those expressing in 25-50% of cells at similar or overexpressed levels compared to the normal brain, Grade 3 for those in 50-75% of cells, and Grade 4 for those in >75% of cells, based on the expression level in nucleus visualized in a high-power field in areas with maximal staining. The expression of the proteins was analyzed as a dichotomous covariate: low immunoreactivity (Grade 0 or 1) versus high expression (Grades 2-4).

Cell culture and cell lines

Human non-small cell lung cancer cell lines NCI-H23 (Adenocarcinoma), NCI-H460 (Large cell carcinoma), NCI-H1299 (Large cell carcinoma), and A549 (Adenocarcinoma) were obtained from American Type Cell Culture (ATCC, USA), and were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. And PC14PE6 cells (Human non-small cell lung cancer cell line, Adenocarcinoma), which were kindly provided by MD Anderson Cancer Center, were maintained in Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Cell viability

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 $0.4X10^4$ cells/well and 100 U/ml for every well. After the incubation, cells were treated with 10 μl CCK-8 solution every well with without AZD7762 (100 nM) or caffeine (0.5 mM) 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 c-radiation source were used. After being incubated for 1–3 days, cells were subjected to the CCK-8 assay.

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Clonogenic assay

For cell survival studies, cells were plated ($8X10^5$ cells/100 mm culture dishes) and incubated for 16 h at 37℃. 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.

Western blotting

Exponentially growing cells were exposed to radiation without or with AZD7762 or caffeine. After treatment cells were harvested, lysed, and prepared for Western blotting analysis, as previously described [52]. Cells were processed 1 h after IR (10 Gy). Anti-ATM, anti-Chk1, anti-Chk2, anti-Rad17, anti-p21, and anti-p53, anti-Cdc25A, anti-Cyclin A, and anti-α-tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Chk1 phospho-Ser317 and anti-Chk2 phospho-Thr68 antibodies were obtained from Cell Signaling Technology (Danvers, MA). Anti-TopBP1 and anti-Nbs1 antibodies were obtained from Bethyl Laboratories, Inc (Montgomery, TX) and Calbiochem (La Jolla, CA),

respectively.

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′.

Establishment of lung cancer brain metastatic xenograft models

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).

Male athymic nude mice, which were 8 weeks of age, were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine, intraperitoneally. Various cell number of NCI-H23 ($5.0X10^3$, $5.0X10^4$, or $5.0X10^5$), NCI-H460 ($5.0X10^3$, $5.0X10^4$, or $5.0X10^5$), NCI-H1299

($5.0X10^3$, $5.0X10^4$, or $5.0X10^5$), A549 ($5.0X10^3$, $5.0X10^4$, or $5.0X10^5$), and PC14PE6 cells ($1.0X10^4$, $1.0X10^5$, or $1.0X10^6$) 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). Mice were sacrificed either when 20% body weight loss or moribund status were observed.

Orthotopic lung cancer brain metastases xenograft studies

Male athymic nude mice, 8 weeks of age were used for this study. To produce orthotopic lung cancer brain metastases models, mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine, intraperitoneally. NCI-H460 cells, PC14PE6 cells, subclones of expressing scramble or Chk1 shRNA ($1.0X10^4$) 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 or doxycycline. AZD7762, which was formulated in 0.9% saline, was injected by i.v. injection on 2 weeks after tumor cells implantation at a dose of 25 mg/kg. Doxycycline, which was formulated in 5% sucrose, was administered for 5 days in drinking water from 9 days after tumor cells implantation at a dose of 3 mg/ml.

For radiation treatment, mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. 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) at 1 h after treatment of AZD7762 or at 2 days after administration of doxycycline.

Statistical analysis

Statistical comparisons were analyzed by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test. Survival analysis of xenograft model was performed using the Kaplan-Meier and log rank tests (SPSS statistical software, version

18.0; SPSS Inc., Chicago, IL). P values <0.05 were considered statistically significant.

The analysis of the relation between the above protein expressions and overall or progression-free survival and was investigated by using the Kaplan-Meier method. The differences between the survival curves were tested using the log-rank test. The results are reported as being statistically significant when 2-sided P<0.05. The Fisher exact test was used to analyze the correlation between IHC variables.

Results

IHC of the component of DNA damage checkpoint signaling and prognostic implications

The progression-free survivals (PFS) of the patients according to expression of components of DNA damage checkpoint signaling are showed in Fig. 2. The patients were divided into two subgroups by expression levels of components of DNA damage checkpoint signaling. There were no significant on PFS according to expression level of ATM, Rad17, Chk1, Chk2, or p53. Median PFS for TopBP1 low expressing tumors was 463 days (95% CI, 328-597 days), compared with 183 days (95% CI, 49-316 days) for TopBP1 high expressing tumors (p=0.013) (Fig. 2C and c). Median PFS for p-Chk1 low expressing tumors was 463 days (95% CI, 240-599 days), compared with 118 days (95% CI, 9-226 days) for p-Chk1 high expressing tumors (p=0.008) (Fig. 2D and d).

Different radio-response in lung cancer cell lines

The radio-sensitivity of five types of lung cancer cell lines (NCI-H23, NCI-H460, NCI-H1299, A549, and PC14PE6) was assessed by CCK-8 assays and clonogenic survival assays after treating them with various doses of radiation (Fig. 3).

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The cell viability of NCI-H23 (about 40% viability at 10 Gy; about 40% viability at 20 Gy) and NCI-H460 (about 70% viability at 10 Gy; about 50% viability at 20 Gy) were decreased at 3 days after 10 or 20 Gy of radiation. The cell viability of NCI-H1299 (about 80% viability at 10 Gy; about 70% viability at 20 Gy), A549 (about 90% viability at 10 Gy; about 100% viability at 20 Gy), and PC14PE6 (about 100% viability at 10 Gy; about 110% viability at 20 Gy) were maintained highly even though 10 or 20 Gy of radiation. The surviving fraction of NCI-H23 and NCI-H460 were decreased by radiation, but, NCI-H1299, A549, and PC14PE6 showed highly maintained under radiation.

The CCK-8 assays (Fig. 3A) and clonogenic survival assays (Fig. 3B) revealed that NCI-H1299, A549, and PC14PE6 cells were the most radio-resistant, and that NCI-H23 and NCI-H460 cells were more sensitive to radiation.

Different response of DNA damage checkpoint pathway on radiation in lung cancer cell lines

In order to estimate the correlation between radio-sensitivity and DNA damage checkpoint signaling, the activation or expression of DNA damage checkpoint signaling in response to radiation in lung cancer cells were examined. The protein expression levels of DNA damage checkpoint signaling (ATM, TopBP1, Chk1, Chk2, Rad17, and p53) were analyzed 6 hours after 10 Gy of radiation (Fig. 4). The activation of DNA damage checkpoint was determined by detection of p-Chk1 (Ser317) and p-Chk2 (Thr68). The level of TopBP1 in NCI-H1299 and PC14PE6 (radio-resistant cells) were higher than those in NCI-H23 and NCI-H460 (radio-sensitive cells). The expression of tumor suppressor gene p53 was very different in the cell lines. The expression of ATM and Rad17 did not show remarkable difference among the cell lines. As shown in Fig. 5B, there were more robust phosphorylations of Chk1 and of Chk2 in radio-resistant cells (NCI-H1299, A549 and PC14PE6) than those in radiosensitive cells (NCI-H23 and NCI-H460). These results suggest that the possibility of correlation between radio-sensitivity and DNA damage checkpoint activity.

Different radio-response in lung cancer brain metastatic xenograft models

The proper cell numbers of five types of lung cancer cells to establish lung cancer brain metastatic xenograft models and estimate efficacy of targeted therapy were estimated. The median survival day and survival curve according to cell numbers in five types of lung cancer cells were shown in Fig. 5 and Table 1. The cell numbers of $5.0X10^5$, $5.0X10^3$, $5.0X10^5$, $5.0X10^5$, and $1.0X10^4$ for NCI-H23, NCI-H460, NCI-H1299, A549, and PC14PE6, respectively, were selected for further studies using xenograft models.

To estimate radio-response in lung cancer brain metastatic xenograft models, the various dose of radiation was delivered into NCI-H460 or PC14PE6 lung cancer brain metastatic xenograft models. In NCI-H460, which showed radio-sensitive *in vitro* study, lung cancer brain metastatic xenograft model, there were median survival gain even though low dose of radiation (Fig. 6A). But, in PC14PE6, which showed radio-resistant *in vitro* study, lung cancer brain metastatic xenograft model, there were median survival gain only high dose of radiation (Fig. 6B).

Correlation between DNA damage checkpoint signaling and radio-sensitization

To investigate the correlation between DNA damage checkpoint signaling and radio-sensitization, we estimated the influence of caffeine, which inhibited ATM and ATR kinase activity, on A549 and PC14PE6 cells. 10 Gy of radiation or 0.5 mM caffeine treatment did not affect in cell growth of A549 and PC14PE6. However, the cell growth was decreased by combination treatment of radiation and caffeine from 2 days after treatment in both of A549 and PC14PE6 cells (Fig. 7A and B). The phosphorylation of Chk1, which is down signal of ATM and ATR kinase, was increased by 10 Gy of radiation and decreased by combination treatment of radiation and caffeine (Fig. 7C). This observation suggests a possibility that components of these DNA damage checkpoint pathways as potential therapeutic targets for enhancing the radio-sensitivity of tumor cells.

Radio-sensitization of lung cancer cells by the treatment of Chk1 inhibitor, AZD7762

The cell proliferation and clonogenic potential of lung cancer cell lines (NCI-H460 and PC14PE6) was assessed under radiation 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 post-radiation. To ensure Chk1 inhibition, 100 nM of AZD7762 was added to cells at 1 h before radiation and incubated on for further 24 h after radiation. In compared to radiation treated cells, the NCI-H460 cell growth was little different by

combination of radiation and AZD7762 (Fig. 8A) and clonogenic potential was a little inhibited by combination of radiation and AZD7762 (Fig. 8B). There was a little radio-sensitizing effect of AZD7762 in radio-sensitive NCI-H460 cells. However, The PC14PE6 cell growth and clonogenic potential were dramatically inhibited by combination of radiation and AZD7762 compared to radiation treated cells (Fig. 9A and B). The radio-sensitizing effect of AZD7762 in radio-resistant PC14PE6 cells was significant to radiation. To confirm that AZD7762 inhibits Chk1 and Chk2, we analyzed Chk1 and Chk2 signaling in PC14PE6 cell lines after radiation (Fig. 9C). Phosphorylation of Chk1 (Ser317) and Chk2 (Thr68) were increased by the addition of AZD7762 to radiation. Radiation alone led to the destabilization of cdc25A, a direct substrate of Chk1, which on Chk1 activation is phosphorylated and consequently degraded and sequentially inhibited cyclin A. Combination of radiation and AZD7762 stabilized cdc25A. Cylin A levels, in contrast, were decreased for the combination, consistent with the abrogation of the checkpoint. Taken together, these results show that AZD7762 inhibits Chk1 and Chk2 in NCI-H460 and PC14PE6 cells. These results show that AZD7762 is a good radiosensitizer.

Chk1 inhibition enhances radio-sensitivity 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 metastases models. We produced orthotopic lung cancer brain metastatic models with injection of NCI-H460 or PC14PE6 cells into athymic nude mice. We estimated the effects of Chk1 inhibition on the survival of two types of lung cancer brain metastases xenograft models in response to radiation. Tumor-bearing mice of NCI-H460 or PC14PE6 lung cancer brain metastatic models were treated with AZD7762 and/or radiation as illustrated (Fig. 10A and 11A, Table 2 and 3). As shown in Fig. 10B and 11B compared with vehicle control (median survival; 20 or 30 days, respectively), AZD7762 treatment (median survival; 18 or 35 days, respectively) had no effect on survival of mice. Radiation treatment (10 or 15 Gy) expanded survival rate

(median survival; 22 or 40 days, respectively), significantly (p = 0.002 or 0.020, compared with Control). And combination of AZD7762 and RT (median survival; 23 or 51 days) further enhanced the survival rate (p = 0.020 or 0.005, compared with RT). 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 metastatic xenograft models.

Depletion of Chk1 enhances radiosensitivity

To address the relative contribution of inhibition of Chk1 by AZD7762 to radio-sensitization, 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. 12A). The clonogenic potential was decreased by depleting Chk1 (Fig. 12B). Relative to nonspecific shRNA expression cells, PC14PE6 (shChk1), Chk1-depeleted lung cancer cells, were sensitized to radiation similarly, consistent with radio-sensitization of inhibition of Chk1 by AZD7762.

Also, we estimate the influence of Chk1 depletion on radio-sensitization in radio-resistant lung cancer brain metastatic xenograft model. The survivals of each treatment group were shown at Fig. 13B and Table 4. The median survival (27 days) was increased about 29% by radiation in the group, which is not treated with doxycycline (median survival; 21 days) (p = 0.002), and the median survival (35 days) was significantly increased about 52% by radiation in the Chk1 depleted group by doxycycline treatment (median survival; 23 days) (p = 0.001). This increase of life span by Chk1 depletion and radiation was significant compared to radiation only treated group (p = 0.002). These results suggest that radio-sensitization is mediated by Chk1 depletion and Chk1 is a good target for an enhancement of sensitivity to radiation.

Discussion

As a central mediator of the cellular response to DNA damage, activation of Chk1 in response to DNA damage results in cell cycle arrest [53,54] as well as promotion of homologous recombination repair (HRR), a process promoted by the binding of the recombinase, Rad51, to sites of DNA double strand breaks [55]. Chk1, an effector kinase in the downstream of ATM and ATR, is able to transiently delay cell cycle progression so that DNA can be efficiently repaired [23,40]. Defects in DNA damage response pathway contribute to increased sensitivity to radiation and other DNA damaging agents [20]. It has been established that the activation of Chk1 in response to radiation also depends upon upstream regulation by ATM [56,57]. 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 [58] and 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 [59]. Chk1 is a crucial effector of those. Based on data demonstrating that Chk1 is an effective target for sensitization to chemotherapy and radiotherapy [43,44,45], we tested the possibility of Chk1 as a target for radio-sensitization. In this study, the activities of Chk1 in response to radiation were higher in radio-resistant lung cancer cell lines than in a radio-sensitive lung cancer cell lines. We also showed the close inverse relationship between PFS of MBT patients and the expression levels of TopBP1 and P-Chk1 by immunohistochemical analysis in the tumor tissues. However, we could not acquire the patients' samples immediately after radiation therapy. Only samples of brain metastases before radiation are usually available in clinical condition. This is a limitation of this study to match *in vitro* data with clinical data. Nonetheless, both basal and post-radiation levels of the two proteins in Western blot tend to be higher in radio-resistant lung cancer cells than in radiosensitive lung cancer cells. In addition, inhibition of Chk1 resulted in enhancement of

radio-sensitivity and increased survival *in vivo*. Considering that radiation therapy is the only viable treatment option in MBTs, clinical prediction for responsiveness of radiation therapy must be important. Thus, this study along with IHC data suggests that the proteins could be important in predicting radio-sensitivity. To the best of our knowledge, this study is the first clinical report that connects DNA damage checkpoints and prognosis of MBTs.

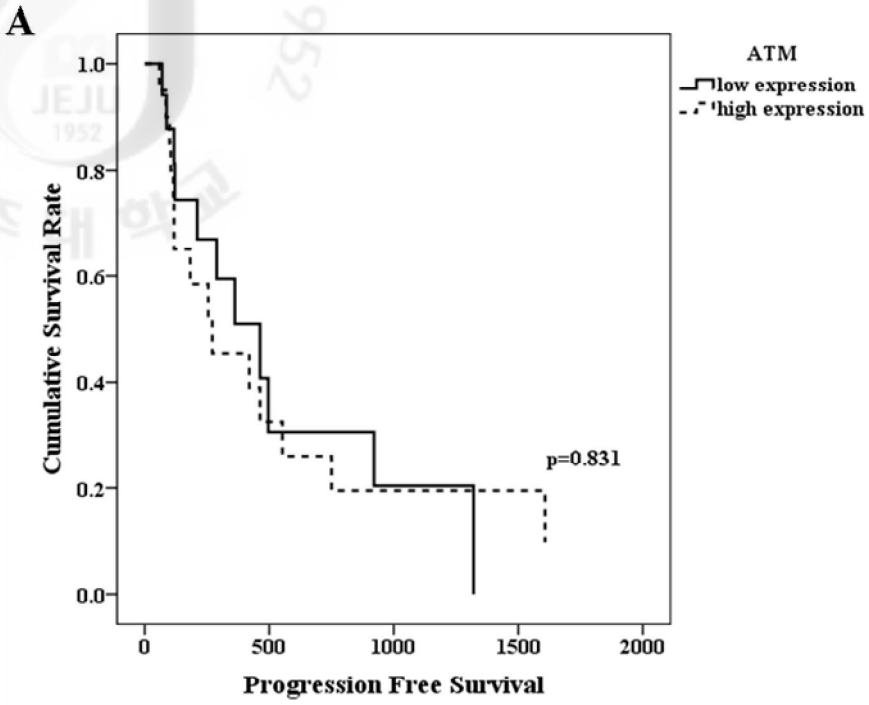
In this study, we have shown that Chk1 or Chk2 inhibition by AZD7762 enhances radio-sensitivity in lung cancer cells and xenograft models of lung cancer brain metastases. ATM and ATR are key upstream players in checkpoint pathways [23]. ATM responds principally to the occurrence of DSB in the genome. By contrast, ATR plays a distinct role in the detection of stalled DNA replication forks, but it also participates in DNA damage responses [23,58,60]. 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 G1 checkpoint and the ATR/Chk1 pathway the S and G2 checkpoints. Functional defects of cell cycle checkpoint showed increased sensitivity to radiation and other DNA-damaging agents [20]. The compound inhibited the ATM signal transduction pathway, disrupted cell cycle checkpoint function, and sensitized tumor cells to radiation [27].

Because AZD7762 is an inhibitor of both Chk1 and Chk2, we made Chk1 shRNA expression stable cell lines. We found that depletion of Chk1 with shRNA increased radio-sensitivity in PC14PE6 cells. Multiple studies using Chk2 siRNA have shown a lack of effect of Chk2 inhibition on radio-sensitization [44,45]. Because Chk2 does have a role in checkpoint signaling especially in response to radiation, the exact mechanism of radio-sensitization 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.

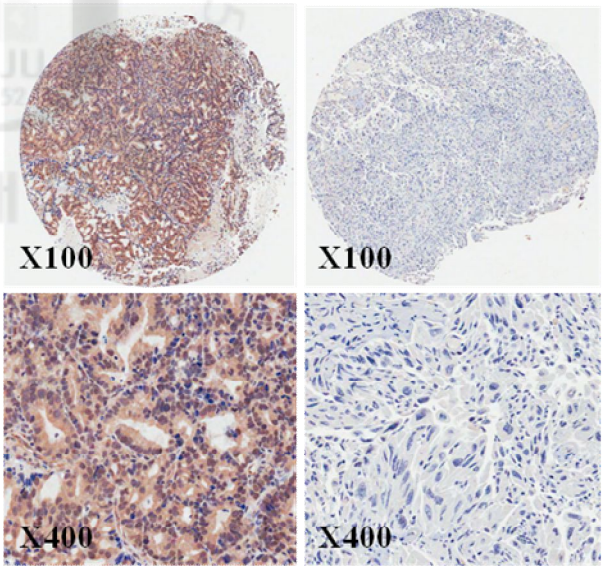
It has been established that activation of Chk1 in response to radiation also depends upon upstream regulation by ATM [56,57,61]. This observation implied that certain DNA

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 [58]. 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 [59]. Claspin has also an essential role for ATR-dependent Chk1 activation [62]. 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 radio-sensitivity.

In conclusion, to overcome the radio-resistance of current radiation therapy to the MBTs, new therapeutic target is mandatory. This study should provide the basis for further trials to target radio-resistance. Although work still remains to further investigate the role of the proteins, the DNA damage checkpoints proteins, especially Chk1 may be promising targets for enhancing therapeutic potentials in MBTs.



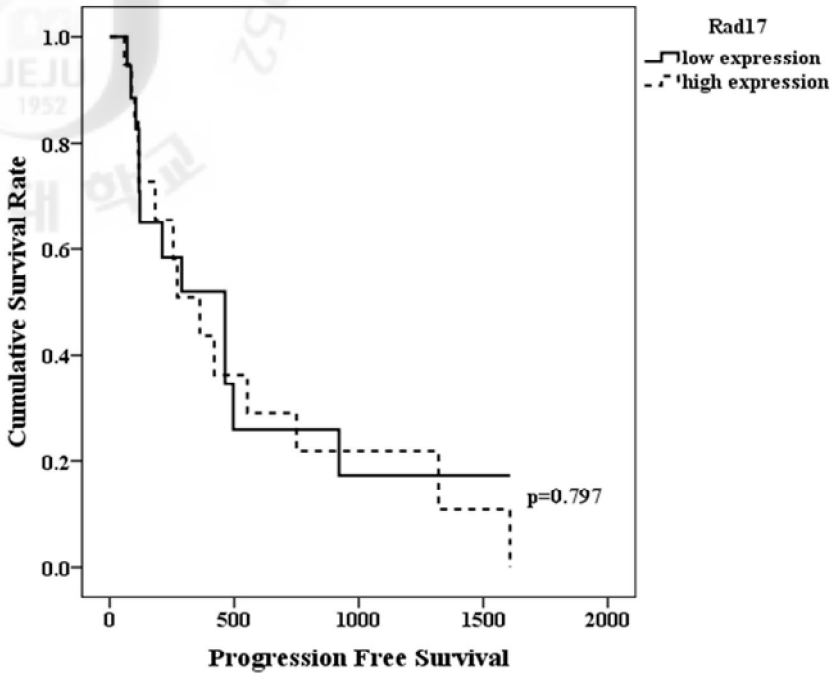
a



ATM
High expression

ATM
Low expression

B



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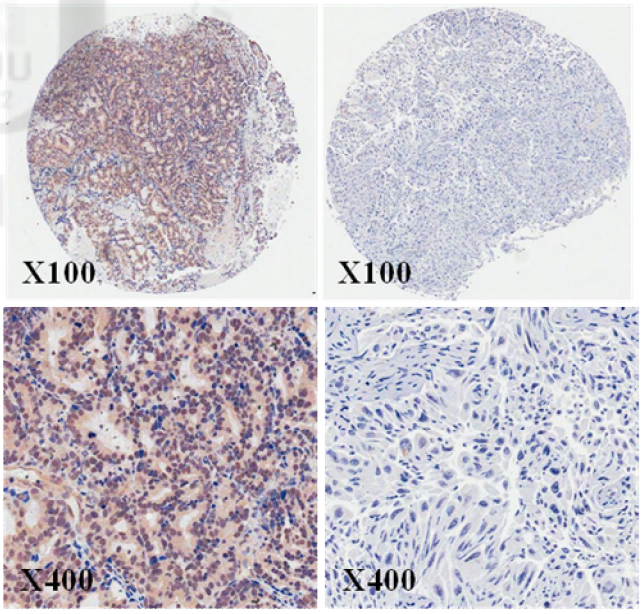
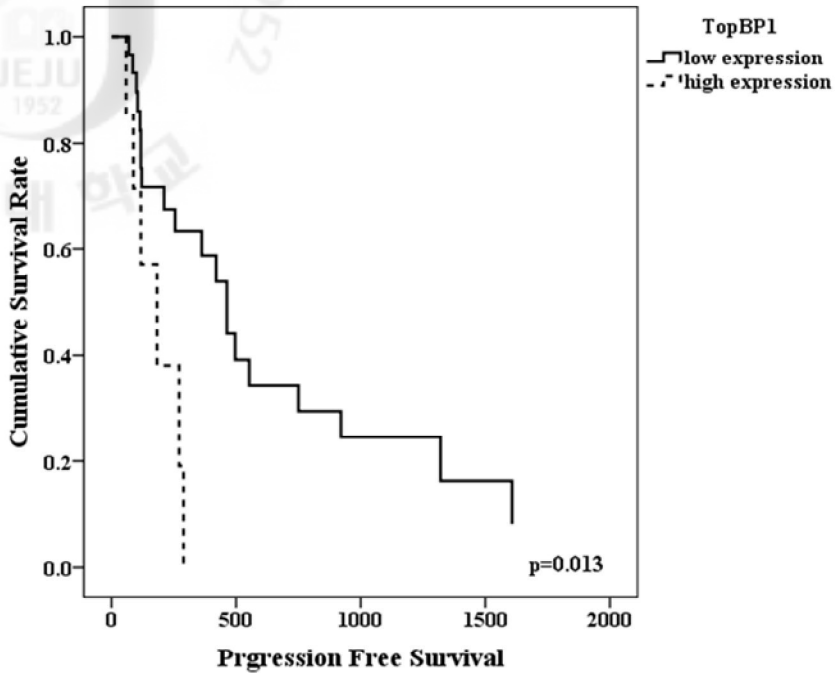


Rad17
High expression

Rad17
Low expression

C



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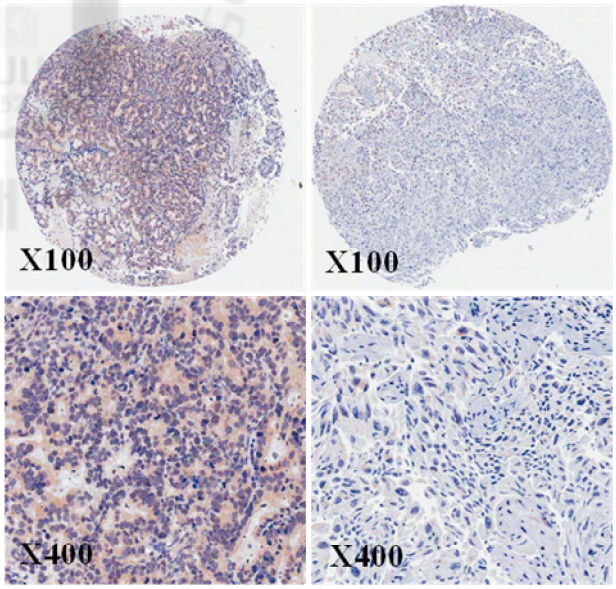
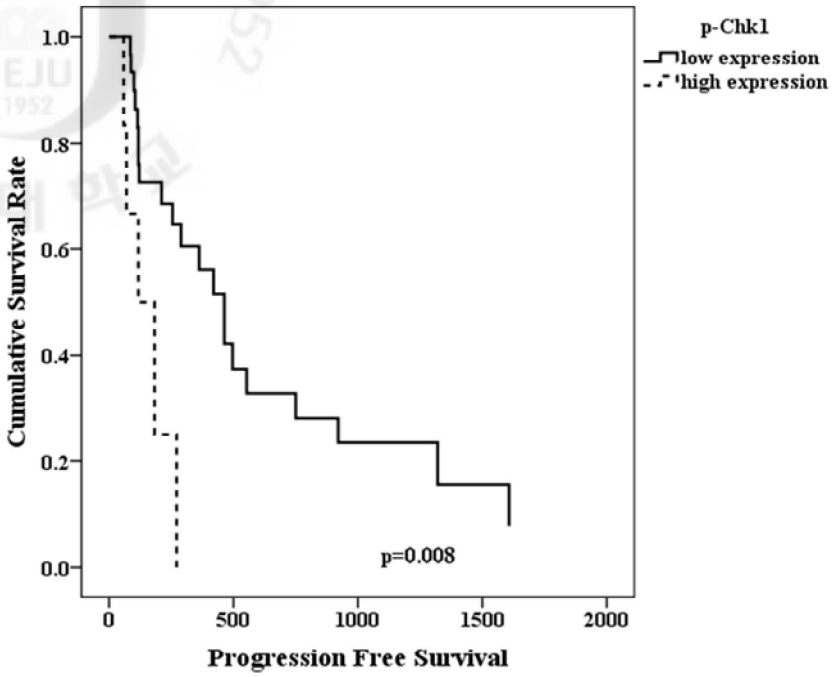


TopBP1 High expression | TopBP1 Low expression

D



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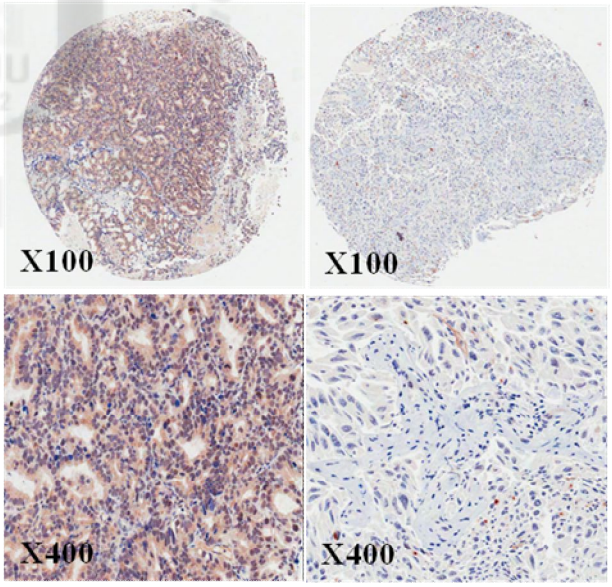
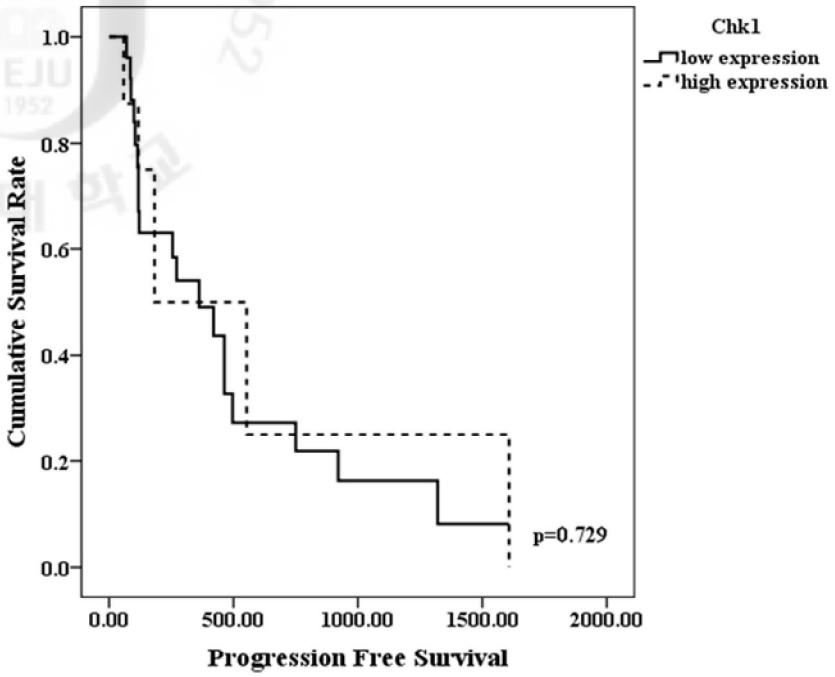


p-Chk1
High expression

p-Chk1
Low expression

E



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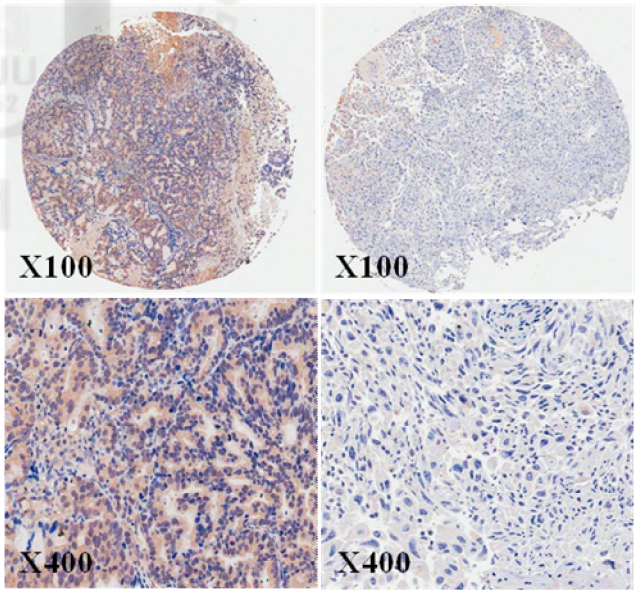
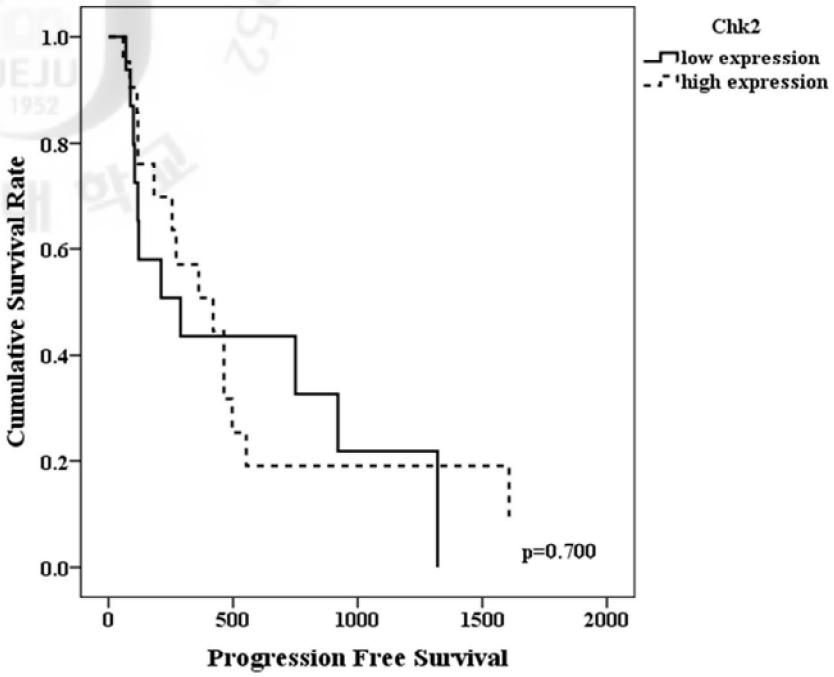


Chk1
High expression

Chk1
Low expression

F



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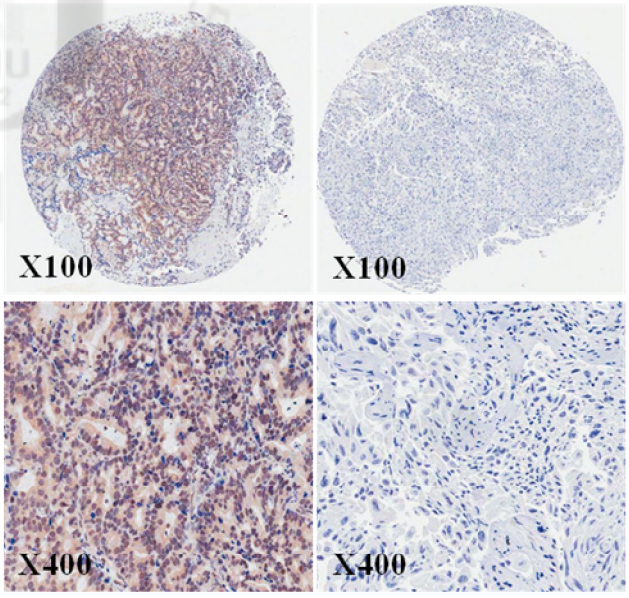
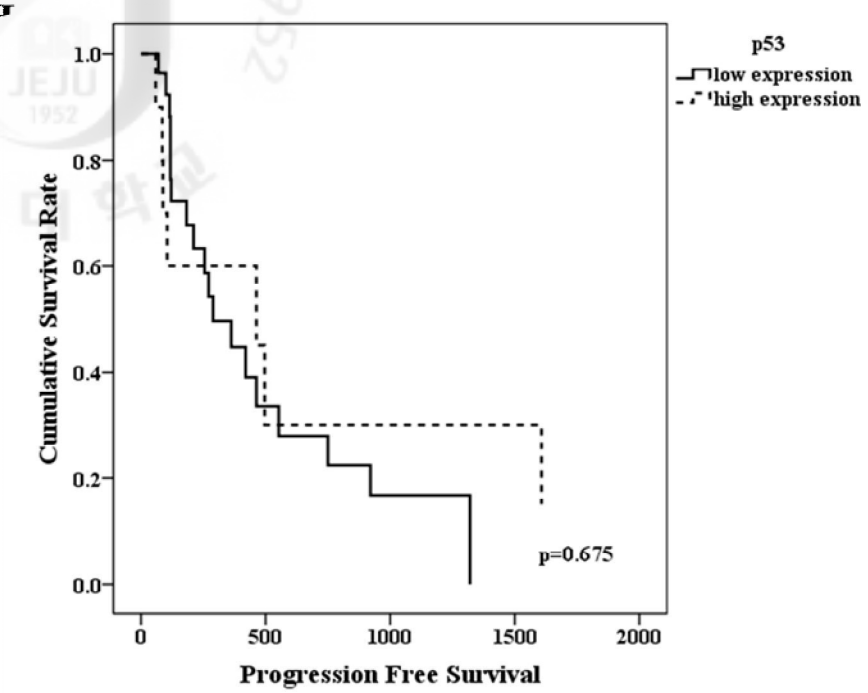


Chk2
High expression

Chk2
Low expression

G



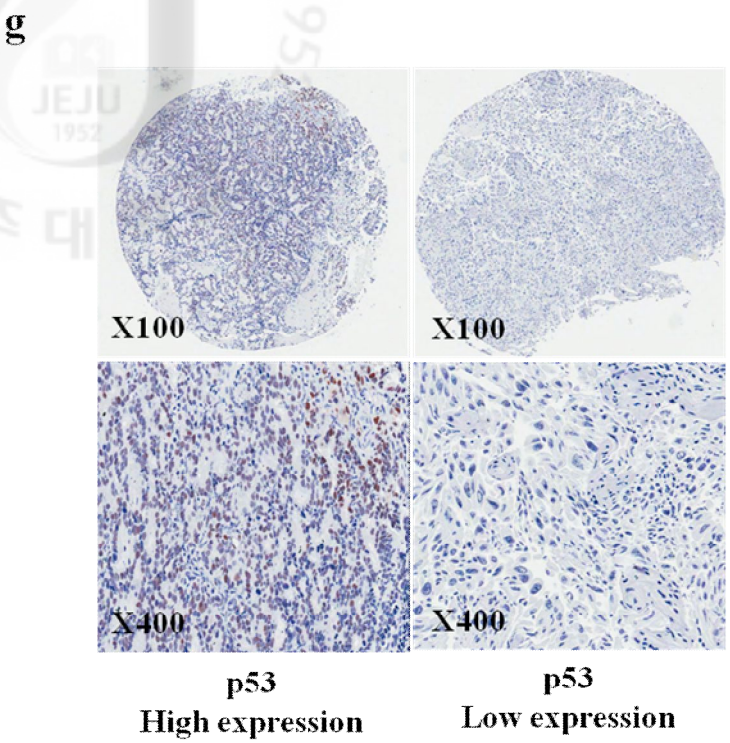
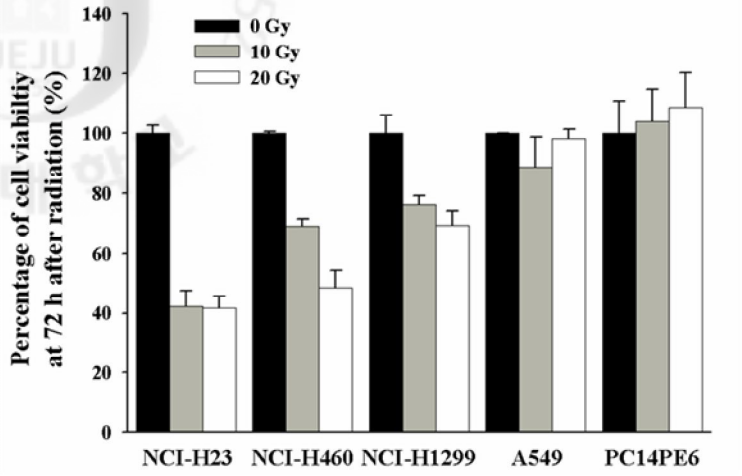


Fig. 2. Relationship between progression-free survival (PFS) and expression level of components of DNA damage checkpoint signaling by IHC analysis. (A-G) PFS according to expression of ATM, Rad17, TopBP1, p-Chk1, Chk1, Chk2, and p53, (a-g) Expression level of ATM, Rad17, TopBP1, p-Chk1, Chk1, Chk2, and p53.

A



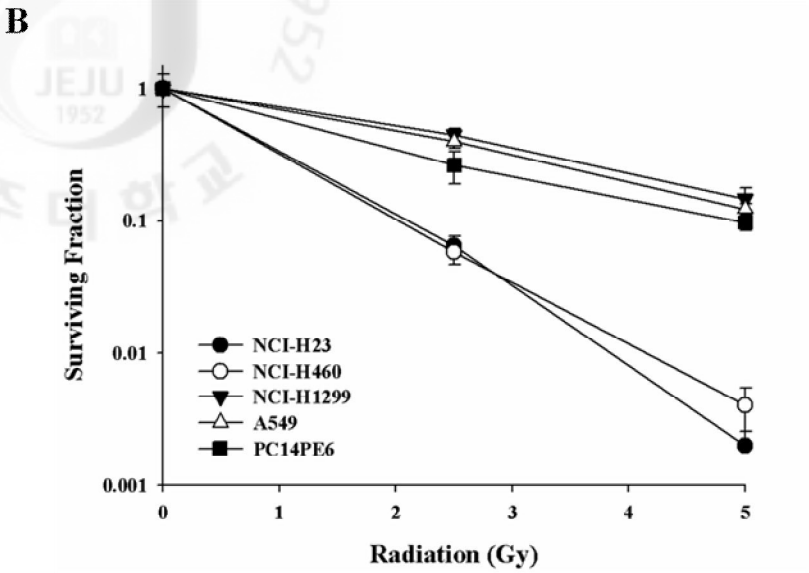


Fig. 3. Radio-sensitivity of five lung cancer cell lines (NCI-H23, NCI-H460, NCI-H1299, A549, and PC14PE6). (A) Cell viabilities of lung cancer cell lines on radiation were assessed by CCK-8 assay. (B) Clonogenic survival assays were performed with radiation. Values are mean ± S.D.

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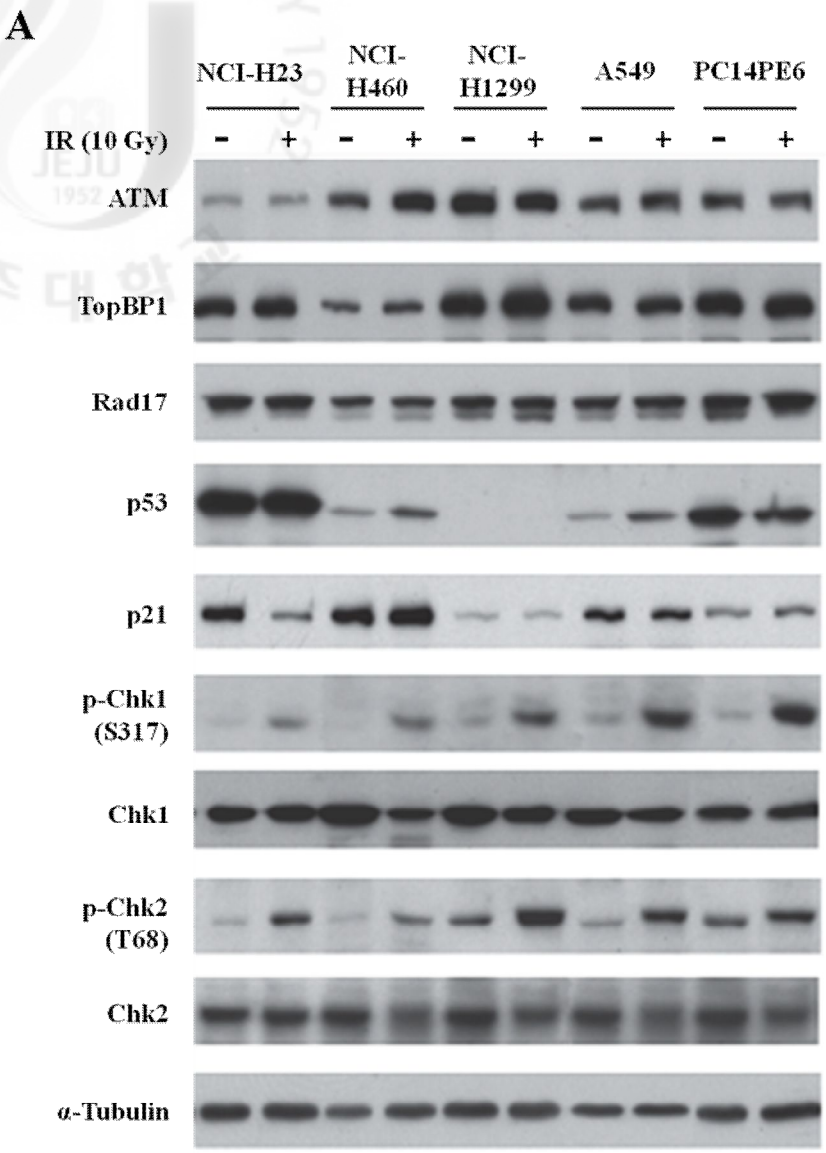


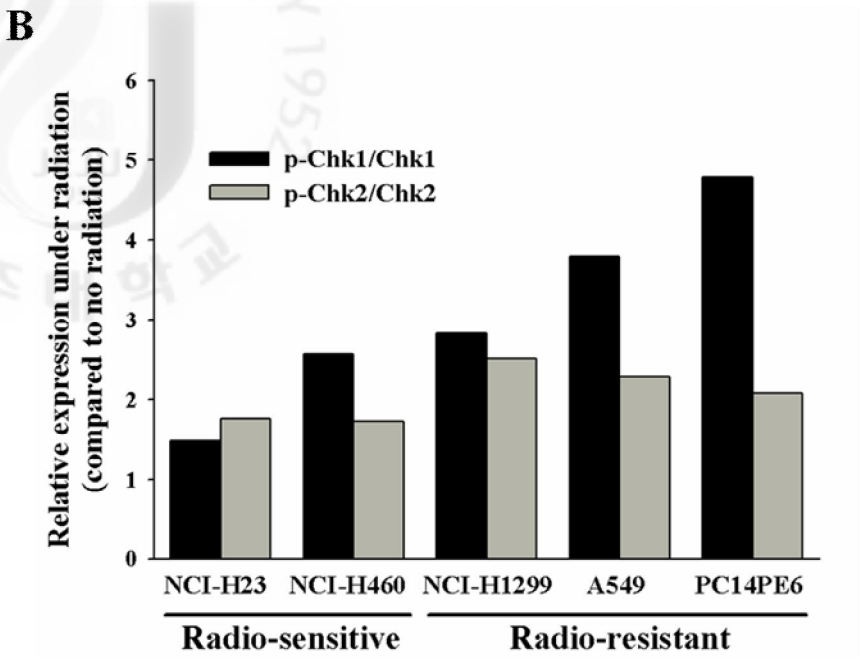
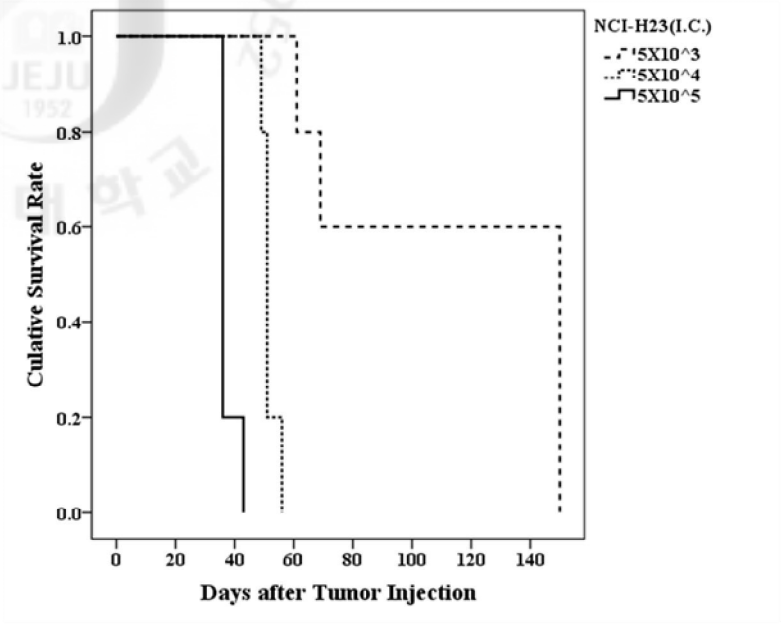


Fig. 4. Changes on expression of the components of DNA damage checkpoint under radiation. (A) Western blot analysis of the components of DNA damage checkpoint, (B) The values from the densitometric analysis were calculated in relation to the concentration of no radiation. Values are mean.

A



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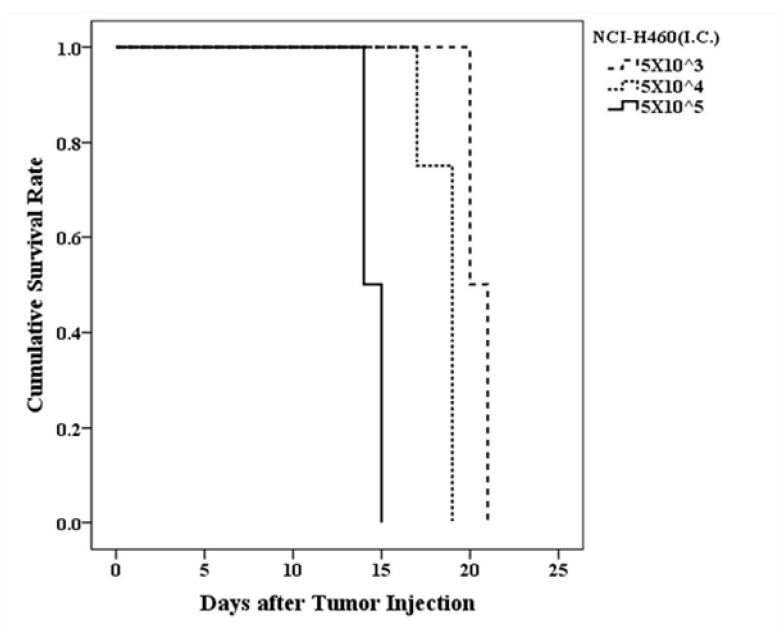

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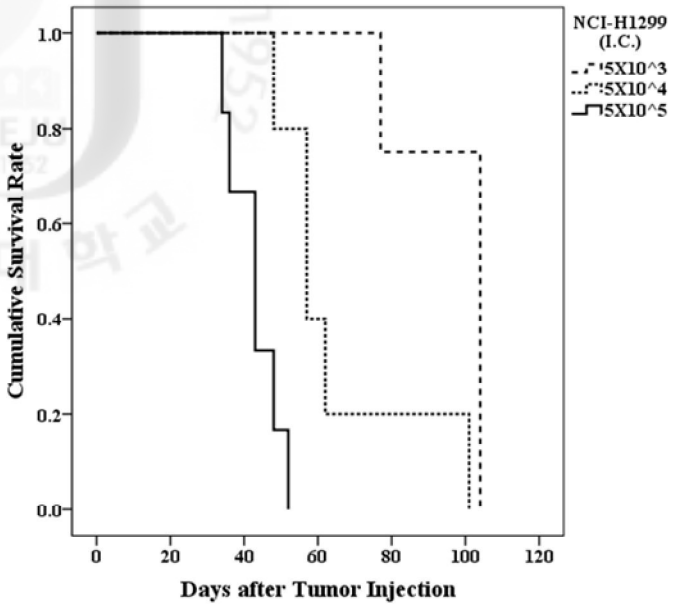

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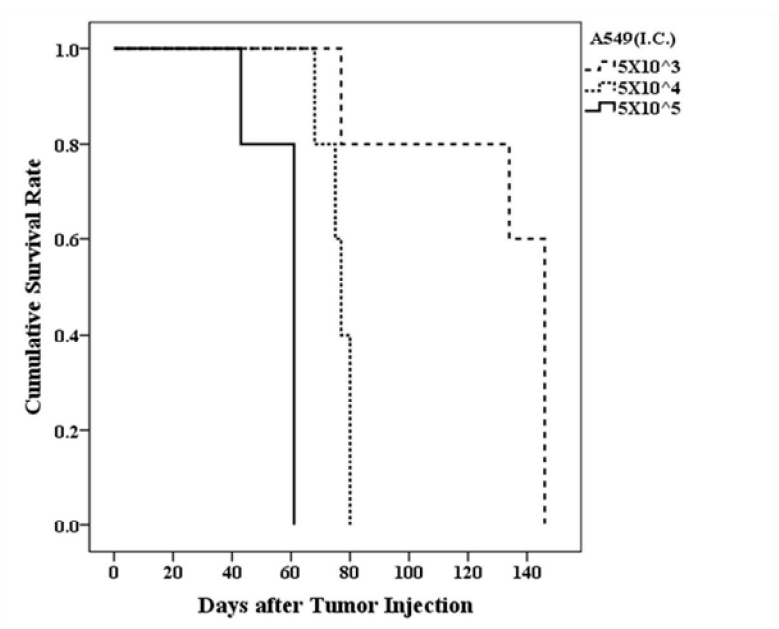


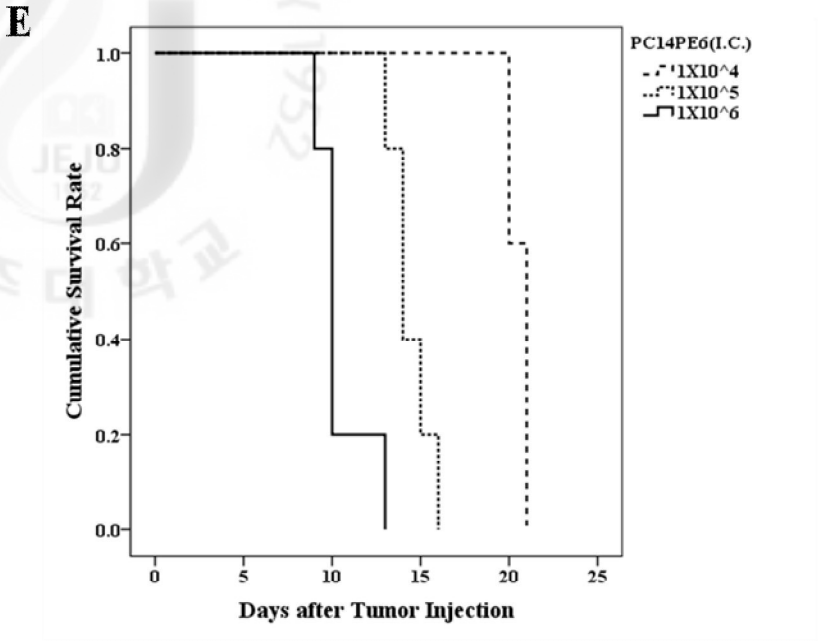


Fig. 5. Survival curves according to various cell number of lung cancer brain metastatic xenograft models using five cancer cell lines. (A) NCI-H23, (B) NCI-H460, (C) NCI-H1299, (D) A549, (E) PC14PE6.

Table 1. The median survival day according to cell numbers in lung cancer brain metastatic xenograft models

Cell dose ($X 10^3$)	Median survival day				Cell dose ($X 10^4$)	Median survival day
	NCI-H23	NCI-H460	NCI-H1299	A549		PC14PE6
5	**150**	**20.5**	**104**	**146**	**1**	**21**
50	**51**	**19**	**57**	**77**	**10**	**14**
500	**36**	**14.5**	**43**	**61**	**100**	**10**

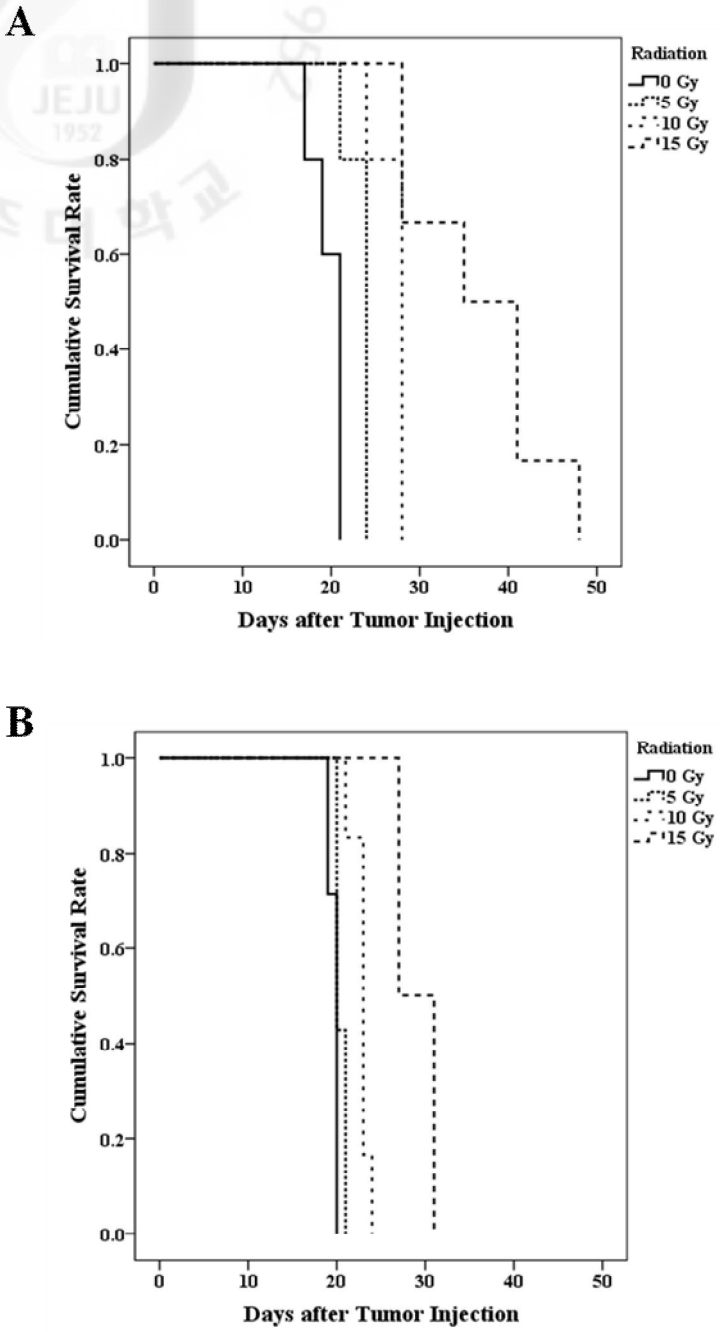
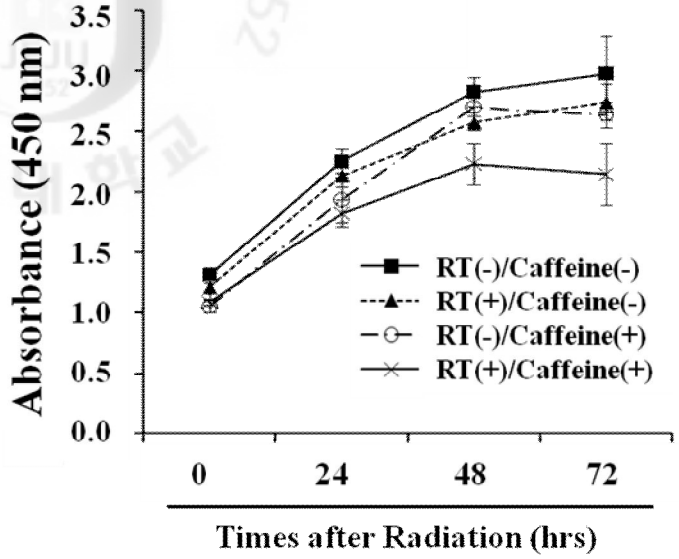


Fig. 6. The *in vivo* radio-response of lung cancer brain metastatic xenograft model. (A)

Survival curve of NCI-H460 lung cancer brain metastatic xenograft model under radiation, (B) Survival curve of PC14PE6 lung cancer brain metastatic xenograft model under radiation.

A



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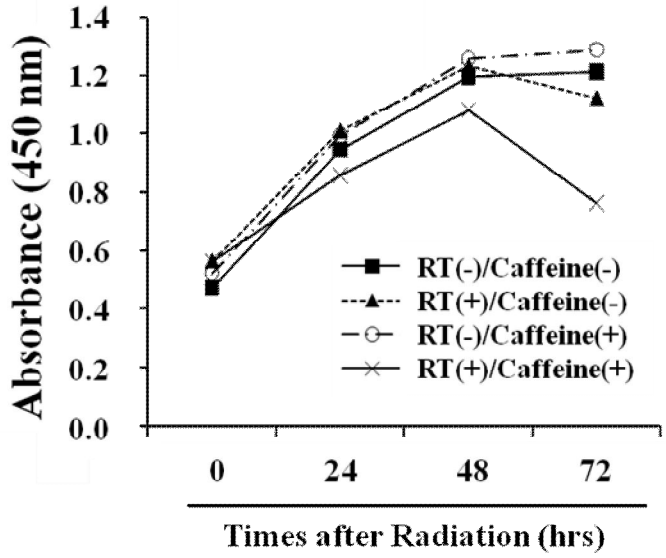


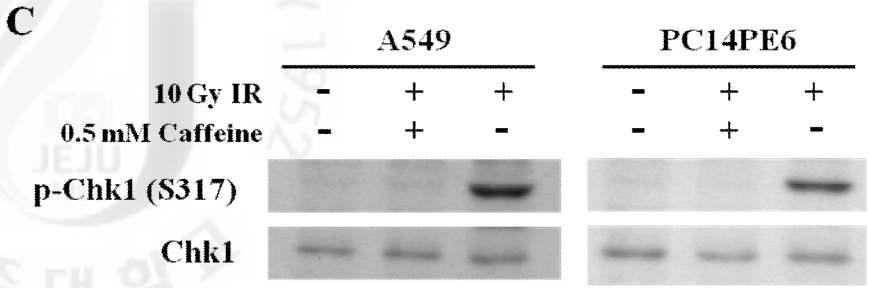
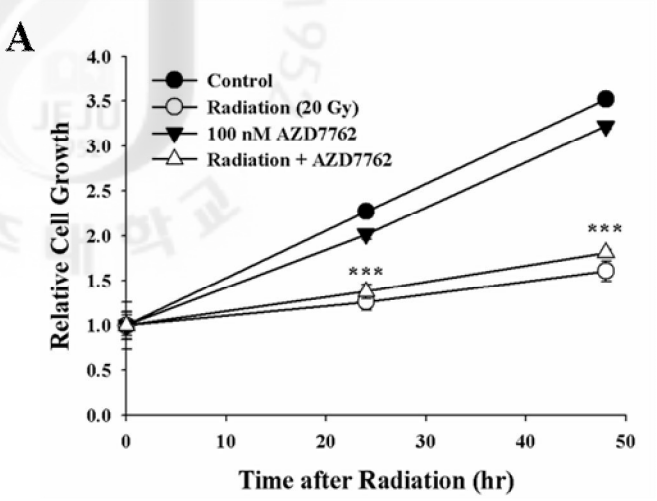


Fig. 7. Radio-sensitizing effect by inhibition of DNA damage checkpoint signaling with caffeine treatment. (A) The growth of A549 lung cancer cells and (B) The growth of PC14PE6 lung cancer cells were decreased by radiation and caffeine treatment. (C) Changes or activation of checkpoint kinase 1 by radiation or caffeine in A549 and PC14PE6 lung cancer cell lines were analyzed by Western blot. Values are mean ± S.D.



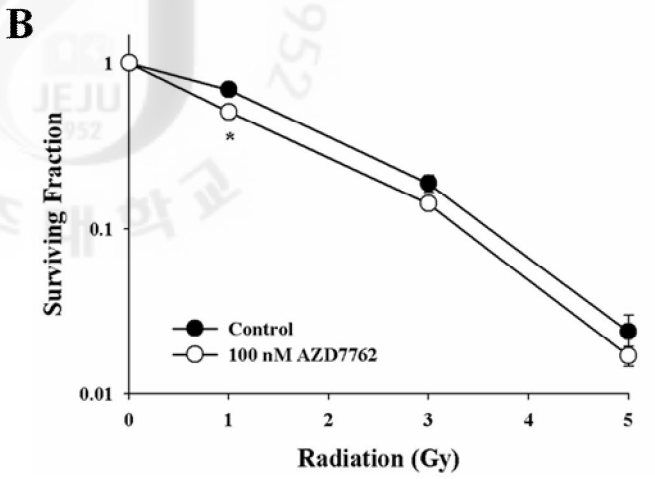
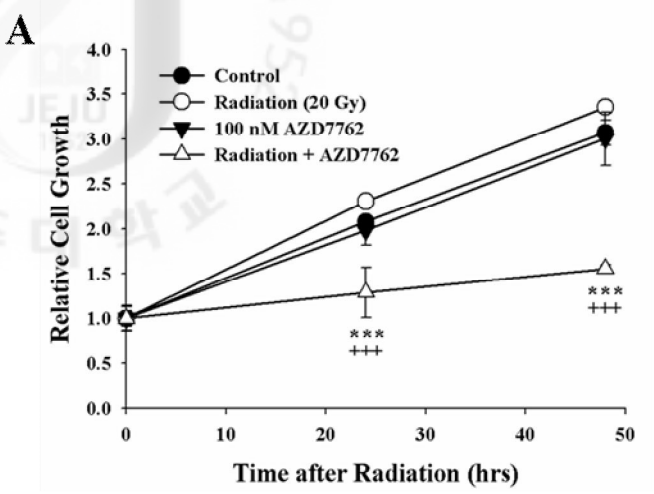
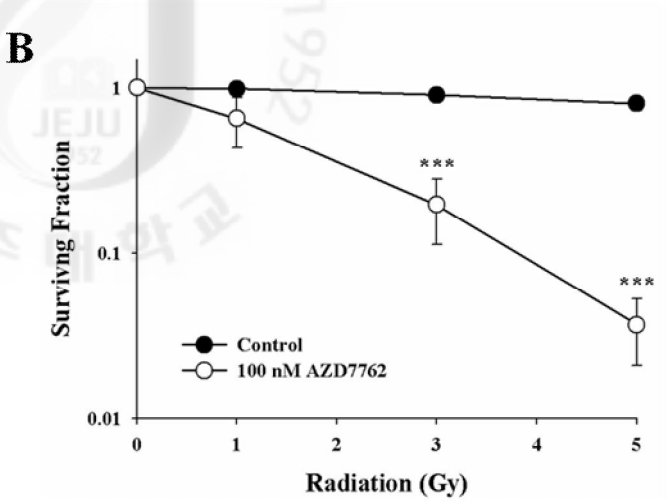


Fig. 8. Radio-sensitizing effect of AZD7762 in radio-sensitive NCI-H460 cells. (A) Cell proliferation and (B) clonogenic potential were assessed. Values are mean ± S.D. $^{*}p<0.05$, $^{***}p<0.001$ vs. Control.





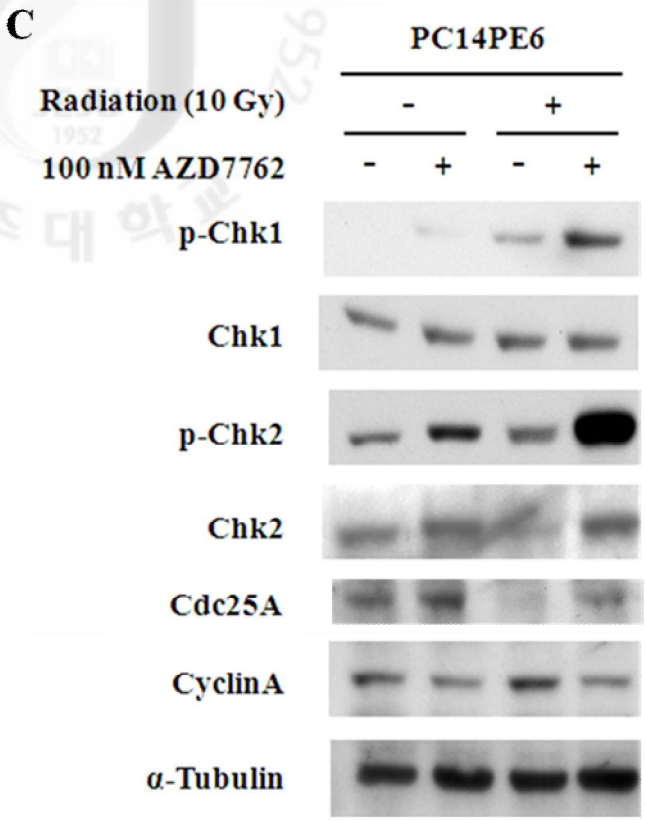
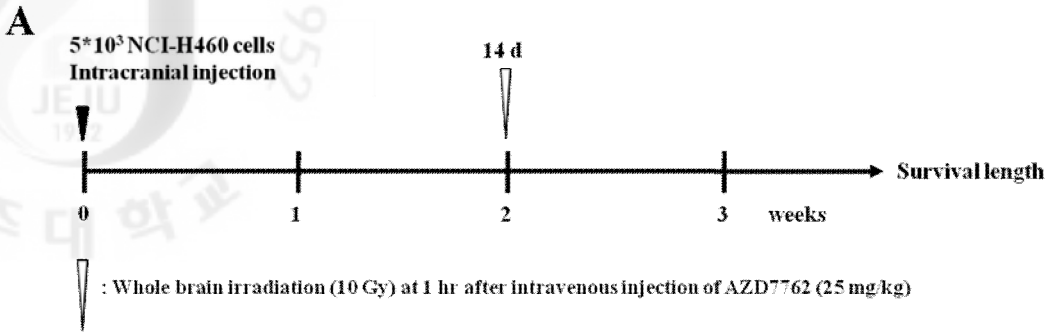


Fig. 9. Radio-sensitizing effect of AZD7762 in radio-resistant PC14PE6 cells. (A) Cell proliferation and (B) clonogenic potential were assessed. (C) Expressions of components checkpoint kinase signaling were analyzed by Western blot. Values are mean ± S.D. $^{***}p<0.001$ vs. Control; $^{+++}p<0.001$ vs. Radiation (20 Gy).



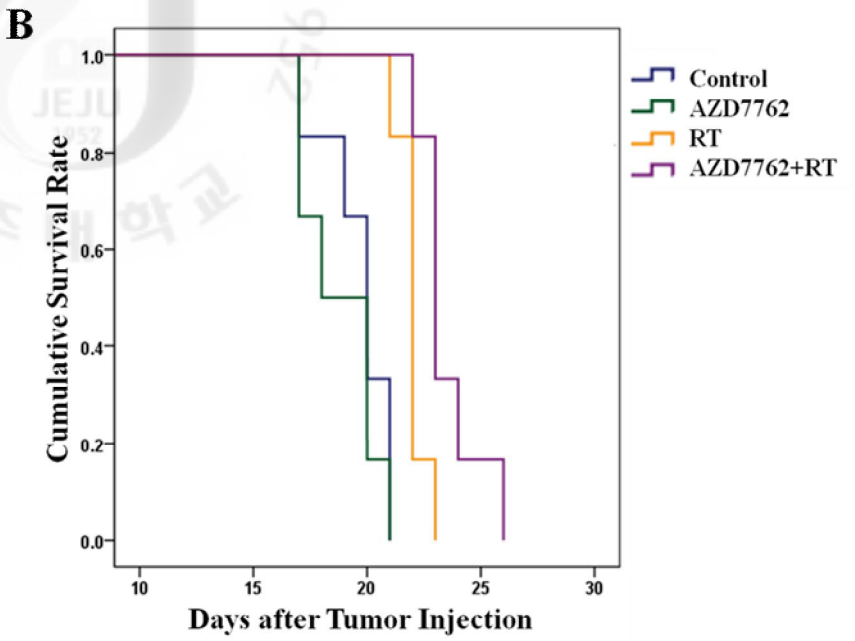
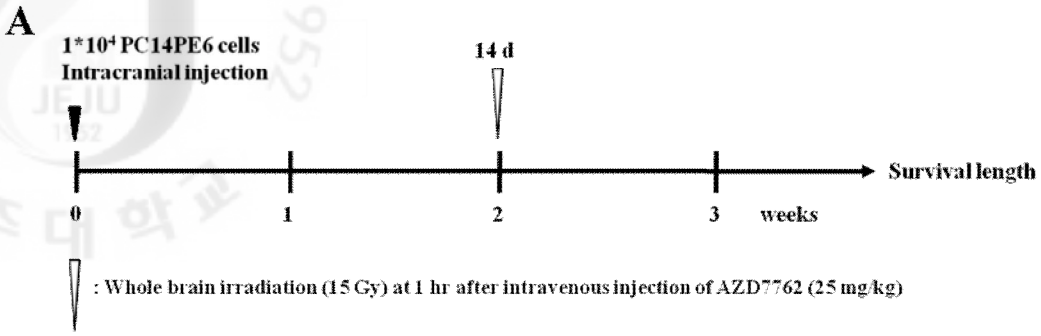


Fig. 10. Effects of AZD7762 on NCI-H460 brain metastatic xenograft in response to radiation. (A) Detailed experimental schedules, (B) Kaplan-Meier plot comparing survival of NCI-H460 brain metastatic xenograft model.

Table 2. Analysis data in NCI-H460 lung cancer brain metastatic xenograft model

Group		Survival			P value	
		Median (D)	Range (D)	ILS* (%)	Compared to Control	Compared to RT
Ⅰ	Control	20	17~21	-		0.002
Ⅱ	25 mg/kg AZD7762	18	17~21	-	0.443	0.001
Ⅲ	10 Gy RT	22	21~23	10	0.002	
Ⅳ	AZD7762+RT	23	22~26	15	0.001	0.020

* ILS; Increase in life span



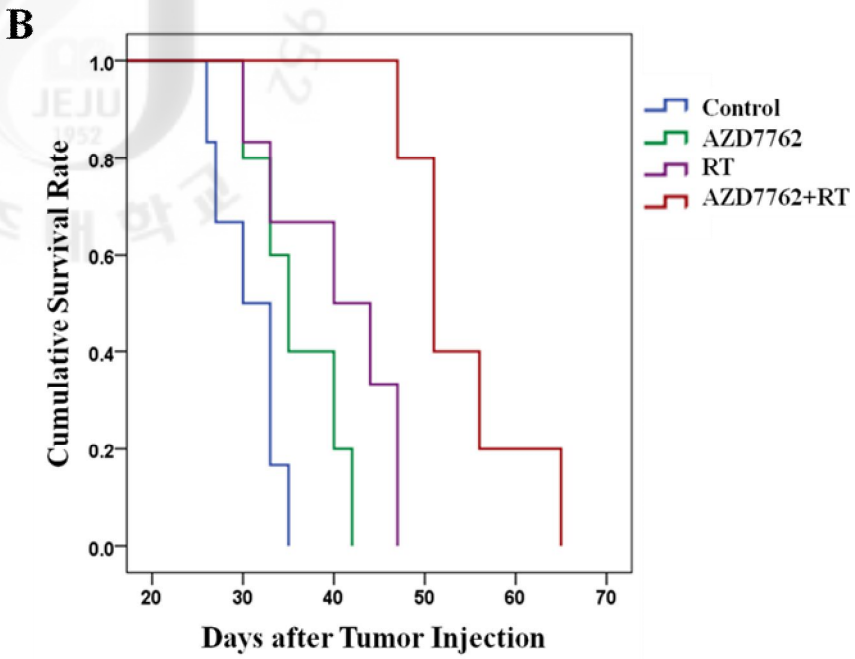
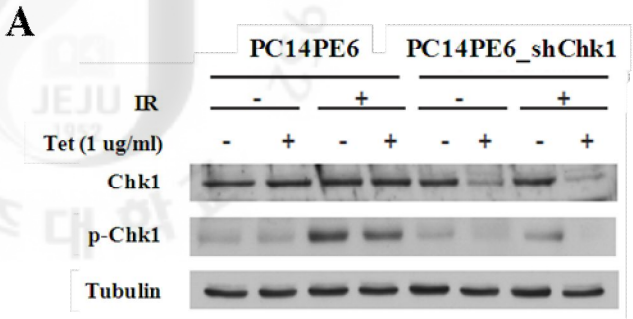


Fig. 11. Effects of AZD7762 on PC14PE6 brain metastatic xenograft in response to radiation. (A) Detailed experimental schedules, (B) Kaplan-Meier plot comparing survival of PC14PE6 brain metastatic xenograft model.

Table 3. Analysis data in PC14PE6 lung cancer brain metastatic xenograft model

Group		Survival			P value	
		Median (D)	Range (D)	ILS* (%)	Compared to Control	Compared to RT
Ⅰ	Control	30	26~35	-		0.020
Ⅱ	25 mg/kg AZD7762	35	30~42	17	0.078	0.150
Ⅲ	15 Gy RT	40	30~47	33	0.020	
Ⅳ	AZD7762+RT	51	47~65	70	0.002	0.005

* ILS; Increase in life span



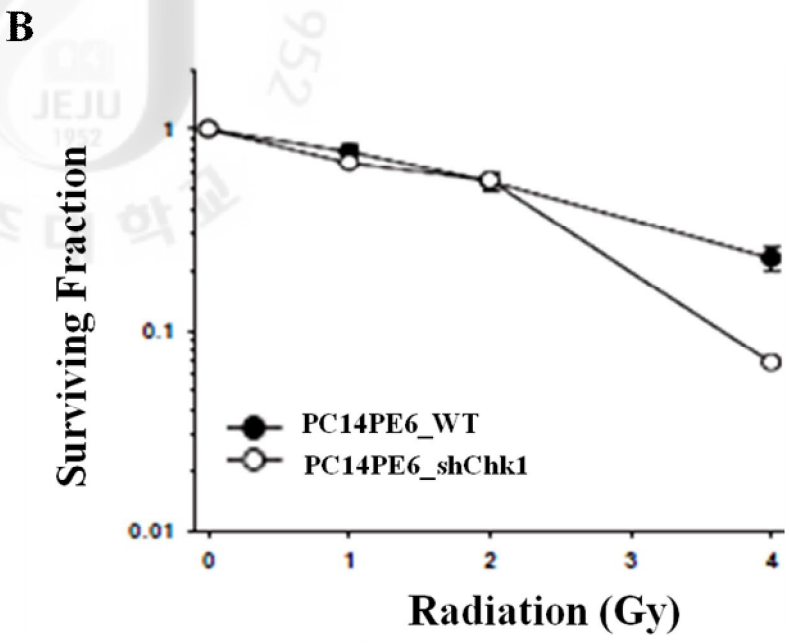
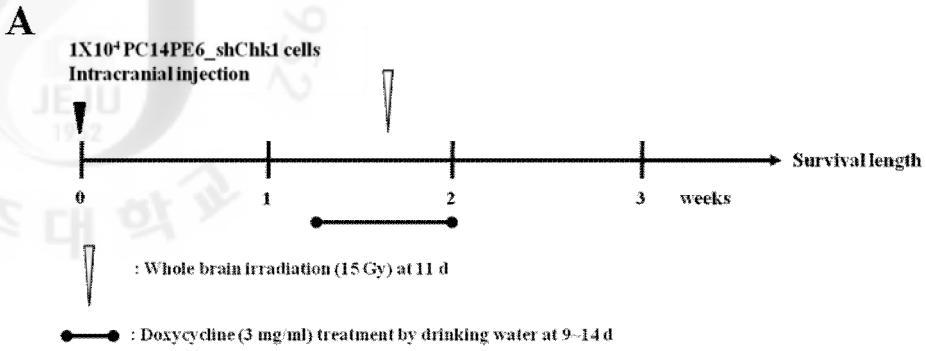


Fig. 12. Depletion of Chk1 enhances radio-sensitivity of PC14PE6 cells *in vitro*. (A) Chk1 protein was selectively depleted in Chk1 shRNA expressing PC14PE6 cells with treatment of 1 ug/ml of tetracycline (Tet). (B) Clonogenic survival assays were performed after shRNA induction.



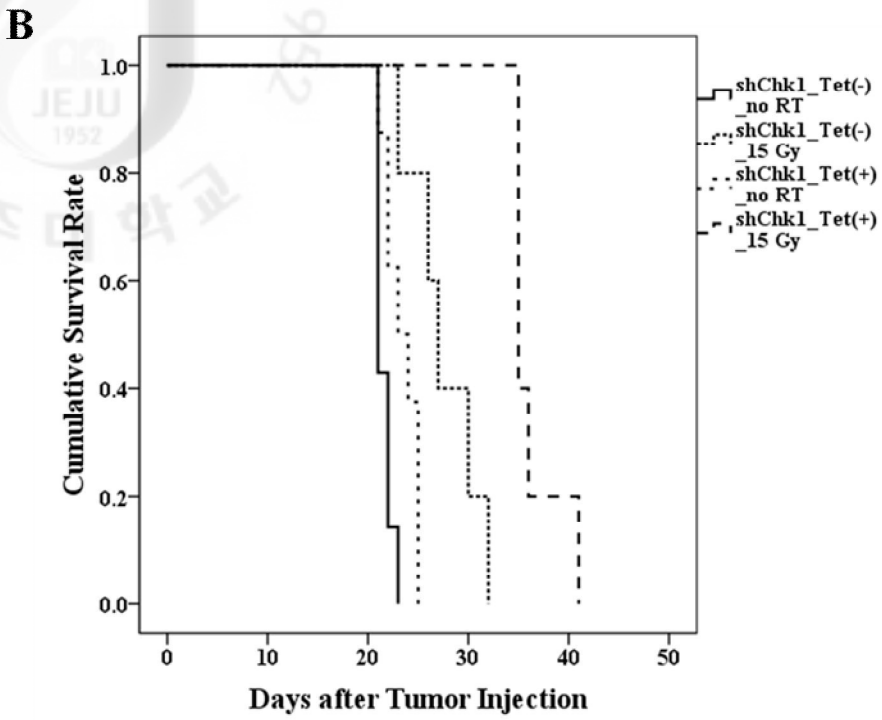


Fig. 13. Radio-sensitizing effect by Chk1 depletion in PC14PE6 brain metastatic xenograft model. (A) Detailed experimental schedules, (B) Kaplan-Meier plot comparing survival of PC14PE6 brain metastatic xenograft model.

Table 4. Analysis data of PC14PE6 lung cancer brain metastatic xenograft model, which is controlled Chk1 expression by tetracycline

Group		Survival			P value	
		Median (D)	Range (D)	ILS* (%)	Compared to Group Ⅰ	Compared to Group Ⅱ
Ⅰ	shChk1_Tet(-)_no RT	21	21~23	-		0.002
Ⅱ	shChk1_Tet(-)_15 Gy RT	27	23~32	29	0.002	
Ⅲ	shChk1_Tet(+)_no RT	23	21~25	-	0.015	0.012
Ⅳ	shChk1_Tet(+)_15 Gy RT	35	35~41	52	0.001	0.002

* ILS; Increase in life span

Part Ⅱ. c-Met enhances radio-resistance in primary breast cancer and breast cancer brain metastatic cancer

Introduction

Historically, brain metastases develop in 15~20% of patients with systemic metastatic disease [63]. At autopsy, asymptomatic metastatic lesions are found in the brains of more than 30% of breast cancer patients [64]. Breast cancer is amongst the leading cause of death in the female population in industrialized countries [65]. Additionally, breast cancer is the solid tumor that most commonly give rise to leptomeningeal metastases, lesions in the tissue that lines the brain and spinal cord [66]. Breast cancer is a disease with a number of subtypes and patients with metastatic triple-negative breast cancer (ER-, PR-, HER2 unamplified) tend to develop brain metastases at a high rate [67]. Despite significant advances in understanding the molecular mechanisms of metastases and development of new diagnostic, prognostic and therapeutic tools, the prognosis of brain metastases is even now very poor [68,69,70]. Current treatments for brain metastases are palliative and centre on surgery and radiation therapy. However, these treatments cause physical and cognitive morbidities, and improvements in patient survival are still measured in weeks or months [7]. Ionizing radiation (IR) is a critical component in the treatments of breast cancer patients with all stages of the disease and a gold standard modality in patients with multiple brain metastases, demonstrating improved overall survival (OS) in recent meta-analysis [71,72,73,74].

However, the problem of recurrent or persistent disease exists due to tumor radio-resistance, and the development of distant metastases after IR has presented a major obstacle to treatment. An important mechanism that underlies the development of such resistance is that cancer cells recognize DNA lesions induced by IR, and repair these lesions by activating

various DNA repair pathways [75]. Constitutive activation of the DNA repair and reduced production of reactive oxygen species (ROS) in response to radiation were reported as main mechanisms of radiation resistance [50,76,77,78]. Among these pathways, the pathway associated with c-Met, a receptor tyrosine kinase (RTK) with high-affinity for hepatocyte growth factor (HGF) that is markedly over-expressed in aggressive forms of a number of major human cancers, has gained particular attention because of its prominent role in invasion, metastases and especially radio-resistance [79]. Based on these multiple roles of HGF/c-Met pathway in the enhanced radio-resistance, selectively targeted blockade of the HGF/c-Met pathway could improve therapeutic efficacy of radiotherapy by improving radio-sensitivity.

In this present study, we found that c-Met over-expression was induced in response to IR in breast cancer cells. The radio-sensitizing effect of c-Met silencing *in vitro* showed that increased c-Met activity is required for radio-resistance. In addition, c-Met inhibition combined with IR showed tumor regression in breast cancer orthotopic xenograft model and lengthened median survival periods in a breast cancer brain metastases xenograft model. Our findings established a basis for a novel strategy to target the HGF/c-Met pathway in combination with radiation, when treating primary and brain metastatic breast cancer and possibly other c-Met pathway dependent cancers.

Materials and Methods

Cell culture

Human breast cancer cell lines (MDA-MB-435 and MDA-MB-231), which were purchased from ATCC, their subclones expressing scrambled or c-Met targeting shRNA, and *ex vivo* cells dissociated form mouse brains were grown in MEM or DMEM supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 ug/ml).

Engineering of breast cancer cells subclones

The constructs of c-Met-targeting shRNA (c-Met shRNA) are 5′-AGAATGTCATTCTACATGAGC-3′. The retroviral vectors (pSuperRetro vector; Oligogene, Seattle, WA) for scrambled or c-Met have a pPGK backbone with an inserted H1 RNA promoter driving shRNA expression. Cells transfected with scramble or c-Met shRNA were selected with 4 ug/ml puromycin (Invitrogen Corporation, Camarillo, CA) over four weeks.

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Isolation and culture of ex vivo cells

For obtaining tumor cells from the xenograft models, five mice per group were sacrificed five days post *in vivo* radiation treatment. Excised brain tissue was minced and then incubated with mixture of collagenase, DNase and deaminase for 30 min. After washing with PBS, minced tissue was filter with 0.2 um strainer and tumor cells were purified with Percoll. *Ex vivo* cells of MDA-MB-435 tumor cells were maintained with MEM medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin, respectively.

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Irradiation

Human breast cancer cells, subclones expressing scrambled or c-Met shRNA, or mice were irradiated using a blood irradiator (IBL 437C blood Irradiator, CIS US, Inc., Bedford, MA) at a dose rate of 2.3 Gy/min.

Clonogenic assay

To evaluate clonogenic potential of irradiated cells, the assay described by Franken et al. [37] was used. Briefly, 50 cells were seeded in 6-well plates. After 18 hours, cells were irradiated (1-4 Gy) and cultured for 14 days. Colonies containing 50 cells or more were considered representative of clonogenic cells. The survival fraction was calculated using the formula: [(number of colonies formed after radiation)/ (numbers of cells seeded X plating efficiency)], where plating efficiency is the ratio of seeded cells that gave rise to clones under no radiation conditions.

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qRT-PCR analysis

RNAs were extracted (QIAGEN) from radiated cells and their complementary DNAs were synthesized (Invitrogen Corporation, Camarillo, CA) per manufacturers' instructions. Quantitative reverse transcriptase PCR was performed using primers (sense; 5'TGGGAAGAAGATCACGAAG-3' antisense; 5'-TGTAGATTGCAGGCAGACAGA-3') in LightCycler 480 (Roche Diagnostics, Indianapolis, IN, USA).

Flow cytometry analysis

For detection of c-Met elevation after radiation, cells were collected at 48 hours after radiation (5 and 20 Gy) and then fixed with 4% paraformaldehyde. c-Met was detected with mouse anti-human c-Met polyclonal antibody (R&D systems, Minneapolis, MN, USA) conjugated with APC (eBiosciences, San Diego, CA, USA).

Xenograft model

To produce the breast cancer orthotopic or the brain metastatic animal model, six-week-old female athymic nude mice were used. 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).

For the orthotopic animal model, anesthetized mice were injected with subclones of expressing scramble or c-Met shRNA (1 X 10^6/50 μl) into second thoracic mammary fat pad. When tumors reached about 250 mm^3, animals were anesthetized and then irradiated with 10 Gy into tumor locally. Tumor diameter was measured using vernier calipers and tumor volume determined by calculating the volume of an ellipsoid using the formula: [length X $(width)^2$ X 0.5].

For the brain metastatic animal model, subclones of expressing scramble or c-Met shRNA (1 X 10^5/5 μl) were stereotaxically injected into the left striata of mice (coordinates; AP +1.0, ML +1.7, DV -3.2 mm from Bregma). At 15 days after cell injection, mice received 10 Gy of whole brain irradiation. Mice were sacrificed either at 20% body weight loss or moribund status were observed. For analysis of tumor mass volume and apoptosis level in the brain metastatic model, 3 mice were sacrificed at 5 days after whole brain irradiation. The brains of these mice were removed and processed for paraffin embedding. For analysis of tumor mass volume, standard H&E staining was performed in the paraffin sections and observed under optical microscope at 40X magnification. The DeadEnd fluorometric TUNEL system (Promega, USA) was used to assay apoptosis and observed under optical microscope at 400X magnification.

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Statistics

Numerical results were expressed as means ± S.D. or means ± S.E. Statistical comparisons were analyzed by one-way analysis of variance (ANOVA) followed by the least significant

difference (LSD) test. Kaplan-Meier estimates of the survival function were plotted, and the significance of differences in overall survival was calculated by the Mantel-Cox log-rank test. A significance level of $p<0.05$ was used for all test. SPSS-PASW statistics software version 18.0 was used for all the statistical analyses.

Results

Response of c-Met under radiation in breast cancer cells

To estimate the change in c-Met expression with radiation in breast cancer cells, c-Met intensity and mRNA level of c-Met were detected using FACS and qRT-PCR, respectively, after *in vitro* radiation in MDA-MB-435 and MDA-MB-231 cells. In both breast cancer cells, intensity and mRNA level of c-Met were dose-dependently increased with radiation. The c-Met intensity was increased 1.48 and 1.45 folds by radiation of 5 Gy and 2.47 and 2.51 folds by radiation of 20 Gy compared to 0 Gy in MDA-MB-435 cells and MDA-MB-231 cells, respectively (Fig. 14A). The mRNA levels of c-Met were elevated 1.82 and 1.62 folds by radiation of 5 Gy and 2.56 and 2.31 folds by radiation of 20 Gy compared to 0 Gy in MDA-MB-435 cells and MDA-MB-231 cells, respectively (Fig. 14B). These results suggest that c-Met have a role in radiation-resistant in breast cancer cells.

Increase of c-Met under radiation in radio-resistant clone of breast cancer brain metastases cells

To investigate the correlation between c-Met and radio-resistance in breast cancer brain metastases, we established an animal model of breast cancer brain metastases using MDA-MB-435 cells and we delivered 10 Gy of radiation into the brain of animal models locally. The radio-response of cells, which were dissociated from animal model of breast cancer brain metastases after *in vivo* radiation, was analyzed by clonogenic assay. The cells from mice with *in vivo* radiation exposure showed more high clonogenic potential than the cells from control mice (Fig. 15A). Therefore, we could establish resistant clone on radiation. The expression of c-Met mRNA in radio-resistant clone was increased 4 fold compared to control (Fig. 15B). These results suggest that c-Met enhances radiation-resistance in breast cancer

brain metastases and c-Met could be functional target to overcome radiation-resistance.

Radio-sensitizing effect through inhibition of c-Met

We established c-Met depleted breast cancer cells using shRNA and conducted clonogenic assay to verify radio-sensitizing effect by inhibition of c-Met. In both MDA-MB-435 and MDA-MB-231 cells, which were depleted c-Met, the response on radiation was more sensitive compared to their control cells (Fig. 16C and D).

In order to confirm the radio-sensitizing effect from *in vitro* study, the responses on radiation were estimated in the breast cancer orthotopic xenograft model and the breast cancer brain metastatic xenograft model. The tumor growth after radiation into mammary tumor was compared between models with or without c-Met depletion (Fig. 17B). The tumor growth was inhibited 24.5% by radiation in the control. However, the tumor growth with c-Met depletion was inhibited 43.2%. Therefore, we observed a radio-sensitizing effect by targeting c-Met in the breast cancer orthotopic model.

Also, we estimated the radio-sensitizing effect of c-Met depletion through comparison of survivals of each group in the breast cancer brain metastatic xenograft model. The survivals of each treatment group are shown at Fig. 18 and Table 5. The median survival was increased 13% by radiation in the group, with no c-Met, inhibition and the median survival was increased 32% by radiation in the c-Met depleted group (Fig. 18B and Table 5). Additionally, the tumor volumes were determined at 5 days after whole brain irradiation in breast cancer brain metastatic xenograft model (Fig. 19). Radiation alone or c-Met silencing reduced tumor mass volume in brain, slightly. In contrast, by combining c-Met silencing and radiation, the tumor mass volume was decreased significantly. The immunohistochemical data demonstrated that the inhibition of tumor growth by a combination of c-Met silencing and radiation had a corresponding enhancement of apoptosis (Fig. 20). Radiation alone modestly induced apoptosis. But by combining c-Met silencing and radiation, the number of TUNEL-positive cells increased, significantly.

These results suggest that radio-sensitization is mediated by c-Met inhibition and c-Met is a functional target for enhancement of sensitivity to radiation.

Discussion

Radiation has been shown to activate multiple signaling pathways, including MAPK, JNK, and PI3K, that in turn affect cell survival and mitogenic responses [80]. The mechanisms causing tumor radio-adaptive resistance are attracting a great deal of interest because of their essential role in the efficacy of clinical anti-cancer radiotherapy. Deeper investigations of the mechanisms of radiation resistance have hinted at the possibility that we can sensitize tumors while sparing normal tissues by specific molecular targeting agents, many of which have shown promising results in preclinical studies thus demonstrating that the combination of radiation therapy and molecular targeting agents can improve outcome without major toxicity. As an example of radio-sensitizing effect of molecular targeting agent, this present study demonstrates that radiation-induced up-regulation and activation of c-Met signaling in a panel of human breast cancer cells is associated with radio-resistance, and that targeting the HGF/c-Met pathway could be a potential therapeutic strategy that generates synergistic antitumor activity in combination with radiation in both primary mass and brain metastases of breast cancer. Hepatocyte growth factor (HGF) is a multifunctional heterodimeric protein typically produced by mesenchymal cells. Its activities are mediated through its cellular receptor, a transmembrane tyrosine kinase encoded by the proto-oncongene c-Met. In malignant cells, HGF has been shown to protect cells from death induced by a variety of DNA-damaging agents, including radiation and topoisomerase inhibitors [81]. Interestingly HGF/SF not only blocked DNA damage-induced apoptosis but also enhanced the rate of repair of DNA strand breaks [82]. HGF and c-Met are co-expressed and often overexpressed in a broad spectrum of human solid tumors including lung, breast, and brain malignancies [83,84]. Therefore, c-Met has been suggested as an independent prognostic factor for breast cancer patients [85,86]. Although the detailed mechanism of induced c-Met up-regulation by

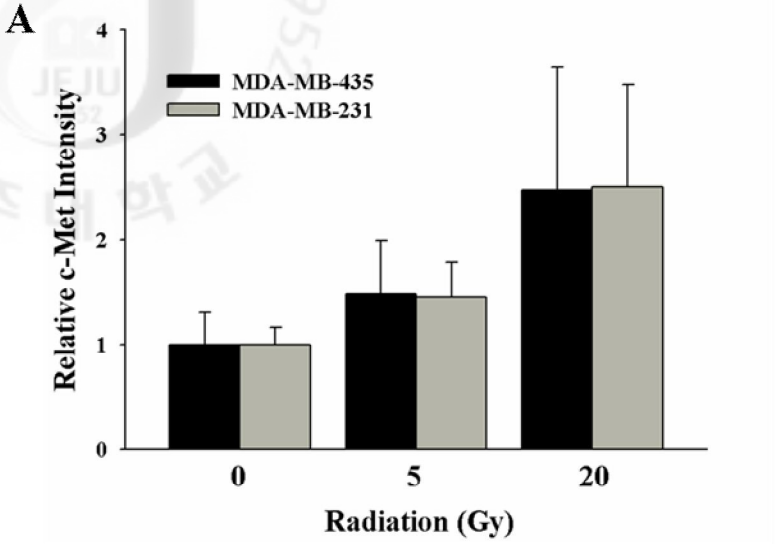
radiation was not elucidated in this study, a recent study showed that c-Met expression is increased via activation of ATM and NF-κB resulting in activation of ligand-independent c-Met signaling and critical downstream effectors, and hypersensitivity to HGF stimulation [87]. The c-Met receptor concomitantly activates multiple pathways, including GRB2-RAS, PI3-K, SRC, and Signal Transducer and Activator of Transcription (STAT) and these transduction pathways have been shown to be critical for the physiological and pathological effects elicited by the *MET* oncogene [88]. Our *in vivo* study suggests additional mechanism for the enhanced tumor regression and apoptosis by combining c-Met silencing and radiation besides radio-sensitizing effect. Therefore, the promising results of the present study suggest that specific targeting of c-Met can provide synergistic anti-tumor activity by acting on both cancer cells and their site-specific microenvironment in addition to radio-sensitizing effects on tumor cell itself.

삭제됨:

Cancer metastases are the single most important factor influencing cancer patient mortality, and therefore, controlling the metastatic spread of tumors remains a crucial target for the successful treatment of cancer. With improvements in control of systemic disease of breast cancer and in survival, the brain is emerging as a sanctuary site of relapse in patients with otherwise controlled breast cancer. Although considerable progress has been made in the treatment of brain metastases, conservative management, chemo- or radiotherapy remains the cornerstone of management in the majority of patients and unfortunately, conventional cytotoxic anti-cancer therapies have shown an unsatisfactory outcome. For the treatment of brain metastases in patients with breast cancer, simultaneously administered agents, which can be used to enhance the effect of radiotherapy aiming either at additive cell kill or true radio-sensitization, are being studied for this poor prognostic subgroup. Given the direct clinical situation, the development of model systems to study brain metastases appears to be a promising strategy for the identification and preclinical development of potential therapeutic leads. Based on these facts, we recapitulated the brain metastases of breast cancer via brain metastases model established by intracranial injection and tested the therapeutic

efficacy of c-Met silencing. The comparably enhanced antitumor radiation responses in both the orthotopic and brain metastases models with distinct tumor microenvironment indicate that the radio-sensitizing response to HGF/c-Met pathway inhibition may not be organ specific, and can be expected in other c-Met-dependent tumors, many of which commonly metastasize to multiple organ sites. In future studies, targetable signaling pathways including c-Met involved in tumor cells-stromal interactions in the brain and respective landscaping role in promoting brain metastases should be investigated in detail. At the same time, novel administration method which can enhance the delivery of the small molecular inhibitor into the brain mass by passing the blood brain barrier effectively remains to be developed.

Finally, the established breast cancer cell lines used in this study have their own disadvantages. Until now, many researchers and pharmaceutical companies have been developing therapeutic drugs by using traditional and artificial model systems such as established cell lines and genetically modified cells. In other words, researchers had to rely on such suboptimal systems that poorly reflect the clinical outcome because of the lack of access to patient-derived cells. Therefore, further study on c-Met radio-sensitizing effects is required *in vivo* model based on patient-derived breast cancer cells for a more clinically-relevant preclinical platform testing newly-developed therapeutics. This study provides sound molecular rationale for targeting c-Met in breast cancer and its metastases to the brain.



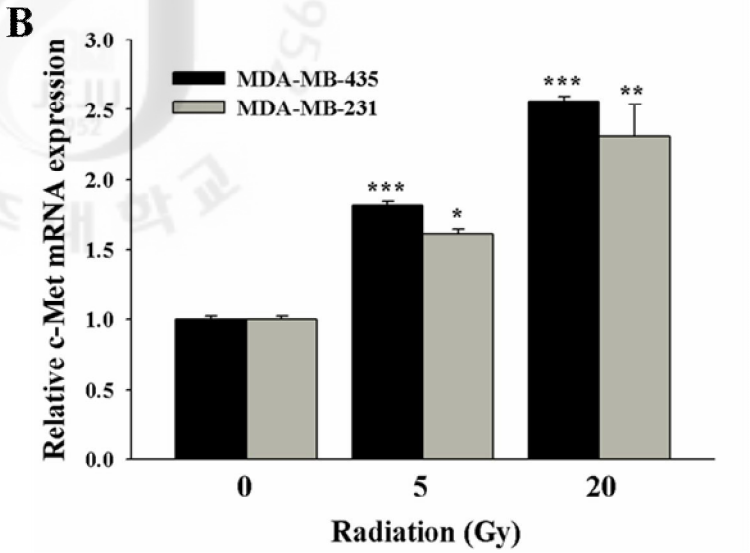
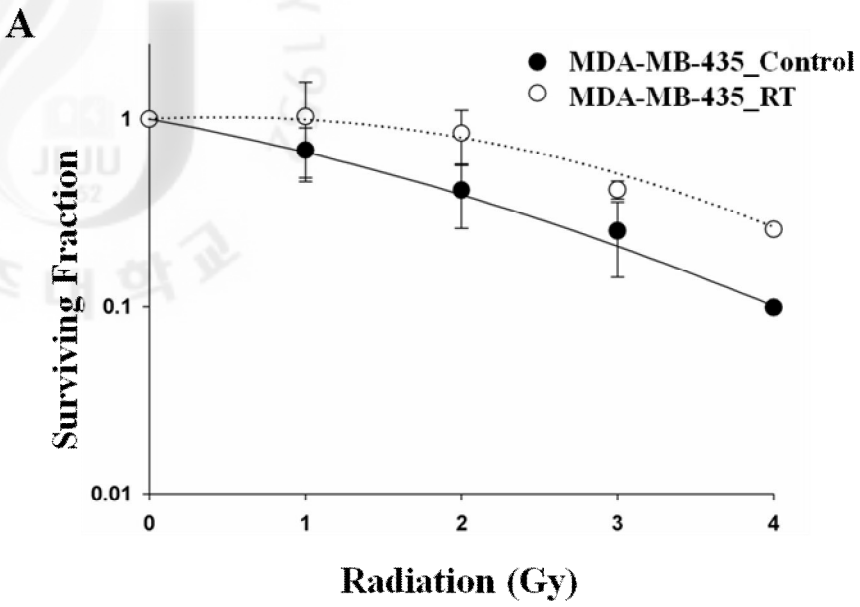


Fig. 14. Increase of c-Met in respond to radiation in breast cancer cells, MDA-MB-435 and MDA-MB-231 cells. (A) Intensity of c-Met was analyzed with FACS. (B) mRNA levels of c-Met were analyzed qRT-PCR. Values are mean ± S.D. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. 0 Gy of each cells.



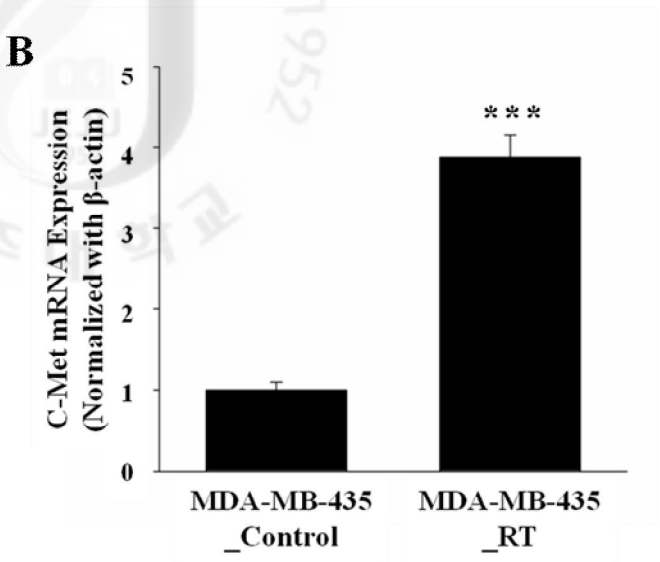
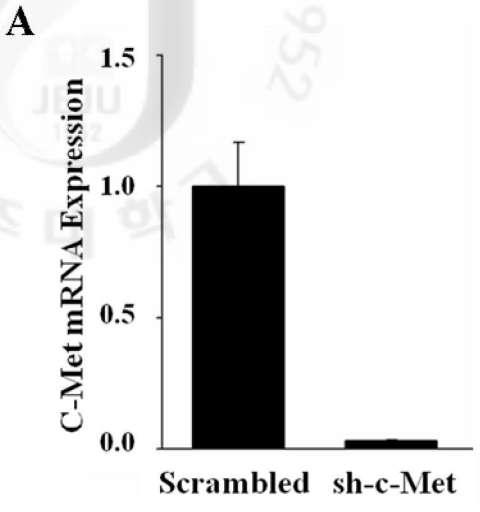
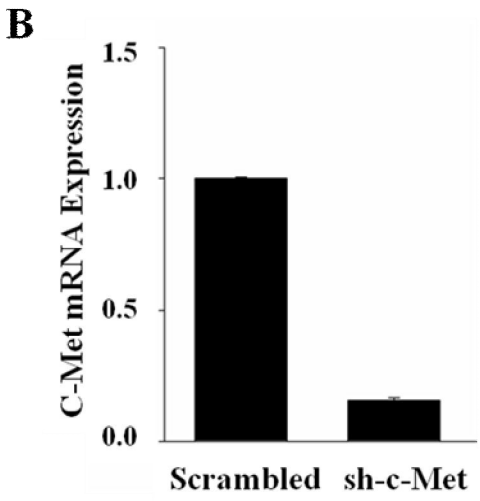
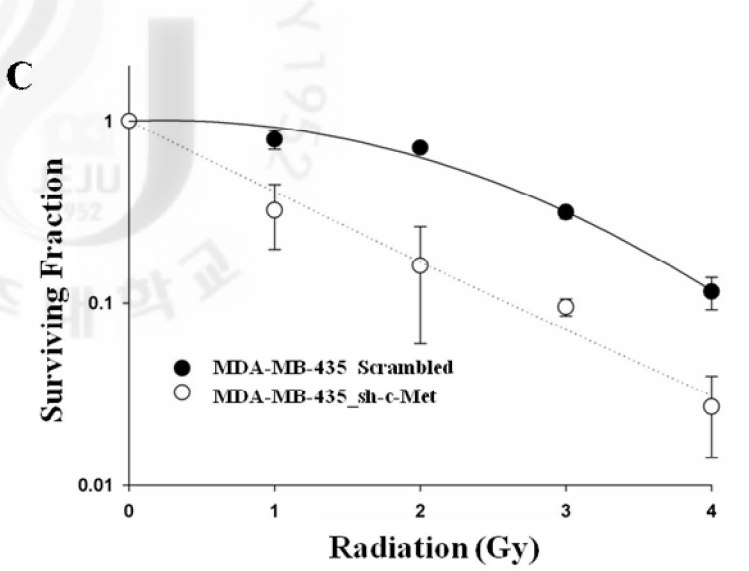


Fig. 15. c-Met was increased in radio-resistant *ex vivo* cells. (A) Estimation of radio-response of *ex vivo* cells by clonogenic survival assay, (B) mRNA levels of c-Met were increased in radio-resistant *ex vivo* cells. Values are mean ± S.D. $^{***}p<0.001$ vs. Control.







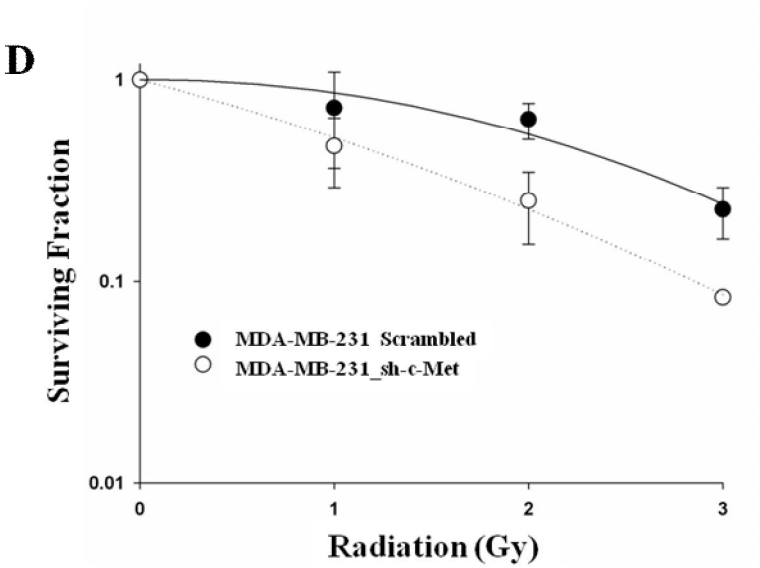
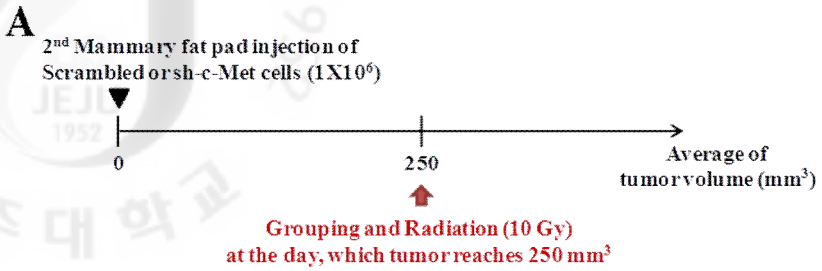


Fig. 16. Depletion of c-Met enhances radio-rensitivity of breast cancer cells *in vitro*. (A and B) c-Met mRNA was decreased by c-Met shRNA expression in MDA-MB-435 and MDA-MB-231 cells, respectively. (C and D) Clonogenic survival assays were performed to estimate radio-response in MDA-MB-435 and MDA-MB-231 cells, respectively. Values are mean ± S.D.



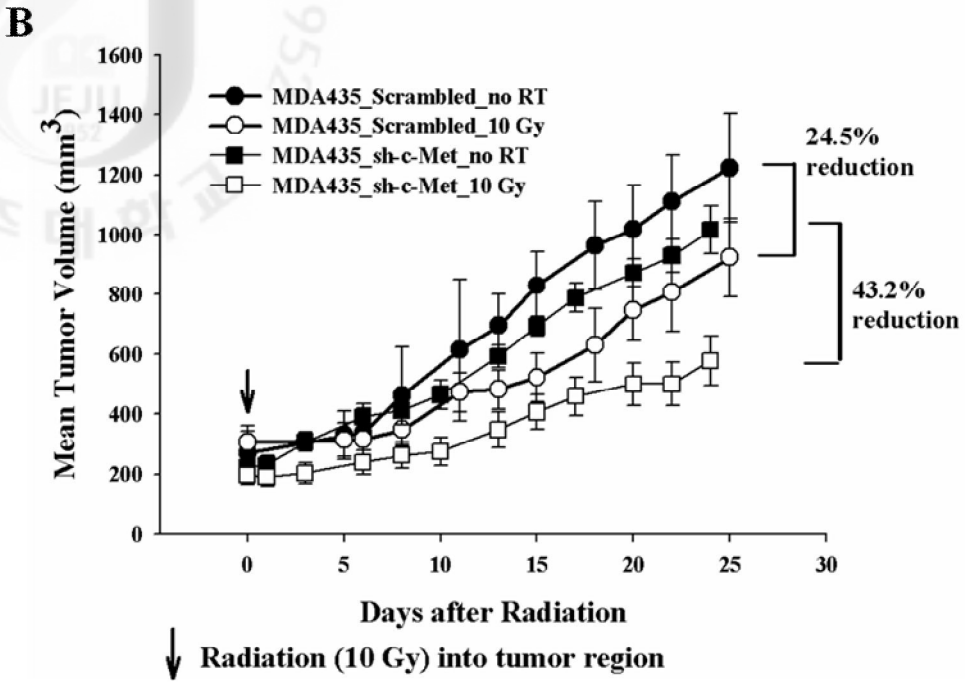
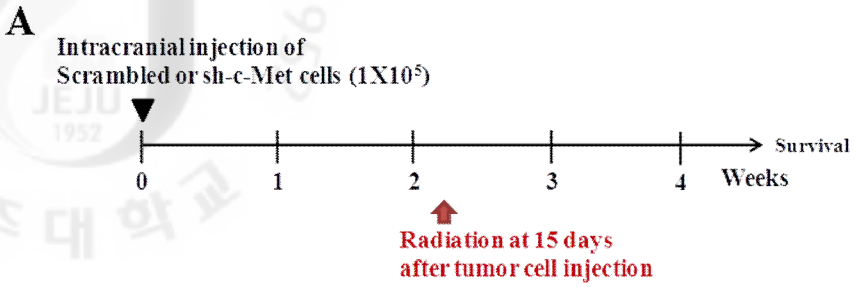


Fig. 17. Inhibition of c-Met enhanced radio-sensitivity in breast cancer orthotopic model. (A) Detailed experimental schedules. (B) Tumor growth after radiation in MDA-MB-435, which is depleted c-Met, breast cancer orthotopic model. Values are mean ± S.E.M.



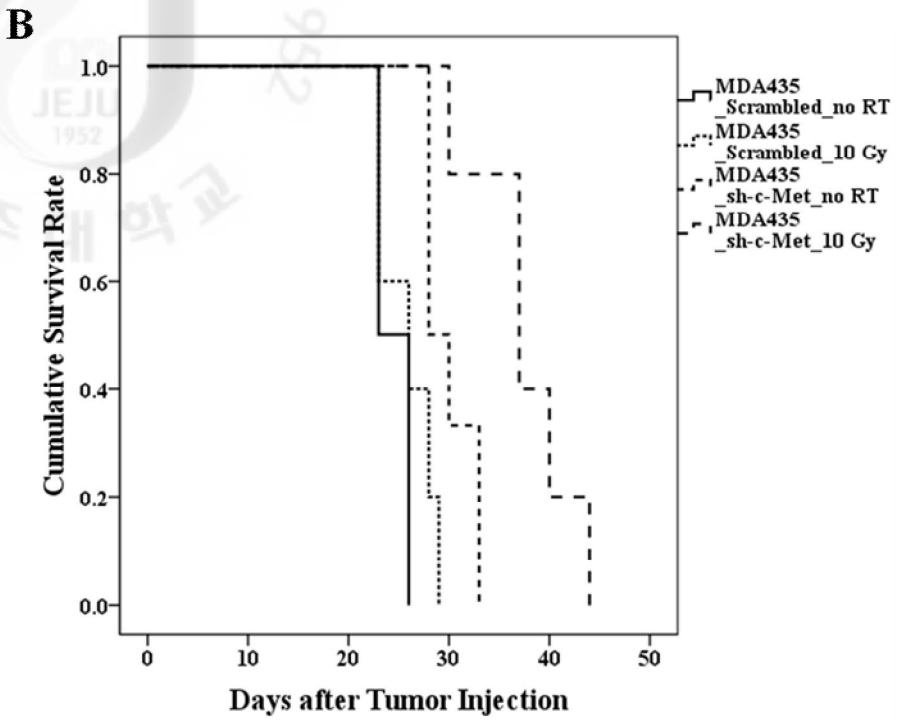
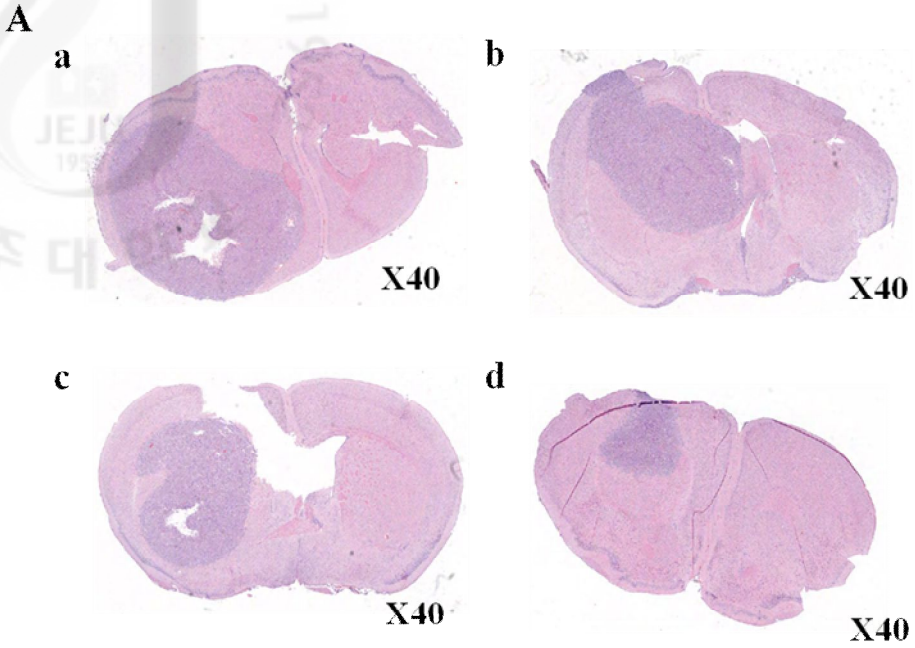


Fig. 18. Inhibition of c-Met enhanced radio-sensitivity in breast cancer brain metastatic model. (A) Detailed experimental schedules. (B) Kaplan-Meier plot comparing survival of MDA-MB-435, which is depleted c-Met, brain metastatic xenograft model.

Table 5. Analysis data of MDA-MB-435, which is depleted c-Met, breast cancer brain metastatic xenograft model

Group		Survival			P value	
		Median (D)	Range (D)	ILS* (%)	Compared to Group Ⅰ	Compared to Group Ⅱ
Ⅰ	Scrambled _no RT	23	23~26	-		0.234
Ⅱ	Scrambled _10 Gy RT	26	23~29	13	0.234	
Ⅲ	sh-c-Met_ no RT	28	28~33	-	0.001	0.027
Ⅳ	sh-c-Met _10 Gy RT	37	30~44	32	0.002	0.002

* ILS; Increase in life span



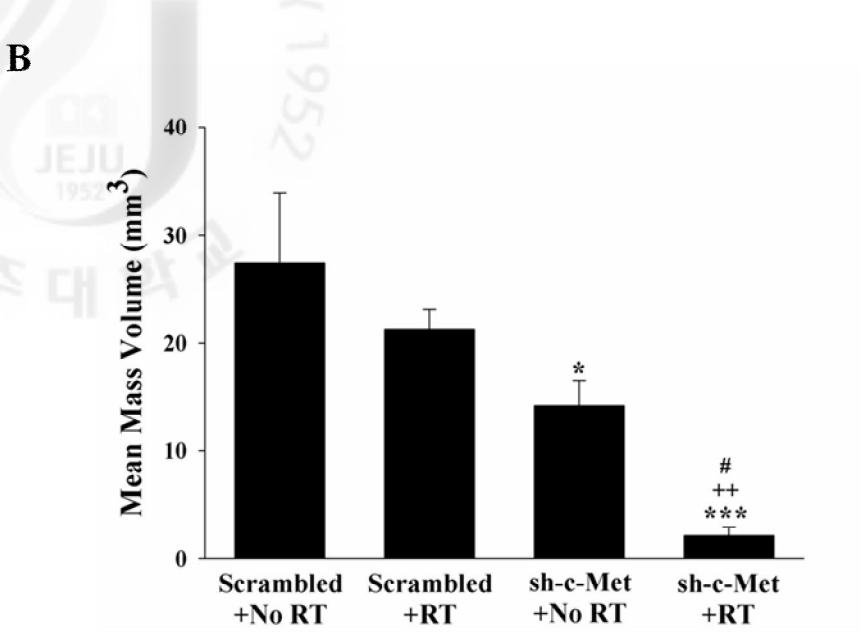
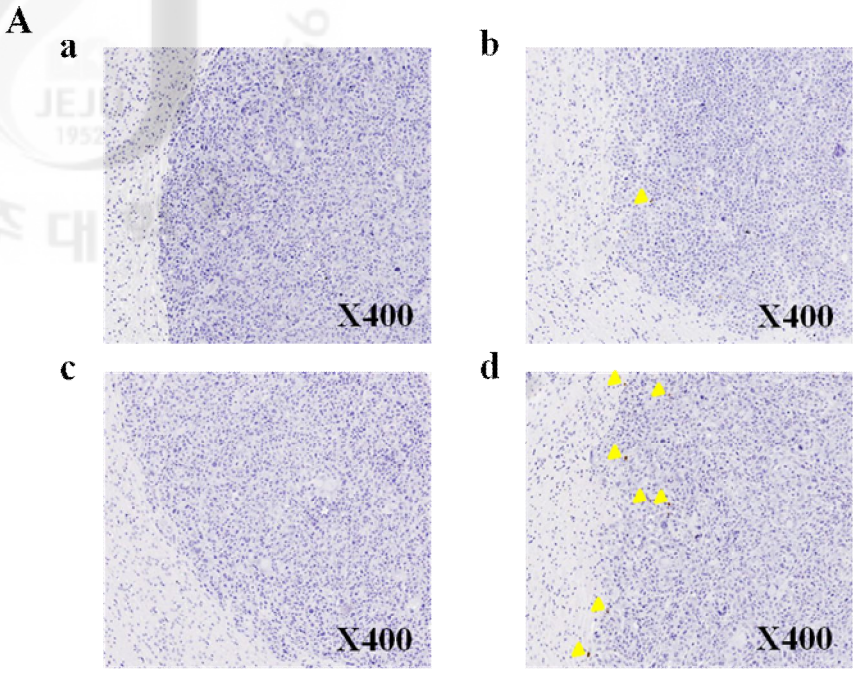


Fig. 19. Regression of tumor by combination of c-Met depletion and radiation in breast cancer brain metastatic xenograft model. The brain was stained with H&E. (A) Representative photos for brain tumors. (a) Scrambled+No RT. (b) Scrambled+RT. (c) sh-c-Met+No RT. (d) sh-c-Met+RT. (B) Tumor volumes of each group. Values are mean ± S.E. $^{*}p<0.05$, $^{***}p<0.001$ vs. Scrambled+No RT; $^{++}p<0.01$ vs. Scrambled+RT; $^{\#}p<0.05$ vs. sh-c-Met+No RT.



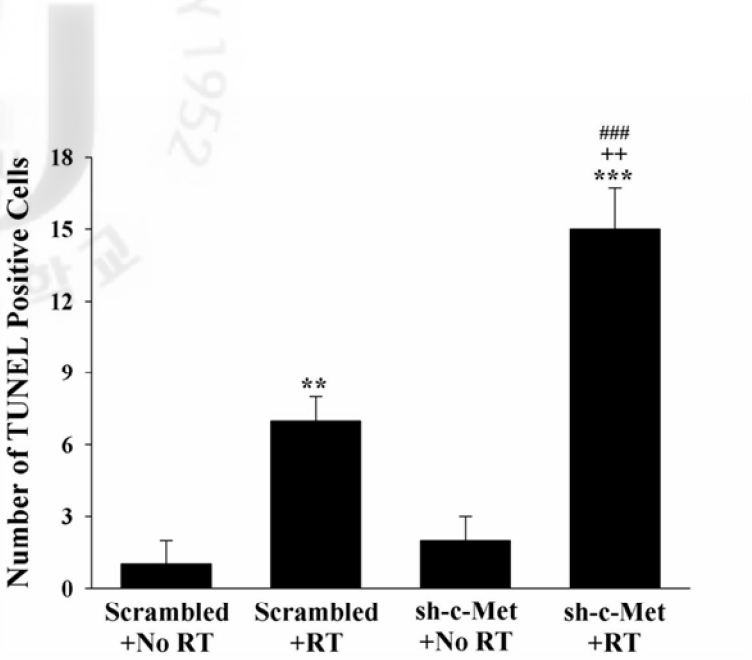


Fig. 20. Induction of apoptosis by combination of c-Met depletion and radiation in breast cancer brain metastatic xenograft model. Apoptosis was detected by TUNEL assay. (A) Representative photos for TUNEL positive cells. (a) Scrambled+No RT. (b) Scrambled+RT. (c) sh-c-Met+No RT. (d) sh-c-Met+RT. Arrow mean TUNEL-positive cell. (B) Number of TUNEL positive cells in each group. Values are mean ± S.E. $^{**}p<0.01$, $^{***}p<0.001$ vs. Scrambled+No RT; $^{++}p<0.01$ vs. Scrambled+RT; $^{###}p<0.001$ vs. sh-c-Met+No RT.

Conclusions

Brain metastases occur late in the progression of multiple types of cancer and are associated with poor patient survival. Current systemic treatments have mostly failed to treat brain metastases effectively because the blood-brain barrier (BBB) remains a formidable obstacle to the entry of most chemotherapeutic agents into the brain parenchyma [89]. Although WBRT leads to stabilization or shrinkage of tumors in at least half of patients, many patients have tumor recurrence either at sites of original disease or in new sites, some of which may have been present at the time of initial treatment but below the threshold of detection [90].

Since the ultimate goal of developing novel radiosensitizers is to improve clinical outcomes in patients with cancer, target identification and testing of potential as a radiosensitizers in more relevant brain metastatic models are very important. In this study, we seek to find out rational target for radiosensitization to translate to the clinic.

Several studies showed that Chk1 and Chk2 of DNA damage checkpoint signaling and c-Met are capable of target in various cancers [22,87,91,92]. But, it is insufficient of clues to translate to the clinic in metastatic brain cancers. For lung cancer brain metastases, we proved that correlation of Chk1 activation and prognosis of brain metastases from patients with metastatic brain tumors and Chk1 is highly activated in respond to radiation in radio-resistant lung cancer cells. We confirmed that the radio-resistance is improved by inhibition of Chk1 in radio-resistant lung cancer cells *in vitro*. Also, we confirmed that targeting of Chk1 using inhibitor or shRNA shows radio-sensitizing effect in radio-resistant lung cancer brain metastatic xenograft models. For breast cancer and breast cancer brain metastases, we found out that expression of c-Met is increased respond to radiation in breast cancer cells and in radio-resistant breast cancer brain metastatic cancer cells. We determined that the radio-

sensitizing effects are shown by targeting c-Met using shRNA in both of breast cancer orthotopic model and breast cancer brain metastatic xenograft model.

Therefore, we suggest that Chk1 or c-Met can be rational target in lung cancer brain metastases or breast cancer brain metastases for developing radiosensitizers, which can be translated to the clinic.

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Abstract in Korean

뇌전이암에서 방사선 내성을 극복할 수 있는 항암치료법 개발

뇌전이암은 성인에서 중추신경계에 발생하는 암중 일반적인 종류 중 하나이며, 전체 뇌종양에서 차지하는 비율이 원발암보다 10배 이상 높다. 원발성 종양에 대한 진단방법의 발달로 진단율이 향상되고, 수술, 방사선 치료 및 항암화학요법의 진보로 원발성 종양 환자의 생존지간이 연장됨에 따라 뇌전이암의 발생과 진단되는 환자수가 증가되고 있으며, 암환자의 사망과 전이에 주요한 원인이다. 뇌전이암의 원발성 암으로는 폐암이 가장 흔하며, 다음으로 유방암, 흑생종 순으로 나타나고 있다. 뇌전이암의 치료는 수술적 절제와 방사선 치료 및 항암화학요법 등이 있고, 이 중 방사선 치료가 표준 치료법으로 이용되고 있다. 하지만 방사선 치료에 대한 종양 세포의 내성이 증가되어 치료에 실패하고 재발하는 환자가 늘고 있다. 따라서 환자의 인식기능 손상을 최소화하면서 뇌전이 환자의 방사선 치료 효율을 극대화시킬 수 있는 radiosensitizer의 개발이 시급하다. 최근들어, 방사선 조사에 의한 DNA damage checkpoint signaling의 활성화에 대한 연구 결과와 방사선 내성과 c-Met의 관련성에 대한 연구 결과가 많이 보고되고 있다. 하지만 이들을 표적으로 하여 뇌전이암 환자 대상의 임상 적용가능성은 불분명하다. 본 실험에서는 폐암으로 인한 뇌전이암에서 방사선 내성을 극복하기 위한 표적으로 DNA damage checkpoint signaling 중 Chk1의 적용가능성을 검증하고자 하였고, 동시에 간세포 성장인자의 수용체인 c-Met의 조절을 통하여 유방암과 유방암으로 인한 뇌전이암에서 방사선 내성을 극복할 수 있는지를 검증하고자 하였다. 뇌전이암 환자의 종양조직에서 면역조직화학염색을 통하여 확인한

DNA damage checkpoint signaling을 구성하는 단백질들의 발현과 환자의 무진행 생존율과 비교분석한 결과, Chk1의 활성이 낮은 환자군에서 무진행 생존율이 길어짐을 알 수 있었다. 또한, 폐암 세포주에서 방사선에 대한 세포의 반응성을 확인하여 방사선에 대하여 민감 (NCI-H23, NCI-460)하거나 내성 (NCI-H1299, A549, PC14PE6)을 보이는 세포주군을 분리하였고, Western blot을 통해 방사선 내성의 폐암 세포에 방사선 조사시, Chk1의 활성이 크게 증가하였다. PC14PE6 세포주에 Chk1 억제제인 AZD7762와 방사선을 병용처리한 결과, 세포의 성장과 클론원성 생존이 유의적으로 감소하였고, Western blot으로 단백질의 발현을 확인한 결과, Chk1의 하위 단백질인 Cdc25A의 발현이 안정화 되었으며, cyclin A의 발현이 감소하였다. 또한, PC14PE6 세포를 이용한 뇌전이암 동물모델에서도 AZD7762와 방사선을 병용처리한 군에서 생존율이 유의적으로 증가하였다. PC14PE6 세포주에서 shRNA를 이용하여 Chk1 유전자를 억제시킨 세포주를 확립하였고, 방사선에 대한 반응성을 클론원성 생존분석과 뇌전이암 동물모델에서 확인한 결과, Chk1 유전자를 억제시킨 세포에서 클론원성 생존이 유의적으로 감소하였으며, 뇌전이암 동물모델에서도 Chk1을 억제시킴으로써 생존율이 크게 증가하였다. 이상의 결과로, 폐암으로 인한 뇌전이암에서 DNA damage checkpoint signaling 중 Chk1이 방사선 내성을 극복할 수 있는 좋은 표적이 될 수 있음을 제시하였다.

방사선 내성과 c-Met의 연관성을 확인하기 위하여, 유방암 세포주인 MDA-MB-435와 MDA-MB-231에서 방사선 조사을 조사한 결과, c-Met의 intensity와 mRNA 발현이 유의적으로 증가하였다. 또한, MDA-MB-435 세포를 이용하여 제작한 방사선 내성 세포주에서도 c-Met의 mRNA 발현이 유의적으로 증가하였다. c-Met의 조절을 통해 방사선 민감도가 증가하는지 확인하기

위하여, MDA-MB-435와 MDA-MB-231 세포에서 shRNA를 이용하여 c-Met 유전자를 억제시킨 세포주를 확립하였다. 각 세포주에서 방사선에 대한 반응성을 클론원성 생존분석을 통하여 확인한 결과, 방사선에 대하여 민감도가 크게 증가하였다. c-Met 유전자가 억제된 MDA-MB-435 세포주를 이용하여 유방암 정위적 동물모델을 제작하여 방사선에 대한 종양성장 억제정도를 평가한 결과, c-Met 유전자의 발현이 억제된 상태에서 방사선 조사시 종양성장 억제효과가 크게 증가하였다. 같은 세포주를 이용하여 뇌전이암 동물모델을 제작한 후 생존율을 비교평가한 결과, c-Met의 발현이 억제되고 방사선을 조사한 동물의 생존율이 유의적으로 하였고, H&E 염색을 통하여 뇌내의 종양 부피를 비교평가한 결과, 종양의 부피 역시 감소되는 것을 확인하였다. 이상의 결과로 유방암 또는 유방암으로 인한 뇌전이암에서 방사선 민감도를 증진시킬 수 있는 표적으로 c-Met을 제시할 수 있다. 이상의 실험결과를 바탕으로, 폐암 또는 유방암으로 인한 뇌 전이 암에서 각각 Chk1 또는 c-Met을 표적으로 하여 방사선 내성을 극복할 수 있음을 확인하였으며, 임상에도 충분히 적용할 수 있는 가능성을 제시할 수 있다.

감사의 글

서식 있음: 글꼴: (영어) HY신명조, (한글) HY신명조, 14 pt, 굵게

서식 있음: 가운데

"수의독성학실험실" 팻말 앞에 서게 된 것이 지금으로부터 약 10년 전입니다. 무료한 예과 1년이라는 시간을 보내고 새로운 경험을 해 보고 싶다는 생각, 선배들과도 친해지면 좋겠다는 생각, 지극히 단순한 생각으로 실험실에 처음 들어가게 되었습니다. 그곳에서 시작된 연구실 생활이, 이영재 교수님의 연구에 대한 열정이, 같은 곳을 바라보며 나아가는 선배들의 노력이, 제가 앞으로 걸어갈 방향을 바꾸어 놓았고 그 꿈을 위해 지금 박사라는 학위를 받게 되었습니다. 학위는 꿈에 다가가기 위한 과정입니다. 저는 아직 많이 서툴고, 더 배워야 하는 것들이 많은 미숙한 열매입니다. 지금까지보다 더 많은 어려움이 있을 것이고, 더 많은 노력이 필요할 것 이라고 생각합니다. 제가 학위를 받는 지금까지 지켜봐 주신 많은 분들이 앞으로 성숙해 가고, 꿈을 이루는 제 모습을 함께 보아 주셨으면 합니다.

제게 연구에 대한 즐거움과 목표를 갖게 해 주신 지도교수이신 이영재 교수님께 감사드리며, 제 부족한 부분을 일깨워 주신 한창훈 교수님, 바쁘신 와중에도 부족한 논문에 대해 아낌없는 격려와 지도를 하여 주신 이경갑 교수님, 강희경 교수님, 주경민 교수님께 깊은 감사의 마음을 전합니다. 아울러, 저의 학부생활과 대학원 생활을 지켜봐 주신 수의학과 교수님들께도 감사드립니다. 제가 박사학위 논문을 마치기 까지 큰 도움을 주신 남도현 교수님과 유해용 교수님, 설호준 교수님께 감사의 마음을 전하고 싶습니다. 그리고 항상 저에게 친동생처럼 아낌없이 충고해 주고 응원해 준 진주연 박사님, 쉽지만은 않았던 실험실 생활에서 큰 힘이 되어준 현주, 원영, 동건이에게 고마운 마음을 전합니다. 제가 어려울 때 서슴지 않고 도와준 김강호 박사님, 김용현 박사님, 김미숙 박사님, 이혜원 선생님, 이시은 선생님, 이예리 선생님, 그리고 신경외과 연구실의 모든 선생님들께

감사 드리고, 제 일인 것처럼 기뻐하여 줄 박민재 선생님, 손은주 선생님, 이경민 선생님, 윤영주 선생님, 함윤희 선생님. 모두들 고마워요. 힘든 마음을 달래주었던 은정이, 상미, 미현언니, 명관오빠..고맙습니다.

마지막으로, 항상 저를 믿어주시고 배려해 주신 부모님께 감사드립니다. 더 멋진 딸이 되어 부모님의 사랑에 보답하겠습니다. 그리고 하나뿐인 오빠, 새언니, 바쁘다고 잘 챙겨주지도 못한 예쁜 우리 조카, 원준이… 고마워요.

저를 아끼시는 모든 분들의 기대가 헛되지 않게 언제나 노력하는 사람이 되겠습니다. 감사합니다.