의학박사 학위논문

폐암 세포에서 복제 단백질 A1 유비퀴틴화를 통한 포스포메발론산 키나아제 녹다운의 방사선 민감도 증진 효과 분석

Radiosensitivity Enhancement by Knockdown of Phosphomevalonate Kinase via Ubiquitination of the Replication Protein A1 in Lung Cancer Cells

울산대학교대학원

의 학 과

김 연 주

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지도교수 송 시 열

이 논문을 의학박사 학위 논문으로 제출함

2024 년 2 월

울산대학교 대학원

의 학 과

김 연 주

김연주의 의학박사학위 논문을 인준함

심사위원 정 성 윤 인

심사위원 송 시 열 인

심사위원 최 은 경 인

심사위원 노 오 규 인

심사위원 정 진 홍 인

울 산 대 학 교 대 학 원

2024 년 2 월

Abstract

Purpose: To evaluate phosphomevalonate kinase (PMVK) as a radiosensitizing target and identify its mechanism of action

Materials and Methods: Human kinase siRNA library screening was done to identify PMVK as a novel radiosensitizing target. To confirm the effect of PMVK knockdown on radiosensitivity, lung cancer cells were transfected with siRNAs against PMVK or the control siRNA. Changes in the γH2AX protein levels were evaluated to confirm the impact of PMVK knockdown on DNA double-strand breakage. To check the mechanisms of radiosensitivity, an investigation for the modulations of nonhomologous end joining (NHEJ) and homologous recombination (HR) factors after PMVK knockdown was done. A tumor growth delay experiment was conducted using a mouse model.

Results: The reduction in cell viability and survival rates in lung cancer cell lines following PMVK knockdown had a notable impact when combined with radiation therapy (RT). Specifically, RT led to an increase in apoptosis, DNA damage, and the arrest of cells in the G2/M phase after PMVK knockdown. Furthermore, PMVK knockdown heightened the cells' sensitivity to radiation by impeding the HR DNA repair pathway, primarily by decreasing the levels of Replication Protein A1 (RPA1) through the ubiquitin-proteasome system. This radiosensitizing effect of PMVK was validated in an in vivo setting using a stable shRNA PMVK mouse xenograft model. Additionally, the study revealed that PMVK expression was elevated in lung cancer tissues, and this increase was significantly associated with both patient survival and the likelihood of cancer recurrence.

Conclusion: PMVK knockdown enhances radiosensitivity via an impaired HR repair pathway by RPA1 ubiquitination in lung cancer. This suggests that PMVK knockdown might be an effective therapeutic strategy to improve the efficacy of RT.

Keywords: Lung cancer, Phosphomevalonate kinase, Radiosensitivity, Replication protein A1, Ubiquitination

Table of contents

List of Figures

Introduction

Lung cancer is the leading cause of cancer-related fatalities worldwide with low survival rates [1]. According to the Korean nationwide survey, patients diagnosed with stage I-II lung cancer have a 5-year survival rate of ≥60%, while those of stage III and IV were limited to 16% and 10%, respectively [2]. Radiation therapy (RT) is an essential component of treatment for locally advanced lung cancer combined with other treatment options such as surgery, chemotherapy, and immunotherapy. However, its efficacy is often hindered by resistance reducing its effectiveness. There have been efforts to overcome radioresistance through escalating total RT, however, the results were not favorable [3]. Hence, there is a demand for a novel strategy such as the use of radiosensitizer to sensitize tumors to RT and combat RT resistance in lung cancer.

Radiotherapy damages DNA and kills cancer cells. Various types of DNA damage are triggered by RT, including single-strand breaks (SSBs), double-strand breaks (DSBs), modifications to sugar and bases, and DNA-protein crosslinks [4]. DSBs are the most lethal form of DNA damage caused by RT and are predominantly repaired through two pathways, non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ does not require sequence homology and cell cycle-independent. Key factors in NHEJ repair include DNA ligase 4, Artemis, XRCC4, Ku70, Ku80, and DNA-PK_{CS} [5]. Conversely, HR repair is limited to the S and G2 phases when a sister chromatid can serve as a template. The Mre11-Rad50-Nbs1 (MRN) complex is involved in recognizing and repairing DSBs in the HR pathway [6]. The ability to repair RT-induced DNA damage is closely linked to tumor radioresistance, so the agent that could disable this DNA damage repair system could be utilized as a radiosensitizer.

Phosphomevalonate kinase (PMVK) plays a key role in the mevalonate pathway for isoprenoid/sterol biosynthesis and protein prenylation. This process provides essential biomolecules like cholesterol, dolichol, ubiquinone, bile acids, prenylated proteins, vitamins, and heme A, which are critical for normal cell growth and signaling. Although the mevalonate pathway has been implicated in various aspects of cancer development and progression, the roles of PMVK remain unclear [7]. Notably, the potential of PMVK as a

radiosensitizer in lung cancer has not been previously explored.

This study identifies PMVK as a novel target for radiosensitization in lung cancer cells using a human kinase siRNA library. Furthermore, the research provides compelling evidence that suppressing PMVK enhances radiosensitivity by impeding HR repair in lung cancer, leading to the degradation of replication protein A1 (RPA1) via the ubiquitin-proteasome system (UPS).

Materials and methods

1) Cell Culture and Irradiation

Human lung cancer (A549, H1299, H460, and EKVX), normal mouse lung (MLE-12), normal human lung (MRC5), and human embryonic kidney (293T) cell lines were purchased from the ATCC. A549 cells were cultured in an F-12K medium (21127022; Life Technologies) containing 10% FBS (16000044; Life Technologies) and antibiotics (15140122; Life Technologies). H1299, H460, EKVX, and MRC5 cells were cultured in RPMI 1640 medium (22400089; Life Technologies) containing 10% FBS and antibiotics. MLE-12 cells were cultured in DMEM/F12 (11320033; Life Technologies) containing 10% FBS and antibiotics. 293T cells were cultured in DMEM (11995065; Life Technologies) containing 10% FBS and antibiotics. RT was administered using a 6-MV photon beam from the linear accelerator (CL/1800, Varian Medical System Inc.).

2) Antibodies and Reagents

Antibodies to detect PMVK (HPA029900), MRE11(HPA002691), and RPA1 (HPA006914) were obtained from Atlas Antibodies. Alpha-tubulin (#2125) and cleaved caspase-3 (#9661) antibodies were purchased from Cell Signaling Technology. Anti-beta-actin (A5441), anti-Flag (F1084), Hoechst 33342 stain (B2261), and cycloheximide (O1810) were purchased from Sigma-Aldrich. NBS1 antibody (NB100-143) was obtained from Novus Biologicals. Ku80 antibody (#556429) was purchased from BD Biosciences. Anti-DNA-PK_{CS} (sc-9051) was purchased from Santa Cruz Biotechnology. PARP (ab32071) and Rad51(ab63801) antibodies were obtained from Abcam. Anti-gamma-H2AX (A300-081A) was obtained from Bethyl Laboratories, Inc. HA antibody (ADI-MSA-106) was purchased from Enzo. Alexa 555-conjugated anti-rabbit IgG (A21429) was obtained from Invitrogen. Anti-rabbit IgG horseradish peroxidase

(711-036-152) and mouse IgG (711-036-151) were purchased from Jackson ImmunoResearch Laboratories, Inc. MG132 (474790) was obtained from Calbiochem.

3) Immunoprecipitation

Cells were co-transfected with Flag-RPA1 and HA-ubiquitin plasmids for 24 hours, followed by transfection with siRNAs for an additional 24 hours. MG132 was added for 4 hours before cell harvesting. Cell lysis was done using NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 0.5% Nonidet P-40). Immunoprecipitation involved incubating cell extracts with Flag M2-agarose beads (A2220; Sigma-Aldrich) at 4°C overnight, followed by the pull-down analyzed by western blotting.

4) Immunocytochemistry

Cells transfected with siRNAs were cultured on sterilized coverslips, exposed to 10 Gy of radiation, and then incubated at 37°C for specified durations. After fixation with 4% paraformaldehyde for 15 minutes, cells were washed with PBS and then permeabilized with 0.5% Triton X-100 diluted in PBS at room temperature for 15 minutes. After being washed with PBS, the cells were blocked with a blocking buffer (5% normal goat serum) at room temperature for 1 hour and incubated with a gamma-H2A histone family member x (γH2AX) antibody (1:200) diluted in blocking buffer at 4°C overnight. After being washed with PBS, cells were incubated with Alexa 555-conjugated anti-rabbit IgG (1:1000) at room temperature for 1 hour in the dark. After being washed, the cells were incubated with Hoechst 33342 stain at room temperature for 10 minutes. The glass slides were washed with PBS and mounted with Dako fluorescence mounting medium. Fluorescence microscopy of the live cells was performed using an inverted confocal laser scanning microscope (LSM880 Airyscan; Carl Zeiss). Fluorescence images were processed using Zen 2012 SP5 software (Carl Zeiss of the LSM880 Airyscan confocal

microscopy system for 3D reconstruction or intensity profile analyses). Foci were counted using ImageJ software.

5) Immunohistochemistry

Human lung tissues (both normal and cancerous) were acquired from the Bio Resource Center at the Asan Medical Center. A Vectastain Elite ABC Kit was obtained from Vector Laboratories and was used for immunohistochemical staining according to the manufacturer's protocol. Briefly, specimen slides were deparaffinized and rehydrated. To expose the antigens, the slides were unmasked in a boiling sodium citrate buffer (10 mM, pH 6.0) for 10 minutes and then treated with 3% H2O2 for 10 minutes. Each slide was blocked with 10% normal goat serum in 1× PBS in a humidified chamber for 1 hour at room temperature. Then, the slides were incubated with PMVK antibody (1:100) at 4°C in a humidified chamber overnight. The slides were washed and hybridized with the secondary antibody from Vector Laboratories (anti-rabbit 1:500) for 1 hour at room temperature. The slides were stained using a Vectastain Elite ABC Kit (PK-6101; Vector Laboratories, Inc.). The stained slides were analyzed under a DP71 microscope (Olympus).

6) RNA Interference and Plasmid Transfections

Cells were transfected with PMVK-specific siRNAs or ON-TARGET plus Non-targeting Control siRNA (#D-001810-10-05; Dharmacon) for 24 hours at 10 nM using Lipofectamine RNAiMAX transfection reagent (13778-150; Invitrogen) in reduced serum medium (31985-070; Opti-MEM, Gibco), according to the manufacturer's reverse transfection protocol. The sense strand sequences of the siRNAs used are as follows: siPMVK #1: GGUGAGUGACACACGGAGA; siPMVK #2: UGGACGAUGCUGAGUCAGA; siRAD51: GGUAGAAUCUAGGUAUGCA; siDNA-PK_{CS}: GCGUUGGAGUGCUACAACA. pDRGFP (#26475), pimEJ5GFP (#44026)

and pCBASceI (#26477) plasmids were obtained from Addgene. shNS and shRNA PMVK plasmids (V3LHS_350080) were purchased from Dharmacon. Plasmids were transfected into cells using the FuGENE HD Transfection reagent according to the manufacturer's protocol.

7) In Vivo Tumor Growth Delay

A549 (parent), A549/shNS (NS), A549/shPMVK #1 (shPMVK #1), and A549/shPMVK #3 (shPMVK #3) cell lines were used to make a xenograft tumor mouse model. A suspension of 1×10^6 cells in 50 μL was injected subcutaneously into the right hind limb of 5-week-old male BALB/c nude mice (SLC). Tumors were grown until the average tumor volume reached 90–100 mm^3. The mice were then divided into groups (5 mice per group). Tumors were irradiated at the dose of 5 Gy using a 6-MV photon beam twice in 3 days. The mice were sacrificed 30 days after RT. The tumor volume was calculated using the following formula:

$$\mathbf{V = (L \times W^2) \times 0.5}$$

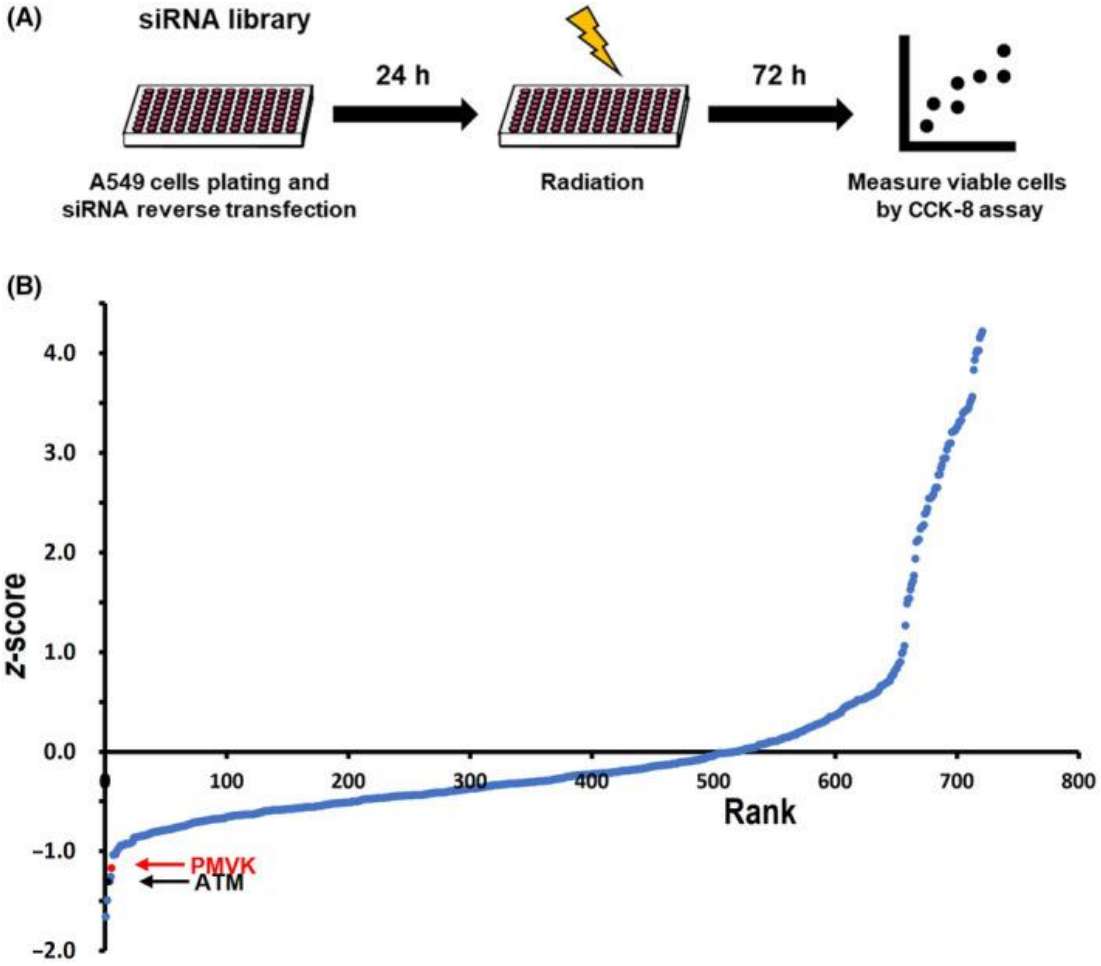
where V = volume, L = length, and W = width.

Results

1) Human kinase siRNA library screening identified PMVK as a novel radiosensitizing target in a lung cancer cell line

Human kinase siRNA library including 720 genes was utilized to uncover potential targets for enhancing the sensitivity of RT. In this screening, siRNAs were used to modify A549 cells, which were then exposed to RT at a dose of 10 Gy or left untreated. Cell viability was assessed 3 days after treatment using a CCK-8 assay (Figure 1). Within the group of genes showing the most significant impact, several were already known to regulate radiosensitivity, including TRIB2, EPHA6, ATM, PIK3C2B, and TPK1 [8-12]. However, the study identified PMVK as a previously unrecognized target for enhancing radiosensitivity, as illustrated in Figure 1B.

Figure 1. Human siRNAs screening and target selection. (A) Schematics of the reverse transfection, radiation, and CCK-8 assay for human siRNAs screening. (B) The mean z-score values for the genes in the human siRNA library screening by rank product. A negative z-score denotes radiosensitization.

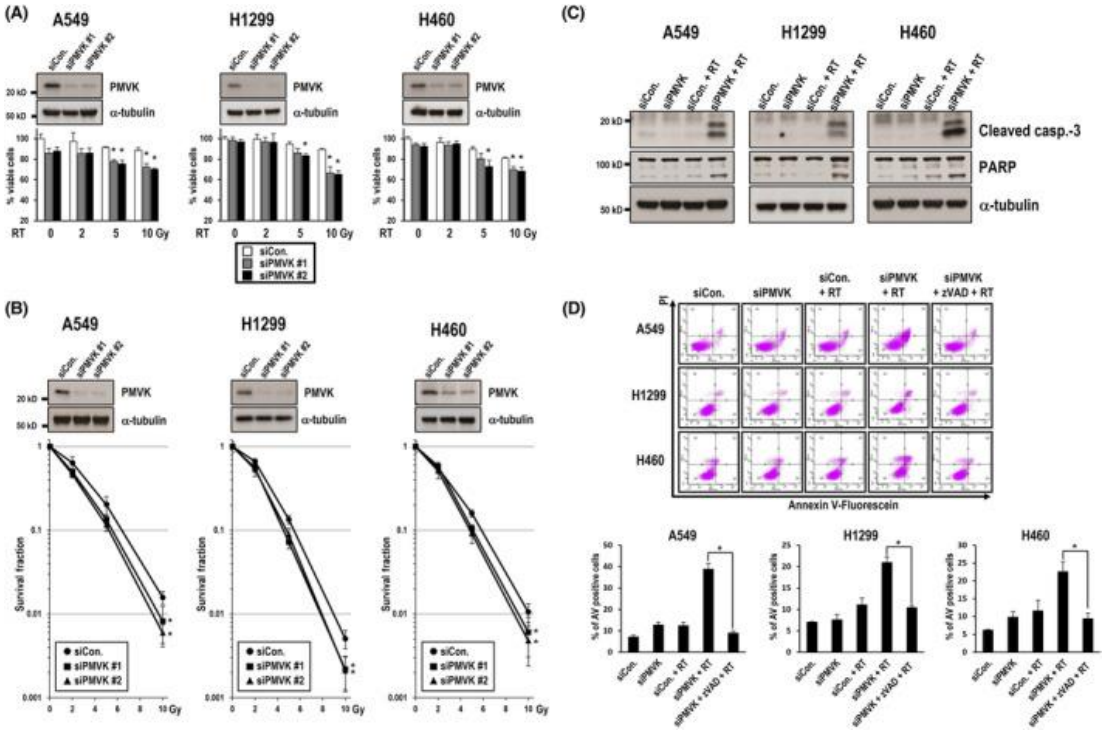


2) Knockdown of PMVK enhances radiosensitivity through apoptosis in lung cancer cell lines

To validate the impact of PMVK knockdown on the radiosensitivity of lung cancer cell lines (A549, H1299, and H460), siRNAs targeting PMVK (siPMVK) or a control siRNA (siCon) were transfected into these cells. RT alone, up to a dose of 10 Gy, led to only limited cell death in these cell lines. However, when PMVK was knocked down using siRNA, a significant decrease in cell viability was observed following radiation treatment (Figure 2A). The survival fraction was notably reduced in the lung cancer cell lines when both siPMVK and RT were administered (Figure 2B). These findings unequivocally demonstrate that the reduction of PMVK enhances the radiosensitivity of lung cancer cells.

Subsequently, investigations were carried out to ascertain whether the increased sensitivity to radiation was linked to apoptotic cell death. This was assessed through Western blotting and flow cytometry analyses. When siPMVK or RT was administered individually, there was no notable increase in the cleavage of caspase-3 or PARP, a caspase-3 substrate, in comparison to the siCon. However, the combined treatment of siPMVK and RT prompted the cleavage of caspase-3 and PARP (Figure 2C). Furthermore, following siPMVK transfection, pre-treatment with zVAD, a broad-spectrum caspase inhibitor, notably reduced the proportion of annexin V-positive cells, which had been increased by the combination of siPMVK and RT (Figure 2D). These findings strongly indicate that the increased sensitivity to radiation resulting from PMVK knockdown is achieved by triggering caspase-mediated apoptosis in lung cancer cells.

Figure 2. Knockdown of PMVK significantly sensitizes RT-resistant lung cancer cells via apoptotic cell death. (A) Lung cancer cell lines were transfected with the siCon 20 nM and, two different PMVK siRNAs (siPMVK #1 and siPMVK #2) 20 nM for 24 hours, and further treated with RT for 48 hours (A549 cells) or 24 hours (H1299, H460 cells) at the indicated doses. Suppression of PMVK expression by siRNA was confirmed by western blotting 24 hours after transfection. Cellular viability was assessed using a CCK-8 assay. Columns are the mean of three experiments; SE. *$p < 0.001$, compared with untreated cells. (B) Cells were transfected with the indicated siRNAs, and then exposed to different doses of RT. Suppression of PMVK expression by siRNA was confirmed by western blotting after transfection for 24 hours. The cells were incubated for 9 days to allow colony formation. Surviving colonies were counted after staining with crystal violet. Columns are the mean of three experiments; SE. *$p < 0.001$, compared with the siCon-transfected cells. (C) Cells were transfected with 20 nM siRNAs (siCon and siPMVK #2) for 24 hours and then exposed to RT (10 Gy) for 48 hours (A549 cells) or 24 hours (H1299 and H460 cells). Cell extracts were prepared for western blotting to detect the changes in the expression of caspase-3 and PARP. Equal loading of the protein samples was confirmed by western blotting of α-tubulin. (D) Cells were transfected with 20 nM siRNAs for 24 hours and then treated with 20 μM z-VAD-fmk for 30 min before RT (10 Gy). After 48 hours (A549 cells) or 24 hours (H1299 and H460 cells), apoptosis was measured using Annexin V-FITC and propidium iodide (PI) staining in flow cytometry (upper) and was quantified (bottom). Columns show the mean of three experiments; SE. *$p < 0.001$.


(A)
A549
H1299
H460
siCon.
siPMVK #1
siPMVK #2
PMVK
α-tubulin
% viable cells
RT
0
2
5
10 Gy
(B)
Survival fraction
(C)
siCon.
siPMVK
siCon. + RT
siPMVK + RT
Cleaved casp.-3
PARP
α-tubulin
(D)
siCon.
siPMVK
siCon. + RT
siPMVK + RT
siPMVK + zVAD + RT
PI
Annexin V-Fluorescein
% of AV positive cells

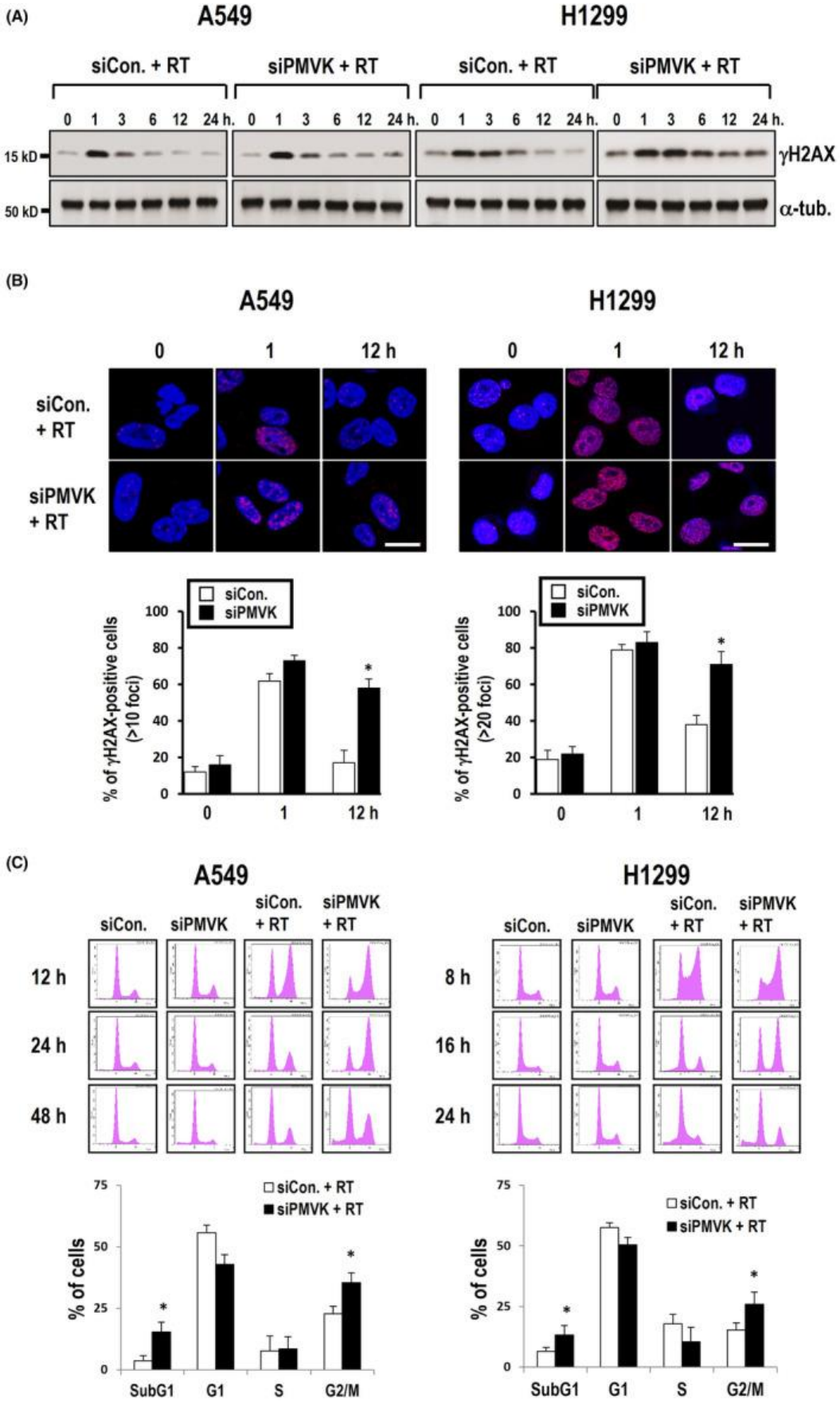
3) Knockdown of PMVK enhances RT-induced DNA DSBs and G2/M arrest

To assess whether PMVK knockdown influenced the DNA damage caused by RT, changes in the levels of γH2AX protein, a specific marker for DNA DSBs, were examined through Western blotting. In A549 and H1299 cells treated with siCon and RT, γH2AX protein levels showed a temporary increase, peaking at 1 hour, and then gradually decreasing. However, in cells treated with siPMVK and RT, the levels of γH2AX protein remained elevated (Figure 3A).

Additionally, the number of nuclear γH2AX foci was compared between cells treated with siPMVK and RT and those treated with siCon and RT. Immunocytochemistry analysis revealed that RT led to a significant increase in the number of cells with γH2AX-positive nuclei at 1 hour, regardless of the presence of siPMVK. Nevertheless, in cells treated with siCon and RT, the number of cells with γH2AX-positive nuclei decreased after 12 hours. In contrast, the number of cells with γH2AX-positive nuclei remained constant in cells treated with siPMVK and RT at 12 hours, indicating a lack of DNA repair and the persistence of DNA damage (Figure 3B).

We then investigated whether the suppression of PMVK had an impact on the alterations in cell cycle distribution induced by RT. In cells treated with siCon, the proportion of cells arrested in the G2/M phase reached its peak at 12 hours for A549 and 8 hours for H1299 after RT and then declined at 24 hours for A549 and 16 hours for H1299. By 48 hours for A549 and 24 hours for H1299, the percentage of G2/M arrested cells had fully returned to normal. Interestingly, in the siPMVK-transfected cells, the percentage of G2/M arrested cells continued to rise even at 48 hours for A549 and 24 hours for H1299 after RT, in contrast to the cells treated with siCon and RT (Figure 3C). These findings demonstrated the pivotal role of PMVK in the induction of DNA DSBs and G2/M phase arrest following RT.

Figure 3. PMVK depletion promotes RT-mediated DNA damage and G2/M cell cycle arrest. (A) Cells were transfected with 20 nM siRNAs for 24 hours and then exposed to RT (10 Gy) for the indicated times. The effect of siPMVK and RT on the γH2AX protein levels was detected by western blotting at the indicated times. Equal loading of the protein samples was confirmed by western blotting of α-tubulin. (B) Cells were transfected with 20 nM siRNAs for 24 hours and then exposed to RT (10 Gy) for the indicated times. The effects of siPMVK and RT on the formation of intracellular γH2AX foci were detected by immunocytochemistry (upper; blue, Hoechst 33342-stained nuclei; red, Alexa 555-stained γH2AX foci; scale bars, 10 μm) and was quantified at the indicated times (bottom). Columns show the mean of three experiments; SE. $*p < 0.001$, compared with the siCon-transfected cells. (C) A549 and H1299 cells were transfected with siCon or siPMVK and then exposed to RT at 10 Gy. The cell cycle distribution was assessed by flow cytometry (upper) and was quantified (bottom) at 48 hours (A549) or 24 hours (H1299). Columns show the mean of three experiments; SE. $*p < 0.001$, compared with the siCon and RT cells.


(A)
A549
H1299
siCon. + RT
siPMVK + RT
siCon. + RT
siPMVK + RT
0 1 3 6 12 24 h. 0 1 3 6 12 24 h. 0 1 3 6 12 24 h. 0 1 3 6 12 24 h.
15 kD
50 kD
γH2AX
α-tub.
(B)
A549
H1299
0 1 12 h
0 1 12 h
siCon. + RT
siPMVK + RT
siCon.
siPMVK
% of γH2AX-positive cells (>10 foci)
% of γH2AX-positive cells (>20 foci)
0 1 12 h
(C)
A549
H1299
siCon.
siPMVK
siCon. + RT
siPMVK + RT
12 h
24 h
48 h
8 h
16 h
24 h
siCon. + RT
siPMVK + RT
% of cells
SubG1 G1 S G2/M

4) Knockdown of PMVK decreases RPA1 protein level through the ubiquitin–proteasome pathway to inhibit HR repair

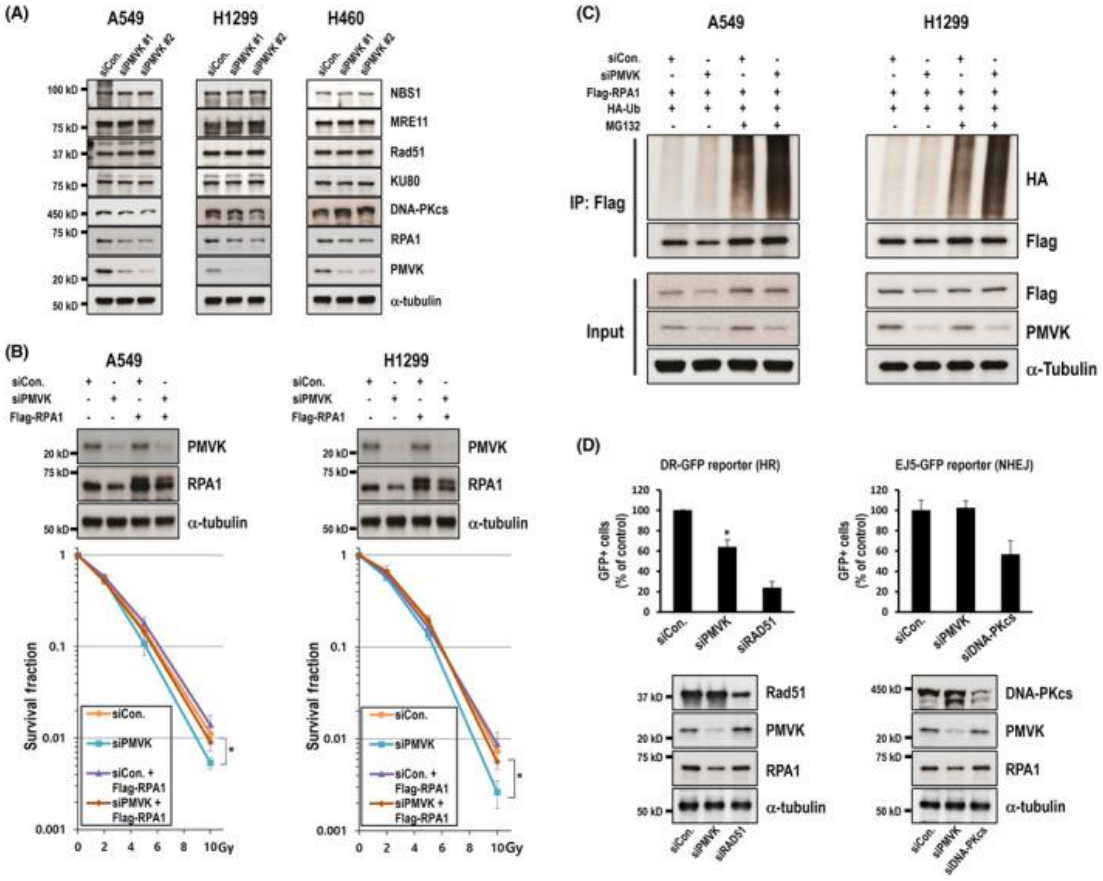
To investigate how PMVK knockdown enhanced the radiosensitivity of lung cancer cells through DNA damage and repair mechanisms, we examined the effects on the factors involved in NHEJ and HR after PMVK knockdown. The expression of RPA1, a key factor in HR and related to DSB repair [13], was significantly reduced in lung cancer cell lines by siPMVK (Figure 4A). To further validate the causative relationship between RPA1 and the radiosensitization brought about by siPMVK, we overexpressed RPA1 and then treated the cells with siPMVK and RT. The overexpression of RPA1 substantially counteracted the decrease in cell survival caused by siPMVK and RT (Figure 4B). These findings highlight that the siPMVK-induced reduction in RPA1 levels plays a role in radiosensitization.

Subsequently, we explored how siPMVK regulated RPA1. Previous studies have indicated that RPA1 stability is controlled through ubiquitination pathways [14]. We investigated whether PMVK knockdown triggered the ubiquitination of RPA1. Lung cancer cells were transfected with Flag-RPA1 and HA-ubiquitin plasmids, in addition to siRNAs, and were treated with the proteasome inhibitor MG132 for 4 hours before analysis through immunoprecipitation using Flag-agarose beads. The reduction in RPA1 caused by PMVK knockdown was counteracted by MG132, and MG132 increased the poly-ubiquitination of Flag-RPA1 (Figure 4C). Furthermore, when we examined the half-life of RPA1 in the presence of cycloheximide, a protein biosynthesis inhibitor, it was found that the half-life of RPA1 was shortened in siPMVK-transfected cells in comparison to siCon-transfected cells. These results demonstrate that PMVK knockdown influences RPA1 degradation through the UPS.

We also verified whether the reduction of RPA1 by siPMVK was associated with HR repair. The efficiency of DNA repair pathways was assessed using DR-GFP (HR) and EJ5-

GFP (NHEJ) reporters. PMVK knockdown reduced the efficiency of HR repair while not significantly affecting the efficiency of NHEJ repair (Figure 4D). Collectively, these findings suggest that the increased radiosensitivity resulting from PMVK knockdown is due to the inhibition of HR repair through the downregulation of RPA1.

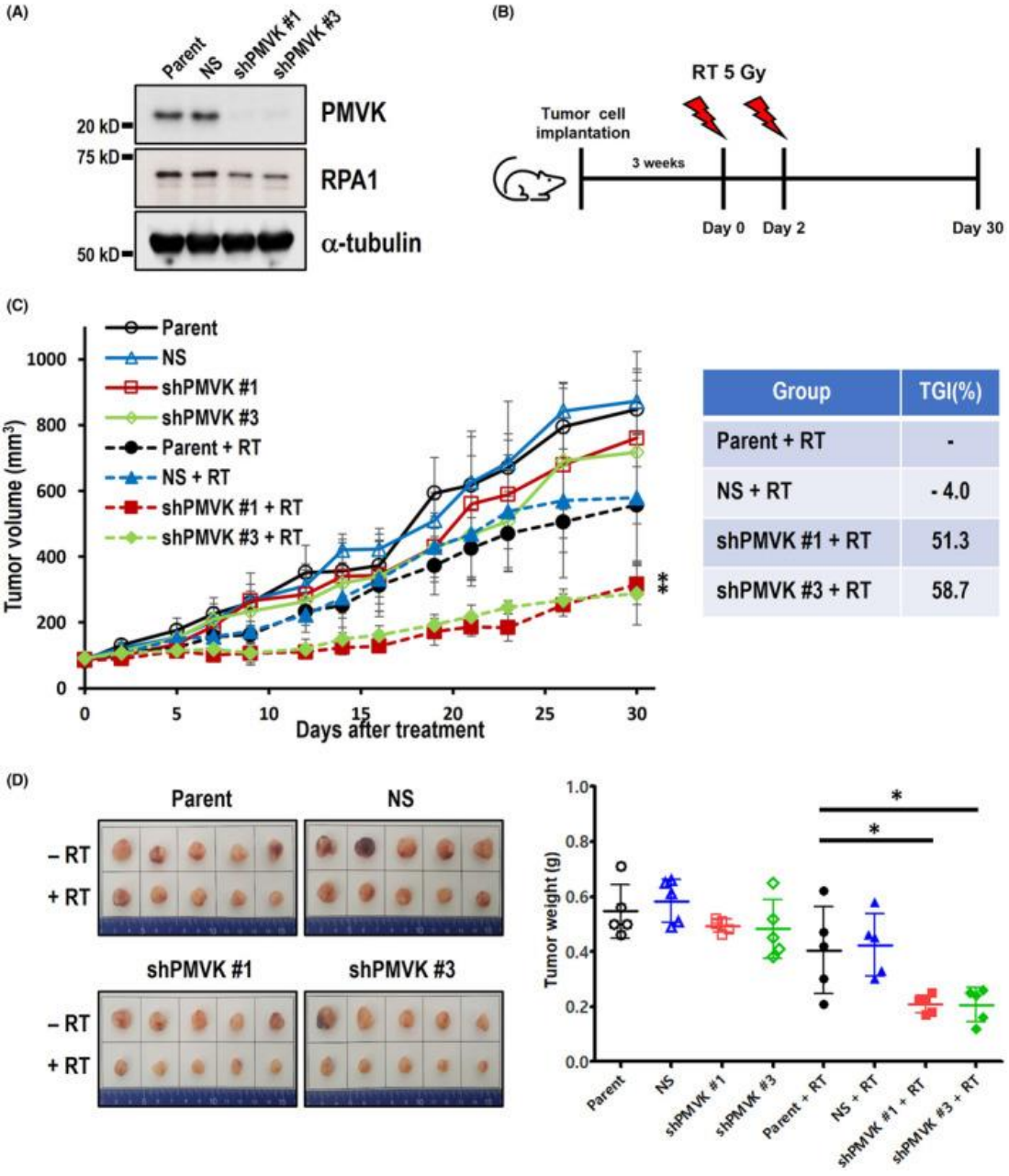
Figure 4. PMVK depletion decreases RPA1 protein level and inhibits HR repair. (A) Cells were transfected with the indicated siRNAs for 24 hours. The cell lysates were subjected to western blotting. Equal loading of the protein samples was confirmed by western blotting of α-tubulin. (B) Cells were transiently transfected with Flag-RPA1 for 24 hours, and then treated with siPMVK and RT. Overexpression of RPA1 and suppression of PMVK expression was confirmed by western blotting. Equal loading of the protein samples was confirmed by western blotting of α-tubulin. The cells were incubated for 9 days to allow colony formation. Surviving colonies were counted after staining with crystal violet. Columns show the mean of three experiments; SE. $*p < 0.001$. (C) Cells were co-transfected with Flag-RPA1 and HA-ubiquitin plasmids for 24 hours and then transfected with indicated siRNAs for another 24 hours. MG132 was added for 4 hours before harvesting, and cells were collected for immunoprecipitation (IP) with Flag-agarose beads and then subjected to western blotting analysis. Equal loading of the protein samples was confirmed by western blotting of α-tubulin. (D) HR and NHEJ repair efficiency was determined with the DR-GFP and EJ5-GFP reporters in 293T cells transfected with the indicated siRNAs using flow cytometric analyses. siRAD51 and siDNA-PK were positive controls of HR and NHEJ, respectively (upper). Protein expression of Rad51, DNA-PK, PMVK, and RPA1, was detected by western blotting. Equal loading of the protein samples was confirmed by western blotting of α-tubulin (bottom). Columns show the mean of three experiments; SE. $*p < 0.001$, compared with the siCon-transfected cells.


(A)
A549
H1299
H460
siCon.
siPMVK #1
siPMVK #2
100 kD
75 kD
37 kD
75 kD
450 kD
75 kD
20 kD
50 kD
NBS1
MRE11
Rad51
KU80
DNA-PKcs
RPA1
PMVK
α-tubulin
(B)
A549
H1299
siCon.
siPMVK
Flag-RPA1
20 kD
75 kD
50 kD
PMVK
RPA1
α-tubulin
Survival fraction
1
0.1
0.01
0.001
0 2 4 6 8 10Gy
siCon.
siPMVK
siCon. + Flag-RPA1
siPMVK + Flag-RPA1
*
(C)
A549
H1299
siCon.
siPMVK
Flag-RPA1
HA-Ub
MG132
IP: Flag
Input
HA
Flag
Flag
PMVK
α-Tubulin
(D)
DR-GFP reporter (HR)
EJ5-GFP reporter (NHEJ)
GFP+ cells (% of control)
120
100
80
60
40
20
0
siCon.
siPMVK
siRAD51
siDNA-PKcs
*
37 kD
20 kD
75 kD
50 kD
450 kD
Rad51
PMVK
RPA1
α-tubulin
DNA-PKcs

5) The depletion of PMVK enhances A549 cell response to radiation and inhibits tumor growth in vivo

We generated A549 sublines (A549/shPMVK) in which PMVK was stably silenced using a PMVK shRNA plasmid. As a control, a nonsilencing (NS) shRNA plasmid was employed. The effectiveness of PMVK shRNA in reducing PMVK and RPA1 expression was confirmed through western blotting (Figure 5A). To evaluate whether the reduction of PMVK had a radiosensitizing impact in a live organism, xenograft mice bearing tumors derived from the parental A549 cells, NS, shPMVK #1, and shPMVK #3 cells received RT (Figure 5B). The groups with PMVK knockdown exhibited a significant delay in tumor growth after RT, without any loss of body weight (Figure 5C). At the 30-day mark, compared to the parent RT groups, the shPMVK #1 RT and shPMVK #3 RT groups displayed tumor growth inhibition ratios (TGI%) of 51.3% and 58.7%, respectively (Figure 5C). Additionally, the two shPMVK RT groups had lower tumor weights compared to the parent RT group (Figure 5D). These results demonstrate that PMVK knockdown substantially increased radiosensitivity in vivo.

Figure 5. The depletion of PMVK increases radiosensitivity in vivo. (A) In the A549 sublines stably transfected with NS and shPMVK plasmids, the protein levels of PMVK and RPA1 were examined by western blotting using anti-PMVK and anti-RPA1 antibodies. Equal loading of the protein samples was confirmed by western blotting of α-tubulin. (B) Schematic of the experimental schedule. Mice bearing xenograft tumors derived from A549 (parent), NS, shPMVK #1, and shPMVK #3 cells were treated with RT. The RT (5 Gy) was performed twice in 3 days. The mice were sacrificed 30 days after RT, and tumor specimens were obtained. (C) Relative tumor volume of shPMVKs xenograft tumors after RT (left) and tumor growth inhibition ratio (TGI%; right). *$p < 0.001$, compared with the parent and RT tumors. (D) At 30 days, all tumors were photographed (left) after isolation, and tumor weights were measured (right). *$p < 0.001$, compared with the parent and RT tumors.

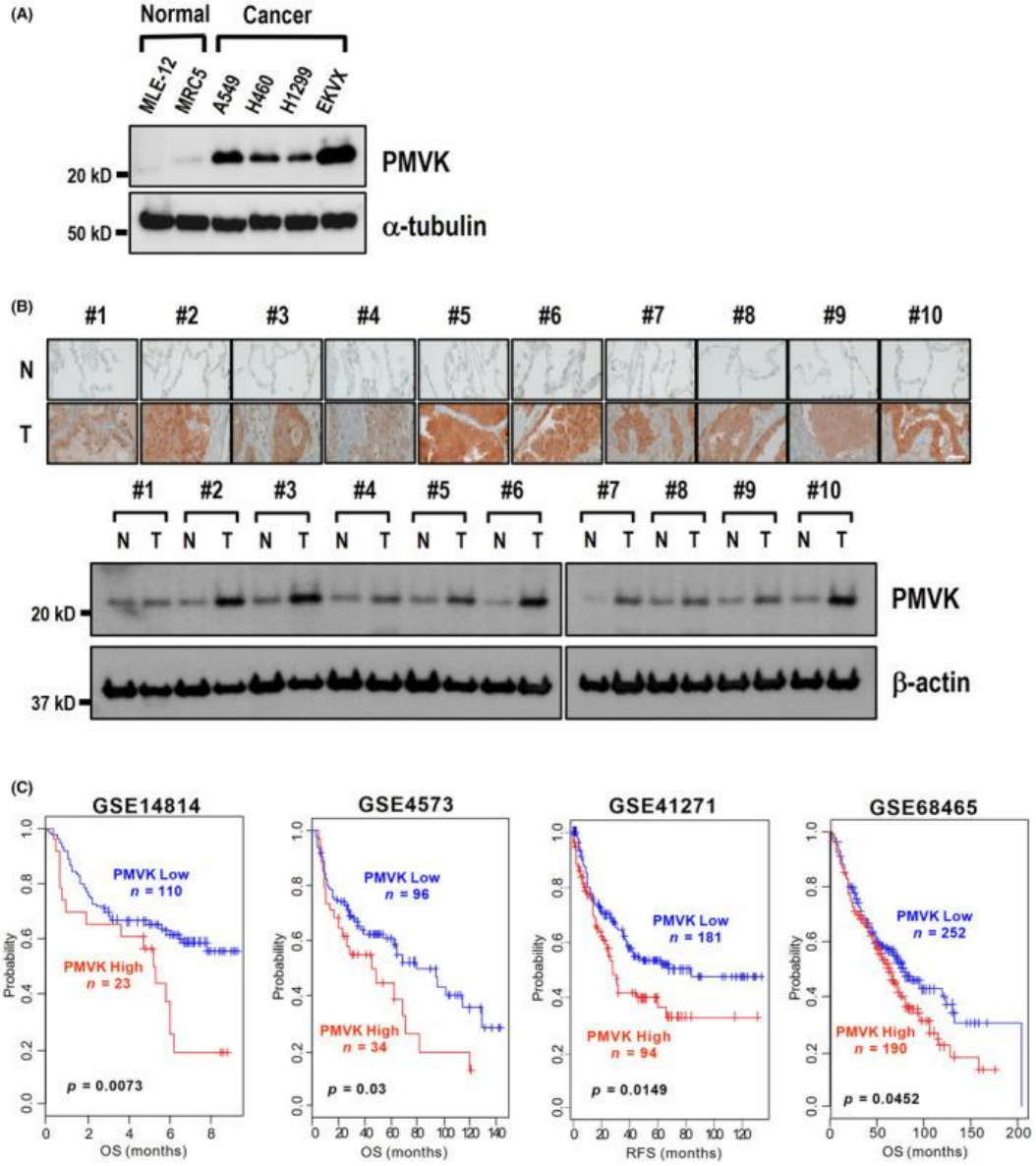


Group	TGI(%)
Parent + RT	-
NS + RT	- 4.0
shPMVK #1 + RT	51.3
shPMVK #3 + RT	58.7

6) PMVK is overexpressed in human lung cancer cells and is associated with lung cancer patient survival probability

PMVK mRNA levels are notably high in the liver, heart, skeletal muscle, kidney, and pancreas, while relatively lower in the brain, placenta, and lung [15]. However, the connection between PMVK expression and human lung cancer remains unclear. Therefore, we conducted an examination of PMVK protein expression in normal lung cell lines and human lung cancer cell lines. The results showed that PMVK expression was elevated in various human lung cancer cell lines when compared to normal lung cells like MRC5 (human) and MLE-12 (mouse) lung cells (Figure 6A). Increased PMVK expression was observed in lung tumor (T) tissues as opposed to normal (N) tissues (Figure 6B). Moreover, PMVK expression was significantly linked to patient survival and the likelihood of recurrence (Figure 6C). These findings suggest that the heightened levels of PMVK protein in lung cancer may be associated with resistance to RT and poorer survival.

Figure 6. PMVK expression is upregulated in human lung cancer cells and is associated with lung cancer patient survival probability. (A) Expression of PMVK in the normal lung and human lung cancer cell lines. (B) Comparison of PMVK expression in lung normal (N) and lung tumor (T) tissues using immunohistochemistry (upper; scale bar, 10 μm) and western blotting (bottom). (C) Patients with lung cancer were divided by relatively high or low PMVK expression and a Kaplan–Meier plot was generated. The differences between these groups were statistically significant in terms of overall survival (OS) and recurrence-free survival (RFS).



Discussion

Radioresistance of cancer cells is the major hurdle in increasing the therapeutic window of RT. There are various mechanisms of radioresistance, such as resistance to cell death, activation of DNA repair mechanisms, tumor hypoxia, and alterations in the cell cycle [16]. To enhance the therapeutic effectiveness of RT, there is a need to develop new radiosensitizers that can maximize its impact without causing resistance and side effects in normal cells.

In this study, the researchers utilized a human kinase siRNA library to identify potential radiosensitizing targets, and they identified PMVK as one such target. PMVK plays a role in the mevalonate pathway by phosphorylating mevalonate 5-phosphate to mevalonate 5-diphosphate [15]. Previous research has indicated that the reduction of PMVK can trigger apoptosis and activate the p53 pathway[17], and it can inhibit farnesyl pyrophosphate and geranylgeranyl pyrophosphate, which are molecules involved in cancer growth [18]. The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) database revealed that PMVK expression tends to be higher in breast[17] and lung tumor tissues (Figure 6C) compared to normal tissues, suggesting a potential link between high PMVK expression and poor treatment outcomes in breast and lung cancer.

While PMVK has been associated with cell growth and signaling in normal cells, its role in cancer biology and treatment has not been fully understood, and the possibility of PMVK as a radiosensitizer also has not been explored. The present study findings showed that knocking down PMVK in lung cancer cells resulted in increased apoptosis, DNA damage, and arrest of the cell cycle at the G2/M phase following RT (Figures 1 and 2). Moreover, the researchers identified a potential connection between PMVK and DNA damage repair, particularly through its effect on the protein RPA1 (Figure 4A). RPA1 is essential for DNA replication, damage repair, and cell cycle regulation, and it is closely

related to the repair of DSBs. The interaction of RPA with BRCA1, Rad51, and Rad52 demonstrated the role of RPA1 in the HR repair of DSBs [19]. Overexpression of RPA1 interfered with the radiosensitivity of human cancer cells and RPA1 was associated with poor survival in cancer patients [20]. Furthermore, after RT, RPA1 knockdown enhanced radiosensitivity, decreased proliferation, inhibited DSB repair, and contributed to G2/M checkpoint arrest in nasopharyngeal cancer cells [21]. However, the regulatory mechanism of RPA1 by PMVK on radiosensitivity has not been reported in lung cancers.

The present study revealed that PMVK knockdown-induced RPA1 downregulation was blocked by MG132 and Flag-RPA1 poly-ubiquitination was increased by MG132 (Figure 4C). Not only for RPA1, the UPS is essential in degrading proteins involved in DNA damage, cell cycle, cell proliferation, and cell death [22]. Ubiquitination is performed in a highly specific manner that labels target proteins via a cascade of enzymatic reactions including Ub-activating (Eq), Ub-conjugating (E2), and Ub-ligating (E3) enzymes [23]. For certain signaling pathways such as mTORC1 and PTEN-AKT, the mechanisms of the UPS have been studied [23], however, there's a paucity of data on the changes in the activity of ubiquitin enzymes/ligases of RPA1 ubiquitination by PMVK knockdown which requires additional studies. In addition, the development of PMVK inhibitors or the siRNA PMVK delivery system will contribute to the application of clinical trials for enhancing the efficacy combined with RT.

Additionally, the study highlighted the significance of the tumor suppressor gene p53, which plays a critical role in regulating cellular responses to RT. p53 is an important tumor suppressor gene involved in apoptosis, cell cycle arrest, and cellular senescence [24]. Radiosensitive cells and tissues have a high basal mRNA expression level of p53, and a decline in radiosensitivity is accompanied by a reduction in p53 mRNA expression [25]. Mutations in the p53 gene have been found in many human cancers, including lung cancer, and p53 inactivation is correlated with RT resistance. Interestingly, p53-deficient H1299

cells have been shown to have enhanced radiosensitivity after PMVK knockdown, similar to p53-wild-type cells (A549 and H460; Figure 1). Notably, the findings suggested that even p53-deficient lung cancer cells could benefit from PMVK knockdown in terms of enhanced radiosensitivity, similar to p53-wild-type cells. This suggests that combining PMVK knockdown with RT could be a promising strategy for treating p53-deficient lung cancers.

Conclusion

PMVK knockdown can serve as a novel approach to sensitize lung cancer cells to RT. This sensitization occurs by inhibiting DNA damage-induced repair mechanisms, leading to the degradation of RPA1 through the UPS. Therefore, PMVK knockdown holds promise for improving the effectiveness of RT in the treatment of lung cancer.

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국문요약

목적: 방사선 민감도 증진 표적으로서 포스포메발론산 키나아제의 효과를 평가하고 그 작용 기전을 규명한다.

방법 및 대상: 방사선 민감도 증진 표적을 찾기 위하여 인간 키나아제 siRNA 라이브러리로 스크리닝을 시행하였다. PMVK 녹다운이 방사선 민감도에 끼치는 영향을 확인하기 위해 폐암 세포를 PMVK 에 대한 siRNA 와 대조군 siRNA 로 형질전환하였다. H2AX 단백질의 변화를 통해 PMKV 녹다운이 DNA 이중나선절단에 끼치는 영향을 확인하였다. 방사선민감도 증진의 작용기전을 확인하기 위하여 PMVK 녹다운 이후 비상동말단연결 및 상동 재조합에 대한 분석을 시행하였다. 마우스 모델을 사용하여 생체에서 종양성장지연실험 또한 수행하였다.

결과: PMVK 녹다운 따른 폐암 세포의 생존율은 방사선치료와 결합 되었을 때 의미있는 감소를 보였다. 특히, 방사선치료는 PMVK 녹다운 후 세포 사멸, DNA 손상 및 G2/M 기에서 정체된 세포 수를 증가시켰다. PMVK 녹다운은 주로 유비퀴틴-프로테아좀 시스템을 통해 복제 단백질 A1 을 줄임으로써 상동 재조합 복구 경로를 방해하여 방사선 민감도를 높였다. 이러한 PMVK 의 방사선 민감도 증진 효과는 shRNA PMVK 마우스 이종이식 모델을 사용하여 생체 내 환경에서도 검증되었다. 또한, PMVK 가 정상 조직 대비 폐암 조직에서 증가되어 있었고, 이러한 증가는 환자의 나쁜 생존율과 암 재발과 관련이 있었다.

결론: PMVK 녹다운은 폐암에서 RPA1 유비퀴틴화에 의해 상동 재조합 복구 경로가 손상되는 기전으로 방사선 민감도을 향상시키며, 이를 통해 방사선치료의 성적을 향상시킬 수 있을 것으로 기대된다.

핵심용어: 폐암, 포스포메발론산 키나아제, 방사선민감성, 복제 단백질 A1, 유비퀴틴화