The effect of YAP inhibition on radiation resistance in head and neck cancer

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The effect of YAP inhibition on radiation resistance in head and neck cancer

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Jina Kim

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ABSTRACT

The effect of YAP inhibition on radiation resistance in head and neck cancer

Purpose: The treatment of head and neck cancer requires a multidisciplinary approach involving surgery, radiotherapy, and chemotherapy. However, despite these multimodal treatments, the local recurrence rate remains high, and effective salvage therapies are urgently needed. Recurrent tumors are often resistant to chemotherapy or radiotherapy, presenting a significant clinical challenge. The Hippo pathway is a major tumor suppressor pathway in many cancers, and its downstream effector molecule Yes-associated protein (YAP) is thought to play a role in various processes, including cell proliferation, cell survival, and treatment resistance. YAP is frequently amplified in head and neck cancer, and previous studies have identified its amplification as a poor prognostic factor for head and neck cancer. This study aims to assess the role of YAP in radiation resistance in head and neck cancer cell lines.

Materials and Methods: After screening 20 head and neck cancer cell lines, we identified HEP-2 as a cell line with relatively high YAP expression and radioresistance. Using short hairpin RNA (shRNA) and small interfering RNA (siRNA) targeting YAP, we investigated the changes in cellular characteristics following YAP knockdown. For pharmacological inhibition of YAP, we used Verteporfin and CA3. In vivo tumorigenicity of YAP knockdown cells was assessed in nude mice. Cell proliferation, clonogenic, and apoptosis assays were performed to assess whether combining YAP inhibition with irradiation induced any synergistic effects. The change in the expression level of Cyclin D1 and γH2AX foci were investigated upon YAP inhibition and irradiation.

Results: YAP knockdown led to reduced cellular proliferation, diminished migration, enhanced radiosensitivity, and increased apoptosis compared with control cells. In vivo tumorigenicity results also confirmed that inhibition of YAP resulted in significantly

decreased tumor volume. The combination of YAP knockdown and irradiation resulted in a synergistic effect compared to YAP knockdown or irradiation alone in terms of cell proliferation and apoptosis. In addition, YAP knockdown resulted in G0/G1 cell cycle arrest. Furthermore, YAP expression level was found to correlate with Cyclin D1 expression, and treatment with both pharmacologic YAP inhibition and irradiation resulted in decreased Cyclin D1 levels, which subsequently led to increased DNA damage.

Conclusion: YAP is associated with cell proliferation, migration, and apoptosis in head and neck cancer cells. Additionally, YAP, through its regulation of Cyclin D1, is involved in cellular DNA damage response following irradiation. Targeting YAP may provide a potential strategy to overcome radioresistance in head and neck cancer.

Key words : Hippo pathway, Yes-associated protein, head and neck cancer, radioresistance, Cyclin D1

1. Introduction

Head and neck cancer is estimated to account for nearly 660,000 new cases globally each year, ranking as the seventh most prevalent malignancy worldwide[1]. Despite significant advancements in treatment, survival rates for head and neck cancer have not improved substantially[2]. Due to its complex anatomical location, a multidisciplinary approach involving surgery, radiation therapy, and chemotherapy, is essential for the effective management of head and neck cancer[3,4].

Radiation therapy is used in over 75% of head and neck cancer patients and plays a pivotal role in tumor control, as well as in the preservation of both morphological and functional aspects[5,6]. However, a major challenge in treating head and neck cancer is the high rate of local recurrence following primary treatment, necessitating the need for effective salvage therapy [7]. In particular, reirradiation is challenging, as recurrent tumors often show resistance to chemotherapy and radiation, making clinical management more difficult[8,9].

The Hippo pathway acts as a major tumor suppressor pathway in several cancer types. The Hippo pathway consists of a kinase cascade, and when it is activated, Yes-associated protein (YAP) is phosphorylated, leading to its degradation or cytoplasmic retention[10-12]. In contrast, when the Hippo pathway is inactive, YAP undergoes dephosphorylation, becomes activated, and translocates to the nucleus where it forms a complex with TEA domain transcription factor (TEAD) to induce the expression of various target genes (Figure 1).

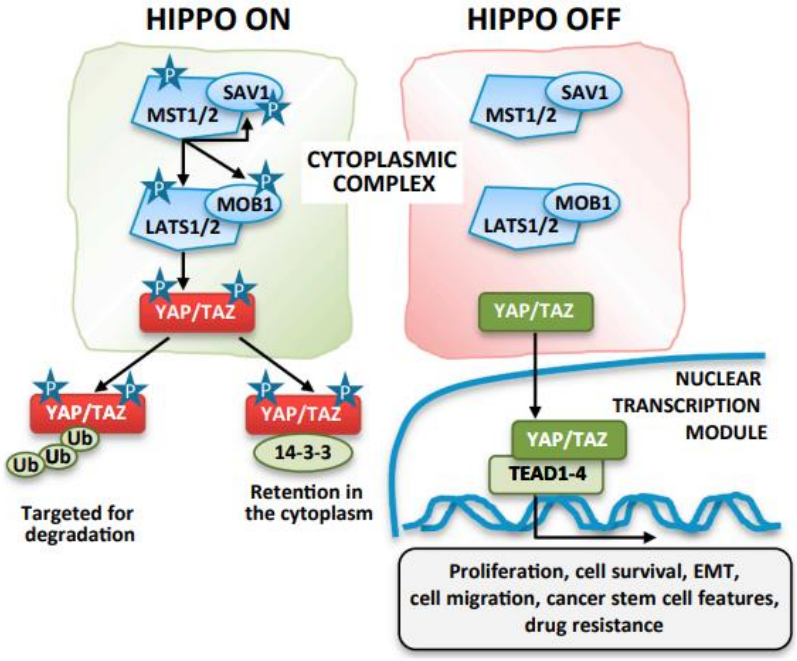


Fig. 1. The Hippo pathway is a major tumor suppressor pathway.

(Oral Oncol 2018)[13]

The Hippo pathway plays a crucial role in head and neck cancer as well. While alterations in the Hippo pathway is known to occur in approximately 10% of human cancers, HPV-negative head and neck cancers exhibit a much higher frequency of alterations of around 42%[14]. In murine models, deletion of Mob1a/b, leading to Hippo pathway inactivation, resulted in the rapid development of tongue carcinoma in situ within two weeks, and invasive squamous cell carcinoma within four weeks, highlighting the pathway's tumor-suppressive function[15].

YAP, a transcriptional co-activator of the Hippo pathway, is a central molecule in several signaling networks. YAP interacts with various tyrosine kinase receptors, mechanical cues, the mTOR pathway, and the KRAS pathway, among others (Figure 2). Upon activation, YAP promotes the transcription of many downstream genes, including genes involved in cell

proliferation, migration, and epithelial-to-mesenchymal transition[16].

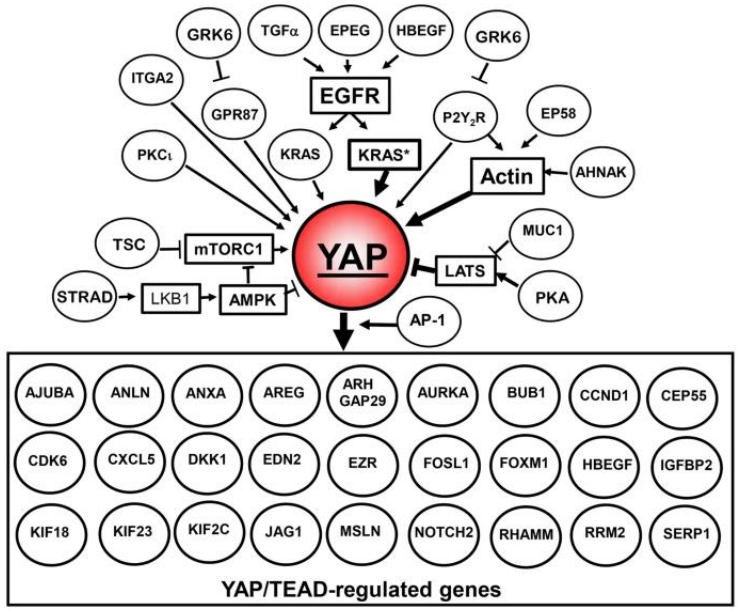


Fig. 2. YAP is a central molecule in several signaling networks.

(Signal Transduct Target Ther 2018)[17]

Under normal circumstances, the Hippo pathway suppresses YAP activity, thereby preventing uncontrolled cell growth[18]. However, in many cancers, including the head and neck squamous cell carcinoma, alteration or amplification of YAP is associated with aggressive tumor behavior and poor prognosis [19,20]. Elevated YAP levels enhance the transcription of genes linked to cancer metastasis and stem cell-like properties, contributing to tumor initiation, recurrence, and resistance to therapy[21,22]. This is especially significant in cancers like esophageal squamous cell carcinoma, where YAP inhibition has been shown to induce significant apoptosis, thereby reducing tumor cell viability[23]. Various cancers have shown amplification of the YAP gene, and head and neck cancers are known to exhibit frequent YAP gene amplification and alterations (Figure 3).

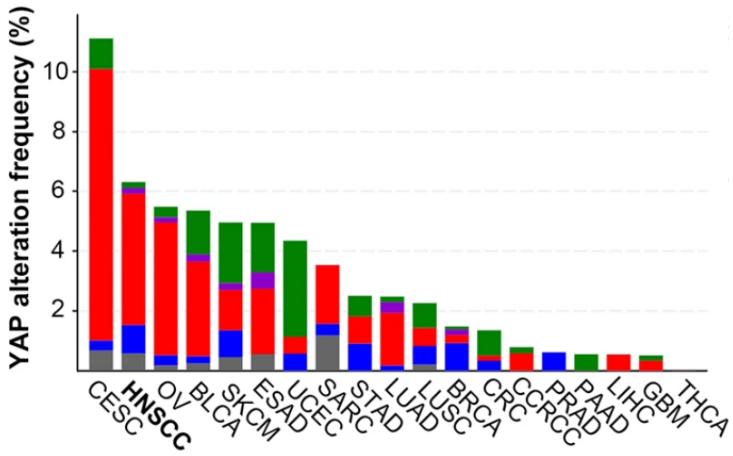


Fig. 3. YAP alteration frequency is high in head and neck squamous cell carcinoma.

(Experimental & Molecular Medicine 2014)[24]

Studies have also shown that YAP is a strong prognostic factor for predicting radiation response. Among individuals receiving radiation therapy for head and neck squamous cell carcinoma, those with high YAP expression showed reduced treatment efficacy[25]. Furthermore, YAP overexpression has been shown to increase radioresistance, as evidenced by studies in glioma cells, where YAP overexpressing cells exhibited better survival and less DNA damage after radiation[26].

In this study, we explored the effect of YAP inhibition on radioresistance in head and neck cancer. Furthermore, we investigated the underlying mechanism by which YAP contributes to radioresistance.

2. MATERIALS AND METHODS

2.1. Cell culture

Human head and neck squamous cell carcinoma cell line HEP-2 was purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were subcultured upon reaching 80% confluence and subsequently maintained at 37 °C in a humidified incubator with 5% CO_2. The cultures were grown in DMEM (Hyclone) and RPMI media (Hyclone), each supplemented with 10% (v/v) fetal bovine serum (GIBCO). Routine assessments for mycoplasma contamination were performed.

2.2. Generation of YAP-knockdown cells

Lentiviral packaging vectors (pCMV-VSVG and pCMV-delta R8.2, Sigma) and the scramble vector (pLKO.1-puro Vector, Sigma) or the transfection vector (pLKO.1-puro-shYAP1 Vector, Sigma) were co-transfected into 293T cells using the CalPhos™ Mammalian Transfection Kit (Takara). Conditioned medium was collected at 48- and 72-hours post-transfection, then filtered through 0.45 μm filters. To enhance transduction, 8 μg/mL polybrene (Invitrogen) was added to the conditioned medium, after which the target cells were infected, and fresh medium was replenished after 6 hours. The short hairpin RNA (shRNA) and small interfering RNA (siRNA) sequences utilized for YAP knockdown are as follows (Table 1).

Table 1. List of shRNA and siRNA sequences used in the study

shRNA	Sequence
shYAP #1	5'-GCCACCAAGCTAGATAAAGAA-3'
shYAP #2	5'-GCCACCAAGCTAGATAAAGAA-3'
siRNA	Sequence
siYAP #1	5'-GACGACCAAUAGCUCAGAUTT-3'
siYAP #2	5'-CUGCCACCAAGCUAGAUAATT-3'

Abbreviations: shRNA, short hairpin RNA; siRNA, small interfering RNA

2.3. Overexpression of CCND1 gene

Using the CalPhos™ Mammalian Transfection Kit (Takara), retroviral packaging vector (pCL10A1) and either the scramble vector (pBABE-puro Vector, Addgene) or the transfection vector (pBABE-puro-CCND1-HA Vector, Addgene) was co-transfected into 293T cells. Conditioned medium was collected 48- and 72-hours post-transfection and subsequently filtered through 0.45 μm filters. 8 μg/mL polybrene (Invitrogen) was added to the conditioned medium to enhance viral transduction, after which the target cells were infected. Fresh medium was replaced 6 hours post-infection.

2.4. Real-time polymerase chain reaction (PCR)

RNA was extracted using Trizol Reagent (Invitrogen), and cDNA was synthesized from the isolated RNA. Real-time reverse transcription PCR was conducted with cDNA, specific primer pairs, and SYBR Green (Roche) on the LightCycler® 96 Instrument (Roche) as described in the user manual. All samples were normalized to GAPDH expression. Primer sequences used for real-time PCR are as follows (Table 2).

Table 2. List of primer sequences used for real-time PCR

	Direction	Sequence
YAP	Forward	5'-CGCTCTTCAACGCCGTCA-3’
	Reverse	5’-AGTACTGGCCTGTCGGGAGT-3’
GAPDH	Forward	5’-GGAGCGAGATCCCTCCAAAA-3’
	Reverse	5’-CACACCCATGACGAACATGG-3’

2.5. Western blot analysis

Cell lysates were prepared using RIPA lysis buffer on ice for 20 minutes. Following incubation, the lysates were collected, and the supernatant was separated by centrifugation at 12,500 rpm for 15 minutes at 4 °C. Protein concentrations were quantified using the BCA assay (Thermo Fisher Scientific) according to the manufacturer’s protocol. Equal amounts of protein (20–50 μg) were mixed with 4X protein sample buffer (Bio-RAD, supplemented with β-mercaptoethanol), and the samples were heated at 95 °C for 5 minutes. Proteins were then resolved on 10–12% SDS-PAGE gels and transferred to 0.45 μm PVDF membranes (Millipore). The membranes were blocked for 1 hour at room temperature with either 5% skim milk in TBST (1M Tris-Cl (pH 8.0), NaCl, TWEEN20) or 5% Bovine Serum Albumin (BSA) in TBST. Primary antibodies were incubated overnight at 4 °C in 5% BSA in TBST. The following primary antibodies were used as detecting proteins: YAP1, pYAP1(S127), caspase-9, caspase-7, caspase-3, pAKT(S473), AKT, γH2A.X, GAPDH (Cell Signaling), phospho-Histone H2A.X(S139) (Millipore), Cyclin D1 (Thermo), and β-actin (Santa Cruz). Following three TBST washes, secondary antibodies were applied to the membranes and incubated for 1 hour at room temperature. Chemiluminescent signals were detected using an ECL solution.

2.6. Cell irradiation

Cells were collected and equal aliquots were cultured overnight at a density adjusted to achieve 40–50% confluency at the time of irradiation. Subsequently, the cells were irradiated to the prescribed dose (2-, 4-, 6-, 8-Gy in a single fraction) using a Versa HD

Linear Accelerator (Elekta, Stockholm, Sweden) with 6 MV photon beam, at a gantry angle of 0°, and a dose rate of 600 MU/min. Cells were placed at the center of the 40 x 40 cm^2 field, with a source-to-surface distance of 90 cm. To ensure uniform dose distribution, the cells were placed on acrylic plates, with two sheets of 1 cm thick bolus placed over them during irradiation.

2.7. Cell proliferation assay

Cells were plated in 96-well plates at a density of 1 x 10^4 cells/mL, with 200 μL of cell suspension per well. To minimize variation due to evaporation, wells located at the periphery of the plate were excluded, and wells containing only medium were used as negative controls. After incubation, 100 μL of medium was removed from each well, and 10 μL of WST solution along with 90 μL of fresh medium was added. The absorbance was measured at 550 nm using a microplate reader.

2.8. Migration assay

Cell migration was assessed using transwell chambers with an 8 μm pore size (Corning Costar). Transfected cells were resuspended in 500 μL of medium containing 0.1% FBS and seeded into the upper chamber at a density of 5 x 10^4 cells per well. The cells were incubated at 37 °C in a 5% CO_2 atmosphere for 30 hours. Following incubation, cells that had migrated to the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with crystal violet. Residual cells on the upper surface were gently wiped off using a cotton swab. The migrated cells were counted in five random microscopic fields at x200 magnification to determine the average number of cells per field.

2.9. Apoptosis assay

Cells were plated in culture dishes and allowed to adhere overnight. 24 hours later, both the cells and supernatants were harvested using 0.25% trypsin-EDTA solution (Sigma-Aldrich). Apoptosis was assessed using the FITC-Annexin V Apoptosis Detection Kit (BD

Bioscience) according to the manual. The cells were then analyzed by flow cytometry on a FACS LSRFortessa (BD Bioscience).

2.10. Cell cycle analysis

Cells were plated in culture dishes and allowed to adhere overnight. After 24 hours, the cells and supernatants were collected using 0.25% trypsin EDTA solution (Sigma-Aldrich). The cells were then washed twice with ice-cold phosphate-buffered saline (PBS) and harvested by centrifugation. To fix the cells, pre-chilled 70% ethanol was applied. Propidium iodide staining solution (Sigma) was used for a 15-minute incubation at 37 °C in the dark. Cell cycle analysis was performed using flow cytometry on a FACS LSRFortessa (BD Bioscience).

2.11. In vivo tumor xenograft

HEP-2 cells transduced with shYAP1 were subcutaneously implanted into the lateral trunk area of 8-week-old female BALB/c nude mice at a concentration of 1 x 10^6 cells per mouse using a 22-gauge needle. Following injection, the mice were monitored for 6 consecutive weeks. Tumor growth was measured with a Vernier caliper, and tumor volume was calculated using the following formula: 1/2 x (minor axis x 2) x major axis.

Animal procedures in this study complied with the guidelines authorized by the Institutional Animal Care and Use Committee (IACUC) of Yonsei University College of Medicine. The animal research facility holds accreditation from AAALAC International.

2.12. Immunofluorescence

Cells were cultured on cover slips, fixed with 4% paraformaldehyde, and then permeabilized with 0.2% Triton X-100 at room temperature. PBS solution with 1% BSA was used for blocking. The following primary antibodies were used for protein detection: YAP1 (Cell Signaling), phospho-Histone H2A.X(S139) (Millipore), Cyclin D1 (Thermo), and β-actin (Santa Cruz). Immunostaining was performed at room temperature with the

aforementioned primary antibodies. Alexa Fluor 488-conjugated anti-rabbit IgG and Alexa Fluor 594-conjugated goat anti-mouse IgG were used as secondary antibodies (Invitrogen). DNA was stained with DAPI in PBS (Sigma). Immunofluorescence images were captured using a Zeiss LSM980 Confocal Microscope (x60 magnification). Image acquisition and processing were performed with the ZEN software (Zeiss), and γH2A.X foci were quantified using the ImageJ software.

2.13. Statistical analysis

Intergroup comparisons were conducted via a two-tailed Student's t-test, and data analysis was carried out using GraphPad Prism (GraphPad software Inc.) and Microsoft Excel (Microsoft Corp.). All data are presented as the mean ± standard deviation, and statistical significance was considered at $p < 0.05$. The level of significance is indicated as * p <0.05, ** p <0.01, *** p <0.001, and **** p <0.0001.

3. RESULTS

3.1. YAP expression level is associated with cell proliferation, migration, and apoptosis

3.1.1. Expression of YAP in head and neck cancer cell lines

Western blot analysis revealed that YAP1 protein was expressed in several head and neck cancer cell lines including HEP-2, SNU-1041, UMSCC-47, UMSCC-104, and YD-32 (Figure 4A). Relative mRNA level results correlated with the western blot results; HEP-2, SNU-1041, UMSCC-47, UMSCC-104, and YD-32 showed relatively high YAP1 mRNA levels (Figure 4B).

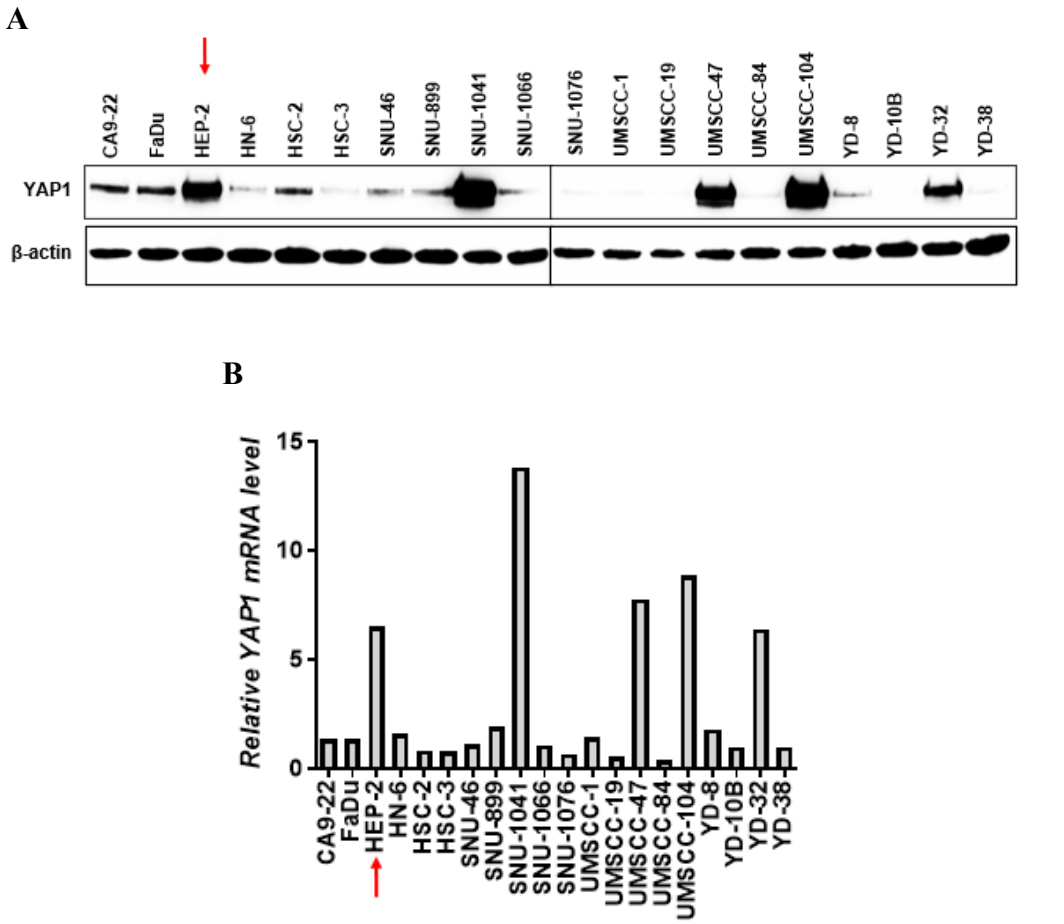


Fig. 4. YAP expression level in head and neck cancer cell lines. (A) Western blot showed increased expression of YAP in several cell lines. (B) Relative mRNA levels correlated with the western blot results.

3.1.2. Radiosensitivity of head and neck cancer cell lines

To investigate the inherent radiosensitivity of the head and neck cancer cell lines, 4 Gy of radiation was administered and the number of colonies formed were counted. As a result, YD-8, UMSCC-1 and HEP-2 cell lines showed relatively radioresistant features.

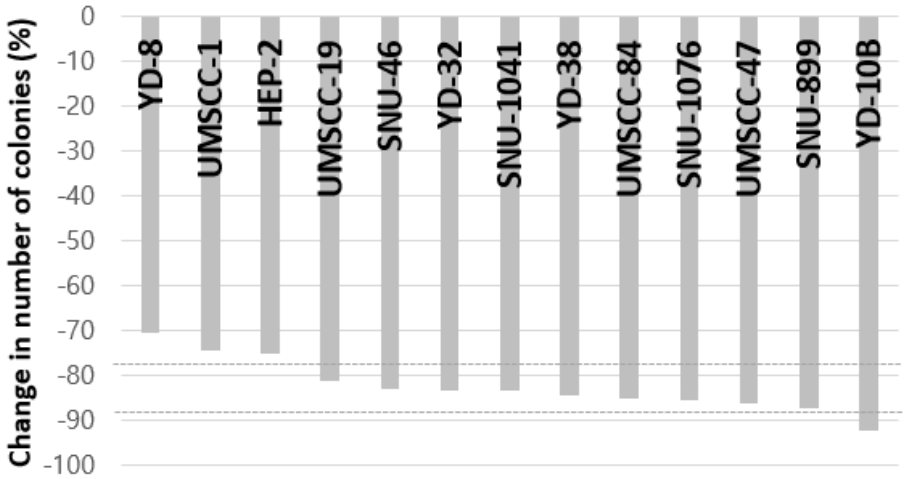
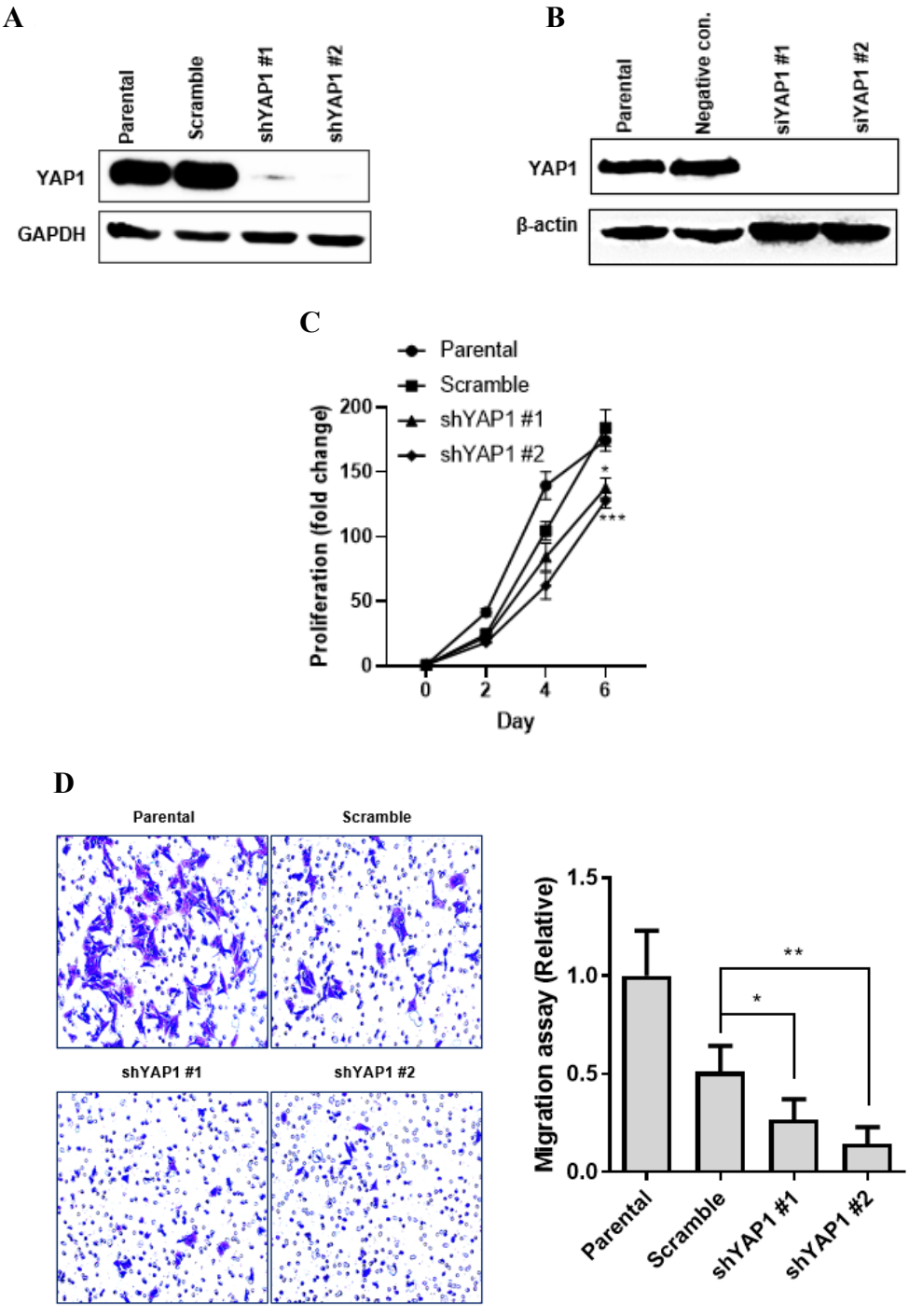


Fig. 5. Waterfall plot showing the inherent radiosensitivity of head and neck cancer cell lines. 4 Gy of radiation was administered to the head and neck cancer cell lines and the change in number of colonies were calculated. Dashed lines denote the upper and lower ranges of median ± one standard deviation.

Since HEP-2 showed relatively high YAP expression and radioresistance, HEP-2 was selected for further experiments in this study.

3.1.3. YAP knockdown affects cellular characteristics

To investigate the change in cellular phenotypes following YAP inhibition, we knocked down YAP from HEP-2 cells using shRNA and siRNA targeting YAP (shYAP and siYAP) (Figure 6A, B). Compared to the parental HEP-2 cells, cell proliferation was significantly decreased by transfection of shYAP in HEP-2 cell lines (Figure 6C). Similarly, cell migration was significantly reduced in HEP-2 cells transfected with shYAP compared to the parental or negative control cells (Figure 6D). Immunofluorescence assay also revealed decreased YAP1 expression in cells transfected with siYAP (Figure 6E). Cell proliferation was significantly decreased in cells transfected with siYAP compared to the parental or negative control cells (Figure 6F).



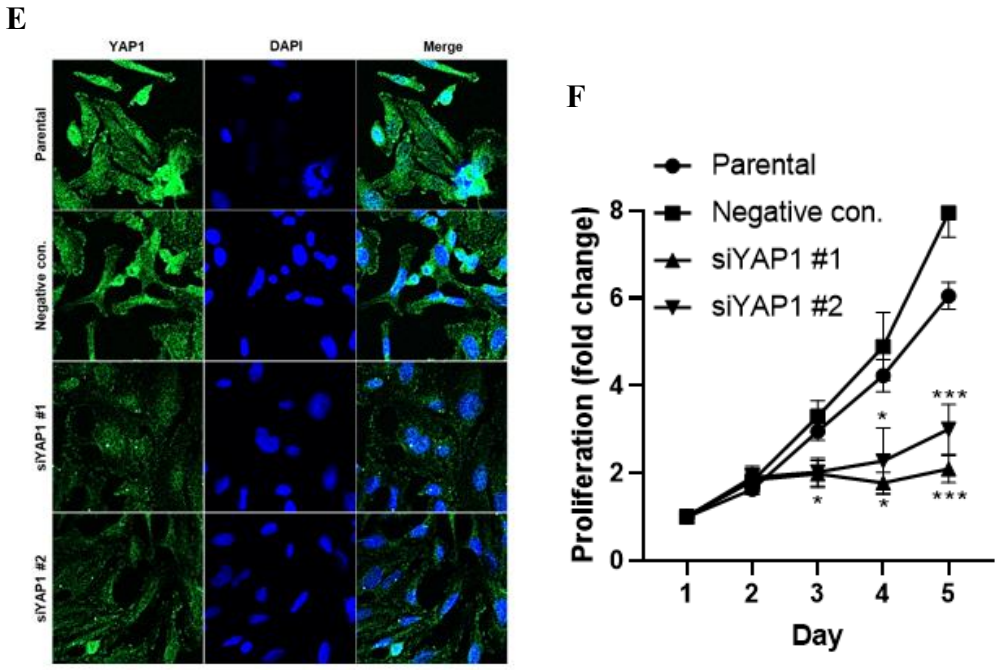


Fig. 6. Reduced cell proliferation and migration in YAP knockdown cells. (A) Western blot results showing decreased expression of YAP1 after transfection with shYAP. (B) Western blot results showing decreased expression of YAP1 after transfection with siYAP. (C) Relative cell proliferation showed decreased cell proliferation after YAP knockdown with shRNA. (D) Cell migration assay results showed decreased cell migration after YAP knockdown with shRNA. (E) Immunofluorescence assay results showing decreased YAP1 expression in cells transfected with shYAP. (F) Cell proliferation decreased after YAP knockdown with siRNA. (* p <.05, ** p <.01, *** p <.001)

We also performed an apoptosis assay to see the change in apoptotic rate following YAP knockdown. We observed a significantly increased rate of apoptosis in cells transfected with siYAP, meaning that YAP knockdown results in increased apoptosis (Figure 7).

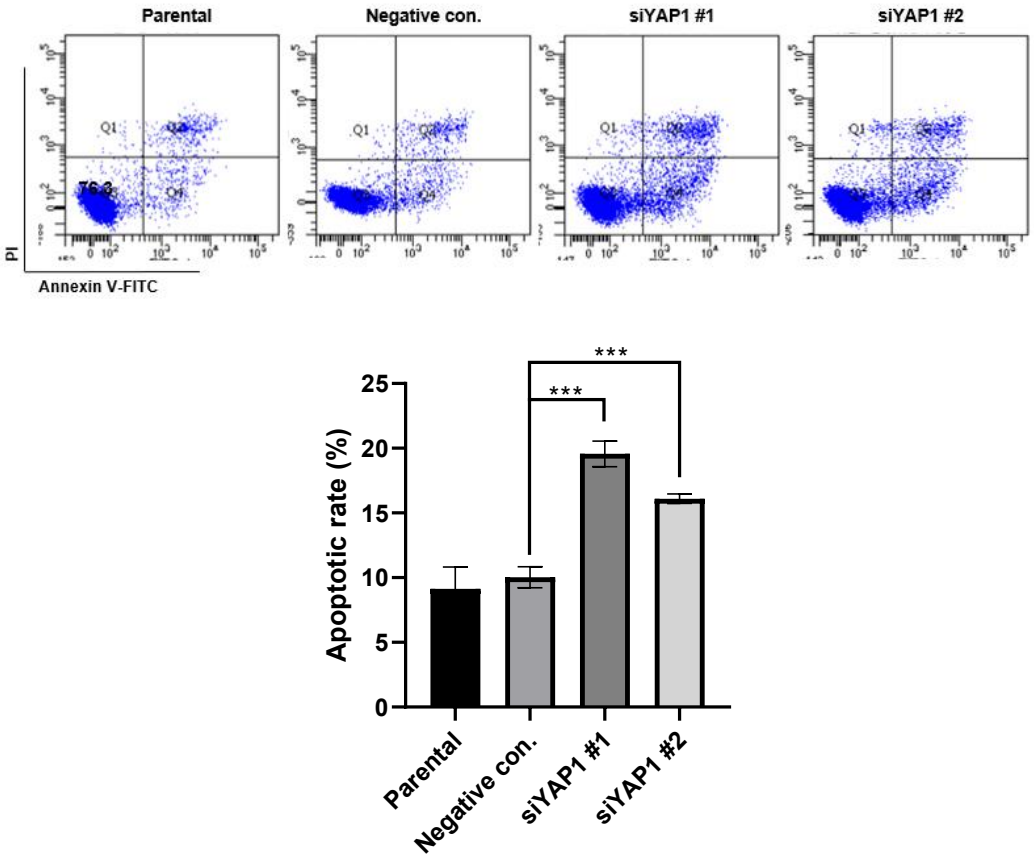


Fig. 7. YAP knockdown results in increased apoptosis. Annexin V apoptosis assay results of non-edited parental, scrambled, or YAP knockdown clones. YAP knockdown resulted in significantly increased rate of apoptosis. (*** p <.001)

In addition, we investigated the tumorigenicity of YAP knockdown cells in vivo using tumor xenograft models. The tumor volumes of implanted tumors were significantly smaller in the mouse with YAP knockdown clones compared with wildtype or scrambled clone (Figure

8A–C).

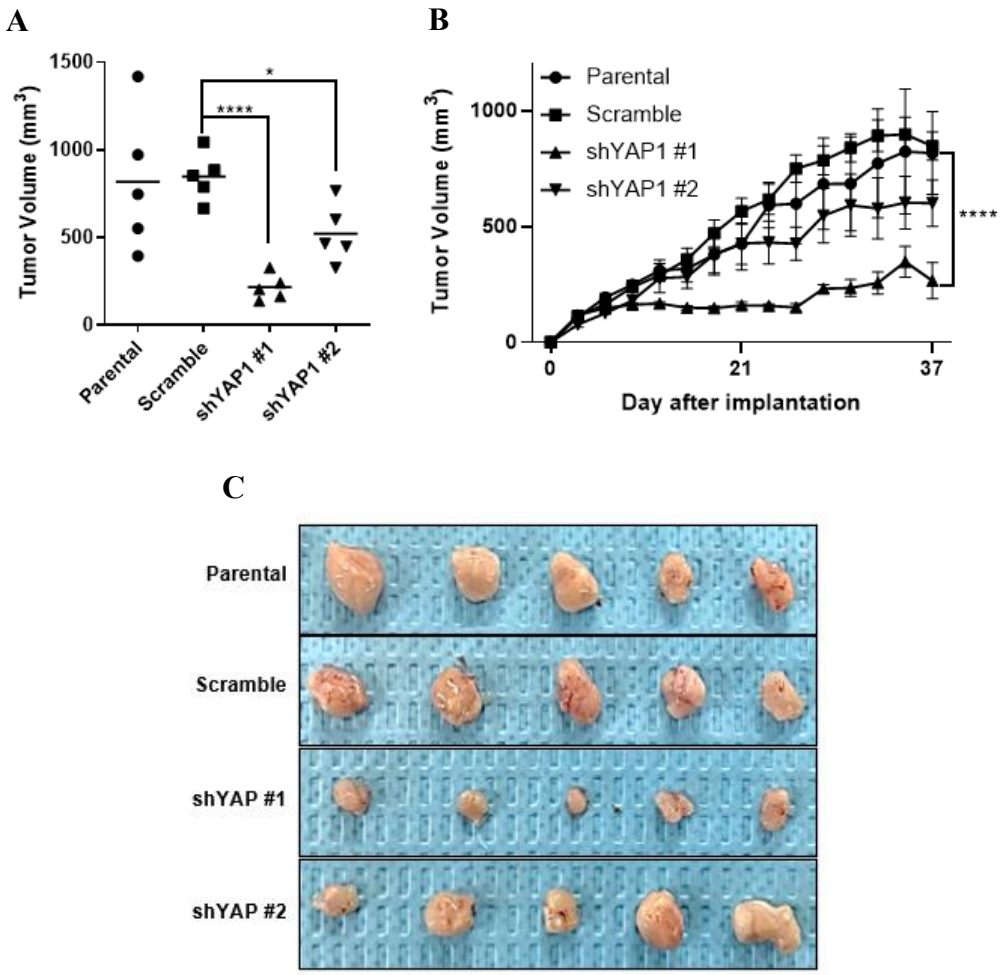


Fig. 8. In vivo tumorigenicity assay showing smaller tumor volumes in YAP knockdown cells. (A) YAP knockdown cells were injected into xenograft model and tumor volume was measured at sacrifice. Tumor volume was significantly smaller in YAP knockdown clones. (B) Longitudinal follow up of tumor growth for 6 weeks after implantation revealed delayed tumor growth in YAP knockdown clones. (C) Gross photography of tumor size. (* p <.05, *** p <.001, **** p <.0001)

Taken altogether, these results confirm that YAP is involved in cell proliferation, migration, and apoptosis, and that inhibition of YAP leads to changes in these cellular phenotypes.

3.2. Combination of YAP inhibition and radiotherapy has a synergistic effect

3.2.1. YAP knockdown enhances radiosensitivity

Next, we wanted to see if YAP knockdown affects radiosensitivity. The non-edited parental, scrambled, or YAP knockdown cells with shYAP were exposed to 0, 2, 4, 6, and 8 Gy of radiation, and colony formation was assessed by counting the number of colonies (Figure 9). As a result, we observed a decreased number of colonies in YAP knockdown clones.

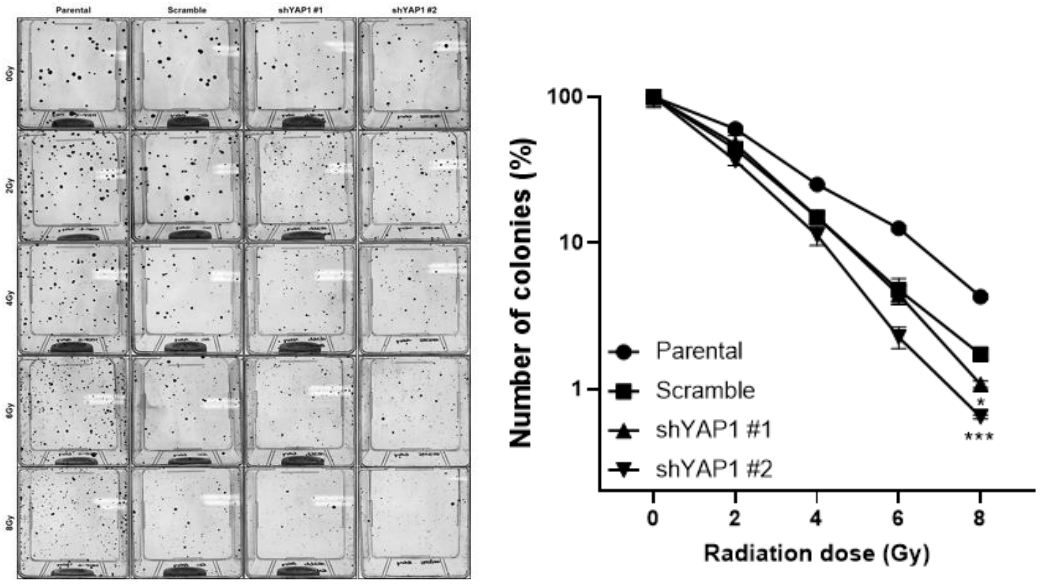


Fig. 9. Clonogenic assay showing increased sensitivity to radiation in YAP knockdown cells. After exposure to 0, 2, 4, 6, and 8 Gy of radiation, colony formation decreased following

YAP knockdown with shYAP compared to parental or scrambled clones. (* p <.05, *** p <.001)

3.2.2. Combination of YAP inhibition and irradiation has synergistic effects

Subsequently, 6 Gy of radiation was administered to both wildtype HEP-2 cells and HEP-2 cells with YAP knockdown, and cell proliferation was assessed longitudinally for five consecutive days. The results showed that the addition of YAP knockdown further decreased cell proliferation compared to radiation alone, indicating a synergistic effect between YAP inhibition and radiation (Figure 10).

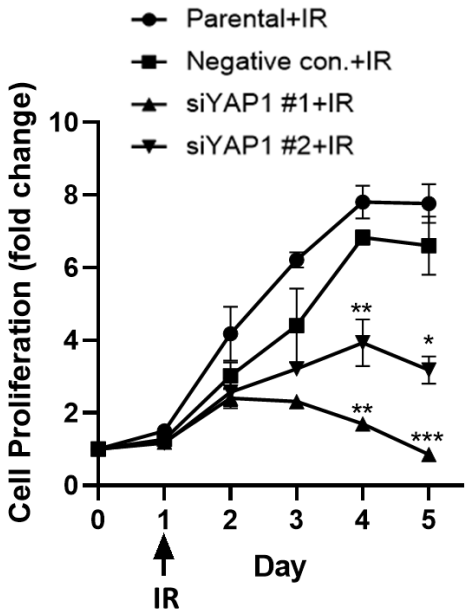


Fig. 10. Reduced cell proliferation in YAP knockdown cells following radiation treatment. Parental and YAP knockdown HEP-2 cells were exposed to 6 Gy radiation on day 1 and cell proliferation was assessed for 5 consecutive days. Compared with radiation alone, YAP knockdown combined with radiation resulted in significant decrease of cell proliferation, showing a synergistic effect between YAP inhibition and radiation. (** p <.01, **** p <.0001)

Based on the previous results, it was confirmed that knockdown of YAP using either siRNA or shRNA enhances radiosensitivity. To explore the potential therapeutic application of YAP inhibition, we conducted similar experiments using YAP inhibitory drugs. Verteporfin is a YAP inhibitory drug that induces YAP phosphorylation and degradation. When HEP-2 cells were treated with Verteporfin and an increasing dose of radiation, the combination of YAP inhibition and radiation reduced colony formation, again demonstrating a synergistic effect between YAP inhibition and radiation (Figure 11).

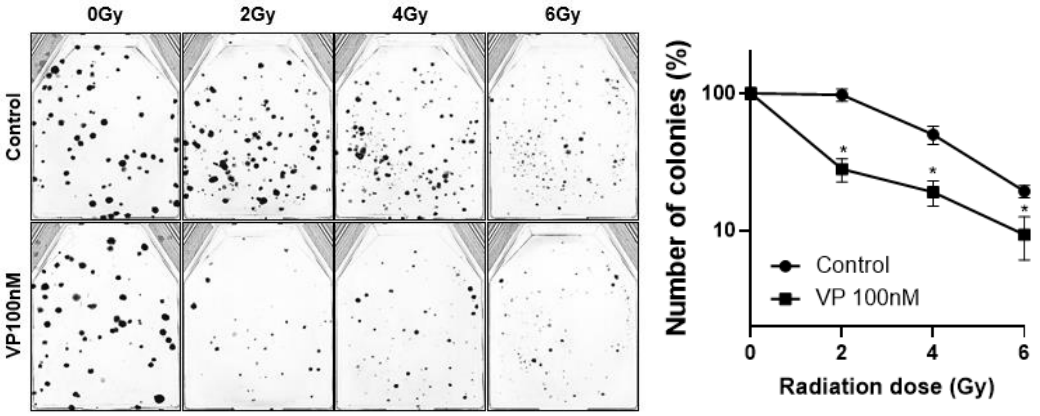


Fig. 11. Colony formation decreased following a combination of Verteporfin, a YAP inhibitory drug, and radiation. Cells treated with a combination of radiation and 100 nM of Verteporfin, a drug inducing YAP phosphorylation and degradation, exhibited significantly decreased colony formation compared to those treated with radiation alone. (* p <.05)

CA3 is another YAP inhibitory drug that blocks YAP-TEAD binding. Compared to cells treated with radiation alone, we also observed a synergistic effect between YAP inhibition and radiation when CA3 was combined with radiation (Figure 12).

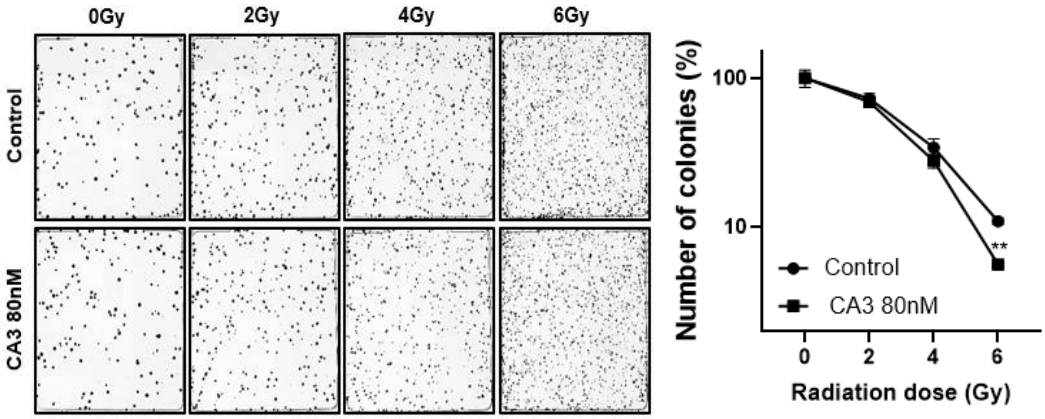


Fig. 12. Colony formation decreased following a combination of CA3, a YAP inhibitory drug, and radiation. Cells treated with a combination of radiation and 80 nM of CA3, a drug inhibiting YAP-TEAD binding, exhibited significantly decreased colony formation compared to those treated with radiation alone. (** p <.01)

3.2.3. YAP knockdown and irradiation results in increased apoptosis

Next, we investigated whether YAP knockdown enhances cellular apoptosis following irradiation. When cells were exposed to 6 Gy of radiation after YAP knockdown with siYAP, we observed a significant increase in the apoptotic rate in YAP knockdown cells compared to both parental and negative control cells (Figure 13). Furthermore, the apoptosis rate was further elevated in cells treated with both siYAP and radiation, compared to those treated with siYAP alone. This suggests a synergistic effect between YAP inhibition and radiation in promoting apoptosis.

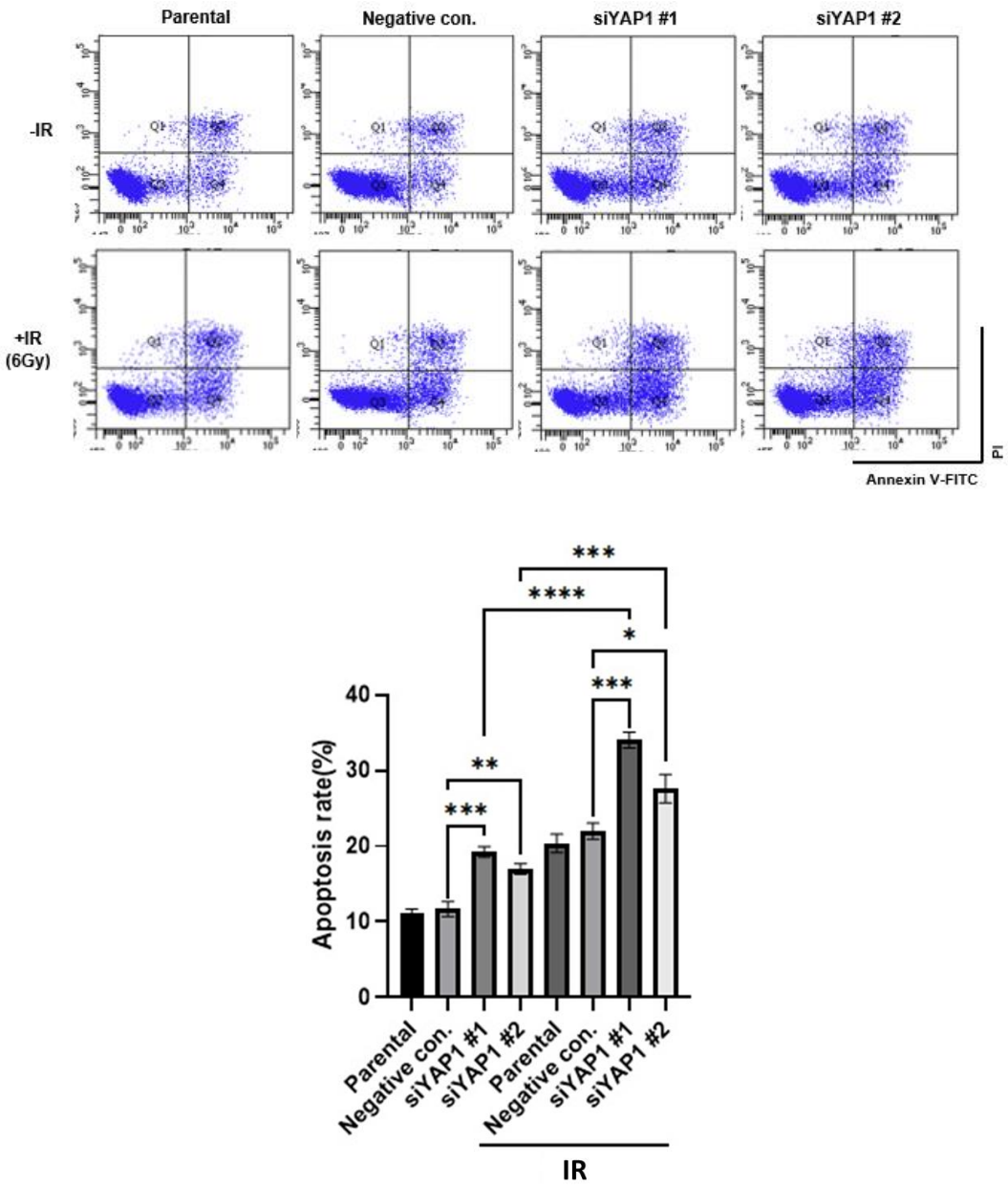


Fig. 13. Apoptosis assay showing increased apoptosis in YAP knockdown cells treated with radiation. Apoptosis rate was significantly higher in YAP knockdown cells compared to parental or negative control cells. A similar trend was observed when YAP inhibition was

combined with 6 Gy of radiation. (* p <0.05, ** p <.01, *** p <.001, **** p <.0001)

Additionally, we examined whether the expression levels of apoptosis-related proteins changed following radiation using western blot analysis. The results showed that, compared to parental or negative control cells, YAP knockdown cells exhibited decreased expression of Bcl-2 and increased expression of BAX, cleaved caspase-3, and cleaved PARP following irradiation, indicating an increased apoptotic rate (Figure 14).

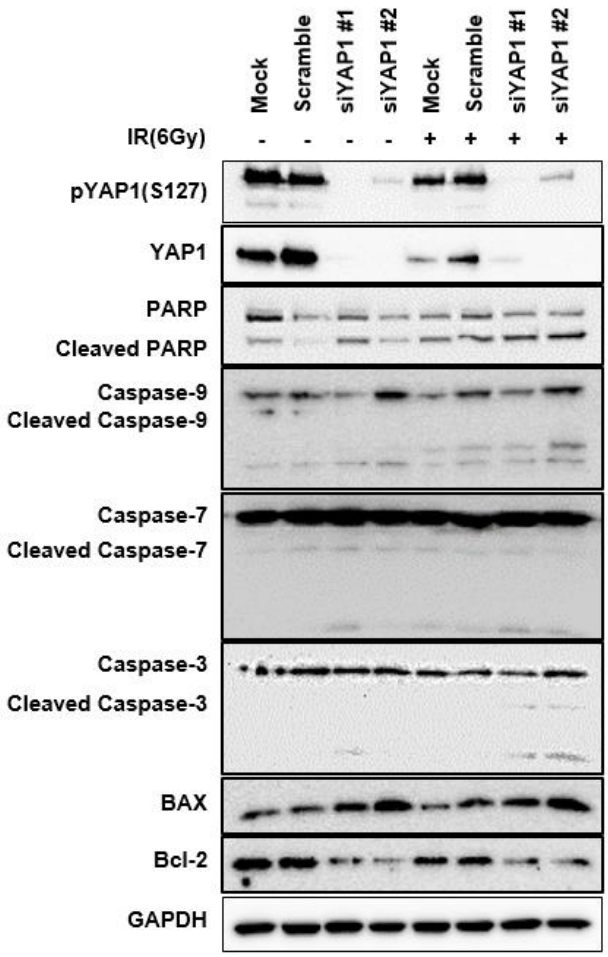


Fig. 14. The expression level of apoptosis-related proteins changed in YAP knockdown cells. Western blot analysis revealed that the protein expression level of Bcl-2 decreased and those of BAX, cleaved caspase-3, and cleaved PARP increased after a combination of YAP knockdown and irradiation.

3.2.4. YAP inhibition and irradiation results in G0/G1 arrest

Lastly, we performed flow cytometry to see the change in cell cycle distribution in YAP knockdown cells following 4 Gy irradiation. As a result, we observed an increased percentage of cells in G0/G1 phase in YAP knockdown cells compared to the parental or negative control

cells (Figure 15).

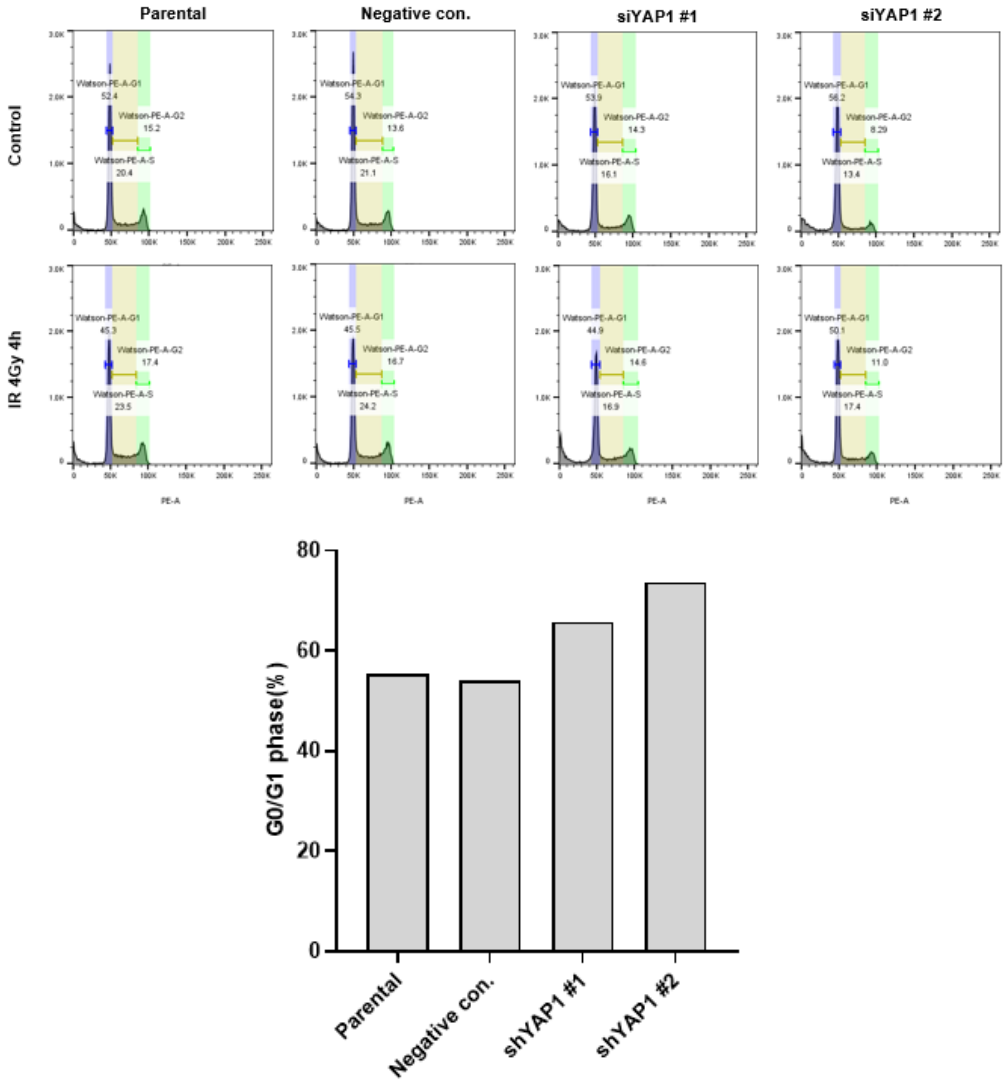


Fig. 15. Flow cytometry results showing an increased percentage of cells in G0/G1 phase in YAP knockdown cells treated with radiation. The percentage of cells in G0/G1 phase was higher in the YAP knockdown cells compared to parental or negative control cells.

3.3. YAP expression level correlates with Cyclin D1 level, and DNA damage

3.3.1. YAP activation increases the expression of Cyclin D1

After discovering that YAP is involved in radioresistance, we sought to elucidate the underlying mechanisms. Noting that YAP inhibition induces G0/G1 cell cycle arrest, we examined the effect of YAP expression on Cyclin D1 levels. Western blot analysis revealed that Cyclin D1 expression decreased upon YAP knockdown, whereas YAP overexpression led to an increase in Cyclin D1 expression (Figure 16).

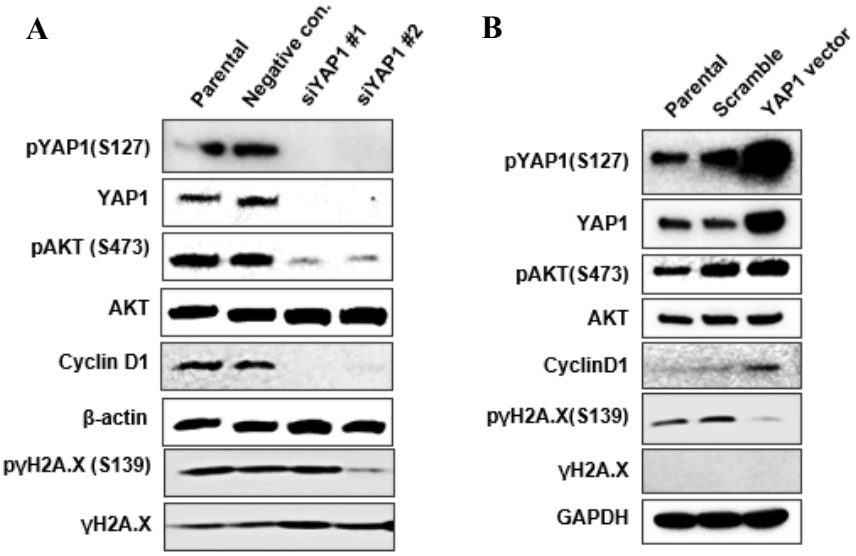


Fig. 16. Western blot analysis showing that the expression levels of YAP and Cyclin D1 are correlated. (A) YAP knockdown resulted in decreased expression of Cyclin D1. (B) YAP overexpression resulted in increased expression of Cyclin D1.

3.3.2. Cyclin D1 level decreases following YAP inhibition and irradiation

Subsequently, we investigated the effect of YAP inhibition by Verteporfin and CA3 on Cyclin D1 expression following 4 Gy radiation treatment. Verteporfin, which induces YAP degradation, resulted in a reduction of both total YAP and Cyclin D1 levels when combined with radiation (Figure 17A). On the other hand, CA3, which inhibits YAP-TEAD binding, did not affect the total YAP level, but a time-dependent decrease in Cyclin D1 expression was observed following radiation exposure (Figure 17B). These findings suggest that YAP is involved in the regulation of Cyclin D1.

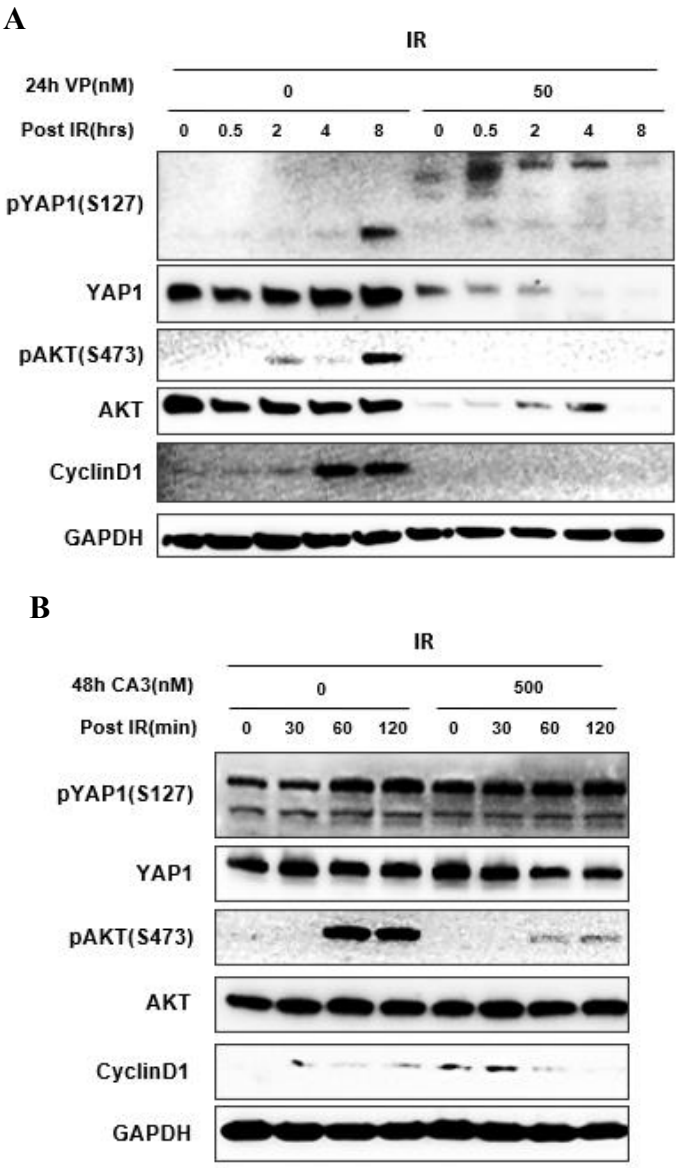
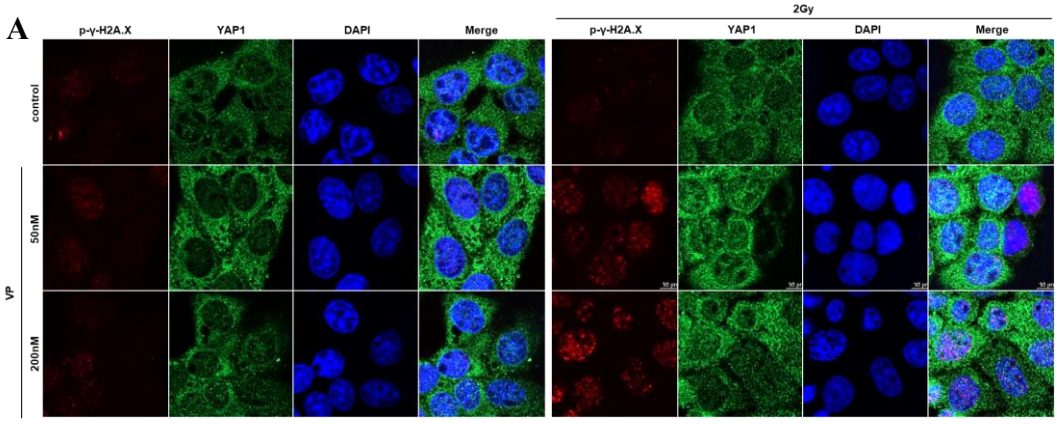
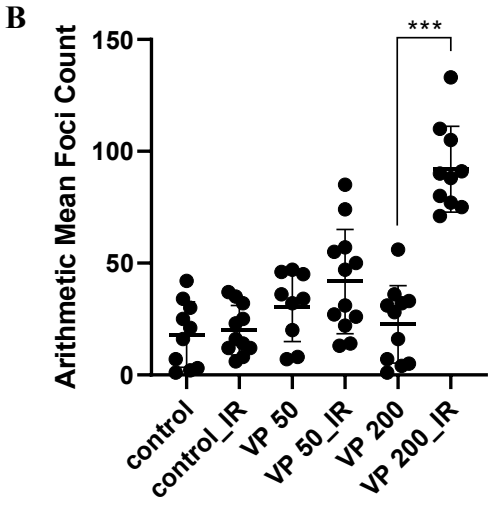


Fig. 17. The expression level of Cyclin D1 decreased following YAP inhibition and radiation. (A) Western blot analysis showed that following a combination of 50 nM of Verteporfin and 2 Gy radiation, expression levels of both total YAP and Cyclin D1 decreased. (B) Following a combination of 500 nM of CA3 and 4 Gy radiation, expression levels of Cyclin D1 decreased in a time-dependent manner.

3.3.3. Nuclear γH2AX foci increases following YAP inhibition and irradiation

Next, we assessed the extent of DNA damage following YAP inhibition and radiation using an immunofluorescence assay. Again, we used Verteporfin and CA3 for the pharmacologic inhibition of YAP, and 2 Gy of radiation was administered. As a result, we observed a significant increase in γH2AX expression when YAP inhibition was combined with radiation, indicating a significant increase in DNA damage (Figure 18).





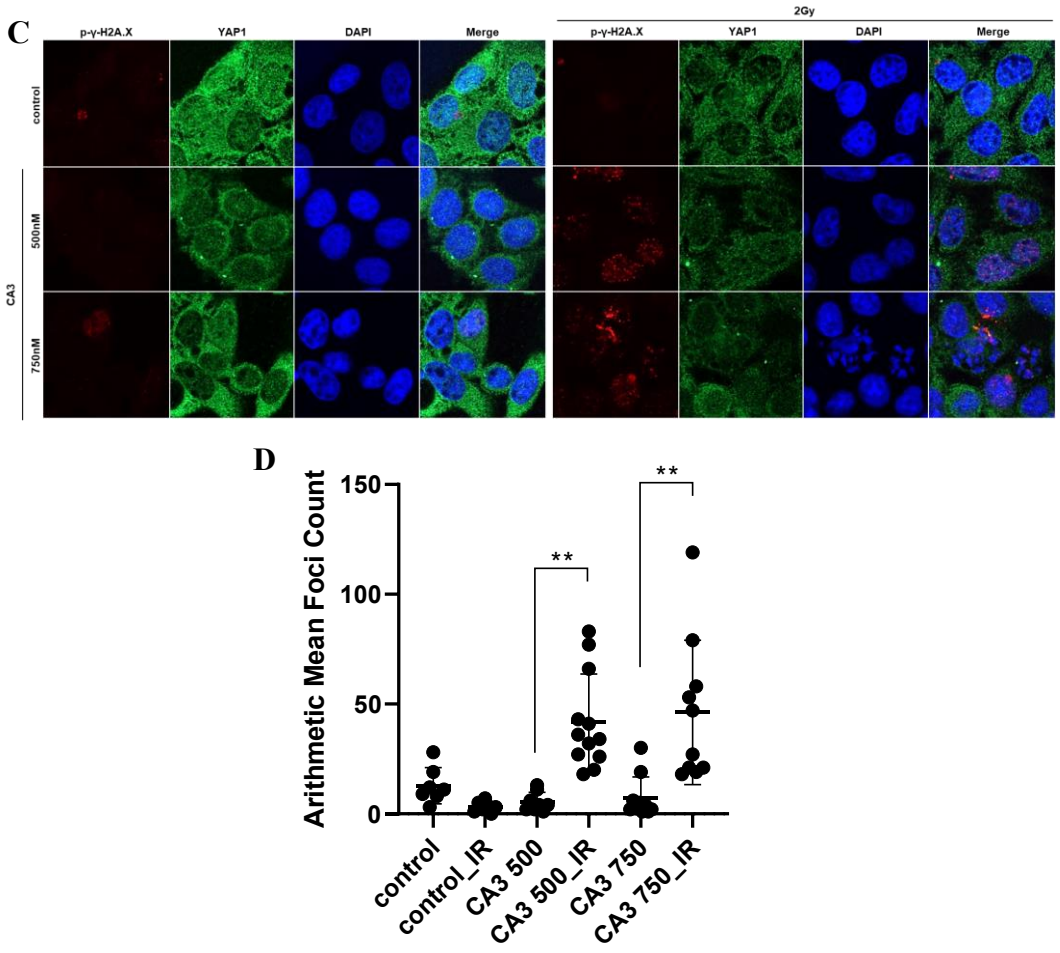
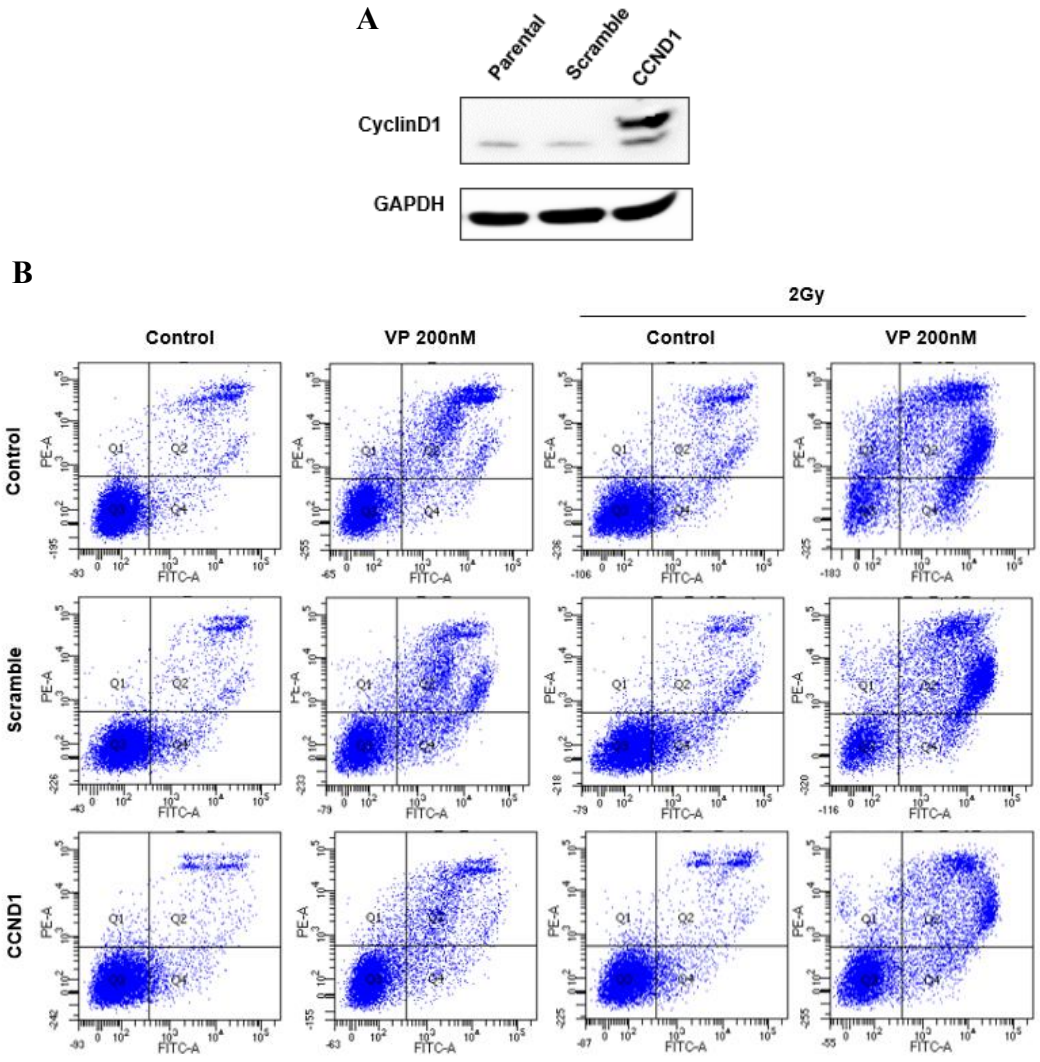


Fig. 18. Immunofluorescence assay showing increased DNA damage following YAP inhibition and radiation. (A) Immunofluorescence assay results following the administration of Verteporfin and radiation. (B) Mean phosphorylated γH2AX foci levels significantly increased following the administration of both Verteporfin and radiation. (C) Immunofluorescence assay results following the administration of CA3 and radiation. (D) Mean phosphorylated γH2AX foci levels significantly increased following the administration of both CA3 and radiation. (** p <0.01, *** p <0.001)

3.3.4. Overexpression of Cyclin D1 reduces apoptosis

To determine whether the cellular characteristics induced by YAP inhibition could be reversed by Cyclin D1 overexpression, we overexpressed Cyclin D1 in HEP-2 cells (Figure 19A) and performed an apoptosis assay. When Verteporfin and radiation were combined in HEP-2 parental cells, scramble cells, and Cyclin D1-overexpressing (CCND1) cells, we observed a reduction in the apoptotic rate in the Cyclin D1-overexpressing cells compared to the Cyclin D1 wildtype cells (Figure 19B-C). Similarly, when YAP inhibition was induced with CA3, we observed a decrease in apoptosis in Cyclin D1-overexpressing cells compared to cells without Cyclin D1 overexpression (Figure 19D-E). Western blot analysis of apoptosis-related proteins revealed that while the expression levels of cleaved caspase-3 and cleaved PARP were increased in YAP knockdown cells treated with radiation, the expression levels of both cleaved caspase-3 and cleaved PARP decreased in Cyclin D1-overexpressing cells (Figure 19F). These results suggest that Cyclin D1 plays a role in regulating cell apoptosis following radiation.



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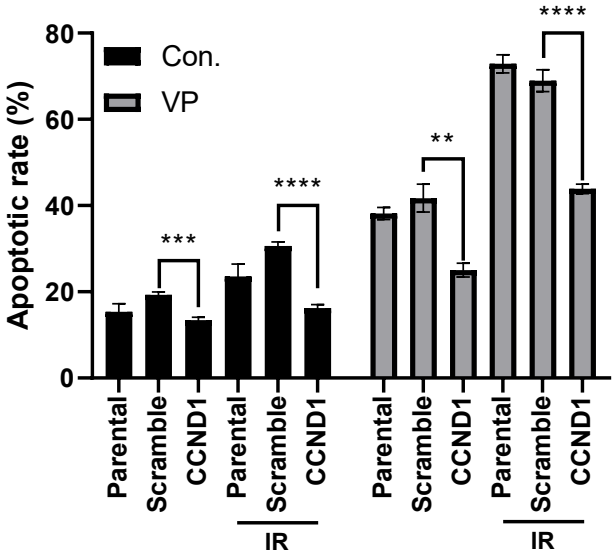

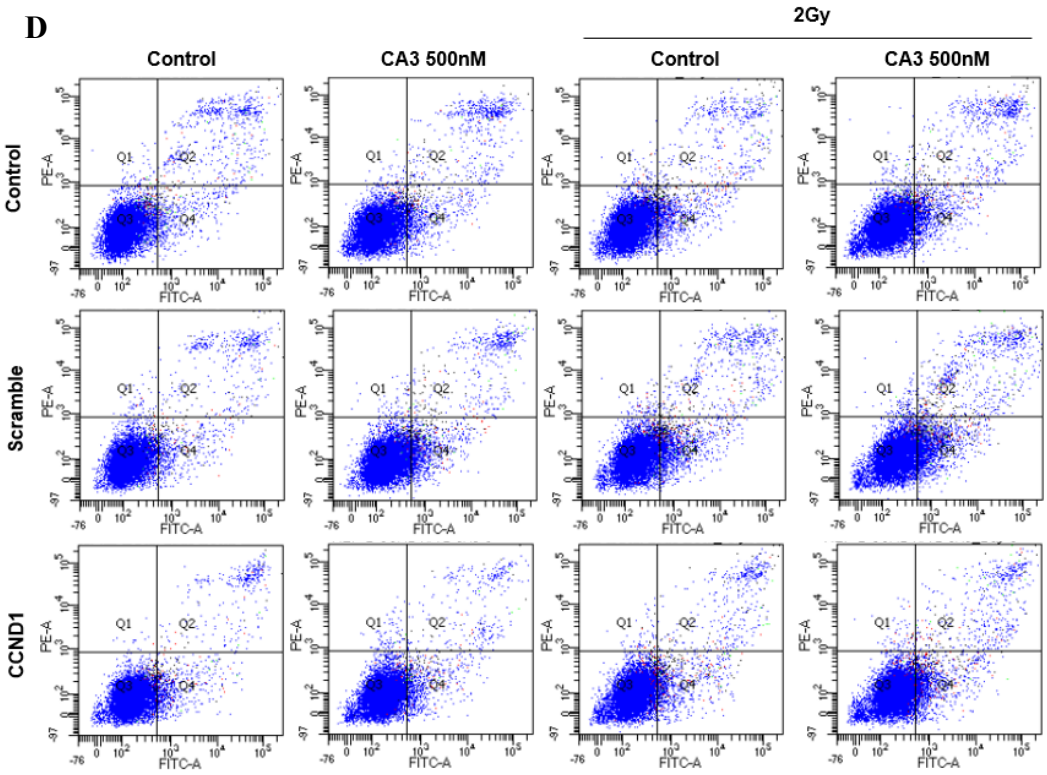

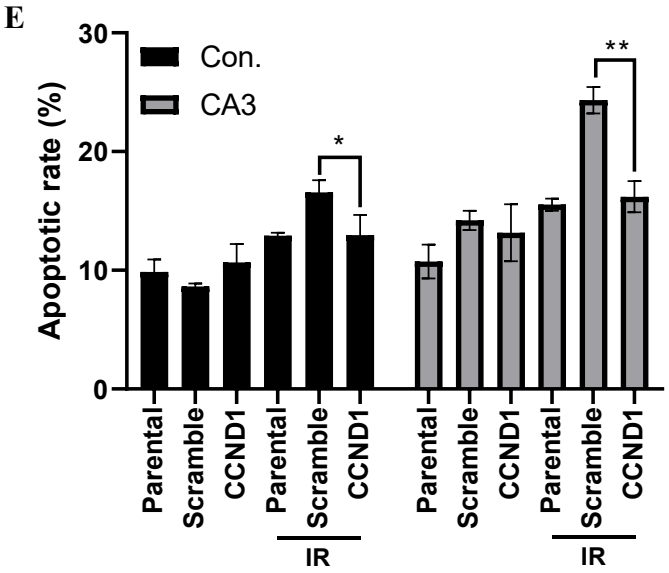

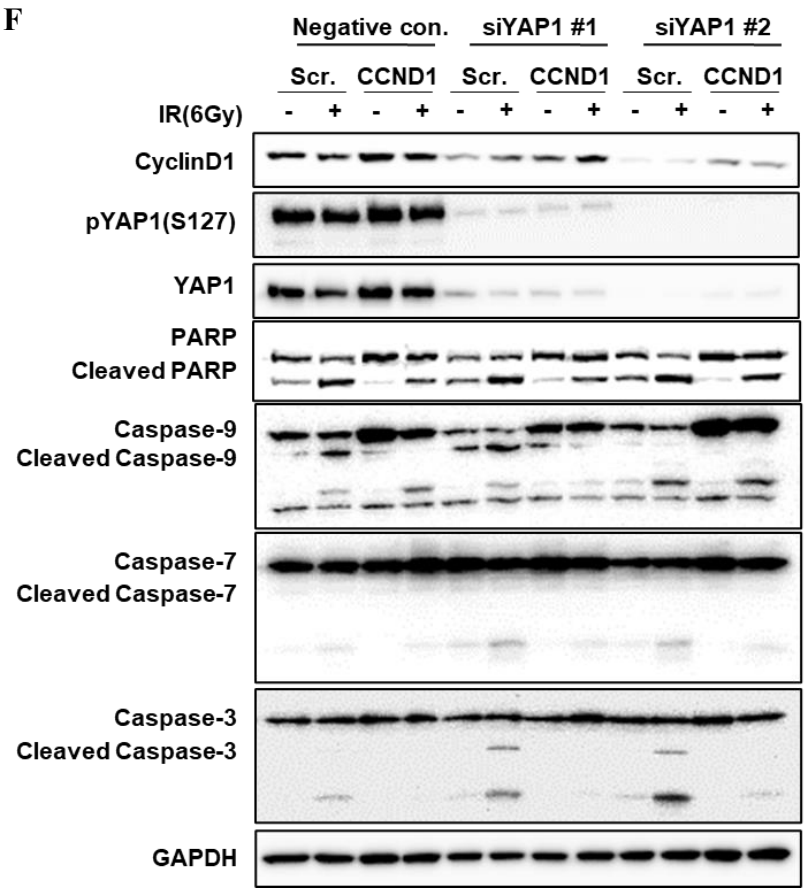

Fig. 19. Cyclin D1 overexpression reversed the increased apoptosis following YAP inhibition and radiation. (A) Cyclin D1 overexpression was confirmed with western blot analysis. (B) Apoptosis assay results of parental, scrambled, and Cyclin D1-overexpressing cells treated with Verteporfin and 2 Gy radiation. (C) Compared to the Cyclin D1 non-overexpressing cells, the apoptotic rate was decreased in Cyclin D1-overexpressing cells. (D) Apoptosis assay results of parental, scrambled, and Cyclin D1-overexpressing cells treated with CA3 and 2 Gy radiation. (E) Compared to the Cyclin D1 non-overexpressing cells, the apoptotic rate was decreased in Cyclin D1-overexpressing cells. (F) Western blot analysis showed that the expression of cleaved caspase-3 and cleaved PARP decreased in Cyclin D1-overexpressing cells following YAP inhibition and radiation compared to negative control cells.

3.3.5. Overexpression of Cyclin D1 reduces DNA damage

Lastly, we performed western blot analysis to assess the extent of DNA damage in Cyclin D1-overexpressing cells treated with a YAP inhibitory drug and radiation. When Cyclin D1 was overexpressed, we observed a reduction in γH2AX expression after treatment with YAP inhibition and radiation, compared to the scramble control (Figure 20).

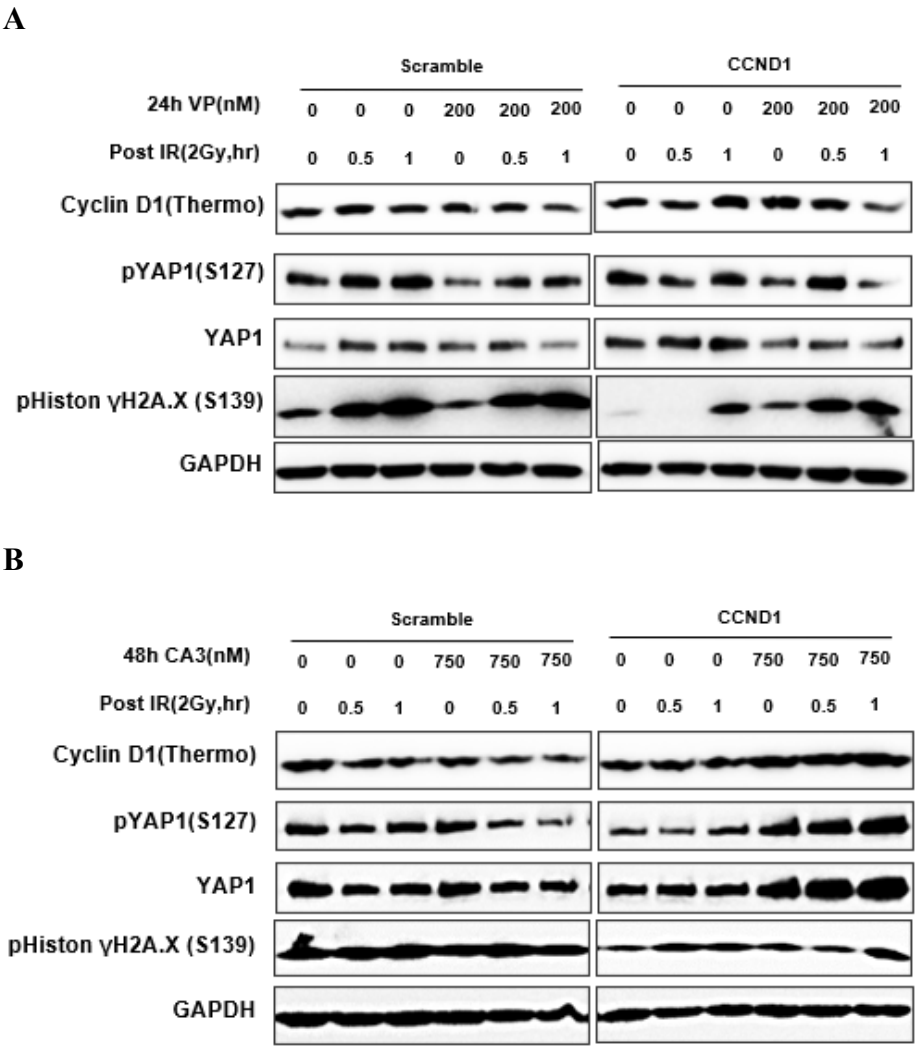

Fig. 20. DNA damage levels were lower in Cyclin D1 overexpressing cells treated with YAP inhibition and radiation. (A) Cyclin D1-overexpressing cells treated with 200 nM of Verteporfin and 2 Gy radiation showed decreased expression of γH2AX compared to scramble cells. (B) Similar results were observed in cells treated with CA3 and 2 Gy radiation.

To assess the extent of DNA damage based on the expression of Cyclin D1 in cells, we performed an immunofluorescence assay of scramble control cells and the Cyclin D1

overexpressed cells following treatments with Verteporfin, radiation, and the combination of Verteporfin and radiation (Figure 21A). As expected, we observed increased expression of green fluorescence, indicating high Cyclin D1 expression, in the Cyclin D1 overexpressed cells (Figure 21B). Additionally, it was observed that cells overexpressing Cyclin D1 showed fewer γH2AX foci expression compared to the scramble cells. Moreover, the number of γH2AX foci inversely correlated with the green fluorescence intensity of Cyclin D1 expression (Figure 21C). These findings suggest that Cyclin D1 regulates DNA damage upon radiation.

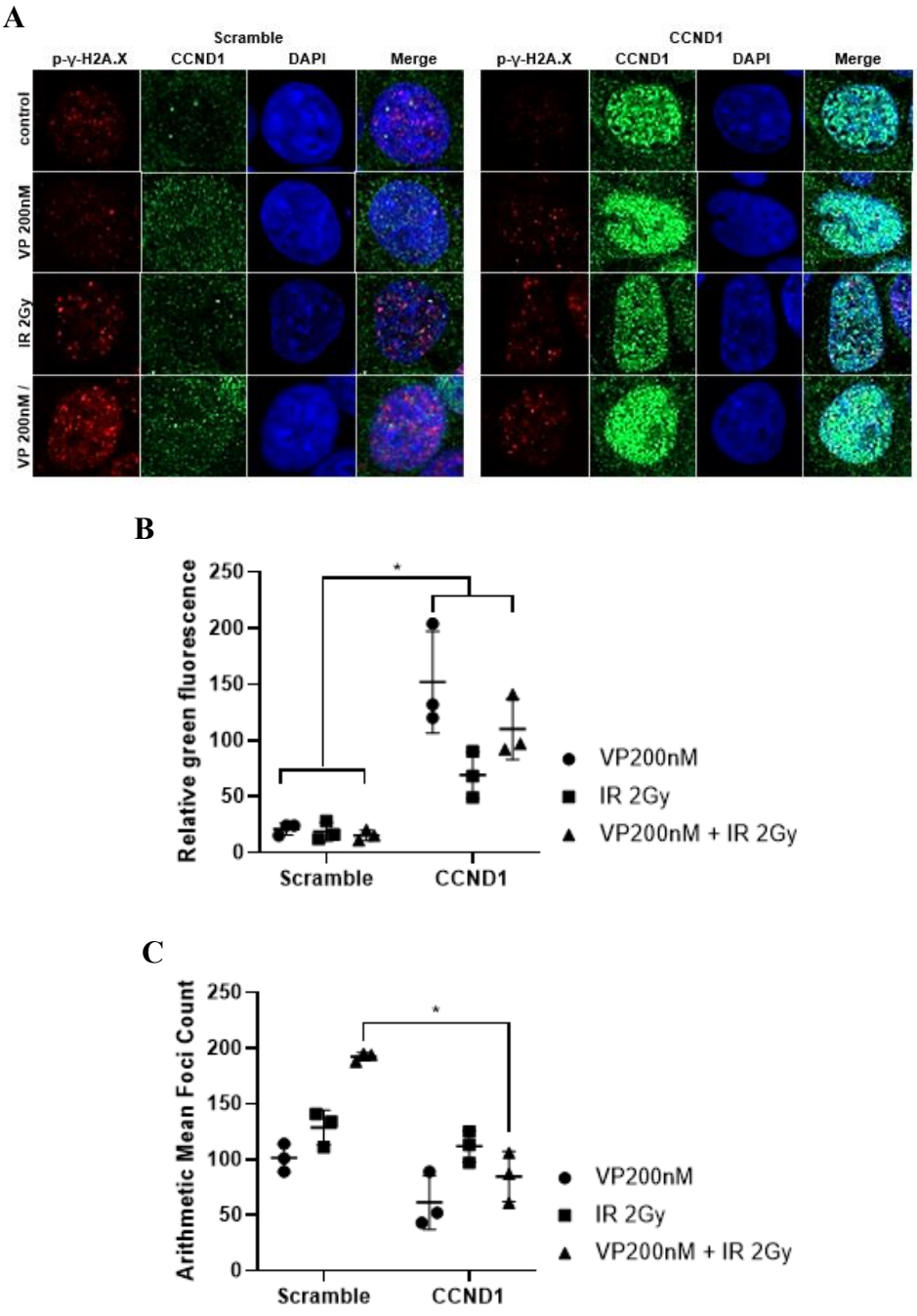

Fig. 21. Cyclin D1 overexpressing HEP-2 cells showed lower levels of phosphorylated

γH2AX. (A) Immunofluorescence assay result of the phospho- γH2AX and CCND1 markers. (B) Relative green fluorescence, indicating Cyclin D1 expression, was significantly higher in CCND1 overexpressed cells. (C) In cells with high CCND1 expression, the level of γH2AX foci expression was significantly reduced. (* p <.05)

4. DISCUSSION

In this study, we observed that YAP is associated with oncogenic features including cell proliferation, migration, apoptosis, and radioresistance, and that radiosensitivity can be enhanced upon YAP inhibition. Moreover, we suggest that YAP regulates cellular DNA damage upon irradiation through Cyclin D1. Although previous studies have suggested that YAP is involved in radioresistance, the precise mechanism underlying this relationship remains unclear. Various signaling pathways, including the PI3K/Akt, MAPK/ERK, and TGF-β pathways have been suggested to crosstalk with the Hippo pathway to confer radioresistance[27-29]. In our study, we aimed to elucidate the mechanism by which YAP contributes to cellular radioresistance by focusing on its interaction with Cyclin D1. In this study, YAP was inhibited not only by siRNA and shRNA, but also through pharmacological methods such as Verteporfin and CA3, with consistent results observed across all approaches.

In our study, YAP inhibition resulted in enhanced radiosensitivity, which is consistent with the findings of several previous studies. For example, in a study involving triple-negative breast cancer cells, genetic and pharmacologic inhibition of YAP using shRNA and Verteporfin led to increased sensitivity to radiotherapy. Furthermore, YAP-inhibited cells showed significantly lower EGFR mRNA levels, suggesting that the EGFR signaling axis may be associated with radioresistance[30]. In another study by Fernandez-L et al., PI3K/Akt signaling has been identified as the pathway responsible for the radioresistant characteristics in YAP overexpressed medulloblastoma cells[27]. However, given that YAP is believed to regulate the transcription of numerous genes, we hypothesized the presence of an alternative

mechanism through which YAP may confer radioresistance.

The synergistic effect of YAP inhibition and radiation observed in our study has also been reported by Zhuang et al., who investigated the immunogenic cell death effects of YAP in murine cancer cells. In their study, they observed that the dual treatment of both radiation and YAP inhibition yielded the best antitumoral response compared to YAP inhibition alone or radiation alone[31]. Moreover, the activation of CD8+ T cells was increased in the tumor microenvironment after the dual treatment, suggesting a potential therapeutic role of immunotherapy combined with YAP inhibition and radiation in the clinical setting. The additive effect of YAP inhibition and radiation has also been observed in human chordoma cells. YAP-inhibited chordoma cells showed significantly increased DNA damage following radiation as well as reduced levels of DNA damage repair proteins, including ATM, ATR, RAD51, BRCA1, BRCA2, and PPP2R4[32].

We also found a significant correlation between YAP expression levels and Cyclin D1 expression. Cyclin D1 is a key regulatory protein of the cell cycle, forming complexes with CDK4/6 and driving the G1 to S phase transition[33]. In addition, Cyclin D1 promotes cell proliferation which in turn results in tumorigenesis[34]. In a study analyzing the expression levels of Cyclin D1 in epithelial odontogenic lesions, it was found that the degree of Cyclin D1 expression was strongly correlated with aggressiveness[35]. Previous studies have suggested that the oncogenic potential of Cyclin D1 may be mediated through mechanisms beyond its CDK-dependent activation and E2F-driven promotion of the cell cycle[36,37]. Moreover, Cyclin D1 has been shown to play an important role in the DNA damage response[38,39]. Cyclin D1 is involved in both the non-homologous end joining (NHEJ) pathway and the homologous recombination (HR) pathway of DNA double strand break repair. Upon DNA damage, Cyclin D1 interacts with key repair proteins including the DNA-PKcs (DNA-dependent protein kinase catalytic subunit) and promotes the NHEJ process[40]. It also participates in the HR process through its direct interaction with RAD51 and BRCA2[41].

Zhou et al. previously reported a correlation between the expression levels of YAP and Cyclin D1. In their study, YAP knockdown significantly reduced Cyclin D1 expression in

a gastric carcinoma cell line[42]. Consistent with this, knocking down YAP has also been shown to induce cell cycle arrest at the G0/G1 phase by inhibiting Cyclin D1 expression in adrenocortical cancer cell lines[43]. Similarly, Ferrick et al. demonstrated that YAP regulates cell cycle entry by modulating the Cyclin D1/p27 ratio in human retinal epithelial cells[44] [preprint]. Mizuno et al. further reported that YAP induces the transcription of CCND1[45]. Our study further extends these findings by suggesting that YAP, through its regulation of Cyclin D1, also plays a role in the DNA damage response.

This study suggests that Cyclin D1 may affect radioresistance by regulating proteins involved in cellular apoptosis. This finding is consistent with other studies. For instance, Nurhidayat et al. divided 44 patients with nasopharyngeal carcinoma into two groups based on their response to radiotherapy: the radiosensitive and radioresistant groups. Immunohistochemical staining results revealed that the radioresistant group exhibited significantly higher levels of Cyclin D1 expression than the radiosensitive group[46]. Additionally, inhibiting Cyclin D1 has been shown to enhance radiosensitivity. Huafang et al. conducted experiments using the esophageal squamous cell carcinoma cell line KYSE-150, and a radioresistant subline, KYSE-150R, which was derived by gradually exposing the parental cell line to radiation[47]. Upon Cyclin D1 knockdown, the KYSE-150 cell line showed no significant change in radiosensitivity, but the KYSE-150R cells exhibited a significant reduction in surviving fraction following radiation exposure compared to the negative control. Furthermore, in a study involving breast cancer cells with Cyclin D1 overexpression, these cells showed increased sensitivity to radiation compared to their counterparts, along with a dose-dependent increase in the apoptotic rate[48]. Yang et al., in their study with breast cancer cell lines, observed reduced levels of cleaved caspase-3 expression following the pharmacologic inhibition of Cyclin D1 and radiation[49].

We also observed a correlation between Cyclin D1 expression and DNA damage levels in our study. Previous studies in other cancer types have reported similar findings. In a study involving mantle cell lymphoma cells, knockdown of Cyclin D1 using shRNA resulted in an increase in γH2AX foci formation[50]. Furthermore, in cells where shRNA expression was

induced by doxycycline, γH2AX foci formation was further increased, confirming that Cyclin D1 knockdown leads to accumulation of DNA damage. Additionally, it has been shown that the level of Cyclin D1 expression is associated with the extent of DNA double-strand breaks following radiation exposure. In prostate cancer cells, after exposure to 4 Gy of radiation, comparison of DNA damage between control and Cyclin D1 knockdown cells revealed that Cyclin D1 knockdown resulted in a significant increase in DNA damage induced by radiotherapy[51]. Immunoblotting analysis showed a significant increase in DNA repair proteins that interact with Cyclin D1, including DNA-PKcs, ATM, and RAD51, following radiation exposure. These findings suggest that Cyclin D1 may play a role in the repair of DNA double strand breaks.

The limitation of this study is as follows. While we have confirmed that Cyclin D1 upregulates the extent of DNA damage, we were unable to determine whether this regulation is due to the promotion of DNA damage repair. Although previous studies have shown that YAP is associated with aggressive behavior in head and neck cancer and that Cyclin D1 plays a role in regulating DNA damage, the connection between YAP and Cyclin D1 in this context has remained unclear. To the best of our knowledge, this study is among the first to explore the potential role of YAP, through Cyclin D1, in regulating DNA damage and apoptosis in head and neck cancer cells. These findings could provide a potential therapeutic strategy targeting YAP to enhance radiation response and improve the efficacy of existing treatments in head and neck cancer. Future studies should investigate whether Cyclin D1 also plays a role in DNA damage repair. Furthermore, exploring the combined anticancer effects of concurrent inhibition of Cyclin D1 and YAP may provide a promising direction for future treatment approaches in cancer therapy.

5. CONCLUSION

In conclusion, our study provides valuable insights into the role of YAP in regulating radioresistance, particularly through its interaction with Cyclin D1. We demonstrate that YAP is associated with cell proliferation, migration, and apoptosis in head and neck cancer cells, and that YAP inhibition enhances radiosensitivity. Additionally, our study suggests that YAP contributes to the DNA damage response upon irradiation by regulating Cyclin D1. Our findings have important clinical implications and offer valuable insights for developing therapeutic strategies aimed at overcoming radioresistance in patients with head and neck cancer.

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Abstract in Korean

두경부암에서 YAP 억제를 통한 방사선 저항성 극복에 관한 연구

목적: 두경부암의 치료는 수술, 방사선치료, 항암화학요법을 포함하는 다학제적인 접근을 필요로 한다. 그러나 이러한 다학제적인 치료에도 불구하고 국소 재발율이 여전히 높아 효과적인 구제적 치료가 필요한 실정이다. 재발한 종양은 종종 항암화학요법이나 방사선치료에 저항성을 보이기에 임상적 접근이 어렵다. Hippo 신호전달경로는 여러 암종에서 주요한 종양 억제 체계로 알려져 있으며, 그 하위 시그널 분자인 Yes-associated protein (YAP)은 세포 증식, 세포 생존 및 치료 저항성 등 다양한 과정에 관여하는 것으로 여겨진다. 이전 연구들에서 YAP이 두경부암에서 종종 증폭되어 있으며, YAP이 과발현된 경우 두경부암의 예후가 좋지 않음을 밝혀낸 바 있다. 본 연구는 두경부암 세포주에서 YAP이 방사선 저항성에 미치는 역할을 평가하고자 하였다.

대상 및 방법: 두경부암 세포주를 스크리닝하여 상대적으로 YAP 발현 정도가 높고 방사선 저항성을 띄는 HEP-2 세포주를 선택하였다. YAP을 타겟으로 한 short hairpin RNA (shRNA) 및 small interfering RNA (siRNA)를 사용하여 YAP을 억제한 후 세포 특성의 변화를 살펴보았다. YAP의 약리학적 억제를 위해서는 Verteporfin 및 CA3를 사용하였다. Nude mouse 모델에서 YAP이 억제된 세포의 생체내 (in vivo) 종양 형성 능력을 평가하였으며, 세포 증식, 집락 형성 및 세포 사멸 분석을 통해서는 YAP의 억제와 방사선 조사의 병용 투여가 시너지 효과를 나타내는지를 확인하였다. 또한, YAP 억제 및 방사선 조사 후 Cyclin D1 과 γH2AX foci의 발현 변화를 확인하였다.

결과: YAP의 억제는 세포 증식의 감소, 세포 이동성의 감소, 방사선 감수성 증가 및 세포 사멸 촉진을 초래하였다. 또한, YAP 억제 시 in vivo에서의 종양 형성 또한

유의하게 감소함을 확인하였다. YAP 억제와 방사선 조사의 병용 투여는 세포 증식 및 세포 사멸 측면에서 YAP 억제 또는 방사선 조사 단독 보다 시너지 효과를 나타내었다. 또한, YAP의 억제는 G0/G1 세포 주기 정지를 유도하였다. 더 나아가 YAP의 발현 정도는 Cyclin D1의 발현과 상관관계를 보였으며, YAP의 억제와 방사선치료의 병용 투여는 Cyclin D1 발현 정도의 감소를 유발하였고, 이는 DNA 손상의 증가로 이어졌다.

결론: YAP은 두경부암 세포에서 세포 증식, 이동 및 세포 사멸에 관여하며, YAP 억제 시 방사선 감수성이 향상되었다. 또한 YAP은 Cyclin D1의 조절을 통해 방사선 조사 후 DNA 손상 반응에 관여하는 것으로 생각된다. YAP을 표적으로 하는 치료는 두경부암에서 방사선 저항성을 극복할 수 있는 잠재적인 치료 전략이 될 것이다.

핵심되는 말: Hippo 신호전달경로, Yes-associated protein (YAP), 두경부암, 방사선저항성, Cyclin D1