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Original Articles

PI3Kα inhibitors sensitize esophageal squamous cell carcinoma to radiation by abrogating survival signals in tumor cells and tumor microenvironment

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ABSTRACT

Radiotherapy is one of the standard therapies for esophageal squamous cell carcinoma (ESCC), but the efficacy is far from desirable. Large scale genome sequencing reveals PI3Kα is frequently hyper-activated in ESCC. We found that ESCC cells harboring alterations in PI3K pathway were more resistant to radiation and combination of a clinical PI3Kα-selective inhibitor CYH33 and radiation synergistically inhibited cell proliferation in 14 ESCC cell lines. Radiation induced phosphorylation of FOXO1 and Akt, which sensitized ESCC cells to PI3Kα inhibitors. Both S1PR3 and DNA-PK contributed to radiation-induced Akt phosphorylation, which were revealed to be collectively dependent on PI3Kα. By contrast, constitutively active Akt abrogated the synergism between PI3Kα inhibitors and radiation. PI3Kα inhibition enhanced radiation-induced DNA damage, G2/M arrest and apoptosis. Combination of CYH33 and radiation significantly inhibited the growth of xenografts derived from ESCC patients, which was accompanied with abrogation of radiation-induced phosphorylation of Akt and filtration of M2-like macrophages. Taken together, combination of CYH33 and radiation possesses synergism in ESCC, which provides promising rationale to test this combinatorial regimen in ESCC patients.

1. Introduction

Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma are the two major types of esophageal cancer, which tend to develop in different parts of the esophagus and are driven by different genetic alterations [1–3]. ESCC is one of the most prevalent and deadly malignant diseases in the world especially in developing countries [4]. For example, 90% of esophageal cancer patients are diagnosed with ESCC and ESCC is the fourth leading cause of cancer-related death in China [5]. While ramucirumab and trastuzumab were approved for the treatment of adenocarcinoma at gastroesophageal junction, no molecularly targeted therapy has been approved for the treatment of ESCC. Surgery, radiation and chemotherapy are currently the standard therapy of ESCC with a 5-year survival rate of 15–25% [6]. By deep sequencing the ESCC samples, multiple studies revealed frequent alteration in PIK3CA via amplification and mutation [5,7–9]. Aberrant activation of PI3Kα also occurs frequently in ESCC through other mechanisms including hyper-activation of upstream receptor tyrosine kinases (RTKs), *RAS* mutations and alteration in downstream effector *AKT*, as well as functional loss of PTEN [5]. Selective targeting PI3Kα has emerged as a promising approach for the treatment of ESCC [10].

Radiotherapy alone or combined with concomitant chemotherapy is accepted as a type of standard therapy in the preoperative setting for resectable tumors or in inoperable patients for non-resectable tumors [11,12], while the efficacy of the therapy is limited by the intolerable toxicity induced by radiation or/and cytotoxic drugs. Recent studies demonstrated that radiation would activate pro-survival pathways including EGFR family members, ERK and Akt, which would compromise its efficacy [13]. On the other hand, the pro-survival signal induced by radiation could sensitize tumor cells to drugs specifically targeting the

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signal. Akt/mTOR pathway has been found activated upon radiation in prostate cancer cells, which sensitized cells to pharmacologic inhibition of mTOR/Akt [14]. In consistency with this observation, combination of radiation with a dual PI3K/mTOR inhibitor PF-05212384 [15] or a β-sparing PI3K inhibitor GDC-0032 [16] displayed synergistic activity in head and neck squamous cell carcinomas. Although hyper-activation of PI3K is frequently found both in ESCC and head and neck squamous cell carcinomas and BYL719, a PI3Kα inhibitor, has been tested in ESCC (NCT01822613), the combinatorial activity of radiation and PI3Kα-selective inhibitors in ESCC remains largely unknown.

CYH33 is a novel PI3Kα-selective inhibitor with a distinctive structure, which was discovered by our group and is currently in clinical trials for the treatment of advanced ESCC (NCT03544905) [17]. Here we reported the potent therapeutic activity of CYH33 against ESCC in preclinical models and found that CYH33 sensitized radiotherapy in ESCC by abrogating survival signals both in tumor cells and tumor micro-environment.

2. Materials and methods

2.1. Compounds and reagents

CYH33 was provided by Shanghai HaiHe Pharmaceutical Co. Ltd. BYL719 was purchased from Selleck Chemicals (Houston, TX, USA) and Cay10444 was obtained from Cayman chemical (Ann Arbor, MI, USA). All these compounds were dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA) at the concentration of 10 mM and stored at −20 °C. Compounds were diluted into desired concentrations in PBS before each experiment. The final concentration of DMSO was no more than 0.1% (v/v). For *in vivo* studies, CYH33 was solved in normal saline containing 0.5% Tween 80 (v/v; Sangon Biotech, Shanghai, China) and 1% CMC-Na (m/v).

2.2. Cell culture

The esophageal squamous cancer KYSE30, KYSE510, KYSE70, KYSE140, KYSE150, KYSE180 and KYSE450 cells were kindly provided by Dr. Hideaki Shimada (Department of Surgery, Toho University School of Medicine). TE6, TE9, TE11, TE14, TT, OE21 and OE33 cells were from RIKEN Cell Bank (constructed by Dr. Nishihira, Tetsuro). THP-1 cells were obtained from Chinese Academy of Sciences Cell Bank (Shanghai, China). All cell lines were authenticated by analyzing short-tandem repeats (STR) by Genesky Biotechnologies Inc. (Shanghai, China). Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

2.3. Cell proliferation assay

Cell proliferation was measured by Sulforhodamine B (SRB, Sigma, St. Louis, MO, USA) assay as described previously [17]. Briefly, exponentially growing KYSE30 and KYSE510 cells seeded in 96-well plates in triplicate were treated with radiation (X-ray irradiator, RS2000, Rad Source Technologies, USA) or concurrently with CYH33 or BYL719. Cells were stained with SRB after 72 h incubation and the OD value was measured at 560 nm with a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The inhibitory rate on cell proliferation was calculated using the formula ($OD560\ nm_{control\ cells} - OD560\ nm_{treated\ cells}$)/$OD560\ nm_{control\ cells} \times 100\%$.

2.4. Combination analysis

ESCC cells were treated with single agent or radiation alone or in combination and inhibitory rate on cell proliferation was determined by SRB assay. Combination Index was analyzed by Calcu syn software [18] (Biosoft, Cambridge, UK). CI < 0.80 indicates synergistic effect, CI = 0.80–1.20 indicates additive effect and CI > 1.20 indicates antagonistic effect.

2.5. Microarray analysis

The microarray analysis was conducted at Shanghai Baygene Biotechnology Co. Ltd (Shanghai, China). Cells were treated with CYH33 or BYL719 alone or concurrently with radiation for 24 h. Total RNA was extracted and subjected to gene expression analysis with Affymetrix Human PrimeView microarrays (Santa Clara, CA, USA) according to the manufacturer's instructions. Transcription factors were enriched with differentially expressed genes (fold change cutoff of 1.5 folds). Molecular pathway analysis was performed using Gene Set Enrichment Analysis (GSEA, http://software.broadinstitute.org/gsea/index.jsp) [19].

2.6. Immunofluorescence

ESCC cells seeded in 24-well plates were treated with radiation (4 Gy) and were fixed with 4% paraformaldehyde 2 h later. Immunofluorescence staining was performed with antibodies against phosphorylated Akt (Ser473), γH2AX (Cell Signaling Technology, Danvers, MA, USA) or phosphorylated FOXO1 (Ser319) (Invitrogen, Carisbad, CA, USA). Slides were mounted with medium containing DAPI and photographed with Olympus FV1000-SIM (Olympus Corporation, Shinjuku, Tokyo, Japan).

2.7. siRNA transfection

siRNA duplexes were synthesized by GenePharma (Shanghai, China). The sequences of siRNAs targeting PIK3CA were as follows: siPIK3CA#1 5′-GAAUGAUAGUGACUUUAGAdTdT-3′ and siPIK3CA#2 5′-UCUUCACCAGAAUUGCCAAdTdT-3'. The negative control sequence was: 5′-UUCUCCGAACGUGUCACGUdTdT-3'. Cells seeded in 6-well plate were transfected with siRNAs using Lipofectamine RNAi max (Invitrogen, Carisbad, CA, USA) according to manufacturer's instructions. Cells were subjected to following experiments 72 h post transfection.

2.8. Plasmids and transfection

Myristoylated tag (ATGGGGTCTTCAAAATCTAAACCAAAGGACCC CAGCCAGCGCCGGCGCAGAATCCGAGGT) was ligated with Akt1 gene and constructed into pBabe-puro vector (#1764, Addgene, Watertow, MA, USA), namely pBabe-puro-myr-Akt1. HEK293T cells seeded in 6-well plates were transfected with pBabe-puro (empty vector) or pBabe-puro-myr-Akt1 along with retrovirus packing plasmid and envelope plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Cell media containing viruses were collected 48 h after infection and filtered through a 0.45 μM filter. KYSE30 and KYSE510 cells were infected with viruses in the presence of 6 μg/mL polybrene (Sigma, St. Louis, MO, USA). Cell stably expressing myr-Akt1 were selected in the presence of 1 μg/mL puromycin. FOXO1 plasmid (#45814) and vector plasmid (#1767) were from Addgene (Watertown, MA, USA). Cell were transfected with the plasmids using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) and subjected to Western blotting or proliferation assay 24 h later.

2.9. Western blotting

Cell lysates were prepared and standard Western blotting was performed with antibodies against Akt, phosphorylated Akt (S473), Akt1, phosphorylated Akt1 (S473), phosphorylated histone H2AX (S139), p110α, FOXO1, phosphorylated FOXO1 (S256) (Cell Signaling Technology, Danvers, MA, USA) and β-Actin (Sigma, St. Louis, MO, USA).

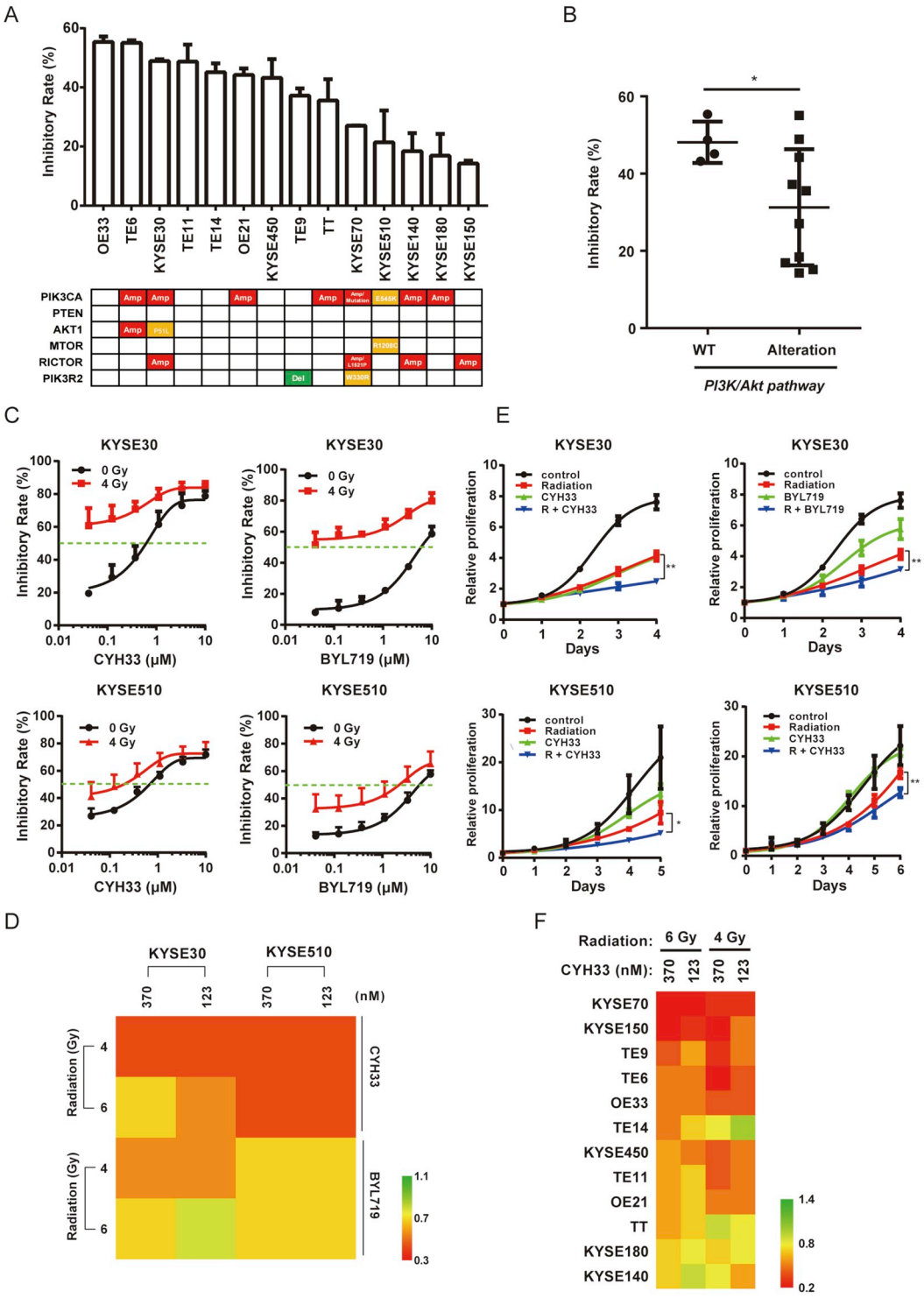

Fig. 1. Combination of CYH33 and radiation synergistically inhibited the proliferation of squamous esophageal cancer cells. (A) A series of ESCC cells were treated 4 Gy of radiation and the inhibitory rates on proliferation were plotted. Boxes below the chart indicate alterations in the PI3K/Akt pathway. (B) Scatter plots of inhibitory rates in cells treated with 4-Gy radiation grouped according to alterations in the PI3K/Akt pathway. p value was measured by two-tailed Student's *t*-test. *: $p < 0.05$. (C) Cells were treated with CYH33 or BYL719 alone or concurrently with radiation (4 Gy) for 72 h and cell proliferation was measured with SRB assay. (D) KYSE30 and KYSE510 Cells were treated with indicated concentrations of CYH33 or BYL719 and radiation. Inhibition of cell proliferation was measured and combination index was calculated by Calcu Syn software. (E) Cells were treated with CYH33, BYL719 or radiation alone or indicated combinations. Cell proliferation was measured with SRB assay at indicated times. (F) Indicated ESCC cells were treated with CYH33 and radiation. Cell proliferation was measured and combination index was calculated by Calcu Syn software. Data shown are mean or mean ± SD from at least two independent experiments. Differences between indicted groups were analyzed using two-tailed Student's *t*-test. *: $p < 0.05$; **: $p < 0.01$.

2.10. Flow cytometry

Cells were prepared for the analysis of cell cycle distribution or apoptosis as previously described [20,21]. Data were collected with a FACS Calibur Instrument (BD Biosciences, Franklin Lake, NJ, USA) and analyzed with the FlowJo software.

2.11. Co-culture of ESCC cells with macrophages

THP-1 cells were treated with 1 μM of phorbol myristate acetate (PMA, Sigma, St. Louis, MO, USA) for 72 h to differentiate into macrophages. Macrophages and ESCC cells were seeded in upper or lower layers of 24-mm Transwell (#3450, Corning Inc., Painted Post, NY, USA) respectively. 24 h later, cells were treated with radiation (4 Gy) and incubated with CYH33 (1 μM) for 96 h. The cell number was counted to calculate relative survival ratio and RNA of macrophages were extracted for RT-PCR.

2.12. Animal studies

The animal studies were conducted at WuXi AppTec (Shanghai, China). All experiments were carried out according to the Institutional Ethical Guidelines on Animal Care and were approved by the Institutional Animal Care and Use Committee at WuXi AppTec. Patient derived xenograft (PDX) models of ESCC (ES-06-0009 and ES-06-0010) were established and preserved at WuXi AppTec (http://onco.wuxiapptec.com/login). Tumor section was cut into small pieces of 20–30 mm^3, which were then implanted into the right back of female nu/nu athymic BALB/cA mice aged 6–8 weeks (Shanghai Sippr-BK laboratory animal Co. Ltd., Shanghai, China). When tumor volume reached a size of 100–200 mm^3, mice were randomized to orally receive vehicle control, CYH33 (12.5 mg/kg, once a day), radiation (day 0, 1, 7, 8, 9, 28) or combination of CYH33 and radiation. Body weight was recorded by electronic balance and the tumor volume was measured using microcalipers twice per week. The tumor volume (V) was calculated using the formula $V = a^2b/2$, and a and b represented the tumor's width and length respectively. Relative tumor volume (RTV) was calculated using the formula $RTV = V_t/V_0$, in which V_t was the tumor volume after treatment and V_0 was the tumor volume at the beginning of treatment. The treatment to control ratio (T/C) was calculated using the formula: $T/C\ (100\%) = (T_{RTV}/C_{RTV}) \times 100\%$, T_{RTV} and C_{RTV} represented the RTV of treatment and control group, respectively.

2.13. Immunohistochemistry

Tumors tissues were collected and fixed in 4% paraformaldehyde at the end of treatment. Paraffin embedding, hematoxylin and eosin (H&E, Sigma, St. Louis, MO) staining and immunohistochemistry against phosphorylated Akt (S473), γH2AX (Cell Signaling Technology, Danvers, MA, USA), F4/80 (eBioscience, Waltham, MA, USA) and CD206 (Abcam, Cambridge, MA, USA) were conducted by Shanghai ZuoCheng Bio Company (Shanghai, China). Slides were observed under a Leica DM6 B microscope equipped with sCMOS camera (Leica, Wetzlar, Germany).

2.14. Statistical analysis

Data are shown as mean ± SD or mean ± SEM. Statistical analysis were performed as indicated. Differences were considered statistically significant when p value was less than 0.05.

3. Results

3.1. Combination of CYH33 and radiation synergistically inhibited the proliferation of squamous esophageal cancer cells

Radiotherapy is one of the most common standard treatments for ESCC, while the clinical efficacy is far from desirable. We detected the sensitivity of a panel of 14 ESCC cell lines to X-ray radiation. As shown in Fig. 1A, these cells displayed different sensitivity to radiation, with the inhibitory rates upon 4-Gy radiation ranging from 14% to 55%. As the PI3K/Akt pathway was among the most frequently deregulated pathways (including gene copy number amplification, deletion and mutation) in ESCC cells (Fig. 1A & S1A), we grouped the tested cell lines according to their genetic alterations in the PI3K/Akt pathway. Cells harboring alterations in this pathway were significantly more resistant to radiation than their counterparts (Fig. 1B), suggesting that deregulation of PI3K/Akt pathway might confer ESCC cells resistant to radiation. CYH33 is a novel PI3Kα-selective inhibitor, which is currently in clinical trials to evaluate its safety and preliminary efficacy in advanced solid tumors patients, and in advanced esophagus cancer patients (NCT03544905). We would like to investigate whether inhibition of PI3Kα would sensitize ESCC to radiation. Inhibition of PI3Kα by BYL719, a PI3Kα inhibitor in phase III clinical trials, or by CYH33 significantly inhibited the proliferation of radiation-sensitive KYSE30 and radiation-resistant KYSE510 cells (Fig. 1C), confirming PI3Kα as a promising target for the treatment of ESCC. Concurrent treatment of the cells with radiation enhanced the activity of BYL719 and CYH33 against cell proliferation. In order to clarify whether the combination of PI3K inhibitors and radiation exerts synergistic activity, KYSE30 and KYSE510 cells were treated with serially diluted CYH33 or BYL719 alone or concurrently with different doses of radiation. The combination index (CI) of each combination was calculated based on the inhibitory rate of cell proliferation (Fig. S1B) and representative CI values were plotted in Fig. 1D. CI values obtained from combination of CYH33 and radiation ranged from 0.4 to 0.7 in KYSE30 cells and from 0.3 to 0.6 in KYSE510 cells, indicating synergism of the combination. Similar results were obtained with combination of BYL719 and radiation. To further demonstrate that inhibition of PI3Kα enhanced the antiproliferative activity of radiation, KYSE30 cells were treated with CYH33 (1 μM), BYL719 (3 μM) alone or concurrently with radiation and cell proliferation was determined at different time points. As shown in Fig. 1E, CYH33 and BYL719 significantly enhanced the activity of radiation to inhibit cell proliferation. Similar results were obtained in KYSE 510 cells. Inhibition of PI3Kα also potentiated the activity of radiation to inhibit the clonogenesis of ESCC cells (Fig. S1C). We further expanded the combination of radiation and CYH33 to other 12 ESCC cell lines and the CI values of representative combinations were plotted in Fig. 1F. Almost all the CI values were less than 0.8 when cells were concurrently treated with CYH33 and radiation at the indicated doses, indicating combination of radiation and CYH33 displayed synergistic against ESCC irrespective their different genetic background.

3.2. Radiation-induced phosphorylation of FOXO1 sensitized ESCC cells to CYH33

In order to dissect the mechanism of the synergism between radiation and PI3Kα inhibitors, we performed microarray analysis in vehicle control cells, cells treated with radiation alone or co-treated with PI3Kα inhibitors. As shown in Fig. S2A, total 193 differentially expressed genes (fold change cutoff of 1.5 folds) were discovered and further subjected to enrichment analysis of transcription factors. A few members of the forkhead box protein O (FOXO) family were statistically enriched (Fig. 2A), indicating altered expression of the genes might be mediated by FOXO family members. We next investigated the level FOXO1 in KYSE30 and KYSE510 cells radiated with 4 Gy of X-ray. As shown in Fig. 2B, the protein level of FOXO1 remained unchanged,

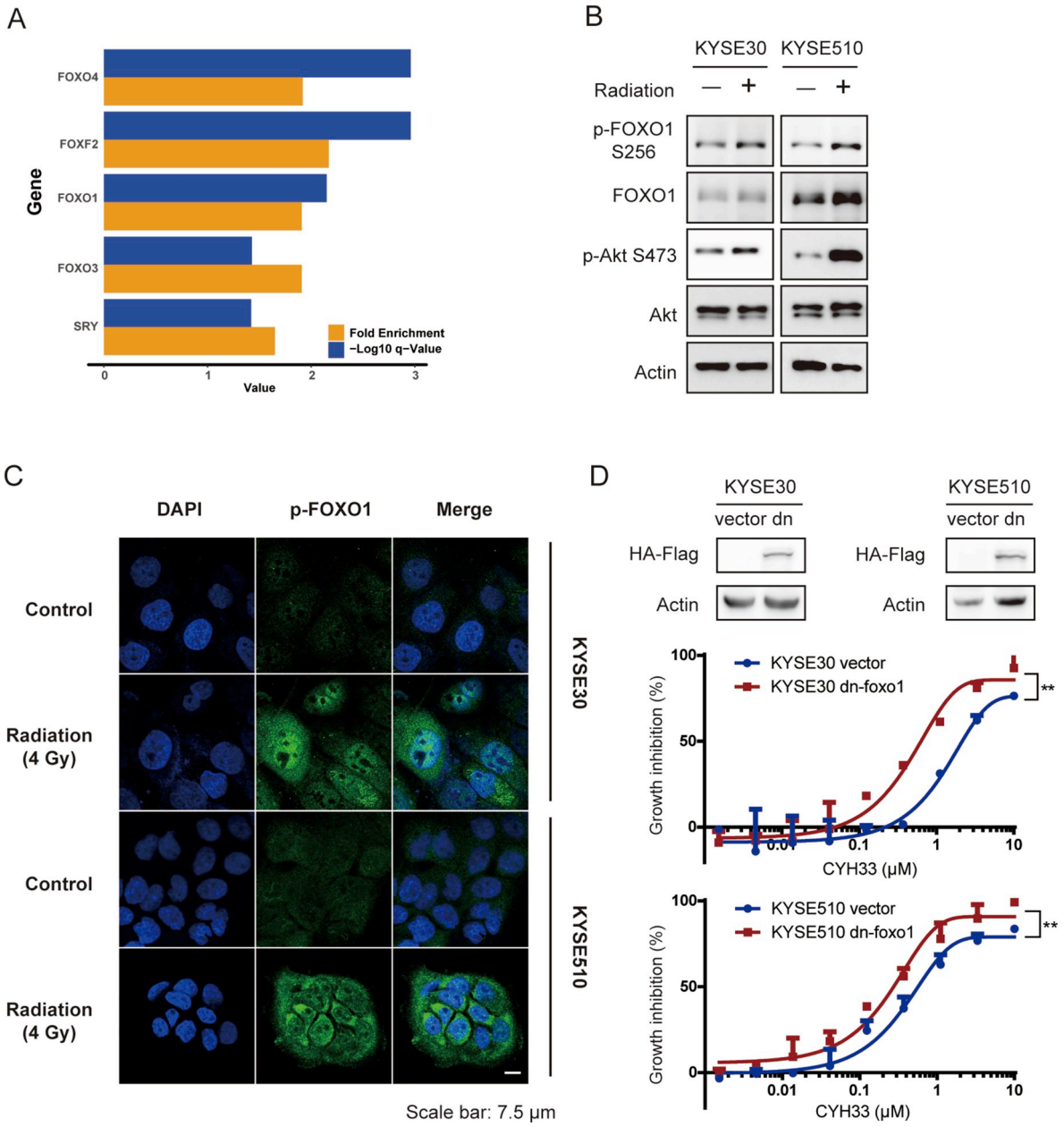

Fig. 2. Radiation-induced phosphorylation of FOXO1 sensitized ESCC cells to CYH33. (A) KYSE30 and KYSE510 cells were treated with radiation (4 Gy) alone or in combination with CYH33 (100 nM) or BYL719 (300 nM) for 24 h. Total RNA was extracted for RNA microarray analysis. Differentially expressed genes were collected for the transcription factor enrichment analysis. There are three replicates in each sample. (B) & (C) KYSE30 or KYSE510 cells were treated with radiation (4 Gy) and cells were subjected to Western blotting (B) or immune-staining for indicated proteins 2 h later (C). (D) Dominant-negative FOXO1 plasmid and vector plasmid were transiently transfected to KYSE30 or KYSE510 cells. Cell lysates were collected for Western blotting and cell proliferation was measured with SRB assay. (B)–(D) Data shown are representative or mean ± SD from at least two independent experiments. Differences between indicted groups were analyzed using unpaired Students *t*-test. *: $p < 0.05$; **: $p < 0.01$.

while phosphorylated form at Ser 256 increased in both tested cell lines. FOXO1 is a well-known substrate of Akt. Accordingly, phosphorylated Akt at S473 significantly elevated upon radiation (Fig. 2B, S2B & S2C), indicating activation of Akt. Phosphorylation of FOXO1 was further confirmed with fluorescent microscopy, which was accompanied with increased localization in cytoplasm (Fig. 2C). FOXO1 act as tumor suppressors by transcriptionally activating genes involved in apoptosis and cell cycle arrest and its function is inhibited by Akt-mediated phosphorylation [22]. As radiation induced FOXO1 phosphorylation and attenuated its activity, we investigated whether inhibition of FOXO1 contribute to the synergism of radiation and CYH33. ESCC cells were transfected with empty vector or plasmid expressing dominant negative FOXO1. As shown in Fig. 2D, CYH33 was more active in ESCC cells expressing dysfunctional FOXO1 than those transfected with empty vector. Therefore, radiation-induced phosphorylation of FOXO1 sensitized ESCC cells to CYH33.

3.3. Radiation-induced Akt phosphorylation was dependent on PI3Kα

We found that radiation induced Akt-mediated FOXO1 phosphorylation, which contributed the synergism between radiation and PI3K inhibition. To explore the mechanism of radiation-induced Akt phosphorylation, genes with altered expression (fold change cutoff of 1.5) upon radiation in ESCC cells were shown in a volcano plot (Fig. 3A).

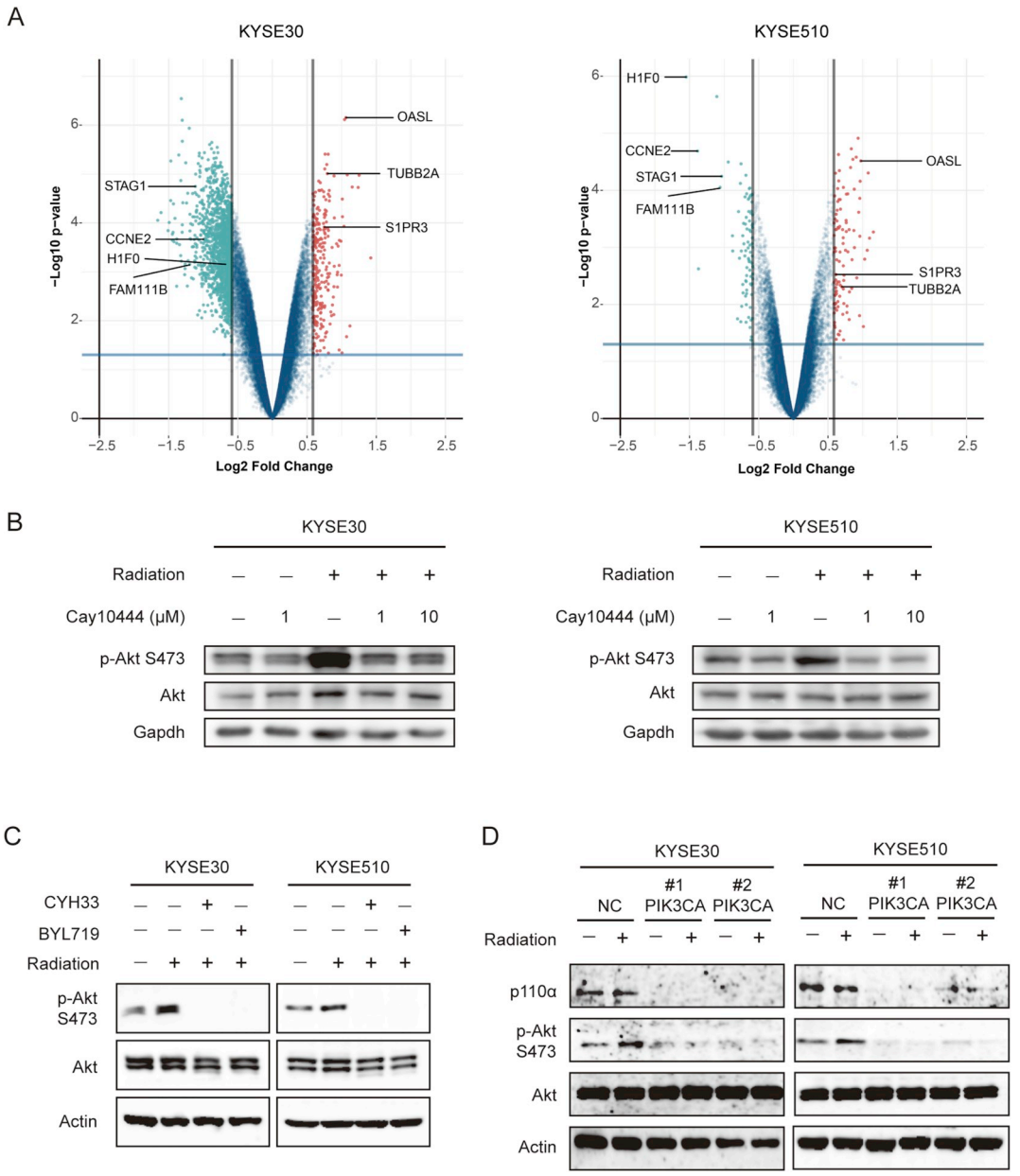

Fig. 3. Radiation-induced Akt phosphorylation was dependent on PI3Kα. (A) Volcano plot of differentially expressed genes in KYSE30 and KYSE510 cells (fold change cutoff of 1.5 folds). The x-axis shows the log2 fold-change in mRNA expression between radiation and control group, whereas the y-axis shows the log10 of the adjusted p value for each mRNA. Dots above the blue line indicates statistically significant ($p < 0.05$) and fold change of mRNA expression by 1.5 folds in log2 was demarcated by the gray line. (B) KYSE30 and KYSE510 cells were treated with radiation (4 Gy) alone or concurrently with Cay10444 for 2 h and subjected to Western blotting. (C) Cells were treated with radiation (4 Gy) alone or concurrently with CYH33 (100 nM) or BYL719 (300 nM) for 2 h and subjected to Western blotting for indicated proteins. (D) KYSE30 and KYSE510 cells transiently transfected with PIK3CA-specific small interfering RNAs were serum-starved for 2 h, then treated with 4 Gy of radiation and Western blotting was performed 2 h later. Data shown are representative from two independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The mRNA level of sphingosine-1-phosphate receptor (S1PR3) increased by 1.68 folds in KYSE30 and by 1.53 folds in KYSE510 cells. Sphingosine-1-phosphate (S1P) has been demonstrated to activate Akt through S1PR3 [23]. To investigate whether the phosphorylation of Akt is due to up-regulation of SIPR3, we treated KYSE30 and KYSE510 cells concurrently with an S1PR3 inhibitor Cay10444 and radiation. As shown in Fig. 3B, Cay10444 partially abrogated radiation-induced Akt phosphorylation. However, Cay10444 failed to enhance the anti-proliferative activity of radiation in both KYSE30 and KYSE510 cells (Fig. S3A), which might be due to its incomplete inhibition of Akt phosphorylation. DNA-dependent protein kinase (DNA-PK) has been reported to mediate Akt phosphorylation in the presence of DNA double strand breaks [24]. Consistently, inhibition of DNA-PK by NU7026 or NU7441 partially abrogated radiation-induced Akt phosphorylation (Fig. S3B). PI3K has been recognized as the immediate upstream activator of Akt. Accordingly, CYH33 or BYL719 completely inhibited radiation-induced Akt phosphorylation in KYSE30 and KYSE510 cells (Fig. 3C). Furthermore, down-regulation of PI3Kα abrogated radiation-induced Akt phosphorylation in ESCC cells (Fig. 3C). Thus, PI3Kα appeared able to converge multiple signals and lead to Akt activation upon radiation.

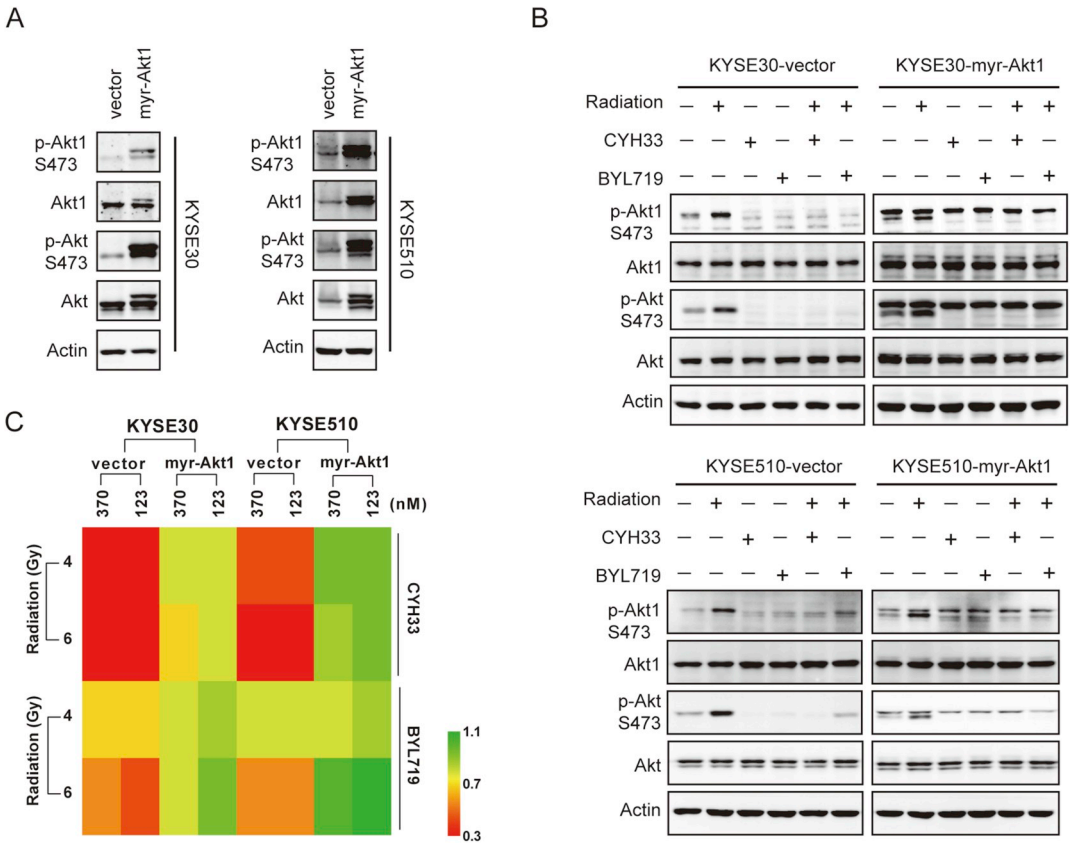

Fig. 4. Constitutively active Akt abrogated the synergism between PI3Kα inhibitors and radiation. (A) Establishment of cells with constitutively active Akt by expressing myristoylated Akt1 (myr-Akt1). (B) Cells were simultaneously treated with radiation (4 Gy) and CYH33 (100 nM) or BYL719 (300 nM) and cells were harvested 2 h later for Western blotting. (C) Cells were concurrently treated with radiation and CYH33 or BYL719. Inhibitory rates of cell proliferation were evaluated and combination index was analyzed by Calcu Syn software. (A) & (B) Data shown are representative from two independent experiments. (C) Data shown are mean from at least two independent experiments.

3.4. Constitutively active Akt abrogated the synergism between PI3Kα inhibitors and radiation

As we found radiation-induced Akt phosphorylation was dependent on PI3Kα, we next sought to investigate whether CYH33 enhanced the anti-proliferative activity of radiation via inhibition of Akt phosphorylation. ESCC cells stably expressing myristoylated Akt1 (myr-Akt1) were established, in which Akt was constitutively active because of its myristoylation (Fig. 4A). Though CYH33 or BYL719 was able to inhibit the phosphorylation of Akt induced by radiation in ESCC cells transfected with empty vector, the compounds failed to impede the phosphorylation of Akt in ESCC cells expressing myristoylated Akt1 (Fig. 4B). ESCC cells with constitutively active Akt were also less sensitive to CYH33 or BYL719 compared to cells transfected with empty vector (Fig. S4A). We next evaluated the combinatorial activity of PI3Kα inhibitors and radiation. ESCC cells transfected with empty vector or vector expressing myr-Akt1 were treated with serially diluted CYH33 or BYL719 alone or concurrently with radiation and inhibition on cell proliferation were measured. The CI values were calculated based on the inhibitory rate of each combination (Fig. S4B). As shown in Fig. 4C,the CI values obtained from the representative combinations were much higher in cells with myr-Akt1than those from cells transfected with empty vector, which were higher than 0.8, indicating no synergism between PI3Kα inhibitors and radiation. Thus, inhibition of Akt activity is important for the synergistic activity between PI3Kα inhibitors and radiation.

3.5. PI3K inhibitors enhanced radiation-induced DNA damage

DNA damage response plays important roles in radiotherapy. To investigate the effect of PI3Kα inhibitors on the DNA repair pathway, GSEA enrichment analysis of differentially expressed genes were performed. As expected, “DNA repair” hallmark gene set was among the top enriched gene sets (Fig. 5A), which was down-regulated after radiation and further decreased in combination with CYH33. To confirm the effect of PI3Kα inhibition on the DNA damage repair upon radiation, phosphorylated H2AX (γH2AX) was employed as a marker for DNA double stand breaks, which is the major form of DNA damage after radiation. As shown in Fig. 5B, radiation resulted in increased γH2AX in ESCC cells and co-treatment of CYH33 or BYL719 further enhanced the level of γH2AX. CYH33 and BYL719 also intensified the γH2AX foci induced by radiation in cell nucleus (Fig. S5A), which was consistent in camptothecin-induced DNA damage (Fig. S5B). DNA damage induced by radiation usually results in cell cycle arrest at G2/M. As shown in Fig. 5C, KYSE30 cells accumulated at G2/M phase 24 h after radiation and recovered 48 h later, indicating partial repair of DNA damage. Co-treatment of CYH33 or BYL719 with radiation significantly increased cell population at G2/M phase 72 h after radiation, indicating delay of DNA damage repair (Fig. 5C), which was consistent with enhanced DNA damage in the presence of CYH33 or BYL719 (Fig. 5B). Similar results were obtained with KYSE510 cells. Failure to repair DNA damage may result in apoptosis. As shown in Fig. 5D, about 10% KYSE30 cells underwent apoptosis 72 h after cells were radiated. Co-treatment of radiation and PI3Kα inhibitors significantly increased the apoptotic cells in a concentration-dependent manner. PI3Kα inhibitors also potentiated the induction of apoptosis by radiation in

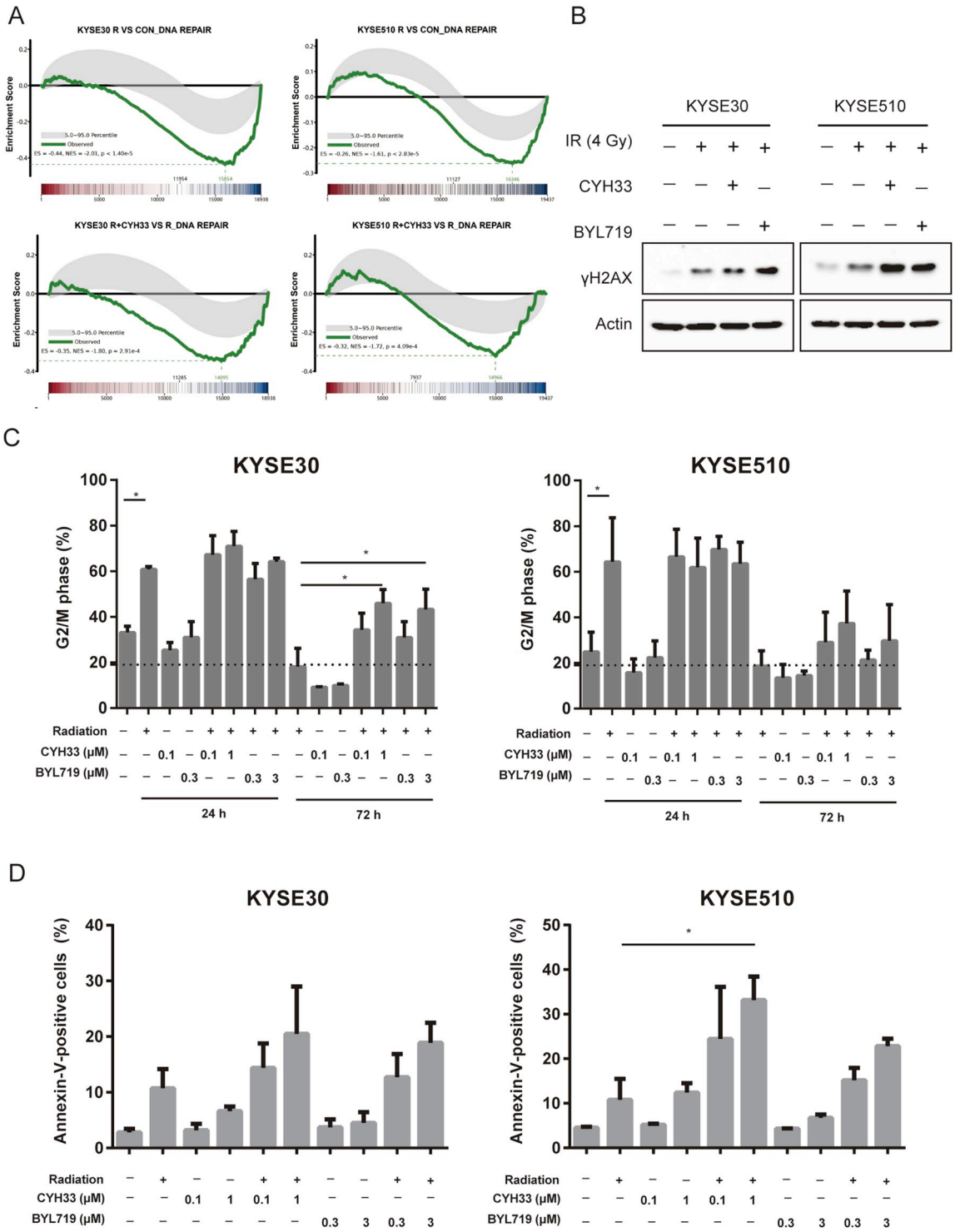

Fig. 5. PI3K inhibitors enhanced radiation-induced DNA damage. (A) GSEA enrichment plot of differentially expressed genes in the gene set of DNA repair after cells were treated with radiation alone or concurrently with CYH33. (B) KYSE30 and KYSE510 cells were co-treated with radiation (4 Gy) and CYH33 (100 nM) or BYL719 (300 nM) and cells were collected 2 h later for Western blotting. Representative from three independent experiments are shown. (C) & (D) KYSE30 and KYSE510 cells were co-treated with radiation (4 Gy) and CYH33 or BYL719. Cell cycle distribution was analyzed by flow cytometry 24 h or 72 h later (C). Annexin V-FITC/PI double labeling assay was performed 72 h later (D). Data shown are mean ± SD from at least two independent experiments. Differences between indicated groups were measured by one-way ANOVA test. *: $p < 0.05$.

KYSE510 cells. These results demonstrated that PI3Kα inhibitors potentiated the anti-proliferative activity of radiation via circumventing DNA damage repair, prolonging cell cycle arrest and promoting apoptosis.

3.6. Combination of CYH33 and radiation significantly inhibited the growth of ESCC PDXs

Combination of PI3Kα inhibitors and radiation was demonstrated to be synergetic against ESCC cells. We next examined the efficacy of

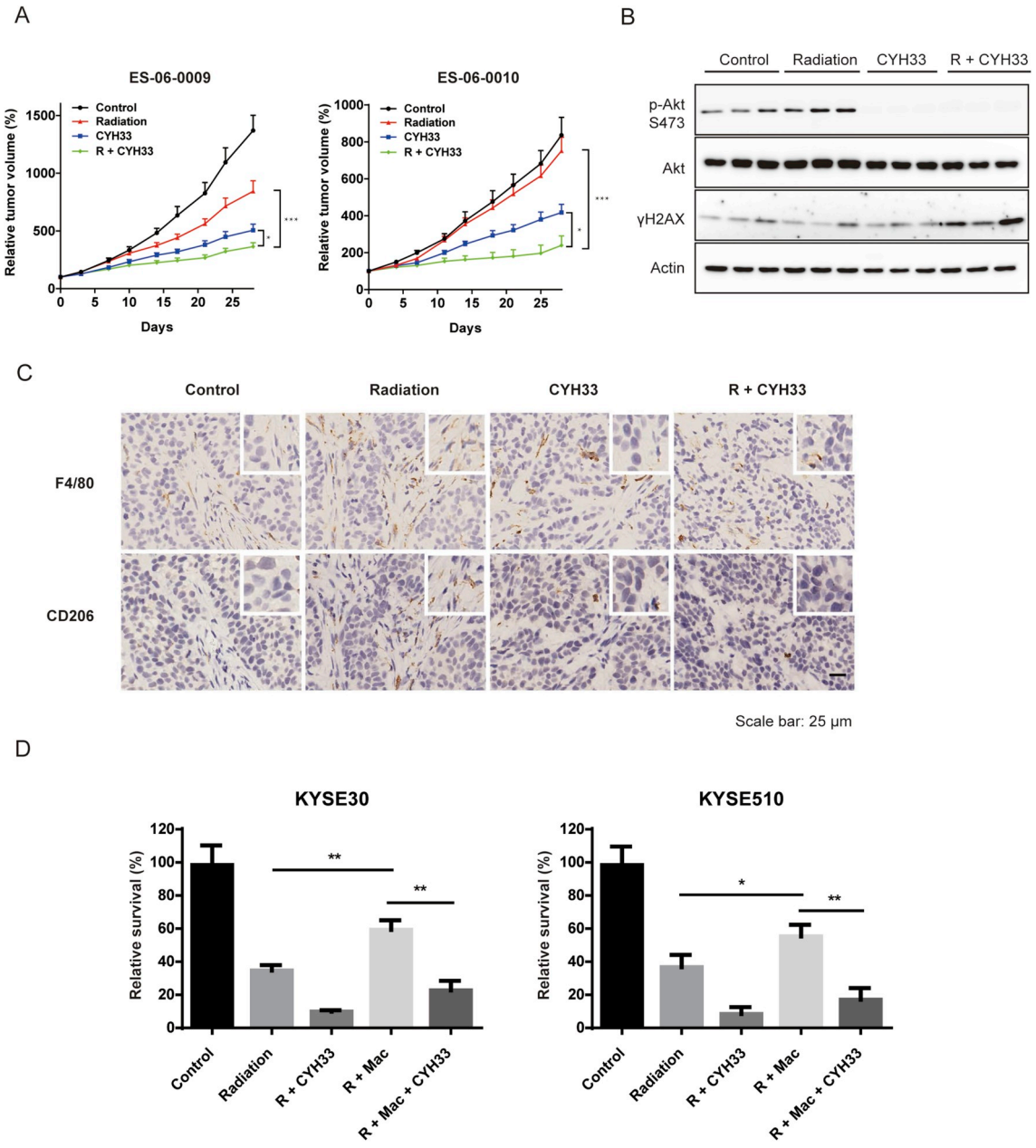

Fig. 6. Combination of CYH33 and radiation significantly inhibited the growth of ESCC PDXs. (A) Randomly grouped nude mice bearing ESCC PDX ES-06-0010 or PDX ES-06-0009 were administered with vehicle control, CYH33 (12.5 mg/kg, QD × 28 day), radiation (6 times) or combination of CYH33 and radiation. Tumor volumes were measured twice a week and T/C values were calculated at the end of treatment. Data presented are mean ± SEM. Differences between indicated groups were measured by Student's *t*-test. *: $p < 0.05$; ***: $p < 0.001$. (B) & (C) On the last day of treatment, mice bearing ESCC PDX were administrated with radiation or CYH33 alone or in combination for 2 h. Tumor tissues were collected for Western blotting (B) or Immunohistochemitry (C) to detect indicated proteins. Representative images from each group are shown. (D) M0 macrophages differentiated from human THP-1 monocytes induced by PMA were co-cultured with ESCC cells in Transwell. CYH33 (1 μM) was added to the Transwell immediately after the Transwell was radiated (4 Gy) and cell numbers of ESCC cells were counted 4 days later. Relative survival rates were calculated with the number of live cells. Data shown are mean ± SD from at least three independent experiments. Differences between indicted groups were analyzed using two-tailed Student's *t*-test. *: $p < 0.05$; **: $p < 0.01$.

CYH33 and radiation in ESCC PDX. ES-06-0009 was originated from a non-smoker ESCC patient, which harbors $PIK3CA^{E545K}$ mutation. As shown in Fig. 6A & Fig. S6B, administration of radiation at 2 Gy slowed down the growth of PDX and yielded a treated/control (T/C) value of 61.19%. CYH33 at the dose of 12.5 mg/kg significantly inhibited the growth of ES-06-0009 with a T/C value of 35.49%. Combination of CYH33 and radiation significantly enhanced the efficacy of monotherapy with a T/C value of 25.93%. To investigate whether CYH33 would potentiate the efficacy of radiation in radiation-resistant PDX, ES-06-0010 was employed. ES-06-0010 was originated from a heavy smoker ESCC patient who received radiation therapy and cancer relapsed. Administration of radiation at 1 Gy had little effect on the growth of ES-06-0010. CYH33 at 12.5 mg/kg exhibited moderate efficacy with a T/C value 49.91% (Fig. 6A). Combination of CYH33 and radiation potently suppressed the tumor growth, with a T/C value of 28.68%. Mice in all groups didn't show significant loss in body weight during the treatment (Fig. S6A). The enhanced efficacy by combination of CYH33 and radiation was accompanied with increased staining of cleaved caspase 3 and γH2AX (Fig. S6C), indicating elevated apoptosis and DNA damage. Furthermore, mice bearing ESCC PDXs were

administrated with radiation or CYH33 alone or in combination and tumor tissues were collected for Western blot on the last day of experiment. As shown in Fig. 6B, radiation significantly induced Akt phosphorylation, which was completely abrogated by CYH33. On the other hand, CYH33 treatment significantly elevated the level of γH2AX. These results further confirmed the synergistic activity between CYH33 and radiation was due to inhibition of Akt activation and enhanced DNA damage.

It has been reported that radiation induced macrophage to an immune-suppressive, M2-like phenotype, which compromised its efficacy in pancreatic ductal adenocarcinoma [25]. Consistently, we found radiation increased infiltration macrophage and the proportion of M2-like type (CD206-positive cells) enhanced in ESCC PDXs (Fig. 6C), while CYH33 abrogated this process. To confirm the observation, M0 macrophages differentiated from human THP-1 monocytes induced by PMA were plated at the top and co-cultured with ESCC cells in the bottom chamber of the Transwell insert. CYH33 was added to the Transwell immediately after it was radiated and cell numbers of ESCC cells were counted 4 days later. As shown in Fig. 6D, Co-culture of radiated macrophages with ESCC cells significantly alleviated the inhibition of radiation on the survival of ESCC cells. However, the pro-survival effect of radiated macrophages was abrogated by CYH33.

4. Discussion

Large scale whole genome sequencing revealed frequently aberrant activation of PI3Kα signaling in ESCC [3,5,9] and proposed PI3Kα as a promising target for the treatment of ESCC. In this study, we found a clinical PI3Kα-selective inhibitor CYH33 possessed potent activity against ESCC and displayed synergistic activity in combination with radiation. The synergism between CYH33 and radiation was revealed to be associated with abrogation of pro-survival signals induced by radiation in tumor cells and tumor-associated macrophages as well as enhancement of DNA damage.

CYH33 is novel PI3Kα inhibitor and currently is in clinical trial in ESCC patients based on its potent activity against ESCC *in vitro* and *in vivo* (Figs. 1 and 6 and data not shown). However, PI3Kα inhibitors usually elicit cytostatic activity in cancer cells and the efficacy is mild in clinical settings [26,27]. As such, combination of PI3Kα inhibitors with current standard therapy is an important strategy for the development of PI3Kα inhibitors. For example, phase III clinical trials of BYL719 with fulvestrant met its primary endpoint in treating patients with HR +/HER2-advanced breast cancer with PIK3CA mutation [28]. We found that combination of PI3Kα inhibitors including CYH33 and BYL719 with radiation displayed synergistic activity against the proliferation of a panel of 14 ESCC cells. Combo therapy of BYL719 and radiation is currently in clinical trials in head and neck squamous cell cancer (NCT02282371). Our study provided rationale to combine radiation based on BYL719 or CYH33 in ESCC in clinical trials. Interestingly, we noticed that the combination indexes of radiation and CYH33 were lower than those of BYL719 (Fig. 1B & Fig. 4C), suggesting better synergistic activity when radiation was combined with CYH33. This superior synergy may reflect the better anti-proliferative activity of CYH33 against ESCC cells. Consistently, CYH33 has been found to possess more potent activity against a series of breast cancer cells and PDXs [17]. The synergism of PI3Kα inhibitors and radiation was attributed to prolonged arrest at G2/M phase and enhanced apoptosis in ESCC cells. Again, we found CYH33 was more potent to than BYL719 induce cell cycle arrest and apoptosis when combined with radiation.

Akt has been reported to be activated in response to genotoxic stress, which is associated with resistance to such therapy [29]. Indeed, we found abrogation of radiation-induced Akt phosphorylation played an essential role in the synergistic activity of CYH33 and radiation in ESCC. Radiation-induced Akt activation led to inaction of FOXO1, which sensitized radiated cells to CYH33. In contrast, constitutively active Akt abrogated the synergism between CYH33 and radiation. Bozulic et al. reported that active Akt acted the direct downstream of DNA-PK in gamma-irradiated cells [24]. However, DNA-PK inhibitor NU7026 or NU7441 partially blocked radiation-induced Akt phosphorylation in radiated ESCC cells. We found elevated expression of S1PR3 upon radiation in ESCC cells and inhibition of S1PR3 signaling by Cay10444, a S1PR3 antagonist, partially prevented radiation-induced Akt phosphorylation. Nevertheless, inhibition of PI3Kα completely blocked radiation-induced Akt phosphorylation. Similarly, knock-down PI3Kα abrogated Akt activation. Therefore, DNA-PK and S1PR3 might sit upstream of PI3Kα, which converge multiple signals to activate Akt. In addition, inhibition of PI3K has been reported to enhance PAPR inhibitor-induced DNA damage via impairing production of nucleotides needed for DNA synthesis and DNA repair [30–32]. Thus, inhibition of PI3Kα would be a favorable strategy to block pro-survival signals induced by radiation-triggered Akt activation.

We found inhibition of PI3K not only abrogated the pro-survival signal in radiated ESCC cells, but also in tumor micro-environment. Filtration of M2-like macrophages increased in ESCC PDX after radiation. M2-like macrophages has been recognized as tumor associated macrophages, which are critical components of tumor microenvironments affecting tumor growth, immune suppression, metastasis and chemoresistance [33]. Consistently, ESCC cells co-cultured with radiated M0 macrophages are less sensitive to radiation. Though we didn't dissect the mechanism of radiation-induced M2-like infiltration of macrophages, it has been extensively studied that Akt signaling pathway plays important roles in macrophage activation and M1/M2 polarization [34]. Therefore, blocking Akt by a PI3K inhibitor CYH33 abrogated enhanced infiltration M2-like macrophages in ESCC PDXs. Moreover, CYH33 is able to enhance anti-proliferative activity of radiation when ESCC cells were co-cultured with M0 macrophages. As other stromal cells and immune cells are important components in tumor microenvironment, it would be worthwhile to evaluate the efficacy of combinatorial radiation and PI3K inhibitors in immune-competent animal models.

5. Conclusions

In summary, we demonstrate a novel PI3K inhibitor CYH33 displays potent therapeutic activity against ESCC and that the combination of CYH33 and radiation possesses synergistic response in ESCC by abrogating radiation-induced survival signals both in tumor cells and tumor micro-environment. Our findings provide promising rationale to test this combinatorial regimen in ESCC patients. However, this combination should be investigated in immune-competent animal models to further elucidate the mechanism of action.Acknowledgements

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Conflicts of interest

Jian Ding is the director of Shanghai HaiHe Pharmaceutical Co. Ltd.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.05.040.

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