Valproic acid, a histone deacetylase inhibitor, enhances radiosensitivity in esophageal squamous cell carcinoma

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Abstract. Histone deacetylase (HDAC) inhibitors have been shown to enhance radiation response in various cancer cell lines. Valproic acid (VPA) has been used in clinical practice for the treatment of epilepsy and other seizure disorders and is also one of the most represented HDAC inhibitors. The aim of this study was to evaluate the radiosensitizing ability of VPA and its mechanisms in four esophageal squamous cell carcinoma (ESCC) cell lines (TE9, TE10, TE11 and TE14). VPA inhibited the viability of all ESCC cells in a dose-dependent manner. The 50% inhibitory concentration (IC_{50}) value of VPA in each cell line was between 1.02-2.15 mM, which is higher than clinically used safe concentrations. VPA induced the hyperacetylation of histones H3 and H4, as well as apoptosis and had a radiosensitizing effect on all four ESCC cell lines at a concentration of 0.5 mM which is equivalent to the therapeutic plasma concentration of anti-epilepsy therapy in humans. The radiosensitization was accompanied by an increase in γH2AX levels, indicating the presence of double-strand breaks (DSBs), and decrease in Rad51 expression, a DSB repair protein. These results suggest that a clinically safe dose of VPA can enhance radiation-induced cytotoxicity in human ESCC cells by chromatin decondensation with histone hyperacetylation and downregulation of Rad51. In conclusion, VPA appears to be a safe and promising radiosensitizer for esophageal cancer radiotherapy.

Introduction

Although esophageal cancer is a relatively uncommon disease, it is one of the deadliest cancers with an average five-year survival rate of approximately 17% (1). Curative treatment is offered in the absence of tumor invasion to other organs or distant metastases. In Japan, surgery is the standard therapy for patients with resectable disease. However, radiotherapy alone has been indicated in unresectable or medically inoperable patients as definitive or palliative treatment. Following the report by an intergroup randomized controlled trial (Radiation Therapy Oncology Group 85-01), which compared chemoradiotherapy (CRT) with radiotherapy alone, the combined modality treatment became a standard treatment for patients who received non-surgical treatment for esophageal cancer (2,3). CRT is currently recognized as the standard therapeutic strategy for unresectable esophageal cancer with locally advanced tumor or distant lymph node metastasis (4,5). The combination of 5-fluorouracil (5-FU) and cisplatin (CDDP) together with radiation, has become the standard treatment, due to the synergism between the two agents and their radiosensitizing effects (6-8). However, these drugs also cause severe toxicity, for example, leukocytopenia, pulmonary dysfunction, pericardial effusion, pleural effusion, radiation pneumonitis, perforation and stenosis (4,9,10). Moreover, irradiation enhances the risk of salvage surgery (11). New strategies to enhance local control and reduce side-effects in performing CRT are required. Various radiosensitizers including new anti-cancer drugs are currently being tested.

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) regulate the acetylation and deacetylation of histone (12,13). Moreover, HDAC is related to the deacetylation of chromatin histone proteins as well as non-histone proteins, which regulate cell differentiation, apoptosis and growth arrest (14). At least 18 human HDACs (HDAC 1-11 and sirtuins 1-7) with varying functions, localizations and substrates have been identified (15). Aberrant HDAC activity has been observed in many human cancers (16). In esophageal squamous cell carcinoma (ESCC), histone H4 has been shown to be significantly hyperacetylated in the early stage of cancer invasion and both the hyperacetylation of histone H4 and the high expression of HDAC1 have been shown to topologically co-localize in the same tumor (17). Silencing the increased expression of HDAC1 by RNAi increases apoptosis and enhances the radiosensitivity of esophageal cancer cells (18). HDACs have been considered

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as one of the most promising therapeutic targets for malignant disease. Numerous studies have reported the antitumor efficacy of HDAC inhibitors. The HDAC inhibitor, vorinostat (SAHA, Zolinza®), was approved by the US Food and Drug Administration only for treating refractory cutaneous T cell lymphoma (19). Diverse HDAC inhibitors have entered clinical trials for a number of malignancies, differing in potency and enzyme specificity.

Valproic acid (VPA) is a well-known HDAC inhibitor as well as an anti-convulsant and it is safely used in the treatment of epilepsy and other seizure disorders. It has been reported that VPA enhances radiosensitivity in various cancer cell lines *in vivo* and *in vitro* (20-27). VPA offers considerable promise as a therapeutic agent for esophageal cancer. However, the radiosensitizing effect of VPA in esophageal cancer has not been confirmed. In the present study, the antitumor and radiosensitizing effects of VPA and its mechanisms were investigated in ESCC cell lines.

Materials and methods

Cell lines and treatment. Four human ESCC cell lines (TE9, poorly differentiated; TE10, highly differentiated; TE11, moderately differentiated; and TE14, moderately differentiated) were kindly provided by Dr Tetsuro Nishihira (Kenotokorozawa Hospital, Saitama, Japan). Each cell line was cultured in RPMI-1640 (Invitrogen, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Nissui Pharmaceutical Co. Ltd., Japan), 100 IU/ml penicillin, 100 μg/ml streptomycin (Invitrogen), 2 mM glutamine (Nissui Pharmaceutical Co. Ltd.), and 0.5 mM sodium pyruvate at 37˚C in a humidified atmosphere of 5% CO_2 in air. VPA (Sigma-Aldrich Co., Japan) was dissolved in phosphate-buffered saline (PBS) to a stock concentration of 100 mM and stored at -20˚C. Cultures were irradiated using MBR-1520R-3 (Hitachi Medicotechnology, Hitachi, Japan) at a dose rate of 1 Gy/min. Power output of X-ray-irradiation was 125 KV, 20 mA. Forward-scattered radiation, 0.5 mm Al and 0.2 mm Cu filters were used.

Cell growth assay. The viability of cells treated with VPA was determined by a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Each cell line was treated with VPA at various concentrations (0.01-5 mM) for 72 h. The percentage inhibition was determined by comparing the cell density of the drug-treated cells with that of untreated controls. All experiments were repeated at least three times.

Clonogenic assay. The cells were plated into dishes and allowed to attach for 6 h. The medium was then replaced by medium with or without VPA. Following incubation for 24 h, the cells were irradiated at various doses (2-6 Gy). The cells were harvested by trypsinization, counted, and known concentrations of cells were re-plated into 100-mm culture dishes and returned to the incubator. After incubation for 7-10 days, the cell colonies were fixed and stained with 0.1% crystal violet. Colonies of >50 cells were manually counted to determine survival.

Western blotting. The cell lysates were prepared in denaturing SDS sample buffer and subjected to SDS-PAGE. Protein (20 μg) from each sample was loaded onto a 5-20% gradient polyacrylamide gel (e-PAGEL, Atto Co. Ltd., Japan). Proteins were transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA) and blocked with commercial gradient buffer (EzBlock, Atto Co. Ltd.) at room temperature for 30 min. The membrane was incubated with the primary antibody overnight. To assess the acetylation of histones, anti-acetyl-histone H3 (Lys 14) (Millipore, Billerica, MA, USA) and anti-acetyl-histone H4 (Lys 12) (Millipore) were used as primary antibodies. β-actin antibody (Sigma) was used as the internal control. Anti-phospho-histone H2AX (Ser 139) antibody (Cell signaling Technology, Beverly, MA, USA) was used for the detection of DNA double-strand breaks (DSBs). Anti-Ku70, anti-Ku80 (Cell signaling Technology), anti-Rad51 (Millipore), and anti-DNA-PKcs antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were used for the detection of DSB repair-related proteins. The immunoblots were visualized using an ECL Plus kit (GE Healthcare Japan Co. Ltd.). Antibody-antigen complexes were detected using an ECL western blotting detection kit (GE Healthcare) and the Light Capture system (Atto Co. Ltd.). Quantification was performed with the CS analyzer program (Atto Co. Ltd.).

Immunofluorescent cytochemistry. Cells were cultured on Lab-Tec chamber slides (Nalge Nunc International, New York, NY, USA). The cells were then treated with VPA for designated times and irradiated. They were fixed in a mixture of methanol and acetone (1:1) for 15 min. The slides were immersed in methanol containing 0.3% H_2O_2 for 30 min, blocked with 3.3% normal goat serum in PBS, and incubated with anti-phospho-histone H2AX (Ser 139) (Cell signaling Technology) and the anti-Rad51 antibody (Millipore) at 4˚C overnight. After sections were washed in PBS, the immunoreactivity was visualized by incubating the sections with anti-rabbit IgG antibody conjugated with Alexa Flour 488 or 594 (Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. The slides were examined with an Olympus immunofluorescence microscope (BX50/BX-FLA, Tokyo, Japan).

Assessment of apoptosis. Annexin V binding assay was used to assess phosphatidylserine externalization as a marker of apoptosis using the Pacific Blue™ Annexin V/SYTOX® AADvanced™ Apoptosis kit according to the manufacturer's instructions (Invitrogen). The extent of apoptosis was quantified by flow cytometry.

Statistical analysis. The results are expressed as the means ± SE. A Student's unpaired t-test was used for comparisons between unpaired groups. The 50% inhibitory concentration (IC_{50}) was assessed by using non-linear regression to fit dose-response curves. A difference was regarded as significant if $P<0.05$. Prism 5 software (MDF Co. Ltd, Japan) was used for all the analyses.

Results

Anti-tumor efficacy of VPA. The effect of VPA on viability of the four ESCC cell lines was assessed by the MTT assay. Cells were treated with various concentrations of VPA for 72 h. VPA inhibited the viability of all ESCC cells in a dose dependent manner (Fig. 1). The IC_{50} value of VPA in each cell line was between 1.02-2.15 mM (Table I).

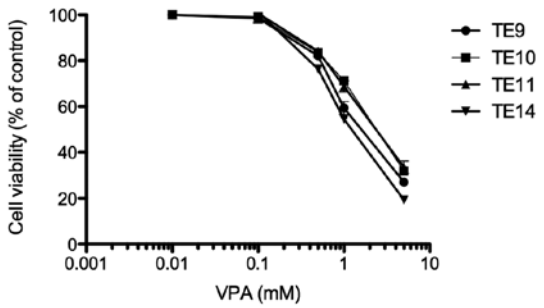


Figure 1. ESCC cell growth inhibition by VPA. Cell viability was assessed by MTT assay. Four cell lines (TE9, TE10, TE11 and TE14) were treated with the indicated doses of VPA (0.01-5 mM). Results represent the means of more than three independent experiments with standard errors.

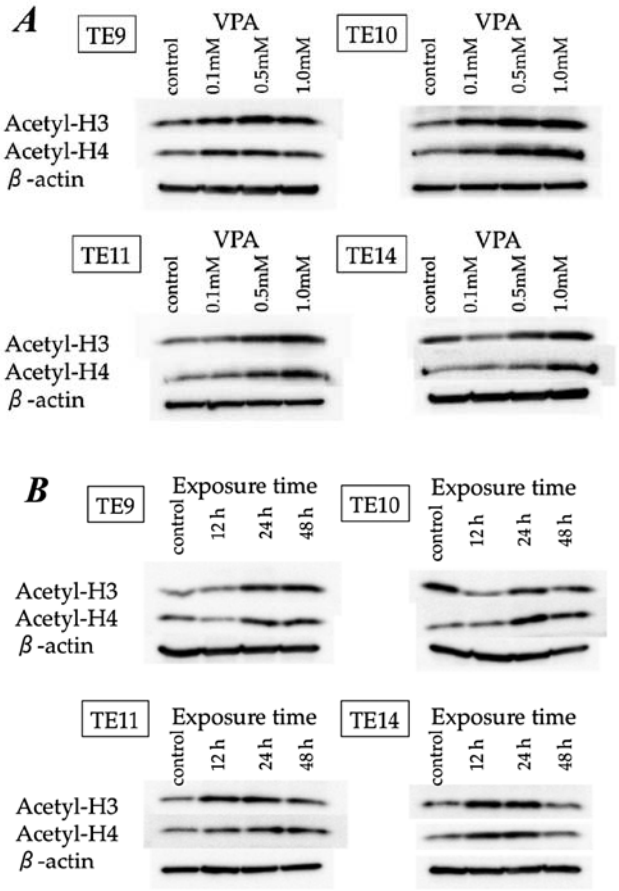
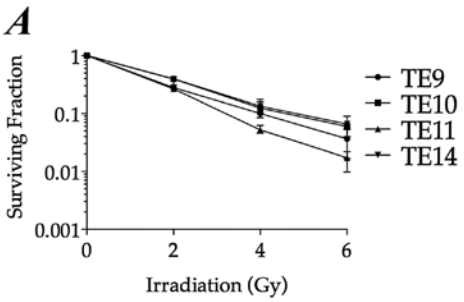


Figure 2. Histone acetylation status determined after exposure to VPA. (A) ESCC cell lines (TE9, TE10, TE11 and TE14) were exposed for 24 h to the designated doses (0.1-1.0 mM) of VPA. (B) Cells were exposed for designated times (12-48 h) to 0.5 mM VPA. After exposure to VPA, cells were collected and subjected to immunoblot analysis for assessment of acetylated histone H3 and H4 expression. Each blot is representative of two or three independent experiments with β-actin used as the loading control.

Inhibitory effect of VPA on HDAC. The expressions of acetylated histones H3 and H4 detected by western blotting were used to examine the inhibitory effect of VPA. First, each cell line was exposed to various concentrations of VPA (0.1-1.0 mM). VPA concentrations higher than 0.5 mM enhanced the hyperacetylation of histones H3 and H4 in all ESCC cell lines (Fig. 2A). The cells were then exposed to 0.5 mM VPA for various times



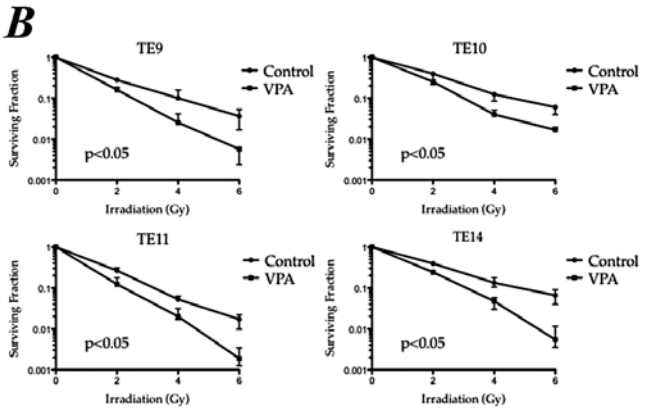


Figure 3. The effect of VPA on ESCC cell radiosensitivity. (A) The survival of each cell line (TE9, TE10, TE11 and TE14) after radiation without VPA was measured by clonogenic assay. (B) Radiosensitization combined with 0.5 mM VPA was examined by clonogenic assay. Results represent the means of two or three independent experiments with standard errors.

Table I. IC_{50} values of VPA on ESCC cell lines.

ESCC cell lines	IC_{50} (mM)
TE9	1.26
TE10	2.15
TE11	1.52
TE14	1.02

(12-48 h). VPA increased histone hyperacetylation after the exposure of cells for >24 h (Fig. 2B).

Radiosensitizing effect of VPA. The radiosensitizing effect of VPA was assessed using a clonogenic assay. Since 0.5 mM VPA showed histone hyperacetylation, it was also used in combination with irradiation. VPA enhanced radiosensitivity in all ESCC cell lines (Fig. 3).

Irradiation-induced apoptosis enhanced by VPA. The apoptotic response to irradiation alone or the combination of irradiation and VPA was assessed. Cells were first cultured with or without 0.5 mM VPA for 24 h, irradiated (6 Gy), and returned to culture for 48 h. The cells were then examined for the detection of apoptosis. VPA significantly increased the proportion of early apoptotic cells represented by Annexin V^+/SYTOX$^-$ after irradiation in all ESCC cell lines. The proportion of late apoptotic

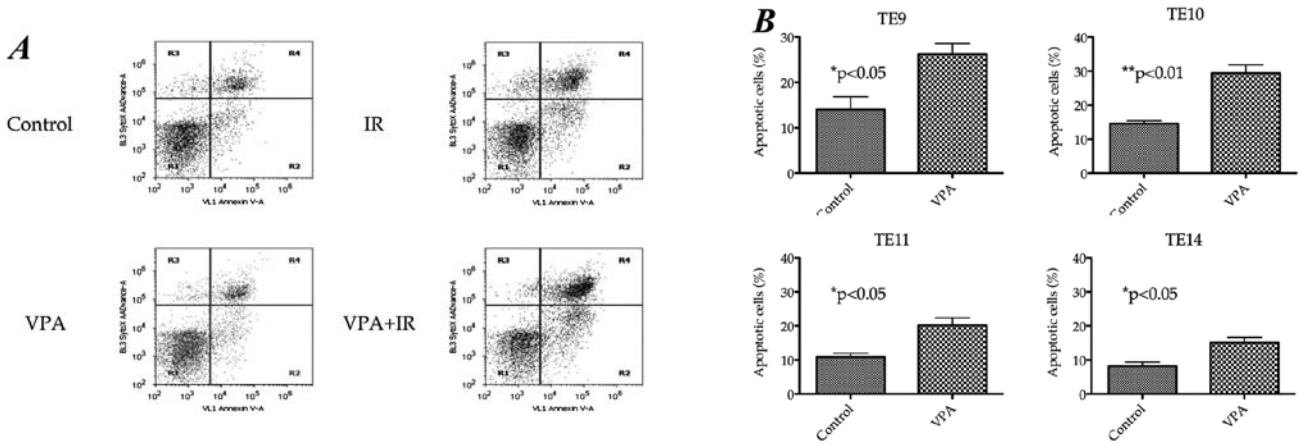


Figure 4. The effect of VPA on apoptosis after irradiation (IR) of ESCC cell lines. The cell lines (TE9, TE10, TE11 and TE14) were treated with or without 0.5 mM VPA for 24 h, irradiated or not, and incubated for 48 h. Annexin V binding assay was used to assess phosphatidylserine externalization as a marker of apoptosis using the Pacific Blue™ Annexin V/SYTOX® AADvanced™ Apoptosis kit. The extent of apoptosis was quantified by flow cytometry. (A) Representative cytogram of TE11. Early apoptotic populations were localized in the lower right quadrants. Late apoptotic or necrotic populations were found in the upper right quadrants. (B) The percentage of early or late apoptotic cells after irradiation in four ESCC cell lines. Results represent the means of two or three independent experiments with standard errors.

cells represented by Annexin V/SYTOX$^+$ was also increased (Fig. 4).

VPA increases DNA DSBs. The expression of γH2AX was analyzed in order to assess DNA DSBs. When ionizing radiation induces DNA DSBs, at the sites of DSBs the histone H2AX is rapidly phosphorylated and forms γH2AX. γH2AX was thus examined as an indicator of DNA DSBs. Western blotting and immunofluorescent cytochemistry were performed. Cells were exposed to either no treatment or 0.5 mM VPA followed by irradiation (6 Gy). After 2 h, proteins were extracted and examined by western blotting. Irradiation caused an increase in the expression of γH2AX. The addition of VPA elevated the expression of γH2AX in all ESCC cell lines (Fig. 5A). Immunofluorescent cytochemistry also showed that the cells treated with a combination of VPA with radiation had a significantly higher expression of γH2AX compared to the controls (Fig. 5B).

VPA attenuates Rad51 expression after irradiation. The effect of VPA on the expression of proteins known to be related to the repair of radiation-induced DSBs (e.g., Ku70, Ku80, Rad51 and DNA-PKcs) was examined by western blotting and immunofluorescent cytochemistry. Cells were pre-treated with or without 0.5 mM VPA for 24 h, irradiated (6 Gy), and re-incubated. After 4 h, proteins were collected. The addition of VPA downregulated the expression of Rad51. The expression levels of Ku70, Ku80 and DNA-PKcs did not change (Fig. 6A). Similarly, immunofluorescent cytochemistry showed that Rad51 foci were decreased by 0.5 mM VPA (Fig. 6B).

Discussion

The results of this study show for the first time that VPA enhances radiosensitivity in ESCC cell lines. The effect of radiosensitizer was seen in various histological types. The increase in DSBs by chromatin modulation and the downregulation of Rad51, a DSB repair protein, were considered as molecular mechanisms enhancing radiation sensitivity. Furthermore, the VPA concentration used as a radiosensitizer was at a low and clinically achievable dose. Thus, VPA as a HDAC inhibitor may provide an additional approach to radiotherapy for esophageal cancer.

Previous laboratory studies have reported HDAC inhibitors are effective as radiosensitizers in many types of malignancies. These studies show that VPA *in vitro* enhances radiosensitivity in malignant glioma (20,22), colorectal carcinoma (21), leukemia (23,24,26,27) and retinoblastoma cells (25). Moreover, a corresponding enhancement of therapeutic efficacy *in vivo* was also reported when VPA was combined with irradiation (20,21). To our knowledge, there are currently four on-going phase I clinical trials assessing the combination of HDAC inhibitors and radiotherapy: VPA with radiotherapy for pediatric glioma, VPA with radiotherapy for pediatric refractory solid and central nervous system (CNS) tumors, VPA or hydralazine with CRT for cervical cancer, and vorinostat with palliative radiotherapy (28-31).

VPA is a short-chain fatty acid and its chemical properties allow easy delivery to the organisms and cells. VPA is rapidly absorbed after oral administration. In the human brain, VPA affects the function of the neurotransmitter, GABA, through several pathways including the inhibition of GABA degradation, increased synthesis of GABA, and decreased GABA turnover. VPA is well known as an anti-convulsant and is safely used in the treatment of epilepsy and other seizure disorders. VPA is in general well-tolerated by patients. Neurological side-effects, such as sedation, dizziness and tremors, as well as mild gastrointestinal toxicities are usually evident early during treatment. The most serious adverse events are liver failure and teratogenicity. In treating epilepsy, the therapeutic plasma concentration ranges from 50-100 μg/ml, equivalent to 0.3-0.6 mM (31,32). Our results showed that IC_{50} values of VPA against ESCC cell lines were 1.02-2.15 mM, which were higher than clinically safe concentrations. However, VPA enhanced the hyperacetylation of histones H3 and H4 in all ESCC cell lines at a low concentration (0.5 mM), which is equivalent to

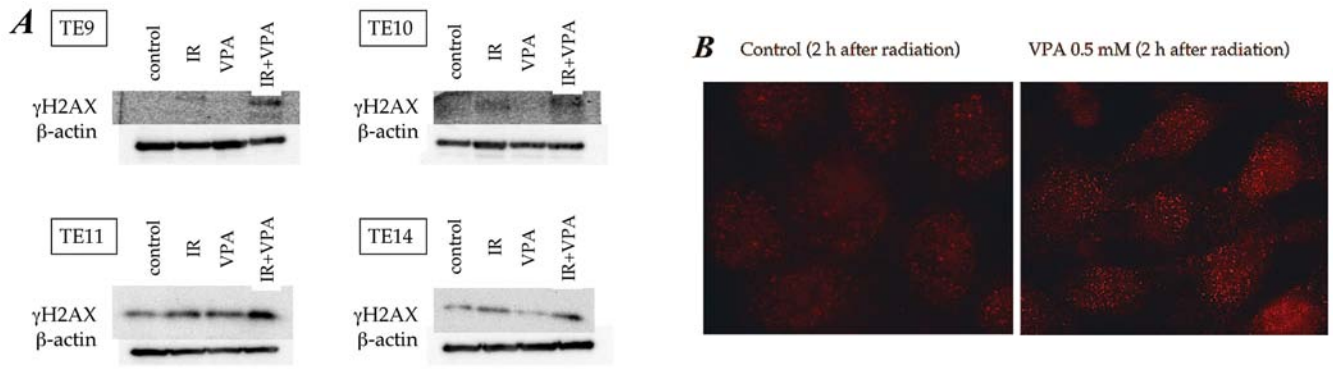


Figure 5. The effect of VPA on radiation-induced DNA DSBs. Phosphorylated histone H2AX (γ-H2AX) was used as an indicator of DNA DSBs. (A) ESCC cells were cultured with or without 0.5 mM VPA for 24 h, and irradiated (6 Gy). After 2 h, lysates were collected. The protein expression level of γ-H2AX was analyzed by western blotting. Each blot is representative of two or three independent experiments with β-actin used as the loading control. (B) Micrographs of γ-H2AX foci on TE11 cells were obtained 2 h after irradiation by immunofluorescent cytochemistry.

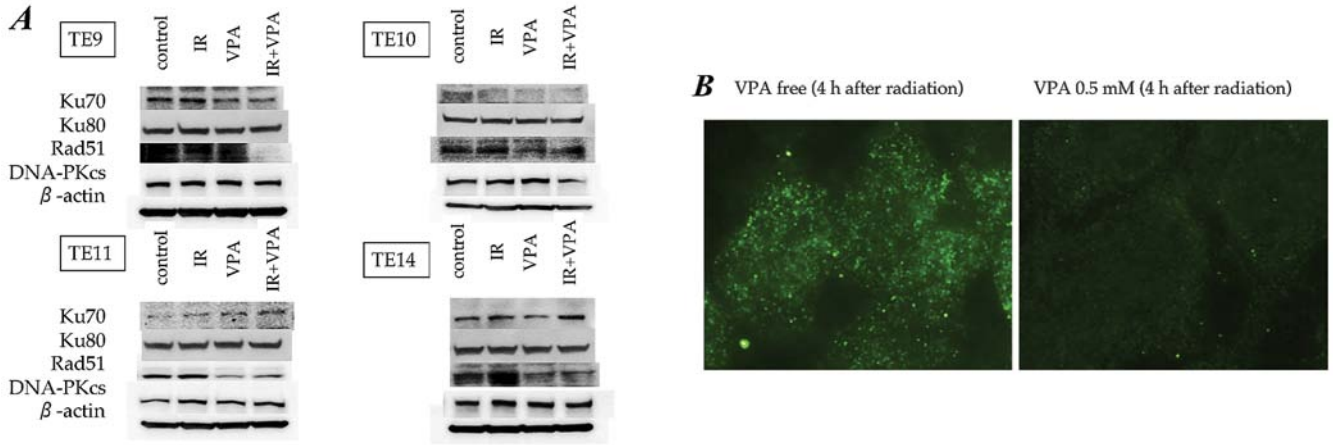


Figure 6. The effect of VPA on radiation-induced DNA DSBs repair protein expression. (A) Cells were cultured with or without 0.5 mM VPA for 24 h, and irradiated (6 Gy). After 4 h, lysates were extracted. The expression of DSB repair-related proteins including Ku70, Ku80, Rad51 and DNA-PKcs was analyzed by western blotting. (B) Micrographs of Rad51 foci on TE11 cells were obtained 4 h after irradiation. VPA decreased the expression of Rad51, a radiation-induced DSB repair protein, after irradiation.

the anti-epilepsy therapeutic plasma concentration in humans. Thus, it can be concluded that pharmacologically relevant levels of VPA can cause the acetylation of histones and lead to the relaxation of chromatin structure. In addition, a low concentration (0.5 mM) of VPA also induced apoptosis and had a radiosensitizing effect.

There are two principal mechanisms by which low concentrations of HDAC inhibitors can radiosensitize tumor cells. First, HDAC inhibitors acetylate histone chromatin enabling access to the chromatin structure. Decondensed chromatin by deacetylation is more likely to cause permanent damage. Harikrishnan *et al* (23) reported that heterochromatin is more resistant to radiation-induced DSBs than euchromatin, and that radiosensitivity of HDAC inhibitors depends on histone modification. Secondly, HDAC inhibitors can suppress the endogenous DNA repair process. In mammalian cells, DSBs are repaired mostly by either homologous recombination (HR) or non-homologous end-joining (NHEJ). Several HDAC inhibitors including VPA can downregulate DNA repair proteins related to HR (Rad51) (33-35) and NHEJ (Ku70, Ku86 and DNA-PKcs) (21,34-40). Munchi *et al* (38) noted that sodium butylate suppresses Ku70, Ku86 and DNA-PKcs at mRNA and protein levels. Chinnaiyan *et al* (35) argued that SAHA suppresses Rad51 and DNA-PKcs at protein levels. In our study, a low dose of VPA suppressed the expression of the Rad51 protein in ESCC cell lines.

Mammalian cells show different levels of radioresistance during the course of the cell cycle. Cells in the G2/M phase are more radiosensitive than in the S phase (41). Flow cytometry was used to assess the effect on different phases of the cell cycle. There was no difference in the distribution of cell cycle phases between untreated control cells and pre-treated cells (data not shown). Therefore, the low concentration VPA was non-toxic and did not induce cell cycle arrest. Taken together, we suggest that a low concentration of VPA sensitizes ESCC cells to irradiation by the modulation of chromatin structure and the downregulation of Rad51, the DNA DSBs repair protein.

While it is known that tumor cell lines differ in their p53 status, all ESCC cell lines used in this study had mutant-type p53 (42). Forty to sixty percent of patients with esophageal cancer have p53 abnormalities, even in early pre-cancerous lesions (43). Previous studies on the relation of p53 status and radiosensitization by HDAC inhibitors have not yielded consistent results. Chen *et al* (44) reported that only wild-type p53 was radiosensitized by VPA in colorectal cancer cells *in vitro* and

in vivo. However, phenylbutyrate radiosensitized only mutant-type p53 *in vitro*. Moreover, phenylbutyrate radiosensitized the knockdown of wild-type p53 in glioblastoma cell lines (45). Kim *et al* (46) showed that trichostatin A significantly radiosensitized various cell lines (adenocarcinoma of the colon, adenocarcinoma of the lung, squamous cell carcinoma of the head/neck and squamous cell carcinoma of the uterus). The effect was also clearer in wild-type p53 than in mutant-type p53. Flatmark *et al* (47) also showed that irrespective of p53 status, trichostatin A sensitized colorectal cancer cell lines. Our results showed the radiosensitization of ESCC cells with mutant-type p53. It is not clear how p53 status influences radiosensitization by HDAC inhibitors, nor how HDAC inhibitors radiosensitize cancer cells. Conceivably, treatment efficacy may depend on histological origin among cancer cells. A currently held idea proposes a classification according to isoform-selective HDAC inhibition in probing for biological functions. A novel isoform-selective HDAC inhibitor will be useful in elucidating the mechanism of action and the development of therapeutic agents with minimal side-effects (48).

HDAC inhibitors can suppress the radiation-induced damage of normal tissue. HDAC inhibitors have been shown to suppress cutaneous radiation syndrome (49) and radiation-induced oral mucositis (50). This mechanism is related to the downregulation of TGF-β and TNF-α and the reduction of oxidative stress. HDAC inhibitors together with radiotherapy can protect normal tissue fibrosis and reduce severe toxicity. Particularly, HDAC inhibitors may prevent fibrosis followed by stenosis in esophageal cancer treatment.

In conclusion, HDAC inhibitors are promising anti-cancer drugs with multiple functions. However, higher doses of HDAC inhibitors have severe toxicities and are difficult to use clinically. With respect to radiosensitization, HDAC inhibitors at low concentrations are effective depending on the cell type. VPA is a representative HDAC inhibitor and its safe use in humans has already been established. Our results show the potential usefulness of VPA in combination with radiotherapy for treating esophageal cancer.

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