Valproic acid suppresses the self-renewal and proliferation of head and neck cancer stem cells

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Abstract. Emerging evidence suggests that cancer cells present profound epigenetic alterations in addition to featuring classic genetic mutations. Valproic acid (VPA), a histone deacetylase inhibitor, can potently inhibit tumor growth and induce differentiation. However, the effect and underlying mechanism of VPA on head and neck squamous cell carcinoma (HNSCC) cancer stem cells (CSCs) remain unclear. In the present study we investigated the effects of VPA on the characteristics of HNSCC CSCs *in vitro* and *in vivo*. As a result, VPA inhibited the self-renewal abilities of HNSCC CSCs during two serial passages and decreased the expression of stem cell markers, such as Oct4, Sox2 and CD44. VPA also potentiated the cytotoxic effect of cisplatin by suppressing the ABCC2 and ABCC6 transporters as well as by inducing caspase-mediated apoptosis. In addition, the combination of VPA and cisplatin attenuated tumor growth and induced apoptosis in a xenograft model. Our results suggest that VPA might be a potential therapeutic strategy in combination with conventional cisplatin for HNSCC patients by elimination of CSC traits.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, with an annual incidence of more than 500,000 cases (1). Despite recent advances in treatment regimens including surgery, radiotherapy, chemotherapy and introduction of novel therapeutic agents, the 5-year survival rate of these patients has been virtually unchanged in the past three decades, remaining at only 50% (2). Further improvements in curative rates in HNSCC will require significant advances in the development of new drugs and treatment strategies. A deeper understanding of the precise molecular mechanisms responsible for HNSCC tumorigenesis and progression will contribute significantly toward the development of new and improved therapeutic agents.

It has been reported that many solid human cancers, including HNSCC, are maintained by a small subpopulation of cells called cancer stem cells (CSCs) (3-6). These CSCs have the unique features of self-renewal, asymmetrical differentiation into multiple lineages and enhanced tumor-initiating capacity in xenograft models (7-9). Accumulating evidence suggests that CSCs are responsible for tumor metastasis and the acquisition of resistance to treatment with conventional chemotherapeutic agents, which leads to tumor relapse (10,11). Therefore, targeting CSCs with conventional or other targeted therapies may be required to effectively treat cancers (12-15).

Epigenetic regulations are required for normal development and gene expression. Disruption of epigenetic regulations often leads to aberrant gene expression and malignant cellular transformation (16). Epigenetic alterations commonly observed in malignant cells include DNA methylation and changes in histone modification patterns as well as expression profiles of chromatin-modifying enzymes (16,17). Histone modification patterns are dynamically regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), which add and remove specific covalent modifications, respectively (18). An imbalance between HATs and HDACs leads to aberrant gene expression and tumorigenesis (19).

Several epigenetic drugs that effectively reverse aberrant DNA methylations and histone modifications have been discovered. Valproic acid (VPA), a branched short-chain fatty acid, inhibits HDACs causing increased chromatin acetylation (20). HDACs have been found to be overexpressed or mutated in many types of human tumors (21,22). A recent study demonstrated that HNSCCs are primarily hypoacetylated which may account for the accumulation and maintenance of CSCs (23). They also showed that inhibition of HDACs using Trichostatin A reduced the number of HNSCC CSCs and inhibited clonogenic sphere forma-

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Table I. PCR primer sequences (5'-3').

Primer	Forward	Reverse
ABCB1	AGAGGGAGGACAAAGTCCCT	CACTTCCTCAGACTGCTCCA
ABCC1	AGAGGGAGGACAAAGTCCCT	CACTTCCTCAGACTGCTCCA
ABCC2	AGAGGGAGGACAAAGTCCCT	CACTTCCTCAGACTGCTCCA
ABCC3	AGAGGGAGGACAAAGTCCCT	CACTTCCTCAGACTGCTCCA
ABCC4	AGAGGGAGGACAAAGTCCCT	CACTTCCTCAGACTGCTCCA
ABCC5	AGAGGGAGGACAAAGTCCCT	CACTTCCTCAGACTGCTCCA
ABCC6	AGAGGGAGGACAAAGTCCCT	AGAGGGAGGACAAAGTCCCT
ABCG2	AGAGGGAGGACAAAGTCCCT	CACTTCCTCAGACTGCTCCA

tion (23). In contrast, a separate report showed that VPA promoted the expansion of breast CSCs through reprogramming of differentiated cancer cells into stem-like cells. The causes of this discrepancy might lie in the different cancer cell systems as well as the different HDAC inhibitors used.

Unfortunately, previous reports regarding the effect of VPA on HNSCC CSCs have been extremely scarce. The objectives of the present study were to evaluate the effect of VPA on HNSCC CSCs and to delineate the mechanisms by which VPA inhibits the characteristics of CSCs derived from human primary HNSCC. Here, we present evidence for the therapeutic value of VPA in combination with conventional cisplatin, which suppressed the self-renewal and proliferation and induced apoptosis of CSCs in HNSCC.

Materials and methods

Isolation and culture of HNSCC stem-like cells. HNSCC stem-like cells (K3 and K5) were isolated and characterized from the primary surgical specimens of HNSCC patients, as previously described (8). The CSC phenotype of HNSCC CSCs was confirmed by functional assays of sphere formation, stemness-associated gene expression and xenograft tumor formation. The cells were grown in serum-free media composed of DMEM supplemented with B27 (Invitrogen), N2 supplement (Invitrogen), basic fibroblast growth factor (bFGF; 20 ng/ml; R&D Systems, Minneapolis, MN, USA) and epidermal growth factor (EGF; 20 ng/ml; R&D Systems).

Sphere formation assays. To assess the self-renewal capacity of HNSCC CSCs *in vitro*, the cells were dissociated into single cells, seeded in a 24-well plate at a density of 200 cells/well, and cultured in serum-free medium, with EGF and bFGF supplementation every other day. Spheres with a diameter exceeding 10 μm were counted after 14 days.

Western blot analysis. Western blot analysis of electrophoretically separated proteins from cells was performed as previously described (8). Specific antibodies against Oct4, Sox2, Bcl-2, Bax and caspase 3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies, anti-rabbit IgG and anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Detection of CD44 expression by flow cytometry. HNSCC CSCs were dissociated into single cells and washed twice in cold phosphate-buffered saline (PBS). Cells were labeled with anti-CD44 and fluorescein isothiocyanate (FITC)-labeled secondary antibodies, then subjected to flow cytometry using a FACSCalibur machine (BD Biosciences). The percentages of $CD44^+$ cells in cultures were determined.

Chemosensitivity assay. HNSCC CSCs were dissociated into single cells and then plated in a 96-well plate at a density of $7X0^3$ cells/well under serum-free culture conditions. Cells were treated with cisplatin at the indicated concentrations and then cultured at 37˚C under a humidified 5% CO_2 atmosphere. Twenty-four hours later, 20 μl of 3-(4,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml in PBS) was added to each well, and the plate was placed at room temperature for 3 h. The absorbance at 570 nm was measured using a SpectraMax 190 (Molecular Devices) instrument.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Total cellular RNA was reverse-transcribed using a reverse transcriptase (RT) kit (Fermentas, Glen Burnie, MD, USA) according to the manufacturer's instructions. For semi-quantitative PCR, cDNA was added to a mixture of specific primers and 1 U of *Taq* DNA polymerase (Roche Diagnostics, Indianapolis, IN, USA), and amplified using an MJ Research MiniCycler (Bio-Rad Laboratories, Waltham, MA, USA). PCR products were separated by agarose gel electrophoresis (1.5% agarose gels) and detected under ultraviolet light (Bio-Rad Laboratories, Hercules, CA, USA). Real-time PCR was performed on an iCycler IQ real-time detection system (Bio-Rad Laboratories), using IQ Supermix with SYBR-Green (Bio-Rad Laboratories). The human sequence-specific primers used are listed in Table I.

Xenograft tumor formation assay. All animal studies were approved by the Institutional Animal Care and Use Committee of Konkuk University. HNSCC CSCs were treated for 48 h with cisplatin (5 μM) alone, cisplatin (5 μM) plus VPA (400 μM) or dimethyl sulfoxide (DMSO; vehicle) *in vitro*. Following this, 10^5 or 10^6 cells were subcutaneously injected into the flank of 8-week-old female BALB/c nude mice using a 22-gauge needle. Mice were visually inspected and palpated weekly to monitor tumor formation. The mice were sacrificed 8 weeks

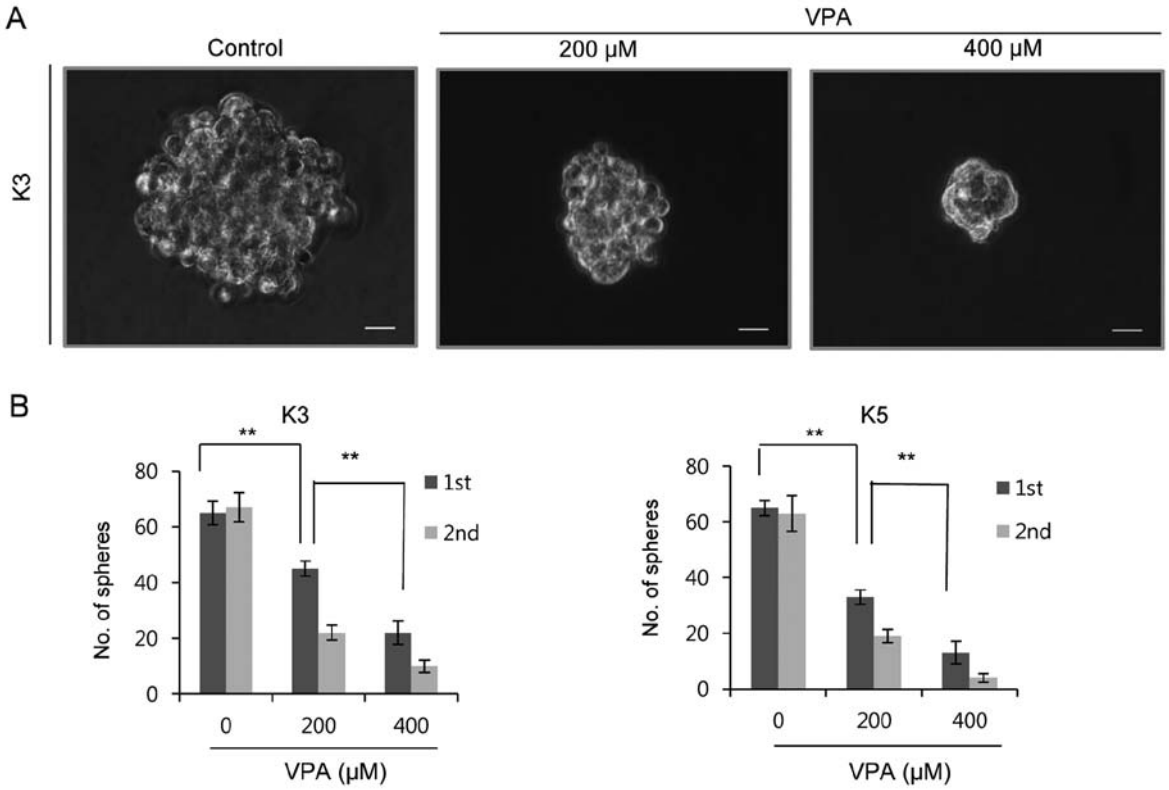

Figure 1. Valproic acid (VPA) reduces self-renewal capacities in HNSCC CSCs. (A) Representative images of spheres after DMSO or VPA treatment. Scale bar, 30 μm. (B) Sphere forming capacity after VPA treatment in HNSCC CSCs (K3 and K5). Cells were grown in serum-free medium conditions at a density of 200 cells/well and treated with VPA for 2 weeks to obtain primary spheres. At the end of the incubation period, the number of spheres with a diameter exceeding 30 μm was calculated. 1st, first passage; 2nd, second passage. Data represent mean ± SD, **P<0.01.

after transplantation, and subcutaneous tumor tissues were harvested for estimating tumor size, weight and apoptosis.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. Tumor tissues collected from mice injected with HNSCC CSCs treated with DMSO, cisplatin (5 μM), or cisplatin (5 μM) and VPA (400 μM) were used for TUNEL assays. Sections (4-μm) from formalin-fixed, paraffin-embedded tumors were deparaffinized and rehydrated using xylene and ethanol, respectively. The slides were rinsed twice with PBS and treated for 15 min at 37˚C with proteinase K (15 μg/ml in 10 mM Tris-HCl, pH 7.4-8.0). Endogenous peroxidases were blocked using 3% hydrogen peroxide in methanol at room temperature for 10 min. The tissue sections were then analyzed with an In Situ Cell Death Detection kit-POD (Roche) following the manufacturer's instructions.

Statistical analysis. The results are presented as mean ± SD. Statistical analyses were performed with the SPSS 10.0 statistical software (SPSS, Inc., Chicago, IL, USA). Statistical significance was determined by one-way ANOVA followed by post hoc tests for multiple comparisons. P<0.05 was considered to indicate a statistically significant result.

Results

VPA suppresses the CSC properties in HNSCC. The sphere formation assay can be effectively used to assess the self-renewal capacity of HNSCC CSCs, and has been reported to correlate closely with tumorigenicity (8). We examined whether VPA had the ability to inhibit the self-renewal capacity of HNSCC CSCs. VPA significantly suppressed sphere formation in two HNSCC CSC cultures during two serial passages in a dose-dependent manner (Fig. 1). CSCs found in HNSCC express typical stem cell markers, including Sox2, Oct4 and CD44. Therefore, we examined whether VPA treatment interfered with the expression of these markers. As evident from the results of the western blot analysis, treatment of HNSCC CSCs with VPA significantly suppressed Sox2 and Oct4 expression (Fig. 2A). Furthermore, VPA treatment significantly reduced the number of CD44$^+$ cells in the HNSCC CSCs (Fig. 2B).

VPA enhances the chemosensitization of cisplatin and apoptosis in HNSCC CSCs. Chemoresistance is one of the most important characteristics of CSCs. We examined whether VPA increases the susceptibility of HNSCC CSCs to cisplatin. A combination of VPA and cisplatin increased the susceptibility of HNSCC CSCs to cisplatin in a dose-dependent manner (Fig. 3A). In order to understand the mechanisms responsible for the increased susceptibility of VPA-treated HNSCC CSCs to cisplatin, we analyzed the changes in expression of ATP-binding cassette (ABC) transporters following VPA treatment. Real-time qPCR analysis revealed that VPA treatment (400 μM) reduced the transcript levels of the ABCC2 and ABCC6 genes in the HNSCC CSCs (Fig. 3B).

To further determine whether the VPA-induced suppression of CSC properties is due to increased apoptosis, we investigated the expression of the apoptosis-related proteins

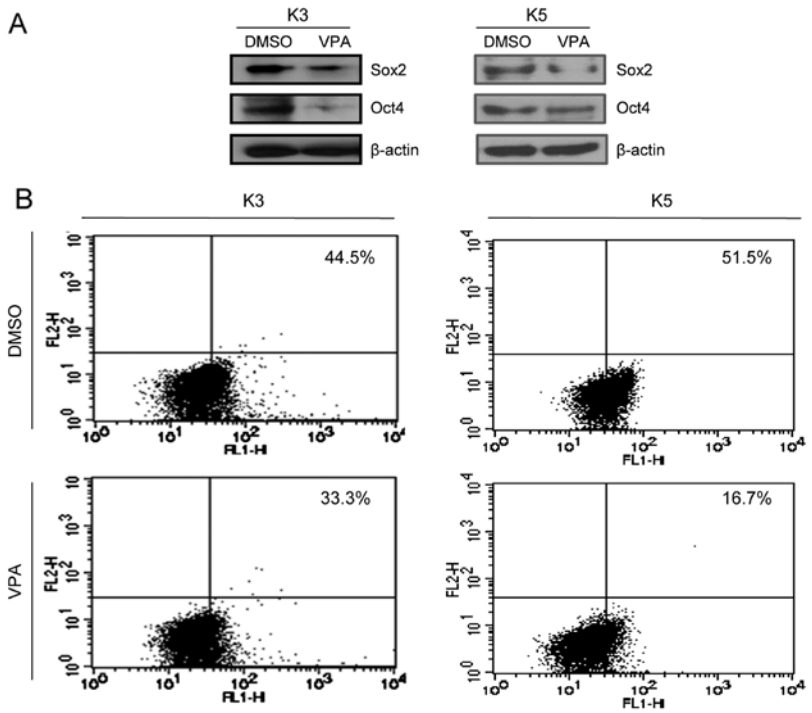

Figure 2. Valproic acid (VPA) reduces stemness-associated marker expression in HNSCC CSCs. (A) Protein levels of Sox2 and Oct4 in HNSCC CSCs treated with DMSO or VPA (400 μM) for 48 h. (B) FACS analysis of CD44 expression in HNSCC CSCs treated with DMSO or VPA (400 μM) for 48 h.

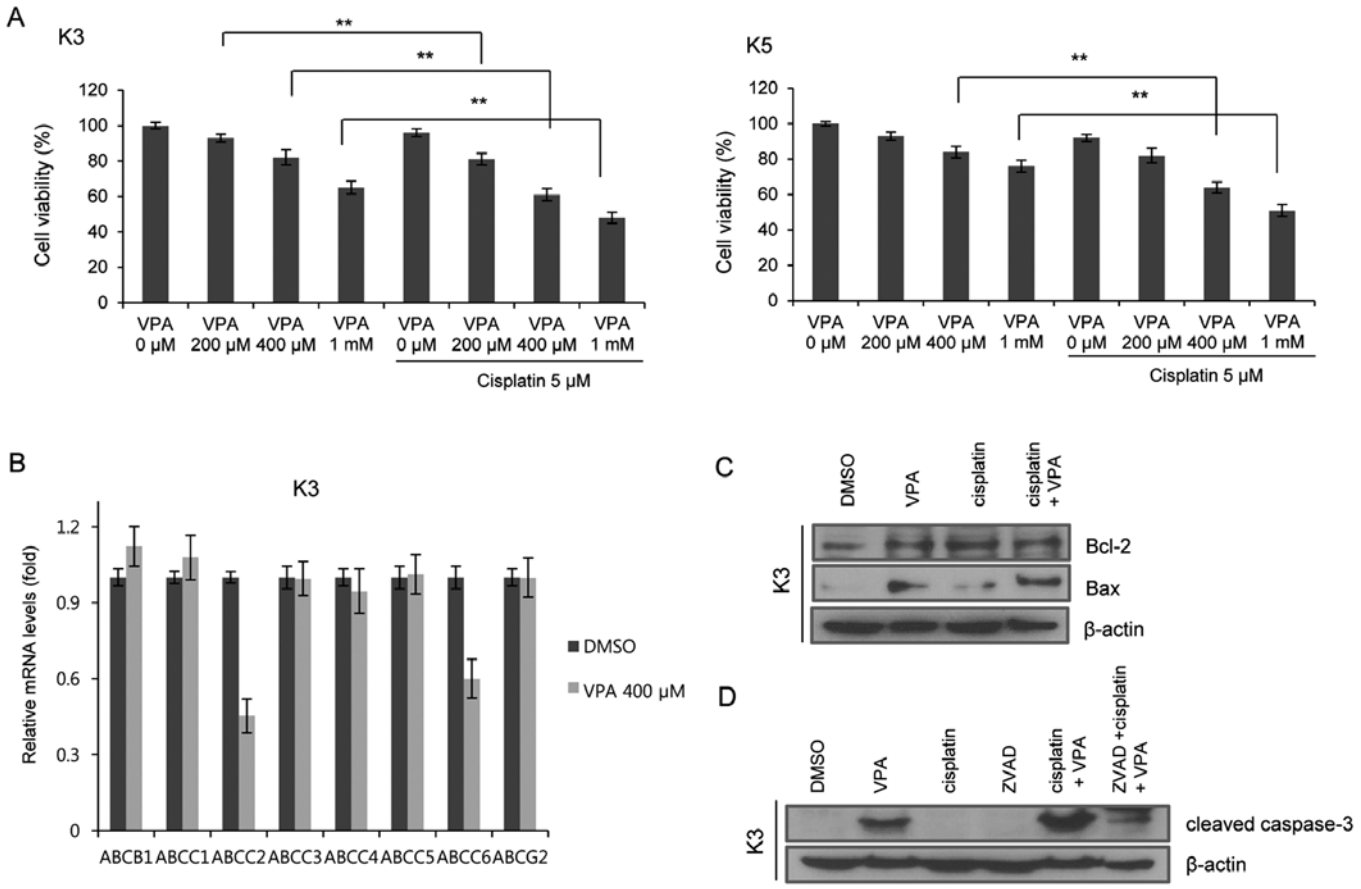

Figure 3. Valproic acid (VPA) enhances the chemosensitivity of cisplatin in HNSCC CSCs. (A) MTT assay of the cell viability after VPA treatment at various concentrations in HNSCC CSCs treated with DMSO or cisplatin (5 μM). Data represent mean ± SD, **P<0.01. (B) mRNA expression levels of various ABC transporter genes after VPA (400 μM) treatment in HNSCC CSCs. Data represent mean ± SD, **P<0.01. (C) Protein expression level of Bcl-2 and Bax in HNSCC CSCs treated with DMSO, VPA (400 μM) alone, cisplatin (5 μM) alone, and cisplatin combined with VPA. (D) Protein expression level of cleaved caspase-3 detected by western blotting in HNSCC CSCs treated with VPA (400 μM) alone, cisplatin (5 μM) alone, zVAD (50 μM) alone, and their combinations. zVAD, a caspase inhibitor.

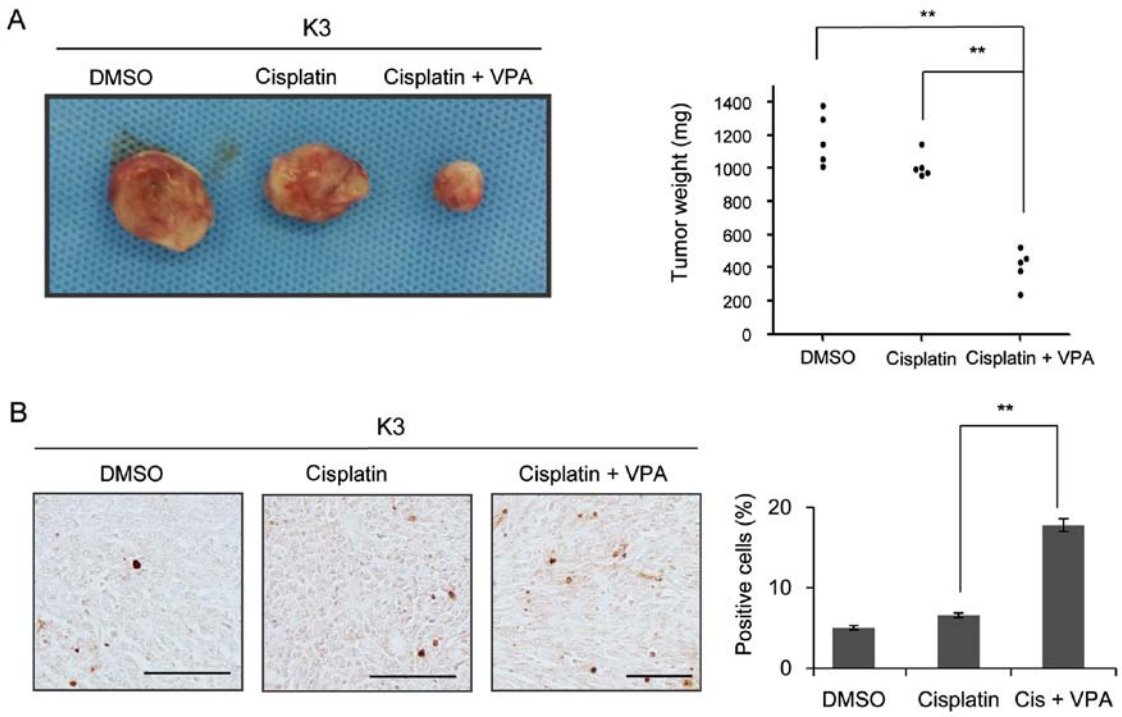

Figure 4. Valproic acid (VPA) attenuates tumor growth of HNSCC CSC-derived xenograft tumors. (A) Representative images of tumors generated after subcutaneous injection of HNSCC CSCs treated with DMSO, cisplatin (5 μM) alone, or VPA (400 μM) plus cisplatin (5 μM) into flank of nude mice (left) and average tumor weight (N=5) (right). Data represent mean ± SD, **P<0.01. (B) TUNEL apoptosis assay of nude mouse tumor tissues generated by HNSCC CSCs treated with DMSO, cisplatin alone, VPA plus cisplatin (left) and its quantification (right). Scale bar, 200 μm. Data represent mean ± SD, **P<0.01.

Table II. Tumor formation ability of injected HNSCC CSCs in xenograft model.

Injected cells	Control	Cisplatin	VPA + cisplatin
10^5	6/6	5/6	1/6
10^6	6/6	5/6	3/6

No. of mice with tumors/no. of mice receiving injections.

Bax and Bcl2. When compared to the cells treated with VPA (400 μM) or cisplatin (5 μM) alone, the expression of Bax in the CSCs was increased following treatment with cisplatin (5 μM) and VPA (400 μM) (Fig. 3C). To test whether caspase activity was required for VPA-mediated apoptosis, we examined the expression of cleaved caspase-3 in the CSCs before and after VPA treatment. Cleaved caspase-3 protein levels were significantly increased in the cells treated with a combination of cisplatin and VPA. A known caspase inhibitor, zVAD, abrogated the VPA-induced changes in cleaved caspase-3 expression (Fig. 3D). These results suggested that VPA is a potent inducer of apoptosis in HNSCC CSCs.

VPA inhibits the growth of HNSCC CSCs in a xenograft model. To confirm the combined effect of VPA and cisplatin on HNSCC CSCs, the inhibitory effect of VPA on the capacity of HNSCC CSCs to initiate tumor formation in nude mice was examined. Palpable tumor masses developed in 100% (6 of 6) of mice injected with 10^5 DMSO-treated cells and 83.3% (5 of 6) of mice injected with 10^5 cisplatin-treated cells; in contrast, only 16.6% (1 of 6) cells with administration of VPA plus cisplatin formed tumors when 10^5 cells were injected (Table II). In contrast to the large tumors generated by HNSCC CSCs treated with cisplatin or DMSO alone, cells treated with 400 μM VPA and 5 μM cisplatin generated tumors that were small, and the average tumor weight in nude mice (N=5) was significantly lower following treatment with cisplatin and VPA (Fig. 4A). TUNEL assay revealed a significant increase in the number of apoptotic cells in tumors generated from CSCs treated with VPA and cisplatin compared to those generated from cells treated with cisplatin or DMSO alone (Fig. 4B). Taken together, these data suggest that combination treatment of ciplatin and VPA induced apoptosis and reduced the growth rate of CSCs *in vivo*.

Discussion

Recent advances in the research of epigenetics have shown that human cancer harbors global epigenetic alterations, in addition to numerous genetic alterations (24). These genetic and epigenetic alterations may have critical effects on all stages of cancer development and progression. Unlike genetic alterations, epigenetic alterations are potentially reversible. This reversibility of epigenetic alterations has led to the possibility of developing a new class of therapeutics that restores the normal epigenetic state in malignant cell populations (16,25). Thus, many drugs that target specific enzymes involved in the epigenetic regulation of gene expression have been introduced, and the utilization of these drugs is emerging as an effective and valuable approach to combination treatment with

conventional chemotherapy (25). Of these, HDAC inhibitors that help in restoring normal histone acetylation patterns have been shown to induce growth arrest, apoptosis, and differentiation by reactivating silenced tumor-suppressor genes (26).

Valproic acid (VPA), a well-known anticonvulsive agent, emerged in 1997 as an antineoplastic agent (27), and has been described to have antiproliferative effects in a variety of human malignancies (28,29). VPA modulates the biology of cancer cells by inducing differentiation, inhibiting proliferation, increasing apoptosis, and decreasing metastatic and angiogenetic potential (30,31). A recent report showed that VPA induced differentiation and apoptosis in ETO-positive leukemic cells (32), and now this drug has been tested in differentiation therapy of acute myeloid leukemia (33). VPA also exerted inhibitory effects on the migration and invasion of prostate cancer cells (34). In HNSCC, VPA has been shown to have acute and chronic growth inhibitory effects (35), and causes a 3- to 7-fold increase in cisplatin cytotoxicity (28).

The recent interest in CSC research has emerged from their expected role in the initiation and progression of cancer. Moreover, CSCs are thought to be responsible for resistance to current anticancer treatment and early recurrence in many cancers (36). According to the CSC hypothesis a different treatment strategy focusing mainly on CSCs is required. However, only few attempts have been made to target CSCs epigenetically. VPA, a known HDAC inhibitor, was found to decrease proliferation potential and multilineage differentiation capability of human mesenchymal stem cells (37). Therefore, we hypothesized that inhibition of HDACs by VPA could suppress CSC activity in HNSCC. In the present study, we demonstrated that VPA interfered with the self-renewal of HNSCC CSCs. Accordingly, VPA effectively suppressed the expression of stem cell markers, including Oct4, Sox2 and CD44. A combination of VPA and cisplatin reduced HNSCC CSC chemoresistance, likely by suppressing ABCC2 and ABCC6 expression and increasing caspase-mediated apoptosis. Furthermore, this combination treatment significantly inhibited the tumor growth and induced apoptosis in a xenograft model.

Several studies have shown that these antitumor and tumor cell differentiation effects of VPA are primarily mediated through inhibition of HDACs (20,38). Furthermore, inhibition of HDACs appears to interact with various other signaling pathways through complex molecular mechanisms. HDAC inhibition is linked to the modulation of phosphatidylinositol-3-kinase/Akt signaling (39). The Akt signaling pathway has been proved to interact with WNT/β-catenin signaling (40). HDAC inhibition was also found to be increased in inhibition-associated phosphorylation of GSK3β (41). Inactivation of the E-cadherin gene was also demonstrated to be triggered by DNA hypermethylation (42).

The present study further elucidated the VPA-induced antitumor effects in HNSCC CSCs. VPA in combination with cisplatin may disrupt the population of CSCs in HNSCC and thus be a potential curative strategy for the management of HNSCC.

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