Oral Oncology 88 (2019) 160–167

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

Down-regulation and nuclear localization of survivin by sodium butyrate induces caspase-dependent apoptosis in human oral mucoepidermoid carcinoma

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ARTICLE INFO

Keywords:
Oral mucoepidermoid carcinoma
Survivin
Sodium butyrate
Acetylation
Apoptosis
Bcl-xL

ABSTRACT

Objective: Sodium butyrate (NaBu) is a histone deacetylase inhibitor that possesses an apoptotic ability. However, the molecular mechanism by which NaBu induces apoptosis in human oral mucoepidermoid carcinoma (MEC), a type of salivary gland tumor, remains unclear.

Materials and methods: The anticancer effects of NaBu and its related molecular mechanisms were determined by trypan blue exclusion assay, 4′-6-diamidino-2-phenylindole staining, live/dead assay, human apoptosis array, RT-PCR, western blotting, immunocytochemistry, preparation of nuclear fractions, and nude mice tumor xenograft.

Results: In this study, we found that NaBu inhibited growth and induced apoptosis in the human oral MEC cell lines MC3 and YD15 with acetylation of histone proteins H2A and H3. NaBu apparently down-regulated survivin protein, as evidenced by the results of the human apoptosis antibody array, and modulated it at the post-translational process. Interestingly, NaBu caused nuclear translocation of survivin protein in both cell lines. NaBu also resulted in decreased expression levels of Bcl-xL mRNA and protein, leading to induction of caspase-dependent apoptosis in human oral MEC cell lines. In addition, NaBu administration inhibited tumor growth *in vivo* at a dosage of 500 mg/kg/day, but it did not cause any hepatic or renal toxicity.

Conclusion: This study provides new insights into the molecular mechanism of apoptotic actions by NaBu in human oral MEC and the basis of its clinical application for the treatment of human oral MEC.

Introduction

Mucoepidermoid carcinoma (MEC), a type of oral cancer, is the most common malignant salivary gland tumor, accounting for 30–35% of all salivary gland malignancies [1–3]. Surgery is the mainstay of treatment for human oral MEC, but it is often not easy to completely remove a tumor [4]. Thus, combination therapy with chemotherapy and X-ray radiation is essential for successful outcome after surgery [4]. To date, several chemotherapeutic drugs including cisplatin have been used in human MECs. However, their therapeutic effects are disappointing due to drug resistance or unwanted side effects.

Epigenetic modifications such as histone modification and DNA methylation are defined as any heritable changes in gene expression that occur without altering the DNA sequences. Histone modifications are covalent post-translational alterations to histone proteins; these modifications include acetylation, methylation, phosphorylation, ubiquitination, and sumoylation [5]. Among them, histone acetylation is regulated by the activity of histone deacetylases (HDACs) and histone acetyltransferases (HATs), which are observed in various degrees of expression in many malignant tumor tissues [6]. Histone acetylation is thought to affect various cellular processes including cell cycle, DNA repair, and apoptosis. Sodium butyrate (NaBu) is the sodium salt of

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https://doi.org/10.1016/j.oraloncology.2018.11.032
Received 14 August 2018; Received in revised form 20 November 2018; Accepted 29 November 2018
Available online 05 December 2018
1368-8375/

butyric acid and exerts various effects on mammalian cell lines including induction of differentiation, suppression of proliferation, and regulation of gene expression [7]. In particular, NaBu can induce histone acetylation in chromatin structure and function as an HDAC inhibitor [8]. It can also activate several anticancer mechanisms including cell cycle arrest, autophagy, and apoptosis [9–11], suggesting that NaBu is an anticancer drug candidate. However, the precise molecular mechanism for the anticancer effect of NaBu in human oral MECs has not yet been clearly elucidated.

In the present study, we attempted to examine the effect of NaBu and the most important molecular mechanism underlying its anticancer activity in human oral MEC cell lines and a nude mouse tumor xenograft model.

Materials and methods

Cell culture and chemical treatment

Human oral MEC cell lines MC3 and YD15 were originally provided by Prof. Wu Junzheng at the Fourth Military Medical University (Xi'an, China) and Prof. Jin Kim at Yonsei University (Seoul, Korea), respectively. MC3 and YD15 cell lines were grown in DMEM and RPMI 1640 (Welgene Inc., Dae-gu, Korea) supplemented with 10% fetal bovine serum and 100 U/mL each of penicillin and streptomycin in a humidified incubator containing 5% CO_2 at 37 °C. NaBu (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was dissolved in distilled water (DW) and the final concentration of DW did not exceed 10%. Z-LEHD-FMK and Z-VAD were purchased from R&D Systems (Minneapolis, MN, USA)

Histone isolation

To confirm the HDAC inhibiting activity of NaBu, acid-soluble proteins were extracted as follows. Briefly, isolated nuclei were mixed with 0.4 N sulfuric acid and incubated for 3 h on ice. After centrifugation at 15,000 rpm for 5 min at 4 °C, the dissolved histones were precipitated with 20% trichloroacetic acid overnight at −80 °C. The tubes were centrifuged and washed with acetone. The pellets were collected by centrifugation at 15,000 rpm for 5 min at 4 °C and then dissolved in DW.

Western blot analysis

Whole cell lysates were prepared with lysis buffer, and protein concentration was measured using a DC Protein Assay Kit (Bio-Rad Laboratories, Madison, WI, USA). After normalization, equal amounts of protein were subjected to SDS-PAGE and transferred to Immuno-Blot PVDF membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween 20 (TBST) at room temperature (RT) for 2 h and incubated with primary antibodies and corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies. Antibodies against acetyl-histone H3 and H2A (Lys5), cleaved caspase 3, cleaved caspase 9, cleaved poly (ADP-ribose) polymerase (PARP), survivin, Bcl-xL and Mcl-1 were purchased from Cell Signaling Technology, Inc. (Charlottesville, VA, USA). Lamin B and actin antibodies were obtained from Santa Cruz Biotechnology, Inc. Immunoreactive bands were visualized by Image Quant TM LAS 500 (GE Healthcare Life Sciences, Piscataway, NJ, USA).

Trypan blue exclusion assay

The trypan blue dye (Gibco, Paisley, UK) was used to determine cell viability. Cells were stained with 0.4% trypan blue and counted using a hemocytometer. Each experiment was carried out in triplicate and the results were expressed as the mean ± SD for each treatment group.

4′-6-Diamidino-2-phenylindole (DAPI) staining

To detect nuclear morphological changes of apoptotic cells, cells were stained with DAPI solution (Sigma-Aldrich, Louis, MO, USA). Briefly, detached cells were fixed in 100% methanol at RT for 10 min, deposited on slides, and stained with DAPI solution (2 μg/ml). The morphological changes of apoptotic cells were observed under a fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Live/dead assay

The cytotoxicity of NaBu was determined using the Live/Dead Viability/Cytotoxicity assay (Life Technologies, Grand Island, NY, USA). The polyanionic dye calcein-AM is retained within live cells, producing an intense green fluorescence through intracellular esterase activity. Ethidium homodimer-1 enters cells with damaged membranes and binds to nucleic acids, producing a bright red fluorescence in dead cells. Briefly, cells were stained with 2 μM calcein-AM and 4 μM ethidium homodimer-1 and then incubated for 30 min at RT. Cells were analyzed under fluorescence microscopy with the appropriate excitation and emission filters.

Human apoptosis array

MC3 and YD15 cells treated with DW or designated concentrations of NaBu for 24 h were harvested for a protein chip assay using the Human Apoptosis Array Kit (R&D Systems, Minneapolis, MN, USA). Briefly, membranes were blocked with an array buffer for 1 h at RT. After washing with 1X washing buffer, membranes were incubated with a detection antibody cocktail for 1hr at RT. The membranes were then washed and incubated with streptavidin–HRP for 30 min at RT. Each capture spot corresponding to the amount of apoptotic protein bound was detected with enhanced chemiluminescence western blotting luminol reagent (Santa Cruz Biotechnology, Inc.)

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted with the easy-BLUE Total RNA Extraction Kit (INTRON, Daejeon, Korea) according to the manufacturer's instructions. One microgram of RNA was reverse transcribed by TOPscript RT DryMIX (Elpis Biotech, Daejeon, Korea), and the resultant cDNA was subjected to PCR using HiPi PCR PreMix (Elpis Biotech, Daejeon, Korea). The PCR conditions for survivin, Bcl-xL, Mcl-1, and β-actin were as follows: 27 cycles of 1 min at 95 °C, 1 min at 60 °C, and 1 min 30 sec at 72 °C; and for Bcl-2 was as follows: (27 cycles of 1 min at 95 °C, 1 min at 55 °C, and 1 min 30 sec at 72 °C. The following primer sequences were used: survivin sense 5′-ATG GCC GAG GCT GGC TTC ATC-3′, survivin anti-sense 5′-ACG GCG CAC TTT CTT CGC AGT T-3′, Bcl-xL sense 5′-AAG CTT TCC CAG AAA GGA TAC A-3′, Bcl-xL anti-sense 5′-TGC TGC ATT GTT CCC ATA GA-3′, Mcl-1 sense 5′-TGC TGG AGT TGG TCG GGG AA-3′, Mcl-1 anti-sense 5′-TCG TAA GGT CTC CAG CGC CT-3′, β-actin sense 5′-GTG GGG CGC CCC AGG CAC CA-3′, β-actin anti-sense 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′. The intensities of the bands were normalized to that of β-actin. The amplified products were analyzed by 1.2% agarose gel electrophoresis and stained with ethidium bromide.

Immunocytochemistry

MC3 and YD15 cells were seeded on 4-well culture plates and treated with DW or NaBu for 18 h. Cells were then fixed and permeabilized using the Cytofix/Cytoperm solution (BD Biosciences, San Jose,

CA, USA) for 1 h at 4 °C. Cells were then blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at RT and incubated overnight at 4 °C with survivin antibody. Subsequently, the cells were exposed to the FITC-conjugated secondary antibodies for 1 h at RT and were visualized using a fluorescence microscope equipped with the appropriate filters for DAPI and FITC dyes.

Preparation of nuclear fractions

Cell pellets were suspended in hypotonic buffer (10 mM N-2-hydroxyethylpiperazine-N′-2′-ethanesulfonic acid, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM NaF, 0.2 mM Na_3VO_4, 0.4 mM toluenesulfonyl fluoride, 1 mM dithiothreitol, leupeptin, aprotinin, and 1.5% Nonidet P-40) for 15 min on ice. After centrifugation at 13,000 rpm at 4 °C for 5 min, the supernatant was removed. The pellets were re-suspended in high salt extraction buffer (50 mM N-2-hydroxyethylpiperazine N′-2′-ethanesulfonic acid, 50 mM KCl, 300 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, and 1 mM dithiothreitol) for 30 min on ice and then washed with a hypotonic buffer. The supernatant containing nuclear proteins for western blotting was centrifuged at 13,000 rpm for 30 min at 4 °C to isolate these protein.

Nude mice tumor xenograft

Six-week-old BALB/c nu/nu male mice were purchased from NARA Biotech (Pyeongtaek, Korea). All mice were handled according to Institutional Animal Care and Use Committee (IACUC) guidelines approved by CHA University (IACUC approval number: 180067). MC3 cells were subcutaneously injected into the flanks of the mice and the mice were then assigned randomly to one of two treatment groups (n = 4 each group). Approximately 10 days after incubation (day 0), vehicle control (PBS) or 500 mg/kg/day of NaBu (i.p.) were administrated to tumor bearing mice five times per week for 21 days. Tumor volume and body weight were measured twice a week. After 21 days, tumor weight was measured. The tumor volumes were measured along the two diameter axes with calipers to allow calculation of tumor volume using the following formula: $V = \pi/6\{(D + d)/2\}^3$, where D and d were the larger and smaller diameters, respectively.

Histopathological examination of organs

Mice organs (liver and kidney) were fixed in 10% neutral buffered formalin. Paraffin-embedded tissue sections were cut at a thickness of 4 μm on a microtome (Leica RM2255, Germany). Slides were dried for 30 min at 42 °C on a slide warmer and stained with hematoxylin and eosin solution. Histopathological changes were analyzed using a Nikon Eclipse E800 microscope.

Statistical analysis

For two experimental comparisons, a two-tailed *Student's t-test* was used and for multiple comparisons, one-way ANOVA was applied to determine the significance of differences between the control and treatment groups. For the *in vivo* study, statistical evaluation was calculated with a Mann-Whitney *U* test in SPSS version 23.0 (SPSS Inc. Chicago, IL, USA) due to the use of non-parametric data. A *p*-value of < 0.05 was considered statistically significant.

Results

NaBu increases histone acetylation and induces apoptosis in MC3 and YD15 cell lines

To confirm the HDAC inhibiting activity of NaBu, we first performed western blot analysis with histone proteins isolated from MC3 and YD15 human MEC cell lines exposed to NaBu for 24 h. NaBu treatment led to a dramatic increase in the expression levels of acetyl histones H3 and H2A in a concentration-dependent manner (Fig. 1A). We next examined the growth-inhibitory effects of NaBu in MC3 and YD15 cell lines using a trypan blue exclusion assay. The results showed that NaBu significantly decreased the number of viable cells (Fig. 1B). To investigate whether NaBu induces apoptosis in both cell lines, we carried out western blot analysis using an antibody against cleaved PARP, DAPI staining, and Live/Dead assay. NaBu clearly increased the expression level of cleaved PARP, and the number of cells with nuclear condensation and fragmentation, and red fluorescence-positive cells in both cell lines compared to the DW-treated control group (Fig. 1C–E). These findings suggest that NaBu inhibition of HDAC may be involved in its anti-proliferative and apoptotic activities in human oral MEC cell lines.

Survivin is involved in NaBu-induced apoptosis in MC3 and YD15 cell lines

Next, to determine what molecule plays a role in NaBu-induced apoptosis, the expression profiles of apoptosis-related proteins between the NaBu-treated group (25 mM for MC3 cells or 50 mM for YD15 cells) and the DW-treated control group were analyzed using a Human Apoptosis Array. As shown in Fig. 2A and B, the human apoptosis array images and graphs derived from two independent experiments showed that NaBu reduced the expression of survivin protein and increased cleaved caspase 3 in both cell lines. The human apoptosis array data were verified by western blot analysis, and NaBu significantly decreased the expression of survivin protein (Fig. 2C). Additionally, the effects of NaBu on survivin protein expression were further confirmed by immunofluorescence staining. Interestingly, survivin protein was significantly transferred from the cytoplasm to the nucleus in NaBu-treated groups of both cell lines (Fig. 3A and B) and western blotting using the nuclear and cytosolic protein fractions verified that treatment with NaBu clearly increased the expression of nuclear survivin protein (Fig. 3C). We then performed RT-PCR to confirm whether NaBu regulates the survivin protein at the transcriptional level. However, the survivin mRNA level did not change (Fig. 2D). Furthermore, a protein turnover assay using cycloheximide (CHX) showed that survivin protein was significantly diminished in the presence of CHX with NaBu compared with a CHX-only treatment group (Supplementary Fig. 1), meaning that NaBu regulates the survivin protein at a post-transcriptional level in MC3 and YD15 cell lines. Collectively, these results suggest that survivin plays an important role in NaBu-induced apoptosis in human MEC cell lines.

Nuclear survivin protein may inhibit the transactivation of STAT3 to induce caspase-dependent apoptosis

According to the previous studies, nuclear survivin/STAT3 binding complex inhibits the transcriptional activity of STAT3 in cancer cells, resulting in the inhibition of downstream molecules of STAT3 signaling [12,13]. To investigate whether NaBu affects the transcriptional activity of STAT3 in MC3 and YD15 cell lines, we first analyzed the mRNA and protein expression levels of Bcl-xL and Mcl-1, which are known to be downstream targets of STAT3 and are associated with apoptosis using both RT-PCR and western blot analysis. NaBu significantly reduced the expressions of Bcl-xL mRNA and protein in both cell lines while not altering those of Mcl-1 mRNA or protein (Fig. 4A and B). Because NaBu regulates Bcl-xL, which may cause the damage to the mitochondrial membrane potential, we next confirmed the cleaved forms of caspase 9 and caspase 3, which are related proteins, using western blotting. Both NaBu-treated cell lines exhibited a concentration-dependent increase in the cleavages of caspase 9 and caspase 3

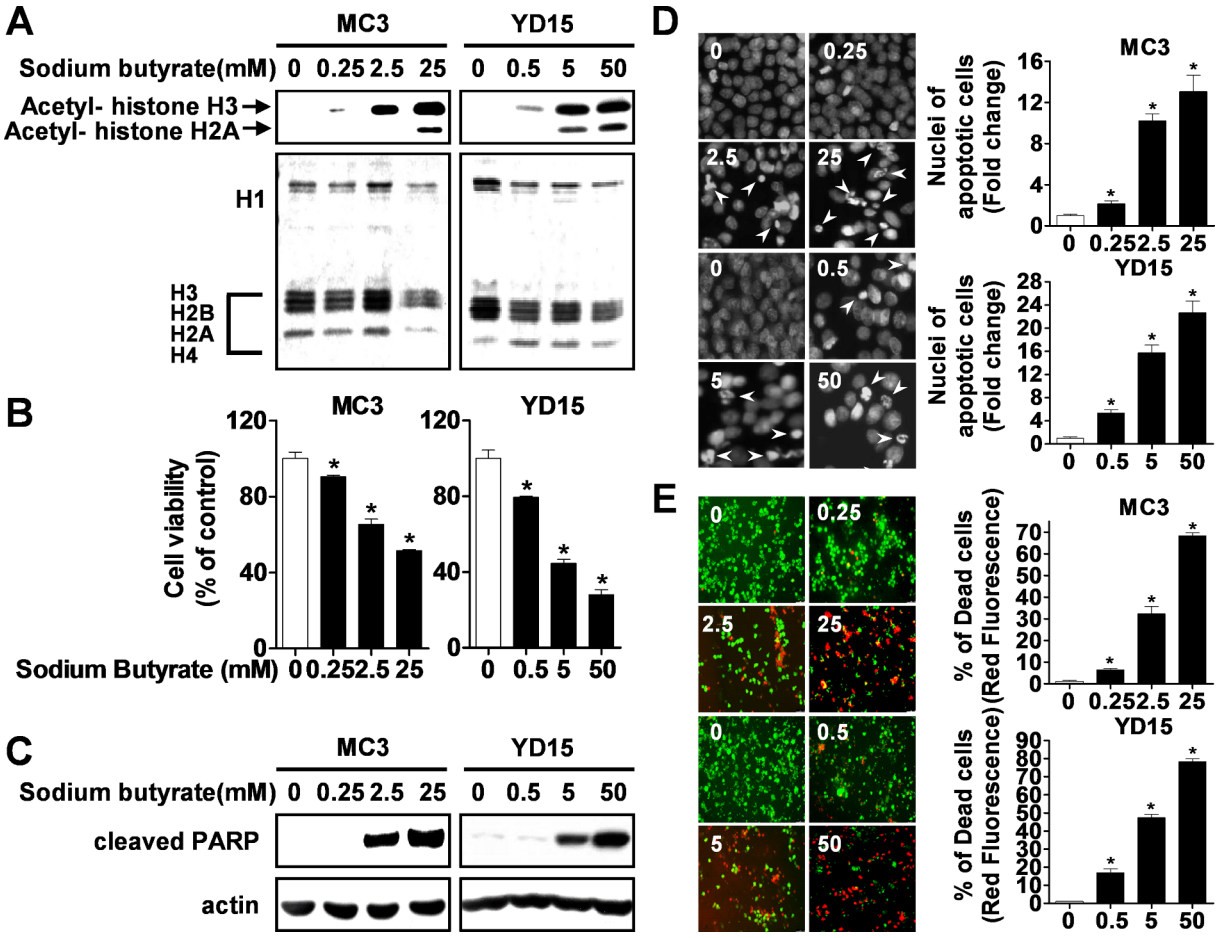

Fig. 1. Effects of NaBu on histone acetylation and apoptosis of MC3 and YD15 cells (A) MC3 (left) and YD15 (right) cells were treated with DW or the indicated concentrations of NaBu for 24 h. Histones were extracted using acid extraction and analyzed to detect acetyl-histones by western blot analysis (Top panel). Equal amounts of proteins were measured by Coomassie Blue staining (Bottom panel). (B) The effects of NaBu on cell viability were analyzed by a trypan blue exclusion assay. (C) The apoptotic effects of NaBu were determined by western blot analysis with an indicated antibody. Actin was used as an internal control. (D) Nuclear condensation and DNA fragmentation were detected using DAPI solution as mentioned in the Materials and Methods. DAPI-stained MC3 and YD15 cells were observed by fluorescence microscopy (Magnification, ×400). (E) Live (green) and dead (red) cells were determined with the Live/Dead assay kit as mentioned in the Materials and Methods (magnification, ×200). The graph represents mean ± SD of three independent experiments and significance ($p < 0.05$) compared with the DW-treated control group is indicated (*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 4C). To confirm the involvement of those two proteins in NaBu-induced apoptosis, MC3 and YD15 cells were pretreated with Z-LEHD-FMK (a caspase 9 inhibitor) or Z-VAD-FMK (a pan-caspase inhibitor) for 1 h and then treated with NaBu for 24 h. As shown in Fig. 4D and E, the cleavage of PARP induced by NaBu was partly reduced in the presence of either Z-LEHD-FMK or Z-VAD-FMK. Together, these data indicate that NaBu induces caspase-dependent apoptosis by inhibiting the transcriptional activity of STAT3 protein in human MEC cell lines.

NaBu inhibits MC3 subcutaneous tumor growth in vivo

Based on the *in vitro* findings, we next investigated the *in vivo* anticancer effects of NaBu using a nude mouse xenograft model bearing MC3 cells. Tumor volume was significantly reduced from the 9th day of NaBu treatment (Fig. 5A and B). Tumor weights of the NaBu-treated group were significantly reduced compared to those of the PBS-treated group (Fig. 5C). However, body weights and absolute and relative organ (liver and kidney) weights of the mice were not significantly altered (Fig. 5D–F). In addition, there was no significant difference in histopathological findings between the PBS- and NaBu-treated groups (Fig. 5G). Collectively, these results suggest that NaBu treatment attenuates tumor growth without hepatic or renal toxicity *in vivo*.

Discussion

Inhibitors of histone deacetylases (HDACs) have been developed as a promising class of anticancer drugs in several human malignancies including oral cancer, since HDAC family members play crucial roles in tumor development [14,15]. Treatment with FR901228 (a HDAC inhibitor) stimulated the expression of maspin mRNA, which is associated with inhibition of tumor angiogenesis and cell invasion in oral cancer cell lines [16]. Suberoylanilide hydroxamic acid (SAHA) also suppressed oral squamous cell carcinoma (OSCC) growth through G1 phase cell cycle arrest [17]. Recently, our research group found that several HDAC inhibitors such as SAHA and A248, induced apoptosis through a variety of signaling pathways in human OSCC cell lines [18,19]. NaBu also induced growth inhibition and apoptosis in several cancer cell lines, including prostate and colorectal cancer [20,21]. Although some studies have shown that NaBu has anticancer efficacy, the antitumor activity of NaBu in salivary gland cancer has not yet been elucidated. Here, we show that NaBu decreased cell viability and induced apoptosis in human MEC cells *in vitro* (Fig. 1B–D). To the best of our knowledge,

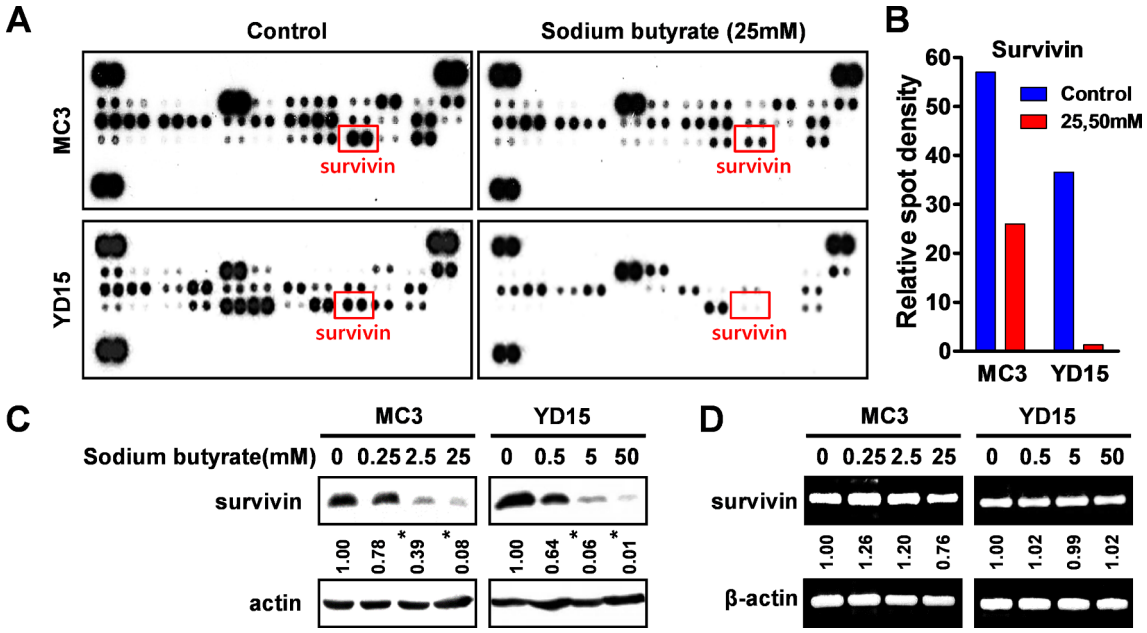

Fig. 2. Involvement of survivin in NaBu-induced apoptosis in MC3 and YD15 cells (A) MC3 and YD15 cells were treated with DW or the indicated concentrations of NaBu for 24 h and then analyzed by human apoptosis array as described in the Materials and Methods. (B) The graph represents the mean of two independent experiments. The effects of NaBu on the expression level of survivin protein (C) or mRNA (D) were determined by western blot analysis and RT-PCR, respectively. Values represent the mean of three independent experiments, and significance ($p < 0.05$) compared with the DW-treated control group is indicated (*).

this is the first study to show the apoptotic effects of NaBu against MEC cell lines. Other researchers have reported that apicidin significantly induces apoptosis through the inactivation of extracellular signal-regulated kinase and AKT/mTOR signaling [22], and histone deacetylase 7 silencing also induces apoptosis in salivary MEC cell lines [23]. Taken together, these findings suggest that HDAC inhibitors can induce apoptosis in oral MEC.

In this work, we applied human apoptosis arrays to analyze expression patterns of 35 proteins associated with apoptosis in human MEC cell lines. We showed that survivin is associated with NaBu-induced apoptosis and confirmed it by western blotting (Fig. 2). Tanaka et al. (2003) showed that survivin protein was positive in 58% of oral

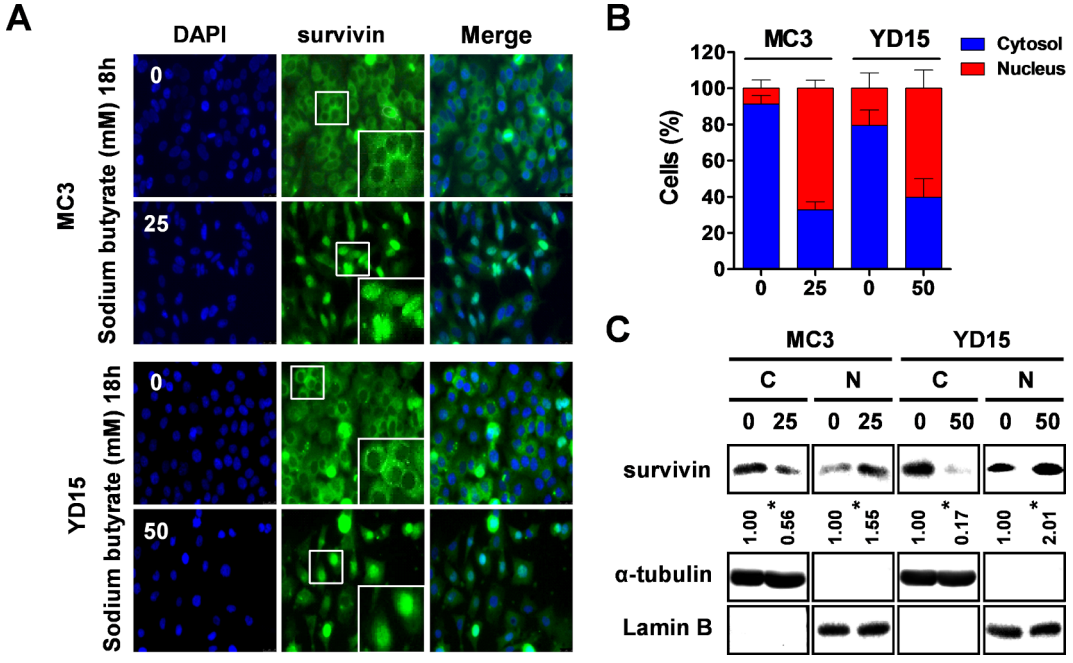

Fig. 3. Effect of NaBu on nuclear localization of survivin protein in MC3 and YD15 cells (A) Both cell lines were treated with DW or the indicated concentrations of NaBu for 18 hr and immuno-stained with survivin antibody. (magnification ×400) (B) Numbers of stained cells in three random fields were counted depending on whether the nucleus staining exceeded staining in the cytoplasm. The relative number of the cells in nucleus or cytoplasm per total number of the cells were calculated and showed as a percentage. The graph represents mean ± SD of three independent experiments. (C) The expression levels of nuclear and cytosolic survivin proteins were detected using western blot analysis. Lamin B or actin were used to normalize the nuclear or cytosolic protein level, respectively. Values represent the mean of three independent experiments and significance ($p < 0.05$) compared with the DW-treated control group is indicated (*).

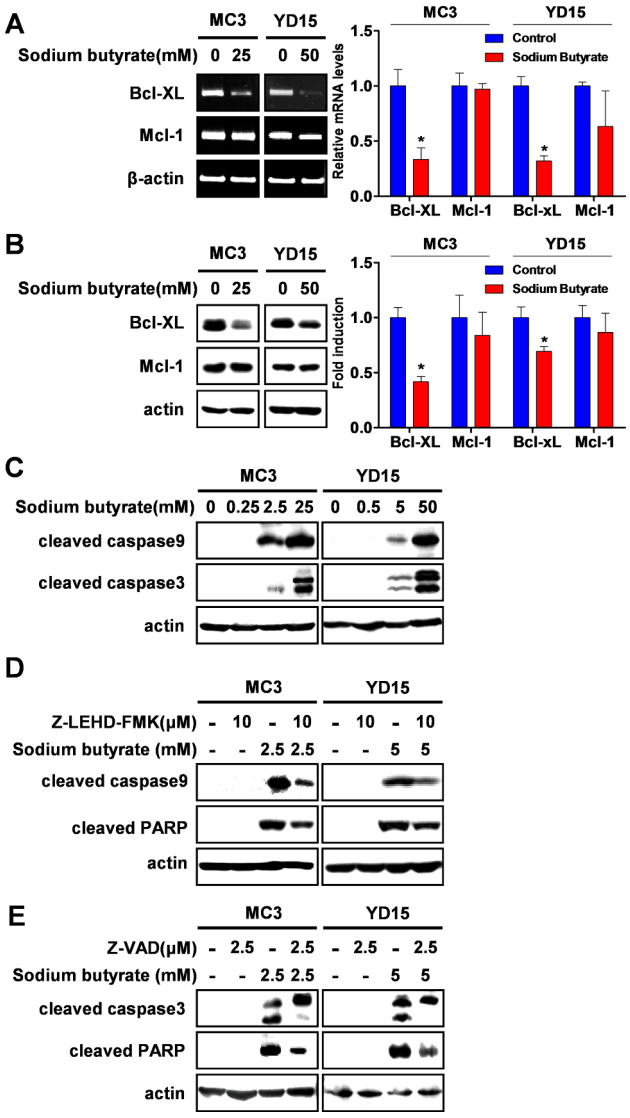

Fig. 4. Effects of NaBu on Bcl-xL and caspase-dependent apoptosis in MC3 and YD15 cells (A) The effects of NaBu on Bcl-xL and Mcl-1 mRNA expression levels were determined by RT-PCR. (B) The effects of NaBu on Bcl-xL and Mcl-1 protein expressions were determined by western blot analysis. The graph represents mean ± SD of three independent experiments and significance ($p < 0.05$) compared with the DW-treated control group is indicated (*). (C) MC3 and YD15 cells were treated with DW or the indicated concentrations of NaBu for 24 hr and cleaved caspase 9 and caspase 3 were determined by western blot analysis. MC3 and YD15 cells were treated with NaBu for 24 hr after 1 hr pretreatment of Z-LEHD (a caspase 9 inhibitor) (D) or Z-VAD (a pan-caspase inhibitor) (E) followed by western blot analysis to determine the involvement of caspases in NaBu-mediated apoptosis. The data represent two independent experiments.

cancer and 37% of pre-malignant lesions, while no immunoreaction was observed in normal oral mucosa [24], suggesting that survivin has a key role during oral carcinogenesis. Our previous studies recently demonstrated that the reduction of survivin by YM155 or naturally occurring substance induced apoptosis in oral cancer [25,26]. Several studies have also reported inhibition of survivin expression by HDAC inhibitors during apoptosis in a variety of cancer cell lines, similar to our current data [27,28]. These findings imply that survivin is a main molecular target involved in HDAC inhibitor-induced apoptosis in oral MEC.

Survivin is known to have distinct functions depending on its cellular location. Cytoplasmic survivin may block caspase-dependent apoptosis as a cytoprotective molecule, and nuclear survivin may indicate impaired survivin function [29]. Several studies indicated that nuclear survivin in cancer cells is a favorable prognostic factor for predicting disease outcomes [30,31]. On the other hand, nuclear localization of survivin may be a negative prognostic factor for survival in several cancers [32,33]. In addition, cytoplasmic survivin expression was significantly correlated with survival as an independent predictor of poor prognosis in patients with salivary gland tumor [34]. Thus, the biological functions of the distinct cellular localization of survivin in cancer are still controversial. Here, we found that NaBu caused survivin protein to transfer into the nucleus from the cytosol (Fig. 3). Previously, Wang et al. (2010) reported that HAT can acetylate survivin in breast cancer cells and acetylation of lysine 129 of survivin may facilitate its homo-dimerization and stability in the nucleus [12]. Recently, it was found that acetylation at lysine 129 of survivin is involved in induction of nuclear translocation of survivin in OSCC cells, while none of the protein kinase inhibitors including STAT3 inhibitor affected the subcellular location of survivin [35]. Thus, we confirmed that NaBu induces survivin acetylation in human MEC cell lines, using an antibody against acetylated lysine. Although NaBu reduced the total expression level of survivin protein, it significantly increased the expression of acetylated lysine on the same location as survivin protein (Supplementary Fig. 2). In addition, we performed immunoprecipitation and acetylated lysine in survivin protein was slightly increased by NaBu (Supplementary Fig. 3). These results suggest that nuclear translocation of survivin protein by NaBu may be due to its acetylation. Other studies have also shown that HDAC6 deacetylated survivin, which controlled its nuclear export, providing a novel target for patients with ER-positive breast cancer; and that SAHA promoted the acetylation of survivin and its nuclear accumulation [36,37]. These results suggest that an HDAC inhibitor may acetylate survivin to promote its nuclear localization in human salivary gland cancer.

Recently, the acetylation of survivin protein has been shown to promote its nuclear accumulation, resulting in inhibition of STAT3 transactivation, providing a good strategy for therapeutic intervention in tumors overexpressing STAT3 [12]. NaBu was shown to suppress the transcriptional activity of Bcl-XL, which is one of the STAT3 downstream molecules (Fig. 4A). This finding confirms the role of nuclear survivin protein in repressing STAT3 oncogenic activity during NaBu-induced apoptosis in human MEC cell lines. In addition, our results showed that caspases were involved in NaBu-induced apoptosis (Fig. 4C–E). Cytoplasmic survivin directly blocks caspase cascades and inhibits apoptosis by binding its BIR1 domain to caspases 3 and 9. Therefore, our findings suggest that NaBu can activate caspase cascades because it regulates the abundance of survivin by regulating its half-life period.

The immune system plays an important role in head and neck cancer (HNC) development and evolution. It is observed that immunosuppressive individuals are more likely to develop HNC and its prognosis is relatively poor [38]. Immune destruction such as the repressive activity of T-regulatory cells on induction and proliferation of T cells have been frequently found in HNC patients [39,40]. Thus, immunotherapy is a promising field in HNC including salivary gland tumor. Vorinostat, a HDAC inhibitor, particularly upregulated PD-1 or PD-L1 expression for immunotherapy and is currently under investigation in a clinical trial (NCT02538510) in metastatic cases of advanced salivary gland cancers [41]. Therefore, future research on the role of NaBu on the regulation of immune checkpoints should be undertaken.

In summary, these observations document the apoptotic activity of NaBu in human oral MEC. We suggest that NaBu reduces the expression of survivin protein and induces its nuclear transfer for NaBu-induced apoptosis. From this work, we conclude that NaBu and its derivatives are promising anticancer drug candidates for the treatment of human oral MEC.

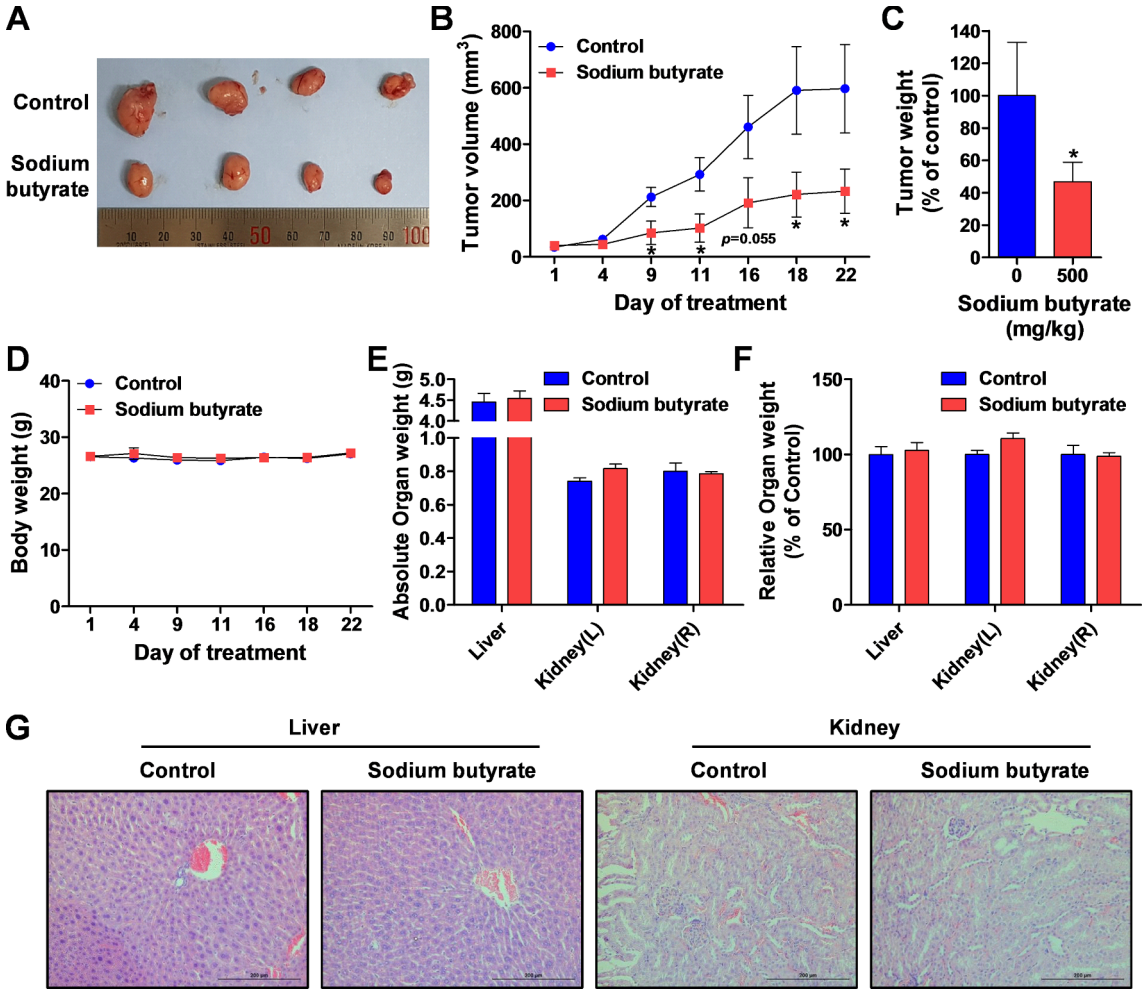

Fig. 5. *In vivo* anti-tumor effects of NaBu on nude mouse xenograft models bearing MC3 cells (A) Nude mice were treated with PBS or NaBu (500 mg/kg/day) for 21 days. The images of tumors are shown. Tumor volume (B), tumor weight (C), and body weight (D) were measured. The graph represents mean ± SD and significance ($p < 0.05$) compared with the vehicle control-treated group is indicated (*). Absolute (E) and relative (F) liver and kidney weights were measured. (G) Histopathological images of liver and kidney tissues (magnification, ×200).

Funding sources

This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science ICT & Future Planning [2017R1D1A1-B03029124 and 2017R1A2B2003491].

Conflict of interest

No potential conflicts of interest are declared.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.oraloncology.2018.11.032.

References

[1] Coca-Pelaz A, Rodrigo JP, Triantafyllou A, Hunt JL, Rinaldo A, Strojan P, et al. Salivary mucoepidermoid carcinoma revisited. Eur Arch Otorhinolaryngol 2015;272:799–819.
[2] Luna MA. Salivary mucoepidermoid carcinoma: revisited. Adv Anat Pathol 2006;13:293–307.
[3] Speight PM, Barrett AW. Salivary gland tumours. Oral Dis 2002;8:229–40.
[4] Andry G, Hamoir M, Locati LD, Licitra L, Langendijk JA. Management of salivary gland tumors. Expert Rev Anticancer Ther 2012;12:1161–8.
[5] Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev Genet 2007;8:286–98.
[6] Chrun ES, Modolo F, Daniel FI. Histone modifications: a review about the presence of this epigenetic phenomenon in carcinogenesis. Pathol Res Pract 2017;213:1329–39.
[7] Kruh J. Effects of sodium butyrate, a new pharmacological agent, on cells in culture. Mol Cell Biochem 1982;42:65–82.
[8] Davie JR. Inhibition of histone deacetylase activity by butyrate. J Nutr 2003;133:2485S–93S.
[9] Shin H, Lee YS, Lee YC. Sodium butyrate-induced DAPK-mediated apoptosis in human gastric cancer cells. Oncol Rep 2012;27:1111–5.
[10] Wang HG, Huang XD, Shen P, Li LR, Xue HT, Ji GZ. Anticancer effects of sodium butyrate on hepatocellular carcinoma cells in vitro. Int J Mol Med 2013;31:967–74.
[11] Verma SP, Agarwal A, Das P. Sodium butyrate induces cell death by autophagy and reactivates a tumor suppressor gene DIRAS1 in renal cell carcinoma cell line UOK146. Vitro Cell Dev Biol Anim 2018;54:295–303.
[12] Wang H, Holloway MP, Ma L, Cooper ZA, Riolo M, Samkari A, et al. Acetylation directs survivin nuclear localization to repress STAT3 oncogenic activity. J Biol Chem 2010;285:36129–37.
[13] Sen N, Che X, Rajamani J, Zerboni L, Sung P, Ptacek J, et al. Signal transducer and activator of transcription 3 (STAT3) and survivin induction by varicella-zoster virus promote replication and skin pathogenesis. Proc Natl Acad Sci USA 2012;109:600–5.
[14] Konsoula Z, Velena A, Lee R, Dritschilo A, Jung M. Histone deacetylase inhibitor: antineoplastic agent and radiation modulator. Adv Exp Med Biol 2011;720:171–9.
[15] Tasoulas J, Giaginis C, Patsouris E, Manolis E, Theocharis S. Histone deacetylase inhibitors in oral squamous cell carcinoma treatment. Expert Opin Investig Drugs 2015;24:69–78.
[16] Murakami J, Asaumi J, Maki Y, Tsujigiwa H, Kuroda M, Nagai N, et al. Effects of demethylating agent 5-aza-2(')-deoxycytidine and histone deacetylase inhibitor FR901228 on maspin gene expression in oral cancer cell lines. Oral Oncol 2004;40:597–603.
[17] Nagumo T, Takaoka S, Yoshiba S, Ohashi M, Shirota T, Hatori M, et al. Antitumor

activity of suberoylanilide hydroxamic acid against human oral squamous cell carcinoma cell lines in vitro and in vivo. Oral Oncol 2009;45:766–70.
[18] Shin JA, Han G, Park SK, Lee K, Kim HJ, Cho SD, et al. The in vitro apoptotic effects of A248 and A1659, newly synthetic histone deacetylase inhibitors in oral cancer cells. Oral Dis 2014;20:482–9.
[19] Jang B, Shin JA, Kim YS, Kim JY, Yi HK, Park IS, et al. Growth-suppressive effect of suberoylanilide hydroxamic acid (SAHA) on human oral cancer cells. Cell Oncol (Dordr) 2016;39:79–87.
[20] Mu D, Gao Z, Guo H, Zhou G, Sun B. Sodium butyrate induces growth inhibition and apoptosis in human prostate cancer DU145 cells by up-regulation of the expression of annexin A1. PLoS ONE 2013;8:e74922.
[21] Zhang J, Yi M, Zha L, Chen S, Li Z, Li C, et al. Sodium butyrate induces endoplasmic reticulum stress and autophagy in colorectal cells: implications for apoptosis. PLoS ONE 2016;11:e0147218.
[22] Ahn MY, Ahn JW, Kim HS, Lee J, Yoon JH. Apicidin inhibits cell growth by downregulating IGF-1R in salivary mucoepidermoid carcinoma cells. Oncol Rep 2015;33:1899–907.
[23] Ahn MY, Yoon JH. Histone deacetylase 7 silencing induces apoptosis and autophagy in salivary mucoepidermoid carcinoma cells. J Oral Pathol Med 2017;46:276–83.
[24] Tanaka C, Uzawa K, Shibahara T, Yokoe H, Noma H, Tanzawa H. Expression of an inhibitor of apoptosis, survivin, in oral carcinogenesis. J Dent Res 2003;82:607–11.
[25] Sachita K, Yu HJ, Yun JW, Lee JS, Cho SD. YM155 induces apoptosis through downregulation of specificity protein 1 and myeloid cell leukemia-1 in human oral cancer cell lines. J Oral Pathol Med 2015;44:785–91.
[26] Choi ES, Cho SD, Jeon JG, Cho NP. The apoptotic effect of the hexane extract of *rheum undulatum* L. in oral cancer cells through the down-regulation of specificity protein 1 and survivin. Lab Anim Res 2011;27:19–24.
[27] Chowdhury S, Howell GM, Teggart CA, Chowdhury A, Person JJ, Bowers DM, et al. Histone deacetylase inhibitor belinostat represses survivin expression through re-activation of transforming growth factor beta (TGFbeta) receptor II leading to cancer cell death. J Biol Chem 2011;286:30937–48.
[28] Yamada T, Horinaka M, Shinnoh M, Yoshioka T, Miki T, Sakai T. A novel HDAC inhibitor OBP-801 and a PI3K inhibitor LY294002 synergistically induce apoptosis via the suppression of survivin and XIAP in renal cell carcinoma. Int J Oncol 2013;43:1080–6.
[29] Stauber RH, Mann W, Knauer SK. Nuclear and cytoplasmic survivin: molecular mechanism, prognostic, and therapeutic potential. Cancer Res 2007;67:5999–6002.
[30] Trieb K, Lehner R, Stulnig T, Sulzbacher I, Shroyer KR. Survivin expression in human osteosarcoma is a marker for survival. Eur J Surg Oncol 2003;29:379–82.
[31] Altura RA, Olshefski RS, Jiang Y, Boue DR. Nuclear expression of Survivin in paediatric ependymomas and choroid plexus tumours correlates with morphologic tumour grade. Br J Cancer 2003;89:1743–9.
[32] Kitamura H, Torigoe T, Hirohashi Y, Asanuma H, Inoue R, Nishida S, et al. Nuclear, but not cytoplasmic, localization of survivin as a negative prognostic factor for survival in upper urinary tract urothelial carcinoma. Virchows Arch 2013;462:101–7.
[33] Qian X, Xi X, Li L. Nuclear survivin is associated with malignant potential in epithelial ovarian carcinoma. Appl Immunohistochem Mol Morphol 2011;19:126–32.
[34] Stenner M, Weinell A, Ponert T, Hardt A, Hahn M, Preuss SF, et al. Cytoplasmic expression of survivin is an independent predictor of poor prognosis in patients with salivary gland cancer. Histopathology 2010;57:699–706.
[35] Liu S, Shi L, Yang X, Ye D, Wang T, Dong C, et al. Nuclear survivin promoted by acetylation is associated with the aggressive phenotype of oral squamous cell carcinoma. Cell Cycle 2017;16:894–902.
[36] Riolo MT, Cooper ZA, Holloway MP, Cheng Y, Bianchi C, Yakirevich E, et al. Histone deacetylase 6 (HDAC6) deacetylates survivin for its nuclear export in breast cancer. J Biol Chem 2012;287:10885–93.
[37] Lee JY, Kuo CW, Tsai SL, Cheng SM, Chen SH, Chan HH, et al. Inhibition of HDAC3- and HDAC6-promoted survivin expression plays an important role in SAHA-induced autophagy and viability reduction in breast cancer cells. Front Pharmacol 2016;7:81.
[38] Schoenfeld JD. Immunity in head and neck cancer. Cancer Immunol Res 2015;3:12–7.
[39] Reichert TE, Strauss L, Wagner EM, Gooding W, Whiteside TL. Signaling abnormalities, apoptosis, and reduced proliferation of circulating and tumor-infiltrating lymphocytes in patients with oral carcinoma. Clin Cancer Res 2002;8:3137–45.
[40] Hoffmann TK, Dworacki G, Tsukihiro T, Meidenbauer N, Gooding W, Johnson JT, et al. Spontaneous apoptosis of circulating T lymphocytes in patients with head and neck cancer and its clinical importance. Clin Cancer Res 2002;8:2553–62.
[41] Alfieri S, Granata R, Bergamini C, Resteghini C, Bossi P, Licitra LF, et al. Systemic therapy in metastatic salivary gland carcinomas: a pathology-driven paradigm? Oral Oncol 2017;66:58–63.