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ORIGINAL ARTICLE

Taurine exhibits an apoptosis-inducing effect on human nasopharyngeal carcinoma cells through PTEN/Akt pathways in vitro

Feng He[1,2,4] · Ning Ma[3] · Kaoru Midorikawa[1] · Yusuke Hiraku[1] · Shinji Oikawa[1] · Zhe Zhang[4] · Guangwu Huang[4] · Kazuhiko Takeuchi[2] · Mariko Murata[1]

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Abstract

Nasopharyngeal carcinoma (NPC) is a distinctive type of head and neck malignancy with a high incidence in southern China. Previous studies have confirmed that taurine shows an anti-cancer effect on a variety of human tumors by inhibiting cell proliferation and inducing apoptosis. However, the underlying molecular mechanism of its anti-cancer effect on NPC is not well understood. To clarify these anti-cancer mechanisms, we performed cell viability and colony formation assays. Apoptotic cells were quantified by flow cytometry. The expression levels of apoptosis-related proteins were evaluated by Western blot. The results showed that taurine markedly inhibited cell proliferation in NPC cells, but only slightly in an immortalized normal nasopharyngeal cell line. Taurine suppressed colony formation and induced apoptosis of NPC cell lines in a dose-dependent manner. Furthermore, taurine increased the active form of caspase-9/3 in a dose-dependent manner. Taurine down-regulated the anti-apoptotic protein Bcl-xL and up-regulated the pro-apoptotic protein Bax and GRP78, a major endoplasmic reticulum (ER) chaperone. These results suggest the involvement of mitochondrial and ER stress signaling in apoptosis. In addition, taurine increased the levels of PTEN (phosphatase and tensin homolog deleted on chromosome 10) and p53, and reduced phosphorylated Akt (protein kinase B). In conclusion, taurine may inhibit cell proliferation and induce apoptosis in NPC through PTEN activation with concomitant Akt inactivation.

Keywords Taurine · NPC · Apoptosis · PTEN · Akt

Introduction

NPC is a distinctive type of head and neck malignancy. Although the incidence of NPC in Western countries is less than 1 per 100,000 (McDermott et al. 2001), the incidence rises to 15–50 cases per 100,000 in the southern regions of China (Yao et al. 2017). Epstein–Barr virus (EBV), environmental carcinogens, ethnic background, and dietary components play important roles in the development of NPC (Xu et al. 2013; Wu et al. 2018). There is an urgent need to identify novel preventive and therapeutic strategies.

Taurine (2-aminoethanesulfonic acid) is a natural amino acid that is expressed widely in all mammalian tissues. It is crucial for proper function of the central nervous system (Kilb and Fukuda 2017), retinal neurons (Gaucher et al. 2012), and cardiac and skeletal muscle (Khalil et al. 2017; Schaffer et al. 2010). Several studies have demonstrated that taurine has anti-inflammatory (Marcinkiewicz and Kontny 2014), antioxidant (Shimada et al. 2015), and hypoglycemic (Pandya et al. 2017) effects. Taurine also possesses

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✉ Kazuhiko Takeuchi
kazuhiko@clin.medic.mie-u.ac.jp

✉ Mariko Murata
mmurata@doc.medic.mie-u.ac.jp

[1] Department of Environmental and Molecular Medicine, Mie University Graduate School of Medicine, Tsu, Japan

[2] Department of Otolaryngology Head and Neck Surgery, Mie University Graduate School of Medicine, Tsu, Japan

[3] Graduate School of Health Science, Suzuka University of Medical Science, Suzuka, Japan

[4] Department of Otolaryngology-Head and Neck Surgery, First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi, China

anti-tumor properties and has been shown to inhibit proliferation and induce apoptosis in certain cancers by differentially regulating pro-apoptotic and anti-apoptotic proteins (Zhang et al. 2014, 2015).

Studies investigating the effect of taurine on NPC remain limited, however, and the mechanism of its anti-tumor action is unclear. In this study, the role on apoptosis in the anti-cancer effect of taurine was examined in the context of NPC. The underlying molecular mechanism was also elucidated to provide evidence of the potential clinical application of taurine in tumor therapy.

Materials and methods

Cell lines

Human NPC cell lines (HK1 and HK1–EBV) and nonmalignant nasopharyngeal epithelial cell line NP460 were a kind gift from Professor Sue-Wah Tsao (Hong Kong University) (Lo et al. 2006; Tsang et al. 2010; Huang et al. 1980; Li et al. 2006). Cells were maintained at 37 °C in a 5% CO_2 incubator. NPC cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco). NP460 cells were maintained in a 1:1 ratio of Defined Keratinocyte SFM (Gibco) and Epilife (Gibco) supplemented with growth factors, 100 U/ml penicillin, and 100 µg/ml streptomycin. Taurine (purity ≥ 98.5%) was purchased from Nacalai Tesque Inc. (Kyoto, Japan). All cell lines were treated with taurine at various concentrations (0–32 mM, dissolved in medium) with various treatment times. The osmotic stress of the 0 mM medium is 291 mOsm/kg and the highest taurine group medium (32 mM) is 320 mOsm/kg H_2O. The useful range of osmolality of cell culture media for vertebrate lines is estimated between 260 and 320 mOsm/kg H_2O (Waymouth 1970). Lang et al. described that osmotic shock by increasing osmolarity to more than 500 mOsm was one of the well-known triggers of apoptosis (Lang et al. 2000). Therefore, even in the highest concentration, taurine may not induce osmotic stress and the following cell death.

Cell viability assay

The anti-proliferative effect of taurine on cells was determined by [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (MTT) assay. Cells were plated at a density of 2×10^3 cells/well into a 96-well plate, treated with taurine at various concentrations (0, 4, 16, 32 mM), and incubated for 24 h, 48 h, and 72 h. Then, 20 µl of 5 mg/ml MTT (Sigma, St Louis, MO, USA) was added to each well, followed by incubation for 4 h at 37 °C. The medium was removed and replaced with 150 µL DMSO, and absorbance values were measured at 570 nm on a Bio-Rad model 680 microplate reader (Bio-Rad laboratories, Hercules, CA, USA). The inhibition rate was calculated as follows:

$$\text{Inhibition rate } (\%) = (1 - (\text{absorbance of experimental group} / \text{absorbance of control group})) \times 100.$$

Colony formation assay

HK1 cells and HK1–EBV cells were counted and plated at 200 cells/well into a six-well plate. After adherence, the cells were treated with taurine (0, 4, 16, and 32 mM) for 48 h and then cultured for another 10 days. Thereafter, the colonies were washed twice with phosphate buffered saline (PBS) and fixed with 70% ethanol for 20 min. The samples were stained with Giemsa solution (Merck, Darmstadt, Germany) and allowed to air dry at room temperature. The numbers of colonies were counted.

Cell apoptosis assay

The Muse Annexin V and Dead Cell Assay Kit (Millipore, MA, USA) was used for quantitative analysis of apoptotic and dead cells with a flow cytometer (Muse™ Cell Analyzer, Millipore) according to the manufacturer's instructions. After incubating with taurine (0, 4, 16, 32 mM) for 48 h, all cells were harvested and diluted to a concentration of 5×10^5 cells/mL in RPMI 1640 medium with 2% FBS. 100 µl of Annexin V and Dead Reagent and 100 µl of single cell suspension were then mixed in a microtube and incubated in the dark for 30 min at room temperature prior to analysis using a Muse Cell Analyzer (Muse™ Cell Analyzer, Millipore). All experiments were performed in quadruplicate.

Western blot analysis

After treatment with taurine for 48 h, cells were harvested and lysed using RIPA buffer (Cell Signaling Technology Inc., Dancers, MA, USA) supplemented with phenylmethylsulfonyl fluoride (PMSF, Nacalai Tesque Inc.). Equal amounts of protein were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (0.45 µm, Millipore). The membranes were blocked with Tris-buffered saline (TBST) containing 0.1% Tween-20 (Nacalai Tesque Inc.) and 5% bovine serum albumin (BSA, Sigma-Aldrich, Saint Louis, MO, USA), and incubated overnight at 4 °C with primary antibodies. The list of antibodies used in this study is summarized in Additional file1:

Table S1. After washing with TBST, the membranes were further incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000, Santa Cruz Biotechnology Inc., CA, USA) for 1 h at room temperature, and finally developed with an electrochemiluminescence system (ECL) (GE Healthcare, Little Chalfont, UK). Protein bands were detected using a LAS4000mini (Fujifilm, Tokyo, Japan), and band intensities of Western blots were quantitatively measured by calculating integrated grayscale densities in consistently sized windows incorporating each band using ImageJ software (ver. 1.48). All experiments were performed in triplicate.

Statistical analysis

Values were presented as mean ± SD. Differences between groups were analyzed using one-way ANOVA followed by Tukey's post hoc analysis using GraphPad Prism5 software (GraphPad Software, San Diego, CA, USA). A p value < 0.05 was considered to be statistically significant.

Results

Taurine inhibits cell proliferation in HK1 and HK1–EBV cells, but not in NP460 cells

To investigate potential cell growth inhibition by taurine in NPC, we examined the effect of taurine on cell proliferation by MTT assay. The results showed that taurine significantly inhibited cell growth in both cancer cell lines in a concentration- and time-dependent manner (Fig. 1a, b). To control for the effect of taurine toxicity on cell growth in normal nasopharyngeal epithelial cells, NP460 cells were treated with taurine using the same dose and incubation time (Fig. 1c). The toxic effect of taurine was significantly lower in NP460 than in HK1 and HK1–EBV cells, especially at 24 h and 48 h. The findings suggest that taurine exerts a significant influence on HK1 and HK1–EBV cell proliferation without toxic effects on normal cells under certain conditions. We performed subsequent experiments using the condition which exhibited no toxic effect on normal cells.

Taurine inhibits cell colony formation in HK1 and HK1–EBV cells

The ability of HK1 and HK1–EBV cells to form colonies in the presence of taurine was investigated using the flat plate colony formation assay (Fig. 2a). The colony counts indicated that taurine inhibited colony formation in a dose-dependent manner. A considerable reduction in colony number was observed at 16 mM and 32 mM taurine (Fig. 2b). The results demonstrated that taurine reduced clonogenic survival of HK1 and HK1–EBV cells in vitro.

Taurine induces apoptosis in HK1 and HK1–EBV cells

To elucidate the mechanisms underlying taurine-induced growth inhibition, we used the Muse™ Annexin V and Dead Cell assay to observe apoptotic rates in the HK1 and

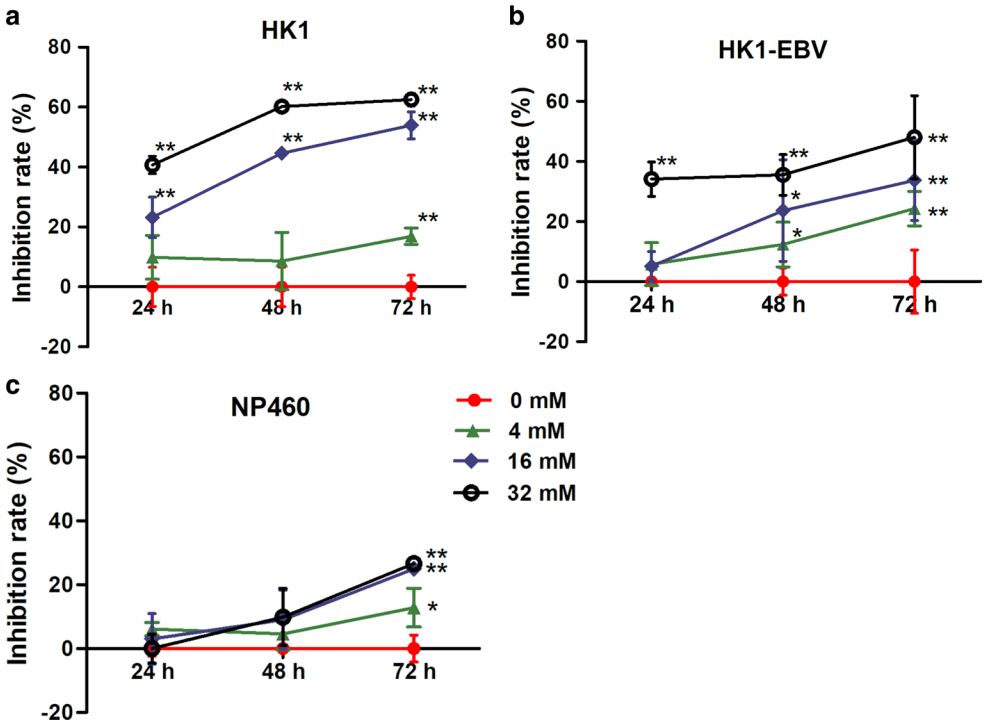

Fig. 1 Taurine inhibits proliferation in human NPC cells in vitro. **a** HK1, **b** HK1–EBV, and **c** NP460 cells were treated with various concentrations of taurine (0–32 mM) for various treatment times. Inhibition rate was assessed by MTT assay. Values are presented as mean ± SD of five independent experiments. *$p < 0.05$, **$p < 0.01$ compared with the 0 mM taurine group

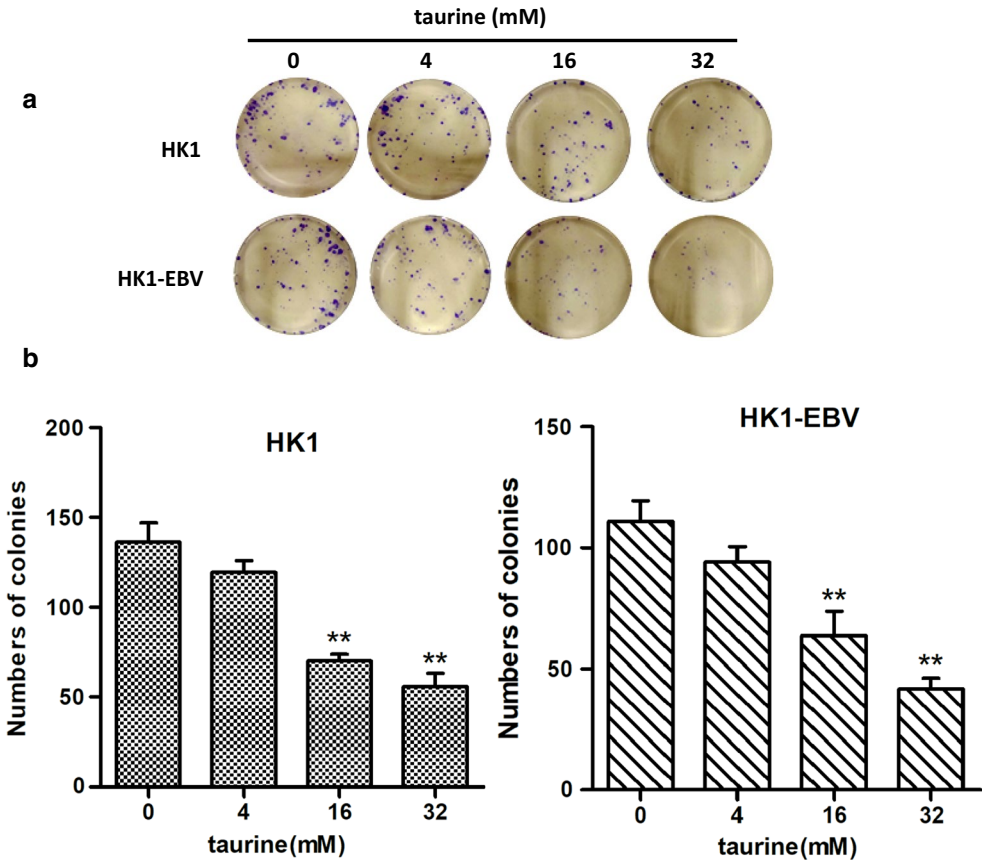

Fig. 2 Taurine inhibits colony formation in HK1 and HK1–EBV cells. **a** Representative colony images and **b** quantification of the colony number. Data are representative of three independent experiments. $*p < 0.05$, $**p < 0.01$ compared with the 0 mM taurine group

HK1–EBV cell lines after treatment with taurine for 48 h (Fig. 3a). In HK1 and HK1–EBV, the percentage of apoptotic cells were 17.0 ± 3.6% and 9.3 ± 2.0% in the 0 mM group, 19.3 ± 3.5% and 9.9 ± 1.7% in the 4 mM group, 31.3 ± 10.1% and 14.0 ± 2.8% in the 16 mM group, and 33.4 ± 1.7% and 20.1 ± 3.4% in the 32 mM group, respectively (Fig. 3b). The groups treated with 16 mM and 32 mM taurine showed significantly increased apoptosis rates compared with the 0 mM group. In addition, we also found no statistical difference in the NP460 cells after taurine treatment (Supplementary Fig. S1a). These results indicate that taurine could trigger apoptosis in the HK1 and HK1–EBV cells, which is consistent with the results from the MTT and colony formation assays.

Taurine affects the expression of proteins associated with apoptosis in the HK1 and HK1–EBV cells

To elucidate how taurine affects apoptosis in NPC cells, apoptosis-related proteins were examined. After 48 h treatment with taurine, Western blot assay showed that the anti-apoptotic gene Bcl-xL was down-regulated (Fig. 4a), and the pro-apoptotic gene Bax was up-regulated compared with untreated cells (Fig. 4b). There were significant increases in the cleaved form of caspase-9 (Fig. 4c) and caspase-3 (Fig. 4d), which is involved in the terminal phase of apoptosis via the mitochondrial pathway (Chen et al. 2018). Interestingly, taurine did not induce significant apoptosis-related proteins in NP460 cells (Supplementary Fig. S1b–e). In addition, we examined the effect of taurine on a major endoplasmic reticulum (ER) chaperone GRP78 expression in HK1 and HK1–EBV cells. As shown in Supplementary Fig. S2, taurine is able to activate the GRP78 in both cell lines. These results suggest that taurine may enhance the apoptosis-inducing effect in NPC through the mitochondrial and ER pathway.

Taurine affects PTEN/Akt pathway in HK1 and HK1–EBV cells

To identify whether the PTEN/Akt pathway is involved in taurine-induced apoptosis, the levels of PTEN and its downstream targets p53 and phosphorylated Akt (p-Akt) were obtained. After cells were incubated with different concentrations of taurine for 48 h, Western blots showed that taurine increased the levels of PTEN and p53 in a dose-dependent manner (Fig. 5a, b), and reduced phosphorylation of Akt (Fig. 5c) compared with the control group in HK1 and HK1–EBV cells. No significant differences of PTEN and p53 were observed in NP460 cells (Supplementary Fig. S1f, g). These results suggest that taurine may stimulate

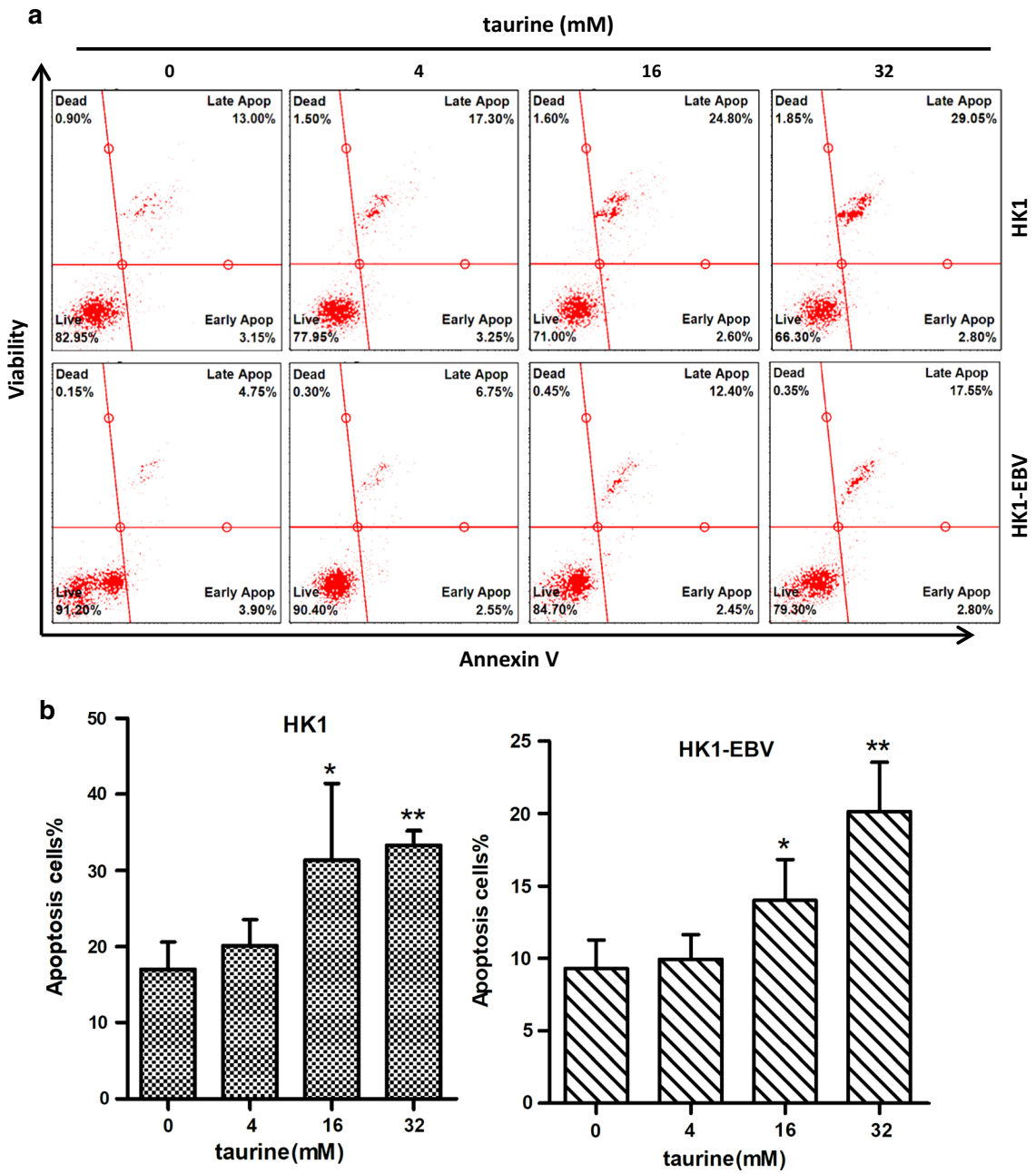

Fig. 3 Taurine induces apoptosis in HK1 and HK1–EBV cells. **a** Cells were separately treated with taurine for 48 h and the apoptosis rate was analyzed by flow cytometry. **b** Rate of apoptotic cells in taurine-treated cells. $*p < 0.05$, $**p < 0.01$ compared with the 0 mM taurine group

apoptosis in NPC cells through regulation of the PTEN/Akt pathway.

Discussion

In this study, the anti-tumor effect of taurine on nasopharyngeal carcinoma was investigated. Our results demonstrate that taurine significantly inhibits the proliferation and colony formation of NPC cell lines in a dose- and time-dependent manner. More importantly, our results demonstrate that taurine does not significantly reduce the viability of the immortalized epithelial cell line NP460 compared to the cancer cell lines. This observation has significant implications, because an ideal chemopreventive agent should be able to eliminate cancer cells while minimizing the toxic effects on normal cells.

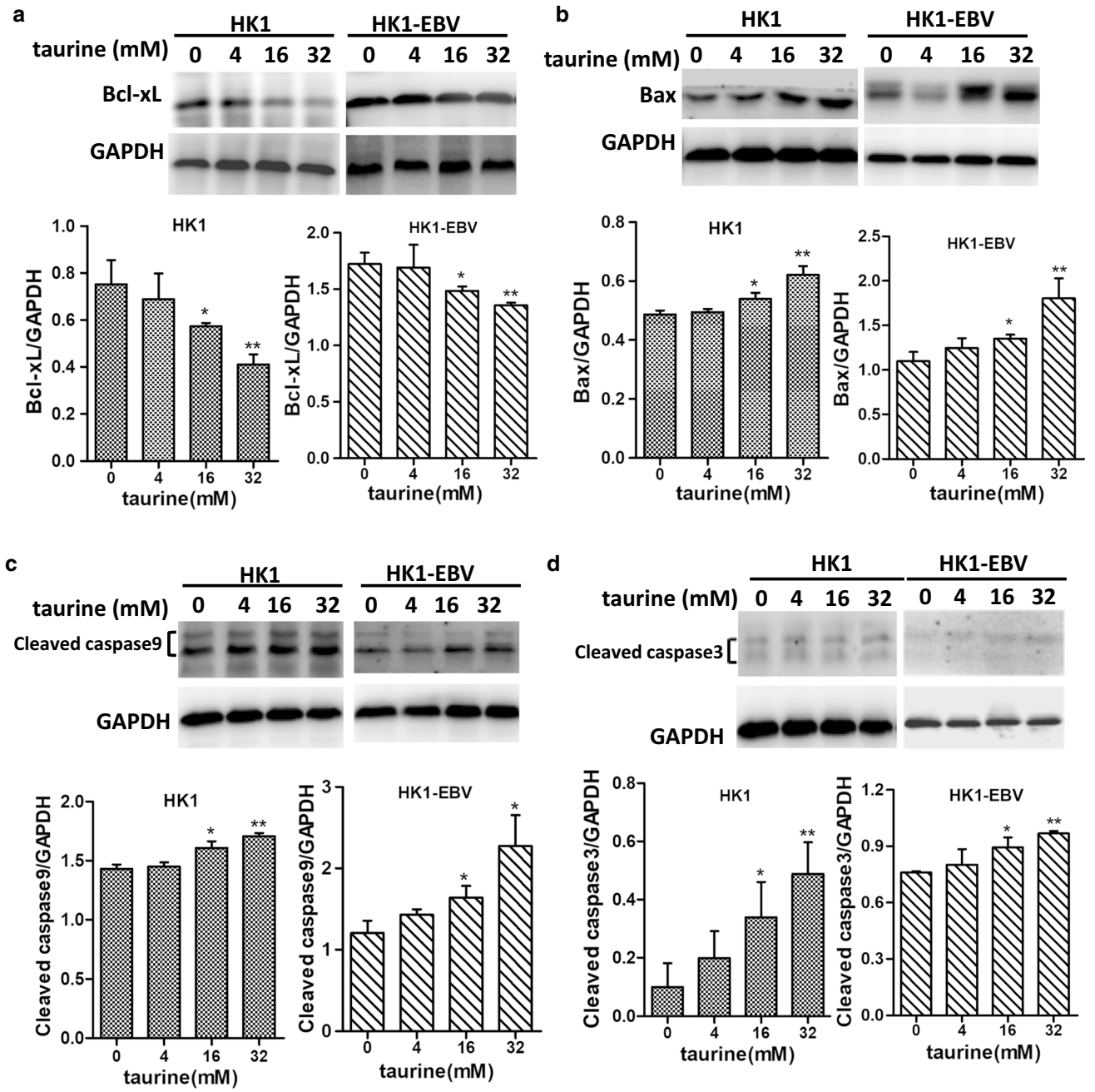

Fig. 4 Taurine induces the activation of apoptosis-related proteins in HK1 and HK1–EBV cells. After treatment with taurine for 48 h, cell lysates were prepared and Western blot analysis was performed against Bcl-xL (**a**), Bax (**b**), and cleaved caspase-9/3 (**c**, **d**). GAPDH was used as a loading control. *$p<0.05$, **$p<0.01$ compared with the 0 mM taurine group

Apoptosis is a well-known process of programmed cell death that can counteract tumor growth; thus, the induction of apoptosis in cancer is a key therapeutic approach (Ghobrial et al. 2005). Currently, one of the important modes of action of anti-cancer drugs is to inhibit tumor cell proliferation and induce apoptosis (Xie et al. 2014; Jiang et al. 2018). It should be noted that taurine showed an opposite effect on cancer and non-cancer diseases. Previous investigations have shown that taurine can cause anti-apoptosis in neurons cells (Leon et al. 2009), kidney cells (Chang et al. 2014) and cardiac myocytes (Das et al. 2011), but induce cell apoptosis in a dose-dependent manner in colon (Zhang et al. 2014) and lung (Tu et al. 2018) cancers. Taurine is known to interfere with the pro-apoptotic activity of many toxic agents or conditions. Taurine deficiency is pro-apoptotic (Jong et al. 2017). On the contrary, taurine including

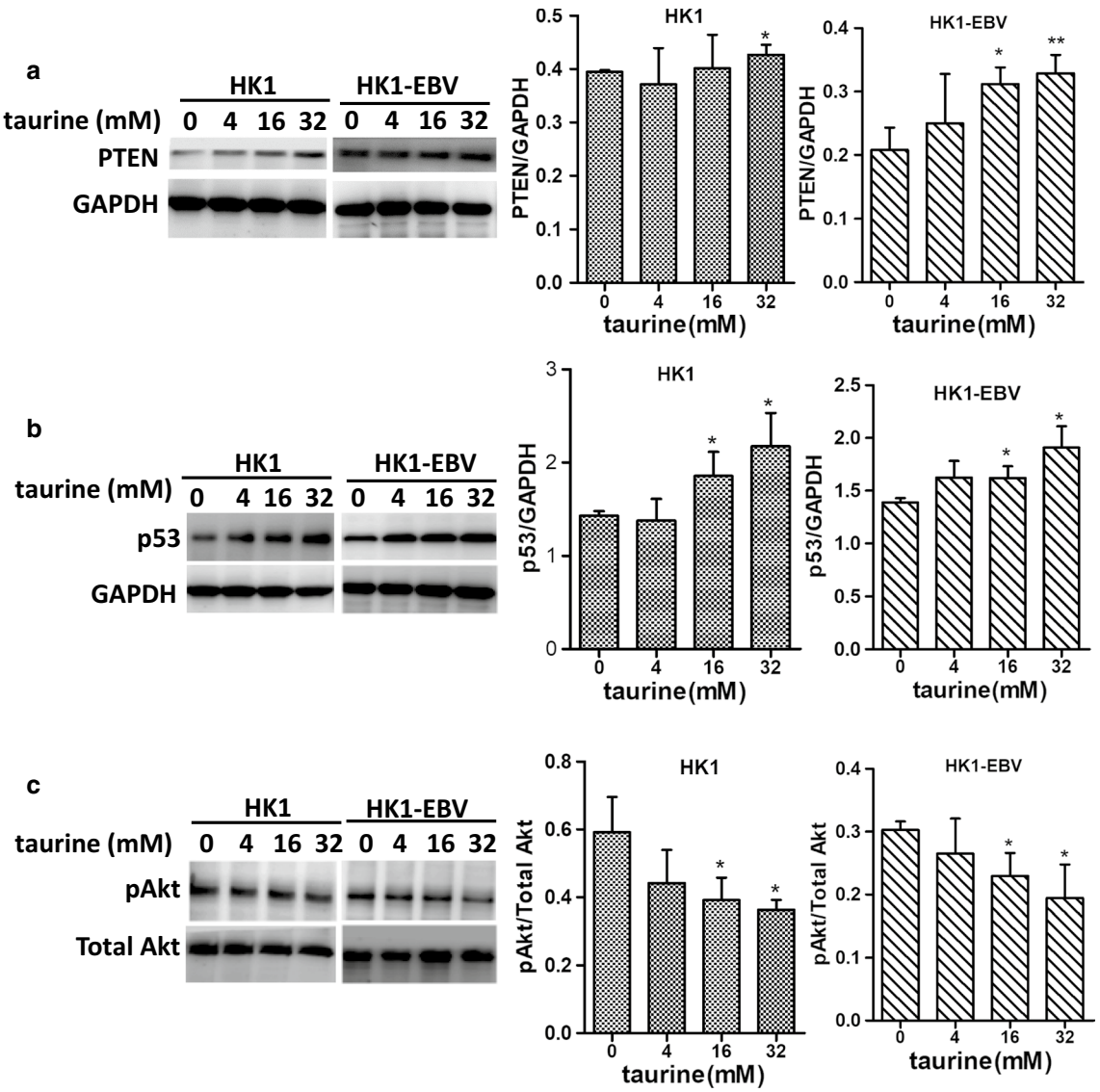

Fig. 5 Taurine activates the PTEN/Akt pathway in HK1 and HK1–EBV cells. Western blot analysis was performed to detect the levels of PTEN (**a**) and its downstream target proteins p53 (**b**) and p-Akt (**c**) were quantitated after incubation with various concentrations of taurine for 48 h. Antibodies to GAPDH and total Akt served as loading controls. $*p<0.05$, $**p<0.01$ compared with the 0 mM taurine group

taurine chloramine has been reported to exhibit pro-apoptotic activity (Emerson et al. 2005). In fact, taurine chloramine inhibits proliferation of rheumatoid arthritis synoviocytes via a p53-dependent pathway (Kontny et al. 2006). It still remains unclear whether taurine affects cancer cells in the condition of taurine deficiency or not. Our present results showed neither anti-apoptotic or apoptosis-inducing effect in the normal nasopharyngeal cell line NP460. Further study is needed to clarify the reasons for the opposite effect of taurine. The present study demonstrates that taurine can reduce the viability of HK1 and HK1–EBV cells, and induce apoptosis in a concentration-dependent manner, but has no effect on NP460 cells. Cells treated with high concentrations of taurine (16 mM and 32 mM) induced a significantly higher apoptotic rate compared with untreated cells. The results revealed that taurine promoted apoptosis of HK1 and HK1–EBV cells dose-dependently, suggesting that it is a potential candidate agent for treatment of NPC.

Apoptosis is regulated by many anti-apoptotic and pro-apoptotic molecules. There are two different main pathways: the mitochondrion-mediated intrinsic apoptotic pathway, and the death receptor-mediated extrinsic apoptotic pathway (Lin et al. 2017; Dillon and Green 2016). To explore the mechanism of taurine-induced apoptosis, changes in expression of several apoptosis-related proteins were examined in HK1 and HK1–EBV cells. Bcl-2 family members

are active primarily in mitochondria, which play a pivotal role in apoptotic cells. The Bcl-2 family can be divided into pro-apoptotic members (Bax) and anti-apoptotic members (Bcl-xL and Bcl-2) (Fmb et al. 2018; Ruefli-Brasse and Reed 2017). Dysregulation of the balance between Bcl-2 family members results in disruption of the mitochondrial membrane and apoptosis through the intrinsic pathway. In the present study, we have shown that taurine can increase the level of pro-apoptotic Bax and decrease the level of anti-apoptotic Bcl-xL. Additionally, Bcl-2 family members act as a gateway for caspase-mediated cell death. A group of cell death enzymes, cysteine aspartases (caspases), play an important role in apoptosis (Chen et al. 2018). This study demonstrates that taurine can significantly enhance the cleaved form of caspase-9 and caspase-3, suggesting that the mitochondrial pathway of apoptosis is involved in taurine-induced apoptosis in HK1 and HK1–EBV cells. The concentrations at which taurine altered the expression of these apoptotic-associated proteins were similar to those at which cell proliferation was suppressed.

To study the molecular mechanism of taurine against NPC, the expression of PTEN was examined. PTEN is a critical tumor suppressor and is a negative regulator of the Akt signaling pathway. The cellular functions of PTEN include regulation of proliferation, migration, apoptosis, and survival (Wang et al. 2015). Phosphorylation of Akt exerts anti-apoptotic effects by regulating downstream substrates, including Bax and Bcl-xL. Furthermore, previous studies have revealed the lower expression of PTEN in NPC cell lines (Zhang et al. 2013) and NPC tissues (Xu et al. 2004). The results of the present study have provided novel evidence demonstrating that the expression of PTEN protein was increased after taurine treatment. The up-regulation of PTEN expression was associated with an increase in p53 expression and decreased Akt phosphorylation. PTEN localizes to the endoplasmic reticulum (ER) and mitochondria-associated membranes. It is responsible for synthesizing, folding, trafficking secretary proteins and regulating intracellular Ca^{2+} homoeostasis. The alterations of the Ca^{2+} concentration result in accumulated unfolded proteins in the ER, a condition called "ER stress" (Bononi et al. 2013). GRP78 is considered as one of the major markers of ER stress-mediated apoptosis (Gil et al. 2018; Wang et al. 2009). In this study, it was found that taurine can activate the expression of GRP78. This supports the hypothesis that taurine may induce apoptosis through the stimulation of PTEN-associated ER stress. This study only focused on the effect of taurine on induction of apoptosis in NPC cell lines in vitro; further studies are necessary to explore its effects in vivo. In addition, the mechanism by which taurine up-regulates PTEN and induces Ca^{2+} flux requires further exploration. Notwithstanding such limitations, we demonstrate that the anti-tumor effect of taurine in NPC may depend on the PTEN/Akt signaling pathway in vitro.

In conclusion, this study has revealed that taurine exhibits apoptosis-inducing effect in NPC via the PTEN/Akt signaling pathway, suggesting one of anti-tumor mechanisms. Taurine transporter (TauT) contributes to cell volume regulation under isotonic and hypertonic conditions (Kubo et al. 2016). TauT utilizes a chemiosmotic Na^+ gradient to couple "downhill" transport of Na^+ with "uphill" transport of taurine across a biomembrane in vivo (Pramod et al. 2013). When intracellular Na^+ concentration increases, Na^+–K^+ ATPase pump may release Na^+ to maintain homeostasis. Our in vitro study has the limitation of different conditions for taurine concentration compared to in vivo. However, the present results may provide the basis for new treatments for patients with nasopharyngeal carcinoma.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent All authors listed have contributed to the conception, design, gathering, analysis, or interpretation of data and have contributed to the writing and intellectual content of the article. All authors gave informed consent to the submission of this manuscript.

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