Biomedicine & Pharmacotherapy 121 (2020) 109602

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

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

Chlorogenic acid inhibits esophageal squamous cell carcinoma growth *in vitro* and *in vivo* by downregulating the expression of BMI1 and SOX2

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ARTICLE INFO

Keywords:
Chlorogenic acid
ESCC
Tumor growth
BMI1
SOX2

ABSTRACT

Esophageal squamous cell carcinoma (ESCC) is one of the most common cancers in China, accompanied by an extremely high mortality rate. Chlorogenic acid (CGA) is a small-molecule compound, that has been shown to have a wide range of biological activities, including antitumor. However, the efficacy and molecular mechanism of CGA on ESCC remains unknown. In this study, we confirmed the inhibition of proliferation by CGA in ESCC cells, as well as the reduction of ESCC xenograft volume by CGA *in vivo*. In addition, CGA also suppressed both the migration and invasion of ESCC cells *in vitro*. In a carcinogen-induced murine model of ESCC, hyperplasia of the esophagus was slowed by CGA, while mice suffering from ESCC that were treated with CGA had longer survival times than mice in the control group. The measurement of pluripotency factors (BMI1, SOX2, OCT4 and Nanog) that are related to poor prognosis revealed reduced expression of both BMI1 and SOX2, but not of OCT4 or Nanog, in ESCC cells, in both a dose- and time-dependent manner. Together, our initial findings demonstrate that CGA suppresses ESCC progression, downregulates the expression of BMI1 and SOX2, and provide an anti-tumor candidate for ESCC therapy.

1. Introduction

Esophageal cancer is the eighth most common cancer and the sixth leading cause of cancer-related death in the world [1]. In 2018, there were over 500,000 new cases of esophageal squamous cell carcinoma (ESCC) [2]. More than 95 % of esophageal cancers consist of squamous cell carcinoma and adenocarcinoma. Among them, ESCC is the most common type. The highest rate of ESCC occurs in China, with the third highest incidence rate and the fourth highest mortality rate in 2015 [3]. Continuous improvement in both diagnosis and treatment (curative surgical therapy, chemotherapy and chemoradiotherapy) has led to a great increase in the overall 5-year survival rate for patients with early stage ESCC. However, progression of ESCC without clear clinical symptoms is an important cause of the high mortality rate, resulting in a 5-year survival rate of less than 25 % for patients with advanced ESCC [4].

Currently available therapies, including surgery, medicine and radiotherapy, are always accompanied by serious side effects, which might greatly affect a patient's quality of life. Moreover, resistance to drugs and radiotherapy is one of the main problems faced by late-stage ESCC patients, as well as metastasis and recurrence, leading to a poor prognosis. Therefore, it is necessary to investigate new candidate drugs for advanced ESCC therapy, especially for late-stage ESCC.

Chlorogenic acid (CGA), the ester formed between caffeic acid and quinic acid [5], is a phenolic compound widely found in the human diet. CGA possesses many health-promoting properties, such as anti-oxidant and anti-inflammatory properties [6]. In recent years, several studies have revealed that CGA also plays an important role in tumor prevention. CGA suppresses glioma growth by repolarizing the M2 phenotype of macrophages to the M1 phenotype [7]. It also decreases the proliferation of A549 human lung cancer cells [8]. In MDA-MB-231 and MCF-7 breast cancer cells, CGA disrupts the cell cycle and induces apoptosis in a dose-dependent manner [9].

As a botanic compound isolated from the cortex of *Foliumeucommiae*

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https://doi.org/10.1016/j.biopha.2019.109602
Received 31 July 2019; Received in revised form 25 October 2019; Accepted 25 October 2019

and the flower bud of *Lonicera confusa*, CGA has recently been approved by the China Food and Drug Administration (CFDA) as an antitumor drug. Phase I clinical trials with late-stage glioma patients showed significant clinical efficacy with few side effects. It is believed that CGA can pass through the blood-brain barrier and induce cell differentiation [10]. However, the molecular mechanism of the antitumor effects of CGA and the antitumor efficacy of CGA for other malignant tumors remains largely unknown.

In this study, we confirmed that CGA inhibited ESCC growth both *in vitro* and *in vivo*. In addition, CGA also suppressed the migration and invasion of ESCC. Using a carcinogen-induced murine model of ESCC, we observed deceleration of esophageal hyperplasia in ESCC mice treated with CGA, as well as a longer survival time. In addition, we observed a decrease in BMI1 and SOX2 after CGA treatment in ESCC cells in both a dose- and time-dependent manner, while knocking down either BMI1 or SOX2 in ESCC cell lines reduced the inhibitory effect on proliferation and cell motility by CGA. Moreover, downregulation of both BMI1 and SOX2 by CGA in tumor tissues was confirmed by both ectopic xenograft tumor and carcinogen-induced murine model of ESCC. Taken together, our findings demonstrate the antitumor efficacy of CGA for ESCC for the first time. Tentative exploration of the molecular mechanism provides a new basis for understanding the pharmacological mechanisms of CGA, suggesting that CGA is a candidate compound for clinically advanced ESCC therapy.

2. Materials and methods

2.1. Cell culture

The ESCC cell lines KYSE30/70/140/150/180/510 were generously provided by Dr. Y. Shimada (Kyoto University, Kyoto, Japan) and verified by short-tandem repeat (STR) profiling in 2015. All cells were cultured at 37 °C and 5 % CO_2 in RPMI-1640 supplemented with 10 % fetal bovine serum (FBS) and antibiotics.

2.2. Cell proliferation

ESCC cells were seeded at 3000 cells per well in triplicate in 96-well plates, and treated with different concentrations (0–200 μM) of CGA. Assessment of cell growth for 4 days was performed by using a Cell Counting kit-8 (CCK-8) reagent (Applygen, Beijing, China) according to the manufacturer's recommendations.

2.3. Colony formation assay

Five hundred cells were seeded into 35-mm culture plates and incubated with complete medium containing different concentrations of CGA at 37 °C with 5 % CO_2 for 10 days. The medium containing CGA was changed every other day. Culture plates were set up in duplicate. After washing with pre-cooled DPBS, cells were fixed and stained with 0.5 % crystal violet. Photographs were taken by a ChemiDoc™ XRS$^+$ electrophoretic imaging system (Bio-Rad Laboratories, Berkeley, USA).

2.4. Migration and invasion assays

Migration and invasion assays were performed using 24-well Boyden chambers (Corning Incorporated, Corning, NY, USA), and the chambers used for invasion assays were coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Cells were pretreated with CGA for 24 h, and 1×10^5 cells in 100 μL RPMI-1640 were planted into the upper chamber. Meanwhile, the lower chamber was filled with complete medium containing CGA, and then cells were cultured at 37 °C (12 h for migration, 24 h for invasion). Cells that remained on the upper side of the filter were removed. The remaining cells were fixed and stained with 0.5 % crystal violet. Cells from at least four randomly selected microscopic fields were counted.

2.5. Oligonucleotide transfection

siRNAs for BMI1 and SOX2 and relevant negative control (NC) were ordered from RiboBio (Guangzhou RiboBio Co., Ltd, Guangzhou, China). Three different oligonucleotides were synthesized for each gene, and then transfected into cells as a siRNA pool. Oligonucleotides transfection were down by using HiperFect (Qiagen, Germantown, MD, USA). The sequences of oligonucleotides are shown below. siBMI1: 5′-GCATTATGCTTGTTGTACA-3′, 5′-CATTGTAAGTGTTGTTTCT-3′, 5′-ATGAAGAGAAGAAGGGATT-3′; siSOX2: 5′-GCAGCTACAGCATGATGCA-3′, 5′-GGAGCACCCGGATTATAAA-3′, 5′−CCACCTACAGCATGTCCTA-3′.

2.6. Flow cytometry

Briefly, cells were digested gently to single cells after 48 h treatment of CGA and then subjected to flow cytometry analysis. To detect cell cycle, we used 75 % ice-cold ethanol to fix the harvested cells and stored them in 4 °C refrigerator. Before analysis, cells suspended in 500 μl PI/RNase Staining Buffer (BD Biosciences) for 30 min in the dark. Apoptosis assay was performed in accordance with FITC-Annexin V Apoptosis Detection kit instruction of manufacturer (BD Biosciences). To detect SP cells, Hoechst 33342 (Sigma, St. Louis, MO) was added to the cell suspension at a final concentration of 5 μg/ml, and the cells were incubated for 2 h in normal culture conditions (37 °C and 5 % CO_2). All experiments were performed on the same Moflo XDP (Beckman Coulter, Atlanta, Georgia, USA).

2.7. Western blotting

Protein was extracted from cells and tissues. Western blotting was performed as described previously [11]. Antibodies that were used are shown below: PCNA (2586, CST, Cell Signaling Technology, Danvers, MA, USA), Survivin (2808, CST), BMI1 (6964, CST, 10832-1-AP, Proteintech, Proteintech Group Inc., Rosemont, IL, USA), SOX2 (3579, CST, 11064-1-AP, Proteintech), OCT4 (60242-1-lg, Proteintech), Nanog (4903, CST), and β-actin (4970, CST)

2.8. Animal model

All research involving animals complied with protocols approved by the Beijing Medical Experimental Animal Care Commission. For the ectopic xenograft model, 4 to 6-week-old NOD/SCID mice were injected subcutaneously with 1×10^6 cells. Ten days after injection, mice were randomly divided into two groups, receiving either normal saline (NS) or 50 mg/kg CGA once a day by intra-peritoneal injection. Tumor size was measured twice a week, and the volume of the tumor was calculated with the formula: $V = 1/2 \times \text{length} \times \text{width}^2$. For orthotopic injection, 2×10^5 cells expressing Luciferase were injected into the muscularis externa of the esophagus of 6-week-old NOD/SCID mice, as described in [12]. Three days after implantation, the mice were divided into two groups (NS or 50 mg/kg CGA) according to *in vivo* bioluminescence imaging using an IVIS Spectrum Imaging System (PerkinElmer, MA, USA). The *in vivo* bioluminescence imaging was performed once a week during CGA treatment. A carcinogen-induced murine model was used, as described in Supplementary Figure S2A, Six-week-old male C57Bl/6 mice were given drinking water containing 100 μg/ml 4-NQO (N8141, Sigma) for 16 weeks, then, the mice were given normal drinking water [13]. CGA treatment started 6 weeks after the change to normal drinking water. Half of the mice were treated with CGA for 6 weeks and then sacrificed and esophageal tissue was dissected. The rest of the mice continued CGA treatment until death.

2.9. Statistical analyses

Statistical analysis was performed using Prism 6 or SPSS21.0 (IBM

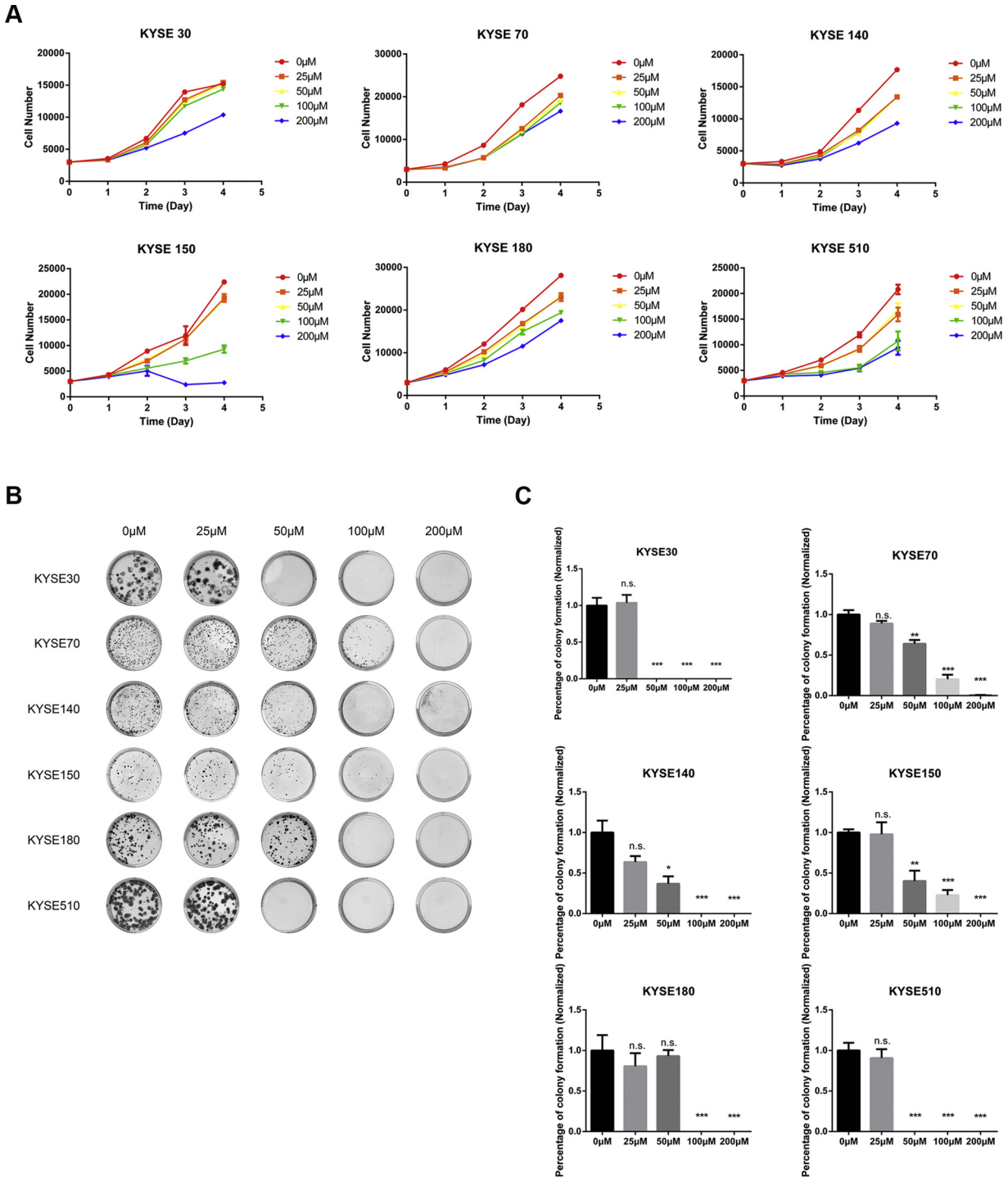

Fig. 1. CGA inhibits proliferation and colony formation of ESCC cells *in vitro*. (**A**) Effects of different concentrations of CGA (from 0 to 200 μM) on the proliferation of six ESCC cell lines. (**B**) Representative photographs of colony formation assays using six different ESCC cell lines treated with different concentrations of CGA (from 0 to 200 μM). (**C**) Number of colonies formed using ESCC cells treated with different concentrations of CGA. The assays were performed in duplicate. Data are represented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

SPSS software, NY, USA). All data are presented as the mean ± SEM unless otherwise stated. Student's *t*-test was used, unless otherwise stated. We considered $P < 0.05$ to be statistically significant.

3. Results

3.1. CGA inhibits ESCC cell proliferation and colony formation in vitro

To detect whether CGA has an antitumor effect on ESCC, we first performed cell proliferation assays in six ESCC cell lines derived from resected specimens of different patients [14], treating cells with different CGA concentrations (from 0 to 200 μM). The growth of the remaining five concentration groups was inhibited to various degrees compared with that of the 0 μM group. When treating the cells with a low CGA concentration (from 25 μM to 100 μM), some of the cell lines exhibited no significant dose-dependent growth inhibition. In contrast, when the treatment concentration reached 200 μM, an obvious inhibitory effect was observed in all six cell lines, compared with the

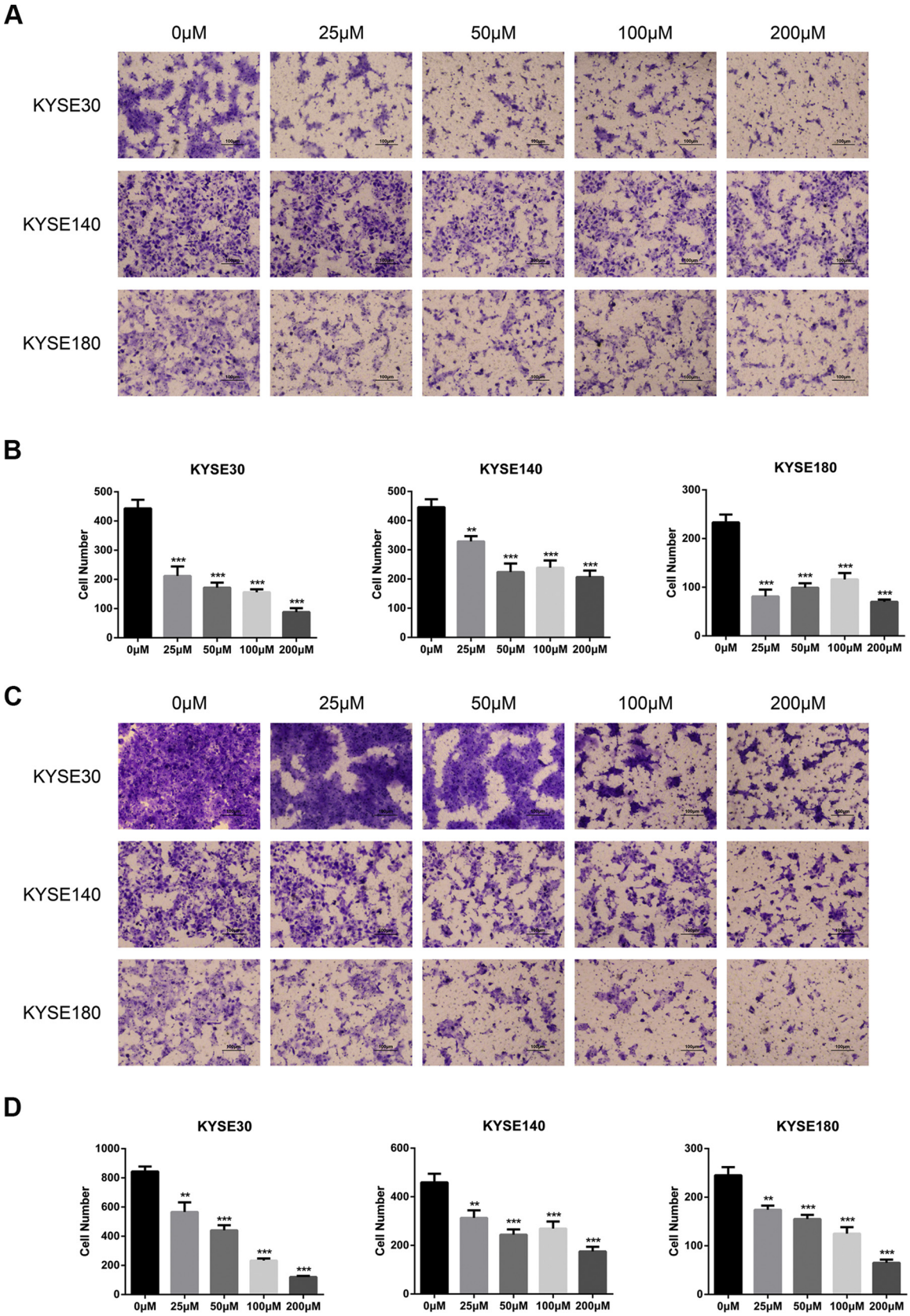

Fig. 2. CGA suppresses ESCC cell migration and invasion*in vitro.* (A) Verification of migratory capability of ESCC cells treated with different concentrations of CGA (from 0 to 200 μM) using a transwell migration assay. (**B**) Quantification of migratory capability of KYSE30/140/180 cells. (**C-D**) Representative micrographs (**C**) and quantification (**D**) of invading cells using ESCC cells treated with CGA. Data are represented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

other concentration groups (Fig. 1A). Next, to further confirm the inhibitory effect of CGA on cells over longer periods of time, we performed colony formation assays using all six cell lines. As expected, CGA suppressed colony formation of all six cell lines in a dose-dependent manner (Fig. 1B and C). These findings confirmed that CGA could inhibit ESCC cell growth, as it can for many other types of cancer. Interestingly, we found that no colonies formed with a CGA concentration of 100 μM and in even lower concentrations. The concentration required for effective inhibition of colony formation seemed to be lower than that of cell proliferative suppression, indicating that CGA may inhibit ESCC cell growth in a long-term, gentle manner.

3.2. CGA suppresses ESCC cell migration and invasion in vitro

Metastasis is considered to be a main cause of death in patients suffering from advanced ESCC. We investigated whether CGA could suppress the motility of ESCC cells *in vitro*. KYSE30/140/180 cells were treated with CGA for 24 h prior to the start of the assay, and then they were used to perform migration and invasion assays as described in Materials and Methods. As we expected, CGA dramatically suppressed both migration and invasion of these three cell lines *in vitro* (Fig. 2). The motility suppression by CGA unexpectedly occurred in a strongly dose-dependent manner, which had no significant correlation with cell proliferative inhibition (Fig. 1A). Together, our findings demonstrated that CGA suppressed both migration and invasion of ESCC cells independent from proliferative inhibition.

3.3. CGA inhibits ESCC growth in vivo

To further assess the anti-tumor efficacy of CGA on ESCC *in vivo*, we established two different animal models to evaluate the growth inhibition of ESCC by CGA. First, we addressed the ability of CGA to inhibit heterotopic xenograft growth. KYSE30/70/140/150/510 cells (KYSE180 was poorly tumorigenic) were subcutaneously injected into NOD/SCID mice. All mice were treated with CGA (ip 50 mg/kg/d) or the same volume of NS. We observed significant reduction of tumor growth of all five ESCC cell lines in mice treated with CGA, compared with that of the control groups (Fig. 3A), yielding a 30.0%–49.1% tumor volume reduction. However, not all of the growth of ESCC cell lines inoculated into NOD/SCID mice could be inhibited significantly by CGA (Supplementary Figure S1). Confusingly, tumor growth of KYSE140 in both the control and CGA group seemed to slow down since 14 days after accepting the treatment (Fig. 3A, middle). It is believed that orthotopic animal models have a more similar microenvironment to that of spontaneous tumors, as the tumors form in the organ of origin [15]. As described in Materials and Methods, we established an ESCC orthotopic animal model by inoculating KYSE140-Luc cells into the muscularis externa of the esophageal wall, near the middle and lower esophagus and below the diaphragm distant from the esophagogastric junction (Fig. 3B). Live animal images were obtained by using an IVIS Spectrum system that revealed an inhibition of tumor growth in mice treated with CGA (ip 50 mg/kg/d), compared with that of the control group (Fig. 3C). The results were further confirmed by quantification of the *in vivo* luciferase activity (Fig. 3D). Taken together, our findings verified the antitumor effect of CGA on ESCC cells *in vivo*.

3.4. CGA slows hyperplasia of the esophagus and prolongs survival time in mice

It has been reported that the administration of 4-nitroquinoline 1-oxide (4-NQO) produces a temporal carcinogenesis progression model that demonstrates multiple dysplastic, preneoplastic and neoplastic lesions after long-term treatment [16]. A murine model of 4-NQO-induced esophageal cancer has also been identified as a useful model that mimics many features and molecular events observed in human ESCC development [13]. Thus, we established a 4-NQO-induced carcinogenesis murine model to further assess CGA efficacy (Supplementary Figure S2A). To determine the time when the mice should begin CGA therapy (ip 50 mg/kg/d), we collected esophageal tissues of mice at different time points that were given 4-NQO drinking water to examine the pathological evidence. H&E staining of these tissues revealed neoplastic growths in mice given 4-NQO drinking water for 22 weeks (Supplementary Figure S2B). Additionally, marked thickness of the esophageal wall was observed compared with that of the control group (Supplementary Figure S2C). Six weeks after CGA treatment, the mice were sacrificed, and the esophagus was excised for pathological analysis. We observed thicker esophageal walls in the control group than in those treated with CGA (Fig. 4A). Pathological examination of H&E staining also confirmed these outcomes (Fig. 4B). The width of the esophagi resected from the CGA group was much thinner than that of the control group (Fig. 4C), and no significant differences were found in the esophagi length of the two groups (Supplementary Figure S2D). These findings suggested a slowing of neoplastic progression of the esophagus due to CGA treatment.

We also hypothesized that CGA could prolong the survival time of mice suffering from ESCC, and to examine this, we established another 4-NQO-induced carcinogenesis murine model in which mice were treated with CGA until they died of natural causes (Supplementary Figure S2A). We found that ESCC mice treated with CGA had longer overall survival than those injected with NS (Fig. 4D). The average survival time increased by 17.04 % (from 60 days to 70.22 days) in the CGA group compared with the NS group. These findings resemble the clinical applicability of CGA for Phase II clinical trials, indicating the possibility of using CGA in advanced ESCC therapy.

3.5. CGA induces cell apoptosis, but has no effect on cell cycle arrest

As we had verified the anti-tumor efficacy of CGA on ESCC, we wanted to also explore the molecular mechanism of CGA in ESCC. It has been reported that CGA can induce breast cancer cell cycle arrest at the G0/G1-S phase, as well as apoptosis *via* the mitochondrial pathway [9]. CGA also induces A549 lung cancer cell apoptosis by upregulating the expression of BAX and CASP3 and downregulating BCL2 [8]. We hypothesized that CGA could affect the cell cycle and apoptosis in ESCC. We evaluated the expression of proliferating cell nuclear antigen (PCNA) and Survivin in these cells. PCNA is a protein expressed in proliferating cells or tumor cells, and its expression has been shown to change periodically throughout the cell cycle. It is considered a well-accepted marker of proliferation [17]. Surprisingly, we observed no significant change in PCNA expression in ESCC cells treated with CGA in either a dose- or time-dependent manner (Fig. 5A and 5B). Detection of cell cycle by flow cytometry also revealed no significant changes of G1, S or G2 phases in ESCC cells after a high concentration of CGA (200 μM) treatment (Supplementary Figure S3A). Moreover, the expression of PCNA in either carcinogen-induced or ectopic xenograft murine models appeared no significant differents between the two groups with or without CGA treatment (Supplementary Figure S5E). These results indicate that no cell cycle arrest was caused by CGA in ESCC cells. Survivin, a member of the inhibitor of apoptosis protein (IAP) family, functions as an inhibitor of apoptosis and is involved in the regulation of cellular proliferation and angiogenesis in cancer [18]. We found that the expression of Survivin decreased in both a dose- and time-dependent manner in CGA-treated ESCC cells (Fig. 5A and 5B), suggesting that CGA caused apoptosis induction in ESCC cells. Moreover, an increased ratio of apoptotic cells was observed in different ESCC cell lines after a moderate concentration of CGA (50 μM) treatment (Supplementary Figure S3B), which was consistent with the regulation of Survivn expression by CGA. Taken together, all our findings indicate that CGA regulates the apoptosis of ESCC cells, rather than cell cycle arrest that was observed in other cancer cell lines.

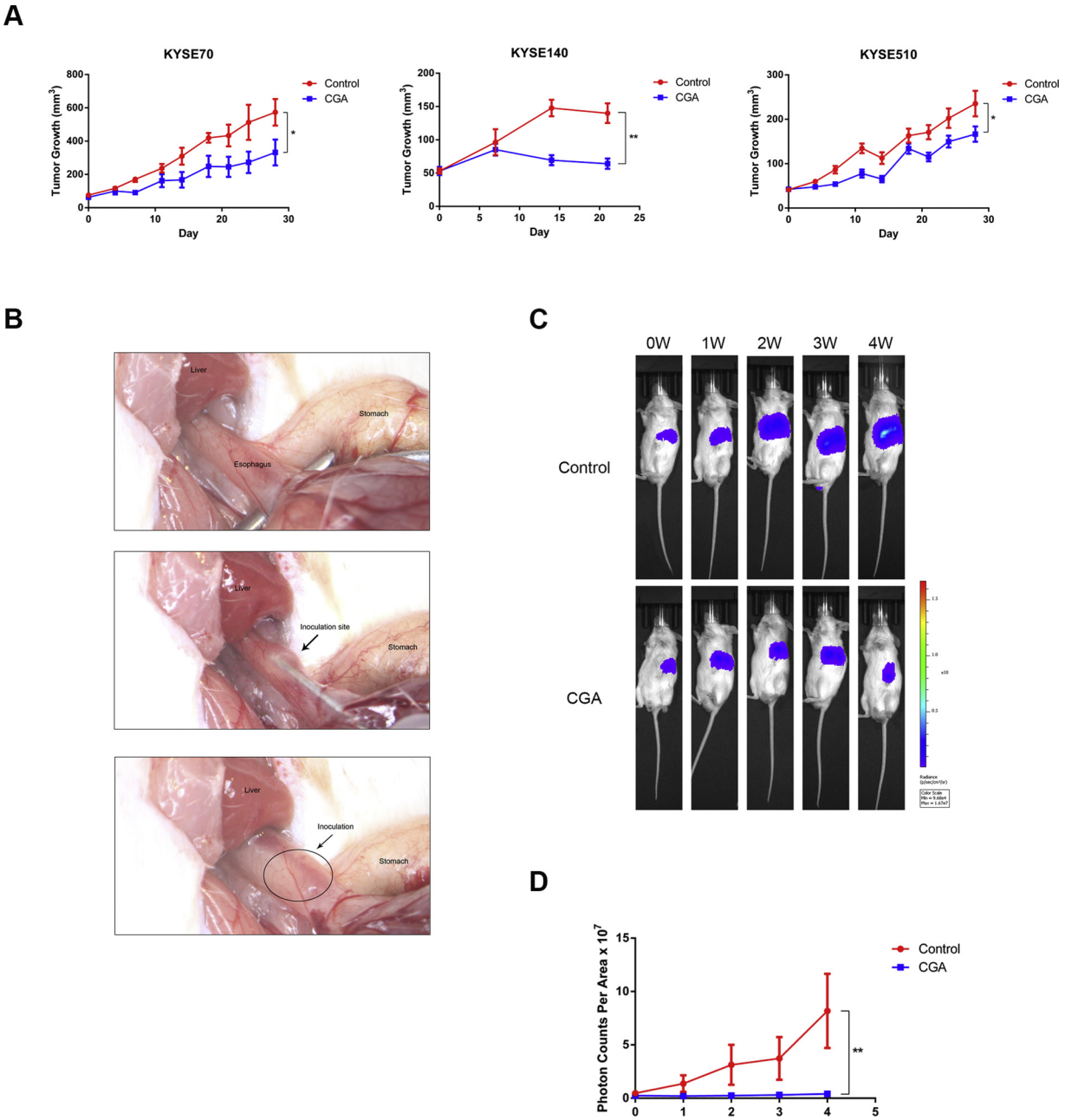

Fig. 3. CGA inhibits ESCC cell growth*in vivo*. (A) Tumor growth measurement of three ESCC cell lines subcutaneously injected into NOD/SCID mice. The CGA group received a dose of 50 mg/kg CGA per day *via* intraperitoneal injection. Tumor size was calculated as length (mm) × width $(mm)^2/2$. (**B**) Surgical inoculation of ESCC cells into the esophagus of mice. Upper, exposure of the anatomical position of the esophageal injection site. Middle,the needle insertion point near the middle-lower esophagus, below the diaphragm and away from the esophagogastric junction, which is indicated by the arrow. Lower, edema formed after successful inoculation of ESCC cells, marked by a circle and an arrow. (**C**) Representative bioluminescent images showing tumor growth in mice from both the control and CGA groups at the indicated time points (weeks). The CGA group received a dose of 50 mg/kg CGA per day *via* intraperitoneal injection. (**D**) Quantitative analysis of tumor growth. The values of the bioluminescent signal from each group were quantified at the indicated time points (weeks) and are expressed as photon counts per area. Data are represented as the mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.6. CGA downregulates the expression of BMI1 and SOX2

The results of Phase I clinical trials suggest that CGA can pass through the blood-brain barrier and induce cell differentiation, transforming tumor cells into healthy cells [10]. On the other hand, aggressive proliferation and high motility are considered to be behaviors that are characteristic of cancer cells in a stemness state [19], which can be considered a state that is opposite to cell differentiation. Hence, we first detected the ratio of side population (SP) cells, which is considered to possess the property of stem cells [20], in ESCC cell lines treated with a moderate concentration of CGA (50 μM). We found that an obvious diminishment of SP cells in KYSE30/70/510 cells with CGA treatment, while there was only a slight decrease of the ratio of SP cells in KYSE140/150/180 treated with CGA (Supplementary Figure S4). The changes of SP cell ratio in these six ESCC cell lines led to an assumption, that is, the regulation of stemness genes might be part of the molecular mechanism of the anti-ESCC effect of CGA. To verify our assumption, we examined the expression level of several master regulators of the stemness state (BMI1, Nanog, OCT4 and SOX2). These genes are considered to be major stemness markers in ESCC, as well as being related

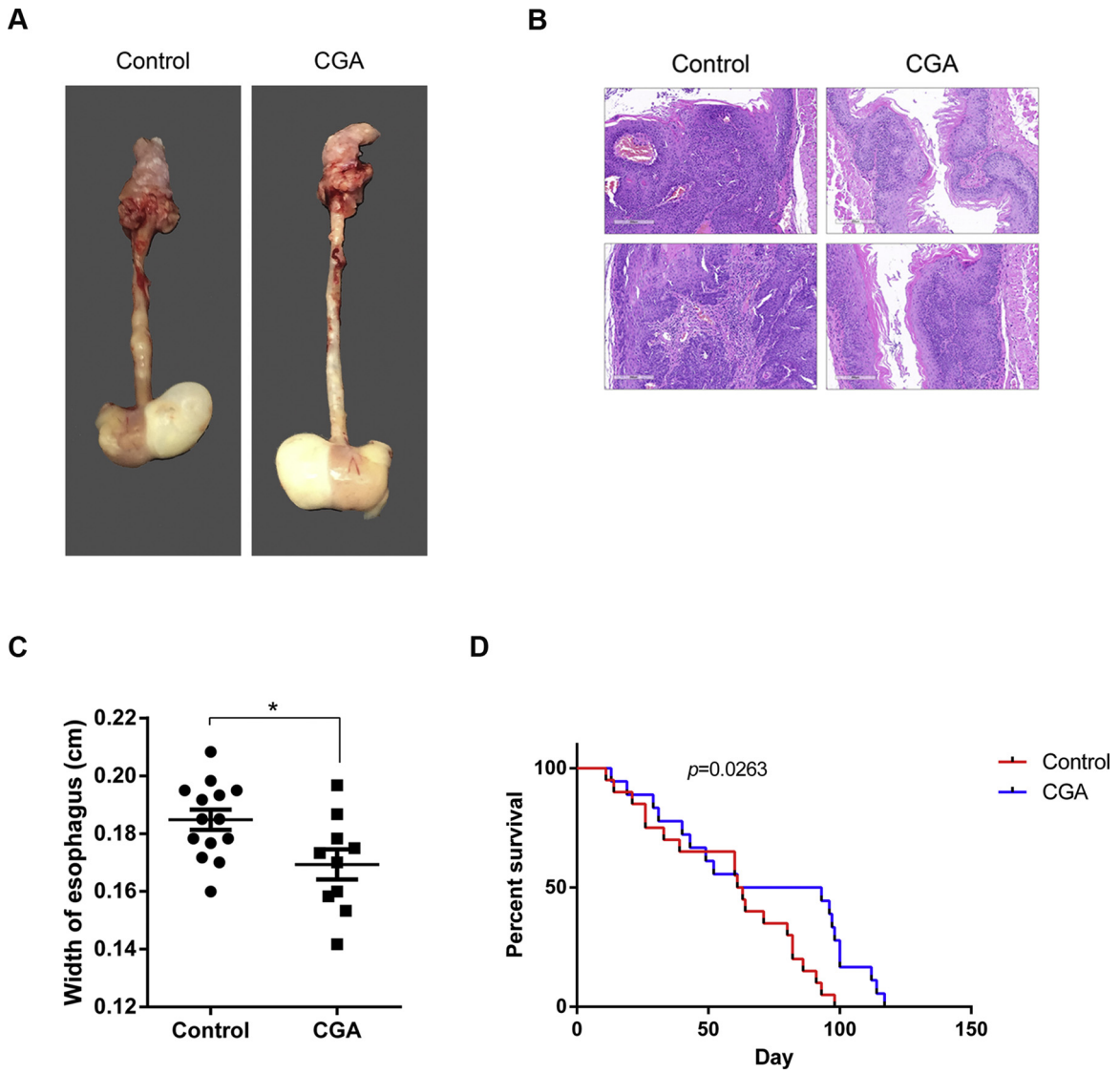

Fig. 4. CGA slows hyperplasia of the esophagus and prolongs survival time in mice. (A) Photos of representative gross esophagi with tongue and stomach from the control and CGA groups (ip 50 mg/kg/d). (**B**) Representative H&E staining of esophagi from the control and CGA groups. (**C**) Measurement of the width of excised esophagi, representing the hyperplasia in esophageal walls. The Mann-Whitney *U*-test was used to analyze the significance. (**D**) Survival curves comparing the control and CGA groups in the 4-NQO-induced ESCC murine model. Data in **D** are represented as the mean ± SEM. $^*P < 0.05$.

to poor prognosis [19,21]. We observed an obvious reduction of BMI1 and SOX2 expression in both a dose- and time-dependent manner in all six ESCC cell lines that were treated with CGA (Fig. 5C and 5D). Downregulation of BMI and SOX2 by CGA occurred more in a time-dependent manner than in a dose-dependent manner, which is consistent with the decrease in Survivin (Fig. 5A and 5B). However, there seemed to be no changes in either OCT4 or Nanog in ESCC cells after CGA treatment (Fig. 5C and 5D). OCT4, Nanog and SOX2 has been reported to work as a transcription complex [19,21], that might be an explanation to the changes of SP cell ratios in different ESCC cell lines.

To further confirm the role of BMI1 and SOX2 played in ESCC cells with CGA treatment, we examined the expression levels of the four stemness markers in esophageal tissues of a 4-NQO-induced ESCC murine model, as well as in tumor tissues of an ectopic xenograft model. Consistent with the expression in ESCC cells, both BMI1 and SOX2 expression decreased in both esophageal tissues and ectopic xenograft tissues of the CGA treatment group compared with those of the control group (Fig. 5E and 5 F, Supplementary Figure S5A and S5B). Meanwhile, no significant changes of Nanog and OCT4 was observed in tumor tissues extracted from either the carcinogen-induced or ectopic xenograft murine models (Supplementary Figure S5C and S5D). Furthermore, when knocking down either BMI1 or SOX2 in ESCC cell lines, the sensitivity to the inhibitory effect of CGA on both proliferation and cell motility was sharply reduced (Supplementary Figure S6). Taken together, our findings confirmed the role of BMI1 and SOX2 as the targets of CGA in ESCC cell growth and motility, suggesting that a potential molecular mechanism of the anti-ESCC effect of CGA was pluripotency regulation.

4. Discussion

Esophageal cancer carries a comparatively poor prognosis among gastrointestinal malignancies, and ESCC is a predominant malignancy worldwide, especially in China. ESCC is characterized by extremely high rates of both incidence and mortality [3]. The deep anatomical position and lack of obvious clinical symptoms make initial diagnoses of late-stage ESCC common. Despite increases in survival rate as a result of advancing treatments, the 5-year survival rate remains dismal, at below 25 % [22]. The available surgical, chemotherapy and chemoradiotherapy options are always accompanied with strong side effects

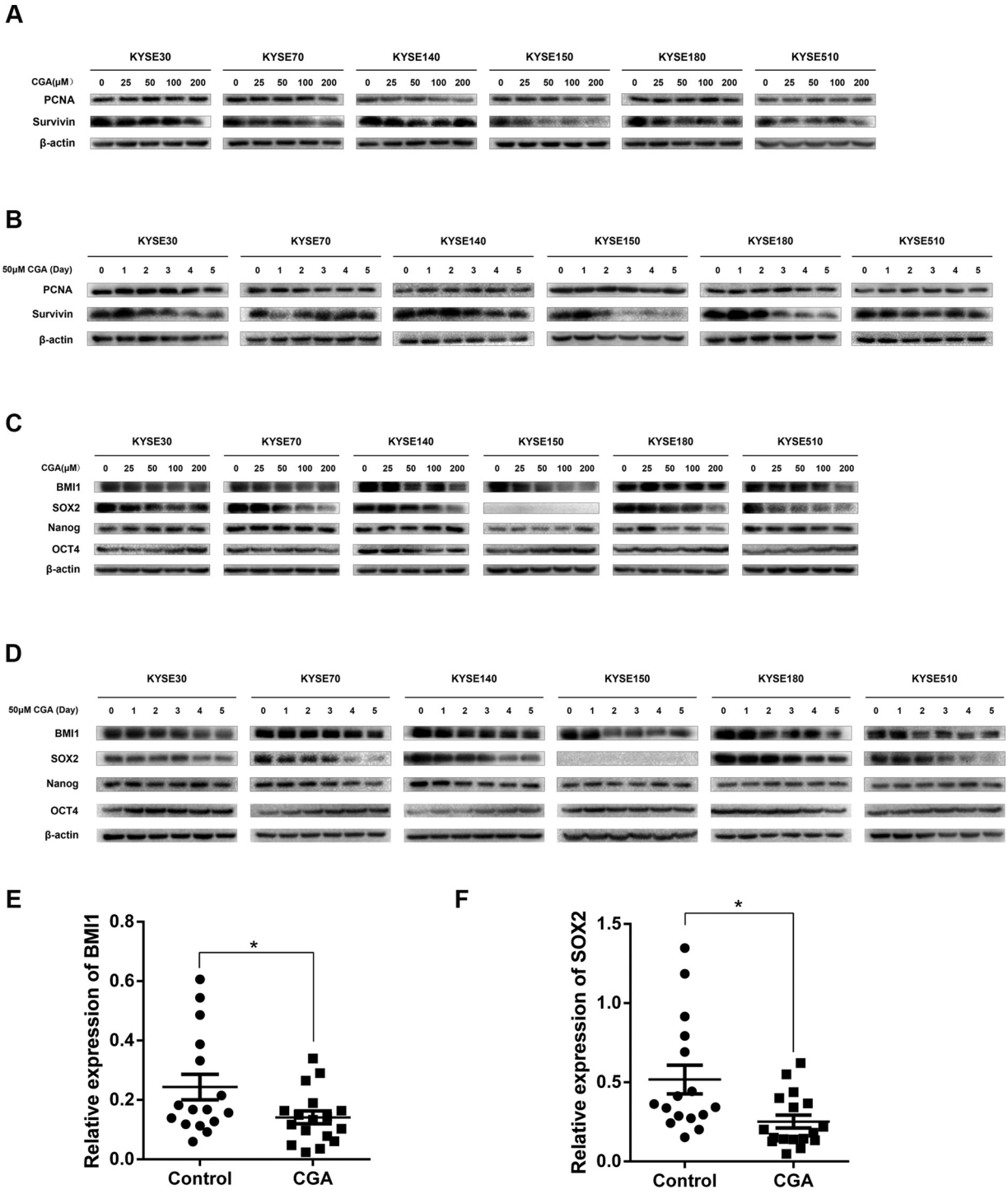

Fig. 5. CGA regulates the expression of several genes in ESCC cells. (**A–B**) PCNA and Survivin protein levels in six ESCC cell lines treated with CGA, shown in a dose- (**A**) or time- (**B**) dependent manner. β-actin is shown as a loading control. (**C-D**) Western blot of stemness transcription factors in six ESCC cell lines treated with CGA, shown in a dose- (**C**) or time- (**D**) dependent manner. β-actin is shown as a loading control. (**E-F**) Quantitative analysis of the protein levels of BMI1 (**E**) and SOX2 (**F**) in esophagi extracted from a 4-NQO-induced carcinogenesis murine model with or without CGA therapy (ip 50 mg/kg/d). Data in **E** and **F** are represented as the mean ± SEM. $^*P < 0.05$.

and a poor quality of life. The development of new anti-ESCC drugs with low toxicities is greatly needed.

CGA, which is derived from traditional Chinese medicine (TCM), exists widely in plants. It is also one of the main polyphenols in the human diet. For example, it is found in apples, coffee beans and tea [6]. Nutrition research has regarded CGA as a nutraceutical for the prevention and treatment of major chronic diseases [23]. Indeed, CGA has been proven to possess many health-promoting properties, such as anti-oxidant [24], anti-inflammatory [25], and anti-microbial properties [26]. In recent years, CGA has been shown to inhibit several types of cancer, such as leukemia, glioblastoma, liver cancer, breast cancer and melanoma, by either inhibiting proliferation or inducing apoptosis *in vitro* [7,9,27–29]. Hence, we asked whether CGA could also inhibit ESCC. To address this problem, we performed a series of assays and established three different animal models. As expected, CGA inhibited ESCC growth both *in vitro* and *in vivo*. In addition, CGA also slowed

hyperplasia of murine esophagi and prolonged the average survival time of mice suffering from ESCC. These findings are similar to the clinical results that are observed when CGA is used to treat other types of cancer, suggesting that CGA is a potential compound for ESCC clinical therapy.

The 4-NQO-induced carcinogenic process produces multifocal tumors, and it is difficult to quantify the hyperplastic and neoplastic. In our study, we observed that the esophageal epithelia of mice in the 4-NQO-treated group were much thicker than those of the control group, which is consistent with a previous report [13]. However, there was no significant difference between the length of esophagi in the two groups (Supplementary Figure S2D). These findings provide us a feasible way for the assessment of the efficacy of CGA on the 4-NQO-induced carcinogenic murine model.

Metastasis, an important symptom of late-stage cancer, is a major cause of death among cancer patients. Thus, we examined the effect of CGA on ESCC cell motility. Indeed, these findings confirmed that CGA suppressed both migration and invasion of ESCC cells *in vitro*, providing an explanation for the prolongation of survival time in mouse models and in patients receiving CGA treatment.

There is a general consensus that CGA plays an antitumor role by arresting cell cycle, inducing cell apoptosis and reducing cell stemness [7–10,30,31]. We wondered whether CGA could inhibit ESCC in the same way. Hence, we performed flow cytometry assays and Western blotting assays to find out the way that CGA works in anti-ESCC. In contrast to what has been reported for other cancer cell types [9], CGA did not induce cell cycle arrest of ESCC cells or affect the expression of PCNA, a marker of the cell cycle, even in a high concentration. However, the results of flow cytometry assays detecting apoptosis and SP cells, as well as the detection of Survivin, an apoptosis-related gene, confirmed that CGA inhibits ESCC *via* inducing apoptosis and reducing cell stemness, which is consistent with the role of CGA played in many other types of cancer, such as lung cancer and leukemia [8,30,31]. Nonetheless, induction of apoptosis might not be able to explain the inhibitory effect of CGA on cell proliferation and motility completely. We consider stemness reduction by CGA in ESCC cells would be a much more important way.

The stemness state of cancer cells is considered to be a major cause of cancer recurrence, drug resistance and metastasis, partly because it is the opposite of cell differentiation. It is reported that SP cells possess the property of stem cells and in some cases function as multipotent stem cells [20]. We first analysed the ratio of SP cells in ESCC cells by flow cytometry. The ratio of SP cells decreased in ESCC cells treated with CGA, compared with those treated with NS, suggesting the possibility of CGA effect on cancer cell stemness. Then, we examined the expression levels of several major stemness transcriptional factors (BMI1, SOX2, Nanog and OCT4) in ESCC. In contrast to the reported that CGA downregulated the expression of SOX2, Nanog and OCT4 in A549 cells [8], we observed an obvious decrease in SOX2 and BMI1, but not OCT4 and Nanog, in both a dose- and time-dependent manner in CGA-treated ESCC cells. Consistently, the expression of SOX2 and BMI1, rather than OCT4 or Nanog, downregulated in tumor tissues extracted from both carcinogen-induced and ectopic xenograft murine models (Supplementary Figure S5), indicating an important role of BMI1 and SOX2 played in antitumor efficacy of CGA on ESCC.

The SP assays revealed a reduction of SP cells in CGA-treated ESCC cells, however, not all the six ESCC cell lines showed significant diminishment of SP cells (Supplementary Figure S4). It is reported that SOX2 is considered a member of the master transcriptional complex, consisting of OCT4/SOX2/Nanog, which can reprogram differentiated cells to generate induced pluripotent stem (iPS) cells [32]. However, in our study, we found no significant changes in Nanog or OCT4 expression (Supplementary Figure S5C and S5D), suggesting that stemness-state regulation by CGA in ESCC might occur by targeting several stemness markers, instead of this transcriptional complex, which might be an explanation to the defective reduction of SP cells in different ESCC cell lines.

BMI1 is associated with tumorigenesis and immortality of cells. The overexpression of BMI1 was correlated with advanced pathological stage and lymph node metastasis in ESCC [19]. SOX2 is amplified at chromosomal region 3q26.3, and its expression is elevated in patients with ESCC. The expression level of SOX2 is significantly associated with higher histological grades and poorer survival in ESCC [19,21]. Downregulation of BMI1 and SOX2 by CGA might provide an explanation for its antitumor efficacy. To identify the possibility of BMI1 and SOX2 functioning as targets of CGA in ESCC cells, we knocked down BMI1 and SOX2 in ESCC cells using siRNAs, respectively, and then we detected cell proliferation and motility of these cells with CGA treatment. As expect, no significant inhibitory effect of CGA was observed in either cell proliferation or motility of these cells after knocking down BMI1 and SOX2, respectively (Supplementary Figure S6).

As a botanical drug, CGA has similar effect on the phenotype of different types of cancer, yet the molecular mechanisms of CGA seems to be quite different from each other. For example, in glioma, CGA could induce the differential of glioma cells to reduce the malignancy [10], as well as regulate the immune cells and the immune microenvironment of glioma [7]. In lung cancer, CGA decreased cell proliferation of A549 cells, and down-regulated gene expression of Nanog, OCT4 and SOX2 [8]. In breast cancer, CGA disturbed the cell cycle and arrested the cancer cell at the G1 phase with a reduction of S-phase *via* binding PKC [9]. Moreover, our study identified that CGA could induce apoptosis and reduce cell stemness instead of cell cycle arrest in ESCC, it downregulated the expression of SOX2 and BMI1, but had no effect on Nanog or OCT4. It seems that CGA functions with multiple-targets synergism mechanisms. However, the molecular mechanisms of CGA in anti-ESCC and other types of cancers need largely more molecular evidence and exploration.

In summary, we demonstrated that CGA inhibits ESCC growth and motility and prolongs survival time. The antitumor effect of CGA is mediated by a reduction in the expression of SOX2 and BMI1. Our findings identify BMI1 and SOX2 as targets for inhibitory regulation of ESCC cell growth by CGA, providing evidence of potential new clinical indications for CGA in ESCC therapy.

Author contributions

Zhan Yun, Jiang Jiandong and Han Yanxing conceived and designed the study. Zhan Yun, Li Rui, Feng Chenlin, Li Xiaolin and Huang Shuai performed experiments. Zhan Yun, Li Rui and Wang Lulu performed the statistics data. Zhan Yun wrote the manuscript with contributions from Wang Lulu, Liu Zhihua and Han Yanxing.

Declaration of Competing Interest

The authors declare no potential conflicts of interest.

Acknowledgements

This work was supported by the Peking Medical School Foundation for Young Scholars (3332015165); the CAMS Innovation Fund for Medical Sciences (No. 2016-I2M-1-001, No. 2017-I2M-1-008, No. 2017-I2M-B&R-09); the Drug Innovation Major Project (No. 2018ZX09711001-003-002, No. 2018ZX09711001-011-003, No. 2016ZX09101017, No. 2015ZX09102-023-004); and Beijing Key Laboratory (No. BZ0150, No. Z141102004414062).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2019.109602.

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