

# Caffeic acid phenethyl ester suppressed growth and metastasis of nasopharyngeal carcinoma cells by inactivating the NF- $\kappa$ B pathway

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**Purpose:** Caffeic acid phenethyl ester (CAPE) is the main polyphenol extracted from honeybee propolis, which inhibits the growth of several kinds of tumor. This study aimed to assess the inhibitory effect of CAPE in nasopharyngeal carcinoma (NPC), evaluate the synergistic action of CAPE in radiotherapy sensitivity of NPC cell lines and further elucidate the possible molecular mechanism involved.

**Materials and methods:** CCK-8 assay was used to analyze cell proliferation ability. Colony formation assay was used to evaluate the clonogenic ability and radio-sensitiveness of NPC cells by CAPE treatment. Wound-healing and transwell assay were used to assess the motility of cells. The expression of key molecules of the epithelial–mesenchymal transition (EMT) was determined by western blot analysis and changes in radiation sensitivity were measured by colony-formation assay. cDNA microarray analysis was used to determine differentially expressed genes with and without CAPE treatment, with Gene Ontology enrichment of gene function and KEGG pathways determined. Cell cycle and apoptosis were detected by flow cytometry and western blot analysis.

**Results:** CAPE suppressed the viability of NPC cell lines time- and dose-dependently. It induced apoptosis in NPC cells along with decreased expression of Bcl-XL and increased cleavage of PARP and expression of Bax. G1 phase arrest was induced by CAPE with over expression of CDK4, CDK6, Rb and p-Rb. The migratory and invasive ability of NPC cells was decreased by the EMT pathway. The irradiation sensitivity of NPC cells was enhanced with CAPE treatment. CAPE specifically inhibited nuclear factor  $\kappa$ B (NF- $\kappa$ B) signaling pathway by suppressing p65 subunit translocation from cytoplasm to nucleus. CAPE treatment was synergistic with chemotherapy and radiotherapy.

**Conclusion:** CAPE may inhibit the proliferation and metastasis of NPC cells but enhance radiosensitivity in NPC therapy by inhibiting the NF- $\kappa$ B pathway. CAPE could be a potential therapeutic compound for NPC therapy.

**Keywords:** nasopharyngeal carcinoma, CAPE, proliferation, metastasis, NF- $\kappa$ B pathway

## Introduction

Nasopharyngeal carcinoma (NPC) is a malignancy derived frequently from the epithelium of Rosenmüller fossa. The racial/ethnic and geographic distribution of NPC is distinctive, with high incidence in some countries including southern China, Southeast Asia, the Arctic and the Middle East/North Africa.<sup>1</sup> The interaction of genetic susceptibility, environmental factors and Epstein–Barr virus (EBV) infection contributes to the pathogenesis and development of NPC.<sup>2</sup>

Radiotherapy is the conventional treatment for NPC because of the cancer's high sensitivity to radiotherapy. With the development of intensity-modulated radiation, 5-year survival in early diagnosed NPC (stage I and II) is more than 80% but remains less than 10% at stage IV.<sup>3</sup> Distant metastasis is the major cause of treatment failure in NPC. Novel therapeutic approaches are needed to improve the outcome with advanced NPC.

Caffeic acid phenethyl ester (CAPE) is the main polyphenol extracted from honeybee propolis and polyphenol and can directly bind to the signaling molecules involved in carcinogenesis and regulate their activity.<sup>4</sup> It has anti-bacterial and viral infection, anti-oxidant, anti-inflammatory and anti-tumor properties.<sup>5-8</sup> In several kinds of human cancer, including prostate cancer, breast cancer, ovarian cancer, oral cancer, CAPE inhibited angiogenesis and metastasis and increased radiation sensitivity.<sup>9-11</sup> Recently, CAPE was found to attenuate NPC cell (TW04) proliferation and invasion by upregulating N-Myc downstream regulated 1 via the mitogen-activated protein kinase pathway and by inhibiting the phosphorylation of signal transducer and activator of transcription 3.<sup>12</sup> However, the effect of CAPE in EBV-positive NPC cells remains unclear, as does the synergistic effect with radiotherapy and chemotherapy.

Pro-inflammatory stimuli in the tumor microenvironment activate the NF- $\kappa$ B pathway. Constitutive activation of nuclear factor  $\kappa$ B (NF- $\kappa$ B) signals mediates critical events in the initiation and progression of human cancers by regulating cell proliferation, apoptosis, cell transformation and immunosuppression.<sup>13,14</sup> Latent-membrane protein 1 (LMP1), as an EBV oncoprotein, is a potent activator of NF- $\kappa$ B signaling.<sup>15</sup> LMP1 can phosphorylate the IKK complex and induce I $\kappa$ B $\alpha$  degradation, thus resulting in constitutive NF- $\kappa$ B activation. Whole-exome sequencing data detected mutation of several NF- $\kappa$ B pathway negative regulators, including CYLD lysine 63 deubiquitinase (CYLD) and TNF receptor-associated factor 3 (TRAF3) in NPC primary tumors.<sup>16,17</sup> Constitutive activation of NF- $\kappa$ B was detected in NPC cell lines and primary tumors,<sup>18</sup> so targeting inhibitors may be a promising therapeutic for NPC.

CAPE can specifically inhibit NF- $\kappa$ B cell signaling.<sup>19</sup> In oral cancer, CAPE remarkably suppressed the proliferation of TW2.6 cells. The half-maximal inhibitory concentration (IC<sub>50</sub>) was 2-4 times higher for normal human oral fibroblast cells and buccal mucosal fibroblast cells than oral cancer cells.<sup>8</sup> Therefore, the toxic effects of CAPE may be lower for normal cells than tumor cells, so CAPE could be a potential agent for cancer therapy.

The aim of this study was to evaluate the effect of CAPE on the biological behavior of NPC cell lines, including proliferation, apoptosis, cell cycle arrest and metastasis, and further elucidate the underlying mechanisms. The synergistic effect of CAPE with radio- and chemotherapy was also investigated.

## Materials and methods

### Cell culture and chemicals

This study was approved by the ethics committee of Guangxi Medical University. CNE2 and HK1 cell line were maintained in our laboratory. CNE2-EBV was kindly provided by Prof Musheng Zeng's group (Sun Yat-sen University Cancer Center, China), and HK1-EBV was kindly gifted by Prof Sai-Wah Tsao (Hong Kong University, China). CNE2-EBV and HK1-EBV cells were established as described previously.<sup>20</sup> These cell lines were cultured in high glucose DMEM supplemented with penicillin G (100 U/mL), streptomycin (100  $\mu$ g/mL) and 10% fetal bovine serum (FBS). Cells were grown at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and were routinely sub-cultured by using 0.25% (w/v) trypsin-EDTA solution. CAPE was purchased from Sigma (St. Louis, MO, USA).

### CCK-8 proliferation assay

An amount of 2 $\times$ 10<sup>3</sup> cells/well were seeded in 96-well plates. Cell viability was assessed by CCK8 assay. All experiments were repeated three times. The amount of yellow-color formazan dye is directly proportional to the number of living cells and was determined by measuring the absorbance at 450 nm with a multi-mode plate reader (SYNERGY-HTX, BioTek Instruments, USA). IC<sub>50</sub> values were calculated by probit regression with GraphPad software.

### cDNA microarray assay

HK1 and HK1-EBV cells were treated with 0.1% DMSO or 100  $\mu$ M CAPE for 24 hrs. cDNA microarray was performed at Shanghai Genminix Informatics. The genes with differential expression were screened, and further GO and KEGG pathway analysis were performed by using the GCBI online platform. The Gene Expression Omnibus (GEO) accession number for the data sets reported in this paper is GSE126608. The following link has been created to allow review of record while it remains in private status: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126608>

## Cell apoptosis assay and measurement of caspase3/7 activity

Cells were seeded at  $3 \times 10^5$  cells/well in 6-well plates for 24 hrs. After CAPE treatment for 24 hrs, cells were harvested. Cell apoptosis was measured by using the APC-Annexin V apoptosis detection kit (BD Biosciences, San Diego, CA) according to the manufacturer's instructions and analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA). Caspase-3/7 activity of NPC cells was measured by using the Caspase-Glo 3/7 Assay Kit (Promega Biotech, USA) according to the manufacturer's instructions. Luminescence of cell lysis was determined by using a multi-mode reader (BioTek SYNERGY-HTX Instruments, USA). Three independent experiments were performed for each assay.

## Cell cycle assay

Cells with and without CAPE treatment (with IC<sub>50</sub> concentration of each cell line) for 24 hrs were harvested, washed with cold phosphate buffered saline (PBS) once, then fixed with 75% cold ethanol for 2 hrs or overnight at 4°C (samples can be stored at -20°C for 1 month). Before staining, fixed cells were harvested and washed in cold PBS. An amount of 100 µL RNase A per sample was added to resuspend fixed cells precipitated in 37°C water bath for 30 mins, and then PI solution was added at 4°C for 30 mins. The percentage distribution of cells in the phases of the cell cycle was determined by flow cytometry with the Cell Cycle Detection Kit (Kaiji, Nanjing, China). Three independent experiments were performed for each assay.

## Western blot analysis

Cells were lysed on ice for 30 mins in RIPA with protease/phosphatase inhibitor cocktail. The protein concentration was measured with a BCA assay kit (Beyotime, Shanghai). Protein samples were separated by SDS-PAGE gel and transferred to NC membrane. The membrane was blocked with 5% bovine serum albumin and incubated with primary antibodies at 4°C overnight. Proteins of interest were detected with appropriate IRDye™ 800/700CW secondary antibodies and detected by using an infrared imaging system (LICOR Odyssey) (Thermo Scientific, Rockford, IL, USA). Protein levels were normalized to that of GAPDH and quantified by using Image J (US National Institutes of Health).

## Colony-formation assay

Colony-formation assay was used to assess the growth inhibitory effects of CAPE on cells in six-well tissue culture plates.

Appropriate numbers of NPC cells were seeded into culture plates, then treated with 20 and 40 µM CAPE for 24 hrs. Plates were maintained in fresh culture medium containing 10% FBS for 10–14 days. Finally, colonies were fixed with 4% paraformaldehyde solution and stained with 1% crystal violet dye; colonies containing more than 50 cells were counted. Experiments were repeated at least three times.

## Clonogenic survival assay

Cells in log phase were counted, and appropriate numbers of NPC cells were seeded into six-well plates in triplicate. The number of cells plated was increased depending on the radiation dose for obtaining a countable number of colonies. After 24 hrs, to calculate radiosensitivity effects, cells were pre-incubated with CAPE for 24 hrs, then exposed to graded doses of X-ray radiation. Irradiation involved use of a 6-MeV X-ray photon produced by a linear accelerator (Elketa, Sweden) at 200 cGy/min. Colony staining was performed as described previously. The surviving cell fraction was calculated as number of colonies/(number of cells × control plating efficiency). All experiments were performed three times.

## Transwell assay and wound-healing assay

The invasiveness of NPC cells was evaluated in 24-well transfected chambers (Costar, Corning, MA, USA) according to the manufacturer's instructions. Briefly, transwell chambers with an 8-µm diameter pore membrane were coated with 200 µL Matrigel at 200 µg/mL and incubated overnight. Cells ( $7 \times 10^4$ ) in 200 µL serum-free medium were seeded into the upper transwell chamber and the lower chamber was filled with 800 µL DMEM containing 10% FBS to induce chemotaxis. After 24–48 hrs of incubation at 37°C in a humidified 5% CO<sub>2</sub> atmosphere, cells were fixed in paraformaldehyde and stained with crystal violet, and cells that invaded the pores to the lower surface of the filter were counted under a microscope. Three invasion chambers were used per condition. The values obtained were calculated by averaging the total number of cells from three filters. Wound healing assay was performed with iBidi Culture-Inserts (iBidi, Germany). NPC cells treated with or without CAPE were seeded in the space of Culture-Inserts; after 12-hr attachment, the insert was removed, then cell patches were overlaid with culture medium. A single wound was created in the plate and wound areas were visualized under an optical microscope at magnification ×100. Cell migration capability was expressed by gap closure.

## Statistical analysis

Statistical analysis was performed with SPSS 22.0 (SPSS Inc., Chicago, IL, USA). All data are presented as mean  $\pm$ SD of at least three independent experiments. Comparisons between two groups involved Independent samples *t* test.  $p < 0.05$  was considered statistically significant.

## Results

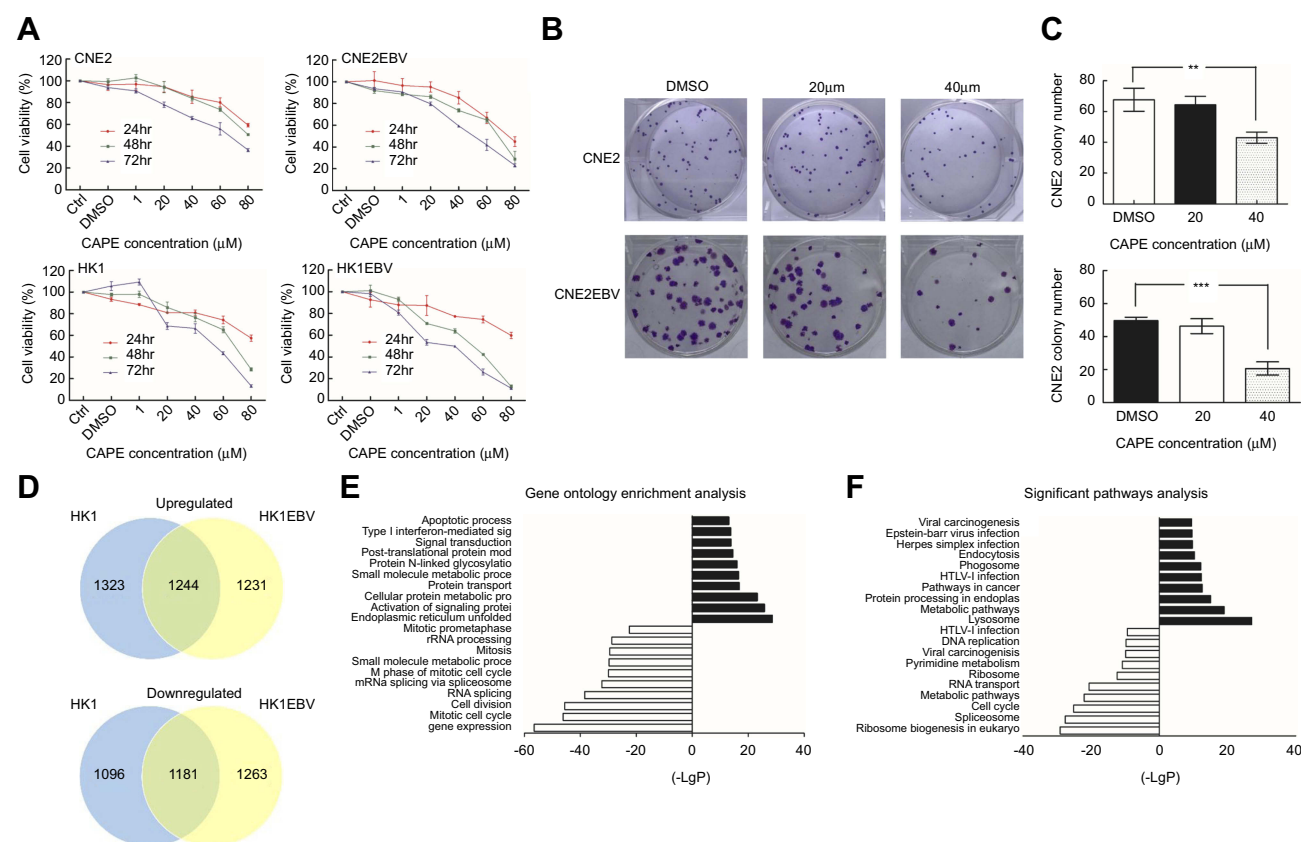
### CAPE suppressed the proliferation and colony-formation ability of NPC cells

To investigate the effect of CAPE on growth of NPC cells, we used CCK8 assay with CNE2, CNE2-EBV, HK1 and HK1-EBV cell lines treated or not with CAPE at different concentrations. As compared with the DMSO control, with increasing CAPE concentration and time of treatment, the viability of these cells remarkably decreased (Figure 1A). The IC<sub>50</sub> values for CAPE with 24-hr treatment were

calculated for the cells: CNE2 (110  $\mu$ M), CNE2-EBV (80  $\mu$ M), HK1 (110  $\mu$ M) and HK1-EBV (110  $\mu$ M). As compared with DMSO treatment, with 40  $\mu$ M CAPE treatment for 24 hrs, lower than the IC<sub>50</sub>, CNE2 and CNE2-EBV cells produced fewer colonies (Figure 1B and C), which suggests that the colony-formation ability of NPC cells was significantly suppressed by CAPE. With 20  $\mu$ M CAPE, the colony number of NPC cells was reduced but not significantly. Non-malignant nasopharyngeal epithelial cell lines NP69 and NP460 were more resistant to CAPE treatment (data not shown).

### Differentially expressed genes regulated by CAPE in NPC cells were mainly involved in apoptosis and cell cycle

To understand the suppressive effect of CAPE on NPC cell lines, we used cDNA microarray assay to screen NPC cell gene profiles regulated by CAPE. We found 4844 and 4919



**Figure 1** Effect of CAPE on viability and colony formation ability of nasopharyngeal carcinoma (NPC) cell lines. **(A)** CNE2, CNE2-EBV, HK1 and HK1-EBV cells were treated with 1–80  $\mu$ M CAPE for 24, 48 and 72 hrs and cell viability was evaluated by CCK8 assay. Data are mean  $\pm$ SD of three independent experiments. **(B)** Clonogenic ability of CNE2 and CNE2EBV cells with concentrations of CAPE. Cells were treated with 20 and 40  $\mu$ M CAPE for 24 hrs, replated, and incubated for 12–14 days to form colonies. Representative colony formation assay of CNE2 and CNE2EBV cells at concentrations of CAPE after crystal violet staining. **(C)** Number of colonies of CNE2 and CNE2EBV cells per microscopic field. Data are mean  $\pm$ SD from three independent experiments. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  compared to control. **(D)** Venn diagram showing the overlap of genes with significantly altered expression after exposure to 100  $\mu$ M CAPE for 24 hrs in HK1 and HK1-EBV cell lines. **(E)** The top 10 significantly upregulated and downregulated Gene Ontology categories in NPC cells after CAPE treatment. **(F)** The top 10 significant KEGG pathways of upregulated and downregulated DEGs.

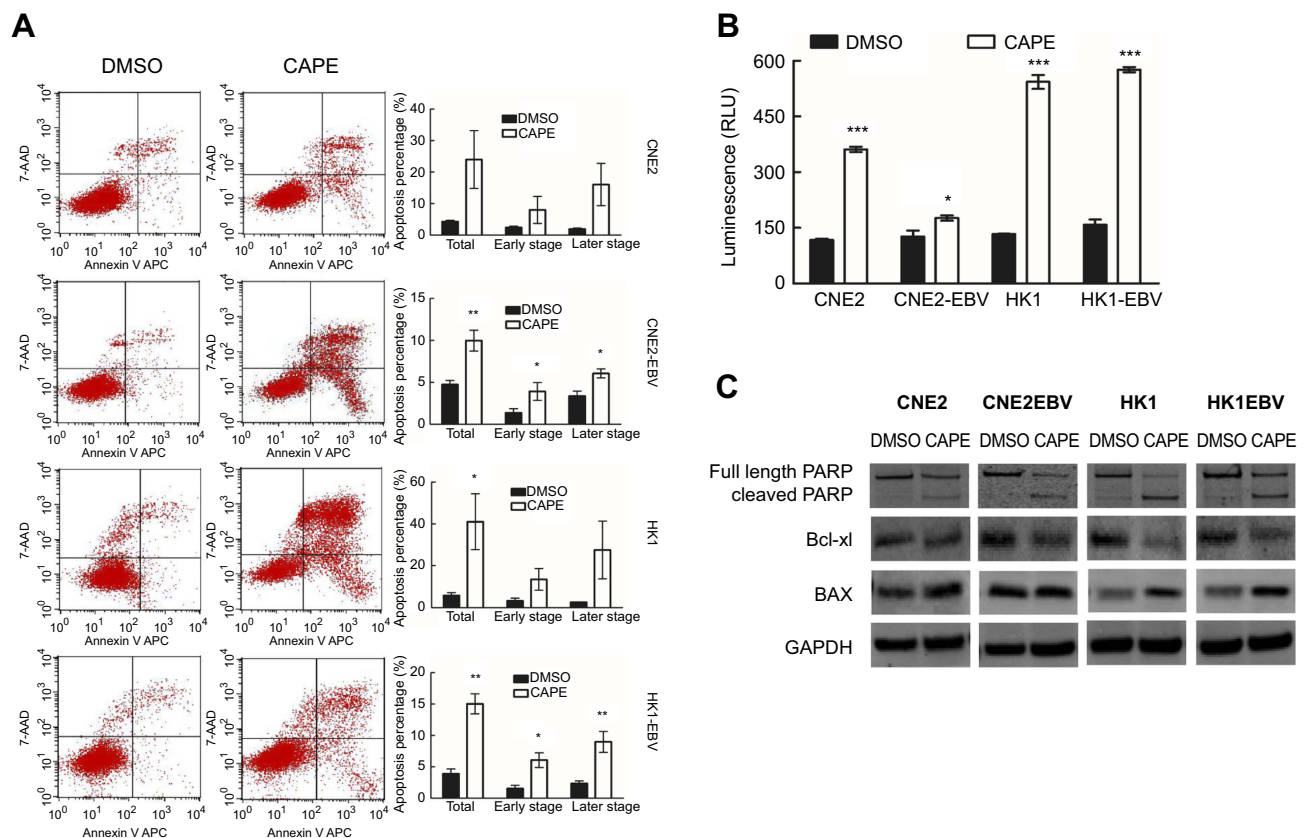


differentially expressed genes (DEGs) in HK1 and HK1-EBV cell lines, respectively: 1244 genes were downregulated and 1181 were upregulated more than 1.2-fold in both cell lines (Figure 1D). The top 10 significantly upregulated and downregulated GO categories were detected by the negative logarithm of the *p*-value (Figure 1E). Downregulated DEGs were mainly involved in the regulation of cell proliferation, such as the mitotic cell cycle, M phase of mitotic cell cycle and mitosis. Upregulated DEGs were involved in tumor cell physiological processes including endoplasmic reticulum unfolded protein response, activation of signaling protein activity involved in unfolded protein response, cellular protein metabolic process, protein transport, small molecule metabolic process, protein N-linked glycosylation via asparagine, post-translational protein modification, signal transduction, type I interferon-mediated signaling pathway and apoptotic process. To identify the key pathways that DEGs were involved in, we used KEGG pathway analysis.

The cell cycle pathway was included in the top 10 significant pathways of downregulated DEGs (Figure 1F). Therefore, the cell cycle pathway plays an important role in CAPE treatment. Next, we further analyzed CAPE-induced change of cell cycle and cell apoptosis.

## CAPE-induced NPC cell apoptosis

From the microarray analysis, to explore whether the decreased cell viability with CAPE treatment resulted from inducing apoptosis, we evaluated the apoptosis of NPC cells with or without CAPE treatment by flow cytometry. As compared with the DMSO control, after treatment with IC<sub>50</sub> doses of CAPE for 24 hrs, the proportion of total apoptotic cells was increased: CNE2, 23.99 ± 9.17%, *p*=0.068; CNE2-EBV, 9.98 ± 1.24%, *p*=0.007; HK1, 41.05 ± 13.33%, *p*=0.037; HK1-EBV, 15.05 ± 1.60%, *p*=0.001. CAPE treatment mainly increased later-stage apoptosis (Figure 2A). With CAPE treatment, caspase 3/7 activity was markedly increased (Figure 2B), which



**Figure 2** CAPE treatment induced apoptosis of NPC cells depending on increasing caspase3/7 activity and regulated apoptotic-related protein expression. After treatment with CAPE for 24 hrs, cells underwent flow cytometry analysis. **(A)** Apoptosis assay of NPC cells by flow cytometry. The histograms on the right show proportion of cells in apoptotic phase. Independent samples *t*-test was performed for the percentage of cell apoptosis. **(B)** After treatment of CAPE or DMSO for 24 hrs, the activity of caspase-3/7 was measured by Caspase-Glo 3/7 Assay. Data are mean ± SD of three independent experiments performed in duplicate. **(C)** Western blot analysis of Bcl-xl, BAX, PARP and cleaved PARP levels in cultured NPC cells treated or not with the indicated concentration of CAPE for 24 hrs; GAPDH was a loading control. Data are mean ± SD from three independent experiments. \**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001, compared to DMSO.

suggests that caspase3/7 are involved in CAPE-induced apoptosis in NPC cells. To investigate the mechanism by which CAPE causes the increase in apoptosis, we analyzed the expression of related proteins that may be involved in the apoptotic pathway, such as cleaved poly ADP ribose polymerase (PARP), Bax and Bcl-x1 (Figure 2C). The protein level of cleaved PARP was upregulated in NPC cells exposed to CAPE. The expression of Bcl-x1 protein was downregulated and that of Bax protein was upregulated (Figure 2C).

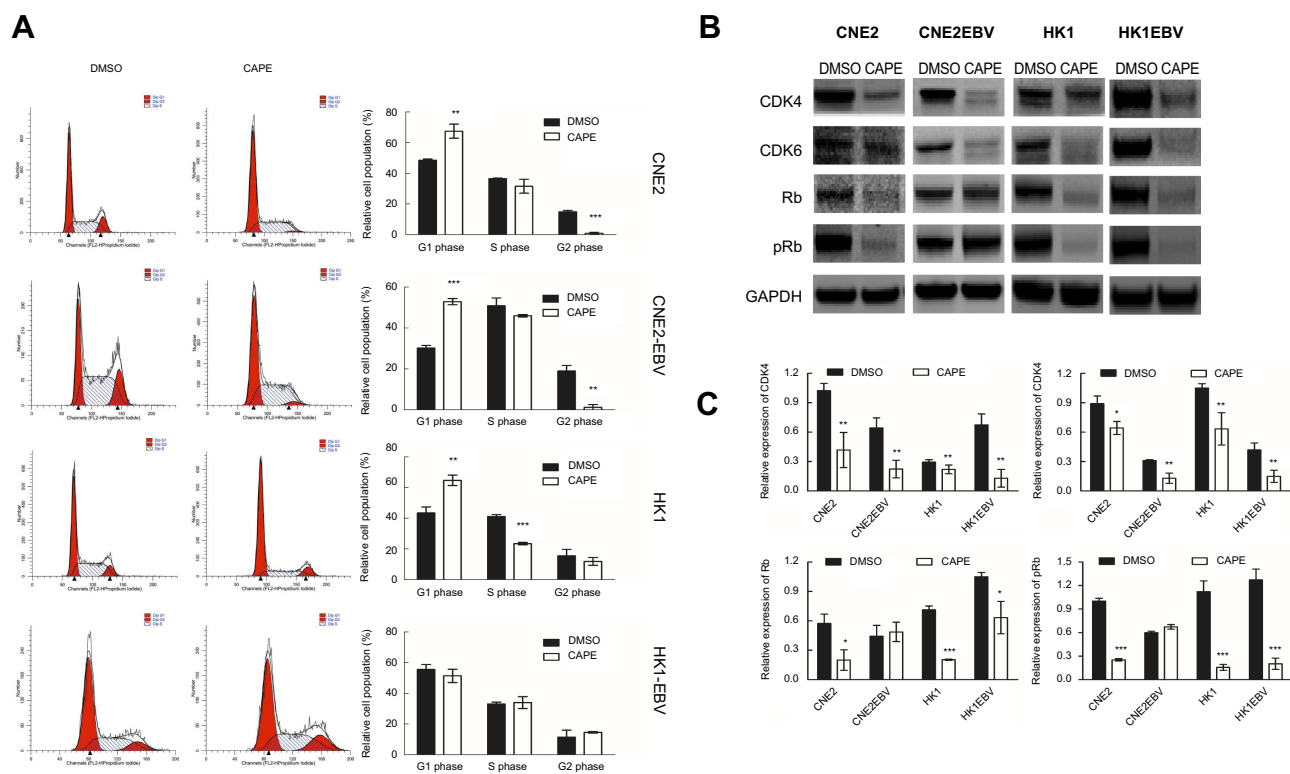
## CAPE-induced G1 cell cycle arrest in NPC cells

To understand the effect of CAPE in the cell cycle of NPC cells, we analyzed the distribution of cells in the phases of the cell cycle by flow cytometry. We found reduced number of cells in the G2/M phase and increased number in the G1 phase in CNE2 and CNE2-EBV cells under CAPE treatment (Figure 3A). In HK1 cells, CAPE treatment led to an increase

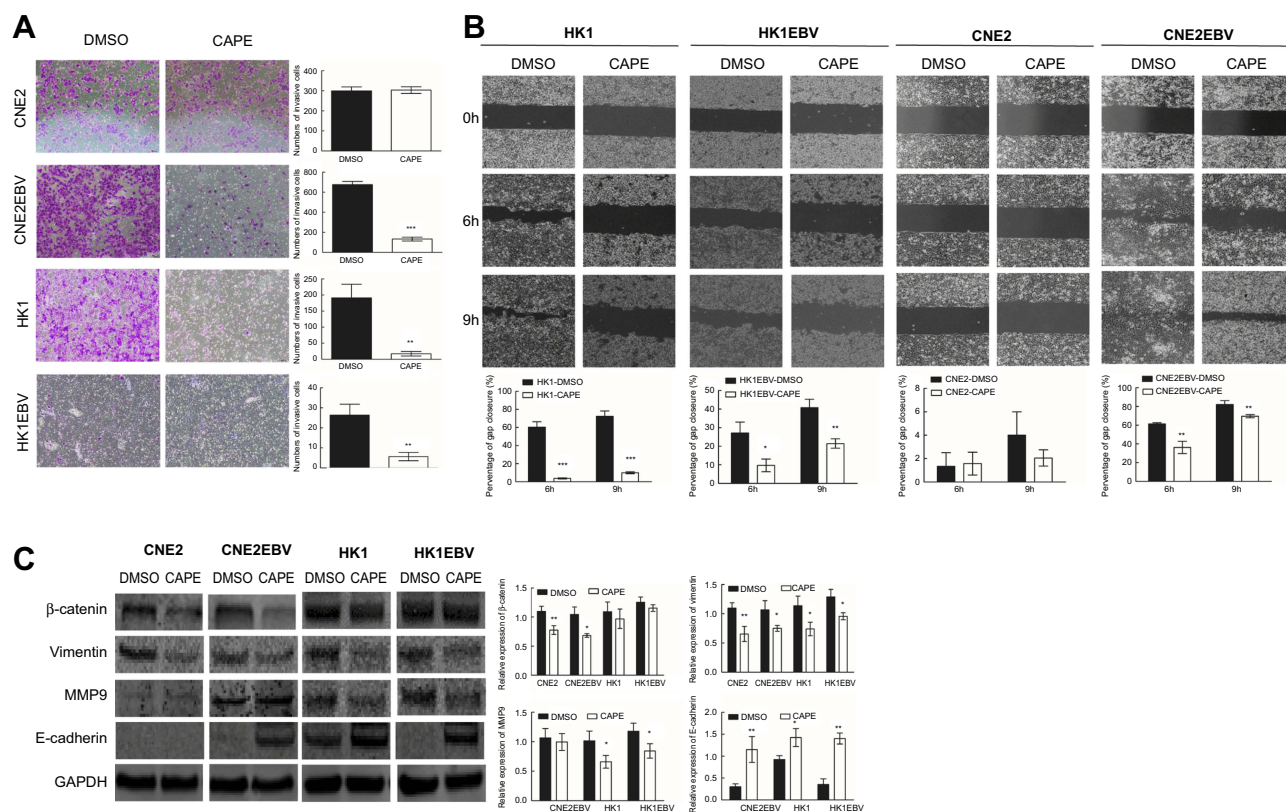
in the G1-phase population and a decrease in the S-phase population (Figure 3A), with no significant effect in HK1-EBV cells. Our data suggest that CAPE induced G1 cell cycle arrest in CNE2, CNE2-EBV and HK1 cells. CDK6 is an important regulatory factor for cell-cycle G1 phase progression and G1/S transition,<sup>21</sup> and CDK4 has been shown to phosphorylate and thus regulate the activity of the tumor suppressor protein Rb. In the early phase of the cell cycle, G1 transition is mainly regulated by cyclin D1 complexed with CDK4 and/or CDK6.<sup>21</sup> Our NPC cells showed downregulated Rb, p-Rb, CDK4, and CDK6 with CAPE treatment (Figure 3B and C).

## CAPE inhibits the invasion and migration of NPC cells in vitro

To further determine the effect of CAPE on NPC cell migration and invasive abilities, we used transwell and wound-healing assay. CAPE treatment markedly attenuated the migratory ability in CNE2-EBV, HK1 and HK1-EBV but



**Figure 3** CAPE treatment inhibits cell cycle progression in NPC cells by downregulating cell cycle-related proteins (CDK4, CDK6, Rb and p-Rb) Cells were treated with CAPE for 24 hrs, harvested, and stained with propidium iodide for flow cytometry of cell cycle distribution. **(A)** Representative flow cytometry of NPC cells; the right histograms represent the proportion of cells in the G1, S and G2/M phases. Student's *t*-test was performed for the percentage of cell cycle distribution. **(B)** Western blot assay of the protein expression of CDK4, CDK6, Rb and pRb in cultured NPC cells treated or not with the indicated concentration of CAPE for 24 hrs; GAPDH was a loading control. **(C)** Distribution of cell cycle. Results are the mean of three independent experiments performed in duplicate. Data are mean  $\pm$  SD from three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , compared with DMSO.



**Figure 4** CAPE inhibits the invasion and migration of NPC cells in vitro. **(A)** Transwell invasion assay of CNE2, CNE2EBV, HK1 and HK1EBV cells with or without CAPE treatment. Images were captured at  $\times 100$ . Data are representative of three independent experiments. The corresponding graphs represent the number of invasive NPC cells per microscopic field. **(B)** Wound-healing assay was used to assess NPC cell motility after CAPE treatment. The corresponding histograms represent the percentage of gap closure per microscopic field. **(C)** Western blot analysis of protein levels of EMT-related factors after CAPE treatment for 24 hrs. Data are mean $\pm$ SD from three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with DMSO. Histograms of the relative expression of  $\beta$ -catenin, Vimentin, MMP9 and E-cadherin.

not CNE2 cells (Figure 4A). Similarly, on wound-healing assay, cellular motility was significantly slower with CAPE than DMSO treatment (Figure 4B). Thus, CAPE could inhibit NPC cell migration and invasion in vitro. Western blot analysis revealed that the expression of E-cadherin was elevated in CNE2-EBV ( $p = 0.008$ ), HK1 ( $p = 0.017$ ) and HK1-EBV ( $p = 0.001$ ), vimentin was remarkably downregulated in all of four cell lines,  $\beta$ -catenin was decreased in CNE2 ( $p = 0.009$ ) and CNE2EBV ( $p = 0.018$ ), while MMP9 was decreased in HK1 ( $p = 0.035$ ) and HK1-EBV ( $p = 0.033$ ) upon the treatment of CAPE (Figure 4C). Our data suggest that CAPE hinders migratory and invasive ability of NPC cells by reversing the epithelial–mesenchymal transition (EMT) pathway.

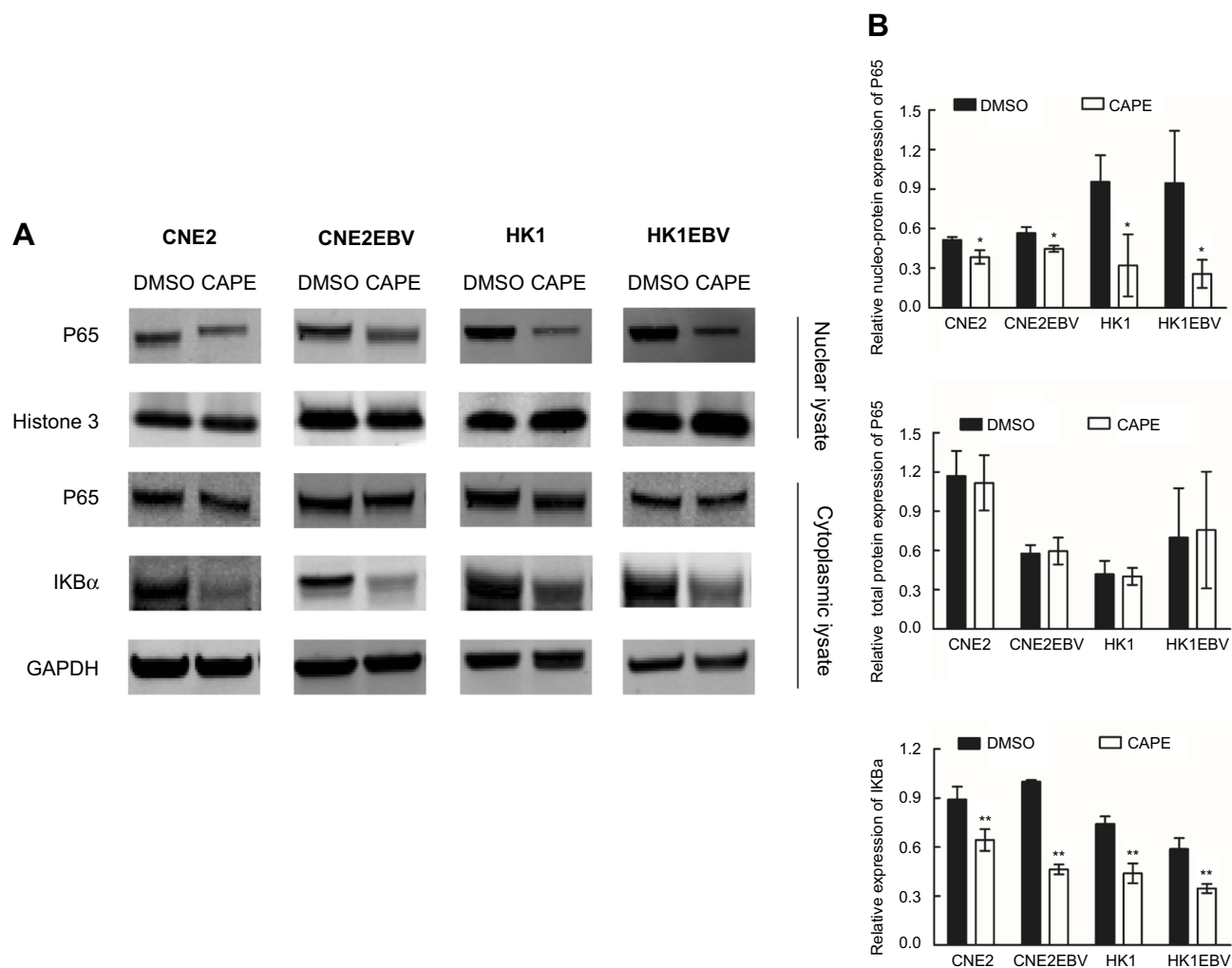
## CAPE impeded nuclear translocation of p65 in NPC cells

Because CAPE has been reported as an NF- $\kappa$ B inhibitor, we thus analyzed the expression of p65 in both cytoplasm and nucleus in NPC cell lines after CAPE treatment. The

expression of I $\kappa$ B- $\alpha$  was decreased significantly (Figure 5A and B), which indicated that more p65 should be free to transfer to the nucleus and act as a transcriptional factor. However, we found a marked reduction of p65 level in the nuclear fraction but not cytoplasm (Figure 5), so CAPE mainly hindered the nuclear translocation of p65, thereby suppressing the NF- $\kappa$ B signaling pathway. The underlying mechanism remains to be further elucidated.

## CAPE enhanced the sensitivity of radiation and cisplatin in NPC cell lines rendering NPC cells more sensitive to cisplatin

To determine the effect of CAPE on the radiosensitivity of NPC cells, CNE2 and CNE2-EBV cells were pretreated with CAPE (20 and 40  $\mu$ M) for 24 hrs, then exposed to ionizing radiation (IR). Treatment with CAPE (40  $\mu$ M) led to significant radiosensitization in both CNE2 and CNE2-EBV cells (Figure 6A). The sensitizing enhancement ratios (SERs) at a surviving fraction of 40 were 1.228 for CNE2



**Figure 5** CAPE treatment suppressed the translocation of NF- $\kappa$ B subunit p65 from cytoplasm to nucleus and downregulated I $\kappa$ B $\alpha$  in NPC cells. NPC cells were preincubated with CAPE for 24 hrs, and cytoplasmic and nuclear extracts were harvested. **(A)** Western blot analysis of cytoplasmic levels of p65 and I $\kappa$ B $\alpha$  expression and nuclear level of p65 expression. GAPDH was a loading control for cytoplasm and Histone 3 for nuclei. **(B)** Histograms of the relative expression of cytoplasmic p65, nuclear p65 and I $\kappa$ B $\alpha$ . \* $p < 0.05$ , \*\* $p < 0.01$  compared with DMSO.

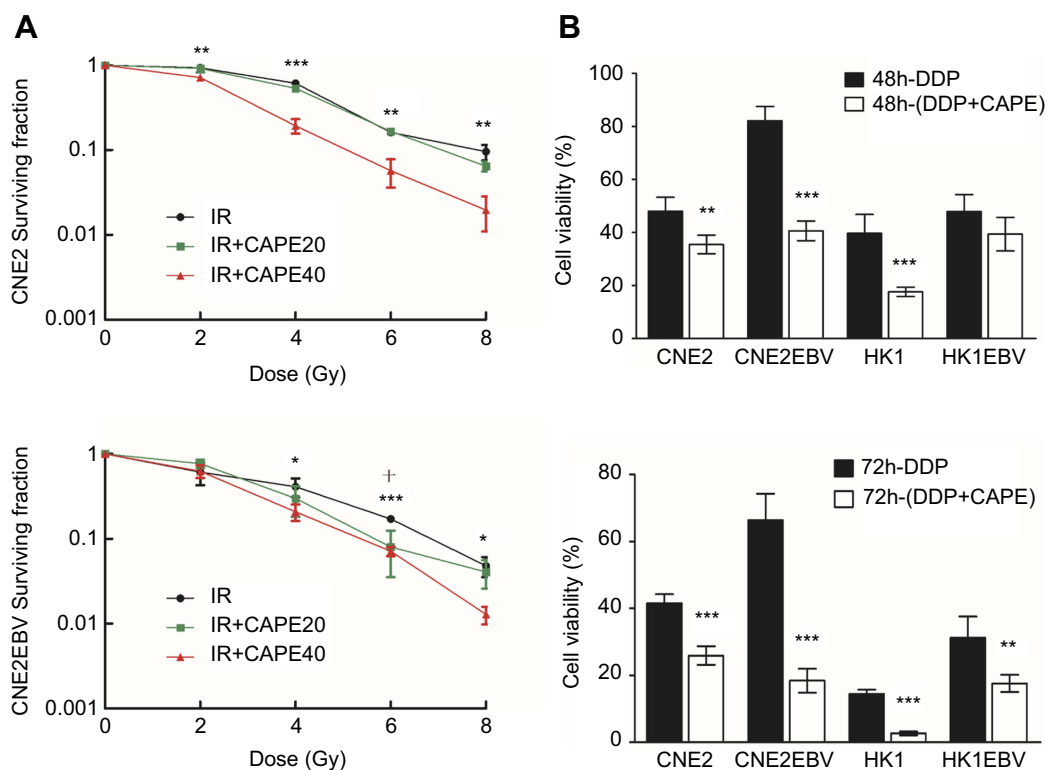
and 1.915 for CNE2-EBV cells. Clonogenic analysis showed that CAPE 20  $\mu$ M treatment significantly decreased the clonogenic survival of CNE2EBV cells combined with 6 Gy X-ray irradiation in a synergistic manner. In contrast, CNE2 cells showed no radiosensitivity enhancement with CAPE 20  $\mu$ M. CAPE also conferred sensitivity to cisplatin in NPC cells (CNE2, CNE2-EBV, HK1 and HK1-EBV). As compared with cisplatin treatment, CAPE combination therapy increased cisplatin sensitivity and decreased the cell viability of NPC cells (Figure 6B).

## Discussion

The anti-cancer effect of CAPE has been reported in several cancers in vivo and in vitro but not in NPC. In this study, we observed dose-dependent growth inhibition of CAPE in NPC cell lines. CAPE reduced the

proliferation of ovarian cancer cells via apoptosis induction, with activation of pro-apoptotic genes (BAD, CASP8, FAS, FADD, p53).<sup>22</sup> By activating oxidative stress, CAPE promotes apoptosis in human multiple myeloma cells.<sup>23</sup> Colorectal cancer cells also showed significant proliferation inhibition and induced cell cycle arrest and apoptosis by decreasing the expression of beta-catenin and the associated signaling pathway target genes.<sup>24</sup> CAPE also induced cell cycle arrest in LNCaP human prostate cancer cell lines and affected the abundance and phosphorylation of proteins involved in PI3K-Akt signaling pathways. In vivo, CAPE retarded the growth of LNCaP 104-R1 xenografts in a nude mouse model without notable toxicity.<sup>25</sup> In addition, CAPE significantly decreased the cell viability of many other cancer cells, including lung carcinoma, fibrosarcoma, melanoma,





**Figure 6** Radiosensitizing and chemotherapy sensitivity effects of CAPE on NPC cells. CNE2 and CNE2EBV cells were preincubated with CAPE for 24 hrs, then exposed to graded doses of X-ray radiation. **(A)** Clonogenic survival assay of CNE2 and CNE2EBV cells with 20 and 40  $\mu$ M CAPE. **(B)** Viability of NPC cells treated with cisplatin plus CAPE and cisplatin alone by CCK8 assay. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  comparing DMSO and IR+CAPE 40  $\mu$ M. + $p < 0.05$  comparing DMSO and IR+CAPE 20  $\mu$ M.

osteosarcoma, breast carcinoma and oral cancer.<sup>26</sup> In line with other studies, we found that CAPE significantly induced cell cycle arrest and apoptosis.

The EMT is crucial to the progression and metastasis of various cancers.<sup>27,28</sup> NPC is an EBV-associated malignancy typically characterized by early metastasis, and metastasis is also a main reason for relapse and death of NPC patients. CAPE effectively suppressed the invasive ability of NPC cells in vitro, which suggests that CAPE is a novel metastatic suppressor for NPC. In addition, we found that the epithelial marker E-cadherin was upregulated and the mesenchymal marker vimentin was downregulated in NPC cell lines with CAPE treatment. Thus, CAPE inhibited cell invasion and metastasis by reversing EMT-like changes in our NPC cells.

Radio-resistance is a major cause of treatment failure in NPC. Radiotherapy, especially intensity-modulated radiation therapy, is the principle and first choice for non-disseminated NPC because of its anatomical location and radiosensitivity.<sup>29</sup> Enhancing the radiosensitivity of NPC may improve the efficacy of radiotherapy in patients with NPC and provide a novel treatment strategy for NPC. We found that CAPE treatment combined with irradiation

significantly decreased the number of colonies relative to irradiation alone in CNE2 and CNE2-EBV cells, so CAPE could enhance the radiosensitivity of NPC. The sensitization of cancer cells to irradiation increased by CAPE has focused on lung cancer and colorectal adenocarcinoma cells, by depleting intracellular glutathione and inhibiting NF- $\kappa$ B signaling.<sup>10,30</sup> Previous studies have demonstrated that CAPE inhibits clone-forming capacity, maintains radiation-induced DNA damage and acts as a radiosensitizer in breast cancer.<sup>11</sup> Cisplatin (CDDP) is the conventional chemotherapeutic for NPC treatment and frequently used for distant metastasis or advanced locoregional recurrence.<sup>31</sup> Cisplatin, as a basic chemotherapeutic agent, can enhance the effectiveness of radiotherapy when combined with other drugs. We also found that CAPE could enhance the sensitivity of NPC cells to chemotherapeutics. Further studies are needed to explore the molecular mechanisms of the radiotherapy and chemotherapy sensitivity influenced by CAPE.

Once NF- $\kappa$ B signaling is stimulated, signal transduction activates the IKK complex, which phosphorylates I $\kappa$ B $\alpha$ , leading to its polyubiquitination and subsequent proteasomal degradation. Subsequently, the p65/p50

heterodimer translocates into the nucleus and regulates the downstream NF- $\kappa$ B signaling pathway. This signaling contributes to tumorigenesis of NPC, which is closely associated with EBV infection and massive lymphoid infiltration. Aberrant activation of NF- $\kappa$ B signaling leads to abnormal expression of various genes regulating apoptosis, proliferation, metastasis, inflammation and radiotherapy resistance,<sup>32</sup> which is a legitimate target for cancer therapy.<sup>33</sup> Different molecule inhibitors, for targeted therapies of the NF- $\kappa$ B pathway, have been discovered and investigated in clinical trials. Bay 11-7082, a highly selective NF- $\kappa$ B inhibitor, mediated blockade of NF- $\kappa$ B by directly inhibiting IKK and enhanced drug sensitivity in therapy-resistant epithelial ovarian cancer cells.<sup>34</sup> Bortezomib, a proteasome inhibitor, suppressed NF- $\kappa$ B activity by preventing the degradation of phospho-I $\kappa$ B $\alpha$ . In vitro, bortezomib reduced the viability of EBV-positive and -negative B cells by inducing apoptosis, and in vivo, inhibited the development of EBV B-cell lymphomas in severe combined immunodeficiency mice. We found that the expression of p65 in cytoplasm of NPC cells, both EBV positive and negative, remained under the condition of degradation of I $\kappa$ B $\alpha$ , but the level in the nucleus was reduced by CAPE treatment. Thus, CAPE may inhibit the p65-dependent activation of NF- $\kappa$ B.

CAPE occurs naturally in propolis. The good bioavailability through the oral route and the good historical safety profile of propolis makes CAPE an ideal adjuvant agent for future NPC treatment.

## Conclusion

In conclusion, our data support that CAPE suppresses the proliferation, survival, metastasis of human NPC cells by inhibiting the NF- $\kappa$ B signaling pathway. CAPE could be a radiosensitizer for NPC and thus could be combined with radiotherapy for NPC treatment. Thus, we suggest CAPE as a potential adjuvant anti-tumor drug for the treatment of NPC.

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## Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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