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Honokiol induces endoplasmic reticulum stress-mediated apoptosis in human lung cancer cells

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ABSTRACT

Aims: Honokiol is a hydroxylated biphenyl natural product and displays potent antitumor activity against several cancers including prostate cancer, melanoma, leukemia, and colorectal cancer. The present study was to investigate the in vitro activity of honokiol against A549 and 95-D human lung cancer cells.

Main methods: A549 and 95-D cells were used with honokiol treatment. Cell viability was determined by CCK-8 assay. The cell migration and apoptosis were evaluated by wound healing assay and TUNEL staining method respectively. The expressions of ER-related proteins were analyzed by western blot and the CHOP siRNA was used to downregulate the CHOP expression.

Key findings: The results demonstrated that treatment of A549 and 95-D cells with honokiol significantly reduced cell viability in a dose- and time-dependent manner. Furthermore, honokiol treatment decreased cell migration and enhanced cell apoptosis, which is accompanied by the upregulation of the expressions of ER stress-induced apoptotic signaling molecules such as GRP78, phosphorylated PERK, phosphorylated eIF2α, CHOP, Bcl-2, Bax, and cleaved Caspase 9. Honokiol treatment-induced increase of ER stress-related signaling molecules and apoptotic proteins in A549 and 95-D cells were reversed by CHOP siRNA.

Significance: Collectively, we conclude that ER stress may participate in the action of the anticancer activity of honokiol in A549 and 95-D cells and induction of ER stress-related apoptosis may represent a novel therapeutic intervention for human lung cancer.

1. Introduction

Lung cancer is the most prevalent cancer with high mortality in China as well as in other countries [1,2]. It was estimated that nearly 1.82 million new cases of lung cancer occurred worldwide according to a cancer statistics in 2012 [1]. Despite advances in diagnosis and treatment of lung cancer, the prognosis of patients with such disease is still poor. Therefore, exploring the precise molecular mechanisms and novel therapeutic strategies against lung cancer are urgently required. Especially, the natural products from Chinese herbs display potent anti-tumor activities against diverse cancers and provide a group of promising leading compounds for the development of effective chemotherapeutic drugs.

Honokiol (HNK, $C_{18}H_{18}O_2$) is originally extracted from the bark of *Magnolia officinalis* and exhibits anti-aging, anti-oxidative, anti-inflammatory and anti-tumor properties. HNK treatment ameliorated age-related learning and memory damages in SAMP8 mice through activation of Akt-mediated prosurvival pathway in the cholinergic neurons [3]. The studies also demonstrated that HNK attenuated cerebral ischemia reperfusion injury via reducing ROS production in rats [4]. In a renal ischemia/reperfusion injury (I/RI) model, HNK pretreatment decreased the expression levels of inflammatory markers such as TNF-α and IL-6 compared to I/R group [5]. Furthermore, HNK displays potent anti-tumor activity in a variety of cancer cells [6]. In human breast

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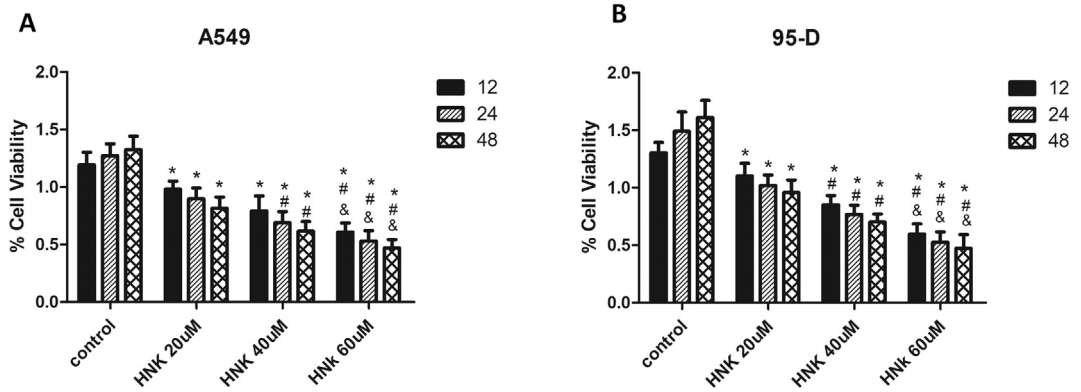

Fig. 1. HNK treatment inhibited cell viability of human lung cancer cells. (A) A549 cells were treated with three different concentrations (20, 40 and 60 μM) of HNK at the set time point (12, 24 and 48 h). (B) 95-D cells were treated with three different concentrations (20, 40 and 60 μM) of HNK at the set time point (12, 24 and 48 h). The data are expressed as the mean ± SD. All the experiments were repeated three times.*, $p < 0.05$ vs. the control group; #, $p < 0.05$ vs. the group treated with 20 μM HNK; &, $p < 0.05$ vs. the group treated with 40 μM HNK.

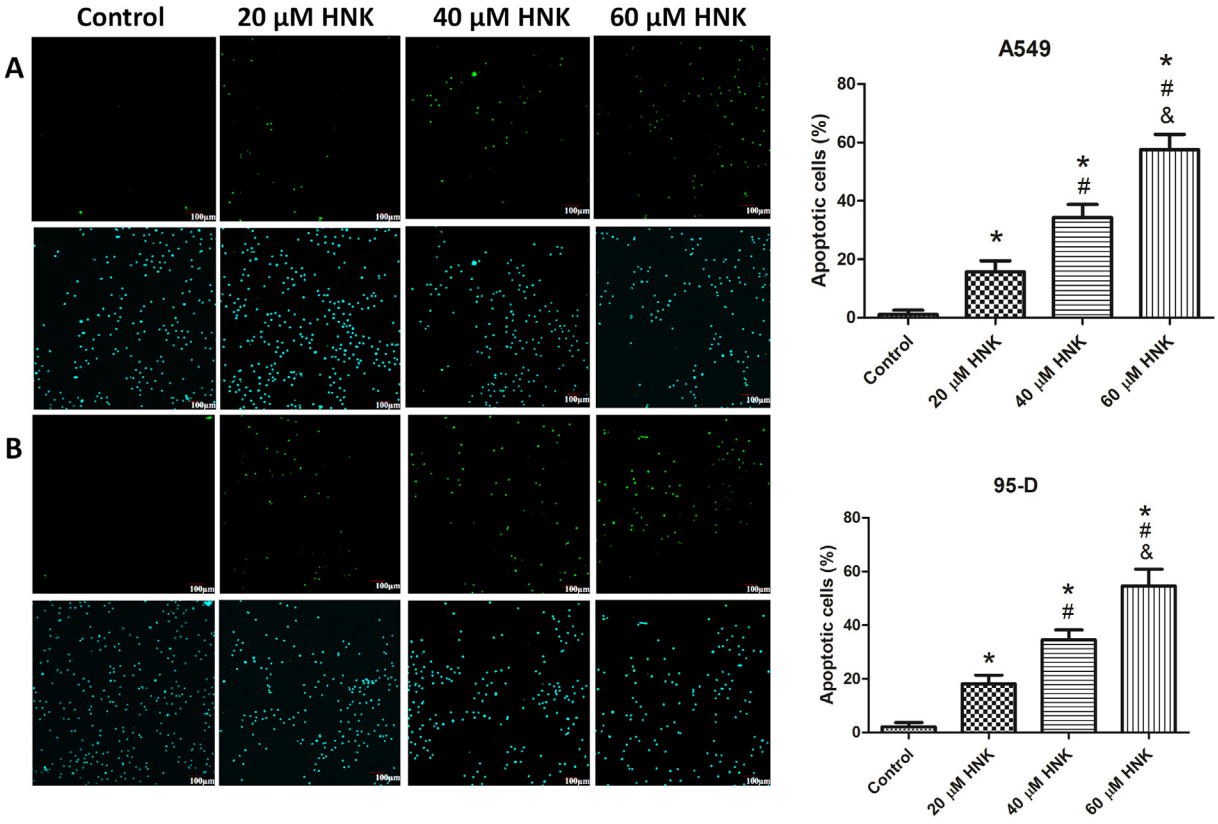

Fig. 2. HNK induced cellular apoptosis of human lung cancer cells. (A) A549 cells were treated with three different concentrations (20, 40 and 60 μM) of HNK for 24 h. (B) 95-D cells were treated with three different concentrations (20, 40 and 60 μM) of HNK for 24 h. The cells with green color indicated the apoptotic cells in the upper panel while the cells with blue color were the cellular nucleus in the lower panel. The data are expressed as the mean ± SD. All the experiments were repeated three times. *, $p < 0.05$ vs. the control group; #, $p < 0.05$ vs. the group treated with 20 μM HNK; &, $p < 0.05$ vs. the group treated with 40 μM HNK. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cancer, HNK resulted in cell apoptosis and cell cycle arrest [7,8]. It was previously reported that HNK not only inhibited lung cancer cell migration but also induced cell apoptosis via activation of PGE2-mediated β-catenin signaling and apoptotic pathway [9–11]. Although accumulating evidence indicates that HNK is a promising anti-tumor agent, the underlying molecular mechanisms still need further elucidation.

Endoplasmic reticulum is an important intracellular organelle to facilitate protein proper folding and serves as the assembly site for most secretory proteins. Endoplasmic reticulum stress (ER stress) also known as unfolded protein response (UPR) is a cellular condition which occurs due to the accumulation of misfolded and unfolded proteins [12]. It can be triggered by diverse physiological and pathological factors such as hypoxia, glucose deprivation, and even tumor growth [13–15]. Up to date, three proteins in the ER membrane function as stress sensors: (1) the double-stranded RNA (PKR)-activated protein kinase-like eukaryotic translation initiation factor 2α (eIF2α) kinase (PERK), (2) inositol-requiring transmembrane kinase/endoribonuclease 1 (IRE1), and (3) activating transcription factor 6 (ATF6) [16]. These proteins normally bind to chaperone glucose-regulated protein 78 (GRP78) to form an inactive protein complex, which also includes the heat shock protein

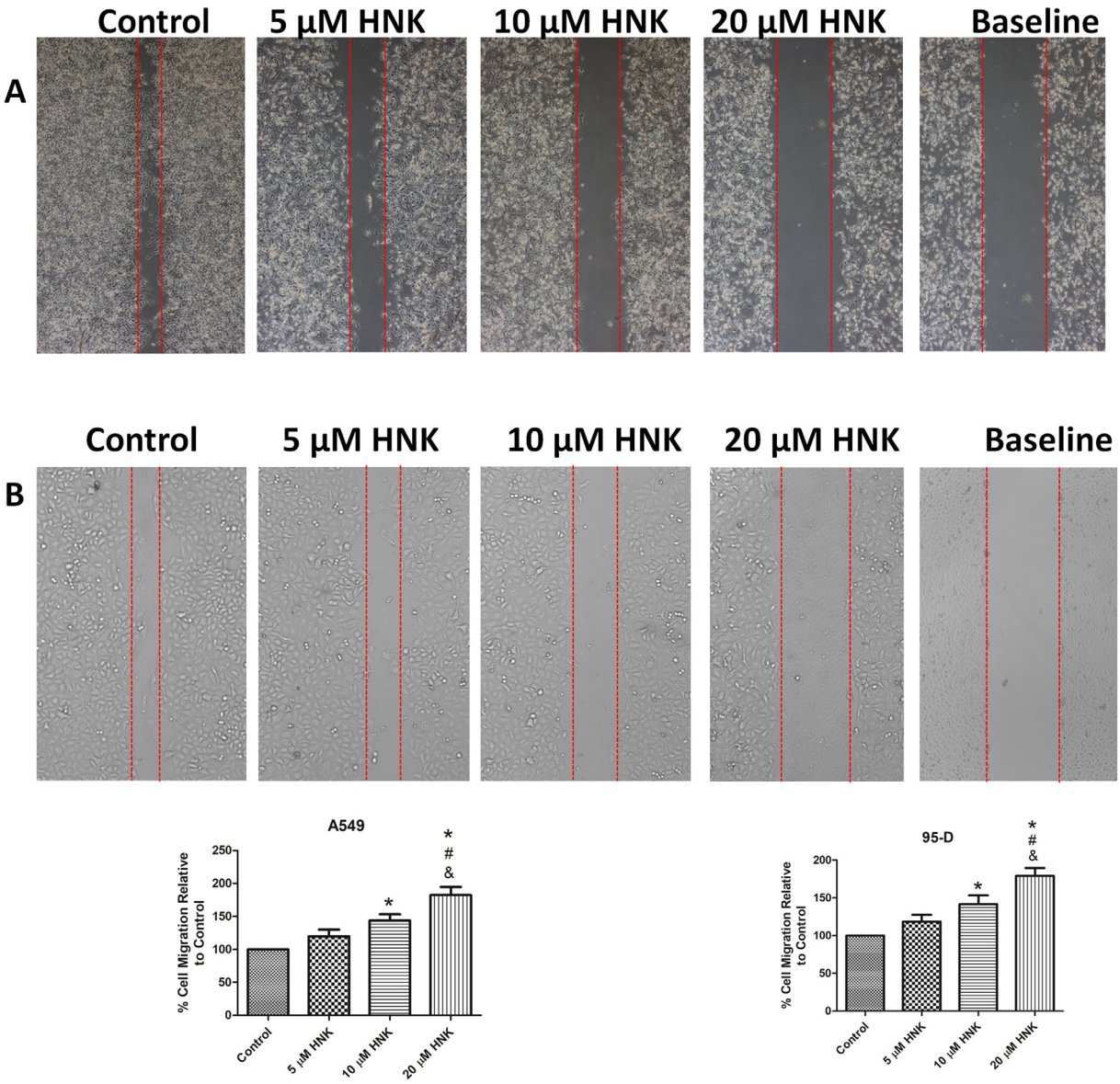

Fig. 3. HNK treatment inhibited cell migration of human lung cancer cells at the low concentrations. (A) A549 cells were incubated with low concentrations (5, 10 and 20 μM) of HNK for 24 h. (B) 95-D cells were incubated with low concentrations (5, 10 and 20 μM) of HNK for 24 h. The results were measured as the distances of the wound edge. The data are shown as the mean ± SD. All the experiments were repeated four times. *, $p < 0.05$ vs. the control group; #, $p < 0.05$ vs. the group treated with 5 μM HNK; &, $p < 0.05$ vs. the group treated with 10 μM HNK.

of 90 kDa (Hsp90) ER homolog, GRP94; protein disulfide isomerase; and calcium binding protein [17]. However, under stress conditions, when misfolded proteins accumulate and cause the sequestration of GRP78, these protein sensors can release from these protein complexes to initiate the ER stress [17]. The ER stress sensor PERK can phosphorylate eIF2α, which further leads to the reduction of protein synthesis and the upregulation of ER stress-related protein expression such as ATF4 and C/EBP homologous protein (CHOP). This signal transduction eventually results in restoration of cellular homeostasis of the protein translation and folding [18]. It was discovered that the activation of eIF2α could selectively promote the protein translation which plays a vital role in stress response [19]. ER stress can further result in cell apoptosis while caspase-9 as well as Bcl-2 family in this signal pathway plays a crucial role. Previous study has shown that rhein induces the apoptosis of SCC-4 human tongue squamous cancer cells through ER stress-induced activation of caspase-9 [20]. Furthermore, HNK induces mitochondrial apoptosis via regulation of Bcl family protein in human squamous lung cancer CH27 cells [10]. However, the role of ER stress-induced apoptosis in the anti-tumor activity of HNK has not been elucidated in human lung cancer cells. Therefore, in this study, the anti-tumor activity of HNK on A549 cells and 95-D cells were investigated and the role of ER stress signal was evaluated in this process.

2. Materials and methods

2.1. Cell culture and drug treatments

Two human lung cancer cell lines (A549 and 95-D cells) were obtained from Chinese Academy of Medical Sciences (Beijing, China). These two cell lines were routinely cultured in high glucose Dulbecco's modified Eagle's medium (DMEM, Hyclone, Thermo Scientific, MA, USA) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, USA) and 1% streptomycin/penicillin. The cells were grown at 37 °C in a humidified atmosphere of 5% CO_2/95% air and passaged with 0.25% trypsin/1 mM ethylenediamine tetraacetic acid (EDTA).

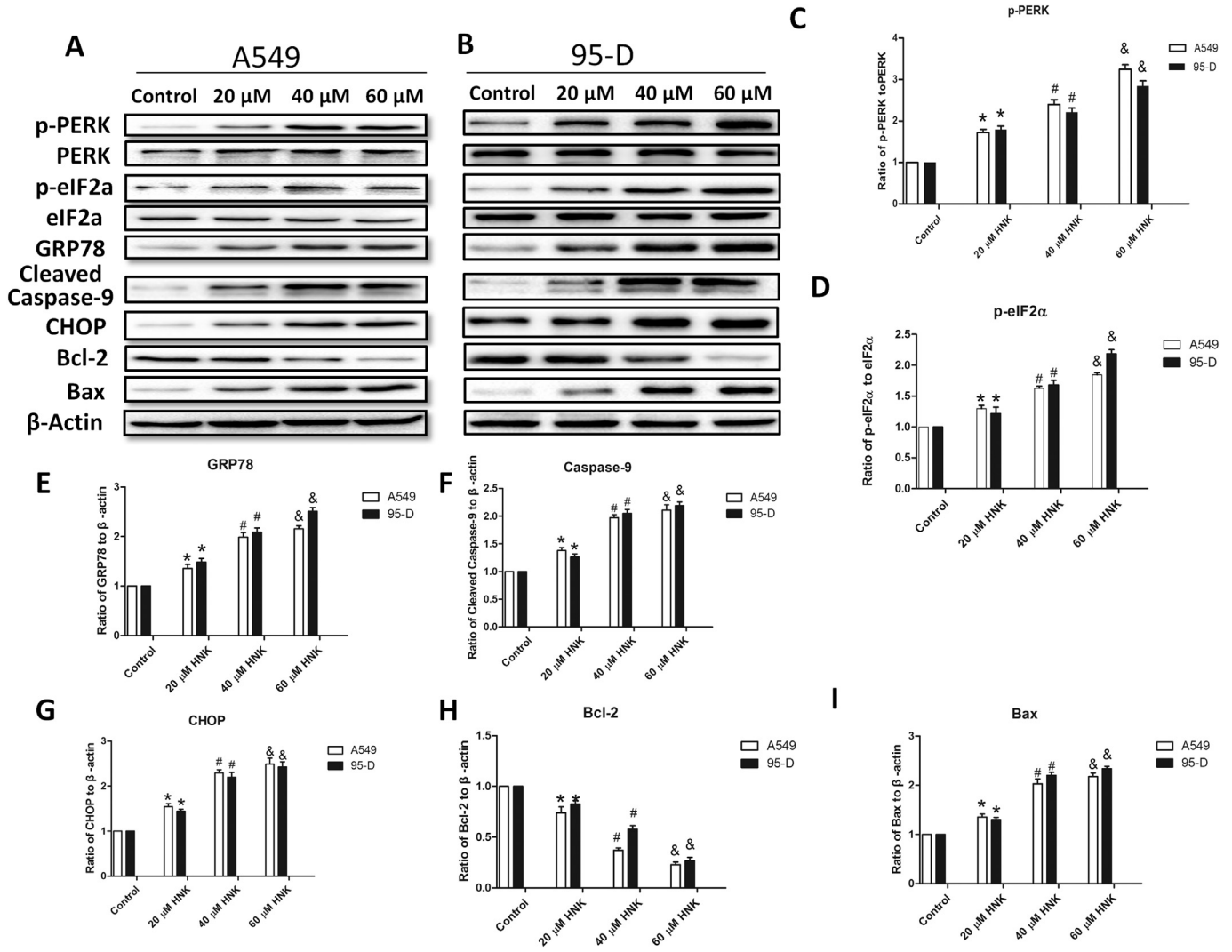

Fig. 4. HNK treatment induced cellular apoptosis via activation of the ER stress signaling pathway. The representative images of western blot were demonstrated when the A549 cells (A) and 95-D cells (B) were treated at the set concentrations of HNK for 24 h; (C) Ratio of p-PERK to PERK; (D) Ratio of p-eIF2αto eIF2α; (E) Ratio of GRP78 to β-actin; (F) Ratio of Cleaved caspase-9 to β-actin; (G) Ratio of CHOP to β-actin; (H) Ratio of Bcl-2 to β-actin; (I) Ratio of Bax to β-actin. The data are shown as the mean ± SD. All experiments were repeated three to five times. *, $p < 0.05$ vs. the control group; #, $p < 0.05$ vs. the group treated with 20 μM HNK; & , $p < 0.05$ vs. the group treated with 40 μM HNK.

HNK (Meiluobio, Dalian, China) was purchased from meilunbio and dissolved in DMSO with the stock concentration of 60 mM. Just prior to each experiment, HNK was freshly diluted in culture medium. Meanwhile, an equal volume of DMSO (the final concentration < 0.1%) was used in the control group. For wound healing assay, the cancer cells were treated with HNK at the concentrations of 5, 10, or 20 μM respectively in our study. For the siRNA experiments, HNK at the concentration of 60 μM was used in the absence or presence of CHOP siRNA. For other tests, the cancer cells were incubated with HNK at the concentrations of 20, 40, or 60 μM.

2.2. Cell viability analysis

The CCK-8 viability assay (7 sea biotech company, Shanghai, China) was employed to evaluate the anti-tumor activities of HNK on A549 and 95-D lung cancer cells. The cultured A549 and 95-D cells were digested by 0.25% trypsin. After centrifugation at 350*g* for 2 min, the cells were suspended and seeded in 96-well plates at a density of 1×10^4 cells per well. HNK at final concentrations of 20 μM, 40 μM and 60 μM respectively were added and DMSO was used to treat the cells as the control group. Subsequently, 10 μL of CCK-8 solution was added to each well and the cells were further cultured at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 2 h. The optical density at 450 nm was detected by using a microplate reader (SpectraMax M5, Molecular Device, USA). All experiments were conducted three times.

2.3. Cellular apoptosis analysis

Cellular apoptosis of A549 and 95-D cells was measured by using an In Situ Cell Death Detection TUNEL Kit (Roche, Indianapolis, USA) according to the manufacturer's instructions. Briefly, cells were seeded on glass coverslips and then incubated with different concentrations of HNK. After 24 h incubation, the cells on the glass coverslips were washed three times with PBS and fixed in 4% paraformaldehyde for 20 min. After wash with PBS, the cells were permeabilized using 0.2% Triton X-100 for 15 min at room temperature. Then the cells were incubated with 80 μL of the TUNEL reaction solution. The cells in the coverslips were incubated for 90 min at 37 °C in a humidified dark chamber. Finally, the nuclei were stained with 10 μg/mL DAPI solution (Beyotime, Shanghai, China) before visualization using a confocal microscope (Olympus, Tokyo, Japan). The positive apoptotic cells were stained and displayed green nuclear staining, and total number of all

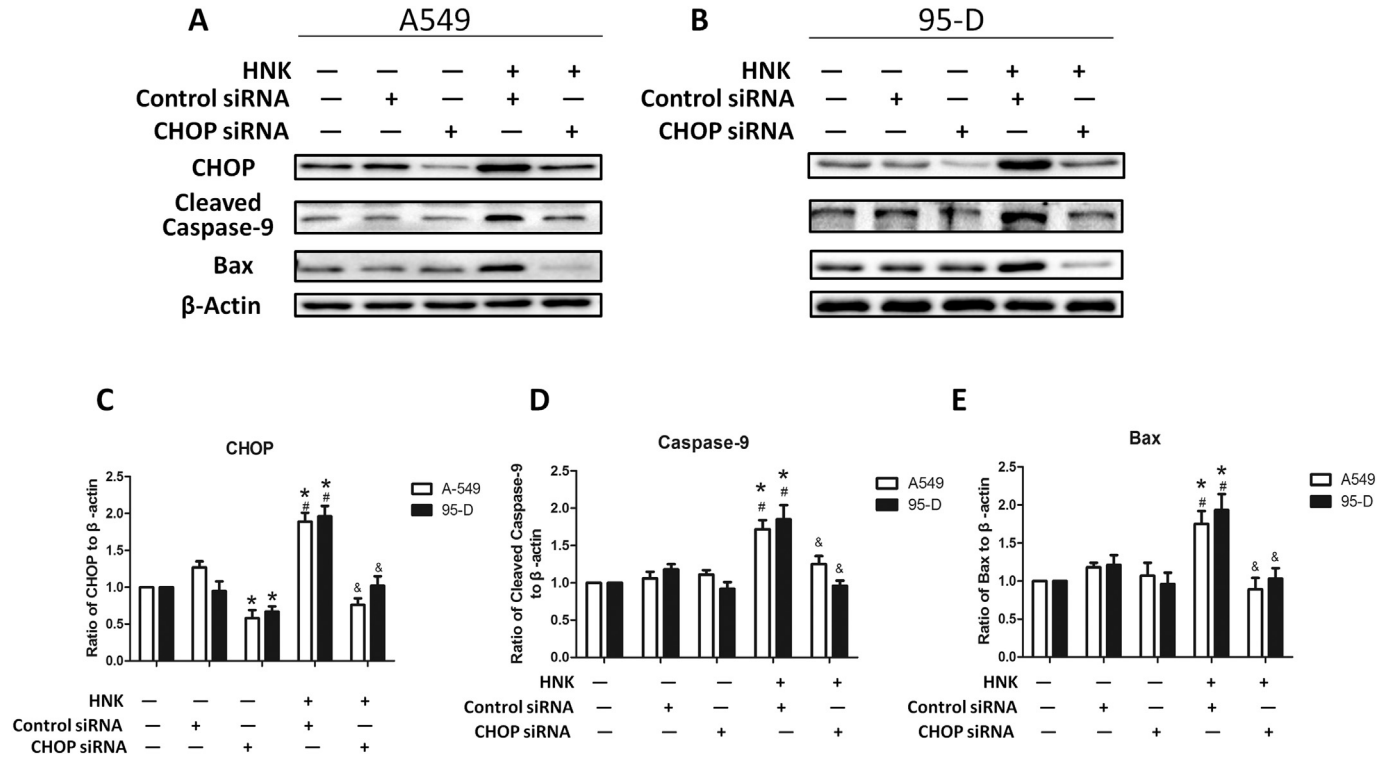

Fig. 5. HNK-induced cellular apoptosis was impaired by CHOP siRNA. The representative blots of CHOP, Cleaved Caspase-9 and Bax were demonstrated when the A549 cells (A) and 95-D cells (B) were treated with CHOP siRNA for 24 h and then incubated with 60 μM HNK; (C) Ratio of CHOP to β-actin; (D) Ratio of Cleaved caspase-9 to β-actin; (E) Ratio of Bax to β-actin. The data are shown as the mean ± SD. All the experiments were repeated three times.*, $p < 0.05$ vs. the control siRNA group; #, $p < 0.05$ vs. the group treated with the CHOP siRNA; &, $p < 0.05$ vs. the group treated with the control siRNA and 60 μM HNK.

cells was counted by blue nuclear DAPI staining. The apoptosis index was calculated as the percentage of ratio of positive apoptotic cells to the total number of cells.

2.4. Wound healing assay

The cells were seeded on 6-well plates in high-glucose DMEM containing 10% FBS and were maintained at 37 °C with a humidified 5% CO_2/95% air incubator before treatment. When the cells reached confluence, the artificial wounds were created by scratching the plate with a sterile pipette tip. After wash with PBS three times to remove cellular debris, the cells were exposed to three different concentrations of HNK (5, 10, or 20 μM) for 24 h. Cell movement was observed with a phase-contrast microscope and the images were photographed by using a 600D camera (Canon, Japan). The distance between the both sides of the scratch was measured and the distance of the control group was normalized to 100%.

2.5. SiRNA transfection

The siRNA transfection experiment was conducted by using Lipofectamine2000 (Invitrogen, San Diego, USA) according to the manufacturer's instruction. Briefly, cells with a density of 2×10^5 cells per well were seeded on 6-well plates in 2 mL normal high-glucose DMEM containing 10% FBS without antibiotics. When the cells grew to approximately 80%–90% confluence, they were transiently transfected with the negative control scramble RNA or CHOP siRNA (GenePharma, Shanghai, China). The siRNA (200 pmol) and 10 μL Lipofectamine 2000 were diluted in 250 μL Opti-MEM (Life Technologies, Rockville, USA). Then the both were gently mixed for 15 min at room temperature. Next, the mixed solution containing siRNA was added to 1.5 mL FBS-free culture medium and the cells were cultured for 6 h. Afterwards, the transfection medium was aspirated, and the cells were cultured in fresh 2 mL DMEM medium supplemented with 10% FBS for another 24 h. Finally, the cells were treated with HNK for protein expression analysis.

2.6. Western blot

The cells were harvested and lysed in RIPA buffer (Beyotime, Haimen, China) supplemented with protease and phosphatase inhibitor cocktail (Roche, Shanghai, China). After the centrifugation, the lysate was mixed with sample buffer (Beyotime, Haimen, China) and then boiled at 95 °C for 7 min. The equal amount of total protein was separated on a 8–15% SDS-PAGE and transferred to the PVDF membrane (Millipore, Bedford, USA). The membranes were blocked with 5% non-fat milk in TBST [50 mM Tris, 150 mM NaCl (pH 7.5), and 0.1% Tween-20] and then probed using primary antibodies (CST, Beverly, USA) against p-PERK, PERK, p-eIF2α, eIF2α, Caspase-9, GRP78, CHOP, Bcl2, Bax, (1:1000 for each), and β-actin (1:5000) at 4 °C overnight. Next, the membranes were washed with TBST, incubated using secondary antibodies (1:5000, Zsbio, Beijing, China) conjugated with horseradish peroxidase (HRP) in TBST buffer at room temperature for 2 h and then washed five times for 5 min each. The signal was detected by chemiluminescent HRP substrate (Millipore, Billerica, USA) and visualized by using a BioRad ChemiDoc imaging system (BioRad, Hercules, USA), and the signal was quantified using Image Lab Software (BioRad, USA).

2.7. Statistical analysis

All the data are presented as means ± Standard Deviation (mean ± SD). The comparisons between groups were performed using one way ANOVA followed by Tukey's Multiple Comparison Test (GraphPad Prism 5). A P value of < 0.05 was considered to be statistically significant.

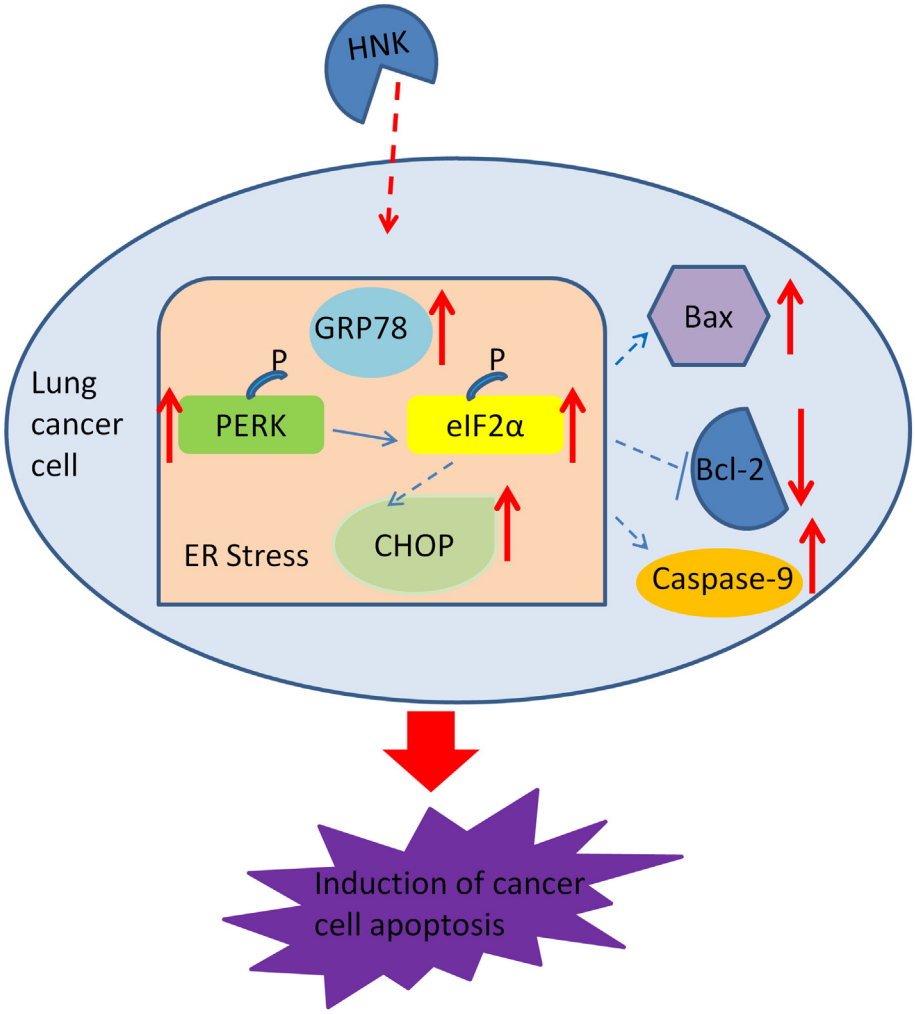

Fig. 6. Schematic diagram for the mechanism of anti-tumor activity of honokiol on lung cancer cells. HNK, honokiol.

3. Results

3.1. HNK treatment inhibited cell viability of human lung cancer cells

To investigate the anti-tumor activity in A549 and 95-D human lung cancer cells, the CCK-8 assay was performed to evaluate the effects of HNK on these cancer cells. Our data showed that treatment of A549 and 95-D cells with HNK at the concentrations of 20, 40 or 60 μM for 12, 24 or 48 h inhibited cell viability in a dose- and time-dependent manner as shown in Fig. 1, which displayed a similar anti-tumor pattern compared to the results in other cancer cells such as pancreatic cancer cells [21]. These data indicate that HNK has potent anti-cancer activity on human lung cancer cells.

3.2. HNK induced cellular apoptosis in human lung cancer cells

To examine the apoptotic effects of HNK on human lung cancer cells, A549 cells and 95-D cells were treated with 20, 40, and 60 μM HNK for 24 h and then stained with TUNEL staining kit. Similar to the effects of HNK on A431 cells, our results in Fig. 2 showed that the apoptotic index reached to 15.7 ± 3.8%, 34.3 ± 4.5%, and 57.5 ± 5.3% in A549 cells and 18.1 ± 3.3%, 34.5 ± 3.7%, and 54.6 ± 6.3% in 95-D cells [22]. The data demonstrated that HNK could induce cellular apoptosis in A549 cells and 95-D cells in a dose-dependent manner.

3.3. HNK inhibited cell migration of human lung cancer cells

Previous study has illustrated that HNK suppressed cancer cell migration through activation of β-catenin and RhoA/ROCK/MLC signaling pathway [9,23]. In our experiments the cell migration was evaluated by wound healing assay with different concentrations (5, 10, 15 μM) of HNK. Our data revealed that the distance between the wound edge significantly increased to 119.6 ± 10.3%, 143.5 ± 9.6%, and 182.4 ± 12.3% in A549 cells and 118.6 ± 8.9%, 141.2 ± 11.8%, and 179.3 ± 10.5% in 95-D cells as shown in Fig. 3. The results showed that HNK significantly inhibited lung cancer cell migration.

3.4. HNK treatment triggered the expressions of apoptotic proteins via activation of ER stress signaling pathway in human lung cancer cells

Multiple studies have discovered the anti-tumor effects of HNK in diverse cancer cells [11,24]. Although it is clear that apoptosis is the main contributor to this effect, whether ER stress pathway is involved in this process remains unclear. To elucidate the role of ER stress signal pathway in the anti-tumor activity of HNK, western blot was used to detect the expressions of ER stress-related proteins in A549 cells and 95-D cells treated with HNK. The results of our experiments in Fig. 4 demonstrated that HNK treatment increased GRP78, phosphorylated PERK, phosphorylated IRE1α, cleaved Caspase-9 and CHOP expression in a dose-dependent manner compared to the control group. Furthermore, HNK reduced the expression of Bcl-2 and increased the expression of Bax and Caspase 9 in these two cancer cells.

3.5. HNK-induced apoptosis is impaired by inhibiting ER stress signaling pathway via CHOP siRNA in human lung cancer cells

To clarify whether manipulation of ER stress signaling affected the anti-cancer activity of HNK, CHOP siRNA was used in our study. The cells were transfected with CHOP siRNA and then incubated with 60 μM HNK for 24 h. The results revealed that CHOP protein expression was significantly downregulated by transfection with CHOP siRNA compared to transfection with scramble siRNA. Compared to combination of HNK and scramble siRNA treatment group, the apoptotic proteins Bax and cleaved Caspase-9 was downregulated and the Bcl-2 was increased in HNK treatment combined with CHOP siRNA (Fig. 5). These data illustrate that HNK has anti-tumor activity via induction of ER stress mediated apoptosis (Fig. 6).

4. Discussion

HNK is a bioactive constituent of *M. officinalis* and displays diverse pharmacological activities. As an anti-tumor compound, HNK has been shown to possess the anti-tumor activity against several cancer cells [7,23–28]. It not only significantly inhibits cell proliferation and cell migration but also induces cancer cell apoptosis [9,23,24]. In human squamous lung cancer CH27 cells, HNK induced apoptosis via the release of mitochondrial cytochrome *c* to cytosol and activation of caspases [10]. Furthermore, cancer cell growth is inhibited while cell apoptosis can be induced through suppression of class I histone deacetylases with HNK treatment in A549, H1299, H460 and H226 NSCLC cells [29]. Consistent with previous studies, the anti-tumor activity of HNK on A549 and 95-D cells was also demonstrated in our in vitro study. HNK significantly inhibited cell viability with the concentrations ranging from 20 μM to 60 μM in a dose- and time-dependent manner. Our data revealed that the apoptotic cells were significantly increased following HNK treatment. Therefore, HNK could exert its anti-cancer activity primarily through the induction of cellular apoptosis. Besides, previous studies showed that HNK treatment inhibits cell migration by activation of β-catenin signaling pathway in human non-small cell lung cancer (NSCLC) cells [9]. Lung tumor progression and lung cancer metastasis are suppressed by HNK via inhibition of STAT3 signaling pathway [30]. Our results showed that HNK indeed inhibited cell migration in A549 and 95-D cells, implying the suppression of tumor metastasis. Thus, the data presented here showed that HNK displayed anti-tumor activity through induction of cell apoptosis and inhibition of cell migration.

In cancer cells, the elevated rate of protein folding in ER is required due to the tumor growth. Besides, the mutant proteins in cancer cells may accumulate in the ER lumen caused by protein misfolding. Therefore, ER stress is easy to occur in cancer cells. Several studies have revealed that ER stress signal pathway is involved in the cell death of cancer cells [31,32]. Bortezomib as a potent inhibitor of the proteasome induced apoptosis through ER stress in pancreatic cancer cells [33,34]. Moreover, curcumin induces cellular apoptosis in human NSCLC NCI-H460 cells partially through ER stress [35]. In human chondrosarcoma cells, HNK resulted in the cell apoptosis through activation of ER stress [36]. However, the role of ER stress on human lung cancer cells has not yet been elucidated. In the study presented here, the most interesting finding is that ER stress signaling molecules were increased in A549 cells and 95-D cells following HNK treatment in a dose-dependent manner. Our results showed that HNK treatment increased GRP78, phosphorylated PERK, phosphorylated eIF2α, and CHOP expression in A549cells and 95-D cells. Furthermore, silencing of CHOP resulted in the reduction of HNK-induced cell apoptosis mediated by CHOP siRNA. Taken together, these results indicate that the anti-tumor activity of HNK in human lung cancer cells was mediated by ER stress pathway.

ER stress can lead to the cellular apoptosis via activation of caspase-9 and caspase-12 [37–39]. The study revealed that PS-341 as a proteasome inhibitor induced cell apoptosis mediated by activation of caspase-9 in head and neck squamous cell carcinoma cells [40]. And gypenosides resulted in cell apoptosis via endoplasmic reticulum stress-mediated activation of caspase-9 and caspase-12 in human tongue cancer SCC-4 cells [41]. Similarly, caspase-9 was activated with HNK treatment in our study, therefore inducing the apoptosis pathway. Moreover, cellular apoptosis was induced by tocotrienols treatment via activation of caspase-9 in breast cancer cell lines [31]. On the other hand, Bcl-2 family proteins link the ER stress and cellular apoptotic pathway and decrease apoptosis via reduction of the release of Ca^{2+} from the ER [42–44]. Our results demonstrated that HNK treatment reduced the Bcl-2 expression and upregulated the cleaved caspase-9 and Bax in A549 cells and 95-D cells. Meanwhile, inhibition of ER stress through CHOP siRNA attenuated the changes of the expression of Bcl-2 and caspase-9 compared to HNK treatment. Thus, in line with previous studies, our data indicate that HNK resulted in cellular apoptosis through Bcl-2 and casapse-9 in A549 cells and 95-D cells.

In conclusion, our results reveal that HNK is an inhibitory compound of human lung cancer cells through the activation of ER stress. Manipulation of ER stress signal pathway via knockdown of CHOP desensitized human lung cancer cells to HNK treatment. Furthermore, this anti-tumor activity of HNK via elevation of ER stress may result from the increase of apoptotic pathway. Therefore, the activation of ER stress signal pathway may provide a potential strategy for prevention of lung cancer cells. And HNK exhibits anti-cancer potential as a chemotherapeutic agent against human lung cancer cells.

Conflict of interest statement

None declared.

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