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ORIGINAL ARTICLE

Honokiol: a promising small molecular weight natural agent for the growth inhibition of oral squamous cell carcinoma cells

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Honokiol (HNK) is a small organic molecule purified from magnolia species and has demonstrated anticancer activities in a variety of cancer cell lines; however, its effect on oral squamous cell carcinoma (OSCC) cells is unknown. We investigated the antitumor activities of HNK on OSCC cells *in vitro* for the first time. The inhibitory effects of HNK on the growth and proliferation of OSCC cells were demonstrated *via in vitro* 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and propidium iodide (PI) assays, and the apoptotic cells were investigated by the observation of morphological changes and detection of DNA fragmentation *via* PI, TdT-mediated dUTP-biotin nick end labeling (TUNEL), and DNA ladder assays, as well as flow cytometry assay. The results showed that HNK inhibited the growth and proliferation of OSCC cells *in vitro* in a time and dose-dependent manner. The inhibitory effect was associated with the cell apoptosis induced by HNK, evidenced by the morphological features of apoptotic cells, TUNEL-positive cells and a degradation of chromosomal DNA into small internucleosomal fragments. The study also demonstrated here that the inhibition or apoptosis mediated by 15 μg·mL^{-1} or 20 μg·mL^{-1} of HNK were more stronger compared with those of 20 μg·mL^{-1} 5-fluorouracil (5-Fu, the control) applied to OSCC cells, when the ratio of OSCC cell numbers were measured between the treatment of different concentrations of HNK to the 5-Fu treatment for 48 h. HNK is a promising compound that can be potentially used as a novel treatment agent for human OSCC.

***Keywords*:** honokiol; oral squamous cell carcinoma; anticancer; apoptosis

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Introduction

Carcinomas of the oral cavity, especially oral squamous cell carcinoma (OSCC), are one of the most leading causes that related to death worldwide. Data from the International Agency for Research on Cancer (IARC) show that there are more than 200 000 new cases of OSCC each year worldwide. The five-year and ten-year relative survival rates are 59% and 48%, respectively [1]. Currently, surgery, radiation and chemotherapy are three major modalities applied in the conventional treatment of this malignant disorder [2]. Chemotherapy (pre- or post-surgery) is beneficial for local control and survival improvement, although it does not always induce a substantially positive response [3]. In fact, the lack of effective chemotherapeutic drug results in a high death rate in patients with OSCC [4]. Therefore, effective chemotherapy medicines for OSCC are highly desirable and demanded.

Honokiol (HNK) is a small organic molecule purified

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from magnolia species. Evidence has demonstrated that HNK has a variety of pharmacological effects, such as anti-inflammatory [4], antithrombotic [5], anti-arrhythmic [4], antioxidative [6] and anxiolytic [7]. Indeed, it has been reported that HNK could inhibit cell proliferation and induce cytotoxicity in a variety of cell lines derived from cancers such as prostate, blood vessel, blood, lung, colon, liver, *etc*. [8-13]. However, little is known about the antitumor effects of HNK on OSCC. Therefore, in this study we chose OSCC cell lines to investigate its possible function on OSCC cell lines *in vitro* and explore the possible mechanism for its inhibitory effects, trying to lay the basis for the potential application in the clinical practice of OSCC treatment.

Materials and Methods

Cell line and Reagents

Two oral sqamous cell carcinoma cell lines, HSC-3 and HSC-4, were purchased from Japanese Collection of Research Bioresources (JCRB) Cell Bank, and maintained in DMEM medium (Gibco, USA) supplemented with 10% heat inactivated fetal bovine serum (APP, Italian) at 37 ℃ in 5% CO_2. HNK was purchased from Sikehua Biotechnology Co. (Chengdu, China), and dissolved in dimethyl sulfoxide (DMSO) at the stock concentration of 20 $g \cdot L^{-1}$. The latter was further diluted in culture medium at the final DMSO concentration <0.1% and the HNK maximum concentration of 20 $\mu g \cdot mL^{-1}$. 5-fluorouracil (5-Fu) was purchased from China National Pharmaceutical Group Co. (Shanghai, China), dissolved in ddH_2O at the stock concentration of 10 $g \cdot L^{-1}$.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Cell growth and viability were assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The kit was purchased from Sigma Chemical Corporation, USA. In brief, the cells (1×10^4 in 100 μL) were seeded on 96-well plates in triplicate. Following a culture at 37 ℃ for 24 h, the medium was replaced with fresh medium at various concentrations of HNK (10, 15 or 20 $\mu g \cdot mL^{-1}$, individually) or 20 $\mu g \cdot mL^{-1}$ 5-Fu (the control) in total volume of 200 μL. Then, cells were incubated further at 37 ℃ for 12, 24, 48 or 72 h, individually. Finally, 20 μL of MTT [5 $mg \cdot mL^{-1}$ in phosphate buffer saline (PBS)] was added to each well, and the cells were incubated for an addition of 4 h, and then the media were removed. MTT formazan precipitate was dissolved in 150 μL of DMSO, shaken mechanically for 30 min and then read immediately at 570 nm in a plate reader (Microplate Reader VersaMax, Molecular Devices Co., USA) [14].

Propidium iodide assay

Cells (3×10^5 in 2 mL) were seeded on six-well plates. Following a 24h-culture at 37 ℃, the media were replaced with fresh medium at various concentrations of HNK (10, 15 or 20 $\mu g \cdot mL^{-1}$, individually), or 20 $\mu g \cdot mL^{-1}$ 5-Fu (the control) in a final volume of 2 mL. Cells were further incubated at 37 ℃ for 48 h. After the treatment, cells were washed with PBS and fixed by 4% methanol-free formaldehyde solution in PBS at 4 ℃ for 25 min, then washed with PBS. 50 μL propidium iodide (PI, 50 $\mu g \cdot mL^{-1}$) was added to the plate and finally the cells were visualized under fluorescence reverse microscope (Leica DMI 6000B, Leica Co., Germany) [15].

TUNEL assay

The DeadEnd^{TM} Fluorometric TUNEL (TdT-mediated dUTP-biotin nick end labeling) system (Promega Inc., USA) was used. In brief, cells (3×10^5 in 2 mL) were seeded on six-well plates. Following a 24 h-culture at 37 ℃, the medium was replaced with fresh medium at 15 $\mu g \cdot mL^{-1}$ of HNK in a final volume of 2 mL. Cells were further incubated at 37 ℃ for 48 h, and washed with PBS and fixed by 4% methanol-free formaldehyde solution in PBS at 4 ℃ for 25 min, and then further washed with PBS and permeablized by 0.2% Triton X-100 in PBS for 5 min at room temperature. Finally the staining was carried out according to the manufacturer's instructions and fluorescence was visualized to count TUNEL-positive cells with Leica DMI 6000B microscope (Leica DMI 6000B, Leica Co., Germany) [16].

DNA ladder assay

To detect DNA fragments, OSCC cells were exposed to 15 $\mu g \cdot mL^{-1}$ HNK for 48 h, then collected and lysed with lysis buffer, containing 50 $mmol \cdot L^{-1}$ Tris-HCl (pH 7.5), 20 $mmol \cdot L^{-1}$ ethylenediamine tetraacetic acid, and 10 $g \cdot L^{-1}$ NP-40. Then 10 $g \cdot L^{-1}$ SDS and RNase (5 $\mu g \cdot mL^{-1}$) were added, incubated at 56 ℃ for 2 h, followed by incubation with proteinase K (2.5 $\mu g \cdot mL^{-1}$) at 37 ℃ for 2 h. Finally, the DNA was precipitated by addition of both ammonium acetate (3.3 $mol \cdot L^{-1}$) and ethanol (99.5%), dissolved in a loading buffer (Takara, Japan), and illustrated the DNA fragmentation under ultraviolet light (Bio-Rad, American) [17] after the electrophoresis on 2% agarose gels (Gene-Tech Co., Shanghai, China).

Flow cytometry assay

Apoptotic rates of the cells were analyzed by flow cytometry (FCM) using Annexin V-FITC/PI Apoptosis

Detection Kit (R & D systems, Abingdon, United Kingdom) according to the manufacturer's instructions. In brief, flow cytometry assay was performed on a FACSAria flow cytometry system (BD Biosciences). Data were analyzed by using BD FACSDiva software, and presented as dot plots showing fluorescence intensity of Annexin-V fluorescein isothiocyanate (Annexin-V FITC) and PI [18].

Statistical analysis

Values were given as mean ± SD. Statistical comparisons were made by Student's *t*-test, and $P<0.05$ was taken as the significance.

Results

HNK inhibited the OSCC cell growth

MTT assays were carried out to evaluate the cell viability upon treatment with three different concentrations of HNK (10, 15 or 20 μg·mL^{-1}, individually), or 20 μg·mL^{-1} 5-Fu (the control), which are based on our pilot studies (Data not shown) and previous report [17]. A pronounced increase in growth inhibition occurred after the treatment with those agents (Figures 1, 2), although the low dosage (10 μg·mL^{-1}) group of HNK also tended to slightly enhancing growth at the early stage in an unknown reason. Those phenomena demonstrated the ability in inhibitory efficiency of HNK on OSCC cells. The positive control (20 μg·mL^{-1} 5-Fu) demonstrated the same results, confirmed the inhibition effects.

To further confirm our observations, a PI assay was performed to visualize the survival OSCC cells and to evaluate cancer cell killing efficiency in those treatment *via* HNK or 5-Fu. Consistent with data in MTT assay, these agents resulted in a marked decrease in the number of cells with statistic significance ($P<0.01$) (Figures 1, 2).

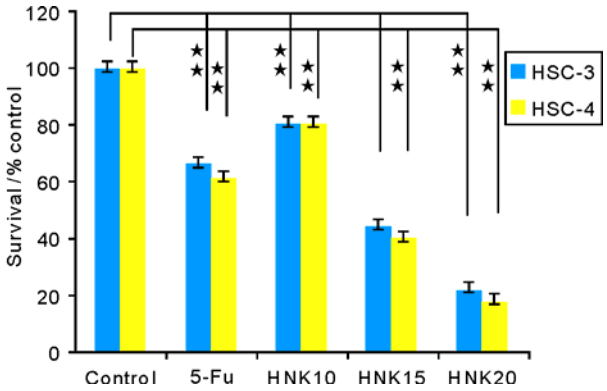

Figure 1 Dose-dependent inhibition effects of OSCC cells exposed to HNK or 5-Fu shown by MTT assay. HSC-3 or HSC-4 cells were treated as the described in Materials and Methods, and the MTT values were detected after 48 h. The values indicated a significant decrease ($P<0.01$) in cell proliferation when the cells were treated with 20 μg·mL^{-1} 5-Fu or treated with three variety of concentrations of HNK. The results also demonstrated that the cytotoxicity increased remarkably with the increase of the concentration of HNK. All experiments were performed in triplicate and the results were expressed as the mean ± SD of the measurements of three experiment. Asterisk (★★) indicated the significance $P<0.01$.

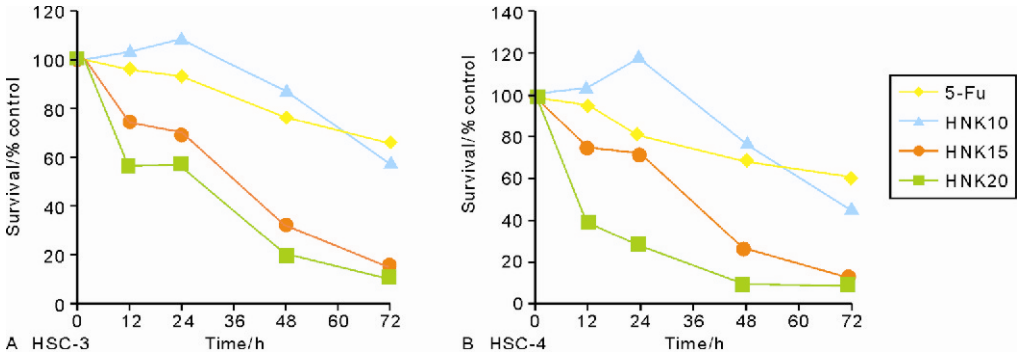

Figure 2 Time-dependent inhibition of OSCC cells exposed to HNK shown by MTT assay. HSC-3 or HSC-4 cells were treated as the described in Materials and Methods, and the MTT values were detected at different time points. After 24 h, the effect of inhibition of OSCC cells proliferation was increased obviously, and the treatment of OSCC cells with 20 μg·mL^{-1} of 5-Fu, 15 μg·mL^{-1} or 20 μg·mL^{-1} of HNK resulted in a sharply growth inhibition at various time points. Moreover, the effect of growth inhibition mediated by three concentrations of HNK were more prolonged compared with that of 20 μg·mL^{-1} 5-Fu in OSCC cells after 72 h. All experiments were performed in triplicate and the results were expressed as the mean ± SD of the measurements of three experiments.

The inhibitory effects of HNK on OSCC cells showed a time and dosage dependent manner

The MTT values were detected from 0 h to 72 h with 12 h or 24 h interval at different time points to test whether the inhibitory effect of HNK on OSCC cells was in a time (Figure 2) dependent manner, and the results demonstrated that the inhibitory effects of cell proliferation were obvious after the exposure of cells to HNK in both cell lines, and the increase in killing efficiency significantly concurred with the time course, although the value was a slight tendency of the cell number to go up at the lower dosage of HNK (10 μg·mL^{-1}) at the early treatment stage (before 24 h).

The MTT assays were also performed to evaluate whether the inhibitory effect of HNK to concur with the dosage increase at three different concentrations of 10, 15 or 20 μg·mL^{-1}, respectively. The results were shown in Figure 1 and demonstrated that HNK-mediated cytotoxicity enhanced remarkably with the increase of the concentration of HNK, indicating a dosage-dependent manner. Indeed, posterior to the 48 h treatment, the cell number decreased by about 60% in the 15 μg·mL^{-1} or 80% in the 20 μg·mL^{-1} HNK group in both cell lines (Figures 1, 2) when compared with that of the untreated cells.

Moreover, another assay (PI assay) was performed to detect of the proliferation inhibition of OSCC cells. Consistent with the data from the above MTT assay, HNK-mediated cytotoxicity grew up dramatically with the increase of the concentration of HNK. A significant decrease (P<0.01) in cell number was observed at the cells treated with three concentrations of HNK for 48 h (Figure 3).

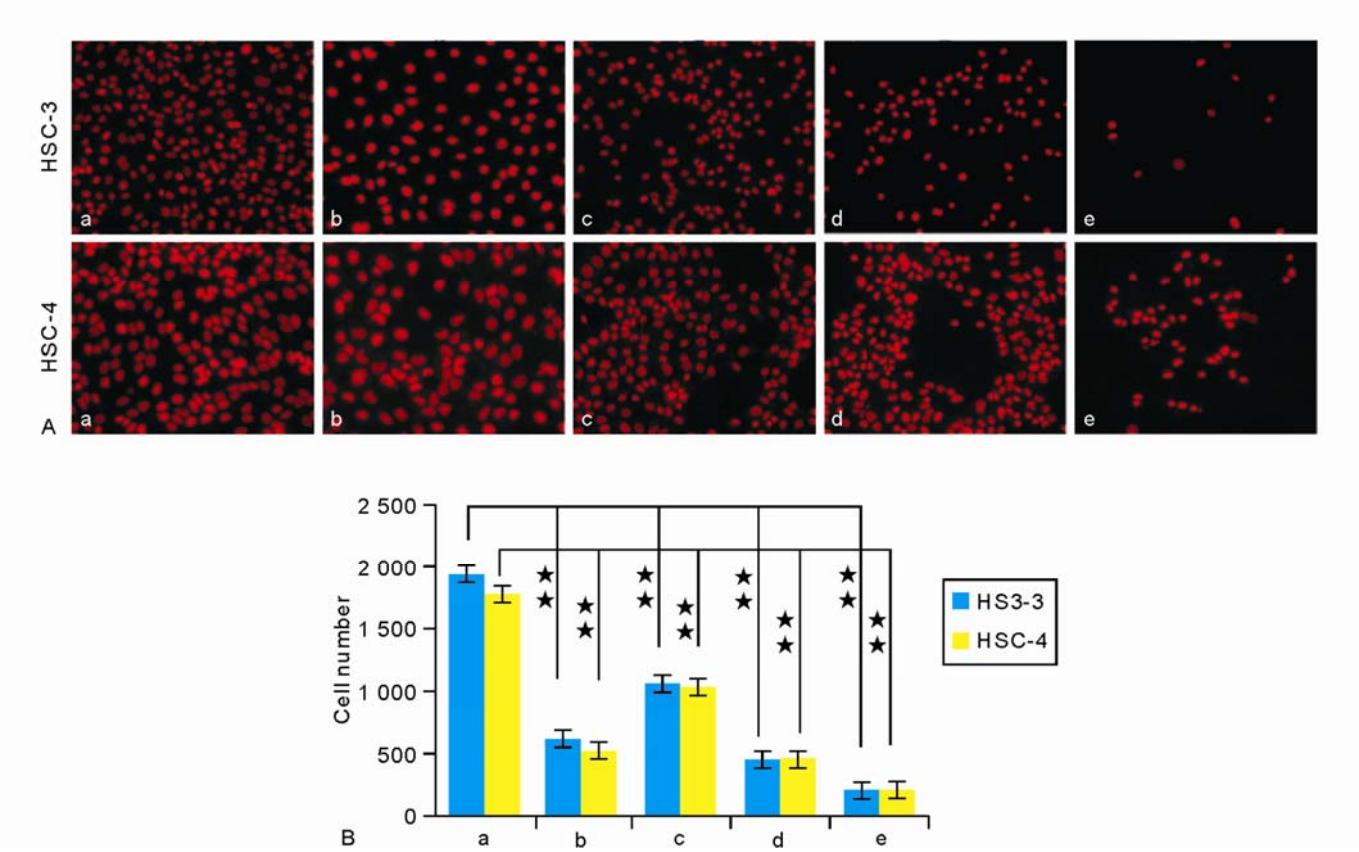

Figure 3 Inhibition of OSCC cells exposed to HNK or 5-Fu shown by PI assay. (**A**) HSC-3 or HSC-4 cells were treated as the described in Materials and Methods, and the inhibition effects of OSCC cells were measured by PI assay. ×100). (**B**) Quantitative analysis of the adherent cell numbers as shown as Figure A. The results indicated a significant decrease (P<0.01) in cell number when the cells treated with 20 μg·mL^{-1} 5-Fu or three concentrations of HNK for 48 h. And, the inhibition of OSCC cells increased remarkably (P<0.01) when the concentration of HNK increased. The results were expressed as the mean ± SD of the measurements (n=5). Asterisk (★★) indicated the significant value less than 0.01. a: The control with drug treatments; b: 5-Fu; c-e: 10, 15 or 20 μg·mL^{-1} HNK, individually.

The inhibitory power of HNK to OSCC cells also showed a dosage dependent manner

The inhibitory power of HNK to OSCC cells was measured by using the ratio of the cell number of OSCC cells between the treatment of different concentrations of HNK (10, 15, or 20 μg·mL^{-1}, respectively) for 48 h to 5-Fu (20 μg·mL^{-1}) treatment for 48 h in both cell lines *via* MTT assays. As shown from the Table 1, the ratio of HNK (10 μg·mL^{-1}) to 5-Fu (20 μg·mL^{-1}) was only around 0.5. However, the value exceeded 1.5 fold at 15 μg·mL^{-1}, and reached 2.13-2.32 fold of 5-Fu at the 20 μg·mL^{-1} HNK, indicating the inhibitory power of HNK to OSCC cells also had a dosage dependent manner.

To further confirm the above inhibitory power of HNK to OSCC cells, the ratio *via* PI assays of the number of OSCC cells between the treatment of different concentrations of HNK (10, 15, or 20 μg·mL^{-1}, individually) for 48 h to 5-Fu (20 μg·mL^{-1}) treatment for 48 h in both cell lines were also counted. As listed in Table 2, the ratio of the PI staining cells between HNK (10 μg·mL^{-1}) to 5-Fu (20 μg·mL^{-1}) was also approximately 0.5, and the value almost doubled to 1.05 to 1.12 at 15 μg·mL^{-1} HNK with a stable tendency around 1.29 to 1.31 at 20 μg·mL^{-1} HNK. These results were consistent with the data from MTT, which confirmed the inhibitory power of HNK to OSCC cells.

HNK induced apoptosis in OSCC cells evidenced via morphological changes and DNA fragmentation detection

According to MTT results, we chose 15 μg·mL^{-1} of HNK dosage group to further observe the possible changes in terms of morphology posterior to the treatment of HNK. Under the fluorescence reverse microscope, the HNK-treated cells clearly exhibited morphological features of apoptosis (Figure 4A) such as karyopyknosis, meniscus as well as cell shrinkage, *etc*. [19].

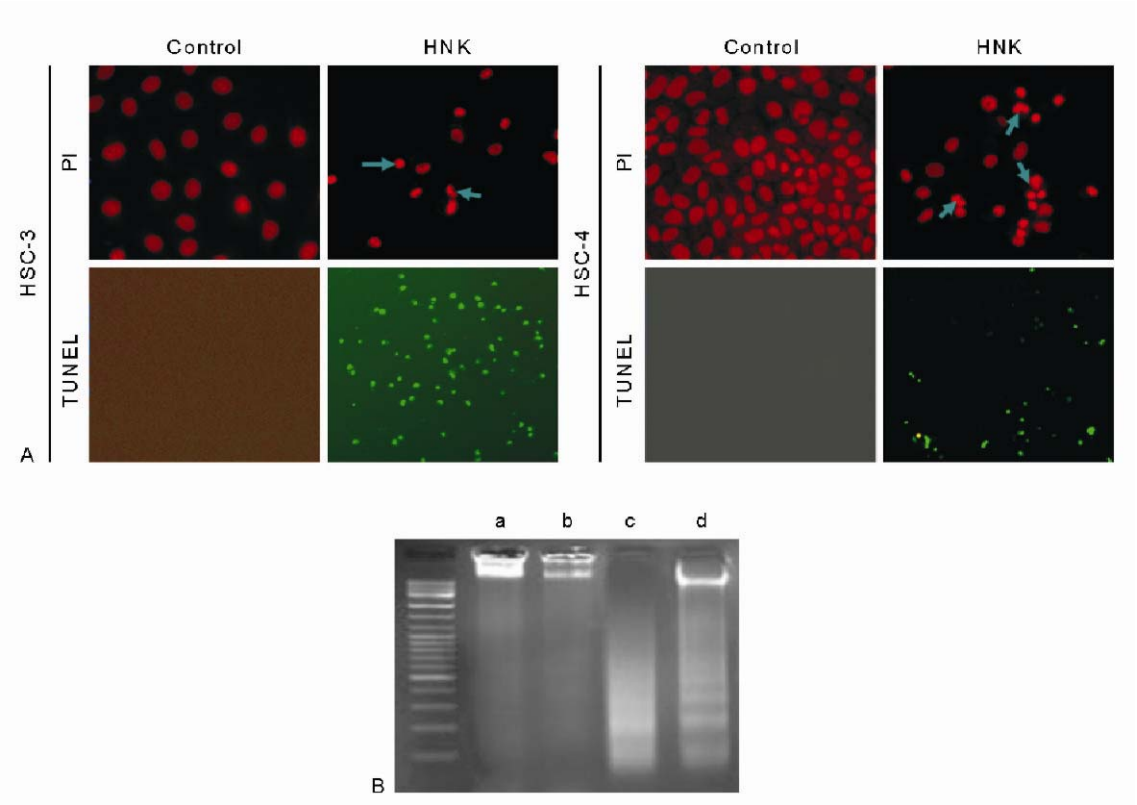

Figure 4 Morphological changes and DNA fragmentation in OSCC cells induced by HNK. HSC-3 and HSC-4 cells were exposed to 15 μg·mL^{-1} HNK for 48 h and then examined by three assays to detected the potential apoptotic effects, individually. (**A**) The examination of cell apoptosis by PI assay in HNK treated cells, exhibited morphological features of apoptosis: karyopyknosis, meniscus, cell shrinkage. Arrows indicate the apoptosis cells. ×200. (**B**) Apoptotic effect was detected by TUNEL assay. ×100. In HNK treated cells TUNEL-positive cells were presented. In HNK treated cells, a degradation of chromosomal DNA into small internucleosomal fragments was evidenced by the formation of 180–200 bp DNA ladders on agarose gels lane c and d. Lane a and b: HSC-3 and HSC-4 blank control; Lane c and d: HSC-3 and HSC-4 cells treated with 15 μg·mL^{-1} of HNK.

Table 1 Ratio of the inhibition effects to OSCC cells between HNK to 5-Fu shown by MTT assay

Group	HNK10		HNK15		HNK20	
	HSC-3	HSC-4	HSC-3	HSC-4	HSC-3	HSC-4
HSC-3*	0.58	-	1.65	-	2.32	-
HSC-4*	-	0.5	-	1.54	-	2.13

*The cells were treated with 20 μg·mL^{-1} 5-Fu.

Table 2 Ratio of the inhibition effects to OSCC cells between HNK to 5-Fu shown by PI assay

Group	HNK10		HNK15		HNK20	
	HSC-3	HSC-4	HSC-3	HSC-4	HSC-3	HSC-4
HSC-3*	0.64	-	1.12	-	1.29	-
HSC-4*	-	0.58	-	1.05	-	1.31

*The cells were treated with 20 μg·mL^{-1} 5-Fu.

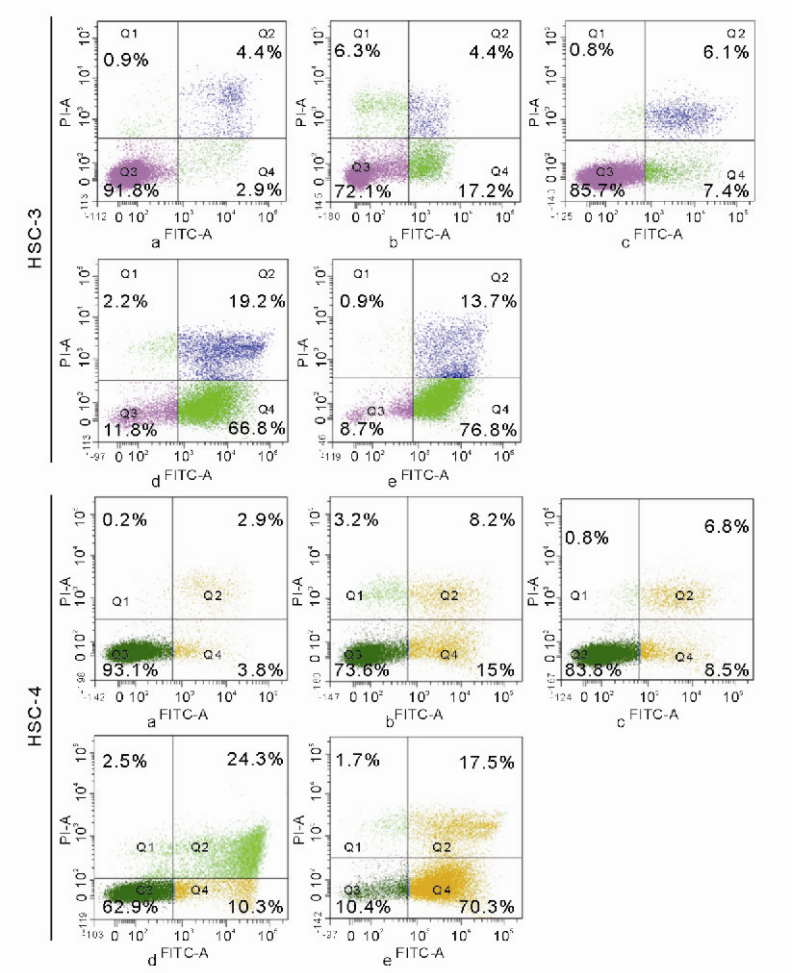

Figure 5 HNK induces apoptosis in OSCC cells exposed to HNK or 5-Fu shown by flow cytometry assay. HSC-3 or HSC-4 cells were treated as the described in Materials and Methods, and flow cytometry assay was used to analysis the apoptotic cells by Annexin-V FITC and PI double staining at 48 h posterior to the treatment. Lower left quadrant, viable cells; lower right quadrant (AV-positive but PI-negative cells), early apoptosis cells; upper left quadrants, necrotic cells; upper right quadrant, late apoptosis cells. The results indicated that HNK induced an apoptotic cell death in a dose-dependent manner in both HSC-3 and HSC-4 cells. And, the apoptotic cells proportion treated with HNK (15 or 20 μg·mL^{-1}) were 1.49-4.19 fold higher than that of 20 μg·mL^{-1} 5-Fu group. a: The control with drug treatments; b: 5-Fu; c-e: 10, 15 or 20 μg·mL^{-1} HNK, individually.

A more sensitive assay, fluorescence TUNEL system, was also conducted to detect the DNA strand breaks by labeling free 3′-OH termini. In HNK treated cells, TUNEL-positive cells were presented (Figure 4A). A degradation of chromosomal DNA into small internucleosomal fragments was remarkably evidenced by the formation of 180–200 bp DNA ladders on agarose gels (Figure 4B), the hallmark of cells undergoing apoptosis. Meanwhile, no DNA ladders were observed in the control cultures (Figure 4B).

These results strongly suggested that HNK induced an apoptotic cell death in OSCC cells.

HNK induced apoptosis in OSCC cells, evidenced by flow cytometry assays

To further confirm that the inhibition of HNK on the growth of OSCC cells was caused by apoptosis, the percentage of apoptotic cells in HNK- or 5-Fu-treated OSCC cells was evaluated by the flow cytometry assay after the double staining of Annexin-V fluorescein isothiocyanate (FITC) and propidium iodide (PI). The ratio of apoptotic cells increased in both of the OSCC cell lines with the increase of the concentration of HNK as compared with the blank control (Figure 5). Furthermore, the ratio of the apoptotic cell proportion (early apoptosis cells and late apoptosis cells, Table 3) of cells between HNK (10 $\mu g \cdot mL^{-1}$) to 5-Fu (20 $\mu g \cdot mL^{-1}$) was near 0.6, and the value grown up to 3.98 (HSC-3) and 1.49 (HSC-4) at 15 $\mu g \cdot mL^{-1}$ treatment, respectively, with a relative stable at 4.19 (HSC-3) or 3.78 (HSC-4) at 20 $\mu g \cdot mL^{-1}$ HNK, concurring with the inhibitory power of HNK to OSCC assessed by the ratio of MTT or PI assay.

Table 3 Ratio of the apoptotic cells proportion of cells between HNK to 5-Fu shown by flow cytometry analysis

Group	HNK10		HNK15		HNK20	
	HSC-3	HSC-4	HSC-3	HSC-4	HSC-3	HSC-4
HSC-3*	0.63	-	3.98	-	4.19	-
HSC-4*	-	0.65	-	1.49	-	3.78

*The cells were treated with 20 $\mu g \cdot mL^{-1}$ 5-Fu.

Discussion

Oral squamous cell carcinoma (OSCC) is one of the most common malignancies that remain incurable with current therapies. As a particular type of head and neck squamous cell carcinoma, OSCC patients have been commonly treated with cisplatin (or carboplatin) and/or 5-Fu, traditionally [20]. However, drug resistance often recurs, accompanied by distressing symptoms [21]. The response rate to most commonly used cytotoxic agents was only about 30%–40% in large studies [22]. Previous studies have demonstrated that the acquired drug resistance of cancer cells was associated with the alterations in apoptosis [23]. In other words, drug resistance to currently used chemotherapeutics was proposed to be partly mediated by the ability of the tumor cells to circumvent apoptosis [24-26]. Therefore, the inhibition of apoptosis is taken as a major contributing factor to drug-resistance in cancer cells. From this point of view, new treatment approaches with different mechanisms of apoptosis induction are all urgently being explored to overcome the limited effectiveness of current treatment modalities for OSCC. It appears that exploiting the apoptotic potential of OSCC would lead to contemporary therapies that might be less toxic to normal cells due to their physiologically-controlled survival pathways.

HNK, a natural product isolated from magnolia grandiflora, has multiple pharmacologic actions including antioxidative, anti-angiogenesis, and antitumor effects and so on [6-7, 10, 17, 27]. Previous studies suggest that growth inhibition by HNK resulted from the induction of apoptosis in several cell lines [9-10, 27-29]. A further study reported that HNK induces a necrotic cell death through the mitochondrial permeability transition pore [30]. Moreover, it is known for its low toxicity in normal peripheral blood mononuclear cell (PBMNC) or primary cultured human cells [17, 31-33]. However, little evidence is available for its effects on OSCC cells.

We have first tested the effects of HNK on oral squamous cell carcinoma cell models in the current study by MTT and PI assays to explore the inhibition potential of HNK on OSCC cells growth and proliferation. We have found that HNK inhibits the growth and proliferation of OSCC cells with a dosage- and time-dependent manner, which is consistent with the previous reports in other cells [17].

Furthermore, we have proposed the inhibitory power as a novel index by using the ratio of the cell number or the inhibitory effects of OSCC cells between the treatment of HNK to 5-Fu (positive group) treatment *via* MTT assays, PI assays, FCM assays to further illustrate the ability of HNK on OSCC cells for the first time.

From this novel index, we have further confirmed and enhanced the preliminary results that HNK inhibits the growth and proliferation of OSCC cells in a dosage- and time-dependent manner, which have obtained solely *via* MTT, PI, or FCM assays. In fact, this novel strategy could be used for the assessment of the inhibitory effects of any new drug that have potential to be candidate reagents for the treatment of oral squamous carcinoma or other tumors compared with conventional ones such as 5-Fu or cisplatin.

Moreover, we have found that HNK induced apoptosis *in vitro* in OSCC cells, evidenced by PI assay, TUNEL assay, DNA ladder assay, and FCM assay, suggesting the HNK may be a novel treatment agent to overcome drug-resistance in OSCC cancer cells, although more studies *in vivo* will be warranted.

On the other hand, some of previous studies have explored two modes of mechanisms in which HNK takes to induce the apoptosis or necrosis in the other cancer cell models [8-13, 17, 27, 33-34]. In brief, HNK have demonstrated the potentiating to activate the extrinsic pathways of cell apoptosis in tumors with p53 depletion or mutation (*i.e*. the activation of caspase 3, 7, and 9 have been noted). At the same time, the alternative (intrinsic) pathway (associated with mitochondrial dysfunction, reactive oxygen generation, and necrosis) may be more important in tumors with wild-type p53. Whether HNK takes the same mechanisms or any other novel pathways to make its antitumor activity in OSCC is currently under further studies *via in vitro* and *in vivo* experiments in our group.

In summary, our study has demonstrated that growth inhibition by HNK resulted from the induction of apoptosis in OSCC cell lines *in vitro*. Our findings suggest that HNK may be a promising agent that can be further studied as a novel treatment agent for human OSCC.

Acknowledgements

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