Original Article
Cytotoxicity of Kaempferol-3-O-rhamnoside against nasopharyngeal cancer via inhibition of EGFR-TK

Dongyan Huang[1], Shiming Yang[1], Zhirao Dai[2], Weimin Li[1], Rongguan Wang[1]

[1]Department of Otolaryngology-Head and Neck Surgery, Clinical Division of Surgery, Chinese PLA General Hospital, Beijing 100853, China; [2]Department of Otolaryngology, The First Affiliated Hospital of PLA General Hospital, Beijing, China

Abstract: Nasopharyngeal carcinoma (NPC) is a cancer arising from the nasopharynx epithelium. Previous studies have shown the potential anticancer properties of kaempferol-3-O-rhamnoside in breast cancer cell lines. In the present study, the anticancer potential of kaempferol-3-O-rhamnoside was investigated in CNE-1 human nasopharyngeal carcinoma cell lines. The inhibition of cell proliferation was investigated using MTT assays. In the present study, we investigated the influence of Kaempferol-3-O-rhamnoside on CNE-1 cells. Cell proliferation, cell invasion and migration and the related biomarkers were all repressed with the treatments of Kaempferol-3-O-rhamnoside in a subset of nasopharyngeal cancer cell lines. Characterization of the role of Kaempferol-3-O-rhamnoside in the development of tumors should lead to a better understanding of the changes occurring at the molecular level during the development and progression of primary human nasopharyngeal cancer.

Keywords: Nasopharyngeal carcinoma, Kaempferol-3-O-rhamnoside, MTT, CNE-1

Introduction

Nasopharyngeal carcinoma (NPC) is a cancer arising from the nasopharynx epithelium. Within the boundaries of the nasopharynx, the tumour epicentre is frequently seen at the fossa of Rosenmüller, from where the tumour invades adjacent anatomical spaces or organs. Despite being of a similar cell or tissue lineage, distinct differences exist between nasopharyngeal carcinoma and other epithelial tumours in the head and neck region. Compared with other cancer types, nasopharyngeal carcinoma is uncommon, albeit with a very unique pattern of geographical distribution. NPC is epidemic in areas of Southeast Asia, Southern China, Hong Kong, and Taiwan [1]. In 1978, the incidence of this disease in Southeast Asia was approximately 15-50/100,000 men per year [2]. In 2010, the data showed that the incidence and mortality rates in Southern China were 19.5, including Hong Kong, with 7.7 per 100,000 persons [3]. Besides geographical variation, some ethnic groups also seem to have a predisposition for nasopharyngeal carcinoma-eg, the

Bidayuh in Borneo, Nagas in northern India, and Inuits in the Artic, in whom age-standardised incidence is reportedly higher than 16 per 100,000 person-years in men [4]. Moreover, In terms of demographic trends, men are two to three times more likely to develop the disease than are women, and peak age of disease occurrence is between 50 and 60 years. Furthermore, the 5-year survival rate of NPC is ~60% in adults and children [5]. Although localized primary disease is often successfully treated by a combination of drugs and radiation therapy, advanced or recurrent malignancy is rarely cured, and toxicity from therapy is often severe and prolonged [6].

So far, the etiology of NPC includes genetic components, such as aberrations in chromosomes 1, 3, 9, 11, 12, and 14; infectious factors, such as Epstein-Barr virus (EBV); and environmental elements, such as the consumption of salt-preserved fish [7]. The pathological type of NPC can be classified into keratinizing squamous cell carcinoma (type I), differentiated nonkeratinizing carcinoma (type II), and undif-

Table 1. The RT-PCR primers for RhoC, MTA1 MMP-7, MMP-9 and GAPDH

Gene	Primers
RhoC	5'-TACGATCGCCTGCGGCCTCT-3'
	5'-AAGGATGGCGTGGGGGAGGG-3'
MTA1	5'-AGCTACGAGCAGCACAACGGGGT-3'
	5'-CACGCTTGGTTTCCGAGGAT-3'
MMP7	5'-TTGACGGTAAGGACGGACTC-3'
	5'-GGCGTTCCCATACTTCACAC-3'
MMP9	5'-AAGGGCGTCGTGGTTCCAACTC-3'
	5'-AGCATTGCCGTCCTGGGTGTAG-3'
GAPDH	5'-CAAGTTCAACGGCACAGTCA-3'
	5'-CACCCCATTTGATGTTAGCG-3'

ferentiated carcinoma (type III) [8]. Radiotherapy and chemotherapy are the main treatments for NPC [9]. However, over several decades, changes in different fields, such as advances in radiotherapy techniques, for example, 2-dimensional radiotherapy to 3-dimensional conformal techniques, which can be used to intensity modulated techniques, dose escalation, and using concurrent cisplatin-based chemotherapy. Unfortunately, approximately 20% of NPC patients still have local recurrence with the above therapeutic schemes [10]. In addition, a well-known cause of local recurrence with poor survival in NPC is radio resistance [11]. Recently, researches showed that radio resistance was related to inducing pathways (e.g., the PI3K/Akt pathway, NF-κB pathway [12], and Wnt pathway [13]), altering molecules (e.g., high mobility group protein box 1 (HMGB1) [14], metadherin (MTDH) [15], and microRNA [16]), mediating tumour anoxic effect [17], facilitating the tumour angiogenesis [18], cancer stem cells [19] and autophagy [20]. In addition, many studies have demonstrated that the biological behavior and prognosis of NPC patients could be significantly different in patients with the same stage, histologic type, or differentiation grade, implying that the existence of other.

The treatments mentioned above often impacts the quality of life due to side-effects or complications [21]. Thus, numerous investigators have focused on discovering novel drugs or treatments. Among all the agents tested, natural products derived from medicinal plants are among the most favorable. In our study, kaempferol-3-O-rhamnoside, the major compound found in the ethyl acetate fractions of the *Schima wallichii* (*S. wallichii*) Korth. Leaves,

was isolated and its properties were investigated against Nasopharyngeal carcinoma cell lines. The results indicated that kaempferol-3-O-rhamnoside was favorable for further exploration of its anticancer therapeutic potential [22]. Therefore, in the present study the anticancer properties and mechanism of kaempferol-3-O-rhamnoside were investigated in Nasopharyngeal carcinoma cell lines.

Method and materials

Extraction and isolation

The *S. wallichii* leaves were dried and extracted with 70% ethanol at room temperature three times for 24 h each. A concentrated extract was obtained in vacuo at 50°C. The ethanol extract was partitioned into n-hexane, ethyl acetate and aqueous phases. Column chromatography on a Wakogel C 200 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) column was performed to the ethyl acetate fraction, as it was previously reported as the most active fraction against cancer cell lines, using a mixture of n-hexane, ethyl acetate and methanol with increasing polarity. The major compound observed was purified using silica G 60 with sulfuric acid ethanol (1:9) and was found to be the most active fraction of *S. wallichii*, which was characterized and analyzed as described previously [22]. The isolate was, however, identified by spectroscopic methods (ultraviolet, infrared and nuclear magnetic resonance) and liquid chromatography mass spectrometry [36].

Cell culture and treatment

The Nasopharyngeal carcinoma cell line (CNE-1) were purchased from ATCC (Virginia, USA), and maintained in RPMI 1640 with 10% (v/v) FBS (Invitrogen, Carlsbad, CA). Cell lines were maintained in a humidified chamber at 5% CO_2, at 37°C. The CNE-1 cells were random divided into four groups with the same cell number under different treatments: Control: 0.1 ml PBS; Low: 0.1 ml Kaempferol-3-O-rhamnoside (10 μM); Medium: 0.1 ml Kaempferol-3-O-rhamnoside (100 μM); High: 0.1 ml Kaempferol-3-O-rhamnoside (1000 μM).

Cell proliferation assays

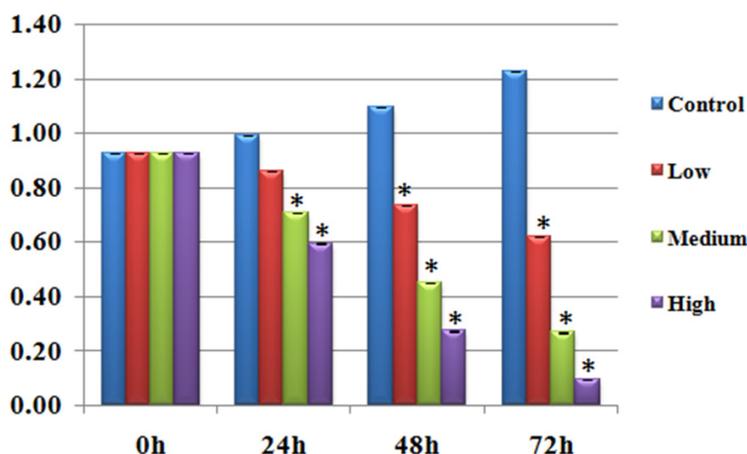A cell proliferation assay was conducted with MTT kit (Sigma) according to the manufactur-

Figure 1. Cell proliferation under four different treatments of Kaempferol-3-O-rhamnoside in 0, 24, 48 and 74 h of CNE-1 cell line. Control: 0.1 ml PBS, Low: 0.1 ml Kaempferol-3-O-rhamnoside (10 μM), Medium: 0.1 ml Kaempferol-3-O-rhamnoside (100 μM), High: 0.1 ml Kaempferol-3-O-rhamnoside (1000 μM). *represents the significant different compared with the control (P<0.05, T-test).

er's instruction. For the colony formation assay, 500 cells were placed into each well of 6-well plate and maintained in media containing 10% FBS for 2 weeks. Colonies were fixed with methanol and stained with 0.1% crystal violet (Sigma) in PBS for 15 minutes. Colony formation was determined by counting the number of stained colonies in 3 randomly selected fields with an inverter microscope. Triplicate wells were measured in each treatment group.

Migration and invasion assay

Cell migration assay was carried out using Transwell Permeable Support (Corning Incorporated, Corning, NY, USA). CNE-1 cells were carefully transferred on the top chamber of each transwell apparatus at a density of 1×10^6 per ml (100 μl per chamber). Cells were allowed to migrate for 24 h at 37°C. Cells that had penetrated to the bottom side of the membrane were then fixed in methanol, stained using hematoxylin and counted at microscope. Cell invasion was analyzed by using Cultrex 24-well BME Cell Invasion Assay (Trevigen Inc., Gaithersburg, MD, USA) according to standard procedures. Briefly, 10^3 CNE-1 cells were seeded in 100 μl serum-free media into the upper wells previously coated with Matrigel basement extract, and 500 μl of media were added in the bottom wells. After 24 h of CO_2 incubation at 37°C, the non-invasive cells on the upper sur-

face were removed and the cells migrated to the lower surface were fixed in 500 μl of Cell Dissociation Solution/Calcein-AM, incubated al 37°C in CO_2 incubator for 1 h and quantified by fluorimetric analysis (485 excitation, 520 nm emission).

RT-PCR

Reverse transcription of mRNA from nasopharyngeal carcinoma cell line under different treatments was carried out in 100 μl final volume from 400 ng total-RNA using high capacity cDNA Archive kit (Applied Biosystems) according to manufacturer's instructions. RhoC, MTA1 MMP-7, MMP-9 and GAPDH mRNA levels were determined by RT-PCR, and primers were utilized as described in **Table 1**. Reactions were performed in 50 μl volumes containing, SYBR Green PCR master mix (Perkin-Elmer Biosystems). Real-time PCR was performed using a GeneAmp PCR System 9600 (Perkin-Elmer Biosystems) in 96-well optical plates. Thermal cycling conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 2 min. Data were collected using the ABI analytical thermal cycler. RNA expression was calculated based on a relative standard curve with ΔΔct method, representing 10-fold dilutions of RhoC, MTA1 MMP-7, MMP-9 PCR products.

Western blot analysis

Total cellular protein in four different treatments was isolated by the addition of 1% PMSF and RIPA lysis buffer (50 mM Tris-HCl (pH7.4), 150 mM NaCl, 1% NP-40, 0.1% SDS). After boiled with SDS-PAGE sample buffer for 5 min, the samples were performed for sodium dodecylsulfate-polyacrylamide gel electrophoresis. Then the proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, USA). After being blocked for 1 h at room temperature, the membrane was incubated with a 1:1000 dilution of rabbit polyclonal anti-mouse RhoC, MTA1 MMP-7, MMP-9, EFGR, p-ERK and GAPDH (ABGENT, USA) overnight. Before detected with an ECL chemiluminescence
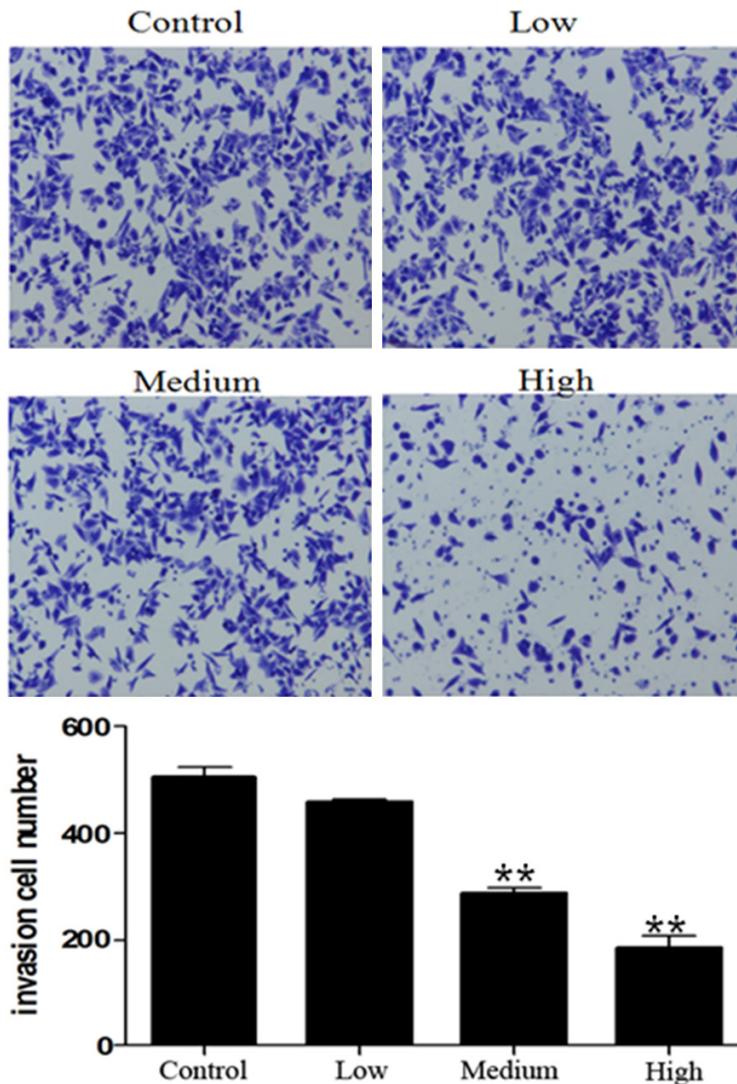
Figure 2. Treatments with Kaempferol-3-O-rhamnoside in CNE-1 cell line reduce cell invasion. CNE-1 cells treated with different concentrations of Kaempferol-3-O-rhamnoside were seeded in the upper chamber of transwell filters. After 24 h incubation, the top of the filters were scraped and cells that Matrigel-invaded through the filters were fixed and stained. Images are representative of invaded cells of one field. Cell number with invasion was statistics under five random fields of microscope. **represents the significant different compared with the control (P<0.01, T-test).

detection kit (Advansta, USA), proteins were incubated with the corresponding secondary antibody for 1 h at room temperature. The bands were obtained by GeneGnome 5 (Synoptics Ltd., UK).

Results

Influence of treatments with Kaempferol-3-O-rhamnoside on CNE-1 cell proliferation

To test whether Kaempferol-3-O-rhamnoside might affect CNE-1 cell proliferation, we intro-

duced Control (0.1 ml PBS); Low (0.1 ml Kaempferol-3-O-rhamnoside (10 µM)); Medium (0.1 ml Kaempferol-3-O-rhamnoside (100 µM)); High (0.1 ml Kaempferol-3-O-rhamnoside (1000 µM)) (**Figure 1**). There were no significant differences of cell proliferation between the four different treatments in 0 h. Of the group in 24 h, High and Medium treatments were significant differences of cell proliferation compared with the control group. However, there was no significant difference between the Low treatments and the control group. Of the group in 48 h and 72 h, Low, Medium and High treatments were all significant differences of cell proliferation compared with the control group. The results mentioned above suggested that different treatments of Kaempferol-3-O-rhamnoside on CNE-1 cells can effectively repress the cell proliferation.

Influence of treatments with Kaempferol-3-O-rhamnoside on CNE-1 cell invasion and migration

To investigate the effect of different treatments with Kaempferol-3-O-rhamnoside on CNE-1 cells, we used transwell chambers to assess the *in vitro* metastatic potential of the tumor cells under different concentrations of Kaempferol-3-O-rhamnoside treatments. The CNE-1 cells were plated in the upper chamber of the transwell. Cells were allowed to migrate and invade for 24 h at 37°C. The results showed that the Kaempferol-3-O-rhamnoside treatments repressed migration and invasion of the CNE-1 cells (**Figures 2** and **3**, respectively). Moreover, the depression effect on cell invasion and migration was significantly enhanced along with the rising concentrations of Kaempferol-3-O-rhamnoside. Of the cell invasion analysis, the number of invasion cells in High dosage treatment group was about one
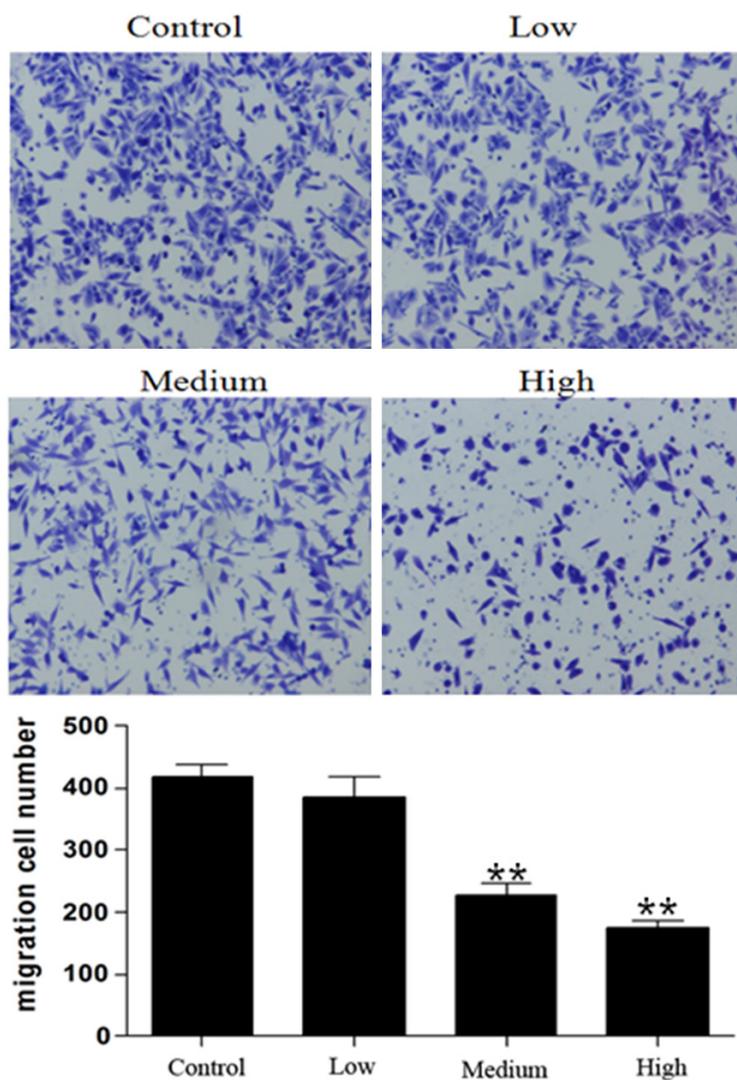
Figure 3. Treatments with Kaempferol-3-O-rhamnoside in CNE-1 cell line reduce cell migration. The cell motility of CNE-1 cells treated with different concentrations of Kaempferol-3-O-rhamnoside was evaluated using the monolayer wound healing assay. CNE-1 cells were seeded in the upper chamber of transwell filters. After 24 h incubation, the top of the filters was scraped and cells that migrated through the filters were fixed and stained. Images are representative of migrated cells of one filed. Cell number with migration was statistics under five random fields of microscope. **represents the significant different compared with the control (P<0.01, T-test).

migration analysis, the number of migration cells in High dosage treatment group was less than half the control group; the number of invasion cells in Medium dosage treatment group was also about a half of the control group; the low dosage group was also minimally affected by the treatment of Kaempferol-3-O-rhamnoside, but the number of migration cell was still decreased when it compared with the control group. The results mentioned above also indicated that Kaempferol-3-O-rhamnoside treatment can effectively repressed the cell migration, which is similar with the cell invasion analysis.

Western blot and RT-PCR and analysis of biomarkers related with invasion and migration of cancer cell

The expressions of biomarkers related with invasion and migration, Including RhoC, MTA1 MMP-7 and MMP-9, were examined by RT-PCR and western blot, respectively. Four key factors related with invasion and migration were selected to study the status of their expressions in Control, Low, Medium and High [23]. Cell extracts were analyzed with Western blotting using RhoC, MTA1 MMP-7 and MMP-9. GAPDH served as the loading control. Western blot analysis showed that Kaempferol-3-O-rhamnoside treatments caused a significant down-regulated protein expression of the four markers (**Figure 4**). The expression of the four markers in Low, Medium and High treatments was significantly retained when they compared with the Control group. Moreover, the results of western blot were confirmed by RT-PCR, the expression level of transcription of the four biomarkers was all repressed in four different groups (**Figure 4**). In summary, there is none of influence on the

third of the control group; the number of invasion cells in Medium dosage treatment group was about a half of the control group; the low dosage group was minimally affected by the treatment of Kaempferol-3-O-rhamnoside, but the number of invasion cell was still decreased when it compared with the control group. The results mentioned above indicated that Kaempferol-3-O-rhamnoside treatment can effectively repress the cell invasion. Of the cell
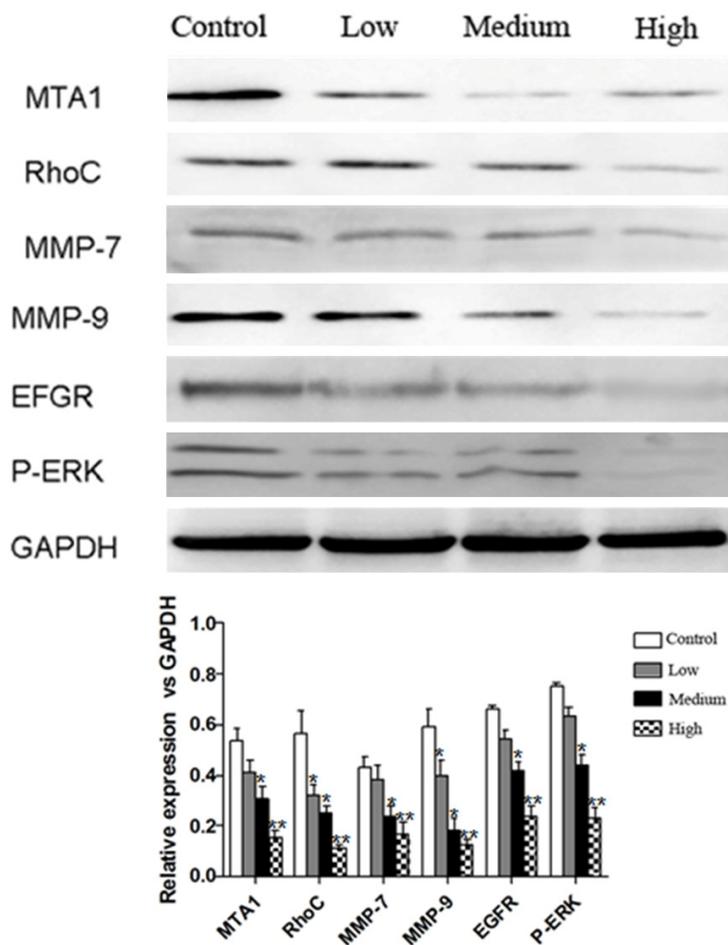
Figure 4. RT-PCR and western blot analysis of migration and invasion related genes with Kaempferol-3-O-rhamnoside treatments in CNE-1 cell. Control: 0.1 ml PBS, Low: 0.1 ml Kaempferol-3-O-rhamnoside (10 μM), Medium: 0.1 ml Kaempferol-3-O-rhamnoside (100 μM), High: 0.1 ml Kaempferol-3-O-rhamnoside (1000 μM). *represents the significant different compared with the control (P<0.05, T-test). **represents the significant different compared with the control (P<0.01, T-test).

loids and tannins, are ethnobotanically used for snake and insect bites [24]. Previous study reported the anticancer properties of kaempferol-3-O-rhamnoside on MCF-7 human breast cancer cell lines [25]. The results indicated that kaempferol-3-O-rhamnoside inhibits the proliferation of MCF-7 cells through the activation of caspase-9 and caspase-3 proteins and that it induced apoptosis [25]. Moreover, research had shown that kaempferol-3-O-rhamnoside inhibited the proliferation of LNCaP cells in a dose-dependent manner but not the noncancerous CHEK-1 cells. Therefore, Kaempferol-3-O-rhamnoside may possibly be considered less harmful to noncancerous cells [26]. Furthermore, this study also showed that the anticancer properties of kaempferol-3-O-rhamnoside occurred via the up-regulation of caspase-8, caspase-9, caspase-3 and finally PARP, the marker of apoptosis. Caspases are synthesized as inactive precursors. There are numerous caspase-activation pathways that promote apoptosis, such as mitochondrial stress by apoptosome pathways, death receptor engagement and granzyme B-induced caspase activation [27]. The results mentioned above showed that the potential anticancer properties of kaempferol-3-O-rhamnoside trigger death.

In order to gain understanding into the influence of kaempferol-3-O-rhamnoside on the development of nasopharyngeal carcinoma, The CNE-1 cells were random divided into four groups with the same cell number under different treatments: Control: 0.1 ml PBS; Low: 0.1 ml Kaempferol-3-O-rhamnoside (10 μM); Medium: 0.1 ml Kaempferol-3-O-rhamnoside (100 μM); High: 0.1 ml Kaempferol-3-O-rhamnoside (1000 μM). In the present study, Cell proliferation was significantly refrained

expressions of RhoC, MTA1 MMP-7 and MMP-9 in the Control group. However, Kaempferol-3-O-rhamnoside treatments could lead to the significantly down-regulated of the expression of RhoC, MTA1 MMP-7 and MMP-9. In addition, we analysis the protein expression of the EFGR and p-ERK, the results revealed that the expression level of both proteins was also repressed under different Kaempferol-3-O-rhamnoside treatments compared with the Control group.

Discussion

*S. wallichi*i plants are found in Asia, from Indochina to Papua New Guinea. Several of the compounds found in *S. wallichii*, including alka-

compared with the Control group up to 72 h. In addition, the invasion analysis by Boyden chamber and migration through polycarbonate membrane showed that different concentrations of kaempferol-3-O-rhamnoside treatments reduced cell invasiveness up to 24 h and cell migration, which were confirmed by western blot and RT-PCR analysis of biomarkers related with cell invasion and migration. Metastasis-associated gene 1 (MTA1) is considered a crit-ical factor in tumor metastasis [28]. Knockdown of MTA1 decreased migratory, invasive, and adhesive capabilities of cervical cancer cells as well as the expression of E-cadherin and p53 [29]. RhoC is involved in the reorganization of the actin cytoskeleton and in the regulation of cell shape, motility, and attachment [30]. Stable expression of RhoC enhanced migratory, invasive, and tumor-forming abilities of cervical cancer cells [31]. MMP-9 (a 92-kDa metalloproteinase also referred to as Gelatinase B) is known to preferentially degrade gelatin, elastin, aggrecan and collagens. It has already been found in high amounts in NP [32], but the precise role of this enzyme regarding the pseudocyst formation remained unclear. In asthma, recent reports suggest a particular role of MMP-9 in lung tissue remodeling [33]. Matrix metalloproteinase-7 (or matrilysin) is known to act as degrading enzyme towards fibronectin, laminin, gelatin, aggrecan and elastin [34]. The expressions of biomarkers (RhoC, MTA1 MMP-7, MMP-9) in the groups of kaempferol-3-O-rhamnoside treatments were down-regulated compared with the Control group. Moreover, we examined the protein expression level of EGFR and p-ERK, which had been proven to play a key role in promotion tumor growth in human cancer cells [35]. The results showed that kaempferol-3-O-rhamnoside treatments caused a significant down-regulated protein expression of the activated key factors with MAPK signaling pathway [35]. In the present study, we investigated the influence of Kaempferol-3-O-rhamnoside on CNE-1 cells. Cell proliferation, cell invasion and migration and the related biomarkers were all repressed with the treatments of Kaempferol-3-O-rhamnoside in a subset of human nasopharyngeal cancer cell lines. Characterization of the role of Kaempferol-3-O-rhamnoside in the development of tumors should lead to a better understanding of the changes occurring at the molecular level during the development and progression of primary human nasopharyngeal cancer.

Disclosure of conflict of interest

None.

Address correspondence to: Dongyan Huang, Department of Otolaryngology-Head and Neck Surgery, Clinical Division of Surgery, Chinese PLA General Hospital, Beijing 100853, China. Tel: +86 01066938322; Fax: +86-01068156974; E-mail: dongyanhuang1@sina.com

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