Original Article
Evodiamine inhibits proliferation of human papillary thyroid cancer cell line K1 by regulating of PI3K/Akt signaling pathway

Zhongchuan Lv1*, Dawei Zhao1*, Ruihua Liu1, Jing Guo2, Yang Lin1, Min Zhang3

1Department of General Surgery, Yantai Yuhuangding Hospital, Yantai 264000, China; 2Department of General Surgery, Qixia City People’s Hospital, Yantai 266500, China; 3Department of General Surgery, Yantai Yuhuangding Hospital of Laishan branch, Yantai 264003, China. *Equal contributors.

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Abstract: This study aims to investigate the antitumor effect of evodiamine against the papillary thyroid cancer cell line K1 and to explore the underlying mechanisms. The effect of evodiamine on K1 cells proliferation was analyzed by MTT assay. Reactive oxygen species (ROS) level and lactate dehydrogenase (LDH) leakage were evaluated by ELISA kit. The expressions of apoptosis related proteins were determined by western blot assay. The results demonstrated that evodiamine significantly inhibited the proliferation of K1 cells and in a dose-dependent manner. Furthermore, evodiamine treatment markedly increased ROS generation and LDH leakage. In addition, evodiamine intervention obviously downregulated the expression of apoptosis related proteins Bax, procaspase-3, procaspase-9 and procaspase-PARP while markedly upregulated the expression of Bcl-2, Bcl-xL cleaved caspase-3, cleaved caspase-9, cleaved PARP. Meanwhile, evodiamine significantly downregulated the expression of PI3K and p-Akt, indicating PI3K/Akt signaling pathway was involved in evodiamine-induced apoptosis in K1 cells. Thus, evodiamine may be a potential chemotherapeutic agent for the treatment of papillary thyroid cancer.

Keywords: Evodiamine, PI3K/Akt, the papillary thyroid cancer, ROS, apoptosis

Introduction
Thyroid carcinoma is a frequently diagnosed malignant tumor of endocrine organs, which accounts for 1.3-1.5% of the whole body malignant tumor [1]. Papillary thyroid carcinoma (PTC) is one of the most frequently occurring forms of differentiated thyroid cancer, representing 75 to 85% of thyroid cancer, and the rate has been increasing in the recent years [2]. It has been generally recognized that surgical resection and radiodine treatments are the most effective therapeutic strategies and have got great improvement in the past few years [3, 4]. However, the survival rate of patients with thyroid cancer is still low due to its rapid progression and metastasis [5]. Hence, there is an urgency to introduce effective therapeutic methods that can reduce the mortality of thyroid cancer patients.

In the past decade, progress has been made in discovering the molecular mechanisms underlying initiation and progression of thyroid cancer. Considerable evidences have shown that the PI3K/AKT pathway plays a fundamental role in thyroid tumorigenesis and is critical to maintenance of malignant phenotype of thyroid cancer cell lines [6, 7]. The accumulation of multiple genetic alterations that can activate PI3K/Akt pathway promotes thyroid carcinoma aggressiveness and progression to poorly differentiated thyroid carcinoma and undifferentiated/anaplastic thyroid carcinoma [8-11]. Suppression of PI3K/Akt pathway can inhibit proliferation of thyroid cancer cells [12, 13]. Therefore, the PI3K/Akt pathway is an attractive therapeutic target for thyroid cancer.

Recently, there is significant interest in developing an agent with the potential to suppress thyroid cancer cells proliferation. An accumulating body of data supports the concept that naturally occurring compounds in the traditional Chinese medicine may have lower toxicity and less possibility of drug resistance and have
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Evodiamine, a quinolone alkaloid, is one of the major bioactive compounds isolated and purified from *Evodia rutaecarpa* Bentham (Rutaceae). It exhibits excellent physiological functions including antitumor [17], anti-allergic [18], anti-inflammatory [19], antiobesity [20], and antinoceptive effects [21], as well as protection against myocardial ischemia-reperfusion injury and regulation of testosterone secretion [22, 23]. Especially, some of these results demonstrate that its anticancer bioactivity is indispensable. Numerous studies demonstrated that evodiamine exerted inhibitory effects on tumor cell migration and induced cell death in several cancer cell lines, including colorectal cancer [24], gastric cancer [25, 26], cervical cancer [27], liver cancer [28], prostate cancer [29], melanoma [30], lung cancer [31], and breast cancer cells [32]. However, whether evodiamine can inhibit the proliferation of papillary thyroid cancer line K1 remains unknown. Based on the above evidence, this study aims to investigate the anticancer activity and the related mechanism of evodiamine in K1 cells.

Materials and methods

Chemicals and antibodies

Evodiamine was obtained from Beijing Institute of Biological Products (Beijing, China) and its purity was determined to be ≥ 98% by HPLC measurement. Chemical structure of evodiamine is shown in Figure 1. It was dissolved in DMSO and stored in aliquots at -20°C and further diluted in the appropriate medium before use. 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was the product of Janssen Chimica Company (New Brunswick, USA). Dulbecco’s Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Logan, UT, USA) and Sijiqing Biological Engineering Materials Co Ltd. (Hangzhou, China), respectively. Bax, Bcl-2, Bcl-xL and β-actin antibodies were purchased from Santa Cruz Biotechnology (Delaware, USA). Caspase-3, cleaved caspase-3, caspase-9, cleaved caspase-9, PARP, cleaved PARP, PI3K, Akt, and p-AKT were purchased from Cell Signaling Technology (Boston, USA). All other chemicals were analytical reagents, and purchased from Sinopharm Chemical Reagent Co Ltd. (Shanghai, China).

Cell line and cell culture

The human papillary thyroid cancer cell line K1 was purchased from the ATCC (Manassas, VA, USA). K1 cells were cultured in DMEM: Ham’s F12: MCDB 105 (2:1:1) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 IU/mL) and streptomycin (100 μg/mL) in a humid atmosphere of 5% (v/v) CO₂ and 95% (v/v) air at 37°C.

Cell proliferation

Cell proliferation was determined using MTT assay. K1 cells were seeded into 96-well plates in culture medium overnight and then treated with various concentrations (0 μM, 1 μM, 5 μM, 10 μM, 20 μM, 40 μM and 60 μM) of evodiamine for 24 h. Then 20 μL MTT (5 mg/mL) was added and cells were continuously incubated for a further 4 h. The formazan crystals converted from tetrazolium salts by viable cells were dissolved in DMSO (150 μL/well) and the absorbance at 570 nm was measured by a microplate spectrophotometer (Bio-Rad, USA).

Lactate dehydrogenase (LDH) leakage

After K1 cells were exposed to 5 μM, 10 μM and 20 μM evodiamine for 24 h, the medium was collected, and the amount of LDH release was measured with a commercial detection kit according to the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

ROS assay

Intracellular ROS production in K1 cells after evodiamine treatment was detected using the 6-carboxy-2’,7’-dichlorofluorescein diacetate (DCFH-DA; Sigma, USA). Cells were pre-
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Figure 2. Effects of evodiamine on cells viability of K1 cells. Cell viability was determined by MTT assay in K1 cells after treatment with 0 μM, 1 μM, 5 μM, 10 μM, 20 μM, 40 μM and 60 μM evodiamine. Data are presented as the mean ± SD, n = 6. *P < 0.05 and **P < 0.01 compared with control group.

Figure 3. Effects of evodiamine on LDH release of K1 cells. LDH release was determined after treatment with 0 μM, 5 μM, 10 μM and 20 μM evodiamine. Data are presented as the mean ± SD, n = 6. *P < 0.05 and **P < 0.01 compared with control group.

treated with different concentration of evodiamine (5 μM, 10 μM and 20 μM) for 24 h. The cells were collected and incubated with DCFH-DA for 30 min at 37°C in the dark. ROS production was detected by the FlexS-tation 11384 fluorometric imaging plate reader (Molecular Devices, Sunnyvale, CA, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Western blot analysis

Cells were washed three times with cold PBS and extracted in the lysis buffer (Tris-HCl pH 7.14, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1 mM Leupeptin, 1 mM PMSF) on the ice for 30 min. Protein concentration was measured using Bradford assay (BioRad Laboratories, Hercules, CA). Equal amounts of protein were subjected to SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. After being blocked with 5% non-fat milk, blots were incubated with primary antibodies overnight at 4°C, and then exerted to incubation with the corresponding secondary antibodies at room temperature for 2 h. The blots were visualized with enhanced chemiluminescence (ECL) detection system (Amersham), and the results were analyzed by LabImage version 2.7.1 (Kapelan GmbH, Halle, Germany).

Statistical analysis

The statistical analysis was calculated by the Student’s t-test, using SPSS version 19.0 for windows (SPSS, Chicago, IL, USA). Data were presented as mean ± SD. The level of statistical significance was set at P < 0.05.

Results

Evodiamine suppressed the viability of K1 cells

The effect of evodiamine on the proliferation of K1 cells was assessed by MTT assay. As shown in Figure 2, the proliferation of K1 cells was suppressed after being treated with different concentrations of evodiamine and in a dose-dependent manner. In addition, the inhibitory effect of low concentration (1 μM) of evodiamine was not significant (P > 0.05). Meanwhile, over 20 μM evodiamine exhibited similar inhibitory effects. Therefore, we choose evodiamine at concentrations of 5 μM, 10 μM and 20 μM for the subsequent experiments.

The pro-apoptotic properties of LE on the viability of K1 cells

The LDH level in medium reflected the impaired level of the cells. As demonstrated in Figure 3, the amount of LDH released from K1 cells were significantly elevated after treatment with evodiamine compared with the control group (P > 0.01).

ROS was involved in cellular apoptosis induced by evodiamine

ROS plays an important role in the apoptosis of many cell types. To elucidate the underlying
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Evodiamine induced apoptosis in K1 cells

To explore the evodiamine-induced apoptosis, the apoptosis related proteins were investigated by western blot analysis. As shown in Figure 6A, the expression of the proapoptotic protein Bax significantly upregulated while the anti-apoptotic proteins Bcl-2 and Bcl-xL obviously downregulated and in a dose-dependent manner. In addition, evodiamine treatment activated caspase-3, caspase-9 and PARP cleavage. The expression of procaspase caspase-3, procaspase caspase-9 and PARP was significantly decreased while cleaved caspase-3, cleaved caspase-9 and cleaved PARP were markedly increased (Figure 6B).

Evodiamine regulated PI3K/Akt signaling pathway in K1 cells

PI3K/Akt signaling pathway plays an important role in cancer progression. Since the activity of AKT is regulated by phosphorylation, the present study examined the phosphorylation status of PI3K/Akt during the evodiamine-induced apoptosis in K1 cells. As illustrated in Figure 7, evodiamine significantly downregulated the expression of PI3K and p-Akt. However, the Akt protein remained constant during the course of evodiamine treatment. These findings revealed that evodiamine induced apoptosis in K1 cells by modulating PI3K/Akt signaling pathway.

Discussion

Epidemiological data support that natural products derived from medicinal plants have lower toxicity, less possibility of drug resistance and long lasting beneficial effects on human health. Recently, natural products are well recognized as having great therapeutic potential for the design of novel drugs in many tumors [33, 34]. Evodiamine, one of the major bioactive compounds of *E. rutaecarpa*, exhibits inhibitory effects on tumor cell migration in vitro and induced cell death in several cancer cell lines [24-32]. In the present study, the results demonstrated that evodiamine exerted the antitumor effects by inducing apoptosis in human papillary thyroid carcinoma cell line K1.

It is reported that apoptosis is the most popular underlying mechanism by which various anticancer and chemopreventive agents including natural compounds exert anticancer effects [35]. LDH is a soluble enzyme located in the...
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A

Evodiamine (μ M)

<table>
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<tr>
<th>Concentration (μM)</th>
<th>Bcl-2</th>
<th>Bcl-xL</th>
<th>Bax</th>
<th>β-actin</th>
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**B**

Evodiamine (μ M)

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<tr>
<th>Concentration (μM)</th>
<th>Procaspe-3</th>
<th>Cleaved caspase-3</th>
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Cytoplasm and is released into the medium upon cell damage or lysis, which occurs during both apoptosis and necrosis. The level of LDH in the cell cytoplasm is a sensitive marker for cell membrane integrity and thus a measurement of cytotoxicity [36]. In this study, evodiamine intervention significantly increased the level of LDH, indicating that evodiamine might induce apoptosis or necrosis in K1 cells.

ROS, an important secondary messenger, exists in many cell types in balance with bio-

Figure 6. Effect of evodiamine on apoptotic related proteins in K1 cells. Apoptotic related proteins were determined by western blot assay after treatment with 0 μM, 5 μM, 10 μM and 20 μM evodiamine. A. The expression of Bcl-2, Bcl-xL and Bax of K1 cells after different treatment. B. The expression of caspase-3, cleaved caspase-3, caspase-9, cleaved caspase-9, PARP, cleaved PARP of K1 cells after different treatment. The expression of apoptotic related proteins are normalized to the corresponding levels of β-actin. The results are presented as means ± SD, n = 3. *P < 0.05 and **P < 0.01 compared with control group.
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chemical antioxidants [37]. Appropriate ROS level is important for cell survival, but excessive ROS production leads to cell death through autophagy, apoptosis and necrosis [38]. It has been reported that excessive generation of ROS could oxidize the mitochondrial pores and disrupt the mitochondrial membrane potential leading to cytochrome c release [39]. Although accumulation of ROS does not kill cells directly, it triggers an apoptotic signaling programme that leads to cell death [40]. In the present study, ROS production significantly elevated after treatment with evodiamine in K1 cells. These results documented that evodiamine treatment might induce apoptosis in K1 cells and ROS might be involved in cellular apoptosis induced by evodiamine, whereas addition of NAC improved the cell viability.

Bcl-2 family plays an important role in apoptosis and is apoptotic regulatory proteins which control the mitochondrial apoptotic process. Functionally, the Bcl-2 related proteins reside upstream of irreversible cellular damage and focus much of their efforts at the level of mitochondria [36]. Therefore, these proteins play a pivotal role in deciding whether a cell will live or die [41]. Bcl-xL interacts with the mitochondrial plasma membrane and protects from apoptotic factors, such as Bax [42]. Bax can directly induce mitochondria to release cytochrome c for its ability to form ion channels indicating that it can open pores in the outer mitochondrial membrane, allowing the exit of cytochrome c [40]. Release of cytochrome c activates caspase-3 and caspase-9 then triggers cleavage of PARP, which finally induced apoptosis [34]. To confirm the evodiamine-induced apoptosis, the apoptosis related proteins were investigated in this study. The results showed that the expression of Bcl-xL and Bcl-2 were significantly downregulated while Bax protein was markedly upregulated. In addition, the expression of procaspase-3, procaspase-9 and PARP were significantly downregulated, the increased expression of cleaved caspase-3 and cleaved caspase-9 simultaneously induced PARP cleavage and resulted in apoptosis.

The PI3K/Akt signaling pathway plays a crucial role in thyroid tumorigenesis and is critical to the maintenance of malignant phenotype of thyroid cancer cell lines [43]. PI3K and its downstream molecules of protein kinase B (PKB or Akt) signaling pathways is closely related to proliferation and survival of human tumor cells [44], abnormal activity can lead to malignant transformation, tumor cell migration, adhesion, tumor angiogenesis and extracellular matrix degradation, and other related PI3K/Akt signaling pathway molecules as a target for therapeutic strategies being developed [45]. PI3K is lipid second messenger that is related to the intracellular signal transduction, Akt is the main effector molecules. Activation of Akt substrate phosphorylation by containing serine threonine residues play a wide range of biological effects, including antiapoptosis, promoting cell survival function [46]. Overactive PI3K/Akt
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pathway would mitigate the process of apoptosis and promote tumor cell cycle progression, which acts as an “on” or “off” switch [34]. In this study, we found that evodiamine significantly downregulated the expression of PI3K and p-Akt. These results indicated that PI3K/Akt signaling pathway is involved in the apoptosis induced by evodiamine in K1 cells.

Conclusion

In conclusion, evodiamine has significant antiproliferation properties and induced apoptosis in human thyroid carcinoma cell line K1. The induction of apoptosis triggered in the evodiamine-treated K1 cells was modulated by PI3K/Akt signaling pathway. Thus, evodiamine may be a potential agent to inhibit tumor progression.

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Disclosure of conflict interest

None.

Address correspondence to: Min Zhang, Department of General Surgery, Yantai Yuhuangding Hospital of Laishan branch, 1181 Gangcheng East Main Street, Yantai 264003, China. Tel: +8613361358663; E-mail: zhangmin151026@163.com

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