Cinnamic Acid (CINN) Induces Apoptosis and Proliferation in Human Nasopharyngeal Carcinoma Cells

Guangying Qi\textsuperscript{a,b} Jian Chen\textsuperscript{a,b} Changrong Shi\textsuperscript{c} Yukun Wang\textsuperscript{c} Sisi Mi\textsuperscript{a} Wenhua Shao\textsuperscript{a} Xiangyuan Yu\textsuperscript{c} Yili Ma\textsuperscript{c} Jiapu Ling\textsuperscript{a} Jian Huang\textsuperscript{c}

\textsuperscript{a}Department of Pathology and Physiopathology, Guilin Medical University, Guilin, \textsuperscript{b}Laboratory of Tumor Immunology and Microenvironmental Regulation, Guilin Medical University, \textsuperscript{c}Department of Biochemistry, Guilin Medical University, Guilin, PR. China

Key Words
CINN • Nasopharyngeal carcinoma (NPC) • Cell proliferation and apoptosis

Abstract
Background/Aims: CINN is the main ingredient of the traditional Chinese medicine cinnamon. The purpose of the present study was to investigate the effects of CINN on the proliferation and apoptosis of NPC cells and to elucidate the underlying molecular mechanisms. Materials and Methods: CNE2 human NPC cells were treated with various CINN concentrations. The effects of CINN on the proliferation and apoptosis of CNE2 NPC cells were examined using the MTT assay and flow cytometric analysis. Additionally, western blotting was performed to analyze the expression of a number of cell cycle- and apoptosis-related proteins. Results: The proliferation of CNE2 cells was significantly inhibited after treatment with different CINN concentrations for various lengths of time. The inhibitory effect of CINN was concentration- and time-dependent. Flow cytometric analysis showed that 2 mmol/L CINN displayed a significant apoptosis-inducing effect. The western blot analysis results showed that KLF6, Fas-L, Bax, P53 and caspase-3 protein expression was drastically increased in the CNE2 cells after treatment with 2 mmol/L CINN, whereas Bcl-2 and cyclin D1 protein expression was markedly reduced. Conclusion: CINN inhibits the proliferation and induces the apoptosis of CNE2 cells. Therefore, CINN possesses a potential anti-tumor effect.

Introduction
Nasopharyngeal carcinoma (NPC) is prevalent in southeast Asia and southern China. The pathogenesis of NPC is a process involving multiple factors and multiple pathways and is closely related to genetic factors, Epstein-Barr virus (EBV) infection and environmental factors.
impacts [1, 2]. NPC is highly invasive and metastasis prone. Although traditional treatment methods have achieved good therapeutic effects, the limitations and non-specificity of these treatment methods render them incapable of completely suppressing NPC development and progression [3, 4]. Therefore, the identification of novel molecular therapeutic targets has become a noteworthy research hotspot.

Cinnamic acid (CINN, also known as beta-phenylacrylic acid) is the main ingredient of the traditional Chinese medicine cinnamon. As early as 1995, Liu et al. discovered that CINN was capable of inhibiting tumor cell proliferation and inducing cell differentiation [5]. Due to its low toxicity, CINN has certain advantages for the treatment of tumors.

Kruppel-like factor 6 (KLF6) is a member of the KLF family. KLF6 is a nuclear factor that contains 3 zinc finger motifs at the end of the carboxyl terminal domain [6, 7]. The KLF6 gene encodes a widely expressed nuclear transcription factor that regulates growth-related signal transduction pathways, cell proliferation, apoptosis, and angiogenesis [8]. Because mutation or deletion of the KLF6 gene has been detected in a variety of malignant tumors, including prostate cancer, stomach cancer, NPC and liver cancer, KLF6 is speculated to be a potential tumor suppressor gene [9-12]. microRNA-181a is highly expressed in gastric cancer tissues. High levels of microRNA-181a expression enhance the potential of gastric cancer to metastasize and spread. KLF6 is a direct target of microRNA-181a. In turn, KLF6 suppresses the expression of microRNA-181a, thereby inhibiting the occurrence of gastric cancer [13]. Moreover, KLF6 inhibits the ability of LN-CaP prostate cancer cells to grow, proliferate, invade and metastasize and induces their apoptosis [14].

The apoptosis-related genes primarily include Fas ligand (Fas-L), BCL2-associated X protein (Bax) and B-cell lymphoma 2 (Bcl-2). Fas-L is a ligand that is capable of binding to the death receptor Fas and mediates cytotoxicity-induced apoptosis [15]. Bcl-2 is capable of inhibiting apoptosis; however, Bcl-2 also induces apoptosis through the mitochondrial pathway [16]. Bax suppresses Bcl-2 activity and antagonizes its anti-apoptotic effect. Therefore, the main function of Bax is to promote apoptosis [16].

Based on the above findings, the present study investigated the effects of CINN on the proliferation and apoptosis of NPC cells and the mechanisms of action of CINN.

Materials and Methods

Cell culture

CNE2 human NPC cells were cryopreserved and provided by the Scientific Research Center of Guilin Medical University. The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS) under standard conditions (37°C and 5% CO₂).

Drugs used in the experiments

CINN was purchased from Sigma-Aldrich Corporation. An appropriate amount of CINN was dissolved in absolute ethanol. Subsequently, a CINN stock solution (1 mol/L) was prepared with a final ethanol concentration of ≤2%. The stock solution was sterilized using membrane filters with pore sizes of 0.22 μm. Prior to the experiments, a series of CINN working solutions (1.0, 2.0, 4.0, 6.0 and 8.0 mmol/L) was prepared. The working solutions were stored at 4°C for future assays.

MTT assay

The cell proliferation inhibitory rate was determined by the MTT assay. CNE2 cells were seeded into 96-well plates at a density of 2×10³ cells per well and cultured adherently for 24 h in an incubator. Subsequently, the cells were overlaid with 1.0, 2.0, 4.0, 6.0, or 8.0 mmol/L CINN (200 μl per well). Each well of cells in the negative control group was overlaid with 200 μl of culture medium. Five replica wells were prepared for each group of cells. After cultivation of the cells for 24, 48, and 72 h, 20 μl of 5 mg/ml MTT for 4 h was added to each well. Light absorbance of the solution was measured at 490 nm on a microplate reader.
Flow cytometry assay

Logarithmically growing CNE2 human NPC cells were seeded into culture flasks. Once the cells fully adhered to the culture surface, they were treated with CINN (final concentration of 2.0 mmol/L) for 12 h. The cells were dual stained with Annexin V-FITC and propidium iodide (PI) for 30 min at room temperature. The stained cells were immediately analysed by flow cytometry (Becton Dickinson, USA). The early apoptotic cells are defined by Annexin V-FITC positive and PI negative.

Western Blot assay

Cells were lysed in lysis buffer and total protein contents were determined by the Bradford method. 30μg of lysis were separated by reducing SDS-PAGE and probed with primary antibodies, followed by their respective secondary antibodies. The primary antibodies against KLF-6, Bcl-2, Bax, FasL, P53, β-actin (Santa Cruz Biotechnology), and Cyclin D1 (Cell Signaling technology) were used. β-Actin was used as the loading control.

Results

The inhibitory effect of CINN on CNE2 cell proliferation

The CNE2 cells were treated with 1.0, 2.0, 4.0, 6.0 or 8.0 mmol/L CINN for 24, 48 and 72 h. The effects of the various CINN concentrations and different treatment durations on CNE2 cell growth were examined using the MTT assay. The results showed that CNE2 cell growth was significantly inhibited by CINN. Moreover, the inhibitory effect of CINN increased gradually with the increasing CINN concentration and prolonged treatment duration (Fig. 1). Cell growth was significantly inhibited after treatment with 2, 4, 6 or 8 mmol/L CINN compared to the control group. In contrast, treatment with 1 mmol/L CINN failed to significantly inhibit cell growth (Fig. 1). Therefore, the CINN concentration of 2.0 mmol/L was used in the subsequent experiments.

Fig. 1. Anti-proliferative effects of CINN on CNE2 cells. Cells in different groups were treated with various concentrations of CINN for 24 h, 48 h and 72 h, and then analyzed for viability by MTT. Changes in the optical density (OD) value at 490 nm in a microplate reader were recorded. (A: 24h; B: 48h; C: 72h).
The effect of CINN on the morphology of CNE2 cells and the apoptosis-inducing effect of CINN on CNE2 cells

CNE2 cells were stimulated with CINN (2 mmol/L) for 24, 48 and 72 h, and the morphological changes induced by the CINN treatments were examined under an inverted microscope. Compared with the control group, the number and proliferation rate of the CNE2 cells were markedly reduced after treatment with 2 mmol/L CINN for 24, 48, or 72 h. The CINN-treated cells became long and thin and displayed an elongated spindle-shaped morphology. Some CINN-treated cells even appeared star-shaped, with significant broadening of the cell body (Fig. 2). The flow cytometric analysis results showed that the CNE2 cells underwent apparent apoptosis after treatment with 2 mmol/L CINN for 24 h. The

Fig. 2. The morphology changes of CNE2 cells treated with CINN for 24h (A: control, B: CINN); 48h (C: control, D: CINN); 72h (E: control, F: CINN).

Fig. 3. Apoptosis-induced effects of CINN on CNE2 cells by flow cytometry. A: Apoptosis rates of CNE2 cells treated with CINN at 2mmol/L for 24h (a.control; b.CINN); B: The column diagram of apoptosis rates of CNE2 cells treated with CINN at 2mmol/L for 24h. **p < 0.01 vs. negative control.

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number of early apoptotic cells increased after CINN treatment compared with the control group (Fig. 3).

Western blot analysis of the effect of CINN on the expression of cell cycle- and apoptosis-related proteins

The western blot analysis results showed that P53 and Bax expression was significantly increased in the CNE2 cells 24, 48 and 72 h after treatment with 2 mmol/L CINN (Fig. 4). KLF6 and Fas-L expression was significantly elevated 12, 24, 48 and 72 h after treatment with 2 mmol/L CINN (Fig. 4). In contrast, Bcl-2 expression exhibited a declining trend after an initial increase. Specifically, Bcl-2 expression was significantly increased 12 and 24 h after CINN treatment, reached an apparent peak value at 24 h and then dropped at 72 h (Fig. 4). Caspase-3 expression was markedly elevated 12, 24, 48 and 72 h after CINN treatment, drastically elevated 48 and 72h, displaying an increasing trend. Whereas Cyclin D1 expression was significantly decreased 24,48 and 72h after treatment with 2 mmol/LCINN, exhibiting a decreasing trend (Fig. 4).

Discussion

At present, primary NPC is primarily treated with radiotherapy. Due to the continuous technological advances in radiotherapy, NPC patients who receive radiotherapy experience a 5-year survival rate of up to 60%. However, the recurrence rate of NPC remains high [17], and post-relapse retreatments are unlikely to achieve satisfactory outcomes [18]. Therefore, recurrence and distant metastasis have become the major causes of death in NPC patients. Because radiotherapy alone is unable to effectively control tumor recurrence and metastasis
and resistance to radiotherapy can readily develop, new methods have been proposed for NPC treatment.

Since the first report by Liu et al. that CINN inhibited the proliferation of tumor cells and induced the differentiation of melanoma [5], scientists have performed some studies on the tumor inhibitory effects of CINN. The present study found that treatment of CNE2 NPC cells with 2 mmol/L CINN markedly reduced the rate of cell proliferation and significantly altered the nuclear-cytoplasmic ratio. Additionally, CINN treatment partially restored normal cell morphology and drove cell differentiation towards a benign phenotype. The flow cytometric analysis revealed significant apoptosis after treatment of the CNE2 cells with 2 mmol/L CINN for 24 h. The number of early apoptotic cells was also increased after CINN treatment. The results of the present study indicate that CINN inhibits the proliferation and induces the apoptosis of CNE2 cells.

To clarify the underlying molecular mechanisms, western blotting was performed to examine the expression of cell cycle- and apoptosis-related proteins. After treatment of the CNE2 cells with 2 mmol/L CINN, the KLF6, Fas-L, Bax, P53 and caspase-3 protein expression levels were significantly upregulated, Bcl-2 protein expression increased initially and then decreased, and cyclin D1 protein expression was markedly reduced.

Caspase-3 is a key executor of apoptosis, and caspase-3 overexpression induces apoptosis [19]. In the present study, caspase-3 protein expression in the CNE2 cells was significantly elevated as the 2 mmol/L CINN treatment duration increased. Therefore, we can infer that CINN is an initiating factor capable of inducing CNE2 cell apoptosis.

Fas-L binds to the death receptor Fas to mediate cytotoxicity-induced apoptosis [15]. Treatment of CNE2 cells with CINN significantly increased the Fas-L protein expression level, suggesting that CINN induced the apoptosis of CNE2 cells through the death receptor pathway; the apoptosis-inducing effect of CINN was concentration- and time-dependent. Bcl-2 is capable of antagonizing apoptosis. However, Bcl-2 also induces apoptosis through the mitochondrial pathway. The direction of the Bcl-2-mediated regulation of apoptosis is affected by the Bcl-2/Bax balance [16]. In the present study, CNE2 cells were treated with 2 mmol/L CINN. The Bcl-2 protein expression level was markedly reduced and the Bax protein expression level was drastically elevated as the treatment duration increased. Therefore, CINN induced the apoptosis of CNE2 cells through the mitochondrial pathway.

As a tumor suppressor gene, P53 promotes the repair of DNA damage. In cases where the damage cannot be successfully repaired, P53 induces apoptosis through promoting the expression of apoptosis-related proteins [20]. In the CNE2 cells treated with 2 mmol/L CINN, the P53 protein expression level significantly increased as the treatment duration increased. Hence, we inferred that CINN induced the apoptosis of CNE2 cells through upregulating P53 expression. Cyclin D1 regulates cell cycle progression from the G1 to S phase, and cyclin D1 overexpression leads to abnormal cell proliferation and tumorigenesis [21]. In the CNE2 cells treated with 2 mmol/L CINN, the cyclin D1 protein expression level was significantly reduced as the treatment duration increased. Hence, CINN induces cell cycle arrest in CNE2 cells and inhibits CNE2 cell proliferation. Moreover, the proliferation inhibitory effect of CINN appears to be time-dependent. A study has shown that KLF6 upregulates P21 through P53-independent pathways, thereby inhibiting the activity of cyclin E/cyclin-dependent kinase 2 (CDK2) [22]. Additionally, KLF6 binds to cyclin D1, thereby reducing the activity of the cyclin D1/cyclin-dependent kinase 4 (CDK4) [23]. Inhibition of either cyclin E/CDK2 or cyclin D1/CDK4 induces cell cycle arrest and suppresses cell proliferation. Tarocchi et al. found that overexpression of the KLF6 gene enhanced P53 protein expression [24]. Moreover, P53 directly downregulates Bcl-2 expression and upregulates Bax expression, thereby promoting apoptosis [25]. P53 also mediates the Fas-L death-receptor pathway [26] and the Bcl-2/Bax-dependent mitochondrial apoptosis pathway [27], which eventually activate the downstream protein caspase-3. Activated caspase-3 executes the apoptotic program [19]. To verify the above mechanisms, we downregulated KLF6 expression in CNE2 cells and found that the Fas-L, Bax, Bcl-2 and tumor necrosis factor (TNF) protein expression levels were significantly downregulated (data not shown). These findings indicate that KLF6 may be
involved in the regulation of apoptosis-related factors, including Fas-L, Bax, and TNF, and that KLF6 may be an upstream gene of the above apoptosis-related factors [28]. Therefore, we preliminarily conjectured the mechanisms by which CINN inhibited cell proliferation and induced apoptosis. It is likely that CINN affects the expression of cell cycle- and apoptosis-related proteins through the upregulation of KLF6 expression, ultimately inhibiting cell proliferation and inducing apoptosis.

After summarizing the findings of the present study, we concluded that CINN inhibited the proliferation of NPC cells through upregulating KLF6 expression and downregulating cyclin D1 expression. However, CINN upregulates the expression of KLF6, P53, Fas-L, Bax and caspase-3 and downregulates the expression of Bcl-2, thereby inducing apoptosis. Additionally, KLF6 plays an important role in the inhibition of the proliferation of NPC cells and the induction of apoptosis of NPC cells. Therefore, KLF6 most likely inhibits cell proliferation and promotes apoptosis through regulating the expression of proteins related to cell proliferation and apoptosis. CINN has the potential to be developed into a differentiation-inducing drug for NPC treatment, and KLF6 may serve as a new target in gene therapies against NPC.

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Disclosure Statement

The authors declare no conflict of interest.

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