ORIGINAL ARTICLE

In vitro and in vivo antitumor activity of neochlorogenic acid in human gastric carcinoma cells are complemented with ROS generation, loss of mitochondrial membrane potential and apoptosis induction

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Summary

Purpose: The main aim of the study was to evaluate in vitro and in vivo the anticancer and apoptotic effects of neochlorogenic acid in human gastric carcinoma cell death and the underlying mechanism of apoptosis induction, reactive oxygen species (ROS) production and loss of mitochondrial membrane potential (MMP), m-TOR/PI3K/AKT signa<mark>lli</mark> pathway, cell migration and cell invasion suppression.

Methods: Fluorescence microscopy using DAPL and annexi V/PI staining in combination with flow cytometry was used to study the apoptotic effects induced by neochloroger on gastric cancer cells. The effects on ROS and MMP were studied by flow cytometry. Western blot assay was used to evaluate the effects of neochlorogenic acid on m-TOR/PI3/ Akt signaling pathway. To examine the anti-cancer activ-ity of neochlorogenic acid ity of neochlorogenic acid in vivo, we used the nude mice xenograft model.

Results: The results indicated that neochlorogenic acid exhibited an IC_{50} of 20 μ M in these cells. The study also showed that apoptosis was due to loss of MMP and increased intracellular ROS production. Neochlorogenic acid downregulated the expression of key proteins of m-TOR/PI3/Akt signaling eathway. After 6 weeks of neochlorogenic acid administran to mice, the average tumor volumes and growth for the untreated control group were significantly higher than the treated groups.

Conclusion: Based on these results, we propose that neochlorogenic acid can be a prospective anti-cancer therapeutic lead for the management of human gastric carcinoma.

Key words: anticancer, apoptosis, flow cytometry, gastric carcinoma, neochlorogenic acid, ROS

Introduction

affects over 28 million people around the globe and caused about 7.5 million deaths in 2008 [1]. Several factors such as the life style and diet are responsible for the development of deadly diseases such as cancer [2]. Gastric cancer (GC) is one of the prevalent types of cancers and more than 50% of all cancers detected in East Asian countries are gastric cancers. Despite a decline in the incidence

Cancer is a devastating health problem which of GC, it is still reported as one of the commonly diagnosed cancers across the world [3]. The treatment of GC generally involves chemotherapy but the clinical outcomes are still very poor. Besides, the adverse effects of the anticancer agents used for the treatment of GC have adverse impact on the overall health of the GC patients [4]. Over the years the dietary polyphenols have gained tremendous attention owing to their strong antioxidant

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properties. Oxidative stress is linked to the development of severe diseases like neurodegenerative, cardiovascular and cancer to name a few. And since polyphenols can relieve the oxidative stress, it is believed that can prove promising in the management of these diseases [5]. Plants produce a wide array of polyphenols as secondary metabolites in response to environmental stresses [6] which are being evaluated for their beneficial health properties [7].

Previous studies have indicated that neochlorogenic acid inhibits the growth of cancer cells [8]. However, the growth inhibitory properties of neochlorogenic acid have not been studied against GC. This was designed for the evaluation of the anticancer properties of neochlorogenic acid, an important plant-derived polyphenol.

Methods

Cell culture conditions

The AGS gastric cancer cell line was obtained from the Cancer Research Institute of Beijing (Beijing, China) and maintained in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Massachusetts, USA), supplemented with 10% fetal bovine serum (EBS) (Invitrogen Life Technologies, Massachusetts, USA), 100 ml streptomycin and 100 U/ml penicillin G (Himedi Pennsylvania, USA) in an incubator at 37°C with 5% CO

MTT assay

For examination of the proliferation rate the AGS cells were cultured in 96-well plates at 5×10^3 cells per well. The cells were then incubated overnight at 37° C followed by replacement of the Dulbecco's modified Eagle's medium with a new media containing neochlorogenic acid at different concentrations (0- 100 μ M) for one day. Afterwards, addition of MTT (0.5 mg/mL) were incubated for 3-4 hrs and followed and the finally the absorbance at 570 nm was determined by spectrophotometer.

Apoptosis, ROS and MMP assays

The nuclear morphology of AGS cancer cells was assessed by fluorescence microscopy after subjecting the cells to DAPI staining. Ten fields with 100 cells/ field were randomly selected for estimation of the cells with condensed nuclei. Annexin V/PI double staining was used for determination of apoptotic AGS cancer cells by flow cytometry. The ROS and MMP levels were determined using standard protocols as described previously [9].

Cell migration and invasion assay

The migration of AGS cells was determined with wound healing assay. The cells were grown till 70% confluence and a scratch was made with a scratching device. The cells were then incubated for 48 hrs again, and the **Figure 1.** Chemical structure of neochlorogenic acid.

wound healing capacity was evaluated by comparing the widths of the wounds. Cell invasion was determined by transwell assay.

In vivo study

Male BALB/c nude mice were maintained in the animal facility following the National Institutes of Health standards for the care and use of laboratory animals and approved by Gansu Provincial Hospital, Lanzhou, Gansu. In this study 4-week-old mice were used and were injected with 5×10⁶ AGS cells sub-cutaneously at the left flank. As the tumors grew, the mice (n=5) were injected intraperitoneally with DMSO (0.1%)-dissolved neochlorogenic acid and diluted with 100 μ L normal saline at 50 mg/kg body weight and taken as the day one of the experiment. Neochlorogenic acid was administered to the mice thrice a week, while the control mice were administered DMSO (0.1%) in normal saline only. At the end of 6 weeks, the mice were euthanized and the tumors were harvested for assessment of their growth and other investigations

Western blo

The GC AGS cells were lysed using ice-cold hypobuffer. After estimating the protein concentrations toni in each of the cell extracts, the samples containing the proteins were loaded and separated on SDS-PAGE. This was followed by transference to a nitrocellulose membrane and incubation with the primary antibody (1:1000) hrs at 4°C. Afterwards, the membrane was incuated with HRP-conjugated secondary antibody (1:1000) for at 24°C for about 1 hr. The visualization of the proteins was carried out by enhanced chemi-luminescence reagent.

Results

Neochlorogenic acid decreases the viability of AGS gastric cancer cells

The impact of neochlorogenic acid (Figure 1) on cell viability was evaluated with MTT assay. The AGS cells were subjected to neochlorogenic acid treatment at varied concentrations. The results of MTT assay revealed that neochlorogenic acid



exhibited considerable antiproliferative effects on the AGS cells and the antiproliferative effects were found to be concentration-dependent (Figure 2). It was observed that the IC_{50} of neochlorogenic acid against AGS cells was 20 μ M. These results clearly show that neochlorogenic acid selectively targets GC cells.

Neochlorogenic acid induces apoptosis in AGS gastric cancer cells

Next we sought to know if neochlorogenic acid induces apoptosis in AGS cells and therefore DAPI staining was performed. The AGS cells were first







Figure 3. Effect of neochlorogenic acid on apoptosis of AGS cells. The figure depicts that neochlorogenic acid induces apoptosis of the AGS cells in a concentration-dependent manner. Experiments were performed in triplicate.



Figure 4. Percentage of apoptotic AGS cells as depicted by annexin V/PI staining. The figure depicts that neochlorogenic acid increases the percentage of the apoptotic AGS cells in a concentration-dependent manner. Experiments were performed in triplicate.



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Figure 5. Effect of neochlorogenic acid on the ROS (%) in treated AGS cells. The figure depicts that neochlorogenic acid increases the ROS levels of the AGS cells in a concentration-dependent manner. The values are mean ±SD (n=3, *p<0.05).



Figure 6. Effect of neochlorogenic acid on the MMP (%) in treated AGS cells. The figure depicts that neochlorogenic acid increases the ROS levels of the AGS cells in a concentration-dependent manner. The values are mean ±SD (n=3, *p<0.05).

exposed to neochlorogenic acid at different concentrations, then subjected to DAPI staining and finally observed under fluorescence microscope. It was found that neochlorogenic acid induced apoptosis in AGS GC cells as evidenced from the increased number of cells with white colored nuclei (Figure 3). Annexin V/PI further revealed that apoptotic cells enhanced from 2.1% in the control to 35.3% at 40 µM of neochlorogenic acid (Figure 4). The effects of neochlorogenic acid were also investigated on the ROS and MMP levels and it was found that neochlorogenic acid caused accumulation of ROS (Figure 5) which was also associated with significant decline in the MMP levels, indicating that neochlorogenic acid might induce ROS-mediated apoptosis in AGS (Figure 6).

Neochlorogenic acid inhibits cell migration and invasion of AGS cells

The impact of neochlorogenic acid at IC_{50} was determined on the migration of AGS cells by

wound healing assay. The results showed that neochlorogenic acid considerably inhibited the migration of the AGS cells (Figure 6). Similar effects were observed on the invasion of the AGS cells (Figure 7).

Neochlorogenic acid inhibits PI3K/AKT signalling pathway

PI3K/AKT signalling pathway is involved in the progression and tumorigenesis of different cancer types [8]. Herein, the effect of neochlorogenic acid was also assessed on PI3K/AKT pathway. What was found was that neochlorogenic acid decreased the p-mTOR, p-PI3K and p-AKT expression dose-dependently. However, the expression of mTOR, PI3K and AKT remained almost unaltered (Figure 9).

Neochlorogenic acid inhibits the tumor growth in vivo

The impact of neochlorogenic acid was also evaluated *in vivo* on the xenografted tumors and it was found that neochlorogenic acid considerably halted the tumor growth (Figure 10A). At the dosage of 50 mg/kg of neochlorogenic acid, the tumor weight was significantly reduced (Figure 10B). In addition, the tumor volume showed a



Figure 7. Effect of neochlorogenic acid on migration of AGS cells. The figure depicts that neochlorogenic acid decreases the migration of the AGS cells. Experiments were performed in triplicate.



Figure 8. Effect of neochlorogenic acid on invasion of AGS cells. The figure depicts that neochlorogenic acid decreases the invasion of the AGS cells. Experiments were performed in triplicate.

Neochlorogenic acid (µM)



Figure 9. Effect of neochlorogenic acid on mTOR/PI3K/AKT pathway and phosphorylated forms in AGS cells as depicted by western blotting. The figure depicts that neochlorogenic blocks the PI3K/AKT/mTOR pathway in the AGS cells. Experiments were performed in triplicate.



Figure 10. Effect of neochlorogenic acid (50 mg/kg) on **(A)** tumor growth **(B)** tumor volume and **(C)** tumor weight. The figure depicts that neochlorogenic acid inhibits the xenografted tumor growth *in vivo*. The values are mean ±SD (n=3, *p<0.05).

time-dependent decline in response to neochloro genic acid (Figure 10C).

Discussion

Over the years plant-derived secondary metabolites have attracted considerable attention as bioactive molecules and have been shown to possess anticancer properties on different types of cancers [10]. Against this background, this study was carried out to investigate the growth-inhibitory effects of a plant-derived polyphenol against AGS GC cells. The results showed that neochlorogenic acid exhibited considerable anticancer activity with an IC_{50} of 20 µM against the AGS GC cells. To further determine the mechanism of the anticancer effects of neochlorogenic acid, DAPI staining was performed, which revealed that neochlorogenic acid exerted anticancer effects via induction of apoptosis. Moreover, the apoptotic effects of neochlorogenic acid were concentration-dependent as evidenced from the annexin V/PI staining. Apoptosis is an important mechanism by which several chemotherapeutic drugs exert their anti-proliferative action [11]. These results are supported by previous investigations wherein polyphenols have been reported into induce apoptotic cell death of cancer cells [12]. We

sought to know if the neochlorogenic acid-induced apoptosis followed the mitochondrial pathway, by estimating the Bax and Bcl-2 expression as well as the ROS production and MMP levels. Western blotting showed that the expression of Bax was increased and that of Bcl-2 was suppressed in response to neochlorogenic acid treatment. Furthermore, upon neochlorogenic acid treatment there was upsurge of ROS production and decline in the MMP levels, ultimately favoring apoptosis. Previous studies have also implicated ROS in the disruption of MMP and induction of apoptosis [13]. Metastasis of cancer cells is initiated by the migration and invasion of cancer cells from the site of their origin to other parts of the body and is an important factor for prognosis of cancer [14]. In the present study we found that neochlorogenic acid inhibited the migration and invasion of the AGS gastric cells. It was reported earlier that many anticancer molecules target mTOR/PI3K/AKT signalling pathway in cancer cells [15]. Therefore, we investigated the effect of neochlorogenic acid on the expression of p-mTOR, mTOR, p-AKT, AKT, p-PI3K and PI3K and found that neochlorogenic acid decreased the expression of p-mTOR, p-PI3K and p-AKT, indicating that the anticancer effects of neochlorogenic acid may in part be due to inhibition of PI3K/AKT signalling pathway. Finally, *in vivo* evaluation showed that neochlorogenic acid inhibits the growth of tumors in xenografted mice, indicative of the potential of neochlorogenic acid in the management of gastric cancer.

Conclusion

The outcomes of this study indicate that neochlorogenic shows considerable anticancer activity against gastric cancer. The anticancer activity can be attributed to its ability to trigger apoptosis, and inhibit mTOR/PI3K/AKT signalling pathway.

The results also suggest that neochlorogenic acid may prove a promising lead molecule for the development of systemic therapy for gastric cancer and thus warrants further evaluation.

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Conflict of interests

The authors declare no conflict of interests.



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