Original Research Article

Cinnamic hydroxamic acid inhibits the proliferation of gastric cancer cells via upregulation of miR 145 expression and down-regulation of P13K/Akt signaling pathway

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Abstract

Purpose: To investigate the anti-proliferative effect of cinnamic hydroxamic acid (CHA) on gastric cancer (GC) cells, and its mechanism of action.

Methods: Two GC cell lines (SGC-7901 and MKN1) and normal human gastric epithelial cells (GES1) were used for this study. The GC cells were cultured in Dulbecco's modified Eagle's medium (DMEM)supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin solution at 37 °C for 24 h in a humidified atmosphere of 5 % CO_2 and 95 % air. GES1 cells were cultured in RPMI medium supplemented with 10 % FBS only. Cell viability and apoptosis were determined using 3 (4,5 dimethyl thiazol 2 yl) 2,5 diphenyl 2H tetrazolium bromide (MTT), and flow cytometric assays, respectively. The level of expression of microRNA-145 (miR-145) was determined using real-time quantitative polymerase chain reaction (qRT-PCR). Protein expressions of c-Myc, p-AKT, PI3K, p21, and matrix metalloproteinase (MMP)-2 and MMP-9were determined using Western blotting.

Results: Treatment of GC cells with CHA for 72 h led to significant and dose-dependent reduction in their viability, and significant and dose-dependent increase in the number of apoptotic cells ($p < 0.05$). It also significantly arrested GC cell cycle at G1 phase ($p < 0.05$). The treatment significantly and dose-dependently decreased SGC-7901 and MKN1 cell migration and invasion, and upregulated miR-145 mRNA expression ($p < 0.05$). The expression of miR-145 mRNA was significantly higher in MKN1 cells than in SGC-7901cells ($p < 0.05$). Treatment of SGC-7901 and MKN1 cells with CHA significantly down-regulated protein expressions of c-Myc, MMP-2/9, PI3K and p-AKT, but upregulated p21 protein expression ($p < 0.05$).

Conclusion: These results show that CHA inhibits the proliferation of GC cells via upregulation of miR-145 expression and down-regulation of P13K/Akt signaling pathway. Therefore, CHA has a good potential as a therapeutic agent for the management of gastric cancer

Keywords: Apoptosis, Cinnamic hydroxamic acid, Gastric cancer, Metastasis, Proliferation

INTRODUCTION

Gastric cancer (GC), a common digestive tract cancer characterized by high morbidity and mortality, is prevalent among South-East Asians [1,2]. Although there has been a drop in the incidence of GC in recent times, its prognosis remains poor [3]. Early detection is key to the treatment of GC, since late diagnosis is associated with poor prognosis [4]. Cell

metastasis is responsible for the failure of chemotherapy [5].

MicroRNAs (miRNAs) consist of approximately 19-25 nucleotides and function through base-pairing with complementary sequences within mRNA molecules. MicroRNAs influence the expression of target genes by pairing with their 3'UTR regions, thereby participating in life processes such as cell growth and differentiation, and apoptosis [6,7]. They have been implicated in the pathogenesis of GC [8]. The expression of miR-145 has been shown to be down-regulated in precancerous lesions of colorectal tissues [9]. Although miR-145 has a tumor-inhibiting effect, the mechanisms underlying its involvement in the proliferation, metastasis, and invasion of tumor cells are not entirely clear. It recruits Myc proto-oncogene protein (c-Myc) and transcription factor-1 (TF-1) in tumor-suppression [10,11]. MicroRNA-145 inhibits GC cell proliferation via regulation of myosin VI (MYO6) expression [12]. Its inhibition of GC cell growth and metastasis has also been reported to occur via suppression of the expressions of specificity protein 1 (Sp1) and N-cadherin [13].

Cinnamic hydroxamic acid (CHA) and its derivatives inhibit the activity of nuclear, as well as cytoplasmic histone deacetylases (HDACs) [14].The presence of para- and meta-substituents in CHAs make these compounds multi-potent inhibitors, while ortho-substituents give them selectivity as inhibitors of HDACs[15]. Studies have shown that the presence of small groups such as methyl, methoxy, and trifluoromethyl in the aromatic ring of CHAs reduces the potency of these compounds, while a large substituent like pyridyl4-oxy group increases their activities and selectivity for HDAC inhibition [16]. This study investigated the anti-proliferative effect of CHA on GC cells, and the mechanism involved.
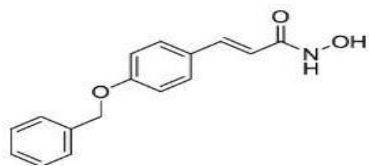

Figure 1: Chemical structure of CHA

EXPERIMENTAL

Materials

Flow cytometer and FACScan software were purchased from BD Biosciences (USA). Anti-c-Myc, p-AKT, PI3K, p21, MMP-2, MMP-9 and GAPDH polyclonal antibodies were products of Cell Signaling Technology (USA). Light microscope was obtained from Olympus Corporation (Japan) while RNAiso Plus was purchased from Dalian (China).

Cell culture

Two GC cell lines (SGC-7901 and MKN1) and normal human gastric epithelial cells (GES1) were obtained from the Chinese Academy of Sciences. The GC cells were cultured in DMEM supplemented with 10 % FBS and 1 % penicillin/streptomycin solution at 37 °C for 24 h in a humidified atmosphere of 5 % CO_2 and 95 % air. The GES1 cells were cultured in RPMI medium supplemented with 10 % FBS only.

Cell viability assay

The viability of MKN1 and SGC-7901 cells in the presence of CHA was determined using MTT assay. The cells were seeded at a density of 1 x 10^5cells/well in 96-well plates and cultured in DMEM for 24 h. Then, CHA (0.75 – 20 μM) was added to the cells and incubated for 72 h. At the end of the third day, 20 μL of MTT solution (5 mg/mL) was added to the wells, followed by incubation at 37 °C for 2 h. The medium was finally replaced with 120 μL of 0.1 % dimethyl sulfoxide (DMSO) to completely dissolve the formazan crystals formed. The absorbance of the samples was read in a microplate reader at 485 nm after shaking for 15 min. The assay was performed in triplicate. Cell viability (V) was calculated as shown in Eq 1.

V (%) = (At/Ac)100 ………….. (1)

where At and Ac are the absorbance of test and control group, respectively.

Assessment of cell apoptosis

The GC cells were seeded at a density of 1.5 x 10^5 cells/well in 6-well plates and cultured for 24 h. Then, CHA (3 – 20 μM) was added to the medium and incubated for another 48 h, followed with washing in phosphate-buffered saline (PBS), and thoroughly mixing with 300 μL binding buffer. The cells were then stained with 5 μL each of annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) within 20 min at room temperature in the dark. Cell apoptosis was determined using a flow cytometer fitted with argon laser operated at 485 nm.

Cell cycle analysis

The effect of CHA on cell cycle distribution in GC cells was determined using a flow cytometer. The

GC cells treated with varied concentrations of CHA (3 – 20 µM) were seeded into 6-well plates and incubated for 48 h. The cells were then washed with PBS, and fixed with 70 % ethyl alcohol at -30 ˚C overnight. Then, tris-hydrochloride buffer (pH 7.4) containing 1 % RNase A was added to the plates. The cells were subsequently stained with PI (5 mg/mL) and injected into the flow cytometer for analysis.

Determination of cell invasiveness

The degree of invasiveness of GC cells was determined using Transwell invasion assay. Cells treated with varied concentrations of CHA (3 – 20 µM) for 48 h in RPMI-1640 medium were seeded at a density of 1 x 10^5 cells/well in Transwell chamber coated with substrate (200 mg/mL Matrigel) and cultured in serum-free medium. Medium containing 20 % FBS was added to the lower chamber. After 24 h, the cells that passed through the matrix gel membrane were stained with crystal violet after fixation, photographed and counted using an inverted microscope.

Cell migration assay

The migratory ability of GC cells was determined using scratch test. Cells in logarithmic growth phase were seeded in 6-well plates until they attained 90 - 100 % confluency, and scratches were made on the cell monolayers. After washing thrice with serum-free medium, the cells were further cultured for 48h, and then observed and analyzed using Image Pro Plus (7.0). Cell motility (M) was calculated as shown in Eq 2.

$$M (\%) = 1 - (S_m/S_i)100 \ \ldots\ldots\ldots\ldots \ (2)$$

where S_m and S_i are the scratch width during measurement and initially, respectively.

Western blotting

The GC cells treated with varied concentrations of CHA (3 –20 µM) were washed with PBS and lysed with Tris-HCl (40 mM, pH 7.4) containing sodium chloride (150 mM), Triton X-100 (1 %*v/v*), and protease inhibitors. The resultant lysate was centrifuged at 15000 rpm for 15 min at 4 °C, and the protein concentration of the supernatant was determined using bicinchoninic acid (BCA) protein kit. A portion of total cell protein (40 µg) from each sample was separated on 12 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 ° C for 120 min.

Subsequently, skimmed milk (5 %) in Tris-buffered saline containing 0.1 % Tween-20 (TBS-T) was added with gentle shaking at 37 ºC, and incubated for 2h to block non-specific binding of the blot. Incubation of the blots was performed overnight at 4 ∘C with primary antibodies of c-Myc, p-AKT, PI3K, p21, MMP-2, MMP-9 and GAPDH, each at a dilution of 1 to 1000. Then, the membrane was washed thrice with TBS-T and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 2 h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using image J (2.0). The respective protein expression levels were normalized to that of standard GAPDH.

qRT-PCR

The level of expression of miR-145 was measured using qRT-PCR. Trizol RNA extraction reagent (RNAiso Plus) was used to extract total RNA from cells in each group, while cDNA synthesis kit (ProSTARt First Strand RT-PCR kit) was used to perform cDNA synthesis reaction according to the instructions of the manufacturer. Light Cycler 1536 RT-PCR detection system was used for the estimation of the mRNA expression of miR-145. Variation in the cDNA content was normalized using GAPDH. The PCR reaction mixture (20 µL) consisted of 6.4 µL of dH$_2$O, 1.6 µL of gene-specific primer (10 µM), 2 µL of synthesized cDNA and 10 µL of SYBR Premix Ex Taq™ II. The amplification process consisted of 94 ˚C for 3 min, 38 cycles at 94 ˚C for 26 s, 53 ˚C for 26 s, 70 ˚C for 26 s and 70 ˚C for 5 min. The Ct value of U6 was taken as the internal parameter, and 2$^{-\Delta\Delta Ct}$ was used to calculate the relative expression level of the protein. The sequence of primers used are shown in Table 1.

Table 1: Primer sequence used for qRT-PCR

Gene	Sequence
miR-145	Forward: 5'-GTC CAG TTT TCC CAG GAA TCC CT-3'
	Backward: 5'-GCT GTC AAC GAT ACG CTA CCT A-3'

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed using SPSS (17.0). Groups were compared using Tukey's post-hoc test. Statistical significance was assumed at *p* < 0.05.

RESULTS

Effect of CHA on the viability of GC cells

As shown in Figure 2, treatment of GC cells with CHA for 72 h led to significant and dose-dependent reduction in their viability ($p < 0.05$).
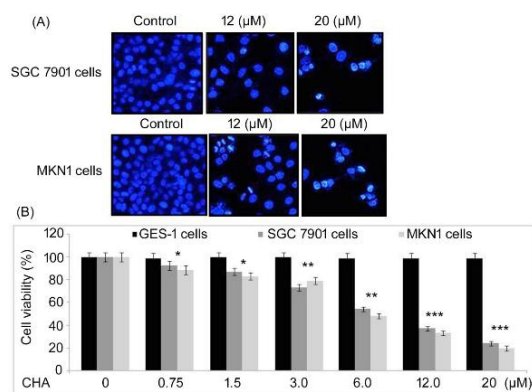

Figure 2: Effect of CHA treatment on the viability of SGC-7901 and MKN1 **cells.** (A): Microscopic examination of GC cell morphology (x250); and (B): results of MTT assay; *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, compared with control cells

Effect of CHA on apoptosis of GC cells

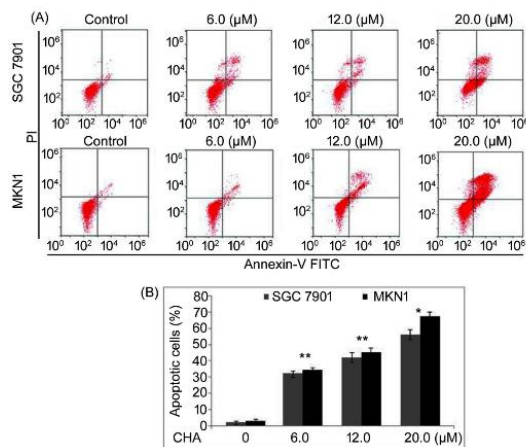Treatment with CHA significantly and dose-dependently promoted apoptosis in GC cells ($p < 0.05$; Figure 3).

Figure 3: Effect of CHA on apoptosis in SGC-7901 and MKN1 cells. (A): Results of flow cytometric analysis of GC cells; and (B): apoptotic cell population in SGC-7901 and MKN1 cells; *$p < 0.05$; **$p < 0.01$, compared with control cells

Effect of CHA on GC cell cycle arrest

As shown in Figure 4, CHA treatment significantly and dose-dependently increased GC

cell population in G1 phase of the cell cycle, but reduced the cell population in S and G2/M phases significantly and dose-dependently ($p < 0.05$). However, CHA significantly arrested GC cell cycle at G1 phase.
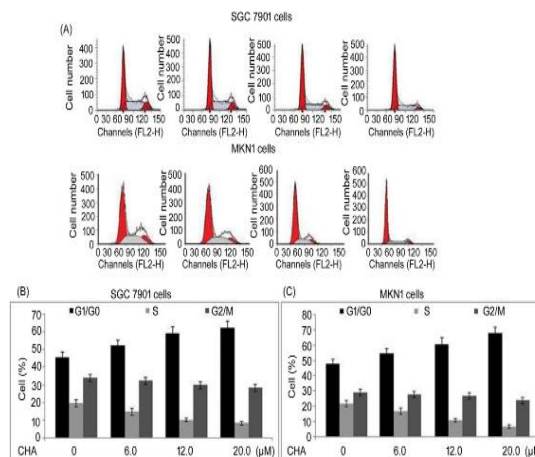

Figure 4: Effect of CHA on GC cell cycle progression. (A): Distribution of cells among different phases of the cell cycle, as measured using a flow cytometer; and (B): Chart showing cell population in various phases of the cell cycle; *$p < 0.05$; **$p < 0.01$, when compared with control cells

Effect of CHA on GC cell metastasis *in vitro*

Cinnamic hydroxamic acid (CHA) treatment significantly and dose-dependently decreased SGC-7901 and MKN1 cell migration and invasion ($p < 0.05$). These results are shown in Figure 5.
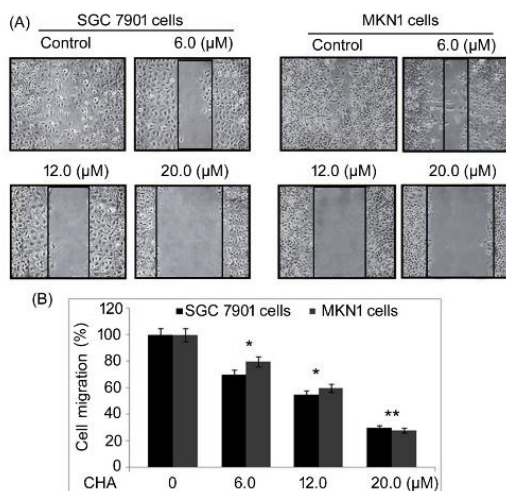

Figure 5: Effect of CHA on migration of SGC-7901 and MKN1 cells. (A): Migration of GC cells as measured using wound healing assay (x250); and (B): chart showing the extent of migration of GC cells; *$p < 0.05$ and **$p < 0.01$, compared with control cells
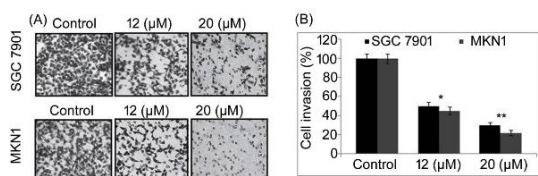
Figure 6: Effect of CHA on SGC-7901 and MKN1 cell invasiveness. (A): Gastric cancer cell invasiveness determined using Transwell assay (x 250); and**(B):** Chart representing adhesive cell counts; *$p < 0.05$; **$p < 0.01$, compared with control cells

Effect of CHA on expression level of miR-145in SGC-7901 and MKN1 cells

As shown in Figure 7, treatment of GC cells with CHA significantly and dose-dependently upregulated miR-145 mRNA expression ($p < 0.05$). The expression of miR-145 mRNA was significantly higher in MKN1 cells than in SGC-7901 cells ($p < 0.05$).
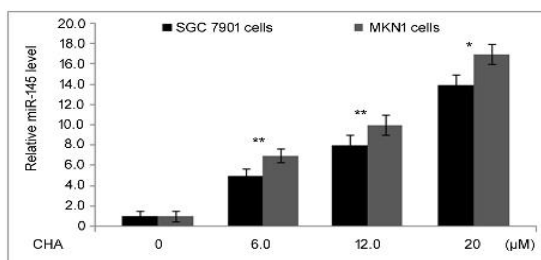

Figure 7: Effect of CHA on miR-145 expressions in SGC-7901 and MKN1 cells. *$p < 0.05$ and **$p < 0.01$, compared with control cells

Regulatory effect of CHA on PI3K/AKT pathway

Treatment of SGC-7901 and MKN1 cells with CHA significantly down-regulated protein expressions of c-Myc, MMP-2/9, PI3K and p-AKT, but upregulated p21 protein expression, significantly and dose-dependently ($p< 0.05$; Figure 8).
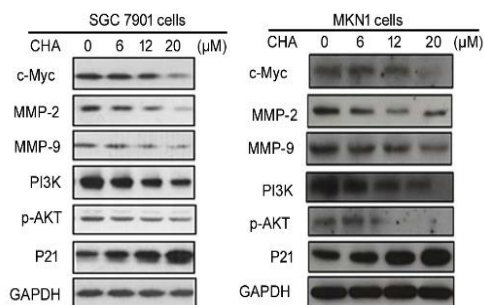

Figure 8: Effect of CHA on PI3K/AKT signaling pathway

DISCUSSION

Globally, approximately one million people come down with GC annually [17]. The pathogenesis of GC is a complex process involving several genes and regulatory pathways. MicroRNAs have been shown to participate in the pathogenesis of GC. However, the precise molecular mechanism involved remains unclear. The initiation of GC involves several signaling pathways and abnormal gene expression patterns which are often accompanied by inactivation of anti-oncogenes and activation of oncogenes. This study investigated the anti-proliferative effect of CHA on GC cells, and the mechanism involved.

Apoptosis, a physiologically significant process for getting rid of damaged cells from the body of a multicellular organism is usually triggered by many antitumor drugs [18]. Natural compounds such as resveratrol and L-securinine engage multiple mechanisms for induction of apoptosis in tumor cells [19].

In this study, CHA treatment significantly and dose-dependently promoted apoptosis in SGC-7901 and MKN1 cells, and significantly arrested the cells at G1 phase. The proliferation of SGC-7901 and MKN1 cells was significantly and dose-dependently suppressed by CHA treatment. These results indicate that CHA may inhibit the proliferation of GC cells via induction of apoptosis and cell cycle arrest at G1 phase.

Alteration in miRNA expression has been implicated in many disease conditions. Studies have shown that miR-145 expression is significantly down-regulated in many types of cancers, and that it serves as a target for a variety of anticancer drugs [20]. The results of this study showed that miR-145 expression was significantly and dose-dependently down-regulated in SGC-7901 and MKN1 cells. These findings are in agreement with those of previous reports [11]. It is likely that the anti-proliferative effect of CHA on GC cells is associated with the upregulation of miR-145 expression.

The PI3K/AKT signaling pathway is a key regulator of normal cellular processes involved in cell growth, proliferation, metabolism, motility, survival, and apoptosis [21]. Aberrant activation of this pathway promotes the survival and proliferation of tumor cells in many human cancers. One of the oncogenes, c-Myc, is associated with cellular transformation and proliferation, as well as regulation of apoptosis [22]. Increased expression of c-Myc leads to DNA damage and ultimately cell apoptosis [23]. Therefore, targeting c-Myc expression

contributes significantly to arrest of cell cycle progression and inhibition of cell proliferation [24]. In this study, treatment of SGC-7901 and MKN1 cells with CHA led to significant and dose-dependent down-regulation of protein expressions of PI3K, p-AKT and c-Myc, an indication that CHA may serve as a down-regulating agent for PI3K/AKT pathway in GC cells.

Matrix metalloproteinases (MMPs) are a group of enzymes that are collectively responsible for the degradation of most extracellular matrix proteins during organogenesis, growth and normal tissue turnover. Matrix metalloproteinase (MMP)-2 and MMP-9 contribute significantly to the decomposition of type IV collagen, thereby promoting cell migration and invasion [25]. The results of this study indicated that CHA significantly down-regulated protein expressions of MMP2 and MMP9 in SGC-7901 and MKN1 cells, an indication that CHA may possess anti-metastatic effect.

CONCLUSION

The results obtained in this study show that CHA inhibits the proliferation of GC cells via the upregulation of miR-145expression and down-regulation of P13K/Akt signaling pathway.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. SenMao Hu designed the study and wrote the manuscript. Shan Ping Li and SenMao Hu performed the experimental work, carried out the literature study and analyzed the data. The research article was thoroughly read by the authors before communication for the consideration of publication.

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