

## Original Research Article

# Inhibition of gastric cancer cell growth, invasion and metastasis by tocotrienolic amide

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Sent for review: 19 July 2020

Revised accepted: 20 November 2020

### Abstract

**Purpose:** To investigate the effect of tocotrienolic amide on gastric cancer (GC) cell growth and metastasis, and the underlying mechanism of action.

**Methods:** Gastric cancer (GC) cell lines MKN28 and NCI-N87 were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin solution at 37 °C in a humidified atmosphere of 5 % CO<sub>2</sub> and 95 % air. Cell invasion and migration were determined using Transwell and wound healing assays, respectively. Real-time quantitative polymerase chain reaction (qRT-PCR) and Western blotting were used for the determination of changes in the levels of expression of Snail, E-cadherin, vimentin, and microRNA-195-5p (miR-195-5p). In vivo tumor growth inhibition was determined 45 days after establishment of GC xenografts in nude mice.

**Results:** Treatment of MKN28 and NCI-N87 cells with tocotrienolic amide significantly and dose-dependently reduced their invasiveness and migratory capacity ( $p < 0.05$ ). It also significantly and dose-dependently downregulated the mRNA and protein expressions of Snail and vimentin, but significantly upregulated E-cadherin expression ( $p < 0.05$ ). The mRNA expression of miR-195-5p was significantly and dose-dependently upregulated in MKN28 and NCI-N87 cells treated with tocotrienolic amide, but was downregulated after transfection with miR-195-5p inhibitor ( $p < 0.05$ ). Transfection of MKN28 and NCI-N87 cells with miR-195-5p inhibitor significantly and dose-dependently upregulated mRNA and protein expressions of Snail ( $p < 0.05$ ). Moreover, treatment of GC mice with TCTA led to significant and dose-dependent reduction in tumor weight and volume ( $p < 0.05$ ).

**Conclusion:** These results suggest that tocotrienolic amide inhibits the growth and metastasis of GC cells by directly targeting Snail and vimentin genes, and thus can potentially be developed for the management of gastric cancer.

**Keywords:** Gastric cancer, Metastasis, Protein expression, Tocotrienolic amide, Tumor volume

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## INTRODUCTION

Gastric cancer (GC), a malignant tumor that develops in the inner lining of the stomach, is the fifth most common cancer worldwide. It is one of

the leading causes of cancer-related death globally [1]. The incidence of GC is higher in developing countries, and accounts for approximately 70 % of total diagnosed cases [2]. Less than 5 % of GC occur in people under 40

years of age, and most of these patients are in the age range of 30 to 39 years, while the others are aged 20 to 29 years. China alone accounts for 25 % of all GC-related deaths. This disease has a quiet onset and progresses slowly, thereby making its early detection difficult since early symptoms are usually not obvious. Gastric cancer (GC) is characterized by poor prognosis, with 5-year survival less than 40 % [3]. The pathogenesis of GC is complex, but it is thought to involve an interplay of factors such as diet, environment, genetics, lifestyle and *H. pylori* infection [2]. Resistance to traditional medicines, high metastasis of gastric tumor cells and recurrence are mainly responsible for poor prognosis of GC, despite availability of improved techniques [4]. Therefore, an understanding of the molecular pathways involved and discovery of efficient therapeutic agents are of great importance for GC treatment.

MicroRNAs (miRNAs) block gene translation by interaction with 3'untranslated portions of mRNAs [5]. Epithelial to mesenchymal transition (EMT) as well as many other processes such as cell migration and proliferation are influenced by miRNAs [6]. Several studies on the molecular mechanisms involved in different kinds of cancers have revealed that the expression of miR-195-5p is dysregulated [7]. During oncogenesis, a process in which normal cells transform into carcinoma cells *in situ*, EMT plays a crucial role [8]. Moreover, carcinoma cells invade and migrate *in situ* into the blood and lymph vessels, leading to metastasis to remote organs [8]. The EMT process and cell metastasis are promoted by Snail, a zinc-finger protein transcription factor, through a shift in equilibrium between E-cadherin and N-cadherin [9]. A previous study reported the clinical importance of Snail in GC [9]. The present study investigated the inhibitory effect of tocotrienolic amide on the migration and invasion of gastric cancer cells.

## EXPERIMENTAL

### Materials

Bicinchoninic acid (BCA) protein assay kit was a product of Biyuntian Biotechnology Institute. Rabbit anti-human Snail, vimentin, E-cadherin,  $\beta$ -actin and horseradish peroxidase-labeled goat anti-rabbit IgG were bought from Abcam Inc. (UK). Trizol reagent was obtained from Thermo Fisher Scientific, Inc. Polymerase chain reaction (PCR) primers were purchased from Qiagen (USA), while SYBR Premix Ex Tap was obtained from Toyobo Co. Ltd. (Japan). Enhanced

chemiluminescence system was a product of Life Sciences, UK.

### Cell lines and culture conditions

Gastric cancer cell lines (MKN28 and NCI-N87) were obtained from Biochemistry and Cell Biology Institute, Shanghai, China. The 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<sub>2</sub> and 95 % air until they attained 80 % confluency. The medium was replaced with fresh one every two days. After 1 week of incubation, the adherent confluent cells were trypsinized with 0.25 % trypsin-EDTA (2 mL), cultured again, and passaged for later use. Cells in logarithmic growth phase were selected and used in this study.

### *In vitro* cell migration assay (scratch test)

Gastric cancer cells (MKN28 and NCI-N87) in logarithmic growth phase were seeded in 24-well plates at a density of  $1 \times 10^6$  cells/mL and cultured in DMEM for 24 h. Tocotrienolic amide (2 - 10 mM) was added to the cells and incubated for 24 h. After achieving 80 % confluency, scratches were made on cell monolayers. The cells were further cultured for 24 h after washing thrice with serum-free medium, and then observed and photographed.

### *In vitro* cell invasion assay

The degrees of invasiveness of MKN28 and NCI-N87 cells were determined using Transwell invasion assay. The cells were placed at a density of  $1 \times 10^6$  cells/mL in 24-well plates containing serum-free DMEM in Transwell chamber. The DMEM (800  $\mu$ L) mixed with 10 % FBS was added the basolateral chamber, whereas 200  $\mu$ L of the cell suspension was added to the upper chamber. Incubation with tocotrienolic amide at 2, 5 and 10  $\mu$ M for 24 h was followed by removal of the medium. The migrated cells were fixed for 35 min with 5 % glutaraldehyde, followed by staining with crystal violet for 25 min, photographing and counted under an inverted microscope. Cell invasion was determined by counting the invaded cells in five randomly chosen fields under the microscope.

### Western blotting

The MKN28 and NCI-N87 cells were treated with varied concentrations of tocotrienolic amide (2 - 10 mM) for 24 h. Changes in expression levels of Snail, E-cadherin and vimentin were determined using Western blotting. The cells were washed

with phosphate-buffered saline (PBS) and lysed with ice-cold radio-immunoprecipitation assay (RIPA) buffer containing protease inhibitor (1 mM phenylmethanesulfonyl fluoride). The resultant lysate was centrifuged at 12,000 rpm for 25 min at 4 °C, and the protein concentration of the supernatant was determined using BCA protein assay 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 90 min. Subsequently, the membrane was incubated with non-fat milk powder (5 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T), with gentle shaking at 37 °C to block non-specific binding of the blot. Thereafter, the blots were incubated overnight at 4 °C with primary antibodies for Snail, E-cadherin, vimentin and  $\beta$ -actin, 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 90 min at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using ImageJ Launcher software. The respective protein expression levels were normalized to that of standard  $\beta$ -actin which served as standard.

#### **Quantitative reverse transcription polymerase chain reaction (qRT-PCR)**

Trizol RNA extraction reagent was used to extract total RNA from the cells, while cDNA synthesis 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 measurement of the expression levels of Snail, E-cadherin, vimentin, and miR-195-5p. Variation in the cDNA content was normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The qRT-PCR reaction conditions were: pre-denaturation at 94 °C for 3 min, PCR reaction at 93 °C for 26 sec, 58 °C for 26 sec, and 71 °C for 26 sec, and a total of 39 cycles. The PCR reaction mixture (20 µL) consisted of 6.4 µL of dH<sub>2</sub>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 Ct value of U6 was taken as the internal parameter, and 2<sup>- $\Delta\Delta$ Ct</sup> method was used for calculating the relative expression levels of the proteins. The forward and reverse sequences of miR-195-5P primer were 5'-CTGGAGCAGC

ACAGCCAATA-3' and 5'AGCT TCCCTGGCTC TAGCA-3', respectively.

#### **Determination of effect of miR-195-5p inhibitor on tocotrienolic amide-induced expression of snail**

Gastric cancer (GC) cells were cultured in serum-free medium with equal volume of miR-195-5p inhibitor or control, each at concentration of 10 µmol/L. Incubation was carried out at room temperature for 6 h. Lipofectamine 2000 was dissolved in serum-free medium and incubated at room temperature for 10 min to form a mixture. The mixture was then added to cells in each group, and cultured at 37 °C in a humidified atmosphere of 5 % CO<sub>2</sub> and 95 % air for 48 h. Normal cell culture without miR-195-5p inhibitor or control served as normal control group. The transfection efficiency was determined with qRT-PCR.

#### **Mice**

BALB/c male mice (n = 40) aged 6 weeks (mean weight = 30 ± 3 g) were obtained from Haisco Pharmaceutical Group, Sichuan, China. The mice were housed in plastic cages in a laboratory with 12-h light/12-h dark cycle, average temperature of 25 °C and 50 % humidity. They were allowed free access to standard feed and clean drinking water. The study protocol was approved by the Institutional Animal Ethics Committee of the People's Hospital of Suzhou New District (no. 2018-182). The study procedures were carried out according to the guidelines of National Institute of Health (NIH) [8].

#### **Establishment of GC xenografts in nude mice**

Gastric cancer (GC) cells (NCI-B87) were trypsinized, washed thrice with RPMI medium, and re-suspended in the same medium. The mice were anaesthetized with ether and placed in supine position prior to inoculation with NCI-B87 cells. A small incision of about 6 - 8 mm in diameter was made in the anorectal region of each mouse through the anorectal wall at the anterior end. A 27-gauge (20 mm) needle was used for subcutaneous injection of NCI-B87 cells (2 x 10<sup>5</sup> cells/mL) suspended in RPMI, into the posterior wall of the mice. The mice were then randomly assigned to 4 groups (10 mice/group): sham, GC, 2 mg tocotrienolic amide/kg body weight (bwt) and 5 mg tocotrienolic amide/kg bwt groups. Mice in sham and GC groups received equivalent volumes of physiological saline in place of tocotrienolic amide. The mice were

sacrificed via cervical dislocation on the 45<sup>th</sup> day of tumor inoculation, under pentobarbital sodium anaesthesia.

### Determination of tumor growth in nude mice

The tumors formed in nude mice were excised and weighed to determine the effect of tocotrienolic amide on tumor growth in the treatment groups.

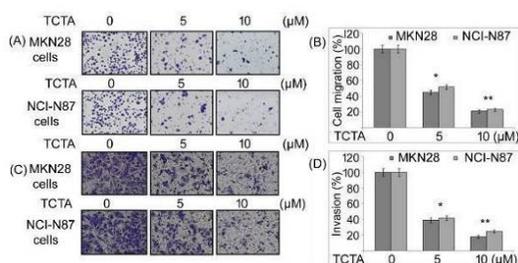
### Statistical analysis

Data are expressed as mean  $\pm$  standard deviation (SD). Statistical analysis was performed using SPSS version 21.0. Groups were compared using Tukey's multiple comparison test. Statistical significance was assumed at  $p < 0.05$ .

## RESULTS

### Effect of tocotrienolic amide on metastasis of GC cells

Treatment of MKN28 and NCI-N87 cells with tocotrienolic amide significantly and dose-dependently reduced their invasiveness and migratory capacity ( $p < 0.05$ ; Figure 1).

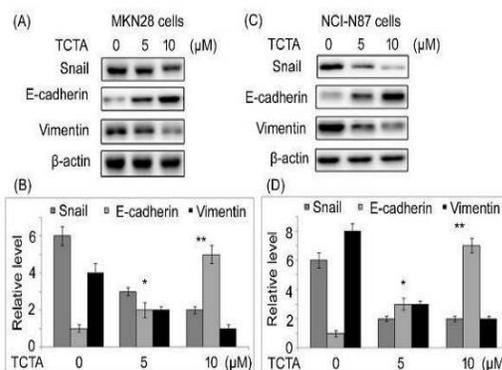


**Figure 1:** Effect of TCTA on GC cell metastasis. (A): photomicrographs of migrating MKN28 cells; (B): photomicrographs of migrating NCI-N87 cells; (C): photomicrographs of invading MKN28 cells; and (D): photomicrographs of invading NCI-N87 cells; \* $p < 0.05$ ; \*\* $p < 0.01$ , compared with control cells

### Effect of tocotrienolic amide on mRNA and protein expressions of Snail, E-cadherin and vimentin in GC cells

The results of Western blotting and qRT-PCR showed that tocotrienolic amide treatment significantly and dose-dependently downregulated the mRNA and protein expressions of Snail and vimentin, but it significantly upregulated the mRNA and protein expressions of E-cadherin ( $p < 0.05$ ). The level of expression of E-cadherin was significantly

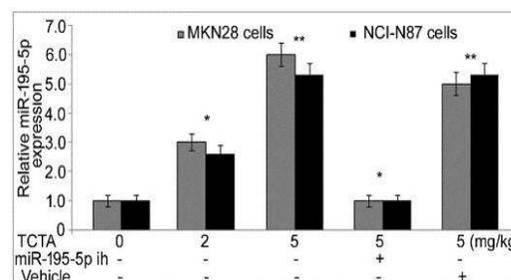
higher in MKN28 cells than in NCI-N87 cells. These results are shown in Figure 2.



**Figure 2:** Effect of tocotrienolic amide on mRNA and protein expressions of Snail, E-cadherin and vimentin. (A): Protein expressions of Snail, E-cadherin and vimentin in MKN28 cells; (B): mRNA expression of Snail, E-cadherin and vimentin in MKN28 cells; (C): Protein expressions of Snail, E-cadherin and vimentin in NCI-N87 cells; and (D): mRNA expression of Snail, E-cadherin and vimentin in NCI-N87 cells; \* $p < 0.05$ ; \*\* $p < 0.01$ , when compared with control cells

### Effect of tocotrienolic amide on miR-195-5p expression

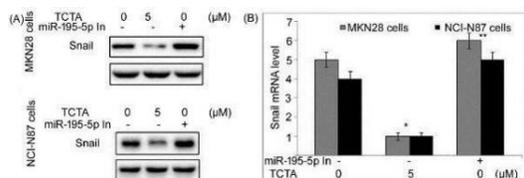
The mRNA expression of miR-195-5p was significantly and dose-dependently upregulated in MKN28 and NCI-N87 cells treated with tocotrienolic amide, but was downregulated by transfection with miR-195-5p inhibitor ( $p < 0.05$ ; Figure 3).



**Figure 3:** Effect of tocotrienolic amide on miR-195-5p expression; \* $p < 0.05$ ; \*\* $p < 0.01$ , compared with control cells

### Effect of miR-195-5p inhibitor on tocotrienolic amide-induced suppression of expression of snail

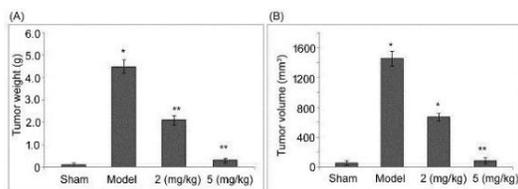
As shown in Figure 4, transfection of MKN28 and NCI-N87 cells with miR-195-5p inhibitor significantly and dose-dependently upregulated mRNA and protein expressions of Snail ( $p < 0.05$ ).



**Figure 4:** Effect of miR-195-5p inhibitor on tocotrienolic amide-induced suppression of mRNA expression of Snail. **(A):** Snail protein expressions in MKN28 and NCI-N87 cells transfected with miR-195-5p inhibitor; and **(B):** Snail mRNA expressions in MKN28 and NCI-N87 cells transfected with miR-195-5p inhibitor; \* $p < 0.05$ ; \*\* $p < 0.01$ , compared with control cells

### Effect of TCTA on tumor growth *in vivo*

Treatment of GC mice with tocotrienolic amide led to significant and dose-dependent reductions in tumor weight and volume ( $p < 0.05$ ). These results are shown in Figure 5.



**Figure 5:** Effect of tocotrienolic amide on tumor growth *in vivo*. **(A):** Weight of isolated tumor; **(B):** volume of isolated tumor; \* $p < 0.05$ ; \*\* $p < 0.01$ , when compared with sham group

## DISCUSSION

Gastric cancer (GC) is the fifth most common cancer worldwide [1]. Helicobacter pylori infection is the most common cause of GC, and accounts for 60 % of diagnosed cases. Other common causes include consumption of pickled vegetables, smoking and genetics. Early symptoms of the disease include heartburn, upper abdominal pain, nausea, loss of appetite, difficulty swallowing, and blood in the stool (hematochezia) [2]. Gastric cancer is treated with surgery, chemotherapy, radiation therapy, and targeted therapy.

However, the outcomes are often poor, with 5-year survival less than 10 % globally [2]. The high metastatic nature of the GC contributes to poor prognosis of patients [10]. The pathogenesis of GC involves several signaling pathways and abnormal gene expression patterns which are often accompanied by inactivation of tumor suppressor genes and activation of oncogenes [2]. This study investigated the effect of tocotrienolic amide on

GC cell growth and metastasis, and the mechanism involved.

Epithelial - mesenchymal transition (EMT) contributes to tumor progression, and a switch from E-cadherin to N-cadherin expression is indicative of EMT. Upregulation of snail protein expression and subsequent downregulation of E-cadherin occur during EMT [11]. Another prominent gene that contributes significantly to EMT is vimentin [12]. The results of previous studies revealed that the upregulation of protein expressions of snail and E-cadherin inhibited EMT in lung cancer cells (A549) [13]. In this study, tocotrienolic amide significantly and dose-dependently inhibited the invasion and migration of MKN28 and NCI-N87 cells. It also significantly and dose-dependently downregulated the mRNA and protein expressions of Snail and vimentin. These results suggest that tocotrienolic amide may inhibit GC cell metastasis via the regulation of EMT.

The role of miR-195-5p in tumor suppression has been reported. Abnormal expression level of this protein leads to chemo-resistance and thus, poor prognosis [14]. It has been reported that in GC patients, miR-195-5p protein level was markedly reduced, while its upregulation resulted in cell apoptosis [15]. MicroRNA-195-5p (miR-195-5p) has been demonstrated to inhibit GC cell proliferation and metastasis by directly targeting fibroblast growth factor (FGF) in nude mice [15]. Overexpression of miR-195-5p suppressed GC cell proliferation and metastasis via inhibition of several signaling pathways [16]. The results of this study indicate that tocotrienolic amide significantly and dose-dependently upregulated miR-195-5p expressions in MKN28 and NCI-N87 cells.

However, transfection of the cells with miR-195-5p inhibitor markedly reversed the effect of tocotrienolic amide. Protein and mRNA expressions of Snail were enhanced after transfection of MKN28 and NCI-N87 cells with miR-195-5p inhibitor. It is likely that Snail may be directly involved in EMT. These results are in agreement with those of previous studies [11,12]. Epithelial-mesenchymal transition (EMT) is partially enhanced by Snail via direct interaction with E-cadherin [17]. Downregulation of E-cadherin expression is considered to be responsible for poor clinical outcomes in cancer patients [17]. High expression level of Snail is indicative of GC progression [18]. In this study, treatment of GC mice with tocotrienolic amide led to significant and dose-dependent reductions in tumor weight and volume.

## CONCLUSION

The results of this study suggest that tocotrienolic amide inhibits the growth and metastasis of GC cells by directly targeting snail and vimentin genes. Thus, the compound can potentially be developed for the treatment of gastric cancer in humans.

## 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. Yi Huang and Shi Xu performed the experimental work, carried out the literature survey, analysed and compiled the data. Shi Xu designed the study and wrote the paper. Both the authors read the paper thoroughly and approved it for publication.

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