The 5,7,2, 5-tetrahydroxy-8,6-dimethoxyflavone up-regulates miR 145 expression and inhibits proliferation of gastric cancer cells

Abstract

Introduction
Gastric cancer is a frequently detected malignancy and its incidence has increased over the past decades in East Asia. The present study investigated the effect of 5,7,2, 5-tetrahydroxy-8,6-dimethoxyflavone (THDMF) on gastric cancer cells and explored the underlying mechanism.

Material and methods
MTT colorimetric assay was used for measurement of MKN28, MKN45 and GES 1 cell proliferation and flow cytometry for detection of apoptosis. Transwell and wound healing assays were used to observe the invasion and migration abilities of MKN28 cells. The expression of p21, MMP2/-9, PI3K and c Myc proteins was detected by western blotting.

Results
The THDMF treatment significantly (P<0.05) reduced MKN28 and MKN45 cell proliferation without changing GES 1 cell viability. A significant increase in apoptotic cell population on treatment with THDMF was observed. Treatment of MKN28 cells with THDMF increased percentage of cells in G1 phase. Exposure of MKN28 cells to THDMF caused a marked decrease in invasion and migration potential. The expression of miR 145 was markedly increased in MKN28 cells on treatment with THDMF. In MKN28 cells expression of c Myc, PI3K, p AKT, MMP-2 and MMP-9 was suppressed markedly. The expression of p21 protein was markedly promoted on exposure to THDMF.

Conclusions
In summary, THDMF exhibits anti-cancer effect on gastric cancer cells in vitro by activation of cell apoptosis and arrest of cell cycle. In addition, THDMF promoted miR 145 expression and down-regulation of PI3K/AKT signaling pathway in MKN28 cells. Therefore, THDMF may be utilized as a potential novel therapeutic agent for the treatment of gastric cancer.
The 5,7,2, 5 -tetrahydroxy-8,6 –dimethoxyflavone up-regulates miR-145 expression and inhibits proliferation of gastric cancer cells

Abstract

Background: Gastric cancer is a frequently detected malignancy and its incidence has increased over the past decades in East Asia. The present study investigated the effect of 5,7,2, 5 -tetrahydroxy-8,6 –dimethoxyflavone (THDMF) on gastric cancer cells and explored the underlying mechanism. The study analyzed cell viability changes, apoptotic features and metastasis potential on treatment with THDMF. Materials and Methods: MTT colorimetric assay was used for measurement of MKN28, MKN45 and GES-1 cell proliferation and flow cytometry for detection of apoptosis. Transwell and wound healing assays were used to observe the invasion and migration abilities of MKN28 cells. The expression of p21, MMP2/-9, PI3K and c-Myc proteins was detected by western blotting. Results: The THDMF treatment significantly (P<0.05) reduced MKN28 and MKN45 cell proliferation without changing GES-1 cell viability. A significant increase in apoptotic cell population on treatment with THDMF was observed. Treatment of MKN28 cells with THDMF increased percentage of cells in G1 phase. Exposure of MKN28 cells to THDMF caused a marked decrease in invasion and migration potential in comparison to control cells. The expression of miR-145 was markedly increased in MKN28 cells on treatment with THDMF. In MKN28 cells expression of c-Myc, PI3K, p-AKT, MMP-2 and MMP-9 was suppressed markedly on exposure to THDMF. The expression of p21 protein in MKN28 cells was markedly promoted on exposure to THDMF. Conclusion: In summary, THDMF exhibits anti-cancer effect on gastric cancer cells in vitro by activation of cell apoptosis and arrest of cell cycle. In addition, THDMF promoted miR-145 expression and down-regulation of PI3K/AKT signaling pathway in MKN28 cells. Therefore, THDMF may be utilized as a potential novel therapeutic agent for the treatment of gastric cancer.

Running title: Inhibition of gastric cancer cell proliferation

Keywords: Metalloproteinase, miR-145, migration, apoptosis
Introduction

Gastric cancer is one among the frequently detected malignancies throughout the world and its incidence has increased over the past decade [1,2]. Despite advancement in the field of chemotherapy and technology gastric cancer is the third leading cause of deaths associated with cancer globally [3,4]. The highest incidence of gastric cancer has been reported in the East Asia [3,4]. The high mortality rate of gastric cancer patients is associated with its detection at advanced stage in most of the cases [5]. The prognosis of gastric cancer patients is very poor [6]. The most common hindrance to the gastric cancer treatment strategies is high rate of infiltration and invasion [7]. Therefore, discovery of the chemotherapeutic agents which can effectively inhibit proliferation and metastasis of gastric cancer cells is highly needed.

MicroRNAs (miRNAs) usually comprise of ~19-25 nucleotides and exhibit their effect by regulating gene expression for inhibiting or cleaving the translation through involvement of one or more mRNA [8,9]. The miRNAs regulate cellular proliferation, death and development of various organs in the body [10,11]. It has been shown that miRNAs play an important role in the regulation of gastric cancer development and progression [12,13]. The expression of miR-145 was found to be down-regulated in the cancerous colorectal lesions [14]. Studies have shown that miR-145 acts as tumor suppressor molecule because it regulates tumor cell growth by targeting expression of Myc proto-oncogene protein (c-Myc) and other transcription factors [15,16]. In gastric cancer cells overexpression of miR-145 has been reported to inhibit growth and metastasis through targeting MYO6 expression [17,18]. Another study has revealed that miR-145 suppresses proliferation, inhibits metastasis and causes arrest of cell cycle in gastric cancer cells by down-regulation of Sp1 expression [19]. Down-regulation of N-cadherin translation by miR-145 has also been found to inhibit metastasis of gastric cancer cells [20]. Therefore, miR-145 employs diverse mechanisms to suppress the proliferation of gastric cancer cells. In the present study effect of 5,7,2,5-tetrahydroxy-8,6-dimethoxyflavone (THDMF) on gastric cancer cells was investigated and underlying mechanism was also explored. The study demonstrated that THDMF caused gastric cancer cell apoptosis, leads to arrest of cell cycle and inhibited proliferation. The THDMF exposure of MKN28 cells promoted expression of miR-145 and down-regulated PI3K/AKT signaling pathway.

Materials and methods

Cell culture

The human gastric cancer cell lines MKN28, MKN45 and the immortalized human gastric epithelial mucosa cell line GES-1 were supplied by the Shanghai Tumor Research Institute, Shanghai, China. The cell lines were grown in DMEM mixed with 10% fetal bovine serum.
The medium also contained 100 U/ml penicillin and 100 μg/ml streptomycin. The cell lines were cultured under a humidified atmosphere of 5% CO₂ atmosphere in an incubator at 37°C.

**Cell proliferation assay**

The proliferation of MKN28, MKN45 and GES-1 cells after 72 h of treatment with THDMF or dimethyl sulfoxide alone (control) was measured by MTT colorimetric assays. The cell lines were exposed to 2, 4, 6, 8, 10, 12 and 15 μM of THDMF at 1x10⁵ cells/well density in 96-well plates for 72 h. Then 5 mg/ml solution of MTT (20 μl) was added to each well of the plate and cells were incubated for 4 h more. Then medium in the wells discarded and dimethyl sulfoxide (150 μl) was put into each well for dissolving any solid crystal formed. The plates were kept in shaker for 20 min at room temperature before the optical density was recorded at 490 nm in scanning multi-well spectrophotometer.

**Apoptosis analysis**

The MKN28 cells were put into six-well plates and grown for 24 h before incubation with 2, 6, 12 and 15 μM of THDMF for 72 h. The cells were harvested, centrifuged for 20 min and the cell pellets were twice washed with cold PBS. After washing, the cells were subjected to incubation under complete darkness with 5 μl of PI at room temperature for 20 min. The cells in each tube were then treated with 350 μl of 1X binding buffer for 15 min. The cell apoptosis was analyzed by flow cytometry using BD Accuri™ C6 Flow cytometer (BD Biosciences, San Jose, CA, USA).

**Cell cycle analysis**

MKN28 cells were put at 2 x 10⁵ cells per well density in 60-mm plates and treated with 2, 6, 12 and 15 μM of THDMF for 72 h. The cells after trypsinizations were subjected washing twice with PBS and subsequently fixed with 70% cold ethyl alcohol for overnight. Then the cells were treated with RNase A (20 μg/ml) at 37°C before staining with PI (10 μg/ml) at 37°C. The flow cytometry employing FACSCalibur instrument (BD Biosciences, San Jose, CA, USA) was used for analysis of DNA content and cell cycle distribution.

**Cell invasion assay**

The 24-well Transwell plates (8 mm pore size) were subjected to coating with 200 mg/ml Matrigel and then dried under sterile conditions for overnight. MKN28 cells were exposed to 2, 6, 12 and 15 μM of THDMF at 2 x10⁵ cell/ml concentration in RPMI-1640 medium on the upper chamber. The lower chamber contained RPMI-1640 medium mixed with 20% FBS. Following incubation for 72 h, the non-invasive cells on the upper chamber were cleaned using cotton wool. The cells were then fixed in methyl alcohol for 15 min at room temperature
followed by staining with hematoxylin-eosin for 25 min. The light microscope (Olympus Corporation, Tokyo, Japan) was used for counting the cells invaded to the lower chamber.

**In vitro wound healing assay**

The MKN28 cells were put at $2 \times 10^5$ cells/ml density in 6-well plate and allowed to attain 100% confluence by incubation at 37°C. The cells were starved for 24 h and then a 100 ml plastic pipette tip was used to draw a wound (straight cell-free) through middle of the wells. The wells were washed with PBS two times followed by treatment with 2, 6, 12 and 15 µM of THDMF for 72 h. The cells after fixing and staining with 3.5% ethyl alcohol containing 1.5% crystal violet dye for 15 min were observed for migration potential. The inverted light microscope (Nikon Corporation) was used for observing the cells at five randomly selected fields.

**Western blot analysis**

The MKN28 cells following 72 h of exposure to 2, 6, 12 and 15 µM of THDMF were collected and then lysed on treatment with lysis buffer [Tris-HCl (40 mM; pH 7.5) mixed with NaCl (150 mM) and Triton X-100 (1%; v/v) and protease inhibitors]. The lysate was freed from cell debris by centrifugation for 20 min at 1200 xg to obtain the supernatants. The concentration of proteins was measured using commercially available bicinchoninic acid protein assay kit in accordance with the instructions from manufacturer. The 20 µg per lane protein samples were resolved using 10% SDS-PAGE transferred subsequently onto the polyvinylidene difluoride membranes. The membranes were prior blocked by incubation with 5% skimmed milk and Tris buffered saline containing Tween-20 (0.1%) for 2 h at room temperature. The samples were probed by incubation with primary antibodies; anti-c-Myc, anti-p-AKT, anti-PI3K, anti-P21, anti-MMP-2, anti-MMP-9 and anti-GAPDH. The membranes after washing with 1X PBST three times were subjected to incubation for 2 h with horseradish peroxidase-conjugated secondary antibody at room temperature. The immunoreactive band visualization was performed using Signal Fire™ Plus ECL Reagent (cat no. 12630; Cell Signaling Technology, Inc.) using GAPDH as loading control. The quantification of intensities was performed using Image J version 2.0 software (Bio-Rad Laboratories Inc, USA).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)**

Total RNA from MKN28 cells following 72 h of exposure to 2, 6, 12 and 15 µM of THDMF was extracted by TRIzol reagent according to manual protocol. The M-MLV and SYBR Green master mix kit were used for reverse-transcription and cDNA amplification, respectively in accordance with the manufacturer's protocol. Amplification of the genes was performed using specific oligonucleotide primers and GAPDH as an endogenous control. The primers used were: miR-145 forward: 5'-GTC CAG TTT TCC CAG GAA TCC CT-3' and backward:
5'-GCT GTC AAC GAT ACG CTA CCT A-3'. The comparative Ct method (2^-\Delta \Delta \text{Ct}) was used for the analysis of data. The experiments were carried out separately in triplicates for each clone. The conditions employed for amplification were: 93°C for 5 min, 40 cycles at 93°C for 30 sec, 54°C for 30 sec, 70°C for 30 sec and 70°C for 10 min.

**Statistical analysis**

The data expressed are the mean ± standard deviation (SD) of experiments carried out independently in triplicates. The data were analysed using SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA). The one-way analysis of variance followed by Tukey's post-hoc test was used for analysis of the data. At P<0.05 differences were taken statistically significant.

**Results**

**THDMF specifically inhibits MKN28 and MKN45 gastric cancer cell proliferation**

Exposure of MKN28 and MKN45 cells to THDMF significantly (P<0.05) reduced proliferation but didn’t affect the normal gastric GES-1 cell viability (Figure 1). Treatment of MKN28 cells with THDMF at 2, 4, 6, 8, 10, 12 and 15 µM reduced proliferation to 87, 72, 64, 55, 43, 36 and 31%, respectively. The proliferation of MKN45 cells was decreased to 89, 75, 68, 56, 45, 38 and 34%, respectively on exposure to 2, 4, 6, 8, 10, 12 and 15 µM of THDMF for 72 h. However, no change in GES-1 cell proliferation was caused by THDMF in the concentration range of 2-15 µM.

**Figure 1.** Effect of THDMF on gastric cancer and normal cell proliferation. MKN28, MKN45 and GES-1 cells were exposed to 2-15 µM of THDMF for 72 h and changes in proliferation were measured by MTT assay. *P<0.05, **P<0.02 and ***P<0.01 vs. control cells.

**THDMF promotes MKN28 cell apoptosis**

The apoptosis induction in MKN28 cells was assessed on exposure to 2, 6, 12 and 15 µM of THDMF at 72 h (Figure 2). The data from flow cytometry showed a significant increase in early as well as late apoptotic cell population in MKN28 cell cultures on exposure to THDMF. Exposure of MKN28 cells to 15 µM of THDMF raised apoptotic cell percentage to 47.68% in comparison to 2.46% in control cells.
**Figure 2.** Effect of THDMF on apoptosis in MKN28 cancer cells. Exposure of cells to different concentrations of THDMF for 72 h was followed by flow cytometry to detect cell apoptosis. The cells were stained with propidium iodide (PI) and fluorescein isothiocyanate (FITC). *p and **p vs. control cells.

**THDMF induces MKN28 cell cycle arrest**

The changes in MKN28 cell cycle progression by 2, 6, 12 and 15 µM of THDMF were detected by flow cytometry (**Figure 3**). Exposure of MKN28 cells to THDMF for 72 h increased proportion of cells in the G1 phase of cell cycle. The cell percentage in G1 phase increased to 67.36% on exposure to 15 µM of THDMF in comparison to 48.25% in control cells. The population of cells in S and G2/M phases was significantly (P<0.02) decreased on exposure to THDMF.

**Figure 3.** Effect of THDMF on cell cycle progression in MKN28 cells. Treatment of the cells with THDMF at different concentrations was followed by analysis of cell cycle distribution using flow cytometry. *p and **p vs. control cells.

**THDMF inhibits MKN28 cell invasion and migration**

The data from Matrigel Transwell assay showed a significant (P<0.05) decrease in invasion of MKN28 cells on exposure to THDMF (**Figure 4**). Exposure of MKN28 cells to 2, 6, 12 and 15 µM of THDMF for 72 h lead to concentration based reduction of cell invasion. The invasion of MKN28 cells was decreased by 8-fold on exposure to 15 µM of THDMF. The migration potential of MKN28 cells was also reduced significantly (P<0.05) on exposure to 2, 6, 12 and 15 µM of THDMF (**Figure 5**). The wound healing assay revealed that reduction of MKN28 cell migration potential was significant from 2 µM and maximum at 15 µM of THDMF.

**Figure 4.** Effect of THDMF on MKN28 cell invasion. (A) The invasion ability of MKN28 cells was detected using Transwell assay following 72 of exposure to different concentrations of THDMF. (B) Quantification of the cell invasion data. *P<0.05 and **P<0.01 vs. control cells.
Figure 5. Effect of THDMF on MKN28 cell migration. (A) The MKN28 cell migration ability was analysed using wound healing assays at 72 h of treatment with different concentrations of THDMF. (B) Quantification of the cell invasion data. *P<0.05 and ***P<0.02 vs. control cells.

**THDMF promotes miR-145 expression in MKN28 cells**

The miR-145 expression in MKN28 cells was markedly increased on exposure to 2, 6, 12 and 15 µM of THDMF at 72 h (Figure 6). The RT-qPCR showed that increase in miR-145 expression was significant in MKN28 cells treated with 2 µM of THDMF. A dose based promotion in miR-145 expression was detected with the increase in THDMF concentration from 2 to 15 µM.

Figure 6. Effect of THDMF on miR-145 expression in MKN28 cells. (A) Relative level of miR-145 in MKN28 cells at 72 h of treatment with different concentrations of THDMF by RT-qPCR. (B) Quantification of the miR-145 expression. *P<0.05 and **P<0.01 vs. control cells.

**THDMF regulates PI3K/AKT signaling pathway in MKN28 cells**

Western blotting showed that exposure of MKN28 cells to THDMF suppressed c-Myc, PI3K, p-AKT, MMP2 and MMP9 expression (Figure 7). There was a concentration based reduction of PI3K, c-Myc, MMP2/9 and p-AKT expression in MKN28 cells on exposure to THDMF. However, the expression of p21 protein in MKN28 cells was markedly promoted on exposure to THDMF.

Figure 7. Effect of THDMF on PI3K/AKT signaling pathway in MKN28 cells. The cells were exposed to different concentrations of THDMF for 72 h and PI3K, p-AKT, c-Myc, MMP2/9 and p21 expression was analysed by western blotting.

**Discussion**

The present study investigated the anti-cancer potential of THDMF against gastric cancer cells in vitro and explored the molecular mechanism involved. The data showed that THDMF caused apoptosis activation and cell cycle arrest leading to suppression of gastric cancer cell proliferation. The anti-proliferative action of THDMF was associated with the promotion of
miR-145 expression and regulation of PI3K/AKT signaling pathway. As the expression of miR-145 in immortalized human gastric epithelial mucosa cells, GES-1 is higher compared to cancer cell lines therefore GES-1 cells were insensitive to THDMF exposure.

Gastric cancer is a commonly detected malignancy diagnosed in around one million people every year throughout the globe [21]. In the present study THDMF exposure significantly reduced MKN28 and MKN45 gastric cancer cell proliferation without affecting the normal gastric GES-1 cell viability. Moreover, THDMF caused apoptosis activation and arrest of cell cycle in G1 phase in MKN28 cells. It has been found that expression of miR-145 is down-regulated in various types of cancer cells and is therefore considered to be an important target for chemotherapeutic intervention [22-25]. It is believed that miR-145 plays an important role as the tumor suppressor factor by inhibition of carcinoma cell proliferation [18-20]. The up-regulation of miR-145 expression in cancer cells inhibits proliferation and causes arrest of cell cycle in the G1 phase [26]. It was demonstrated that HuR expression is elevated in gastric cancer tissues as well as cell lines and this over-expression has significant relation to lymph node metastasis. Targeting HuR by RNA interference inhibited viability and enhanced apoptosis via promotion of pro-apoptotic factors (Bcl-2 and Bax). Moreover, HuR expression was found to be inversely correlated with miR-145 expression in gastric cancer tissues and HuR was shown to be a direct target of miR-145 [27]. The MKN45 cell proliferative potential and metastasis is inhibited by lidocaine through up-regulation of miR-145 [28]. The present study showed down-regulation of miR-145 expression in MKN28 gastric cancer cells in consistence with the reported literature [18]. However, treatment of MKN28 cells with THDMF markedly promoted the expression of miR-145 in dose based manner. These findings suggest that THDMF exhibits cytotoxicity effect on MKN28 cells through up-regulation of miR-145 expression.

The oncogene, c-Myc is linked to the uncontrolled proliferation, regulation of cell apoptosis and transformation of cells [29]. The higher levels of c-Myc generate apoptotic signals leading to DNA damage in the epithelial cells [30]. It has been found that inhibition of c-Myc expression plays a vital role in the arrest of cell cycle [31]. In the present study THDMF treatment of MKN28 cells markedly down-regulated the expression of c-Myc. Exposure of MKN28 cells to THDMF lead to arrest of cell cycle in G1 phase. The data from flow cytometry showed that THDMF exposure of MKN28 cells markedly increased MKN28 cell percentage in G1 phase and decreased the population in S and G2/M phases. Therefore, THDMF arrested MKN28 cell cycle in G1 phase through down-regulation of c-Myc expression. The PI3K/AKT pathway regulates metastasis, proliferation and apoptosis of the cells through inhibition of downstream phosphorylation [32,33]. In the present study THDMF exposure of MKN28 cells suppressed the PI3K and p-AKT expression. Thus THDMF
treatment regulates the PI3K/AKT signaling pathway in MKN28 cancer cells. The most important members of metalloproteinases are MMP-2 and MMP-9 which play a leading role in invasion, metastasis and vascularization of the tumor cell through degradation of type IV collagen [34]. The results from present study showed that exposure of MKN28 cells to THDMF down-regulated the levels of MMP-2 and MMP-9. Thus, THDMF inhibited the invasion and migration of MKN28 cells through suppression of MMP-2 and MMP-9 levels.

Conclusion

The present study demonstrated that THDMF exhibits anti-cancer effect on gastric cancer cells in vitro through apoptosis activation and cell cycle arrest. Moreover, the anti-proliferative action of THDMF involved promotion of miR-145 expression and down-regulation of PI3K/AKT signaling pathway. Therefore, THDMF may be utilized as a potential novel therapeutic agent for the treatment of gastric cancer.

Conflict of interest

The authors declare no conflict of interest.

References


Figure 1. Effect of THDMF on gastric cancer and normal cell proliferation. MKN28, MKN45 and GES 1 cells were exposed to 2-15 µM of THDMF for 72 h and changes in proliferation were measured by MTT assay. *P<0.05, **P<0.02 and ***P<0.01 vs. control cells.
Figure 4. Effect of THDMF on MKN28 cell invasion. (A) The invasion ability of MKN28 cells was detected using Transwell assay following 72 hours of exposure to different concentration of THDMF. (B) Quantification of the cell invasion data. *P<0.05 and ***P<0.01 vs. control cells.
Figure 5. Effect of THDMF on MKN28 cell migration. (A) The MKN28 cell migration ability was analysed using wound healing assays at 72 h of treatment with different concentration of THDMF. (B) Quantification of the cell invasion data. *P<0.05 and ***P<0.02 vs. control cells.
Figure 6. Effect of THDMF on miR 145 expression in MKN28 cells. (A) Relative level of miR 145 in MKN28 cells at 72 h of treatment with different concentration of THDMF by RT qPCR. (B) Quantification of the miR 145 expression. *P<0.05 and **P<0.01 vs. control cells.
Figure 7. Effect of THDMF on PI3K/AKT signaling pathway in MKN28 cells. The cells were exposed to different concentration of THDMF for 72 h and PI3K, p AKT, c Myc, MMP2/9 and p21 expression was analysed by western blotting.
Figure 2. Effect of THDMF on apoptosis in MKN28 cancer cells. Exposure of cells to different concentrations of THDMF for 72 h was followed by flow cytometry to detect cell apoptosis. The cells were stained with propidium iodide (PI) and fluorescein isothiocyanate (FITC). *p and **p vs. control cells.
Figure 3. Effect of THDMF on cell cycle progression in MKN28 cells. Treatment of the cells with THDMF at different concentrations was followed by analysis of cell cycle distribution using flow cytometry. *p and **p vs. control cells.