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Kaempferia galanga L. extract and its main component, ethyl *p*-methoxycinnamate, inhibit the proliferation of Ehrlich ascites tumor cells by suppressing TFAM expression

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ABSTRACT

Kaempferia galanga L. shows anti-cancer effects; however, the underling mechanism remains unclear. In this study, we explored the underlying mechanism of the anti-cancer effects of *Kaempferia galanga* L. *Kaempferia galanga* L. rhizome extracts (KGEs) suppressed Ehrlich ascites tumor cell (EATC) proliferation by inhibiting S-phase progression. The main component of KGE is ethyl *p*-methoxycinnamate (EMC), which exhibits the same anti-proliferative effect as KGE. Furthermore, EMC induced the downregulation of cyclin D1 and upregulation of p21. EMC also decreased the expression of mitochondrial transcription factor A (TFAM) but did not significantly change mitochondrial DNA copy number and membrane potential. Phosphorylation at Ser62 of c-Myc, a transcription factor of TFAM, was decreased by EMC treatment, which might be due to the suppression of *H-ras* expression. These results indicate that EMC is the active compound responsible for the anti-cancer effect of KGE and suppresses EATC proliferation by regulating the protein expression of cyclin D1 and p21; TFAM may also regulate the expression of these genes. In addition, we investigated the anticancer effects of KGE and EMC *in vivo* using EATC bearing mice. The volume of ascites fluid was significantly increased by intraperitoneal administration of EATC. However, the increase in the volume of ascites fluid was suppressed by oral administration of EMC and KGE. This study provides novel insights into the association between the anti-cancer effects of natural compounds and TFAM, indicating that TFAM might be a potential therapeutic target.

1. Introduction

Cancer is a serious health problem as it is the leading cause of death in both developed and developing countries [1]. The number of cancer cases is increasing annually, with 10 million deaths in 2020. Breast cancer is the most common cancer in women, and its

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incidence has rapidly increased in recent years. The number of new cancer cases is estimated to increase to 27.5 million by 2040; hence, it is important to develop effective approaches for cancer treatment. Current cancer treatments (chemotherapy, radiotherapy, and surgery) have several effects [2]. Chemotherapeutic agents are toxic to cancerous and normal cells, resulting in damage to various organs [3]. Therefore, compounds with fewer side effects have attracted attention for the development of novel chemotherapeutic agents.

Plants contain many bioactive compounds that act on a variety of targets and exhibit different mechanisms of action [4]. Flavonoids, phenolic acids, tannins, alkaloids, and terpenoids, which are natural compounds in plants, exert anti-cancer activity [5]. In addition, natural compounds are less toxic to normal cells despite their high anti-cancer activity [6]. Therefore, natural compounds can be developed into novel anti-cancer drugs.

Kaempferia galanga L. is a monocotyledonous plant in the ginger family that is used as a spice and herbal medicine. *Kaempferia galanga* L. contains various natural compounds, such as terpenoids, phenols, and flavonoids [7]. *Kaempferia galanga* L. rhizome extract (KGE) has various physiological effects, including antioxidant [8] and anti-inflammatory [9] effects. Previous studies reported that intraperitoneal administration of KGE decreased the number of tumor cells and extended the lifespan of mice inoculated with Ehrlich ascites tumor cells (EATCs) [10]. KGE decreases the survival rate and fragmentation of the nucleus in EATCs; however, the detailed mechanism of the anti-cancer effect of KGE on EATC remains to be elucidated.

Ethyl *p*-methoxycinnamate (EMC; Fig. 1) is the major bioactive secondary metabolite of *Kaempferia galanga* L. Ethanolic (70%) extract of KGE contains EMC (87.40%), ethyl cinnamate trans (5.05%), methyl 4-methoxycinnamate (4.02%), and benz[A]azulene-1,4-dione,2,3-dimethyl- (1.49%) [11]. EMC has a variety of physiological effects, including anti-inflammatory [12], antioxidant [13], and bactericidal effects [14]. Previous studies have reported that EMC reduces the viability of MCF-7 breast cancer cells at concentrations over 625 μM [15]; however, this concentration of EMC is very high and may induce apoptosis of cancer cells and toxicity to normal cells. Indeed, EMC not only inhibits cell proliferation but also induces apoptosis in human oral squamous cell carcinoma Ca992 cells at a concentration of 425 μM [16]. Therefore, to avoid the toxic effects of EMC, it is necessary to evaluate its anti-cancer effect and cytotoxicity on normal cells at low concentrations.

Mitochondrial transcription factor A (TFAM), a mitochondrial DNA (mtDNA)-binding protein, is a transcription initiator for mtDNA and regulates mtDNA copy number by maintaining mtDNA replication and stability [17,18]. TFAM is also present in the nucleus and regulates the expression of genes encoded by the nuclear DNA [19,20]. ONCOMINE, a database of cancer microarrays, has reported that TFAM expression is enhanced in many types of tumor tissues [21]. Furthermore, TFAM overexpression promoted the proliferation of MCF-7 breast cancer cells [22]. Therefore, targeting TFAM may be a promising approach to inhibit the growth of cancer cells.

Although TFAM is closely associated with cancer growth, reports describing the effects of natural compounds with anti-cancer properties on TFAM expression are still limited. Therefore, we aimed to elucidate the mechanism underlying *Kaempferia galanga* L.'s anti-cancer effect by targeting TFAM in EATCs derived from breast cancer. The anti-cancer effects of KGE and EMC were also demonstrated in a mouse tumor model, in which EATC was administered intraperitoneally.

2. Materials and methods

2.1. Preparation of KGEs

Kaempferia galanga L. was purchased from Indonesian vendor. Crushed *Kaempferia galanga* L. was extracted with 99.5% ethanol (JAPAN ALCOHOL TRADING COMPANY LIMITED, Tokyo, Japan) for 2 h at room temperature (25−28 °C) with stirring, and the extract was filtered through a filter paper. The extract was subsequently concentrated at 45 °C using a vacuum evaporator and lyophilized. The obtained extract was stored at −20 °C.

2.2. Cell culture

EATC (JCRB9090) and 3T3-L1 cells (JCRB9014) were purchased from Japanese Cancer Research Resource Bank (Tokyo, Japan). EATC is a spontaneous mouse-derived mammary adenocarcinoma cells line collected from ascites to accommodate ascites formation. EATC is undifferentiated cancer that is 100% malignant, highly proliferative, non-regressive, and highly transplantable to mice, making it a well-established tumor model that is used to examine the anti-cancer effects of many compounds [23].

3T3-L1 fibroblasts were isolated from mouse embryos and used as normal cells in this study. Cells were cultured in Dulbecco's

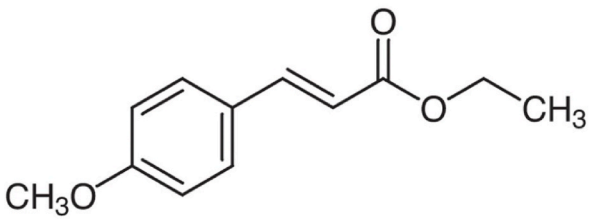

Fig. 1. Chemical structure of EMC.

modified Eagle's medium (DMEM; 05915, Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; S1810, Biowest, Nuaille, France) and 0.1% penicillin/streptomycin (00906/02008, Meiji Seika Kaisha, Tokyo, Japan) in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Trypan blue assay

The proliferation and viability of EATC and 3T3-L1 cells were evaluated using a trypan blue assay. EATCs (1.0×10^6 cells/ml/φ 45-mm dish) were cultured with or without KGE (0−50 μg/ml), EMC (0−400 μM; M1204, purity >98.0%, Tokyo Kasei Kogyo Co., Tokyo, Japan), hesperidin (400 μM; 088–0734, FUJIFILM Wako Pure Chemical, Osaka, Japan), 5-fluorouracil (5-FU; 100 μM; 068–01401, FUJIFILM Wako Pure Chemical), cisplatin (6.25 μM; 033–20091, FUJIFILM Wako Pure Chemical) or PD0332991 (20 μM; PZ0199, Sigma-Aldrich, St. Louis, MO, USA) for 24 h. EATCs were collected and subsequently stained with trypan blue (0.4%). 3T3-L1 cells (1.0×10^5 cells/ml/φ 35-mm dish) were cultured with or without KGE (0−200 μg/ml) and EMC (0−800 μM) for 24 h 3T3-L1 cells were collected and subsequently stained with trypan blue (0.4%). Viable and dead cells were counted under a microscope. The cell viability was calculated using the following formula: % cell viability = [(total cells – dead cells)/total cells] × 100. The 50% lethal concentration (LC50) was determined by regression analysis of cell viability data. The selectivity index (SI) was calculated using the following formula: Selectivity index (SI) = LC50 of 3T3-L1 cells (normal cells)/LC50 of EATC.

2.4. Bromodeoxyuridine (BrdU) assay

BrdU assay was performed according to previously published methods [24]. EATCs were cultured in a medium containing BrdU (027–15561, FUJIFILM Wako Pure Chemical) for 24 h and fixed with 70% ethanol. After acid denaturation (2 N HCl for 30 min), cells were incubated with 0.1 M Tris-HCl for 5 min, followed by 0.1% Triton-X-100 in PBS for 5 min. The cells were then sequentially incubated with an anti-BrdU antibody (M0744, DakoCytomation, Glostrup, Denmark), biotin-conjugated secondary antibody (E0433, DakoCytomation), and horseradish peroxidase-coupled streptavidin (P0397, DakoCytomation) for 1 h. Next, the cells were washed twice with PBS. Finally, the cells were stained with 3,3′-diaminobenzidine tetrahydrochloride dye as a substrate, and BrdU-positive cells were observed under a microscope.

2.5. High -pressure liquid chromatography (HPLC) analysis of KGE

The percentage of EMC in KGE was determined using a previously reported method [25]. HPLC was performed on a JASCO 1500 Series HPLC system (JASCO, Tokyo, Japan). The standard curve was prepared using commercially pure EMC. The extract was separated using a Shodex 5C8 4E column (5 μm particle size, 4.6 × 250 mm, Showa Denko KK, Tokyo, Japan). The mobile phase consisted of methanol/water (58:42, v/v) at a flow rate of 1.0 ml/min for 30 min. The column temperature was 40 °C, and the sample injection volume was 10 μl. The HPLC spectra were recorded at a wavelength of 270 nm.

2.6. Cell cycle

Cell cycle analysis of EATCs was performed using a Muse Cell Cycle Kit (MCH100106, MUSE, Millipore, Bedford, MA, USA) according to the manufacturer's instructions. EATCs (1.0×10^6 cells/ml/φ 45-mm dish) were cultured with or without KGE (25 μg/ml) and EMC (100 μM) for 24 h, collected, and centrifuged (300×*g*, 5 min, 4 °C). After washing the EATCs twice with PBS, the cell suspension was added to 70% ethanol and incubated overnight at −20 °C. The fixed cells (200 μL) were mixed with an equal volume of Muse Cell Cycle Reagent and incubated for 30 min at room temperature (25−28 °C) in the dark. The cell cycle was analyzed using a Muse cell analyzer.

2.7. Western blotting

EATCs were washed twice with PBS and then suspended in radioimmunoprecipitation assay buffer containing phosphatase and protease inhibitors, such as leupeptin, pepstatin, NaF, Na_3VO_4, and phenylmethylsulfonyl fluoride. Total protein was extracted by sonicating the EATC, followed by centrifugation. The concentration of the extracted protein was measured using a Pierce BCA protein assay kit (23227, Thermo Fisher Scientific, Waltham, MA, USA), and equal amounts of each sample (protein content: 30 μg) were separated by SDS-PAGE using an AE-8155 myPowerII 500 (ATTO, Tokyo, Japan). The samples were then transferred onto polyvinylidene difluoride membranes (IPFL00010; Merck Millipore, Billerica, MA, USA). Membranes were blocked with 3% bovine serum albumin buffer for 1 h and then incubated with the primary antibody for 1 h at room temperature (25−28 °C). The following primary antibodies were used: anti-cyclin D1 (1:250, A-12, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p21 (1:500, F-5, Santa Cruz Biotechnology), anti-c-Myc (1:1000, 9E10, Santa Cruz Biotechnology), anti-p-c-Myc (Ser62) (1:1000, #13748, Cell Signaling Technology, Danvers, MA, USA), anti-TFAM (1:250, F-6, Santa Cruz Biotechnology), and anti-β-actin (1:40000, #3700, Cell Signaling Technology). Membranes were incubated sequentially with biotin-conjugated secondary antibody (1:3000, E0433, DakoCytomation) and horseradish peroxidase-coupled streptavidin (1:10000, P0397, DakoCytomation) for 1 h. Protein bands were visualized using EzWestLumi Plus (2332638, ATTO).

2.8. Quantitative reverse transcription PCR (qRT-PCR) analysis

Total RNA from EATCs was purified using the High Pure RNA Isolation Kit (11828665001; Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. RNA quality and quantity were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and RNA was synthesized into cDNA using the ReverTra Ace qPCR RT Master Mix (FSQ-201, Toyobo, Osaka, Japan). qRT-PCR was performed on a Stratagene Mx3005P instrument (Agilent Technologies) using THUNDERBIRD SYBR qPCR Mix (QPS-201, Toyobo). The expression level of each gene was normalized to that of the housekeeping gene (*β-Actin*), and the fold changes relative to the control group were calculated using the ΔΔCt method. All primers were designed using the Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), and the specificity of the qRT-PCR amplification products was confirmed by melting curve analysis. The primer sequences are shown in Table 1.

2.9. Measurement of mtDNA copy number

The mtDNA copy number was determined using qRT-PCR as described in the "Quantitative reverse transcription PCR (qRT-PCR) analysis" section. Briefly, DNA was extracted from EATCs using a GenElute Mammalian Genomic DNA Miniprep Kit (1003037774, Sigma-Aldrich) according to the manufacturer's protocol. The mtDNA copy number was defined as the ratio of mtDNA (*ND1*) to nuclear DNA (*β-Actin*) and was measured using qRT-PCR.

2.10. Measurement of mitochondrial membrane potential ($\Delta\Psi m$)

Changes in the mitochondrial membrane potential were measured using rhodamin-123 (rho-123) dye. After culturing with or without EMC, rho-123 (final concentration, 26 μM) was added to all the groups and incubated at 37 °C for 30 min. After incubation, cells were collected and washed twice with PBS. The fluorescence intensity of rho-123 uptake was measured using a fluorescence microplate reader at excitation and emission wavelengths of 485 and 535 nm.

2.11. Animal treatment

Animal experiments were conducted in accordance with the guidelines of the Osaka City University Laboratory Animal Committee, which were approved by the Laboratory Animal Ethics Committee (permission number: S0056). Twenty-four female ICR mice (25–27 g body weight) were obtained from Japan SLC, Inc., (Shizuoka, Japan). All mice were kept in a temperature-controlled room (25 °C) with a 12 h light/12 h dark cycle. Mice were fed standard pellets diet (Labo MR stock; Nihon Nosan, Kanagawa, Japan) and water *ad libitum*. All mice were pre-fed for 1 day to acclimate to the laboratory and then divided into 4 groups; (1) Control, EATC (−) group: mice were injected intraperitoneally with 0.5 ml of PBS., (2) Control, EATC (+) group: mice were injected intraperitoneally with EATC (0.5 ml of 2.0×10^6 cells/ml PBS), (3) KGE + EATC (+) group: mice were intraperitoneally injected with EATC and orally administered with KGE in 0.2 ml (80 mg/kg body weight), and (4) EMC + EATC (+) group: mice were intraperitoneally injected with EATC and orally administered with EMC in 0.2 ml (66 mg/kg body weight). Groups (1) and (2) were orally administered 0.2 ml of 1% (w/v) Tween-80 (164–21591, FUJIFILM Wako Pure Chemical) solution every day. KGE and EMC were dissolved in 1% (w/v) Tween-80 solution and orally administered to mice every day. During the 13 days treatment, the body weight of each mouse was measured every 3 days. After 13 days of treatment, mice were sacrificed under isoflurane inhalation anesthesia, and volume of ascites fluid was measured.

2.12. Statistical analysis

All quantitative data were expressed as mean ± standard deviation (SD) or mean ± standard error (SE). Multiple groups were compared using one-way ANOVA followed by Tukey−Kramer test using Statcel-4 (OMS Inc., Tokorozawa, Japan). Statistical significance was set at $p < 0.05$.

Table 1
Primer sequences used in real-time PCR analysis.

Gene[a]	Forward primer (5'→3')	Reverse primer (5'→3')
Tfam	CCGTATTGCGTGAGACGAAC	GACAAGACTGATAGACGAGGGGA
ND1	CTAGCAGAAACAAACCGGGC	CCGGCTGCGTATTCTACGTT
H-ras	ACATCCATCAGTACAGGGAGCAGA	GGTGGGTTCAGTTTCCGCAAT
β-Actin	GGAGATTACTGCCCTGGCTCCTA	GACTCATCGTACTCCTGCTTGCTG

[a] *Tfam*, Mitochondrial transcription factor A; *ND1*, NAD(P)H dehydrogenase 1; *H-ras*, Harvey rat sarcoma virus oncogene; *β-Actin*, Beta-actin.

3. Results

3.1. Effect of KGE on viability and proliferation of EATC and 3T3-L1 cells

We evaluated the effect of KGE on the viability and proliferation of EATCs after 24 h using trypan blue assay. KGE (0−25 μg/ml) did not significantly change cell viability (Fig. 2A) but decreased the cell number in a dose-dependent manner (Fig. 2B). In addition, we evaluated the effect of KGE on the viability of 3T3-L1 cells (normal cells). KGE (0−25 μg/ml) did not significantly decrease the viability or number of 3T3-L1 cells (Fig. 2C and D). The LC50 of KGE against EATC and normal cells was 54.07 and 173.33 μg/ml, respectively, with an SI of 3.21.

3.2. Effect of KGE on DNA synthesis in EATCs

We investigated the effect of KGE on DNA synthesis using the BrdU assay. BrdU was used to label S-phase cells because it integrates into the newly synthesized DNA in the S-phase. As shown in Fig. 3, the number of BrdU-positive EATCs significantly decreased by KGE treatment. These results indicate that KGE inhibits the S-phase progression of EATCs.

3.3. Measurement of EMC content in KGE using HPLC

We measured the percentage of EMC in the KGE (used in this study). After comparing the peak areas of EMC and KGE on the chromatogram, the EMC percentage was 78.3% in KGE (Fig. 4A and B). The calibration curve was obtained from the concentration and peak area of EMC ($y = 3.0 \times 10^7 x + 115088$, $R^2 = 0.9997$, linear range: 0.2−0.8 mg/ml).

3.4. Effect of EMC on viability, proliferation, and DNA synthesis

To determine whether EMC is the active compound responsible for the anti-proliferative effect of KGE on EATCs, we performed trypan blue and BrdU assays. EMC (0–100 μM) did not significantly decrease EATC viability (Fig. 5A) but decreased the cell number in a concentration-dependent manner (Fig. 5B). In normal cells, EMC (0–100 μM) did not significantly reduce cell viability and numbers (Fig. 5C and D). The LC50 of EMC against EATC and normal cells was 387.66 and 759.50 μM, respectively, with an SI of 1.96. EMC treatment significantly decreased the number of BrdU-positive cells (Fig. 5E). Notably, these results were similar to those observed in

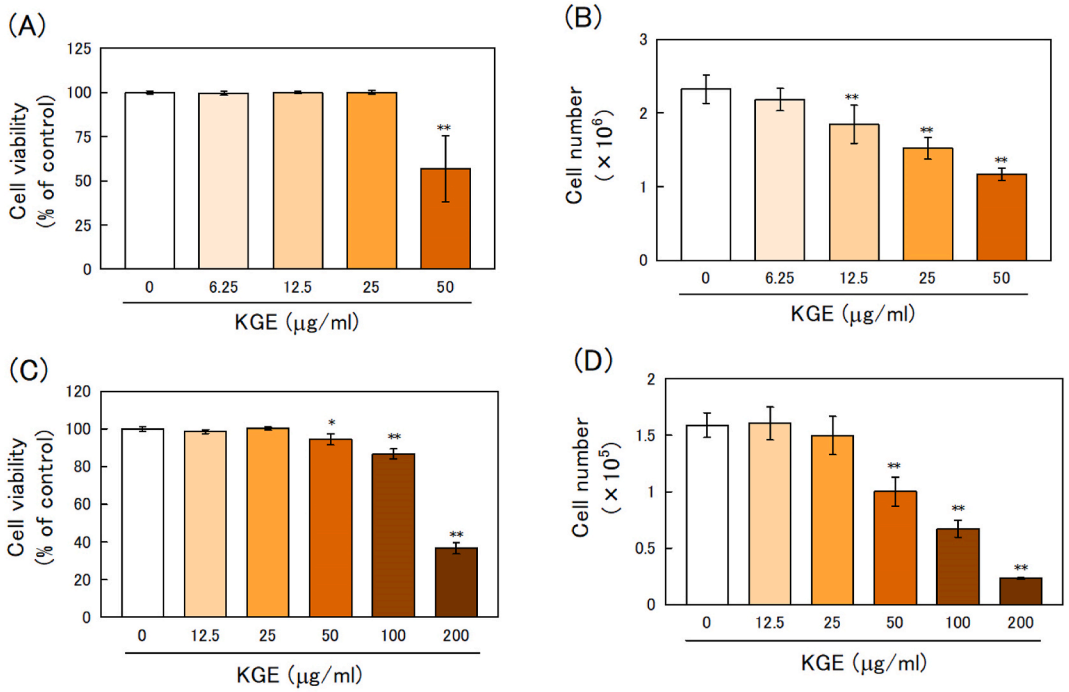

Fig. 2. Effect of KGE on the viability and number of EATCs and 3T3-L1 cells. EATCs were cultured with different concentrations of KGE (0−50 μg/ml) for 24 h 3T3-L1 cells were cultured with different concentrations of KGE (0−200 μg/ml) for 24 h. The (A) viability and (B) number of EATCs and the (C) viability and (D) number of 3T3-L1 cells were assessed using a trypan blue assay. Data are shown as the mean ± SD (n = 5). Statistical analysis was performed using one-way ANOVA, followed by the Tukey−Kramer test. The data were considered significantly different relative to the control (*$p < 0.05$ and **$p < 0.01$).

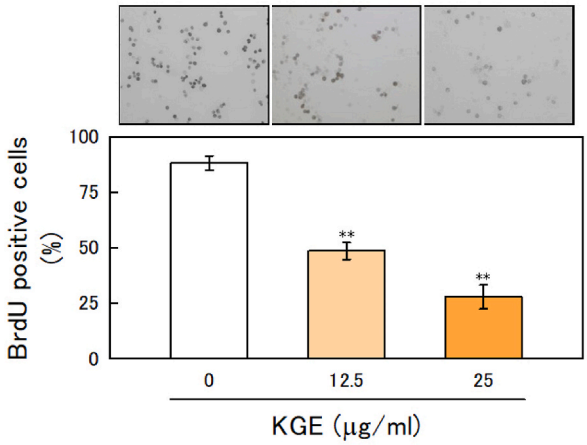

Fig. 3. Effect of KGE on DNA synthesis in EATCs. EATCs were cultured with BrdU (100 μM) and different concentrations of KGE (0−25 μg/ml) for 24 h. DNA synthesis was assessed using the BrdU assay. Immunostaining images show BrdU-positive cells. The percentage of BrdU-positive cells was calculated by BrdU-positive cell number relative to the total cell number. The number of cells per field was 60–150. Data are shown as mean ± SD (n = 6). Statistical analysis was performed using one-way ANOVA, followed by the Tukey−Kramer test. The data were considered significantly different relative to the control (**$p < 0.01$).

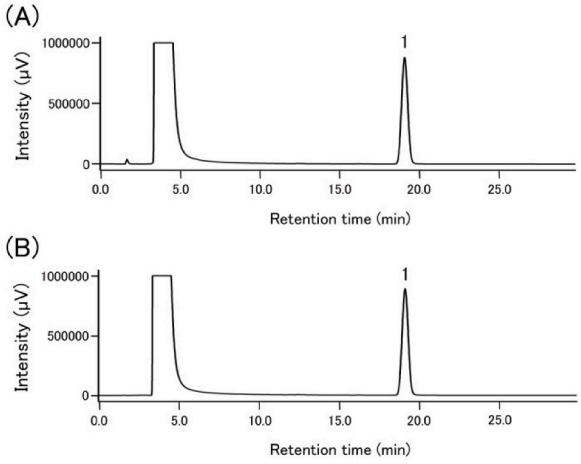

Fig. 4. HPLC profile of KGE. (A) HPLC spectrum of EMC standard compound. The retention time of the EMC standard compound was 19.075 min (peak 1). (B) HPLC spectrum of KGE. The retention time of KGE was 19.092 min (peak 1). The HPLC spectrum was detected at 270 nm.

the cells treated with KGE.

3.5. Effect of KGE and EMC on the progression of the cell cycle in EATCs

To confirm that KGE and EMC blocked the S-phase progression in the cell cycle, we examined their effects on the cell cycle using flow cytometry. As shown in Fig. 6A and B, the number of G0/G1 phase cells was significantly increased by incubating EATCs with KGE or EMC. In contrast, the number of cells in the S phase and G2/M phase decreased.

3.6. Effect of EMC on expression levels of cell cycle regulators in EATCs

To elucidate the mechanism of cell cycle arrest by EMC, we measured the expression levels of cell cycle regulators in EATCs. Cyclin D1 is an important regulator of the G1/S transition, whereas p21 suppresses cell cycle progression by inducing G1 arrest. As shown in Fig. 7A and B, EMC treatment significantly decreased cyclin D1 expression. In contrast, p21 expression was significantly increased following EMC treatment.

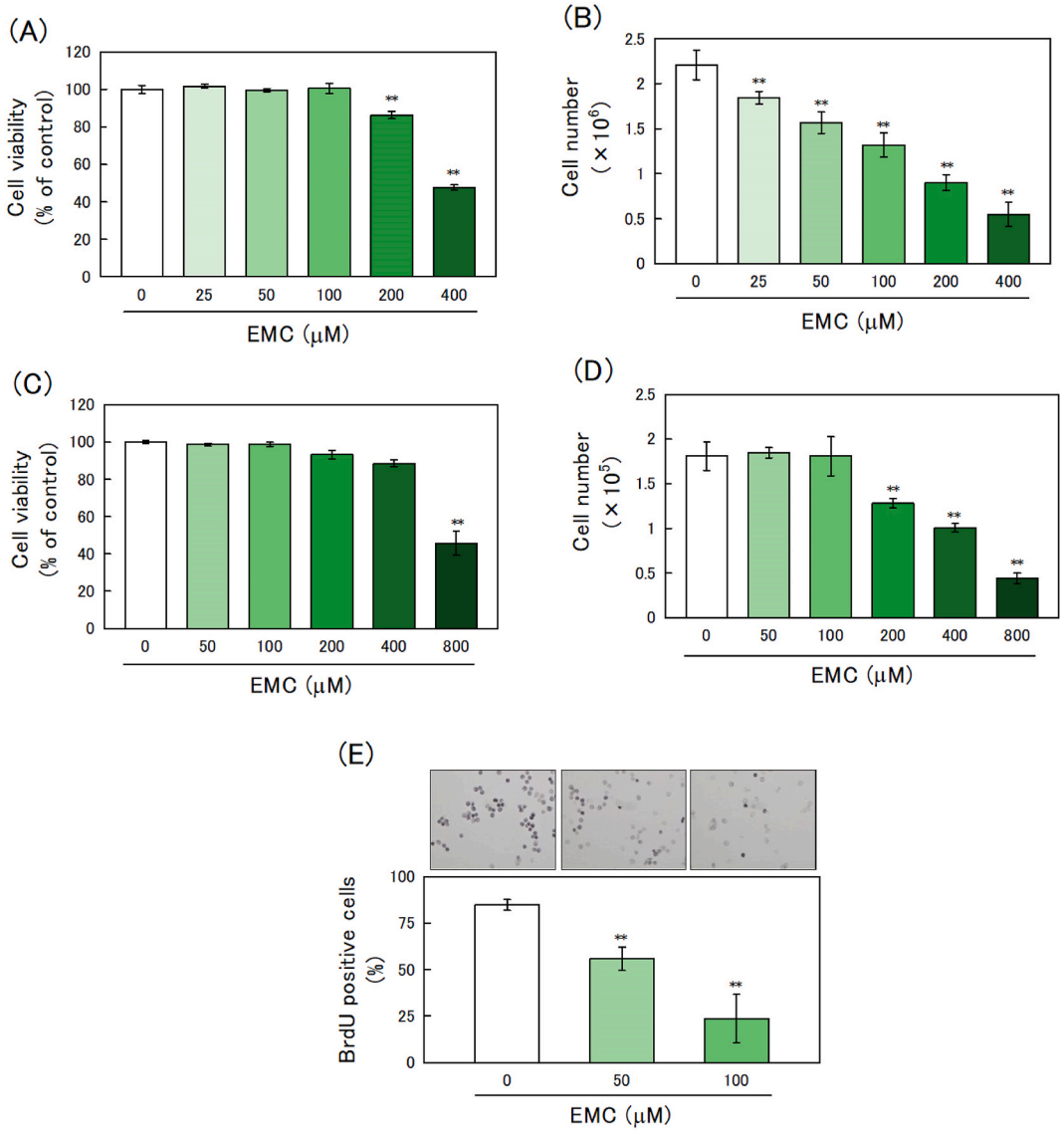

Fig. 5. Effect of EMC on viability, number, and DNA synthesis. (A and B) EATCs were cultured with different concentrations of EMC (0−400 μM) for 24 h. (C and D) 3T3-L1 cells (normal cells) were cultured with different concentrations of EMC (0−800 μM) for 24 h. The (A) viability and (B) number of EATCs and the (C) viability and (D) number of 3T3-L1 cells were assessed using a trypan blue assay. Data are shown as mean ± SD (n = 5). (E) EATC were cultured with BrdU (100 μM) and different concentrations of EMC (0−100 μM) for 24 h. DNA synthesis was assessed using the BrdU assay. Immunostaining images show BrdU-positive cells. The percentage of BrdU-positive cells was calculated by BrdU-positive cell number relative to the total cell number. The number of cells per field was 50–250. Data are shown as mean ± SD (n = 6). Statistical analysis was performed using one-way ANOVA, followed by the Tukey−Kramer test. The data were considered significantly different relative to the control (**$p < 0.01$).

3.7. Effect of EMC on TFAM expression, mtDNA copy number, and mitochondrial membrane potential in EATCs

To further investigate the mechanism underlying the anti-proliferative effect of EMC, we measured the mRNA and protein expression levels of TFAM in EATCs. EMC treatment significantly decreased the mRNA and protein expression levels of TFAM (Fig. 8A and B). In addition, we assessed the effect of EMC on mtDNA copy number and mitochondrial membrane potential, which is an indicator of mitochondrial function. As shown in Fig. 8C and D, EMC had no significant effect on mtDNA copy number or mitochondrial membrane potential.

3.8. Effect of EMC on the mRNA expression levels of H-ras and phosphorylation of c-myc at Ser62 in EATCs

Next, we investigated the mechanism underlying the EMC-induced decrease in TFAM expression. c-Myc is a Tfam transcription

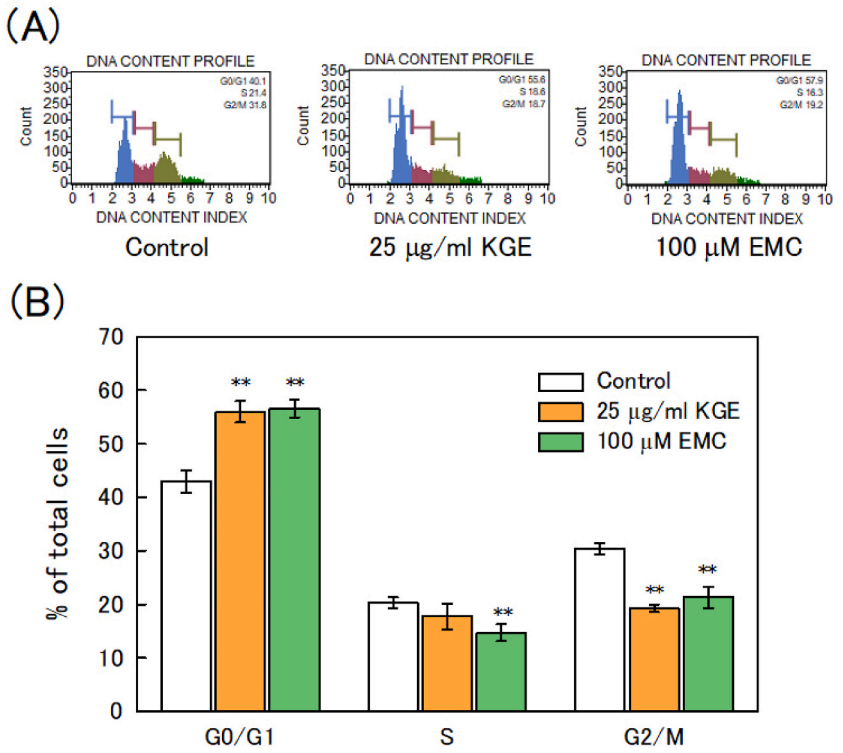

Fig. 6. Effect of KGE and EMC on the progression of the cell cycle in EATCs. EATCs were cultured with different concentrations of KGE (25 μg/ml) or EMC (100 μM) for 24 h. Cell cycle analysis was performed using the Muse Cell Cycle Kit and Muse cell analyzer. (A) Histograms of DNA content show the distribution of cell cycle phases (G0/G1, S, and G2/M) of EATCs. (B) The ratio of the cell cycle stages in EATCs was analyzed. Data are shown as mean ± SD (n = 4). Statistical analysis was performed using one-way ANOVA, followed by the Tukey−Kramer test. The data were considered significantly different relative to the control (**$p < 0.01$).

factor, and its transcriptional activity is increased by Ras-mediated phosphorylation of Ser62 [26,27]. Therefore, we investigated the effect of EMC on the phosphorylation of c-Myc at Ser62 and the mRNA expression of *H-ras*, which encodes Ras protein. EMC significantly suppressed *H-ras* mRNA expression (Fig. 9A). In addition, EMC treatment significantly decreased c-Myc phosphorylation at Ser62 (Fig. 9B).

3.9. Effect of KGE and EMC on the body weight and ascites fluid volume of EATC bearing mice

We investigated the anticancer effects of KGE and EMC *in vivo* using EATC bearing mice. We confirmed by HPLC analysis that 80 mg of KGE contained 66 mg of EMC. The body weight and volume of ascites fluid in mice of control, EATC (+) group were significantly increased compared to those in mice of control, EATC (−) group (Fig. 10A and B). However, the volumes of ascites fluid were significantly reduced with the treatment of EMC (66 mg/kg body weight), and the treatment of KGE (80 mg/kg body weight) showed a tendency to decrease the volume of ascites fluid.

3.10. Effect of natural compounds and anticancer drugs on the mRNA expression levels of Tfam in EATC

We measured the mRNA expression level of *Tfam* to determine whether TFAM is involved in the anti-cancer mechanism of natural compounds, such as hesperidin, and known three anti-cancer drugs, such as 5-FU, cisplatin and PD0332991. Hesperidin is a flavonoid and inhibits the growth of many types of cancer cells [28]. EMC, hesperidin and three anti-cancer drugs significantly decreased cell number of EATC to the same levels without reducing cell viability (Fig. 11A and B). EMC and hesperidin decreased the mRNA expression levels of *Tfam*, but the anticancer drugs did not (Fig. 11C).

4. Discussion

This study provides, for the first time, a detailed mechanism of the anti-cancer effects of KGE and EMC (the main component of the extract) using EATCs. The SI values of KGE and EMC were 3.21 and 1.96, respectively, indicating that these substances are selectively cytotoxic to cancer cells. KGE and EMC also inhibit tumor cell proliferation by blocking the G1/S phase transition. In addition, we demonstrated that EMC suppressed cell proliferation by upregulating p21 and downregulating cyclin D1 expression. EMC also

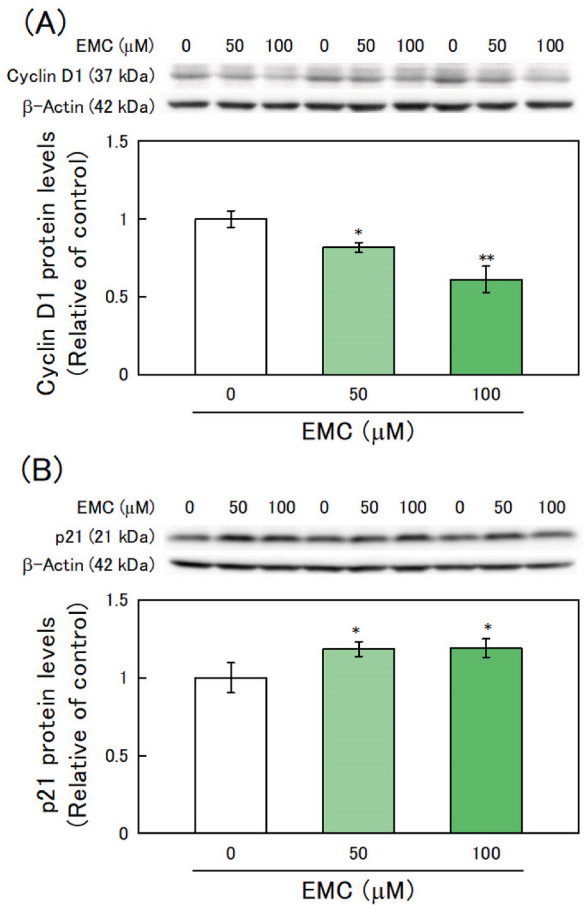

Fig. 7. Effect of EMC on expression levels of cyclin D1 and p21. EATCs were cultured with different concentrations of EMC (0−100 μM) for 12 h. Expression levels of cyclin D1 (A), p21 (B), and β-actin protein were measured using Western blot analysis. Data are shown as mean ± SD (n = 3). Statistical analysis was performed using one-way ANOVA, followed by the Tukey−Kramer test. The data were considered significantly different relative to the control (*$p < 0.05$ and **$p < 0.01$). Non-adjusted full images of immunoblots were shown in the supplemental material (Fig. S1).

decreases the expression of TFAM, a regulator of mitochondrial function, and is closely associated with cancer cell proliferation. Interestingly, despite the decreased expression levels of TFAM, EMC did not induce mitochondrial dysfunction, including a reduced mtDNA copy number and mitochondrial membrane potential. These results indicate that the downregulation of TFAM might inhibit the proliferation of EATC by regulating the expression of nuclear genes, rather than inducing mitochondrial dysfunction. To the best of our knowledge, this study is the first to report that the natural compound-induced decrease in TFAM expression correlates with the expression levels of cyclin D1 and p21.

Cancer cells exhibit uncontrolled proliferation and damage to the surrounding normal tissues. Therefore, arresting cell proliferation and inducing apoptosis is important for inhibiting cancer cell growth. In this study, KGE (0−25 μg/ml) decreased the number of cancer cells in a dose-dependent manner without reducing cell viability. Furthermore, the LC50 of KGE for 3T3-L1 cells was much higher than that for EATCs, with an SI of 3.21 for KGE. A high SI value indicates that a substance is more toxic to cancer cells than to normal cells, meaning that it may have potential as an anti-cancer agent. These results indicate that KGE might induce anti-proliferative effects in EATC without cytotoxicity to normal cells. In addition, we found that the anti-proliferative mechanism of KGE involves inhibition of the G1/S phase transition of EATCs. We identified the active compounds responsible for the anti-proliferative effects of KGE. The EMC content of KGE was calculated to be 78.3% using commercially available pure EMC. Therefore, the EMC concentration was calculated to be 100 μM at 25 μg/ml of KGE. EMC (0−100 μM) decreased cell number and inhibited the G1/S phase transition without reducing EATC viability. These results are similar to those obtained with KGE treatment. Therefore, EMC was identified as the active compound responsible for the anti-proliferative effect of KGE on EATCs. Furthermore, EMC (0−100 μM) was not cytotoxic to 3T3-L1 cells. Our results indicated that low-dose EMC (100 μM) suppressed cell proliferation without inducing apoptosis and could safely provide anti-cancer effects.

Cell proliferation is regulated by the cell cycle, which is divided into four stages: G1, S, G2, and M [29]. Cancer has an aberrant cell cycle, which induces a lack of differentiation and unregulated cell proliferation [30,31]. A previous study reported that oncogenic alterations in cell cycle regulators are observed in more than 90% of human cancers and frequently occur during the G1 phase [32].

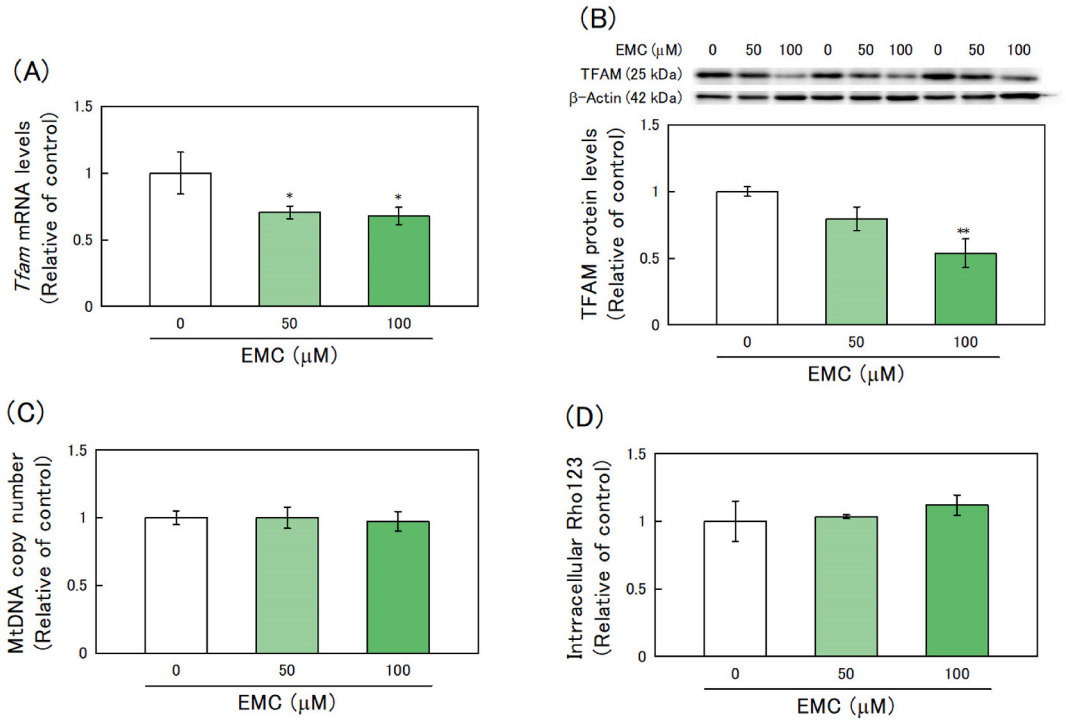

Fig. 8. Effect of EMC on the expression level of TFAM and mitochondrial function. EATCs were cultured with different concentrations of EMC (0−100 μM) for 12h. (A and C) The expression level of *Tfam* mRNA and mitochondrial DNA (mtDNA) copy number were measured using quantitative reverse transcription PCR. (B) Expression levels of TFAM and β-actin protein were measured using Western blot analysis. Non-adjusted full images of immunoblots were shown in the supplemental material (Fig. S2). (D) Mitochondrial membrane potential was measured by rhodamin-123 (rho-123) dye. Data are shown as mean ± SD (n = 3). Statistical analysis was performed using one-way ANOVA, followed by the Tukey−Kramer test. The data were considered significantly different relative to the control (*$p < 0.05$ and **$p < 0.01$).

Cyclin D1 expression is important for progression from the G1 to the S phase. Cyclin D1 induces progression from the G1 to the S phase by phosphorylating retinoblastoma protein after forming a complex with cyclin-dependent kinases (CDKs) [33]. The expression levels of cyclin D1 are enhanced in human malignancies [34]. In contrast, p21, a well-known anti-cancer factor, causes G1 cell cycle arrest by inhibiting the cyclin D1-CDK4/6 complex [33,35]. Jinhua et al. reported that ferulic acid induced G1/S phase cell cycle arrest by downregulating cyclin D1 and upregulating p21 in CaSki and HeLa cervical carcinoma cell lines [36]. In this study, EMC decreased the expression levels of cyclin D1 and increased those of p21. These results suggest that the regulation of cyclin D1 and p21 expression is involved in the suppression of EMC-induced S-phase progression by causing G1 arrest in EATCs.

TFAM, identified as a transcription factor for mtDNA, regulates cell proliferation, survival, and migration [37,38]. Overexpression of TFAM promotes cell proliferation, metastasis, and apoptosis resistance in colon cancer cells by enhancing mitochondrial function derived from increased mtDNA copy number, mitochondrial membrane potential, and intracellular ATP levels, whereas knockdown of TFAM reverses these effects [39]. Hesperidin and chlorogenic acid inhibit cell proliferation in correlation with decreased TFAM expression levels, mtDNA copy number, and intracellular ATP levels in MCF-7 breast cancer cells [40]. TFAM functions not only as a regulator of mitochondrial function but also as a nuclear transcription factor. TFAM is also localized in the nucleus, and some amounts of TFAM bind strongly to the nuclear chromatin. TFAM regulates p21 expression, as TFAM depletion induces G1 cell cycle arrest via p21 upregulation in PC3 human prostate cancer cells [19,41]. Furthermore, TFAM is involved in the regulation of cyclin D1 expression as the knockdown of TFAM induces a decrease in cyclin D1, whereas the overexpression of TFAM shows the opposite effect [42,43]. In this study, EMC significantly decreased the expression level of TFAM. EMC also did not significantly alter mtDNA copy number and membrane potential. These results suggest that the EMC-induced reduction in the expression level of TFAM inhibits cell proliferation by regulating the expression of cyclin D1 and p21 rather than by inducing mitochondrial dysfunction.

The oncogene c-Myc plays a pivotal role as a transcription factor for genes involved in various biological processes, including cell proliferation, apoptosis, and differentiation [44]. c-Myc directly induces TFAM expression by binding to the promoter region of TFAM encoded by the nuclear DNA [26]. The synthesized c-Myc is unstable because of its short half-life; however, phosphorylation of its Ser62 residue increases its stability and transcriptional activity [45,46]. Ras, composed of H-ras, K-ras, and N-ras isoforms, is activated by stimulation with growth factors and induces the phosphorylation of c-Myc at Ser62 via its downstream effector, the ERK pathway [45]. This study suggests that EMC suppressed TFAM expression by decreasing the transcriptional activity of c-Myc due to the decreased expression of *H-ras* mRNA. Further studies are needed to elucidate the mechanism underlying the regulation of *H-ras* mRNA expression by EMC.

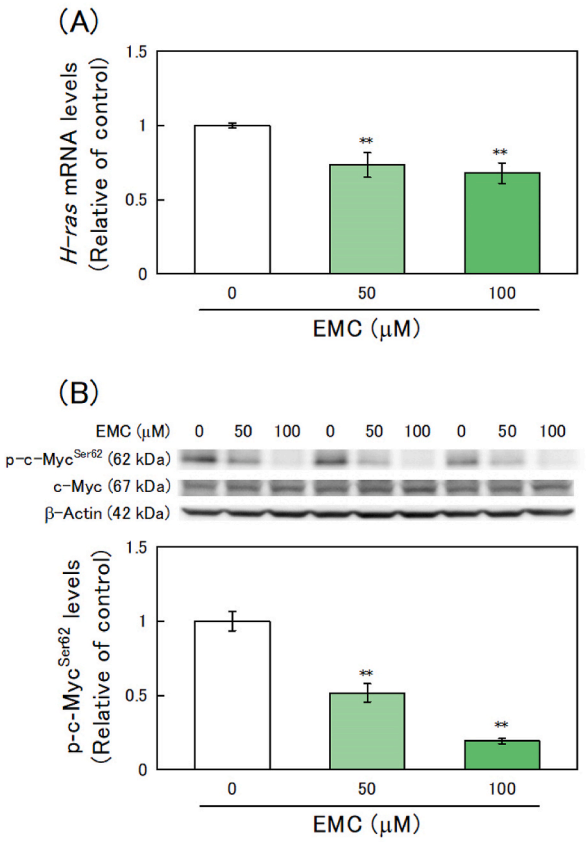

Fig. 9. Effect of EMC on expression levels of *H-ras* mRNA and phosphorylation at Ser62 of c-Myc. EATCs were cultured with different concentrations of EMC (0−100 μM) for 12 h. (A) The expression level of *H-ras* mRNA was measured using quantitative reverse transcription PCR. (B) The levels of phosphorylation at Ser62 of c-Myc, c-Myc and β-actin protein were measured using Western blot analysis. Non-adjusted full images of immunoblots were shown in the supplemental material (Fig. S3). Data are shown as mean ± SD (n = 3). Statistical analysis was performed using one-way ANOVA, followed by the Tukey−Kramer test. The data were considered significantly different relative to the control (**$p < 0.01$).

Intraperitoneal administration of EATC rapidly increases ascites fluid in mice. The volume of ascites fluid is an index of anti-cancer effect [47,48]. In this study, the volume of ascites fluid was significantly increased by intraperitoneal administration of EATC. However, the increase in the volume of ascites fluid was suppressed by oral administration of EMC and KGE. The reason why the lower reduction in the volume of ascites fluid with the treatment of KGE than that of EMC, is not clear. However, these results suggest that KGE and EMC show anti-cancer effect *in vivo* animal model.

Finally, we investigated whether TFAM is involved in the anticancer effects of natural compounds other than EMC and the anti-cancer drugs. Hesperidin suppresses proliferation of cancer cell by regulating intracellular signaling pathways [28]. On the other hand, anti-cancer drugs suppress the proliferation of cancer cells by acting directly on nuclic acids or specific proteins involved in cell cycle. 5-FU replaces thymine in DNA and uracil in RNA, leading to damage induction in these molecules [49]. Cisplatin directly interacts with DNA and causes the formation of crosslinks [50]. PD0332991 is a CDK4/6 inhibitor and inhibits the formation of the cyclin D1-CDK4/6 complex by directly binding to CDK4/6 [51]. In our study, hesperidin, 5-FU, cisplatin, and PD0332991 inhibited the proliferation of EATC similarly to EMC. The mRNA expression level of *Tfam* was decreased by treatment with EMC and hesperidin, which are natural compounds, but not by treatment with these anti-cancer drugs. As shown in Fig. 12, the anti-cancer drugs exert their anti-cancer effects at the downstream of TFAM. Furthermore, it has been reported that various anti-cancer drugs, including 5-FU, enhance resistance to drug-induced apoptosis by increasing TFAM expression [52,53]. In our study, 5-FU and PD0332991 also increased *Tfam* expression, which may enhance apoptosis resistance in EATC. In contrast, suppression of TFAM expression may be involved in the anti-cancer mechanisms of natural compounds that target signaling pathways at the upstream of TFAM. However, we need further investigation on the differences with natural compounds and anti-cancer drugs in the action mechanisms of TFAM.

In summary, this study revealed that EMC is the active compound responsible for the anti-cancer activity of KGE and inhibits the G1/S phase transition of EATC by regulating the expression of cell cycle regulators (cyclin D1 and p21). Furthermore, EMC suppressed the expression of TFAM, which plays an oncogenic role by decreasing the transcriptional activity of c-Myc; a decrease in TFAM inhibits cell growth by regulating the expression of these cell cycle regulators (Fig. 12). This study also provides novel insights into the association between the anti-cancer effects of natural compounds and TFAM, indicating that TFAM might be a potential therapeutic target. However, further studies, including clinical trials, are needed to establish whether KGE and EMC exert anti-cancer effects in

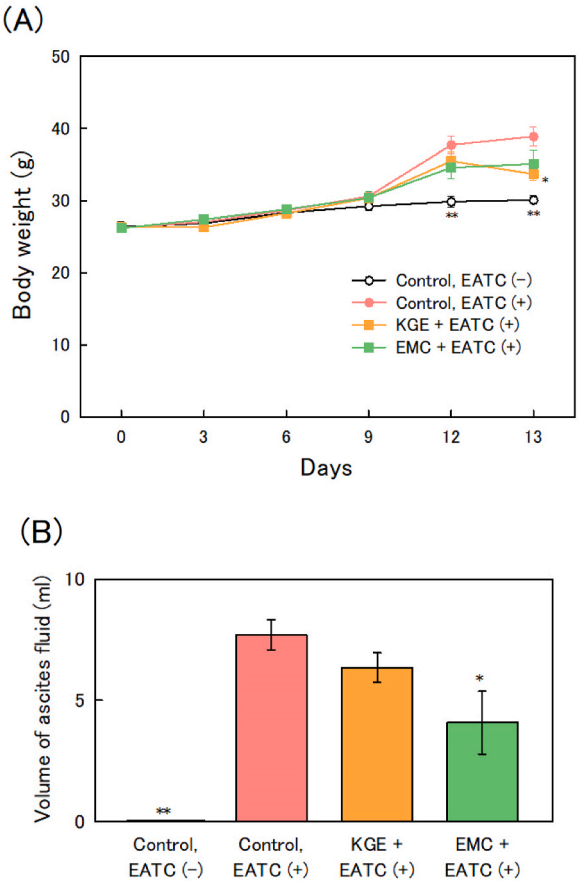

Fig. 10. Effect of KGE and EMC on the body weight (A) and the volume of ascites fluid (B) in EATCs bearing mice. KGE (80 mg/kg body weight) or EMC (66 mg/kg body weight) was orally administered daily. Data are shown as mean ± SE (n = 5–7). Statistical analysis was performed using one-way ANOVA, followed by the Tukey−Kramer test. The data were considered significantly different relative to the EATC (+) (*$p < 0.05$ and **$p < 0.01$).

patients with breast cancer.

Author contribution statement

Yutaro Sasaki, Isao Matsui-Yuasa, Akiko Kojima-Yuasa: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Toshio Norikura: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Ritsuko Fujii, Leenawaty Limantara: Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

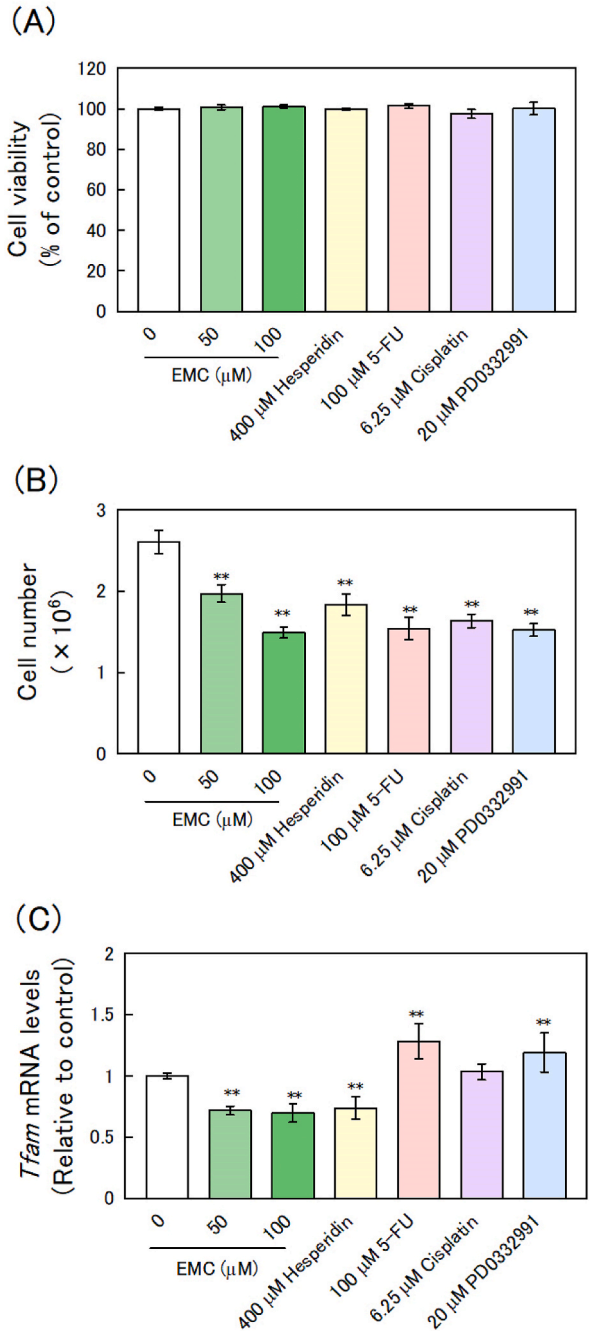

Fig. 11. Effect of natural compounds and anticancer drugs on the mRNA expression levels of *Tfam* in EATC. (A and B) EATCs were cultured with EMC, hesperidin, 5-FU, cisplatin or PD0332991 for 24 h. The (A) viability and (B) number of EATCs were assessed using a trypan blue assay. Data are shown as mean ± SD (n = 5). (C) The expression level of *Tfam* mRNA was measured using quantitative reverse transcription PCR after 12 h of culture. Data are shown as mean ± SD (n = 3). Statistical analysis was performed using one-way ANOVA, followed by the Tukey−Kramer test. The data were considered significantly different relative to the control (**$p < 0.01$).

Abbreviations

EATC	Ehrlich ascites tumor cell
KGE	*Kaempferia galanga* L. rhizome extract
TFAM	Mitochondrial transcription factor A
MtDNA	Mitochondrial DNA

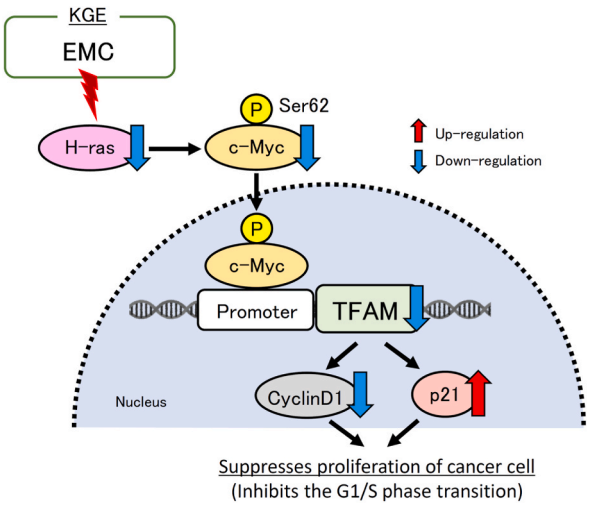

Fig. 12. The proposed underlying mechanism of the anti-proliferative effect of EMC against EATCs. EMC decreases the expression level of *H-ras* and suppresses the phosphorylation of c-Myc at Ser62. As a result, the transcriptional activity of c-Myc is reduced, and the expression level of TFAM, the target gene of c-Myc, is decreased. Decreased expression of TFAM induces cyclin D1 downregulation and p21 upregulation, inhibiting EATC proliferation by blocking the S-phase progression.

DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
EMC	Ethyl *p*-methoxycinnamate
BrdU	Bromodeoxyuridine
HPLC	High-pressure liquid chromatography
qRT-PCR	Quantitative reverse transcription PCR
rho-123	rhodamin-123
CDK	cyclin-dependent kinase

Appendix B. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heliyon.2023.e17588.

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