# Fatty acids inhibit anticancer effects of 5-fluorouracil in mouse cancer cell lines

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**Abstract.** The present study investigated the effects of two major dietary fatty acid components, linoleic acid (LA) and elaidic acid (EA), on the antitumor effects of 5-fluorouracil (5-FU) in the LL2, CT26 and CMT93 mouse cancer cell lines. Concurrent treatment with LA and 5-FU elicited a decreased cell viability compared with treatment with 5-FU alone. In addition, increased inhibition of growth was observed following concurrent treatment with EA and 5-FU. Sequential treatment of LA followed by 5-FU abrogated the anticancer effects of 5-FU, and treatment with EA followed by 5-FU increased cancer cell growth in addition to abrogating the anticancer effects of 5-FU. The expression of the stem cell markers CD133 and nucleostemin (NS) increased in all three cell lines treated concurrently with 5-FU and either LA or EA when compared with cells treated with 5-FU alone. Aldehyde dehydrogenase activity in the cancer stem cells (CSCs), in response to concurrent treatment with 5-FU and either LA or EA, was increased compared with 5-FU treatment alone. 5-FU inhibited the growth of CT26 tumors, but co-treatment with either LA or EA abrogated this effect. NS-positive CSCs were more abundant in CT26 tumors treated with 5-FU and either LA or EA compared with those treated with 5-FU alone. The results of the present study suggested that, rather than altering the sensitivity of cancer cells to 5-FU, LA and EA may promote the survival of CSCs. The results indicated that dietary composition during chemotherapy is an important issue.

## Introduction

High intake of fatty food is associated with an increased risk of cancer, particularly for colorectal cancer (CRC) (1-3). A high-fat diet is associated with aggressive prostate cancer and n-6 fatty acids, including linoleic acid (LA), enhance breast cancer invasion and metastasis (4). LA in combination with azoxymethane results in the upregulation of insulin-like growth factor-I/II receptors (5), Ras (6), cyclooxygenase-2 (7) and high mobility group box-1 (8), which act to accelerate colon carcinogenesis (6,7,9,10). Prolonged treatment with LA induces a quiescent state in CRC cells and the dormancy of subcutaneous tumors in mice, which is thought to be associated with low proliferation of cancer stem cells (CSCs) (11,12).

Trans fatty acids (TFAs) are reported to increase the risk of certain types of cancers, including CRC and prostate cancer (13,14). TFAs, in particular elaidic acid (EA), enhance cancer cell growth, invasion, survival and metastasis through activation of the Wnt signaling pathway and the induction of epithelial-mesenchymal transition (15-17). TFAs were previously recognized as safe for human consumption; however, the Food and Drug Administration has proposed that TFAs must be removed from prepared foods by June 2018 (18).

LA and EA are abundant dietary fatty acids, and are part of a normal diet eaten by patients with cancer receiving chemotherapy. The present study focused on the effect of LA and EA on the action of 5-fluorouracil (5-FU), a common anticancer agent, with a particular emphasis on cancer stemness.

## Materials and methods

*Cell culture.* The mouse colon cancer CT26 cell line was provided by Professor Isaiah J. Fidler (MD Anderson Cancer Center, Houston, TX, USA). The mouse rectal carcinoma CMT93 cell line and the mouse lung cancer LL2 cell line were purchased from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37°C in a humidified condition with 5% $CO_2$ atmosphere.

Cells in 6 dishes ($1x10^5$ in 3.5 cm-diameter dishes) were treated with LA (50 $\mu$g/ml) or EA (20 $\mu$g/ml) and concurrently with 5-FU (1 $\mu$g/ml) at 37°C for 24 h. For sequential treatment, cells were seeded in 6 dishes ($1x10^5$ in 3.5 cm-diameter dishes) and treated with LA (50 $\mu$g/ml) or EA (20 $\mu$g/ml) at 37°C for 24 h prior to 5-FU treatment (1 $\mu$g/ml at 37°C for 24 h).

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The cell viabilities were assessed using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) Assay kit (Promega Corporation, Madison, WI, USA). Following treatment with LA or EA and/or 5-FU, MTS solution was added to each well for 2 h at 37°C in 5% $CO_2$. The absorbance at 490 nm was recorded using a microplate reader.

*Animals*. Male BALB/c mice (4-weeks-old) were purchased from Japan SLC, Inc. (Shizuoka, Japan). The animals were maintained in the pathogen-free animal facility with a 12/12 h light/dark cycle in a temperature (22°C) and humidity-controlled environment, according to institutional guidelines approved by the Committee for Animal Experimentation of Nara Medical University (Kashihara, Japan; approval no. 9559), in accordance with the current regulations and standards of the Japanese Ministry of Health, Labor and Welfare.

*Animal models*. To establish a subcutaneous tumor model, CT26 cancer cells ($1x10^7$) were inoculated into the scapular subcutaneous tissue of BALB/c mice. Mice were euthanized and observed using immunohistochemistry 4 weeks following inoculation. 5-FU (10 mg/kg; Wako Pure Chemical Industries, Ltd.) was injected intraperitoneally twice a week for 2 weeks following inoculation. LA (Sigma-Aldrich; Merck KGaA) or EA (Wako Pure Chemical Industries, Ltd.) were administrated at 1.0 and 0.1% w/w, respectively, through the daily supplementation of a standard CE-2 diet (CLEA Japan, Inc., Tokyo, Japan). The mean intake of LA and EA was 4.2±0.78 and 0.31±0.07 mg/day, respectively. A total of five mice were used for per treatment group. The mean weight of the mice was 21.5±1.4 g at the start of treatment.

*Semi-quantitative reverse transcription-quantification polymerase chain reaction (RT-qPCR)*. Total RNA (1 µg) was extracted using an RNeasy extraction kit (Qiagen, Inc., Valencia, CA, USA). RNA was extracted from $1x10^7$ CT26 cells and eluted in 50 µl RNase-free water. cDNA was synthesized using ReverTra Ace RT-qPCR kit (Toyobo, Tokyo, Japan). Quantification of PCR products was performed with QuantiTect Primer Assays using QuantiFast, QuantiTect, Rotor-Gene, and FastLane kit for SYBR-Green-based detection (Qiagen, Inc.), according to the manufacturer's protocol. mRNA quantification was performed according to the $2^{-\Delta\Delta cq}$ method (19). The number of replicates was 30 cycles. The primer sets used for amplification were as follows: Mouse CD133 (prominin 1; accession no., BC028286.1) forward, 5'-GAAAAGTTGCTCTGCGAACC-3' and reverse, 5'-TCTCAAGCTGAAAAGCAGCA-3'; mouse nucleostemin (NS; accession no. AY181025.1) forward, 5'-CAGGATGCTGACGATCAAGA-3' and reverse, 5'-TTGATTGCTCAGGTGACAGC-3'; and mouse β-actin (accession no. NM_007393.4) forward, 5'-AGCCATGTACGTAGCCATCC-3' and reverse 5'-CTCTCAGCTGTGGTGGTGAA-3'. The primers were synthesized by Sigma Genosys (Sigma-Aldrich; Merck KGaA). The PCR conditions were set according to the manufacturer's protocol. The number of replicates was 30 cycles. PCR products were electrophoresed on a 2% agarose gel and visualized using ethidium bromide.

*Immunohistochemistry*. Consecutive 4 µm tissue sections were immunohistochemically stained using the immunoperoxidase technique, as described previously (8). Mouse CD133 antibody (AG13328; ProteinTech Group, Inc., Chicago, IL, USA) and mouse nucleostemin antibody (sc-166430; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were used at a concentration of 0.5 µg/ml at 37°C for 2 h. Color development was performed using 3-3'-diaminobenzidine (Dako; Agilent Technologies, Inc.), and the specimens were counterstained with Mayer's hematoxylin (Sigma-Aldrich; Merck KGaA) to visualize the nuclei. Following immunostaining, all slides were assessed to measure the number of positively stained nuclei using an all-in-one microscope (BZ-X700; Keyence, Osaka, Japan).

*Aldehyde dehydrogenase (ALDH) activity*. ALDH activity was measured using the ALDEFLUOR kit (Veritas Technologies LLC, Tokyo, Japan) according to the manufacturer's protocol. The activity was normalized to the negative control, diethylaminobenzaldehyde.

*Statistical analysis*. Statistical significance was calculated using the two-tailed Fisher's exact test, $\chi^2$ test and unpaired Student's t-test, with the assumption of Gaussian distributions, by Kolmogorov and Smirnov tests Statistical analysis was performed using InStat software (GraphPad Software, Inc., La Jolla, CA, USA). The data are presented as the mean ± standard deviation. P<0.05 (two-sided) was considered to indicate a statistically significant difference.

## Results

*Effect of LA and EA on the 5-FU-induced reduction of cancer cell viability*. Three cancer cell lines, CT26 (colon cancer), LL2 (lung cancer) and CMT93 (rectal cancer), were concurrently treated with either LA or EA and 5-FU, or with LA, EA or 5-FU alone. Treatment with LA alone decreased cell viability in all 3 cell lines (Fig. 1). LA also augmented the tumor cell inhibition observed following 5-FU treatment in all 3 cell lines. In contrast, EA alone did not significantly suppress tumor viability in any of the 3 cell lines (Fig. 1). In addition, concurrent treatment of EA with 5-FU did not recover or enhance 5-FU-induced growth inhibition in the three cell lines.

Sequential treatment with LA or EA followed by 5-FU was then examined (Fig. 1). Concurrent treatment of 5-FU and LA revealed a more pronounced decrease of cell viability in the 3 cell lines. In contrast, temporal treatment of LA to 5-FU demonstrated no additional decrease of viability compared with that of 5-FU treatment alone. (Fig. 1). In addition, EA pretreatment reversed the effect of 5-FU, increasing the viability of LL2 and CMT93 cells (Fig. 1).

*Effect of LA or EA with 5-FU on the stemness of cancer cells*. Previous studies have demonstrated that the resistance of cancer cells to anticancer drugs is associated with CSCs (20,21). Therefore, alterations of stemness in LA- or EA-treated cancer cells were examined (Fig. 2). Expression of CD133 and NS was examined in cells treated with 5-FU alone, or concurrently with LA or EA (Fig. 2A-D). The expression of CD133 and NS was increased in cells treated with LA or EA when compared with untreated cells. Although expression of
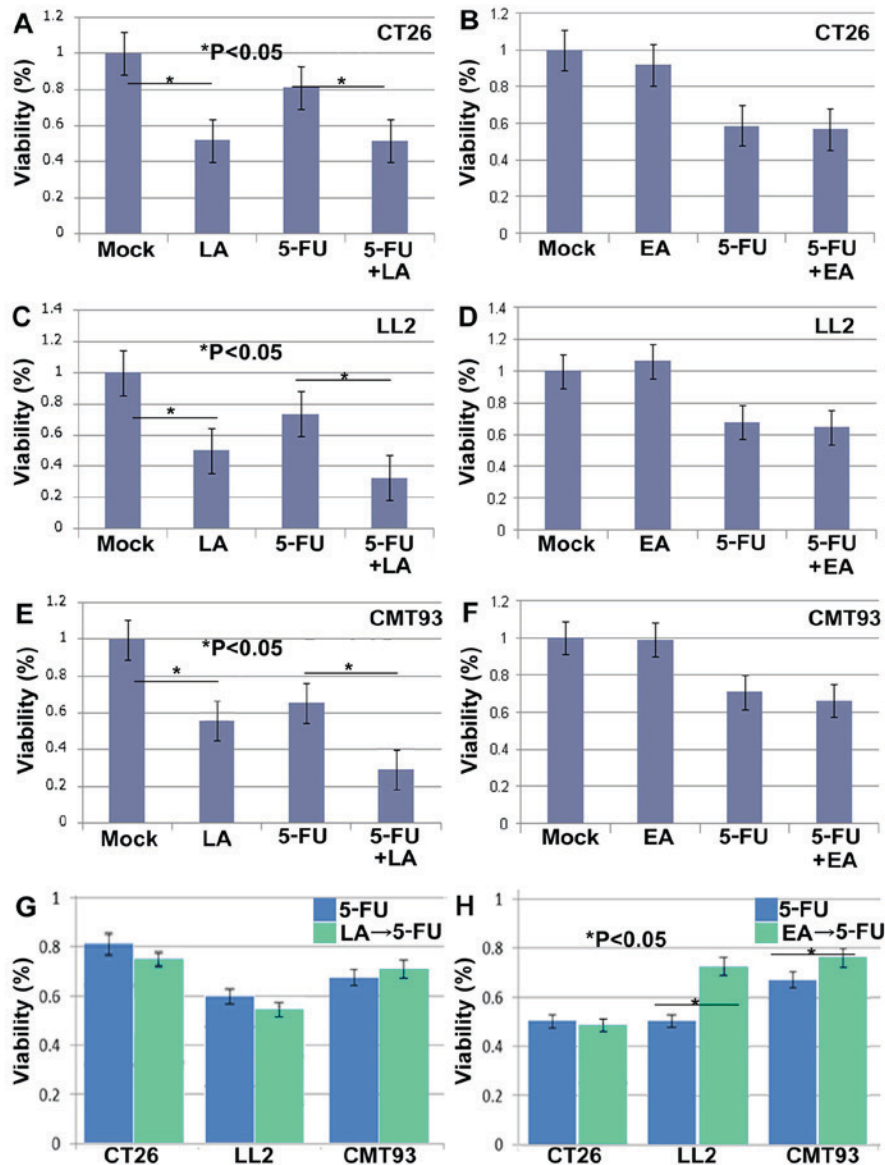
Figure 1. Effect of LA or EA on 5-FU-induced reduction of cancer cell viability. CT26 cells were treated with (A) LA (50 µg/ml) or (B) EA (20 µg/ml), administered concurrently with 5-FU (1 µg/ml). LL2 cells were treated with (C) LA (50 µg/ml) or (D) EA (20 µg/ml), administered concurrently with 5-FU (1 µg/ml). CMT93 cells were treated with (E) LA (50 µg/ml) or (F) EA (20 µg/ml), administered concurrently with 5-FU (1 µg/ml). (G) LA (50 µg/ml) (H) or EA (20 µg/ml) was administered 24 h prior to 5-FU treatment (1 µg/ml) in 3 cancer cell lines. Data are expressed as the mean ± standard deviation. LA, linoleic acid; EA, elaidic acid; 5-FU, 5-fluorouracil; Mock, vehicle treated with 70% ethanol.

NS appears to be higher in cells treated with 5-FU and LA concurrently compared with cells treated with LA alone in CT26 and CMT93 cells, the expression of CD133 and NS was lower in cells treated concurrently with 5-FU and either LA or EA compared with cells treated with LA or EA alone. However expression was increased in cells treated concurrently with 5-FU and either LA or EA compared with cells treated with 5-FU alone. Activity of ALDH, a stem cell-associated enzyme, was increased in cells treated with LA or EA compared with in untreated cells (Fig. 2E). ALDH activity was higher in cells treated concurrently with 5-FU and either LA or EA compared with cells treated with 5-FU alone (Fig. 2E).

*Effect of LA or EA with 5-FU on tumor growth*. Finally, the effect of oral intake of LA or EA on the antitumor effects of

5-FU was examined in BALB/c mice inoculated with CT26 cells (Fig. 3). Treatment with 5-FU inhibited the growth of CT26-derived tumors, and this was abrogated by concurrent treatment with either LA or EA (Fig. 3A and B). The expression of NS in CT26-derived tumors was examined by immunohistochemistry (Fig. 3C and D). NS-positive CSCs were more abundant in tumors treated with 5-FU and either LA or EA compared with tumors treated with 5-FU alone.

**Discussion**

Fatty acids are dietary components, which are known to be involved in colon carcinogenesis (6,7). LA and EA are known to be risk factors for CRCs (14,22-24), enhancing inflammation
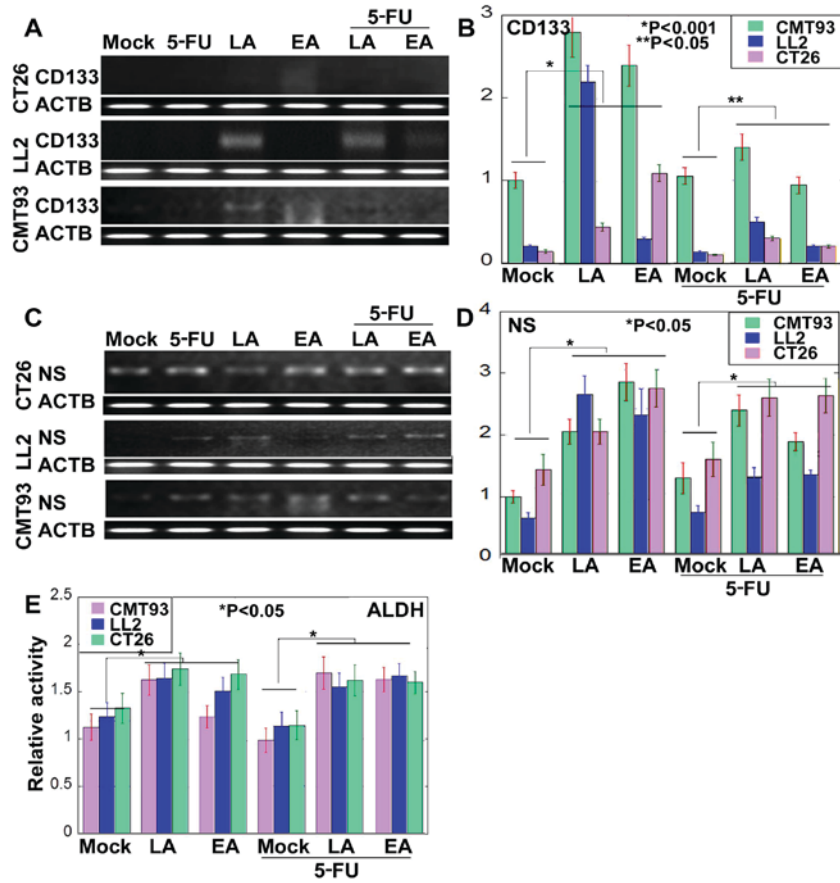
Figure 2. Effect of LA or EA with 5-FU on the stemness of cancer cells. Expression of (A) CD133 was examined in cells treated with 5-FU (1 $\mu$g/ml) and either LA (1 $\mu$g/ml) or EA (20 $\mu$g/ml) concurrently, with (B) quantification. Expression of (C) NS was examined in cells treated with 5-FU (1 $\mu$g/ml) and either LA (1 $\mu$g/ml) or EA (20 $\mu$g/ml) concurrently, with (D) quantification. (E) ALDH activity of cells treated with 5-FU and either LA or EA concurrently. Data are expressed as the mean ± standard deviation. LA, linoleic acid; EA, elaidic acid; 5-FU, 5-fluorouracil; NS, nucleostemin; ALDH, aldehyde dehydrogenase; ACTB, $\beta$-actin.
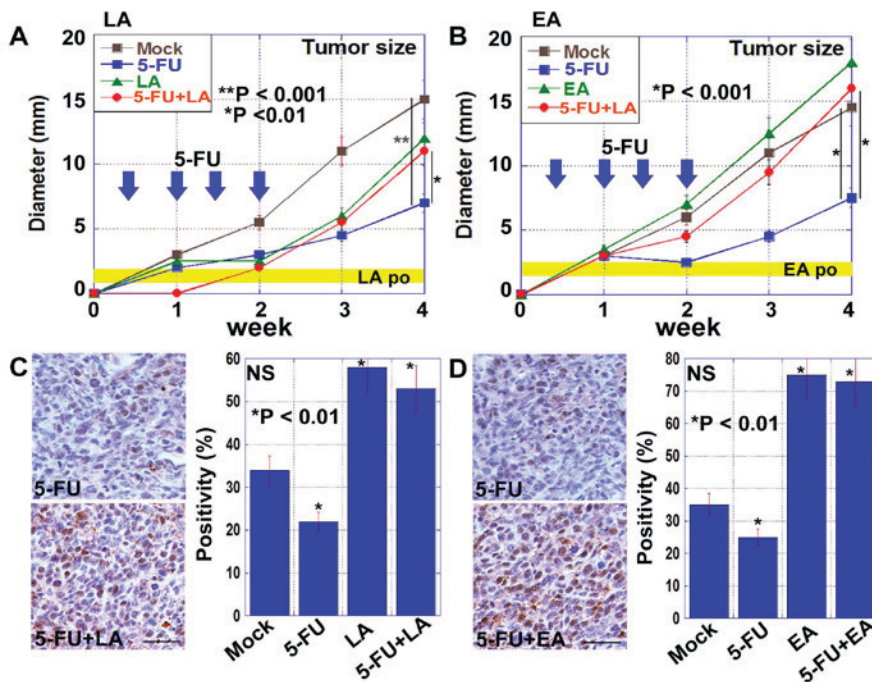


Figure 3. Effect of LA or EA with 5-FU on tumor growth. Size of subcutaneous CT26 derived tumors treated with 5-FU (10 mg/kg) and oral administration of (A) LA or (B) EA (10% w/w in CE-2 diet). Expression of (C and D) NS in CT26 derived tumors was examined by immunohistochemistry (microphotography) following (A) LA and (B) EA treatment. NS positivity was examined by observing 1,000 tumor cell nuclei. Scale bar, 50 $\mu$m. Data are expressed as the mean ± standard deviation. LA, linoleic acid; EA, elaidic acid; 5-FU, 5-fluorouracil; NS, nucleostemin.

and suppressing mucosal immunity (25-28). LA-derived prostaglandin E2, produced by cyclooxygenase-2, induces chronic persistent inflammation, which produces reactive oxygen species (25,26). Likewise, EA induces secretion of TNF-α, activation of nuclear factor-κB, and inhibition of cluster of differentiation 8+ T-lymphocytes (27,28).

LA and EA affect CSCs. LA induces senescence in cultured CRC cells and dormancy in CRC cells inoculated into mice (11). Conversely, EA increases the proliferation of cells in spheres via the activation of epidermal growth factor receptor and the Wnt signaling pathway, and the upregulation of stem cell markers (16,17). EA also induces epithelial-mesenchymal transition and enhances tumor growth and metastasis (16,17).

Previous studies have revealed that cancer stemness is responsible for resistance to anticancer chemotherapy (20,21). CSCs possess refractoriness to chemotherapy through low proliferative activity, advanced DNA repair and the expulsion of chemotherapeutic agents from the cytosol (29). Therefore, factors affecting the stemness of cancer cells may also affect the efficacy of chemotherapy.

Sequential treatment with LA or EA followed by 5-FU abrogated growth inhibition by 5-FU. Pretreatment with LA or EA may select and condense CSCs or increase the stemness of these cells. ALDH activity, a marker for stem cell activity (30), was enhanced by LA and EA. Upregulation of CD133 and NS by LA or EA varied by cell line. CD133 expression is associated with stem cells (31,32), whereas NS expression is associated with stem cells and progenitor cells (33). Therefore, LA and EA may affect populations of stem cells and progenitors in a cell or tissue-dependent manner (34).

The anti-chemotherapeutic effect of ingested LA or EA was confirmed using a mouse subcutaneous tumor model. Oral intake of LA or EA abrogated the antitumor effects of 5-FU. This result may be an example of the anti-chemotherapeutic properties of dietary components. The anti-chemotherapeutic effects of LA or EA were observed not only in CT26 and CMT93 CRC cells, but also in LL2 lung cancer cells. LA and EA are not thought to be risk factors for lung cancer; however, the present data indicated that the anti-chemotherapeutic effects of LA or EA may affect the efficacy of treatment of various malignancies. The effect of dietary components on chemotherapeutic effects, or on cancer stemness, should be the focus of wide ranging studies concerning human cancers.

## Acknowledgements

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