Effects of an elemental diet, Elental®, may differ between healthy oral cells and oral cancer cells

KOJI HARADA[1], TARANNUM FERDOUS[1], KENJI WATANABE[2], YOICHI MIZUKAMI[2] and KATSUAKI MISHIMA[1]

[1]Department of Oral and Maxillofacial Surgery, Yamaguchi University Graduate School of Medicine;
[2]Center for Gene Research, Science Research Center, Yamaguchi University, Ube, Yamaguchi 755-8505, Japan

Received February 5, 2020; Accepted July 23, 2020

DOI: 10.3892/or.2020.7896

Abstract. The effectiveness of an elemental diet (ED), Elental®, against radiotherapy- or chemoradiotherapy-induced oral mucositis was previously reported. However, the administration of additional nutrition or an ED in patients with oral cancer may also provide extra nutrition for cancer cells, which could result in cancer development. At present, it remains unclear whether the beneficial effects of an ED are likely to surpass its potential harmful effects on oral cancer treatment. In the present study, we aimed to clarify whether Elental® has different effects on a healthy human oral keratinocyte (HOK) cell line compared with its effects on oral squamous cell carcinoma (OSCC) cell lines (HSC2, HSC3, HSC4). The efficacy of Elental® was compared in relation to the growth and migration ability of HOK and OSCC cell lines using MTT assay and migration assay, respectively. In addition, whole transcriptome analysis and network analysis were performed to determine the difference in the mechanism of action of Elental® between HOK and HSC2 cells. In addition, Elental® promoted growth and migration ability of-malnourished and 5-fluorouracil (5-FU)-treated damaged HOK cells cultured in low nutrition medium (0% growth supplement). However, Elental® did not affect the growth ability of 5-FU-treated damaged HSC2 cell line in low nutrition medium (0 or 1% fetal bovine serum (FBS), as well as the growth ability of HSC3 and HSC4 cell lines in medium containing 0% FBS. Elental® pre-treatment also enhanced the apoptosis-inducing effect of anticancer agents against OSCC cells. In addition, whole transcriptome analysis and Ingenuity Pathways Analysis (IPA) data suggested that Elental® may help in the proliferation and survival of HOK through the induction of *ERK*. Moreover, Elental® added stress to HSC2 cells through the induction of the endoplasmic reticulum stress response marker, *BiP* and *GRP 94*. The results showed that Elental® may add stress to HSC2 cells and provide growth stimulation to HOK. These findings suggest that the effects of Elental® on healthy oral cells and oral cancer cells may differ.

Correspondence to: Dr Koji Harada, Department of Oral and Maxillofacial Surgery, Yamaguchi University Graduate School of Medicine, 1-1-1 Minamikogushi, Ube, Yamaguchi 755-8505, Japan
E-mail: harako@yamaguchi-u.ac.jp

Key words: elemental diet, whole transcriptome analysis, network analysis, oral keratinocyte, oral cancer cell

Introduction

Oral mucositis is one of the typical treatment-limiting side effects of chemotherapy or chemoradiotherapy in patients with cancer. The condition is characterized by inflammation, ulceration, lesioning, and bleeding of oral mucosa (1). Oral mucositis can cause several problems, including acute oral pain, difficulty in swallowing, reduced nutritional intake, and malnutrition, all of which can significantly affect the oral hygiene and quality of life of patients (2,3). Severe oral mucositis can be a costly and dose-limiting side effect of intensive treatment for cancer, which may lead to the discontinuation of cancer therapy and limit the completion of treatment, thereby adversely affecting the prognosis and survival rates of patients (1,4-6). There may be several factors responsible for the onset and increase of oral mucositis in patients with cancer who are receiving chemotherapy or chemoradiotherapy. However, the detailed mechanisms and the complex pathobiology underlying oral mucositis are not yet fully understood.

Chemotherapeutic agents, including 5-fluorouracil (5-FU), can damage the mouth epithelium, which is a key factor in the development of mucositis. Sometimes, the basal cell layers of epithelia are most affected, leading to the loss of epithelial renewal capacity, which may result in ulceration. Chemotherapeutic agents can also harm rapidly dividing, immature keratinocytes and stem cells (1,7-9). There are various treatments available for chemotherapy-induced oral mucositis, which can reduce the frequency and severity of the disease. However, most of these treatments are only partially effective against established lesions and do not prevent the development of new lesions. Therefore, the efficacy of these treatments is limited (6,10-18).

Elental® (EA Pharma Co., Ltd.) is an elemental diet (ED) that includes a high proportion of L-glutamine and has been used in Japan as a treatment for patients who are malnourished. Elental® is cost-effective, clinically safe, and easily digestible. It contains a mixture of amino acids, carbohydrates, vitamins, minerals, and a small amount of fat (19,20). Elental® has been

successfully used in the treatment of Crohn's disease (21-24), as well as in the management of chemotherapy-induced mucositis in patients with cancer (25,26). Previous clinical studies revealed the efficacy of Elental® for the treatment of chemotherapy-induced oral mucositis in patients with oral cancer (27,28). In addition, Elental® may accelerate the healing process of 5-FU-induced oral mucositis and dermatitis by promoting the production of fibroblast growth factor and by suppressing the expression of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) via the suppression of NF-κB in keratinocytes (29,30). However, the exact mechanism of its action remains unknown.

It is assumed that any nutritional supplement taken by patients with oral cancer might not only provide an extra source of nutrition for healthy (non-cancerous) cells but also for cancer cells, which could promote the development of cancer (31,32). Therefore, it is essential to clarify whether the beneficial effect of an ED is sufficient to negate its possible harmful effects in patients with oral cancer who are suffering from mucositis or dermatitis.

In the present study, the effects of Elental® were compared between human oral keratinocytes (HOK) and human oral squamous cell carcinoma (OSCC) cell lines and the effectiveness of Elental® in the treatment of malnourished or damaged cells was investigated. In addition, the mechanism of action of Elental® was analyzed in different cell types, specifically healthy HOK and OSCC cell lines.

Materials and methods

Cell lines and cell culture. Human oral keratinocyte cell line, HOK, was purchased from ScienCell Research Laboratories, and cells of the human OSCC cell lines, HSC2, HSC3 and HSC4 were purchased from Cell Bank, Riken BioResource Center. HOK cells were cultured in Oral Keratinocyte Medium-New Zealand Origin BPE medium (OKM-NZ, cat. no. 2611-NZ), containing OKM basal medium supplemented with 1% oral keratinocyte growth supplement, New Zealand Origin BPE (OKGS-NZ, cat. no. 2652NZ) and 1% penicillin/streptomycin solution (cat. no. 0503), referred to hereafter as complete medium for HOK. HSC2, HSC3 and HSC4 cells were cultured in Dulbecco's modified Eagle's medium (D-MEM)/Ham's F-12 (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml streptomycin/100 U/ml penicillin (Thermo Fisher Scientific), which will be referred to as complete medium for OSCC cells. All the cells were cultured and maintained in a humidified atmosphere containing 5% CO_2 at 37˚C.

Cell proliferation assay. Cells ($5x10^3$ cells per well) were seeded in 96-well plates (Becton-Dickinson Labware) in OKM-NZ basal medium with 1% OKGS-NZ (complete medium for HOK) or D-MEM/Ham's F-12 medium with 10% FBS (complete medium for HSC2 cells). The next day, for HOK cells, the above medium was replaced with OKM basal medium containing 0% OKGS-NZ growth supplement (low nutrition medium), OKM basal medium with 1% OKGS-NZ growth supplement (complete medium), or OKM basal medium containing 1% OKGS-NZ and 5-FU (final concentration 2 μg/ml). Similarly, for OSCC cells, the medium was replaced with D-MEM/Ham's F-12 medium with 0% or 1% FBS (low nutrition medium), or D-MEM/Ham's F-12 with 10% FBS plus 5-FU (2 μg/ml). After 24 h, the cells were treated with different concentrations of Elental® (0, 0.1, 0.5, 1, 5, 10, 50 and 100 μg/ml), which was dissolved in OKM basal medium containing 0% OKGS-NZ (low nutrition medium) or 1% OKGS-NZ (complete medium), or D-MEM/Ham's F-12 medium with 0% or 1% FBS (low nutrition medium). After 24 h, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 25 μl/well) was added to the 96-well plates and incubated for 4 h at 37˚C. Next, the culture medium containing MTT was removed and replaced with dimethyl sulfoxide (100 μl/well), and the absorbance was measured using a spectrophotometer (BioRad Laboratories) at 490 nm. Growth stimulating effects were compared among the treatment groups. All assays were run in triplicate.

Another protocol for MTT was used to determine the effect of Elental® sequential treatment with anticancer agents on OSCC cell proliferation. Cells ($5x10^3$ cells per well) were seeded in 96-well plates (Becton-Dickinson Labware) in D-MEM/Ham's F-12 supplemented with 10% FBS. After 24 h, the cells were subjected to single or sequential treatments with Elental® (5.0 μg/ml), 5-FU (1.0 μg/ml) and/or DOC (docetaxel; 1.0 ng/ml). In case of untreated controls, the cells were cultured in D-MEM/Ham's F-12 supplemented with 10% FBS for 48 h without any treatment. In case of single treatments, the cells were cultured without any treatment; i.e., no treatment (trt.) for 24 h followed by Elental® (No trt.→ Ele), or 5-FU (No trt.→ 5-FU), or DOC (No trt.→ DOC) for 24 h. In case of sequential treatments, the cells were treated with Elental® for 24 h followed by 5-FU for 24 h (Ele → 5-FU), Elental® for 24 h followed by DOC for 24 h (Ele → 5-FU), 5-FU for 24 h followed by Elental® for 24 h (5-FU→ Ele), or DOC for 24 h followed by Elental® for 24 h (DOC→ Ele). D-MEM/Ham's F-12 supplemented with 10% FBS was used with 5-FU or DOC; and D-MEM/Ham's F-12 supplemented with 0% FBS was used with Elental®. Then, MTT was added followed by DMSO, and the absorbance was measured using the same procedure described above. All assays were run in triplicate.

Cell migration assay. Cell migration assays were performed using a Boyden chamber, according to the manufacturer's instructions (Neuro Probe). Briefly, 25 μl OKM basal medium (containing 0 or 1% OKGS-NZ) or D-MEM/Ham's F-12 (with 0 or 10% FBS) plus different concentrations of Elental® (0, 0.1, 0.5, 1, 5, 10, 50 and 100 μg/ml) was added to the lower chamber as a chemoattractant. Next, 50 μl OKM basal medium with 0% OKGS-NZ or D-MEM/Ham's F-12 medium with 0% FBS containing $5x10^3$ cells was seeded on a gelatin-coated polycarbonate membrane in the upper chamber. The cells were incubated for 24 h at 37˚C in a 5% CO_2 atmosphere, then the membrane was washed with PBS, and the cells on the top surface were removed using a cotton swab. Cells adhering to the lower surface were fixed with methanol, stained with hematoxylin solution, and counted under a microscope in five predetermined fields (magnification, x200). All assays were independently repeated at least three times.

Whole transcriptome analysis. Total RNA was isolated from samples using an RNeasy Mini Kit (Qiagen). The isolated RNA was examined with an Agilent 2100 Bioanalyzer (Agilent) using an RNA 6000 Nano Kit (Agilent), once the concentrations had been determined using a Qubit (Thermo Fisher Scientific). The RNA integrity number (RIN) values were >7 for all samples, indicating that the samples contained high-quality RNAs. The expression libraries were produced using an Ion Ampliseq Transcriptome Human Gene Expression kit (Thermo Fisher Scientific). Total RNAs (70 ng) were reverse-transcribed to cDNAs and then amplified using the primer set of the Ion Amplicon Transcriptome Human Gene Expression Core Panel under the following PCR conditions: 11 cycles of denaturation for 15 sec at 99˚C, then annealing and extension for 16 min at 60˚C. Barcodes were inserted into the amplicons and adaptor sequences were inserted into both ends using an Ion Barcode Adaptor 17-32 Kit (Thermo Fisher Scientific). The libraries were amplified by PCR reactions of five cycles of denaturation for 15 sec at 98˚C and then annealing and extension for 1 min at 64˚C. The products were determined using a quantitative PCR method, and the qualities were confirmed with a Bioanalyzer (Agilent). Following emulsion PCR using the Ion Chef system (Thermo Fisher Scientific), the sequence reaction was carried out using Ion Proton with an Ion PI Hi-Q Chef Kit and an Ion PI Chip Kit v3 BC (Thermo Fisher Scientific); approximately 93 million reads and 10.5 gigabases were detected from the reaction. The reads were trimmed and mapped with Torrent Suit (Thermo Fisher Scientific) using hg19 AmpliSeq Transcriptome ERCCv1 as the reference sequences. The mapping ratio was >99.7%, and the read count in each sample was >10 million.

Ingenuity network analysis. The gene sets significantly increased or decreased by co-relation analysis using Prism8 were subjected to a network analysis using Ingenuity Pathways Analysis software (IPA version 8.6; Qiagen). IPA indicates molecular and cellular functions and canonical pathways on the basis of data from millions of molecular interactions reported in the literature; this software is updated weekly. IPA uses Fisher's exact test to determine whether the input genes are significantly related to pathways by comparison with the entire ingenuity knowledge base.

Western blotting. Cells ($2.0x10^6$ cells in a 100-mm dish) were treated with different concentrations of Elental® (0, 0.1, 0.5, 1, 5, 10, 50 and 100 μg/ml) dissolved in OKM basal medium containing 0% OKGS-NZ or D-MEM/Ham's F-12 medium with 0% FBS. For time-dependent experiments, the same number of cells were treated with 5 μg/ml Elental® dissolved in OKM basal medium containing 0% OKGS-NZ or D-MEM/Ham's F-12 medium with 0% FBS for 0, 12, 24, 36, 48 or 60 h. The cells were lysed with RIPA (radioimmunoprecipitation) buffer (Thermo Fisher Scientific). Whole-cell lysates were subjected to electrophoresis on 10% SDS-polyacrylamide gels (Thermo Fisher Scientific) and then transferred to a PVDF membrane (Thermo Fisher Scientific). After blocking, the membranes were incubated at 4˚C overnight with anti-ERK rabbit polyclonal antibody (dilution 1:500, cat. no. 4695; Cell Signaling Technology), anti-p-ERK rabbit polyclonal antibody (dilution 1:250, cat. no. 9101; Cell Signaling Technology), anti-BiP mouse monoclonal antibody (dilution 1:500, cat. no. 66574-1-Ig; Proteintech Group, Inc.), anti-GRP94 rabbit polyclonal antibody (dilution 1:250, cat. no. 20292; Cell Signaling Technology, Inc.), or anti-α-tubulin mouse monoclonal antibody (dilution 1:500, cat. no. sc-5286; Santa Cruz Biotechnology, Inc.). Then the membranes were washed with a buffer and incubated with Novex® alkaline-phosphatase conjugated (goat) anti-rabbit immunoglobulin G (IgG) secondary antibody (no dilution, cat. no. WB20007; Thermo Fisher Scientific) or (goat) anti-mouse IgG secondary antibody (no dilution, cat. no. WB20006; Thermo Fisher Scientific). The antibodies were detected using a chromogenic immunodetection system, WesternBreeze (Thermo Fisher Scientific), according to the manufacturer's instructions. Quantification of protein bands was performed using ImageJ v1.51h software available at http://rsb.info.nih.gov/ij/. The fold change of expression of each protein of interest was calculated relative to the internal control and expressed as a percentage.

TUNEL (terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling) assay. To detect apoptotic cells, a TUNEL assay was performed by labeling 3'-OH DNA ends generated by DNA fragmentation. Cells ($5x10^3$ cells per well) were seeded on chamber slides (Iwaki & Co., Ltd.) in D-MEM/Ham's F-12 containing 10% FBS. After incubation at 37˚C for 24 h, the cells were subjected to single or sequential treatments with Elental® (5.0 μg/ml), 5-FU (1.0 μg/ml) and/or DOC (docetaxel; 1.0 ng/ml). In case of untreated controls, the cells were cultured in D-MEM/Ham's F-12 supplemented with 10% FBS for 48 h without any treatment. In case of single treatments, the cells were cultured without any treatment for 24 h followed by Elental® (No trt.→ Ele), or 5-FU (No trt.→ 5-FU), or DOC (No trt.→ DOC) for 24 h. In case of sequential treatments, cells were treated with Elental® for 24 h followed by 5-FU for 24 h (Ele→ 5-FU) or DOC for 24 h (Ele→ 5-FU), 5-FU for 24 h followed by Elental® for 24 h (5-FU→ Ele), or DOC for 24 h followed by Elental® for 24 h (DOC→ Ele). D-MEM/Ham's F-12 supplemented with 10% FBS was used with 5-FU or DOC; and D-MEM/Ham's F-12 supplemented with 0% FBS was used with Elental®.

The treated cells were washed twice with phosphate-buffered saline (PBS), air dried, and fixed in 4% paraformaldehyde at room temperature for 30 min. The TUNEL assay was performed using a DeadEnd™ Colorimetric TUNEL System according to the manufacturer's instructions (Promega Corporation). Briefly, the cells on the cover glass were immersed in 0.2% Triton® X-100 in PBS for 5 min. After being washed with PBS, the cells were incubated with equilibration buffer (0.05 M phosphate buffer containing 0.145 M sodium chloride, pH 7.4) and then Tdt enzyme in a humidified chamber at 37˚C for 90 min. The cells were subsequently put into pre-warmed working strength stop wash buffer for 15 min. After being rinsed in PBS, the cells were incubated with antidigoxigenin-peroxidase conjugate for 30 min. Peroxidase activity in each cell was demonstrated by the application of 3,3'-diaminobenzidine. The number of TUNEL-positive cells (apoptotic cells) was counted under a microscope in three random fields, and the result was expressed as a percentage of TUNEL-positive cells.

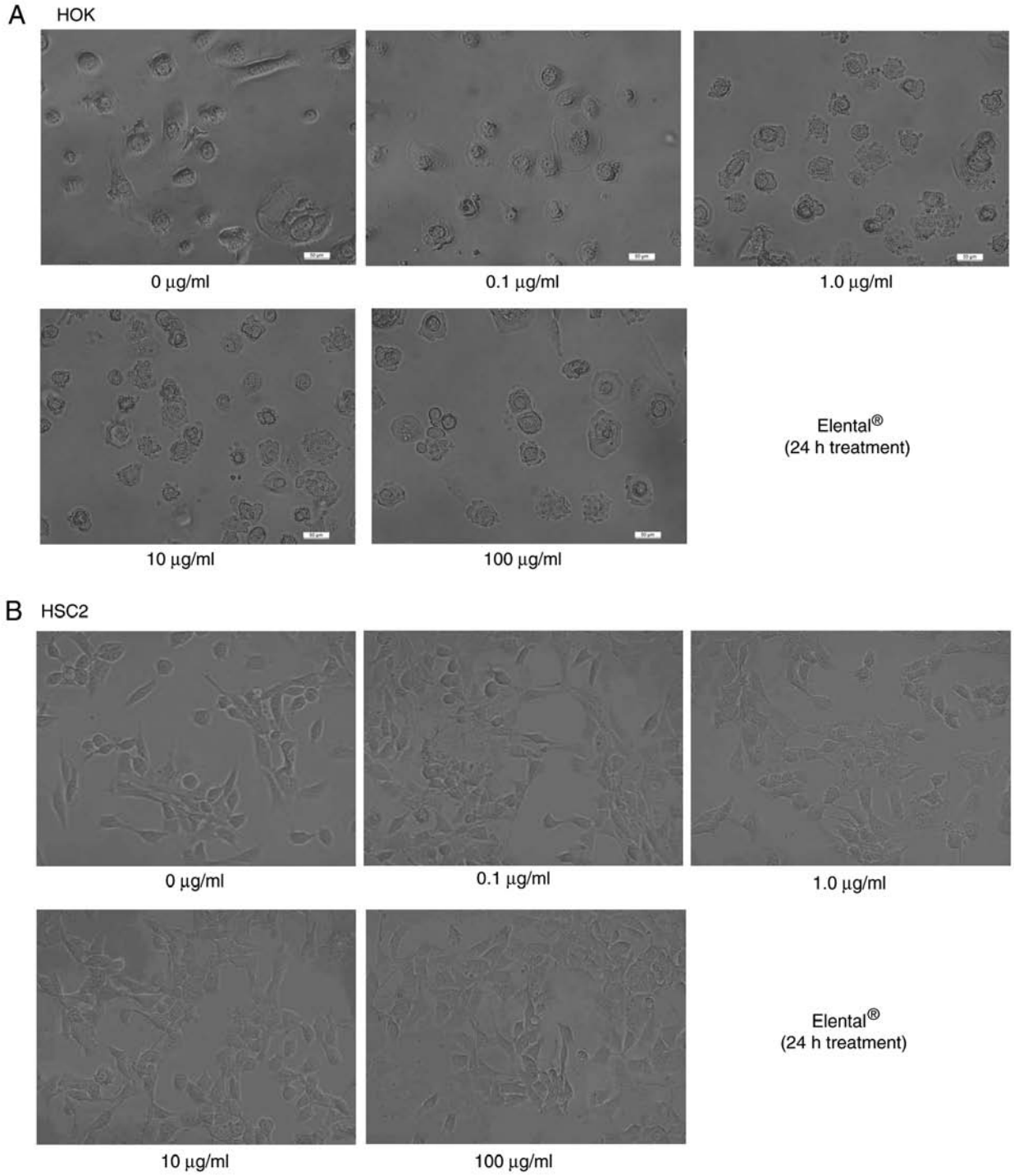

Figure 1. Effect of Elental® on cell morphology. (A) Untreated HOK and Elental®-treated HOKs showed a similar round morphology. (B) The morphology of Elental®-treated HSC2 cells was slightly different from untreated HSC2 cells. Elental® did not markedly affect the morphology of HOK or HSC2 cells.

Statistical analysis. Data were expressed as means ± SD. The significance of the experimental results was determined using one-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparison tests. The significance of the TUNEL assay data was determined by Mann-Whitney U test. The differences were considered statistically significant when $P<0.05$. The datasets measured by whole transcriptome analysis were normalized by each transcripts per million (TPM) in GAPDH and were analyzed using a correlation assay and principal component analysis.

Results

Effect of Elental® on cell morphology. We did not observe any differences between the morphology of untreated HOK and Elental®-treated HOK. As shown in Fig. 1A, the majority of cells in the two groups exhibited the same round shape. There was a slight difference between the morphology of untreated HSC2 and Elental®-treated HSC2 cells. Briefly, Elental® treatment increased granular vesicles in cytoplasm slightly (Fig. 1B). Essentially, however, Elental® had almost no

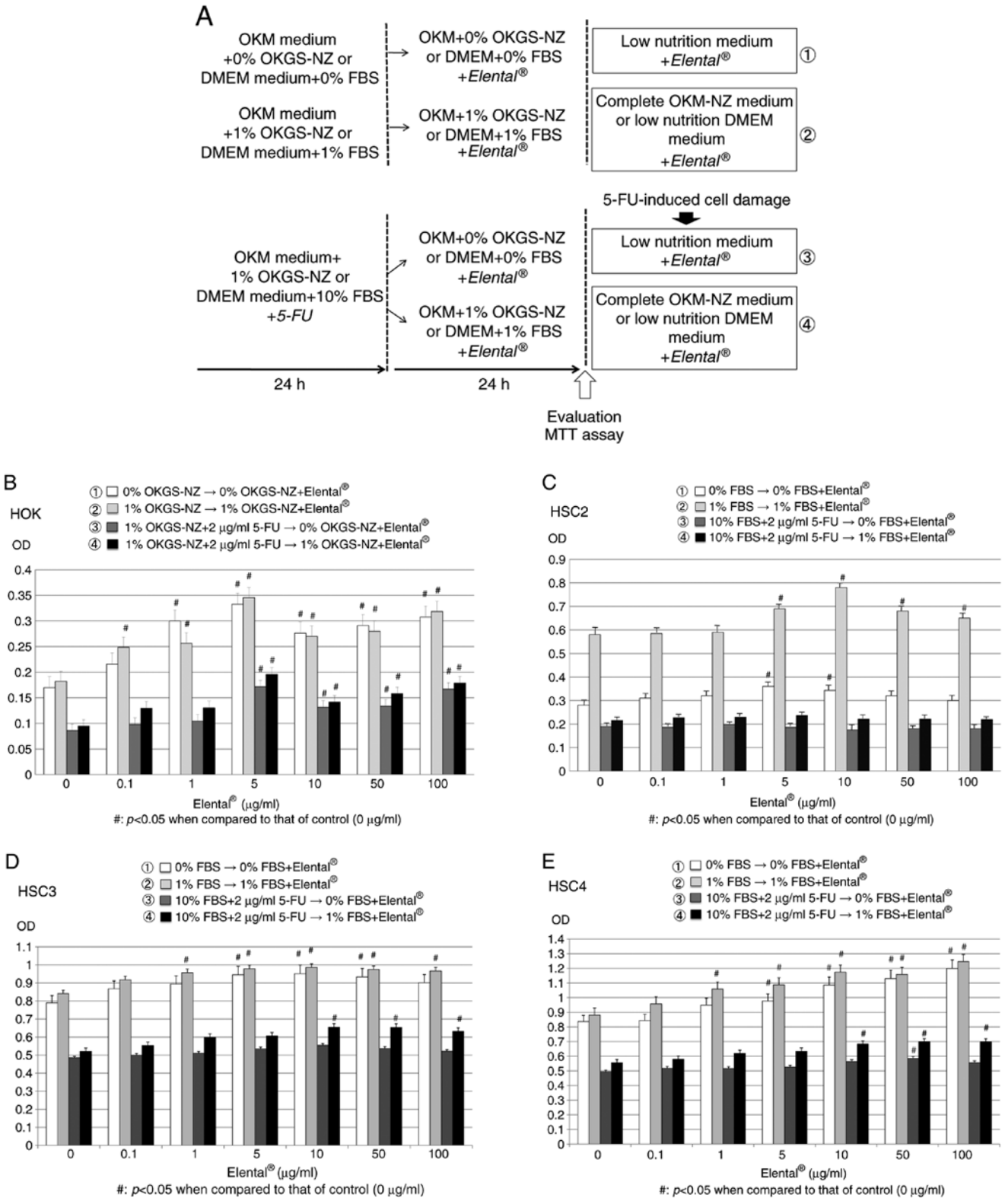

Figure 2. Effect of Elental® on cell proliferation. (A) Experimental methodology of the MTT assay. Twenty-four hours after cell seeding, HOK were cultured in OKM basal medium containing 0% OKGS-NZ (low nutrition medium) or 1% OKGS-NZ growth supplement (complete medium), or 1% OKGS-NZ and 2 μg/ml 5-FU; HSC2 cells were cultured in D-MEM/Ham's F-12 medium with 0% or 1% FBS (low nutrition medium), or D-MEM/Ham's F-12 with 10% FBS plus 5-FU (2 μg/ml). Then, both 5-FU-treated and -untreated HOK cells were cultured in OKM basal medium containing 0% OKGS-NZ (low nutrition medium) or 1% OKGS-NZ growth supplement (complete medium) plus different concentrations of Elental® (0, 0.1, 0.5, 1, 5, 10, 50 and 100 μg/ml). Similarly, 5-FU-treated and -untreated OSCC cells were cultured in D-MEM/Ham's F-12 medium with 0% or 1% FBS (low nutrition medium). After 24 h, the proliferation ability of the cells was evaluated by an MTT assay. (B) Elental® exerted a growth-stimulating effect on HOK even under poor nutritional conditions (OKM basal medium containing 0% OKGS-NZ) or in damaged HOK (5-FU-treated HOK). (C) Elental® improved the growth ability in malnourished HSC2 cells under poor nutritional conditions (D-MEM/Ham's F-12 medium with 0% or 1% FBS), but did not affect the growth ability of 5-FU-damaged HSC2 cells. (D and E) Elental® (1-100 μg/ml) improved the growth rate of HSC3 and HSC4 in poor nutritional condition, and only D-MEM/Ham's F-12 medium with 1% FBS Elental® (10-100 μg/ml) could increase the growth rate of 5-FU-treated HSC3 and HSC4, but medium with 0% FBS could not. #P<0.05 when compared with the control (one-way analysis of variance and Tukey-Kramer multiple comparisons tests). 5-FU, 5-fluorouracil.

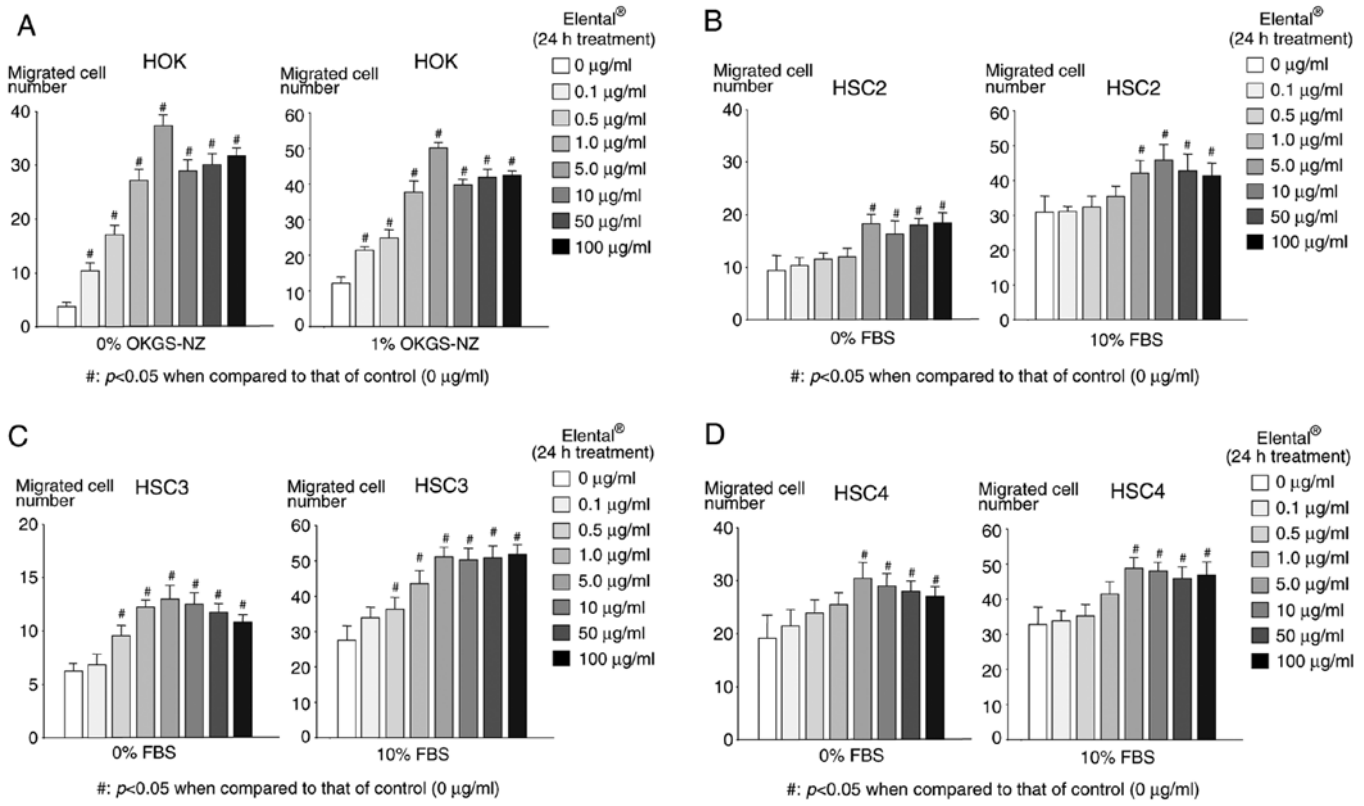

Figure 3. Effect of Elental® on cell migration. (A) Elental® stimulated the ability of HOKs to migrate, regardless of the nutritional conditions. (B) Elental® also improved the ability of HSC2 cells to migrate. However, a low concentration of Elental® could not improve the ability of HSC2 cells to migrate in either nutritional condition. (C) The migration activity of Elental® (1.0-100 μg/ml)-treated HSC3 cells was significantly higher than untreated cells, both in poor nutritional conditions (medium with 0% FBS) and substantial nutrient conditions (medium with 10% FBS). (D) Elental® (5.0-100 μg/ml)-treated HSC3 cells showed higher migration ability than untreated cells regardless of the nutritional conditions. #P<0.05 when compared to that of control (0 μg/ml).

effect on the morphology of HOK or HSC2 cells. Similarly, the morphology of HSC3 and HSC4 cells remained almost unchanged after Elental® treatment (data not shown).

Elental® affects the cell proliferation ability of HOKs differently to that of OSCC cell lines. MTT assays were used to measure the growth rate of Elental®-treated and untreated HOKs or OSCC cell lines (HSC2, HSC3 and HSC4). Fig. 2A summarizes the experimental methodology used for the MTT assay. Briefly, healthy cells or 5-FU-damaged cells were treated with Elental® dissolved in low-nutrition culture medium (OKM basal medium containing 0% OKGS-NZ, or D-MEM/Ham's F-12 with 0 or 1% FBS medium) or in complete medium (OKM basal medium containing 1% OKGS-NZ).

Both in nutritionally poor conditions (medium with 0% OKGS-NZ growth supplement) and substantial nutrient conditions (complete medium with 1% OKGS-NZ), the growth rate of Elental® (0.1-100 μg/ml)-treated HOK was significantly higher than that of untreated HOK. In addition, Elental® (5-100 μg/ml) could stimulate the proliferation of 5-FU (2 μg/ml)-pretreated HOK even under poor nutritional conditions (medium with 0% OKGS-NZ). Elental® improves cell growth ability in malnourished or damaged HOK (Fig. 2B).

In the case of HSC2 cells, the growth rate of Elental® (5-100 μg/ml)-treated HSC2 cells was higher than that of untreated HSC2 cells after 48 h in poor nutritional conditions (medium with 0 or 1% FBS). However, Elental® (0.1-100 μg/ml) did not significantly affect the rate of proliferation of 5-FU (2 μg/ml)-pretreated damaged HSC2 cells in poor nutrition conditions. In short, Elental® improved the growth ability of malnourished HSC2 cells, but did not affect growth ability in damaged HSC2 cells (Fig. 2C). On the other hand, the growth rates of Elental® (1-100 μg/ml)-treated HSC3 and HSC4 were higher than that of untreated HSC3 and HSC4 at 48 h in poor nutritional condition (medium with 0-1% FBS). In addition, Elental® (10-100 μg/ml) could increase the growth rate of 5-FU (2 μg/ml)-pretreated HSC3 and HSC4 only in medium with 1% FBS (poor nutritional condition), whereas the medium with 0% FBS could not. In short, a high dosage of Elental® significantly improved cell growth ability in malnourished or damaged HSC3 and HSC4; however, the proliferation rate was still low compared to HOK cells in poor nutritional condition (Fig. 2D and E).

Elental® has different effects on the ability of HOK and OSCC cell lines to migrate. The migration activity of Elental®-treated HOK and OSCC cells was measured using a Boyden chamber. Fig. 3A shows that Elental® (0.1-100 μg/ml)-treated HOK had a significantly higher migration ability compared with that of untreated HOK, regardless of the nutritional conditions, with a concentration of 5 μg/ml Elental® showing the most noticeable effect on migration. In the case of HSC2 and HSC4, the migration activity of Elental® (5.0-100 μg/ml)-treated cells was significantly higher compared with that of untreated HSC2

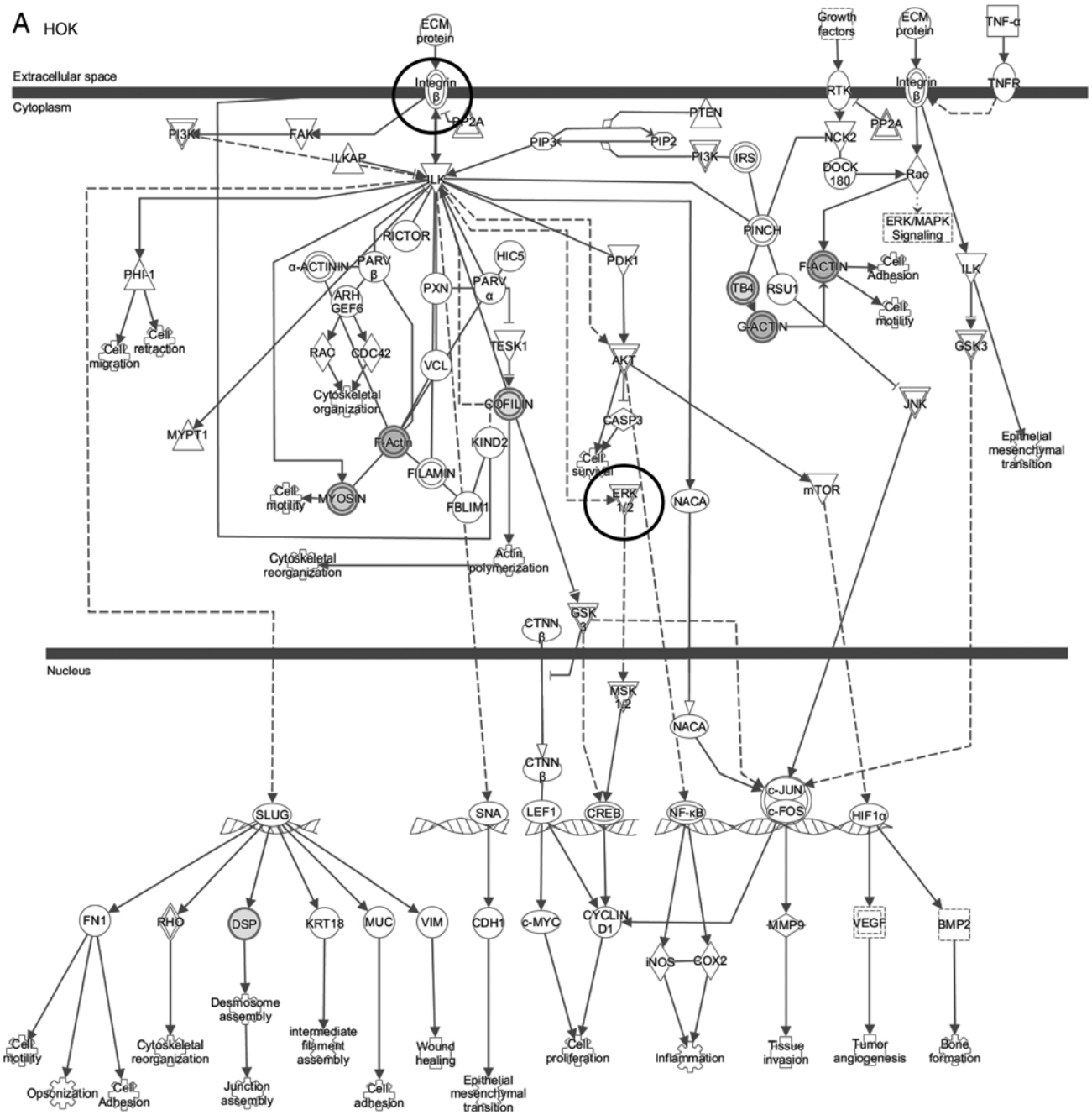

Figure 4. Pathway analysis by IPA was performed using the differentially expressed genes identified in Elental® (5 μg/ml)-treated cells versus untreated cells. (A) A pathway via the integrin-mediated activation of *ERK* was detected in the first pathway of comparison between Elental® 12 h-treated HOK and untreated HOK (0 h). IPA, Ingenuity Pathways Analysis; HOK, human oral keratinocyte.

or HSC4 cells, both in poor nutritional conditions (medium with 0% FBS) and substantial nutrient conditions (complete medium with 10% FBS) (Fig. 3B and D). Similar results were observed in the case of Elental® (1.0-100 μg/ml)-treated HSC3 cells, both in poor nutritional and substantial nutrient conditions (Fig. 3C). Briefly, Elental® improved the migration ability of HOK and OSCC cell lines, although low concentrations of Elental® (0.1-0.5 μg/ml) did not improve the migration ability of OSCC cell lines, regardless of the nutritional condition.

Mechanism of action of Elental® in HOK and HSC2 cells. Our cell proliferation and migration assay results showed that Elental® affected the growth and migration ability of HOK more effectively than that of OSCC cell lines, particularly HSC2 cells. To clarify the reasons behind this difference in the action of Elental® in HOK and HSC2 cells, we examined the mechanism of action of Elental® in these two cell types. Whole transcriptome analysis was performed followed by network analysis (IPA) using differentially expressed genes identified between Elental® (5 μg/ml)-treated cells and untreated cells. We supplied the results of Heatmap and Whole transcriptome analysis as supplement data (Fig. S1). An interesting pathway was found in the first pathway of comparison between Elental® HOK treated for 12 h and untreated HOK (0 h), i.e., a pathway via the integrin-mediated activation of ERK (Fig. 4A). In addition, *ERK* may be one of the genes of focus as detected from

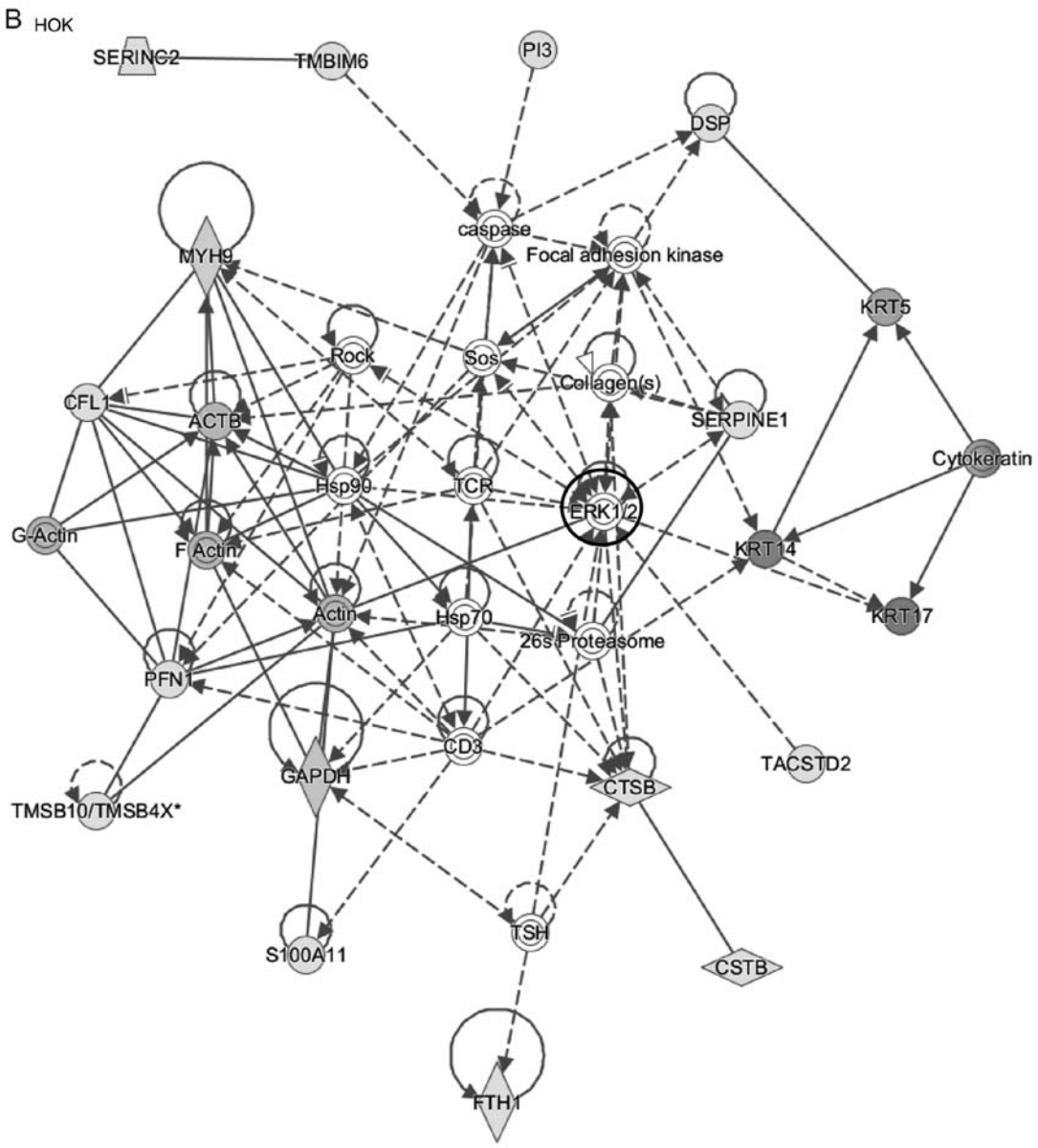

Figure 4. Continued. Pathway analysis by IPA was performed using the differentially expressed genes identified in Elental® (5 μg/ml)-treated cells versus untreated cells. (B) *ERK* could be the gene of focus as found from the first network analysis when comparing Elental® 12 h-treated HOKs and untreated HOK. IPA, Ingenuity Pathways Analysis; HOK, human oral keratinocyte.

the first network analysis comparing HOK treated for 12 h with Elental® and untreated HOK (Fig. 4B). An interesting pathway was also found in the first pathway of comparison between HSC2 cells treated for 12 h with Elental® and untreated HSC2 cells. This pathway showed the possibility of activation of endoplasmic reticulum response marker genes, including *BiP* and *GRP94*, which may lead to the induction of apoptosis (Fig. 4C). The activation of *HSP*, as well as *BiP*, *GRP94* and other genes, was also found further downstream, which suggests that Elental® treatment could be involved in the induction of apoptosis by adding a great deal of stress to HSC2 cells (Fig. 4C). Furthermore, *Akt* and *HSP* may be genes of focus as indicated from the first network analysis data when comparing HSC2 cells treated for 12 h with Elental® and untreated HSC2 cells. This suggests that Elental® treatment may not only cause stress but also partially activate survival signals (Fig. 4D).

Expression of ERK in Elental®-treated cells. To confirm the results of whole transcriptome analysis and IPA, we examined the expression of ERK in HOK and HSC2 cells using western blot analysis. Fig. 5 shows that Elental® (50-100 μg/ml) enhanced the expression of p-ERK in HOK compared with its expression in untreated HOK. On the other hand, we could only detect faint bands of p-ERK in Elental® (1.0-5.0 μg/ml)-treated HSC2 cells. Although the p-ERK expression was a bit higher in treated cells than in untreated HSC2 cells, the expression level of p-ERK/ERK in HSC2 was low compared with its expression level in HOK (Fig. 5). Briefly, Elental® induced p-ERK expression in HOK, but not in HSC2 cells.

Expression of BiP and GRP94 in Elental®-treated cells. Western blot analysis was used to examine the expression of BiP and glucose-regulated protein 94 (GRP94) in HSC2 cells and further check the results of the whole transcriptome

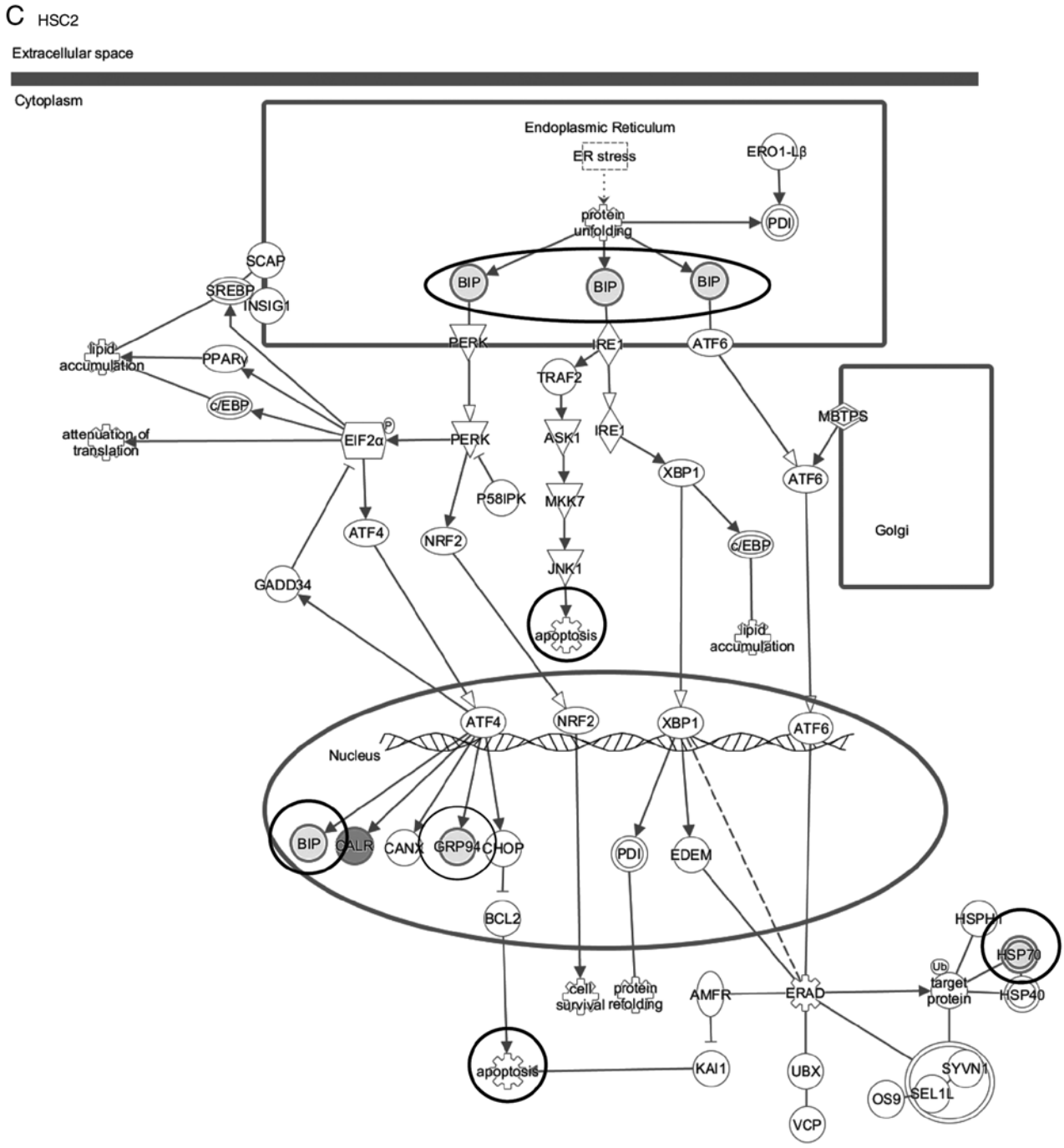

Figure 4. Continued. Pathway analysis by IPA was performed using the differentially expressed genes identified in Elental® (5 μg/ml)-treated cells versus untreated cells. (C) The pathway of comparison between Elental® 12-h treated HSC2 cells and untreated HSC2 cells (0 h) showed the possible activation of endoplasmic reticulum stress markers, including *BiP*, which may induce apoptosis. (C) Activation of *HSP*, *BiP*, *GRP94* and other genes was detected further downstream, which suggests that Elental® treatment may be involved in the induction of apoptosis by subjecting HSC2 cells to considerable stress. IPA, Ingenuity Pathways Analysis; HOK, human oral keratinocyte.

analysis and IPA. Fig. 6A shows that Elental® (1.0-100 μg/ml) enhanced the expression of BiP in HSC2 cells after 24 h of treatment than in untreated cells. Elental® (1.0-100 μg/ml) also induced BiP expression in HSC3 and HSC4 (Fig. 6B and C). Additionally, Elental® (5.0 μg/ml) enhanced the expression of BiP in treated cells after 12=60 h of treatment compared with its expression in untreated cells. Therefore, Elental® could induce BiP expression in OSCC cells.

On the other hand, 24 h treatment of Elental® (5.0-100 μg/ml) increased the expression of GRP94 in HSC2 cells than in untreated cells; whereas 12-24 h of treatment with Elental® (5.0 μg/ml) enhanced the expression of GRP94 in treated cells. Briefly, 12-24 h of Elental® treatment could induce GRP94 expression in HSC2 cells; whereas treatment >24 h could not (Fig. 7).

Sequential treatment effects of Elental® and anticancer agents on OSCC cell proliferation and apoptosis in vitro. We aimed to determine whether Elental® pre-treatment can enhance the growth-limiting and apoptosis-inducing ability of

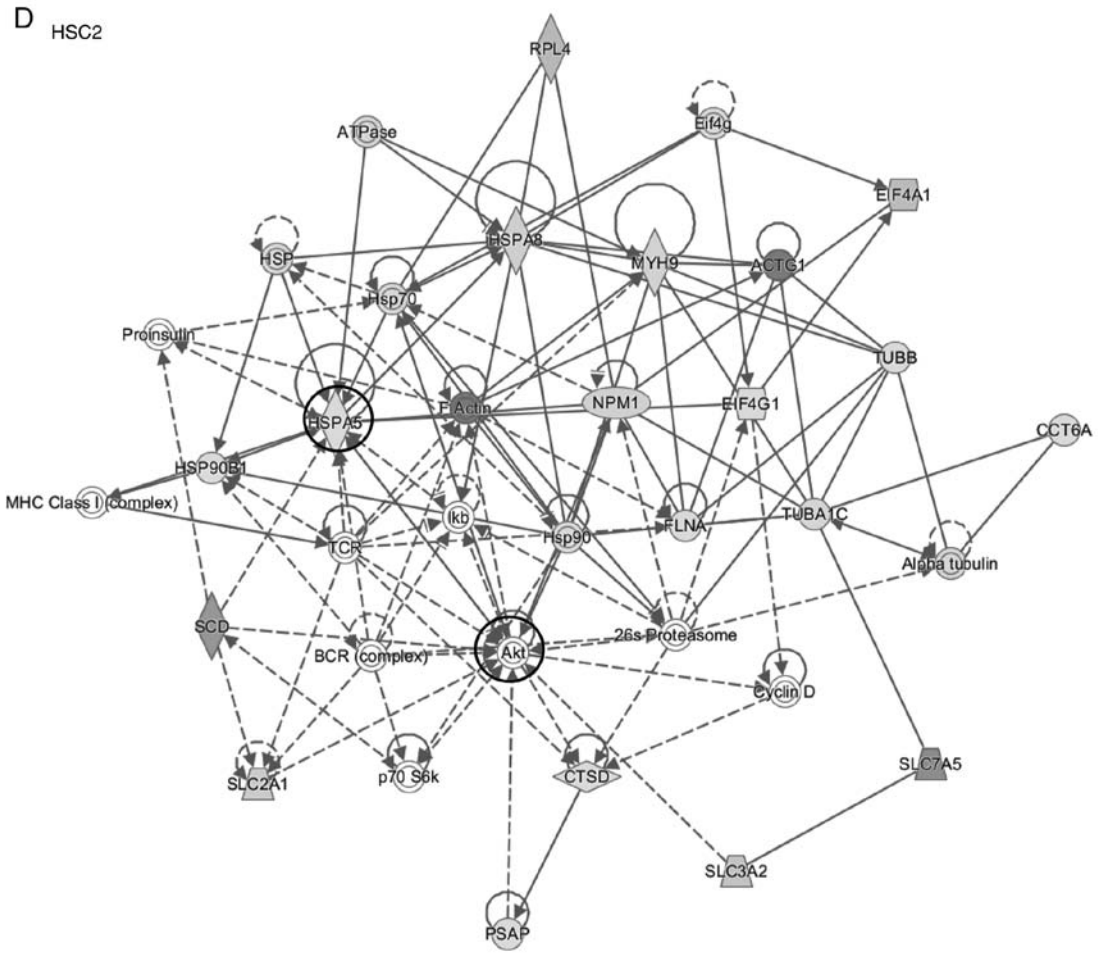

Figure 4. Continued. Pathway analysis by IPA was performed using the differentially expressed genes identified in Elental® (5 μg/ml)-treated cells versus untreated cells. (D) *Akt* and *HSP* could be the genes of focus as found from the first network analysis comparing HSC2 cells treated with Elental® for 12 h with untreated HSC2 cells, which suggests that Elental® treatment not only exerted stress but also partially induced survival signals. IPA, Ingenuity Pathways Analysis; HOK, human oral keratinocyte.

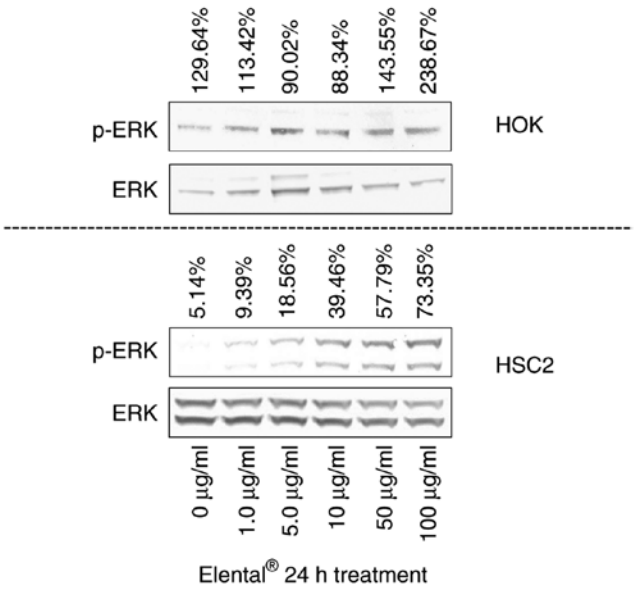

Figure 5. Expression of ERK in Elental®-treated cells. Western blot analysis revealed that Elental® enhanced the expression of p-ERK in treated HOK compared with its expression in untreated HOK. However, bands in Elental® (1.0-5.0 μg/ml)-treated HSC2 cells were faint. Although the expression in treated cells was a bit higher than in untreated HSC2 cells, the expression level of p-ERK/ERK in HSC2 was low compared with its expression level in HOK.

widely used anticancer agents or not. The growth inhibitory effect of Elental® sequential treatment with 5-FU or DOC on HSC2, HSC3 and HSC4 cells was analyzed by MTT assay. Fig. 8A summarizes the experimental methodology used for the MTT assay. As shown in Fig. 8B-D, Elental® (5.0 μg/ml) pre-treatment for 24 h followed by 5-FU (Elental®→ 5-FU) or DOC (Elental®→ DOC) for 24 h slightly inhibited cell growth in all three cells than in 5-FU (No trt.→ 5-FU) or DOC (No trt.→ DOC) alone; however, we could not find any significance. Elental® pre-treatment did not significantly increase the growth inhibitory effect of 5-FU or DOC in HSC2, HSC3 and HSC4 cells.

Therefore, we assumed that sequential or pre-treatment of Elental® may increase apoptosis-inducing ability of anticancer agents, and we performed TUNEL assay to detect DNA fragmentation and chromatin condensation in treated cells using the same experimental protocol described in Fig. 8A. As shown in Fig. 9A and B, Elental® pre-treatment significantly enhanced the apoptosis-inducing ability of 5-FU (Elental®→ 5-FU) or DOC (Elental®→ DOC) in HSC2 and HSC3 cells. In the case of HSC4 cells (Fig. 9C), Elental® pre-treatment could significantly increase apoptosis-inducing ability of 5-FU (Elental®→ 5-FU), but not DOC (Elental®→ DOC). The highest number of apoptotic cell were observed in the case

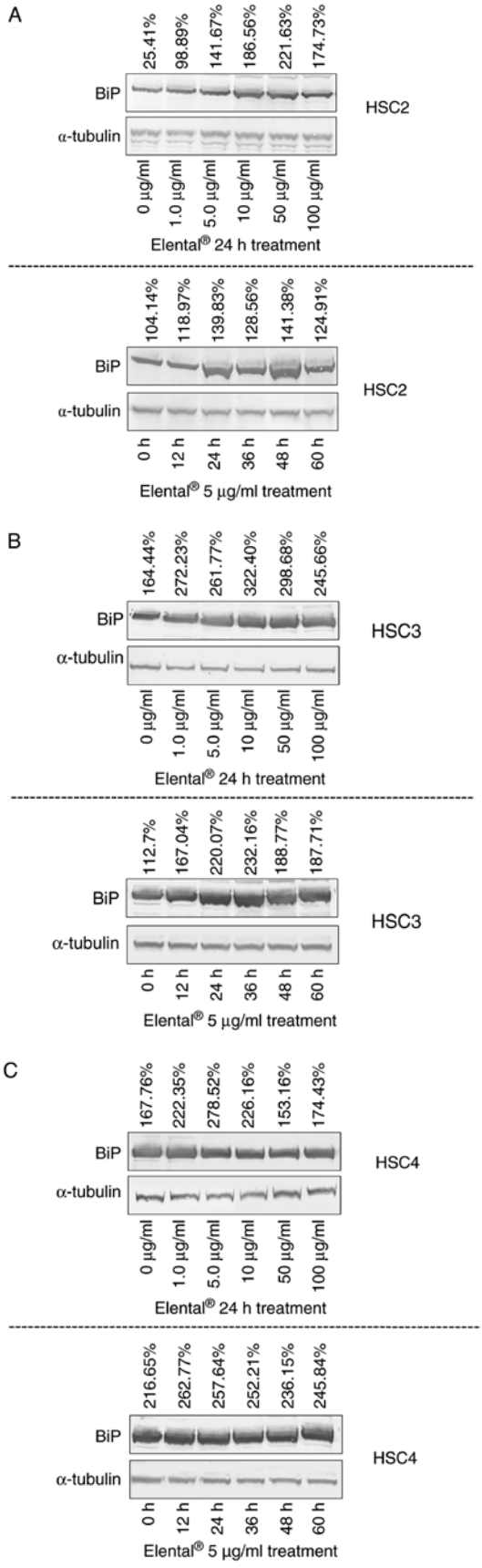

Figure 6. Expression of BiP in Elental®-treated OSCC cells. Western blot analysis revealed that Elental® (1.0-100 μg/ml) treatment enhanced the expression of BiP in OSCC cell lines compared with its expression in untreated OSCC cells. It also showed that BiP expression was enhanced in Elental® (5.0 μg/ml, 12-60 h)-treated OSCC cell lines compared with its expression in untreated cells. In summary, Elental® could induce BiP expression in all OSCC cell lines: (A) HSC2, (B) HSC3 and (C) HSC4.

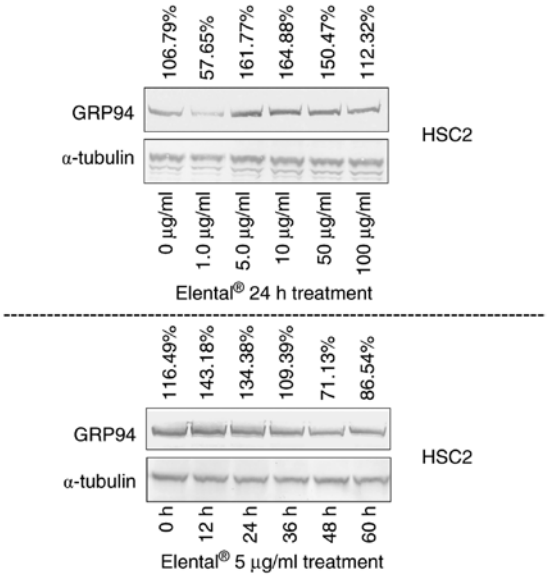

Figure 7. Expression of BiP in Elental®-treated HSC2 cells. Western blot analysis revealed that Elental® (5.0-100 μg/ml) enhanced the expression of GRP 94 in treated HSC2 cells compared to untreated HSC2 cells. Elental® (5.0 μg/ml) enhanced the expression of GRP 94 in HSC2 cells after 12-24 h of treatment.

of Elental® (5.0 μg/ml) sequential treatment for 24 h followed by 5-FU (1.0 μg/ml) for 24 h (Elental®→ 5-FU) on the three types of cells. Briefly, pre-treatment of Elental® may prompt apoptosis by anticancer agents.

Discussion

Elental® is used in Japan as a treatment for patients who are malnourished, or who have inflammatory bowel disease. Elental® has been shown to be useful in the management of chemotherapy-induced mucositis in patients with various types of cancer (7-9,23-29,33). We have also reported that Elental® reduced chemotherapy-induced oral mucositis and dermatitis in patients with OSCC (25,26). Therefore, we should consider more extensive use of Elental® for patients with cancer in the future. However, although, to the best of our knowledge, there have been no reports about the possible harmful effects of nutritional supplements or ED intake in cancer patients, we cannot exclude the possibility that an ED could act as an extra source of nutrition for cancer cells, which might facilitate cancer progression. Therefore, it is important to weigh the beneficial effects of Elental® against any possible harmful effects.

In the present study, we aimed to examine the efficacy of Elental® administration during treatment for oral cancer by comparing the action of Elental® in HOK and HSC2 cells. The aim also was to clarify whether Elental® works differently on healthy oral cells compared with its action on oral cancer cells. We observed that Elental® promoted growth and migration of malnourished and 5-FU-treated damaged HOK but did not significantly affect the proliferation of 5-FU-treated damaged HSC2 cells. On the other hand, a high dosage of Elental® (10 μg/ml) could increase the growth rate of 5-FU-treated HSC3 and HSC4 even in some poor nutritional condition (1% FBS); however, the proliferation

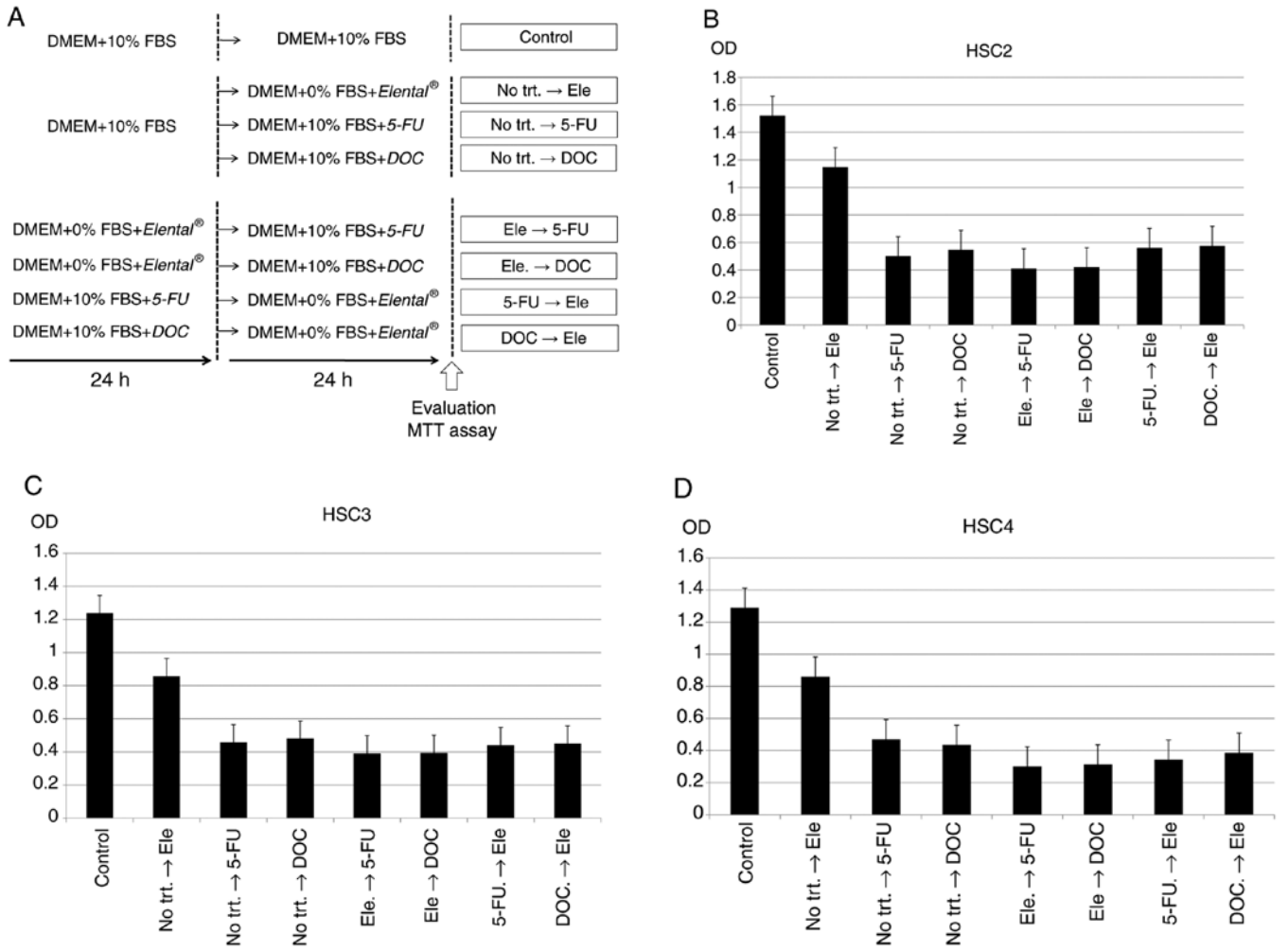

Figure 8. Sequential treatment effects of Elental® and anticancer agents on OSCC cell proliferation *in vitro*. Inhibition of cell growth was evaluated by MTT assay. (A) Experimental methodology of the MTT assay. Twenty-four hours after cell seeding, the cells were subjected to single or sequential treatments with Elental® (5.0 μg/ml), 5-FU (1.0 μg/ml) and/or DOC (docetaxel; 1.0 ng/ml). Untreated control cells were cultured for 48 h without any treatment. In case of single treatments, the cells were without any treatment for 24 h followed by Elental® (No trt.→ Ele), or 5-FU (No trt.→ 5-FU), or DOC (No trt.→ DOC) treatment for 24 h. In case of sequential treatments, the cells were treated with Elental® for 24 h followed by 5-FU for 24 h (Ele → 5-FU) or DOC for 24 h (Ele → DOC), 5-FU for 24 h followed by Elental® for 24 h (5-FU → Ele), or DOC for 24 h followed by Elental® for 24 h (DOC → Ele). (B-D) Elental® pre-treatment for 24 h followed by 5-FU (Elental® → 5-FU) or DOC (Elental® → DOC) for 24 h could slightly inhibit cell growth in OSCC cell lines compared to 5-FU (No trt.→ 5-FU) or DOC (No trt.→ DOC) alone; however Elental® pre-treatment did not significantly increase the growth inhibitory effect of these anticancer agents against (B) HSC2, (C) HSC3 and (D) HSC4. Ele, Elental®; 5-FU, 5-fluorouracil; DOC, docetaxel; No trt., no treatment.

rate was still low compared to HOK cells in poor nutritional condition (0 or 1% FBS). HOK and HSC2 were used for whole transcriptome analysis and IPA analysis, as Elental® affects the proliferation rate of the two cells differently. Extensive gene analysis using whole transcriptome analysis followed by IPA resulted in noteworthy data (Fig. 4A-D). Briefly, Elental® may play a role in the growth and survival of HOK through the integrin-mediated activation of *ERK* and may induce heat shock protein via endoplasmic reticulum stress in HSC2 cells. Crucially, under certain conditions Elental® may stress cancer cells and stimulate the growth of healthy cells. This finding could be important for patients with cancer, as it indicates that Elental® may not exert any harmful effects on healthy cells. We showed that Elental® enhanced the expression of p-ERK in HOK but not so in HSC2 cells, the expression level of p-ERK/ERK in HSC2 was low compared with its expression level in HOK (Fig. 5). Western blot analysis data also revealed that Elental® enhanced the expression of BiP in HSC2 cells compared with its expression in untreated cells (Fig. 6A). Almost identical results were observed in the case of HSC3 and HSC4 (Fig. 6B and C). From our findings, we can conclude that Elental® may accelerate wound healing and reduce oral mucositis via the integrin-mediated activation of ERK, without promoting cancer progression.

GRP94 expression was analyzed in HSC2 because it is another stress-related protein and is also involved in cellular protein metabolic process. We observed that, Elental® increased the expression of GRP94 in HSC2 cells; whereas 12-24 h of treatment with Elental® (5.0 μg/ml) showed the best results (Fig. 7). Moreover, Elental® pre-treatment could enhance the apoptosis-inducing ability of 5-FU against OSCC cell lines, although they could not exert significant growth inhibitory effects on OSCC cell lines compared to 5-FU alone (Figs. 8 and 9). Our results indicate that Elental® may add stress to HSC2 and other OSCC cells, but could provide growth stimulation to HOK.

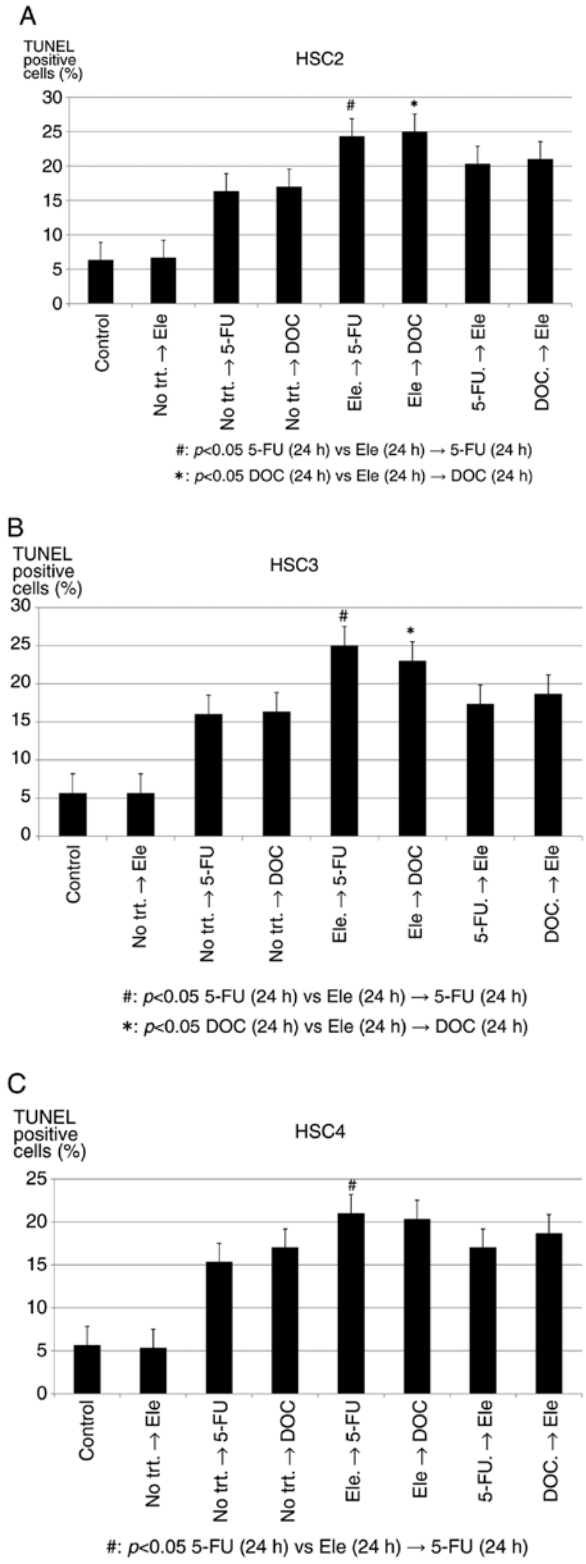

Figure 9. Sequential treatment effects of Elental® and anticancer agents on OSCC cell apoptosis *in vitro*. The experimental protocol of TUNEL was the same as the MTT assay protocol shown in Fig. 8A. (A and B) Elental® pre-treatment for 24 h followed by 5-FU (Elental®→ 5-FU) or DOC (Elental®→ DOC) significantly increased the apoptotic-inducing ability of 5-FU or DOC in (A) HSC2 and (B) HSC3 cells compared to 5-FU (No trt.→ 5-FU) or DOC (No trt.→ DOC) alone. (C) Elental® significantly increased the rate of apoptosis of 5-FU (Elental® → 5-FU) in HSC4 compared to 5-FU (No trt.→ 5-FU) alone. Error bars represent the standard deviation of the mean of TUNEL positive cells in three random fields. #P<0.01 when compared to that of 5-FU alone; *P<0.05 when compared to that of DOC alone (Mann-Whitney's U test). Ele, Elental®; 5-FU, 5-fluorouracil; DOC, docetaxel; No trt., no treatment.

It has been reported that the administration of additional nutrition during perioperative periods in patients with cancer could help with the maintenance of general health and enhance wound healing (33). According to the guidelines of the American Society for Parenteral and Enteral Nutrition (ASPEN), early enteral feeding is strongly recommended (advisability A) following surgery for digestive organ cancer (34). However, until now there has been no useful and decisive nutrition administration strategy available for patients with cancer. Currently, we have to develop nutritional supplement administration strategies based on the state of the disease in individual cancer patients, after carefully evaluating any potential beneficial or harmful effects on cancer treatment. Our findings suggest a solution to this problem through the use of an effective nutritional supplement that has no adverse side effects for patients with cancer. Amino acid-based dietary formulations such as Elental® may be able to exert an incredible healing effect on oral mucositis and dermatitis in patients with oral cancer, although this effect may vary depending on the amino-acid formulation of each particular ED. Further investigations into Elental® and other amino-acid formulations may be necessary to clarify their usefulness in the treatment of oral cancer. Moreover, in this study, the cells were directly exposed to different concentrations of Elental®. However, whether there is any positive correlation between *in vivo* absorption levels of ingredients and *in vitro* dissolution level of Elental® remains to be determined and further investigation to clarify this point in the future should be conducted.

Acknowledgements

Not applicable.

Funding

This study was supported in part by a Grant-in-Aid from the Japanese Ministry of Education, Science and Culture, grant no. 15K11292.

Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

KH and YM designed the study. KH, TF, and KW performed the experiments, analyzed the data, and wrote and revised the manuscript. YM and KM revised the manuscript and provided valuable suggestions during the study. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest and are fully responsible for the content of this paper. All authors reviewed and approved the paper.

References

1. Duncan M and Grant G: Oral and intestinal mucositis-causes and possible treatments. Aliment Pharmacol Ther 18: 853-874, 2003.
2. Burdelya LG, Gleiberman AS, Toshkov I, Aygun-Sunar S, Bapardekar M, Manderscheid-Kern P, Bellnier D, Krivokrysenko VI, Feinstein E and Gudkov AV: Toll-like receptor 5 agonist protects mice from dermatitis and oral mucositis caused by local radiation: Implications for head-and-neck cancer radiotherapy. Int J Radiat Oncol Biol Phys 83: 228-234, 2012.
3. Sonis ST: Oral mucositis. Anticancer Drugs 22: 607-612, 2011.
4. McCarthy GM, Awde JD, Ghandi H, Vincent M and Kocha WI: Risk factors associated with mucositis in cancer patients receiving 5-fluorouracil. Oral Oncol 34: 484-490, 1998.
5. Miyano K, Ueno T, Yatsuoka W and Uezono Y: Treatment for cancer patients with oral mucositis: Assessment based on the mucositis study group of the multinational association of supportive care in cancer in international society of oral oncology (MASCC/ISOO) in 2013 and proposal of possible novel treatment with a Japanese herbal medicine. Curr Pharm Des 22: 2270-2278, 2016.
6. Lalla RV, Sonis ST and Peterson DE: Management of oral mucositis in patients who have cancer. Dent Clin North Am 52: 61-77, 2008.
7. Skubitz KM: Glutamine as a potential treatment for the prevention of chemotherapy induced mucositis. J Infusional Chemotherapy 4: 64-67, 1994.
8. Kyllo RL and Anadkat MJ: Dermatologic adverse events to chemotherapeutic agents, part 1: Cytotoxics, epidermal growth factor receptors, multikinase inhibitors, and proteasome inhibitors. Semin Cutan Med Surg 33: 28-39, 2014.
9. Shou J, Lieberman MD, Hofmann K, Leon P, Redmond HP, Davies H and Daly JM: Dietary manipulation of methotrexate-induced enterocolitis. JPEN J Parenter Enteral Nutr 15: 307-312, 1991.
10. Campos MI, Campos CN, Aarestrup FM and Aarestrup BJ: Oral mucositis in cancer treatment: Natural history, prevention and treatment. Mol Clin Oncol 2: 337-340, 2014.
11. Keefe DM, Schubert MM, Elting LS, Sonis ST, Epstein JB, Raber-Durlacher JE, Migliorati CA, McGuire DB, Hutchins RD, Peterson DE, *et al*: Updated clinical practice guidelines for the prevention and treatment of mucositis. Cancer 109: 820-831, 2007.
12. Peterson DE, Bensadoun RJ and Roila F; ESMO Guidelines Working Group: Management of oral and gastrointestinal mucositis: ESMO clinical recommendations. Ann Oncol 20 (Suppl 4): S174-S177, 2009.
13. Quinn B, Potting CM, Stone R, Blijlevens NM, Fliedner M, Margulies A and Sharp L: Guidelines for the assessment of oral mucositis in adult chemotherapy, radiotherapy and haematopoietic stem cell transplant patients. Eur J Cancer 44: 61-72, 2008.
14. Henke M, Alfonsi M, Foa P, Giralt J, Bardet E, Cerezo L, Salzwimmer M, Lizambri R, Emmerson L, Chen MG and Berger D: Palifermin decreases severe oral mucositis of patients undergoing postoperative radiochemotherapy for head and neck cancer: A randomized, placebo-controlled trial. J Clin Oncol 29: 2815-2820, 2011.
15. Bensinger W, Schubert M, Ang KK, Brizel D, Brown E, Eilers JG, Elting L, Mittal BB, Schattner MA, Spielberger R, *et al*: NCCN Task force report. Prevention and management of mucositis in cancer care. J Natl Compr Canc Netw 6 (Suppl 1): S1-S21, quiz S22-S24, 2008.
16. Svanberg A, Ohrn K and Birgegard G: Oral cryotherapy reduces mucositis and improves nutrition-a randomised controlled trial. J Clin Nurs 19: 2146-2151, 2010.
17. Scully C, Epstein J and Sonis S: Oral mucositis: A challenging complication of radiotherapy, chemotherapy, and radiochemotherapy. Part 2: Diagnosis and management of mucositis. Head Neck 26: 77-84, 2004.
18. Cowen D, Tardieu C, Schubert M, Peterson D, Resbeut M, Faucher C and Franquin JC: Low energy Helium-Neon laser in the prevention of oral mucositis in patients undergoing bone marrow transplant: Results of a double blind randomized trial. Int J Radiat Oncol Biol Phys 38: 697-703, 1997.
19. Online EA Pharma Co., Ltd. Products Information, Elental®. http://www.eapharma.co.jp/medicalexpert/product/elental/elental_information.html, Webpage in Japanese. Accessed July 25, 2019.
20. Yamamoto T, Nakahigashi M, Umegae S, Kitagawa T and Matsumoto K: Impact of elemental diet on mucosal inflammation in patients with active Crohn's disease: Cytokine production and endoscopic and histological findings. Inflamm Bowel Dis 11: 580-588, 2005.
21. Johtatsu T, Andoh A, Kurihara M, Iwakawa H, Tsujikawa T, Kashiwagi A, Fujiyama Y and Sasaki M: Serum concentrations of trace elements in patients with Crohn's disease receiving enteral nutrition. J Clin Biochem Nutr 4: 197-201, 2007.
22. Yamamoto T, Nakahigashi M, Saniabadi AR, Iwata T, Maruyama Y, Umegae S and Matsumoto K: Impacts of long-term enteral nutrition on clinical and endoscopic disease activities and mucosal cytokines during remission in patients with Crohn's disease: A prospective study. Inflamm Bowel Dis 13: 1493-1501, 2007.
23. Hanai H, Iida T, Takeuchi K, Arai H, Arai O, Abe J, Tanaka T, Maruyama Y, Ikeya K, Sugimoto K, *et al*: Nutritional therapy versus 6-mercaptopurine as maintenance therapy in patients with Crohn's disease. Dig Liver Dis 44: 649-654, 2012.
24. Yamamoto T, Nakahigashi M, Umegae S, Kitagawa T and Matsumoto K: Impact of long-term enteral nutrition on clinical and endoscopic recurrence after resection for Crohn's disease: A prospective, non-randomized, parallel, controlled study. Aliment Pharmacol Ther 25: 67-72, 2007.
25. Fukui T, Itoh Y, Orihara M, Yoshizawa K, Takeda H, Kawada S and Yoshioka T: Elental prevented and reduced oral mucositis during chemotherapy in patients esophageal cancer. Gan To Kagaku Ryoho 38: 2597-2601, 2011 (In Japanese).
26. Ogata Y, Takeuchi M, Ishibashi N, Kibe S, Takahashi K, Uchida S, Murakami N, Yahara T and Shirouzu K: Efficacy of Elental on prevention for chemotherapy-induced oral mucositis in colorectal cancer patients. Gan To Kagaku Ryoho 39: 583-587, 2012 (In Japanese).
27. Harada K, Minami H, Ferdous T, Kato Y, Umeda H, Horinaga D, Uchida K, Park SC, Hanazawa H, Takahashi S, *et al*: The Elental® elemental diet for chemoradiotherapy-induced oral mucositis: A prospective study in patients with oral squamous cell carcinoma. Mol Clin Oncol 10: 159-167, 2019.
28. Harada K, Ferdous T, Horinaga D, Uchida K, Mano T, Mishima K, Park S, Hanazawa H, Takahashi S, Okita A, *et al*: Efficacy of elemental diet on prevention for chemoradiotherapy-induced oral mucositis in patients with oral squamous cell carcinoma. Support Care Cancer 24: 953-959, 2016.
29. Harada K, Ferdous T, Kobayashi H and Ueyama Y: Elemental diet accelerates the recovery from oral mucositis and dermatitis induced by 5-Fluorouracil through the induction of fibroblast growth factor 2. Integr Cancer Ther 17: 423-430, 2018.
30. Harada K, Ferdous T, Mizukami Y and Mishima K: Elemental diet inhibits pro-inflammatory cytokine production in keratinocytes through the suppression of NF-κB activation. Oncol Rep 40: 361-368, 2018.
31. Luo M, Brooks M and Wicha MS: Asparagine and Glutamine: Co-conspirators fueling metastasis. Cell Metab 27: 947-949, 2018.
32. Knott SRV, Wagenblast E, Khan S, Kim SY, Soto M, Wagner M, Turgeon MO, Fish L, Erard N, Gable AL, *et al*: Asparagine bioavailability governs metastasis in a model of breast cancer. Nature 554: 378-381, 2018.
33. Liu H, Ling W, Shen ZY, Jin X and Cao H: Clinical application of immune-enhanced enteral nutrition in patients with advanced gastric cancer after total gastrectomy. J Dig Dis 13: 401-406, 2012.
34. ASPEN Board of Directors and the Clinical Guidelines Task Force: Guidelines for the use of parenteral and enteral nutrition in adult and pediatric patients. JPEN J Parenter Enteral Nutr 26 (Suppl 1): 1SA-138SA, 2002.