Effects of aged garlic extract may differ between normal cells and cancer cells

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Abstract. Aged garlic extract (AGE) is one of the widely used garlic-based products that are extensively studied and are commercially available. However, there are no reports that compare the effects of AGE on normal cells and oral cancer cells. The present study tried to investigate the effects of AGE on normal healthy cells and cancer cells. The effect of AGE on the proliferation of normal cell lines HaCaT and fibroblast, in addition to cancer cell lines SCC7, HSC2, HSC3 and Ca9-22 were evaluated by MTT assay. The effects of AGE on cell migration were examined using wound healing and migration assays. Whole transcriptome analysis, ingenuity pathways analysis (IPA) and western blot analysis were used to investigate the mechanism of action of AGE in HaCaT and SCC7 cells. Data from the aforementioned assays were then evaluated and compared to assess if AGE affects normal cells differently compared with cancer cells. AGE was found to promote the proliferation and migration of normal cells, especially HaCaT, in the absence of FBS more markedly compared with those of cancer cells. However, AGE could not promote wound healing in fibroblast cells at the same rate as in HaCaT cells. In normal cells, sequential or combination AGE treatment with 5-fluorouracil (5-FU), cisplatin (CDDP) and docetaxel (DOC) somewhat counteracted the proliferation-limiting effects of anti-cancer agents. However, in cancer cells, AGE treatments enhanced the inhibitory effects of anti-cancer agents when used in combination with 5-FU, CDDP or DOC. This observation was more evident in the case of pre-treatment with anticancer agents followed by AGE sequential treatment. Subsequently, whole transcriptome analysis and IPA data suggested that AGE facilitated the proliferation and survival of normal cells through the induction of protein kinase B family protein and brain-derived neurotrophic factor pathways, whilst suppressing ferroptosis These data were also confirmed by western blotting to see if the genetic changes shown in whole transcriptome analysis and IPA are also induced at the protein level. In addition, AGE may reduce cancer cell proliferation through the suppression of cancer metastasis signaling and enhancement of phagocytic activity according to whole transcriptome analysis and IPA data. Taken together, these findings suggested that AGE may prompt the proliferation of normal cells and suppress the that of cancer cells through different cellular processes and signaling pathways.

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Introduction

Garlic (*Allium sativum*) has been used in food preparation and for medicinal purposes for centuries (1). In particular, aged garlic extract (AGE) is one of the more widely studied and accepted garlic products, since it has a higher safety profile compared with other garlic preparations (2,3). AGE is typically prepared from fresh garlic by extraction and aging in an aqueous ethanol mixture for >10 months at room temperature (2). This aging process is necessary to increase the levels of beneficial compounds in garlic extracts, such as pyruvate, S-allylcysteine, S-allylmercaptocysteine (SAMC) and the antioxidant compound N-α-(1-deoxy-D-fructos-1-yl)-L-arginine (2,4). AGE has a number of reported health benefits, because of its high antioxidant activity (5,6), in addition to anti-inflammatory (7,8), immunomodulatory (9-11) and anti-cancer activities (4). A multitude of clinical studies have shown that AGE can alleviate hypertension (3), atherosclerosis (12), metabolic syndrome (13) and periodontal disease (14).

Cancer is the second leading cause of mortality and was responsible for 10 million deaths worldwide in 2020 (15). There is ample evidence of the anticancer activity of AGE in several types of cancer in previous human studies (16-20). Specifically, garlic has been reported to suppress carcinogenesis and inhibit the proliferation of skin, esophageal, lung, gastric, colorectal and prostate cancer cells both *in vivo* and *in vitro* (21). AGE contains the key component, SAMC, which can inhibit cell

proliferation and promote apoptosis in colorectal cancer cells, prostate carcinoma cells, breast cancer cells and ovarian cancer cells (4). In addition, to the best of the authors' knowledge, reports on toxic side-effects of AGE or any unfavorable interactions with other medications have remained scarce (17). However, the benefits of applying AGE for the treatment of oral squamous cell carcinoma (OSCC), a major type of head and neck squamous cell carcinoma (HNSCC), has not been previously evaluated.

AGE is also a documented potent anti-fatigue agent (22). There are several AGE-related products available commercially, since it has numerous applications in the health industry. Kyoleopin® (Wakunaga Pharmaceutical Co., Ltd.) is one such product. The main composition of Kyoleopin® includes AGE, several amino acids (glycine, proline and alanine), vitamin B1 and vitamin B12 (23). It has been used in Japan as a nutritional supplement for treating physical fatigue or physical weakness after illness (23). However, any nutritional supplement administered to patients with cancer may not only provide an extra source of nutrition for normal (non-cancer) cells but also for cancer cells, which may promote the development of the latter. Therefore, it is essential to clarify if AGE can exert possible harmful effects in patients with cancer or unfavorable interactions with anticancer agents. The most common chemotherapeutic agents for HNSCC and OSCC treatments are fluoropyrimidine-based drugs (such as Tagafur and 5-FU), platinum-based drugs [carboplatin and cisplatin (CDDP)] and taxane-based drugs [paclitaxel and docetaxel (DOC)] (24,25). The combination therapy of CDDP + 5-FU remains the standard regimen for HNSCC treatment (24). How AGE may interact with such medications and how it affects patients with OSCC receiving these drugs all remain unknown. Therefore, it is important to understand how AGE affects normal and cancer cells in patients with OSCC.

In the present study, the effectiveness of AGE against oral cancer cells were investigated. In addition, its effects between normal cells and OSCC cancer cells were compared, with focus on its mechanism of action differs in both cell types.

Materials and methods

Cell lines and cell culture. OSCC cell lines (HSC2, HSC3 and SCC7) and the human gingival carcinoma cell line (Ca9-22) were obtained from Cell Bank (Riken BioResource Center). The immortalized human keratinocyte cell line HaCaT was purchased from Funakoshi Co., Ltd. A primary dermal fibroblast (passage number 3), normal, human, Adult HDFa (ATCC® PCS-201-012™) was obtained from American Type Culture Collection. HaCaT and Ca9-22 were tested by STR. They were judged to be authentic as evaluation value was ≥0.8.

Cells were cultured and maintained DMEM/Ham's F-12 (Merck KGaA) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.) and 100 μg/ml streptomycin/100 units/ml penicillin (Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere containing 5% CO_2.

Cell proliferation assay. Cells ($3x10^3$ cells/well) were seeded into 96-well plates in D-MEM/Ham's F-12 containing 10% FBS. AGE was obtained from Wakunaga Pharmaceutical Co., Ltd. The effects of AGE (1 pg/ml-10 mg/ml) in the medium without or with 10% FBS were first tested for 24-48 h (data not shown). Subsequently, 0.001-10 mg/ml AGE without or with 10% FBS was focused upon, where the cells were treated for 24-48 h to assess the effects of AGE. The cells were also treated with different concentrations of AGE in medium with 0% FBS for 24 h and anticancer agents (in medium with 10% FBS) for 24 h sequentially or simultaneously. The anticancer agents used were 5-FU (Kyowa Hakko Kirin Co., Ltd.), CDDP (FUJIFILM Wako Pure Chemical Corporation) and DOC (FUJIFILM Wako Pure Chemical Corporation). The assay was performed as previously described (26). Briefly, 0.5 mg/ml MTT (Merck KGaA) solution was added to each well (25 μl/well) and the 96-well plates were placed inside an incubator at 37˚C for 4 h. MTT solution was then removed completely and dimethyl sulfoxide was added to each well (100 μl/well). After 5 min, the absorbance was measured with a spectrophotometer (Bio Rad Laboratories, Inc.) at 490 nm. All assays were run in triplicate.

Wound healing assay. Cells ($1.5x10^4$ cells per well) were seeded into a 24-well plate and were cultured in the absence or presence of AGE (0.1-100 μg/ml) dissolved in DMEM/Ham's F-12 with 0% FBS and 1% penicillin/streptomycin until a monolayer of cells was formed. A 200-μl pipette tip was then used for making a wound in the cell layer through the central axis of the plate. The migration of cells into the wounded area was examined after 24 h using a light microscope (BX-51-33-FLD2; Olympus Corporation). All assays were independently repeated ≥3 times.

Cell migration assay. A cell migration assay was performed using a Boyden chamber set (Neuro Probe, Inc.), according to the manufacturer's instructions. Polycarbonate membranes (Neuro Probe, Inc.) of 8-μm pore sizes were pre-coated with gelatin (0.1 mg/ml, FUJIFILM Wako Pure Chemical Corporation) rinsed in sterile water and were used for the assay. Cells were suspended to a final concentration of $1.0x10^4$/50 μl DMEM/Ham's F-12 without FBS medium. Bottom wells of the chamber were filled with of 0% FBS DMEM/Ham's F-12 (25 μl/well) containing different concentrations (0.1 μg-1 mg/ml) of AGE as a chemoattractant. The chamber was covered with a gelatin coated membrane, before then 50 μl cell suspension was added to the top wells. After 24 h incubation at 37˚C in a 5% CO_2 atmosphere, the polycarbonate membrane was washed with PBS. Cells on the upper surface of the membrane were carefully removed with a cotton swab. The cells on the lower surface of the membrane were then fixed with absolute methanol for 10 min at room temperature and stained with a hematoxylin solution for 15 min at room temperature. The stained cells were counted in three different fields (magnification, x200) under a microscope (BX-51-33-FLD2; Olympus Corporation). All assays were independently repeated ≥3 times.

Whole transcriptome analysis. Only HaCaT and SCC7 cells were chosen for genomic analysis. Total RNA was isolated from each sample using the RNeasy Mini kit (Qiagen GmbH). The quality of RNA was examined with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) using a RNA 6000 Nano kit (Agilent Technologies, Inc.) after the concentrations were determined by Qubit (Thermo Fisher

Scientific, Inc.). The RNA integrity number values were found to be >7 in all samples, indicating that the samples contained high-quality RNA. The expression libraries were produced using the NEBNext ultra II RNA library prep kit (cat. no. E7770L; New England BioLabs, Inc.) and NEBNext Multiplex Oligos for Illumina (cat. no. E7335S; New England BioLabs, Inc.). According to the manufacturer's instructions, total RNA (500 ng) extracted from each sample was reverse transcribed into cDNA using the NEBNext Ultra II RNA First Strand Synthesis Module (cat. no. E7771; New England BioLabs, Inc.), before the index sequences were inserted during PCR amplification. Following initial denaturation at 98˚C for 30 sec, amplification was performed for 9 cycles of a denaturation at 98˚C for 10 sec, annealing at 65˚C for 75 sec and extension at 65˚C for 75 sec, with a final extension at 65˚C for 5 min. The primer sequences were as follows: NEBNext Index 6 Primer for Illumina, 5'-CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'; NEBNext Index 7, 5'-CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'; NEBNext Index 8, 5'-CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'; NEBNext Index 9, 5'-CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'; NEBNext Index 10, 5'-CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'; NEBNext Index 11, 5'-CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3' and NEBNext Universal PCR Primer, 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'. The quality of the library was examined using a Bioanalyzer (Agilent Technologies, Inc.) after purification using AMPure XP beads (cat. no. A63882; Beckman Coulter, Inc.). The libraries were sequenced on an Illumina Next-seq sequencer (Illumina, Inc.) with an Illumina NextSeq High Output 150 bp pair-end cycle sequencing kit (cat. no. 20024907; Illumina, Inc.). In total, >30 million reads in each sample were detected in the reaction, which were trimmed and mapped with the human reference genome GRCh38 and mouse reference genome GRCm38 release-92 using CLC Genomics Workbench software (ver. 8.01; Qiagen GmbH), where the mapping ratio was 98%.

Ingenuity network analysis. The gene sets markedly increased or decreased according to co-relation analysis using GraphPad Prism (ver. 9.0.3; GraphPad Software, Inc.; Dotmatics) were subjected to a network analysis using Ingenuity Pathways Analysis software (IPA; ver. 8.6; Qiagen GmbH). IPA reports molecular and cellular functions and canonical pathways on the basis of data from millions of molecular interactions reported in the literature, where this software is updated weekly. IPA uses Fisher's exact test to determine whether the input genes are markedly associated with pathways by comparison with the entire ingenuity knowledge base. (https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-ipa/).

Western blotting. HaCaT and SCC7 cells ($2.0x10^6$ cells in 100 mm dish) were treated with different concentrations of AGE (0, 0.1, 1, 10, 100 μg/ml and 1 mg/ml) dissolved in DMEM/Ham's F-12 medium with 0% FBS. The cells were lysed with RIPA buffer (Thermo Fisher Scientific, Inc.). The protein content of the whole cell lysates was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.). A total of 20 μl protein sample containing 50 μg/ml protein was loaded into each well of a NuPAGE 4-12% Bis-Tris gel (Thermo Fisher Scientific, Inc.) and was subjected to electrophoresis by 10% SDS-PAGE, before being transferred onto a polyvinylidene fluoride (PVDF) membrane using iBlot gel transfer stacks (Thermo Fisher Scientific, Inc.). A blocking solution was prepared with WesternBreeze Blocker/Diluent part A and B (Thermo Fisher Scientific, Inc.) according to manufacturer's instruction. The PVDF membranes were first incubated in the blocking solution at room temperature for 30 min, then the membranes were incubated with the anti-protein kinase B family protein (Akt1) mouse monoclonal antibody (dilution, 1:200; cat. no. sc-5298), the anti-p-Akt1 mouse monoclonal antibody (dilution, 1:100; cat. no. sc-52940), anti-brain derived neurotrophic factor (BDNF) rabbit polyclonal antibody (dilution, 1:100; cat. no. sc-20981), anti-glutathione peroxidase 4 (GPX4) mouse monoclonal antibody (dilution, 1:50; cat. no. sc-166570), anti-glutathione mouse monoclonal antibody (dilution, 1:100; cat. no. sc-133136), or anti-α-tubulin mouse monoclonal antibody (dilution, 1:500; cat. no. sc-398103). These antibodies were purchased from Santa Cruz Biotechnology, Inc. The membranes were washed using 1X WesternBreeze wash solution (Thermo Fisher Scientific, Inc.) and incubated with ready-made Novex alkaline-phosphatase-conjugated (goat) anti-mouse (no dilution; cat. no. WB7104) or anti-rabbit IgG secondary antibodies (no dilution; cat. no. WB7105; both from Thermo Fisher Scientific, Inc.). The antibodies were detected using Novex® AP Chromogenic substrate (cat. no. WP20001; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/WesternBreezeChemiLum_WesternBlotImmun_protocol.pd'). ImageJ v.1.51h software (National Institutes of Health) was used to quantify the average intensities of each protein band, which were then compared with the band intensities of the control protein, α-tubulin.

Statistical analysis. All data are indicated as the mean ± standard deviation. The significance of the experiment results was determined by one-way analysis of variance and Tukey-Kramer multiple comparisons tests. The datasets measured by whole transcriptome analysis were normalized by each transcript per million to GAPDH and were analyzed using correlation assay such as determination of fold change, unpaired Student's t-test and principal component analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

AGE affects the cell proliferation ability of normal cell differently compared with cancer cells. MTT assay was used to measure the viability of AGE-treated and untreated normal cells and cancer cells. AGE can serve as a type of nutrient in a manner not dissimilar to FBS. The present study first assessed

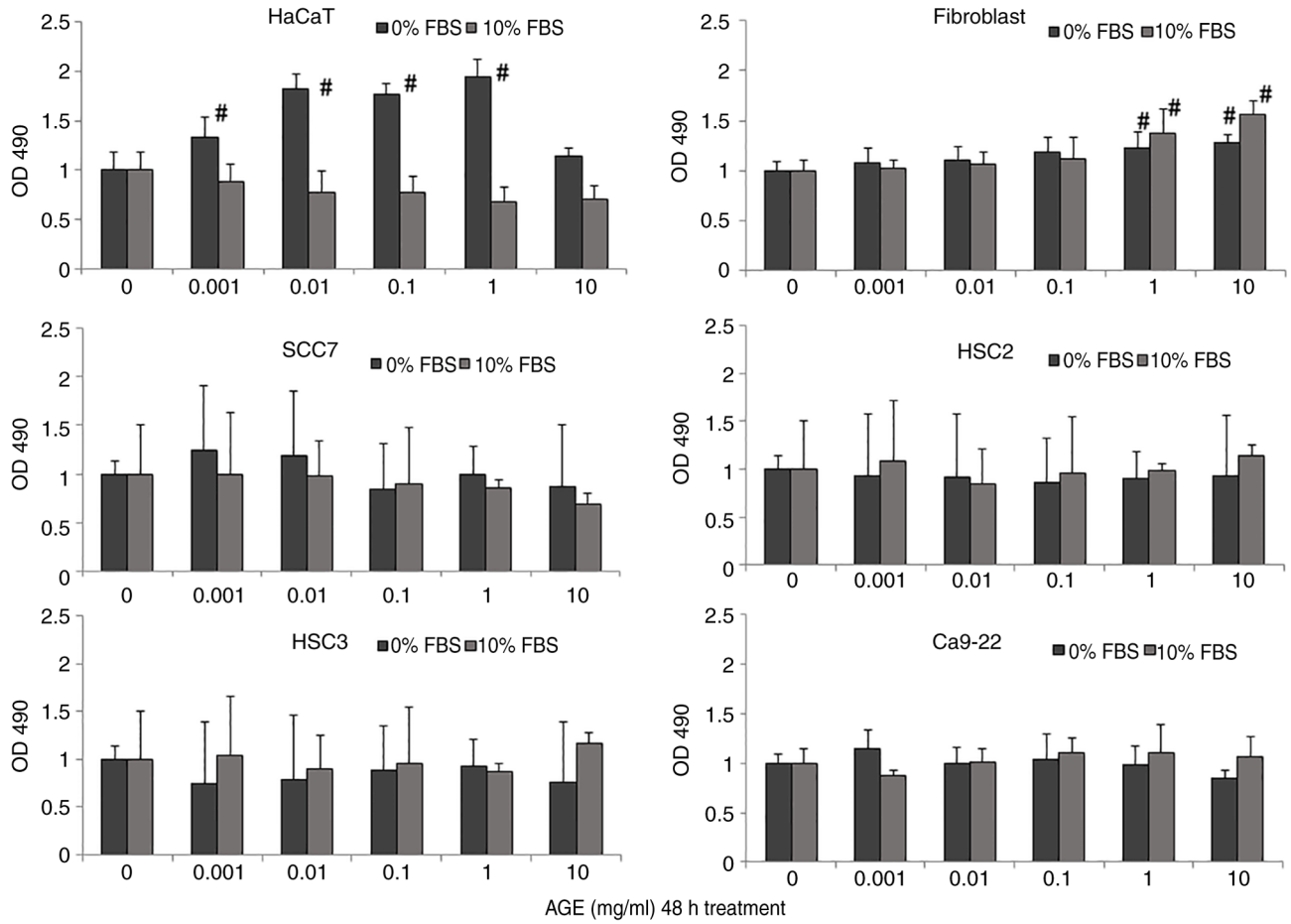


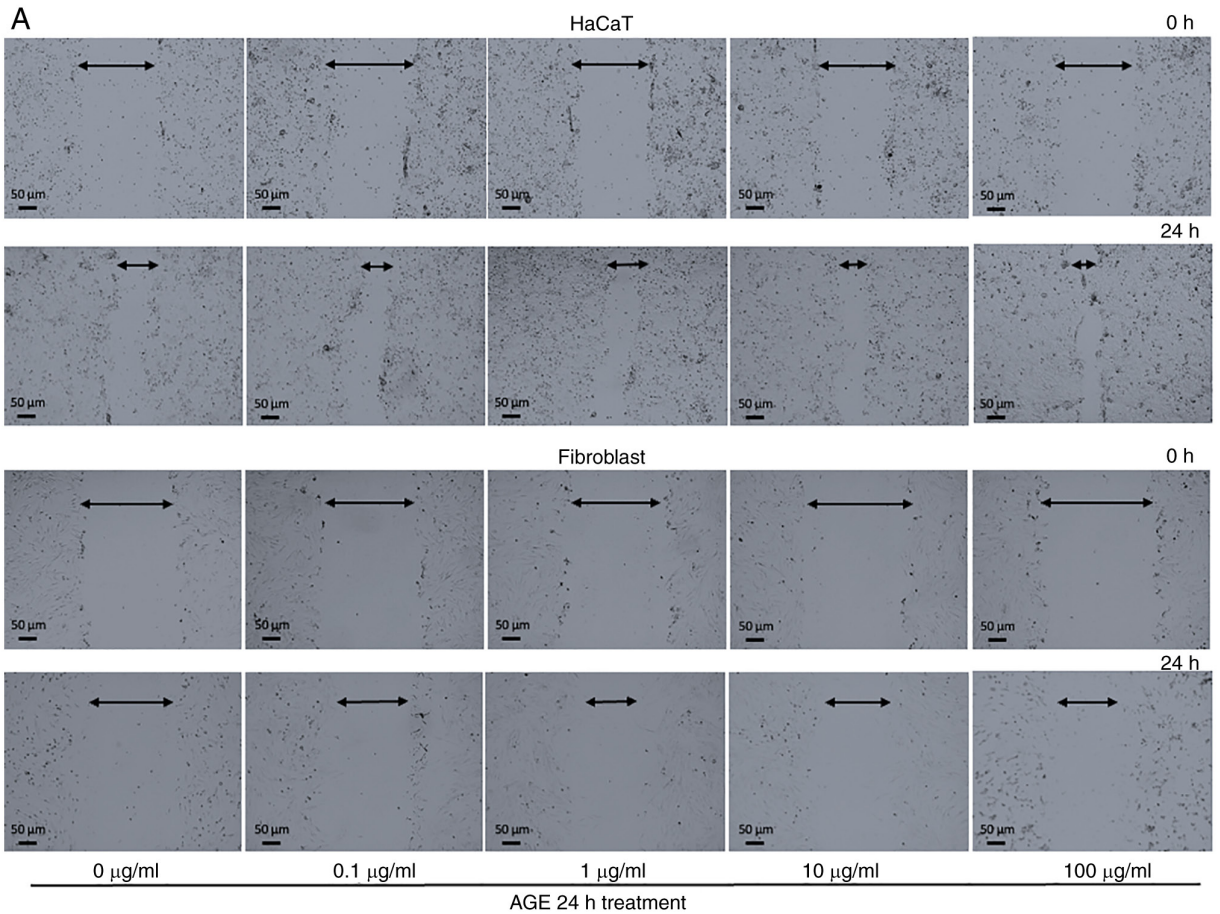
Figure 1. Effect of AGE on cell proliferation. AGE exerted growth-stimulating effect on HaCaT (AGE 0.001-1 mg/ml) in the absence of FBS (0% FBS) and on Fibroblast (AGE 1-10 mg/ml) in medium with 0% or 10% FBS. However, higher concentrations (0.01-1 mg/ml) of AGE did not promote the growth of cancer cells. #P<0.05 vs. control (AGE 0 mg/ml) one-way analysis of variance and Tukey-Kramer multiple comparisons tests. AGE, aged garlic extract.

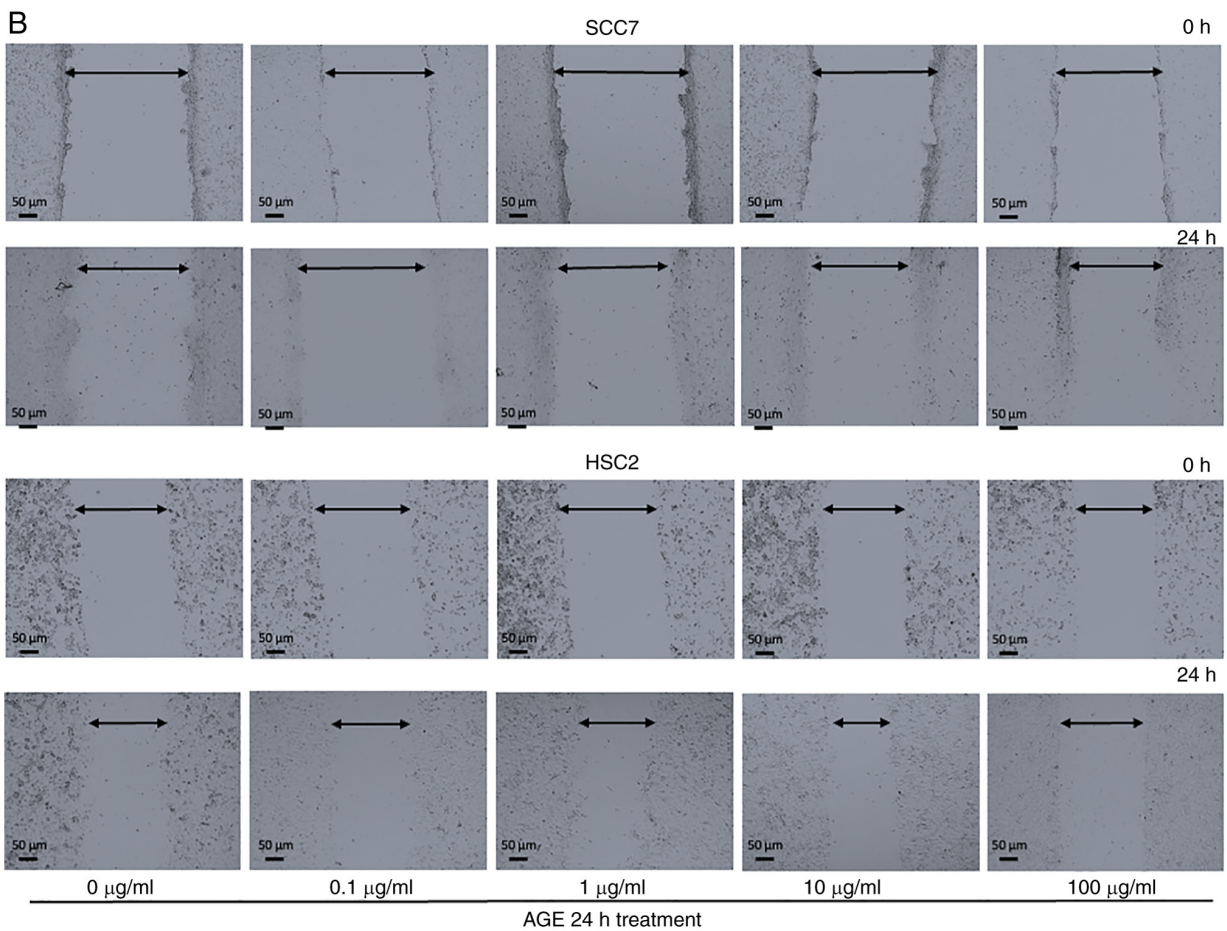
the effects of AGE on cell viability without FBS and with FBS as an extra source of nutrition. Briefly, 0% FBS equated to the starvation state, whereas 10% FBS was used to mimic adequate nutrition. In nutritionally-poor conditions (medium with 0% FBS), the viability of AGE (0.001-1 mg/ml)-treated HaCaT cells was found to be markedly higher compared with that of untreated HaCaT cells (Fig. 1). In addition, AGE (1-10 mg/ml) was found to stimulate the proliferation of fibroblasts in medium containing 0% or 10% FBS. By contrast, 0.01-1 mg/ml AGE did not markedly increase the viability of cancer cells (Fig. 1). Notably, morphological changes among the AGE-treated and untreated normal cells and cancer cells were not observed (data not shown). It was also noticed that cell proliferation effect due to AGE treatment is stronger in medium without FBS (0% FBS) than in medium containing 10% FBS.

AGE affects the wound healing ability of normal cells differently compared with cancer cells. Horizontal movement ability of AGE-treated and untreated cells was next evaluated using wound healing assay. AGE-treated HaCaT cells exhibited notably higher wound healing capabilities compared with those in the untreated control group (Fig. 2A). AGE-treated fibroblasts also showed increased wound healing abilities compared with those in the untreated control group, but it was not to the same rate as that observed in HaCaT cells (Fig. 2A). By contrast, although AGE-treated cancer cells showed limited wound healing ability in some cases, the overall wound healing ability was observed to be weaker in all cancer cell lines tested compared with that in HaCaT cells (Fig. 2B and C).

AGE affects the migratory ability of normal cells differently compared with cancer cells. The migratory activity of the AGE-treated cells was measured using Boyden chamber assays. Fig. 3 showed that AGE (1 μg-1 mg/ml)-treated HaCaT and fibroblasts had markedly higher migratory ability compare with that of untreated cells. In the case of cancer cells, low concentrations of AGE increased their migratory ability, but higher concentrations of AGE in contrast decreased their migration ability in AGE (10 μg-1 mg/ml)-treated cancer cells compared with that in the corresponding untreated cells. Markedly decreased migratory ability was observed in SCC7 and HSC2 cells following treatment with AGE 10 μg-1 mg/ml.

Combined effects of AGE and anticancer agents. The combined effects of AGE and anticancer agents were found to be different depending on the cell type in question. The combination (sequential or simultaneous) of AGE and anticancer agents was not able promote the proliferation of normal





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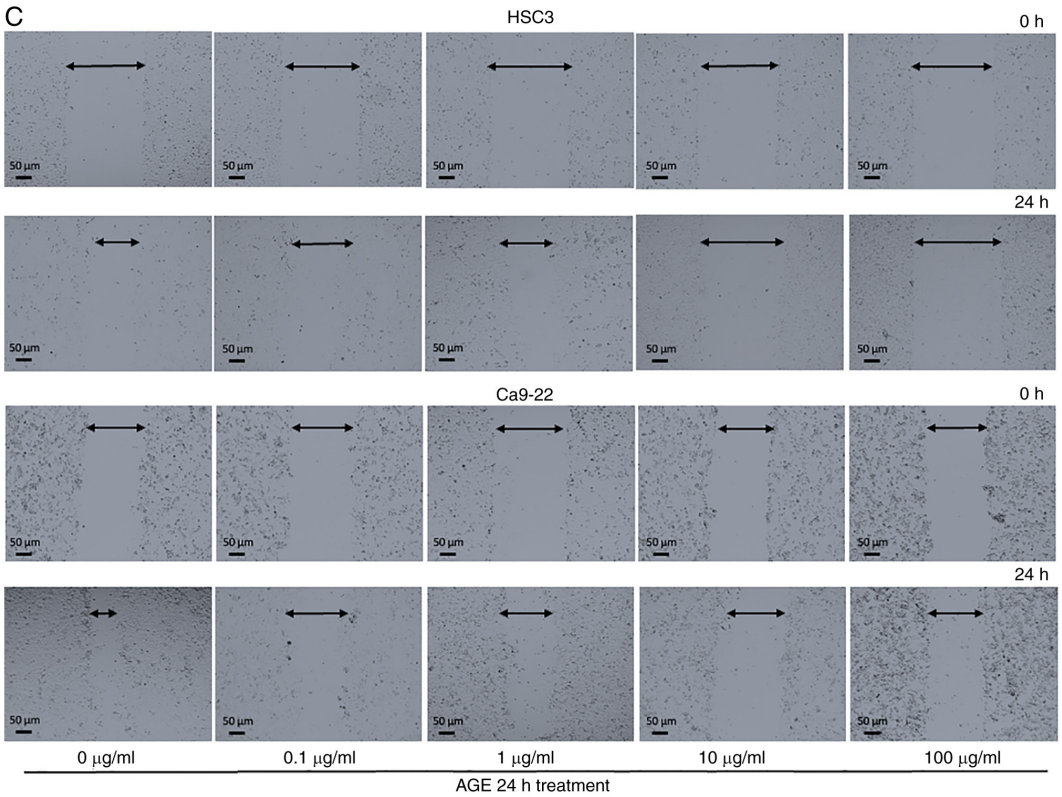


Figure 2. Effect of AGE on wound healing. The wound healing ability of AGE-treated cells was examined by a wound healing assay. Black arrow shows the width of each wound area. (A) AGE increased the wound healing ability of HaCaT more markedly than Fibroblast cells. (B) AGE did not affect the wound healing ability of SCC7, but AGE slightly affected HSC2. (C) AGE did not affect the wound healing ability of HSC3, but had medium effect on Ca922. AGE- treated cancer cells showed weaker wound healing ability than AGE- treated HaCaT cells. This assay was run in triplicate. AGE, aged garlic extract.

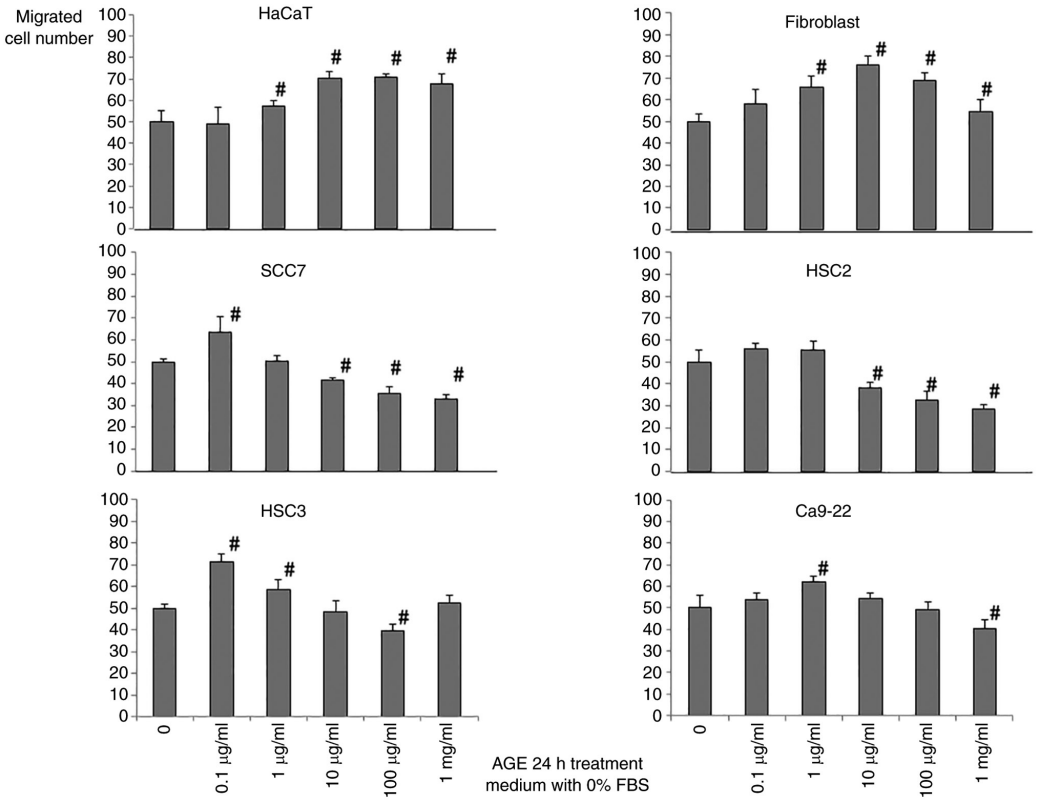
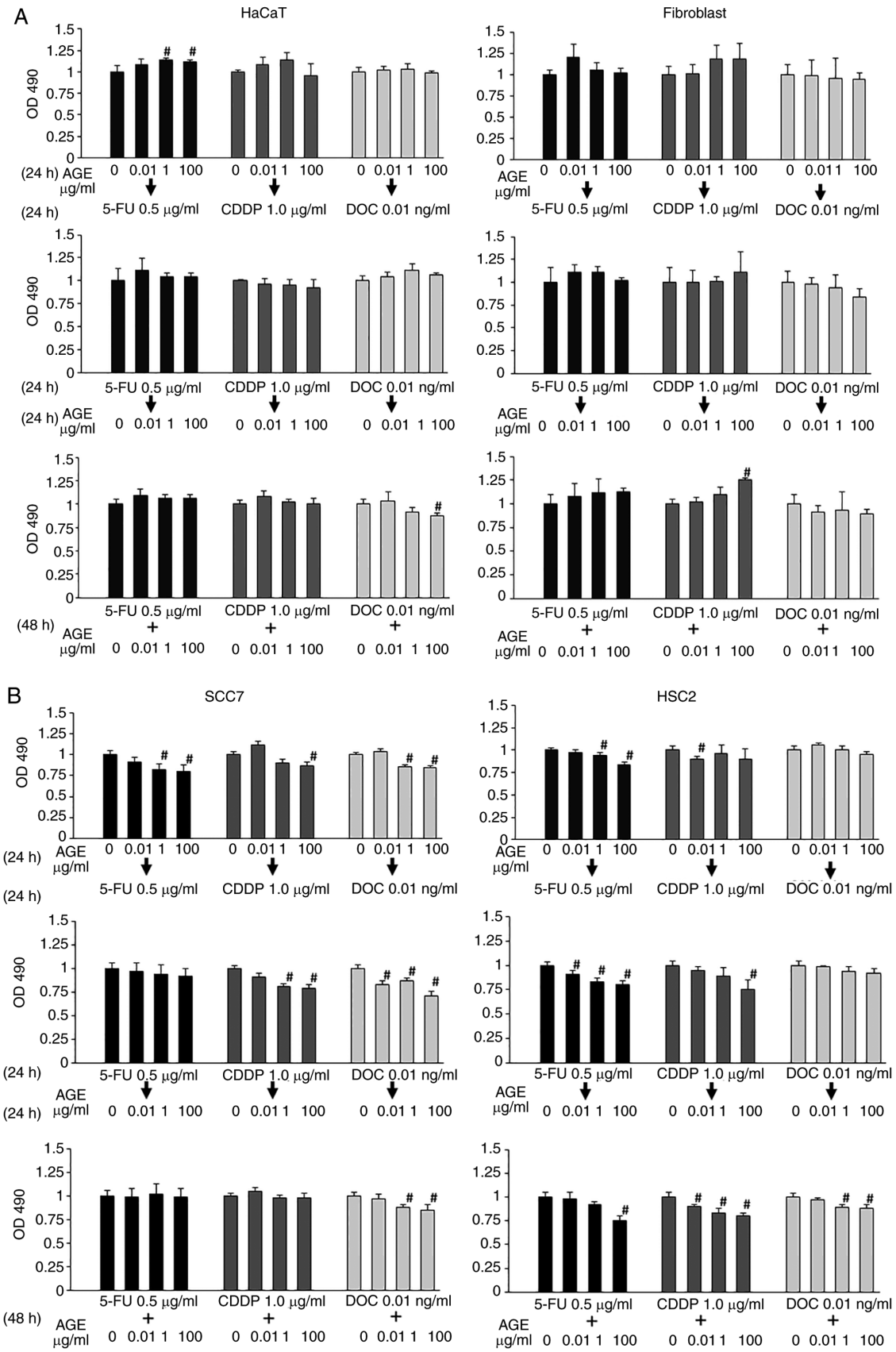


Figure 3. Effect of AGE on cell migration. AGE (1 μg-1 mg/ml) stimulated the migration ability of normal cells (HaCaT and Fibroblast). Low concentration of AGE increased the migration ability of cancer cells. However, high concentration of AGE decreased the migration ability of cancer cells in some cases, especially in SCC7 and HSC2 cells. $^{\#}P<0.05$ vs. control (AGE 0 mg/ml) one-way analysis of variance and Tukey-Kramer multiple comparisons tests. AGE, aged garlic extract.



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cells, although its rate was observed to be slightly increased in AGE (1 μg or 100 μg/ml) followed by 5-FU (0.5 μg/ml)-treated HaCaT (Fig. 4A). In the majority of cases, inhibition on proliferation was not observed. However, AGE pre-treatment in HaCaT and fibroblast cells, in addition to combined treatment with AGE, appeared to have counteracted the inhibitory

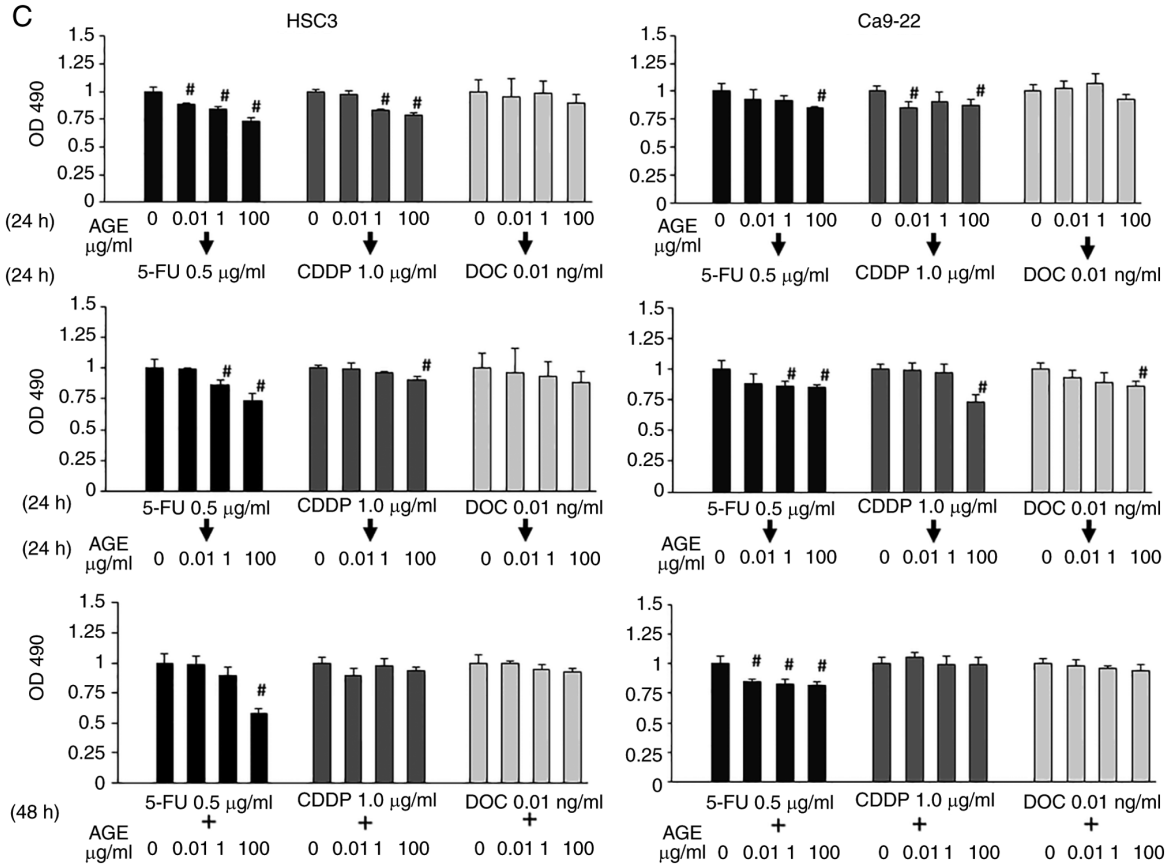
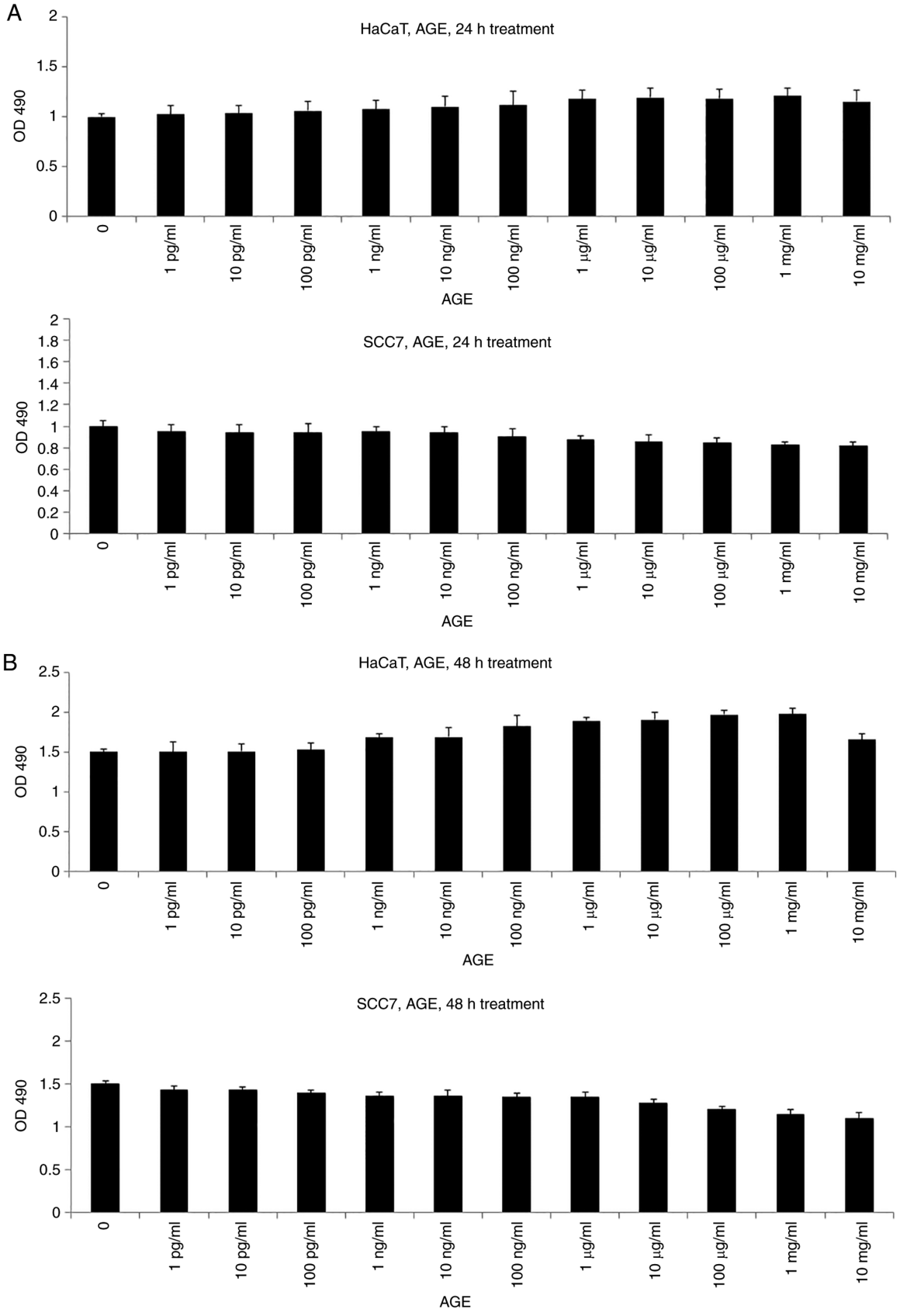


Figure 4. Combined effects of AGE and anticancer agents. Combined effects of AGE and anticancer agents were different depending on the cell types. (A) Sequential or simultaneous treatment with AGE and anticancer agents did not affect growth of normal cells that much; however, cell growth rate was slightly increased in few cases. In most cases, growth inhibition was absent. (B and C) Pre-treatment with anticancer agents tend to improve the growth inhibitory effect of AGE against cancer cells. However, the data was not the same for all cancer cell types. $^{\#}P<0.05$ vs. control (AGE 0 mg/ml); one-way analysis of variance and Tukey-Kramer multiple comparisons tests. AGE, aged garlic extract.

effects of 5-FU and CDDP on proliferation in fibroblast cells (Fig. 4A), although these results are not conclusive.

In the cancer cell lines tested, the effects of the same combination treatments were different compared with those in normal cells. In SCC7 cells, AGE slightly improved the inhibitory effects of 5-FU, whereas pre-treatment with CDDP or DOC followed by AGE conferred superior inhibitory effects (Fig. 4B). In HSC2 cells, pre-treatment with 5-FU or CDDP followed by AGE, in addition to the simultaneous treatment with CDDP and AGE, yielded superior results than pre-treatment with AGE followed by 5-FU or CDDP (Fig. 4B). In HSC3 cells, AGE pre-treatment improved the inhibitory effects of CDDP, but all types of combination (sequential or simultaneous) treatments with 5-FU and AGE exerted superior inhibitory effects compared with those in the untreated control group (Fig. 4C). In Ca9-22 cells, pre-treatment with all anticancer agents resulted in superior findings than pre-treatment with AGE followed by each anticancer agent in addition to the simultaneous treatment with AGE and each anticancer agent, but AGE also improved the inhibitory effects following simultaneous treatment with 5-FU (Fig. 4C). Briefly, pre-treatment with anticancer agents followed by AGE tended to potentiate the inhibitory effects of these agents against cancer cell proliferation more effectively compared with AGE pre-treatment or concurrent treatment (Fig. 4B and C). However, since the data varied considerably among the different cell types, these results at present remain inconclusive.

Mechanism of action of AGE in HaCaT and SCC7. The aforementioned cell proliferation and migration assay results showed that AGE promoted proliferation and migration in normal cells, but not cancer cells. To clarify the reasons underlying this difference in the action of AGE in normal cells and cancer cells, the mechanism of action of AGE in HaCaT and SCC7 was next examined. Whole transcriptome analysis was performed, followed by IPA using the differentially expressed genes identified in between AGE (100 μg/ml for 48 h)-treated cells and untreated cells. This was because this treatment promoted cell proliferation strongly in HaCaT cells but inhibited cell proliferation considerably in SCC7 compared with that exerted by lower doses (Fig. 5A and B). Between AGE-treated (48 h) HaCaT and untreated HaCaT cells, a pathway through inhibition of Ferroptosis was detected (Figs. 5C and S1). In addition, *Akt* could be one of the genes of focus, since it was detected from the first network analysis on comparison between AGE-treated HaCaT and untreated HaCaT cells (Figs. 5D and S2). Moreover, *BDNF* could be one of the genes of focus according to the third network analysis on comparison between AGE-treated and untreated HaCaT cells (Figs. 5E and S3). By contrast, comparing AGE-treated (48 h) SCC7 and untreated SCC7 cells revealed the possibility



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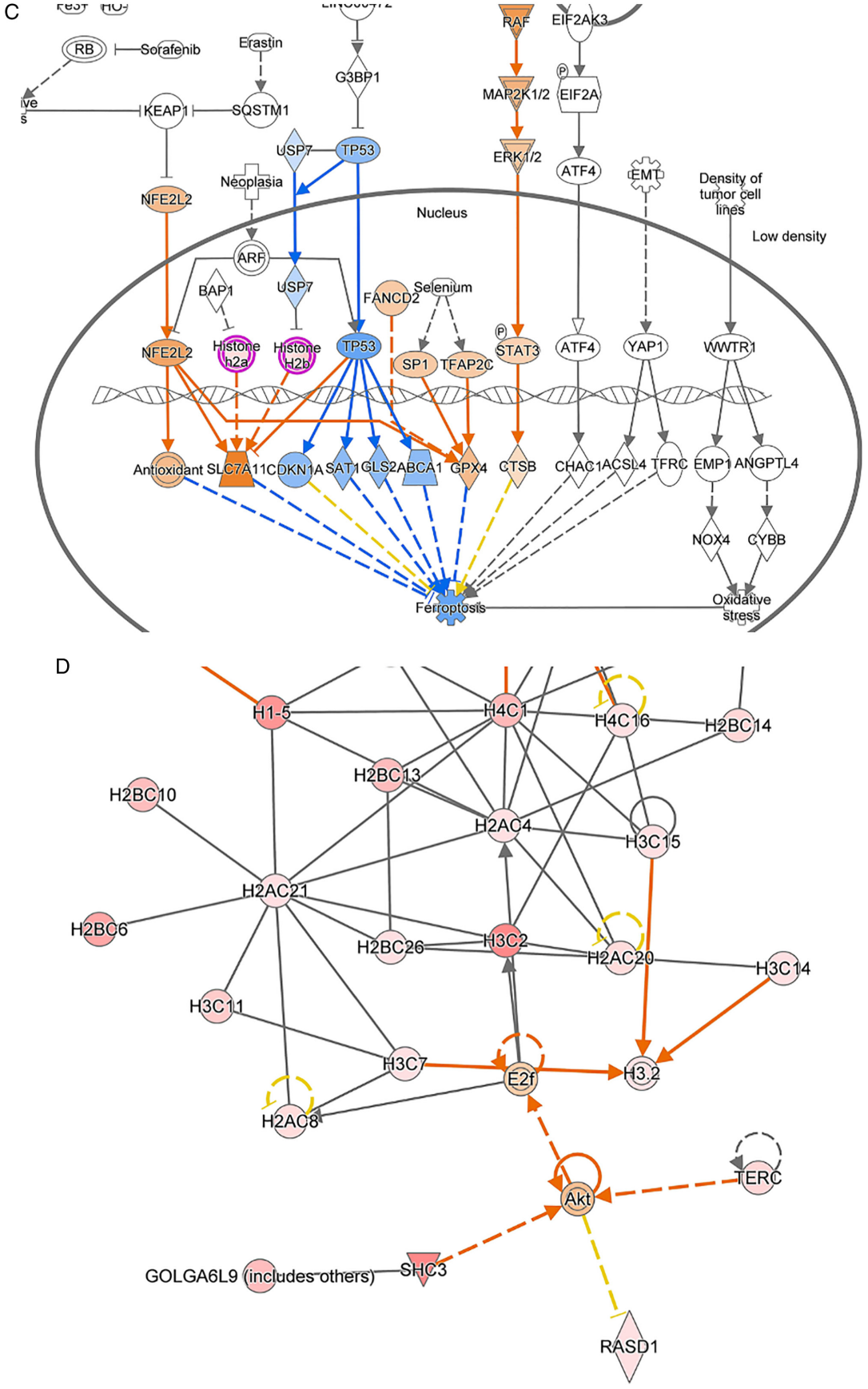
of cancer cell proliferation inhibition by AGE through the suppression of cancer metastasis signaling (Fig. 5F) and enhancement of phagocytic activity (Fig. 5G)

Expression of Akt, BDNF and ferroptosis related factors in AGE-treated cells. To verify the results of whole transcriptome analysis and IPA, the protein levels of Akt, p-Akt, BDNF, GPX4



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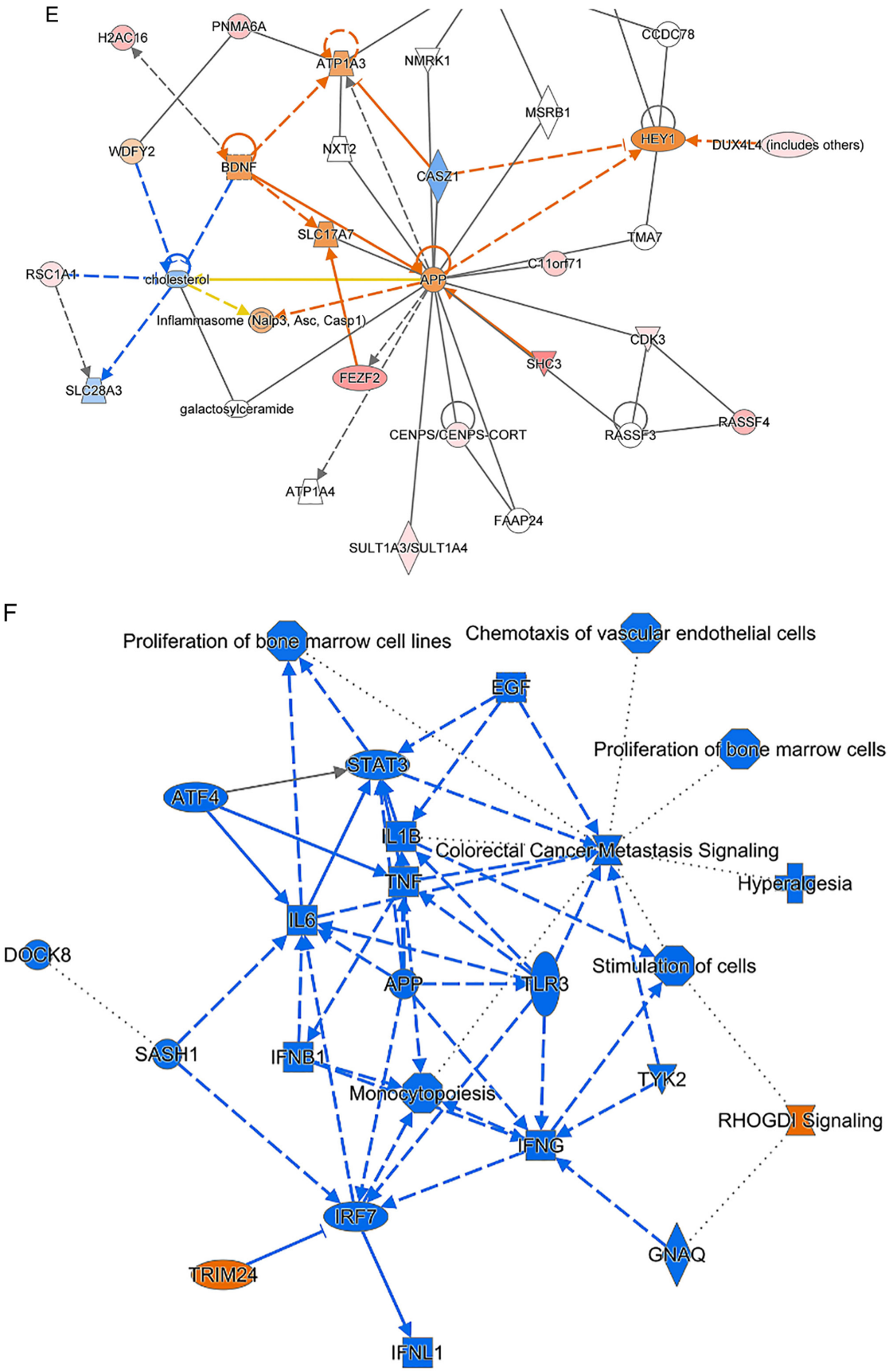
and Glutathione in HaCaT and SCC7 cells were examined by western blotting. Fig. 6F shows that AGE (0.1 μg-1 mg/ml) enhanced the expression of Akt, BDNF, GPX4, Glutathione and Akt phosphorylation in HaCaT cells compared with those



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in the untreated HaCaT cells. By contrast, the levels of these proteins were not enhanced in SCC7 cells (Fig. 6). Briefly, AGE induced Akt and BDNF, whilst suppressing the expression of ferroptosis-related factors, in HaCaT cells but not in SCC7 cells.

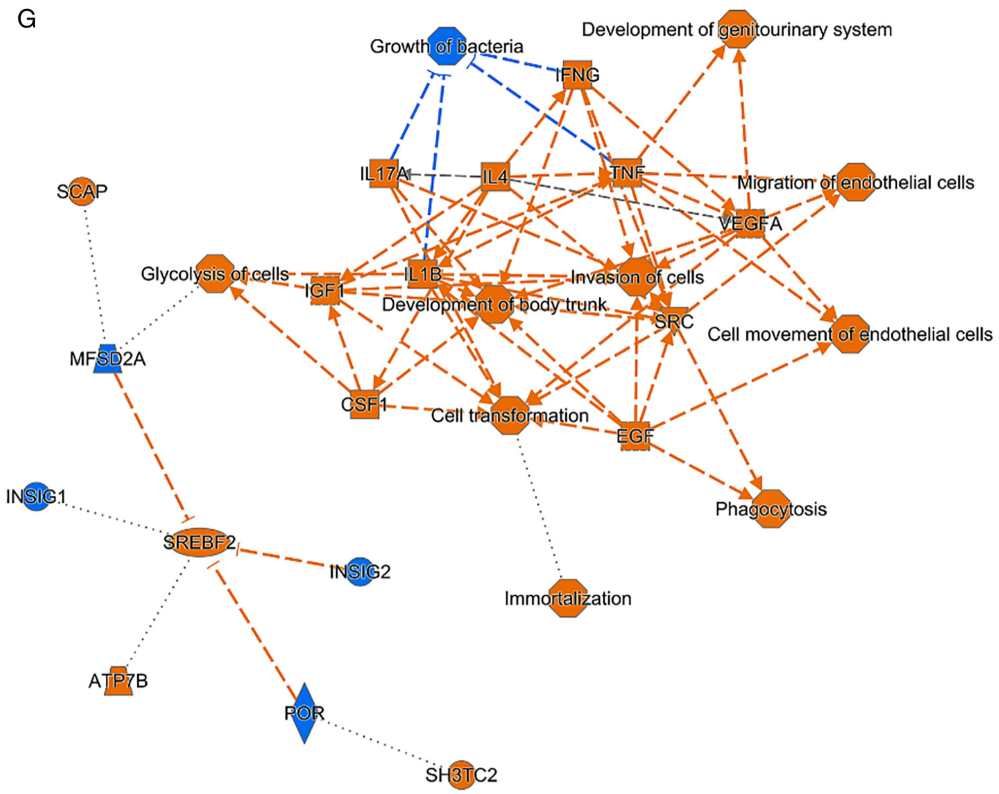

Figure 5. Mechanism of action of AGE in HaCaT and SCC7. (A) MTT assay of 24 h treatment. AGE promoted proliferation in HaCaT, but not SCC7. (B) MTT assay of 48 h treatment. AGE promoted proliferation in HaCaT, but not SCC7, and 48 h treatment showed improved results vs. 24 h treatment. (C) The pathway through inhibition of ferroptosis was detected in the pathway of comparison between AGE (100 μg/ml, 48 h)-treated HaCaT and untreated HaCaT. (D) *Akt* could be one of the genes of focus as detected from first network analysis on comparison between AGE (100 μg/ml, 48 h)-treated and untreated HaCaT. (E) *BDNF* could be one of the genes of focus as detected from third network analysis on comparison between AGE (100 μg/ml, 48 h)-treated HaCaT and untreated HaCaT. (F) The possibility of cancer cell growth inhibition by AGE through the suppression of cancer metastasis signaling between AGE (100 μg/ml, 48 h)-treated and untreated SCC7. (G) The possibility of cancer cell growth inhibition by AGE through the enhancement of phagocytic activity between AGE (100 μg/ml, 48 h)-treated SCC7 and untreated SCC7. AGE, aged garlic extract.

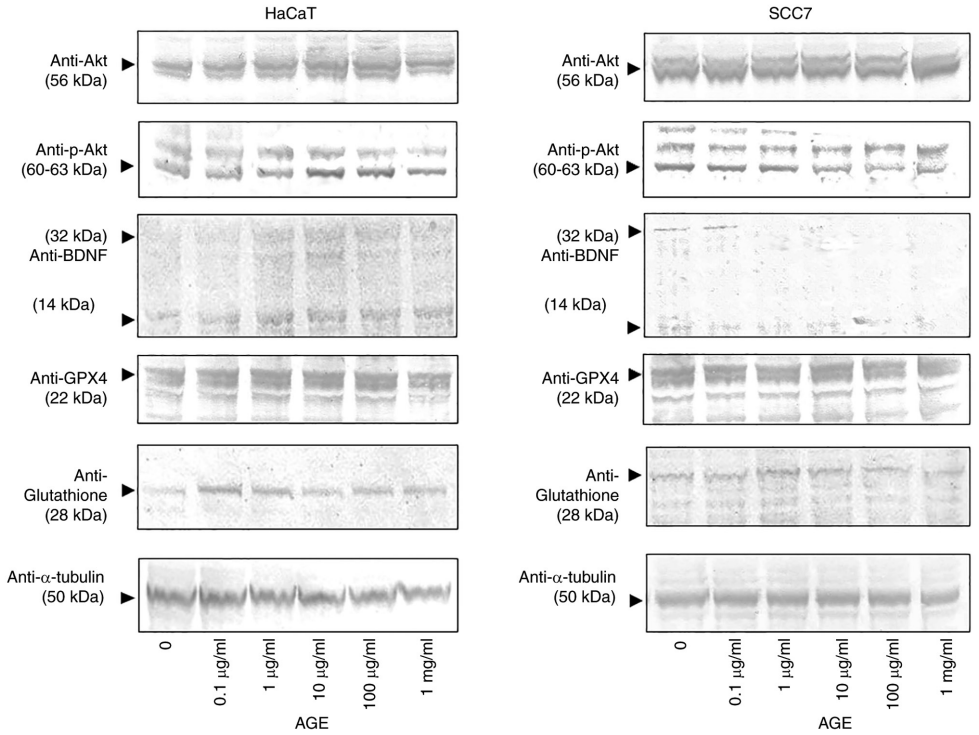

Figure 6. Expression of Akt, BDNF and ferroptosis related factors in AGE-treated cells. Western blotting showed that AGE (0.1 μg-1 mg/ml) enhanced the expression of Akt, p-Akt, BDNF, GPX4 and glutathione in HaCaT compared to the untreated HaCaT. On the other hands, those expressions were not enhanced in AGE-treated SCC7. Akt, protein kinase B family protein; BDNF, brain derived neurotrophic factor AGE, aged garlic extract; p-, phosphorylated.

Discussion

Garlic is considered to be a promising food source that can improve quality of life. In particular, AGE is one garlic supplement that is available commercially (1,20). AGE supplements and tonics are popular amongst patients suffering from physical fatigue, as AGE lacks the typical unpleasant and pungent odor of raw garlic. It is gentle on the gastric mucosa and to the to the best of the authors' knowledge, there have been no reports of severe side effects (2,22,23). Several studies have previously explored the mechanism underlying the anti-fatigue activity of AGE (22,23). However, the possibility that a nutritional supplement including AGE can become an extra source of nutrition for cancer cells to facilitate cancer progression cannot be excluded. Therefore, it is important to clarify whether AGE is beneficial or harmful for patients with cancer and how it affects normal cells and cancer cells alike. Nevertheless, it has been reported that AGE can exert anticancer activity against a number of cancers such as skin, esophageal, lung, gastric, colorectal and prostate cancer (17-21). AGE contains several important components, such as SAMC, which are effective in cancer prevention and can inhibit cancer cell proliferation and progression (4,21). Therefore, there is also a possibility that AGE can improve the efficacy of standard chemotherapeutic drugs in patients with cancer, or AGE itself may exert anticancer effects against OSCC. In the present study, the value of AGE administration in OSCC treatment was examined by evaluating its effectiveness against oral cancer cells and by comparing the action of AGE between normal cells and cancer cells. The ultimate aim was to clarify whether AGE affects normal cells and cancer cells differently.

AGE was found to promote proliferation and migration in normal cells, but not in cancer cells. In the majority of cases, it tended to inhibit the proliferation and migration of cancer cells. However, lower concentrations of AGE may not be able to exert migration inhibitory effects in some cancer cells. No morphological changes were observed among the normal cell lines and cancer cell lines following AGE treatment at this time (data not shown). Moreover, it was observed that AGE enhances cell proliferation in starvation state (0% FBS) compared with adequate nutritional state (10% FBS). The reason for this is not clear, although it is assumed that all cells including cancer cells have a certain limit of growth. Probably within that limit, when 10% FBS is present, adding an extra source of nutrition (such as AGE) may not improve cell growth rate any further.

In addition, the combination of AGE and anticancer agents, especially pre-treatment with 5-FU or CDDP followed by AGE, somewhat improved the inhibitory effects of these drugs against cancer cells in few cases. However, similar treatments did not inhibit the proliferation of normal cells. Extensive gene analysis using whole transcriptome followed by IPA resulted in beneficial data. Briefly, AGE may be involved in the proliferation and survival of normal cells through the suppression of ferroptosis and the induction of Akt and BDNF signaling. Ferroptosis is a type of programmed cell death that is iron-dependent and lipid peroxidation-driven. The roles of glutathione and GPX are well known in ferroptosis and even therapy-resistant cancer cells are particularly vulnerable to ferroptosis (27). By contrast, BDNF belongs to a family of neurotrophic factors and it increases cancer cell proliferation and migration (28,29), the activation of which leads to the activation of several downstream signaling pathways, including PI3K/Akt. The PI3K/Akt pathway is directly associated with cell survival, proliferation and angiogenesis (28).

In addition, IPA data revealed that AGE may reduce cancer cell proliferation through the suppression of cancer metastasis signaling and enhancement of phagocytic activity. Phagocytosis checkpoints are essential checkpoints for cancer immunotherapy, where it links innate and adaptive immunity. Targeting these phagocytosis checkpoints and blocking their signaling pathways has been previously found to enhance phagocytosis and reduce tumor size (30). Therefore, it could be concluded that AGE may suppress cancer cell proliferation under certain conditions but may stimulate the proliferation of normal cells in nutritionally poor conditions (0% FBS). This finding is more ideal for patients with cancer, since the present study indicated that AGE may not exert any harmful effects on normal cells. Western blot analysis confirmed the IPA data and showed that AGE increased the levels of Akt, p-Akt, BDNF, GPX4 and Glutathione in HaCaT cells, but not in SCC7 cells, compared with those in untreated cells.

Therefore, it can be concluded that the effects of AGE on normal cells may vary from those on cancer cells, where AGE may prompt the proliferation of normal cells and suppress the that of cancer cells through different cellular processes and signaling pathways. AGE may also accelerate wound healing and reduce oral mucositis without promoting cancer progression. These findings may also suggest a solution to the problem of choosing a useful and effective nutritional supplement in patients with cancer who are not suffering from adverse side effects or doesn't interfere negatively with cancer medications.

The present study may have some limitations. Although several normal cell lines and cancer cell lines were used in this study, other types of normal cells and cancer cells may behave differently under the same cultural and experimental conditions involving AGE. The mechanism of action of AGE was only investigated in two cell lines (HaCaT and SCC7). Similarly, the expression of Akt, BDNF and ferroptosis related factors were studied only in these two cell lines. More importantly, the exact molecular mechanisms by which AGE inhibits cancer cell growth and metastasis still remain unclear. Moreover, this study did not examine the effect of AGE on the cell cycle and apoptosis of these cell lines. Therefore, additional research is necessary to confirm our data. Future investigations will address the aforementioned limitations to understand the anticancer effects of AGE more elaborately and clearly. Moreover, they will focus on the long-term effects of AGE on cell survival, proliferation and tumor progression with a broader range of cell types and *in vivo* models to understand its therapeutic potential.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article. All whole transcriptome sequencing data are available in this public database: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE294094. These data are available for viewing with the permission of the authors upon request.

Authors' contributions

KH designed the study. KH, TF, KK and KW performed the experiments, analyzed the data, wrote and revised the manuscript. KH and YM confirm the authenticity of all the raw data. YM revised the manuscript and provided valuable suggestions during the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Human Dermal Fibroblast (HDFa), a primary cell line, was purchased from American Type Culture Collection. This cell line was collected with informed consent by ATCC and donor privacy is protected. This cell line can be used for non-commercial research purposes without special permission from the authors' institutions at present.

Patient consent for publication

Not applicable.

Competing interests

Financial support was received from Wakunaga Pharmaceutical Co., Ltd.

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